# STUDIES OF CANINE ACIDOPHIL CELL HEPATITIS

by

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## ABSTRACT

Canine Acidophil Cell Hepatitis (CACH), the subject of this study, was first described only relatively recently by Jarrett and O'Neil (1985). The aims of this study were to increase understanding of the disease, to investigate its experimental transmission to dogs and various laboratory animal species and to search for the aetiological agent.

The introduction to this thesis describes the structure and function of normal and diseased liver, before concentrating on viruses known to cause liver disease in animals or humans and their resultant illnesses. A wide range are discussed since the aetiological agent of CACH has still to be isolated or identified. All of the protocols and techniques employed in this study are covered in the general materials and methods chapter.

A sample of twenty CACH field cases described in chapter three, demonstrate the wide age range and variety of dogs which can be affected as well as the extensive geographical distribution of the disease throughout the country. The series was not a comprehensive coverage of all the CACH cases and suspected cases studied.

Three canine transmission studies investigated the transmissibility of CACH, following the course of infection with frequent clinical, biochemical and haematological examinations. They provided material for other investigations including electron microscopy and serological studies. The last of the three used material from experimentally-infected rats to transmit CACH back to dogs. Transmissibility to various laboratory species was investigated, some were found to be more susceptible to CACH infection than others.

Extensive electron microscopical examinations, of experim-

ental and field case material, located inumerable interesting particles but none could be definitely identified as virions.

Preliminary serological studies, involving agar gel immunodiffusion (AGID) and peroxidase-anti-peroxidase (PAP) immunocytochemistry, produced some interesting results but were not extensive enough to estimate the possibililty of developing a diagnostic test for use in living animals.

To the memory of

Spot
Blackie
Patch
Stripe
Star
Tiny
Fatso
Snowy
Tan
Scruff
Tiger
Susie
Rex
Di
Broon

and last though never least, except in stature,

Mo

# LIST OF CONTENTS


| | | Page |
|---|---|---|
| Title Page | | 1 |
| Abstract | | 2 |
| List of Contents | | 5 |
| List of Tables | | 11 |
| List of Figures | | 12 |
| Acknowledgement | | 16 |
| Author's Declaration | | 17 |
| Definitions | | 17 |
| CHAPTER 1 | GENERAL INTRODUCTION | 18 |
| 1.0 | INTRODUCTION | 19 |
| 1.1 | NORMAL LIVER | 19 |
| 1.1.1 | Physiology | 19 |
| 1.1.2 | Histology | 20 |
| 1.1.3 | Ultrastructure | 23 |
| 1.2 | GENERAL LIVER DISEASE | 26 |
| 1.2.1 | Aetiology | 26 |
| 1.2.2 | Pathophysiology | 26 |
| 1.2.3 | Biochemical Evaluation | 29 |
| 1.2.4 | Histopathology | 35 |
| 1.2.5 | Ultrastructural Changes | 36 |
| 1.3 | HEPATITIS VIRUSES AND THEIR DISEASES | 39 |
| 1.3.1 | Hepatitis A Virus | 40 |
| 1.3.2 | Hepatitis B Virus | 41 |
| 1.3.3 | Hepatitis C Virus | 41 |
| 1.3.4 | Hepatitis D Virus | 42 |
| 1.3.5 | Hepatitis E Virus | 43 |
| 1.3.6 | Other Viruses Causing Hepatitis in Humans | 44 |
| 1.3.7 | Arenaviruses | 45 |
| 1.3.8 | Animal Hepadnaviruses | 53 |
| 1.3.9 | Canine Adenovirus | 54 |
| 1.3.10 | Canine Herpesvirus | 56 |
| 1.3.11 | Canine Acidophil Cell Hepatitis Agent | 57 |
| 1.3.12 | Other Unidentified Agents | 58 |
| 1.4 | ACUTE HEPATITIS | 59 |
| 1.4.1 | Clinical and Biochemical Features | 59 |
| 1.4.2 | Histopathology | 60 |
| 1.5 | CHRONIC HEPATITIS | 61 |
| 1.5.1 | Human Classification | 61 |
| 1.5.2 | Canine Classification | 64 |
| 1.5.3 | Clinical and Biochemical Features | 65 |
| 1.5.4 | Histopathology | 67 |
| 1.5.5 | Aetiological Types | 68 |
| 1.6 | CIRRHOSIS | 77 |
| 1.6.1 | Classification | 77 |
| 1.6.2 | Pathogenesis | 78 |
| 1.6.3 | Clinical and Biochemical Features | 80 |
| 1.6.4 | Gross Pathology | 81 |
| 1.6.5 | Histopathology | 82 |
| 1.7 | THERAPY | 83 |
| CHAPTER 2 | GENERAL MATERIALS AND METHODS | 87 |
| 2.0 | INTRODUCTION | 88 |
| 2.1 | FIELD CASES | 88 |

| | | Page |
|---|---|---|
| 2.1.1 | Clinical Details | 89 |
| 2.1.2 | Postmortem Examination | 89 |
| 2.1.3 | Haematological and Biochemical Examinations | 90 |
| 2.2 | TRANSMISSION EXPERIMENTS | 90 |
| 2.2.1 | Experimental Dogs | 90 |
| 2.2.2 | Experimental Laboratory Animals | 91 |
| 2.2.3 | Inocula | 92 |
| 2.2.4 | Clinical Investigations | 93 |
| 2.2.5 | Haematological Procedures | 93 |
| 2.2.6 | Biochemical Procedures | 94 |
| 2.2.7 | Preparation of Azothioprine Solutions | 94 |
| 2.2.8 | Postmortem Examination | 95 |
| 2.3 | HISTOLOGICAL PROCEDURES | 96 |
| 2.4 | ULTRASTRUCTURAL PROCEDURES | 97 |
| 2.4.1 | Tissue Blocks | 97 |
| 2.4.2 | Dissociated and Other Cell Preparations | 100 |
| 2.4.3 | Purified Preparations | 101 |
| 2.5 | IMMUNOLOGICAL PROCEDURES | 104 |
| 2.5.1 | Raising Antiserum | 104 |
| 2.5.2 | Absorption of Antiserum | 105 |
| 2.5.3 | Agar Gel Immunodiffusion (AGID) | 107 |
| 2.5.4 | Enzyme Immunocytochemistry | 108 |
| 2.5.5 | Immune Electron Microscopy | 111 |
| CHAPTER 3 | CANINE FIELD CASES | 113 |
| 3.0 | INTRODUCTION | 114 |
| 3.1 | MATERIALS AND METHODS | 114 |
| 3.2 | CASE HISTORIES | 115 |
| 3.2.1 | Field Case 1 / Dog A | 115 |
| 3.2.2 | Field Case 2 / Dog F | 117 |
| 3.2.3 | Field Case 3 / Dog G | 119 |
| 3.2.4 | Field Case 4 | 120 |
| 3.2.5 | Field Case 5 | 120 |
| 3.2.6 | Field Case 6 | 122 |
| 3.2.7 | Field Case 7 | 123 |
| 3.2.8 | Field Case 8 | 124 |
| 3.2.9 | Field Case 9 | 125 |
| 3.2.10 | Field Case 10 / Dog B | 125 |
| 3.2.11 | Field Case 11 | 128 |
| 3.2.12 | Field Case 12 | 129 |
| 3.2.13 | Field Case 13 | 129 |
| 3.2.14 | Field Case 14 | 130 |
| 3.2.15 | Field Case 15 | 131 |
| 3.2.16 | Field Case 16 | 132 |
| 3.2.17 | Field Case 17 | 133 |
| 3.2.18 | Field Case 18 | 134 |
| 3.2.19 | Field Case 19 | 135 |
| 3.2.20 | Field Case 20 | 136 |
| 3.3 | CONCLUSION | 137 |
| CHAPTER 4 | DOG TRANSMISSION EXPERIMENTS | 139 |
| 4.0 | INTRODUCTION | 140 |
| 4.1 | MATERIALS AND METHODS | 141 |
| 4.2 | NORMAL VALUES | 142 |
| 4.3 | DOG EXPERIMENT ONE | 143 |

| | | Page |
|---|---|---|
| 4.3.1 | Experiment Design | 143 |
| 4.3.2 | Source Case | 145 |
| 4.3.3 | General Clinical History | 147 |
| 4.3.4 | Rectal Temperatures | 152 |
| 4.3.5 | Biochemical Results | 155 |
| 4.3.6 | Haematological Results | 161 |
| 4.3.7 | Postmortem Findings | 166 |
| 4.3.8 | Histological Results | 167 |
| 4.3.9 | Conclusion | 169 |
| 4.4 | DOG EXPERIMENT TWO | 171 |
| 4.4.1 | Experiment Design | 171 |
| 4.4.2 | Source Case | 172 |
| 4.4.3 | General Clinical History | 173 |
| 4.4.4 | Immunosuppressive Regimes | 175 |
| 4.4.5 | Rectal Temperatures | 176 |
| 4.4.6 | Biochemical Results | 181 |
| 4.4.7 | Haematological Results | 195 |
| 4.4.8 | Postmortem Findings | 204 |
| 4.4.9 | Histological Results | 205 |
| 4.4.10 | Conclusion | 208 |
| 4.5 | DOG EXPERIMENT THREE | 210 |
| 4.5.1 | Experiment Design | 210 |
| 4.5.2 | Source Cases | 212 |
| 4.5.3 | General Clinical History | 213 |
| 4.5.4 | Rectal Temperatures | 217 |
| 4.5.5 | Biochemical Results | 219 |
| 4.5.6 | Haematological Results | 225 |
| 4.5.7 | Postmortem Findings | 230 |
| 4.5.8 | Histological Results | 231 |
| 4.5.9 | Conclusion | 233 |
| CHAPTER 5 | RAT TRANSMISSION EXPERIMENTS | 236 |
| 5.0 | INTRODUCTION | 237 |
| 5.1 | MATERIALS AND METHODS | 239 |
| 5.2 | RAT CLINICAL BIOCHEMISTRY | 240 |
| 5.3 | RAT EXPERIMENT ONE | 241 |
| 5.3.1 | Experiment Design | 241 |
| 5.3.2 | Source Case | 242 |
| 5.3.3 | Results | 243 |
| 5.3.4 | Conclusion | 245 |
| 5.4 | RAT EXPERIMENT TWO | 246 |
| 5.4.1 | Experiment Design | 246 |
| 5.4.2 | Source Case | 247 |
| 5.4.3 | Results | 247 |
| 5.4.4 | Conclusion | 249 |
| 5.5 | RAT EXPERIMENT THREE | 251 |
| 5.5.1 | Experiment Design | 251 |
| 5.5.2 | Results | 253 |
| 5.5.3 | Conclusion | 254 |
| 5.6 | RAT EXPERIMENT FOUR | 254 |
| 5.6.1 | Experiment Design | 254 |
| 5.6.2 | Source Cases | 255 |
| 5.6.3 | Histological Results | 256 |
| 5.6.4 | Biochemical Results | 258 |
| 5.6.5 | Conclusion | 259 |
| 5.7 | RAT EXPERIMENT FIVE | 260 |

| | | Page |
|---|---|---|
| 5.7.1 | Experiment Design | 260 |
| 5.7.2 | Source Case | 262 |
| 5.7.3 | Histological Results | 263 |
| 5.7.4 | Biochemical Results | 264 |
| 5.7.5 | Conclusion | 265 |
| 5.8 | RAT EXPERIMENT SIX | 266 |
| 5.8.1 | Experiment Design | 266 |
| 5.8.2 | Histological Results | 267 |
| 5.8.3 | Biochemical Results | 268 |
| 5.8.4 | Conclusion | 269 |
| CHAPTER 6 | MOUSE AND GUINEA PIG TRANSMISSION EXPTS. | 270 |
| 6.0 | INTRODUCTION | 271 |
| 6.1 | MATERIALS AND METHODS | 272 |
| 6.2 | MOUSE EXPERIMENT ONE | 272 |
| 6.2.1 | Experiment Design | 272 |
| 6.2.2 | Results | 273 |
| 6.2.3 | Conclusion | 275 |
| 6.3 | GUINEA PIG EXPERIMENT ONE | 276 |
| 6.3.1 | Experiment Design | 276 |
| 6.3.2 | Results | 277 |
| 6.3.3 | Conclusion | 278 |
| 6.4 | GUINEA PIG EXPERIMENT TWO | 280 |
| 6.4.1 | Experiment Design | 280 |
| 6.4.2 | Histological Results | 281 |
| 6.4.3 | Haematological and Biochemical Results | 282 |
| 6.4.4 | Conclusion | 283 |
| CHAPTER 7 | HAMSTER TRANSMISSION EXPERIMENTS | 284 |
| 7.0 | INTRODUCTION | 285 |
| 7.1 | MATERIALS AND METHODS | 286 |
| 7.2 | HAMSTER CLINICAL BIOCHEMISTRY | 288 |
| 7.3 | HAMSTER EXPERIMENT ONE | 289 |
| 7.3.1 | Experiment Design | 289 |
| 7.3.2 | Histological Results | 291 |
| 7.3.3 | Biochemical Results | 292 |
| 7.3.4 | Conclusion | 292 |
| 7.4 | HAMSTER EXPERIMENT TWO | 292 |
| 7.4.1 | Experiment Design | 292 |
| 7.4.2 | Histological Results | 294 |
| 7.4.3 | Biochemical Results | 295 |
| 7.4.4 | Conclusion | 296 |
| 7.5 | HAMSTER EXPERIMENT THREE | 297 |
| 7.5.1 | Experiment Design | 297 |
| 7.5.2 | Histological Results | 297 |
| 7.5.3 | Biochemical Results | 298 |
| 7.5.4 | Conclusion | 298 |
| 7.6 | HAMSTER EXPERIMENT FOUR | 299 |
| 7.6.1 | Experiment Design | 299 |
| 7.6.2 | Histological Results | 299 |
| 7.6.3 | Biochemical Results | 301 |
| 7.6.4 | Conclusion | 302 |
| 7.7 | HAMSTER EXPERIMENT FIVE | 302 |
| 7.7.1 | Experiment Design | 302 |
| 7.7.2 | Histological Results | 304 |

| | | Page |
|---|---|---|
| 7.7.3 | Biochemical Results | 304 |
| 7.7.4 | Conclusion | 305 |
| CHAPTER 8 | ELECTRON MICROSCOPICAL INVESTIGATIONS | 306 |
| 8.0 | INTRODUCTION | 307 |
| 8.1 | TISSUE SECTIONS | 307 |
| 8.1.1 | Tissues Collected and Processed for Electron Microscopy | 308 |
| 8.1.2 | Examination of Tissue Blocks | 309 |
| 8.2 | DISSOCIATED CELLS AND OTHER PREPARATIONS PROCESSED AS SOLIDS | 310 |
| 8.2.1 | Dissociated Cells | 310 |
| 8.2.2 | Buffy Coat | 310 |
| 8.2.3 | Lymphocytes | 311 |
| 8.2.4 | Abdominal Fluid Preparations | 311 |
| 8.2.5 | Cell Culture | 312 |
| 8.3 | NEGATIVE STAINS OF PURIFIED PREPARATIONS | 313 |
| 8.3.1 | Isopycnic Centrifugation | 313 |
| 8.3.2 | Fractions from Liver Preparations | 314 |
| 8.3.3 | Fractions from Lymph Node Preparations | 317 |
| 8.3.4 | Abdominal Fluid Preparations | 318 |
| 8.3.5 | Cell Culture Medium | 319 |
| 8.4 | CONCLUSION | 319 |
| CHAPTER 9 | SEROLOGICAL STUDIES | 321 |
| 9.0 | INTRODUCTION | 322 |
| 9.1 | ANTIBODIES FOR IMMUNOLOGICAL INVESTIGATIONS | 322 |
| 9.1.1 | Raising Antiserum in Rabbits | 322 |
| 9.1.2 | Absorbed Serum | 323 |
| 9.2 | AGAR GEL IMMUNODIFFUSION THEORY AND METHOD | 323 |
| 9.3 | AGID EXPERIMENT ONE | 325 |
| 9.3.1 | Experiment Design | 325 |
| 9.3.2 | Results | 327 |
| 9.3.3 | Conclusion | 327 |
| 9.4 | AGID EXPERIMENT TWO | 328 |
| 9.4.1 | Experiment Design | 328 |
| 9.4.2 | Results | 330 |
| 9.4.3 | Conclusion | 330 |
| 9.5 | PAP IMMUNOCYTOCHEMISTRY THEORY AND METHOD | 331 |
| 9.6 | PAP IMMUNOCYTOCHEMISTRY EXPERIMENTS | 322 |
| 9.6.1 | PAP Experiment One | 333 |
| 9.6.2 | PAP Experiment Two | 333 |
| 9.6.3 | PAP Experiment Three | 334 |
| 9.6.4 | PAP Experiment Four | 334 |
| 9.6.5 | PAP Experiment Five | 336 |
| 9.6.6 | Conclusion | 337 |
| 9.7 | IMMUNE ELECTRON MICROSCOPY | 338 |
| 9.7.1 | Samples investigated by immune electron microscopy | 338 |
| 9.7.2 | Results | 339 |
| 9.8 | SEROLOGICAL TESTING FOR EVIDENCE OF HEPATITIS B INFECTIONS | 339 |
| CHAPTER 10 | CONCLUSION | 341 |

Page

APPENDIX 1 HISTOLOGICAL PROCEDURES 344

APPENDIX 2 ACIDOPHIL CELL SCORING SYSTEM 347

REFERENCES 350

# LIST OF TABLES


| | | Page |
|---|---|---|
| 3-1 | Summary of histological features of CACH field cases | fp137 |
| 4-1 | Dog experiment 1 antibody titres | fp148 |
| 4-2 | Dog experiment 1 early faecal egg counts and anthelmintic regime | fp149 |
| 4-3 | Dog experiment 1 urine biochemistry results | 167 |
| 4-4 | Dog experiment 1 liver histology results - acidophil cell scores | fp170 |
| 4-5 | Dog experiment 2 antibody titres | fp173 |
| 4-6 | Dog experiment 2 early faecal egg counts and anthelmintic regime | fp174 |
| 4-7 | Dog experiment 2 urine biochemistry results | 205 |
| 4-8 | Dog experiment 2 liver histology results - acidophil cell scores | fp209 |
| 4-9 | Dog experiment 3 antibody titres | 213 |
| 4-10 | Dog experiment 3 early faecal egg counts and anthelmintic regime | fp214 |
| 4-11 | Dog experiment 3 urine biochemistry results | 231 |
| 4-12 | Dog experiment 3 liver histology results - acidophil cell scores | fp234 |
| 5-1 | Rat experiment 1 liver histology results | fp245 |
| 5-2 | Rat experiment 2 liver histology results | fp250 |
| 5-3 | Rat experiment 3 liver histology results | fp254 |
| 5-4 | Rat experiment 4 liver histology results | fp256 |
| 5-5 | Rat experiments 5 and 6 liver histology results | fp264 |
| 6-1 | Mouse experiment 1 liver histology results | fp275 |
| 6-2 | Guinea pig experiment 1 liver histology results | fp278 |
| 6-3 | Guinea pig experiment 2 liver histology results | fp282 |
| 7-1 | Hamster experiments 1,2 and 3 liver histology results | fp295 |
| 7-2 | Hamster experiment 4 liver histology results | fp299 |
| 7-3 | Hamster experiment 5 liver histology results | fp304 |

# LIST OF FIGURES


| | | Page |
|---|---|---|
| 1-1 | Structure of liver lobules and acini | fp 20 |
| 1-2 | Example of an accessory portal circulation | fp 28 |
| 1-3 | Liver of dog V | fp 76 |
| 1-4 | Gross appearance of macronodular cirrhosis | fp 78 |
| 3-1 | Liver biopsy of CACH Field Case 13 | fp 130 |
| 3-2 | Liver biopsy of cirrhotic CACH Field Case 14 | fp 130 |
| 3-3 | Liver biopsy of CACH Field Case 15 | fp 132 |
| 3-4 | Liver of CACH Field Case 16 | fp 132 |
| 3-5 | Liver biopsy of CACH Field Case 19 | fp 135 |
| 3-6 | Liver biopsy of CACH Field Case 19 | fp 135 |
| 4-1 | Liver of source dog B (FC10) | fp 146 |
| 4-2 | Time sequence of early events & premises used | fp 150 |
| 4-3 to 4-8 | Dog expt. 1 rectal temperatures | fp 153-153 |
| 4-9 to 4-26 | Dog expt. 1 biochemistry results | fp 158-160 |
| 4-27 to 4-44 | Dog expt. 1 haematology results | fp 163-165 |
| 4-45 | Liver of control dog 34 | fp 167 |
| 4-46 | Liver of control dog 35 | fp 167 |
| 4-47 | Liver of dog 31 | fp 168 |
| 4-48 | Liver of dog 30 | fp 168 |
| 4-49 | Liver of dog 32 | fp 169 |
| 4-50 | Liver of CACH field dog G/FC3 | fp 172 |
| 4-51 | Liver of CACH exptl dog E | fp 172 |
| 4-52 to 4-62 | Dog expt 2 rectal temperatures | fp 179-fp 181 |
| 4-63 to 4-98 | Dog expt 2 biochemistry results | fp 189 - 194 |
| 4-99 to 4-131 | Dog expt 2 haematology results | fp 199-fp 204 |
| 4-132 | Liver of uninoculated dog 39 | fp 205 |
| 4-133 | Liver of dog 36 | fp 205 |
| 4-134 | Liver of dog 37 | fp 206 |
| 4-135 | Liver of dog 37 | fp 206 |
| 4-136 | Liver of dog 38 | fp 207 |
| 4-137 | Incl. body in liver of dog 38 | fp 207 |
| 4-138+139 | Liver of dog 41 | 207 |
| 4-140 | Liver of dog 40 | 208 |
| 4-141 | Comparison of enzyme peaks | fp 210 |
| 4-142 | Liver of experimtal rat (139) | fp 213 |
| 4-143 to 146 | Dog expt 3 rectal temperatures | fp 218-fp 219 |

| | | Page |
|---|---|---|
| 4-147 to 162 | Dog expt 3 biochemistry results | fp222-fp225 |
| 4-163 to 174 | Dog expt 3 haematology results | fp228-229 |
| 4-175 | Liver of dog 43 | fp230 |
| 4-176 | Liver of dog 44 | fp231 |
| 4-177 | Liver of dog 44 | fp231 |
| 4-178 | Liver of dog 45 | fp232 |
| 4-179 | Liver of dog 45 | fp232 |
| 4-180 | Liver of dog 42 | fp233 |
| 4-181 | Liver of dog 42 | fp233 |
| 5-1 | Liver of source dog A/FC1 | fp242 |
| 5-2 | Liver of source dog A/FC1 | fp242 |
| 5-3 | Liver of rat 19, 4 wks post dog A's liver inoc | fp243 |
| 5-4 | Liver of rat 23, 6 weeks post dog A's liver | fp243 |
| 5-5 | Liver of 6-week nil control rat 33 | fp244 |
| 5-6 | Liver of rat 40, 8 weeks post dog B's liver | fp247 |
| 5-7 | Liver of rat 53, 8 weeks post dog B's serum | fp247 |
| 5-8 | Liver of rat 55, 10 weeks post dog B's liver | fp248 |
| 5-9 | Liver of 10-week PBS-control rat 67 | fp248 |
| 5-10 | Liver of rat 44 (source rat A) 12 weeks post dog B's liver inoculum | fp249 |
| 5-11 | Liver of rat 45 (source rat B) 12 weeks post dog B's liver inoculum | fp249 |
| 5-12 | Liver of 12-week control rat 76 | 249 |
| 5-13 | Liver of rat 90, an in-contact dam | fp253 |
| 5-14 | Lilver of 14-week PBS-control rat 126 | 256 |
| 5-15 | Liver of 14 week nil control rat 133 | fp257 |
| 5-16 | Liver of rat 112, 8 week postinoculation with normal dog liver | fp257 |
| 5-17 | Liver of rat 118, 14 weeks post normal dog | 257 |
| 5-18 | Liver of rat 98, 14 weeks post rat A's liver inoculum | fp258 |
| 5-19 | Liver of rat 105, 14 weeks post rat B's liver | fp258 |
| 5-20 | Liver of CACH field case dog F (FC2) | fp262 |
| 5-21 | Liver of (source case)experimental dog D | fp262 |
| 5-22 | Liver of rat 137, 6 wks post dog D's liver inoculum | fp263 |
| 5-23 | Liver of rat 156, 18 wks post dog D's liver | fp263 |

| | | Page |
|---|---|---|
| 5-24 | Liver of 6-week control rat 173 | fp267 |
| 5-25 | Liver of rat 165, an in-contact dam | fp267 |
| 6-1 | Liver of 4 wk nil control mouse 49 | fp273 |
| 6-2 | Liver of 4-wk liver inoculated mouse 30 | fp273 |
| 6-3 | Liver of 4-wk serum inoculated rat 40 | fp274 |
| 6-4 | Liver of 4 week liver inoculated guinea pig 9 | fp277 |
| 6-5 | Liver of 4-week control guinea pig 10 | fp277 |
| 6-6 | Liver of 8-week control guinea pig 19 | fp281 |
| 6-7 | Liver of 8-week liver-inoculated guinea pig 14 | fp281 |
| 6-8 | Liver of in-contact dam guinea pig 20 | 282 |
| 7-1 | Liver of hamster 24, 4-wks post dog A's liver | fp294 |
| 7-2 | Liver of in-contact hamster 76 | fp303 |
| 8-1 | Liver of dog 38 of experiment 2 | fp309 |
| 8-2 | Crystalline intranuclear inclusion body in hepatocyte nucleus of CACH field case dog G | fp310 |
| 8-3 | Crystalline intranuclear inclusion body in hepatocyte nucleus of experimental CACH dog J | fp311 |
| 8-4 | Pelleted peritoneal fluid prep of dog 38 | fp309 |
| 8-5 | Exptl CACH dog 42 liver fract 3 | fp316 |
| 8-6 | Experimental CACH dog 42 fract 4 liver | fp316 |
| 8-7 | Exptl dog 45 liver fract 2 | fp316 |
| 8-8 | Dog 45 liver fract 2 | fp316 |
| 8-9 | Dog 45 liver fract 2 | fp316 |
| 8-10 | Dog 45 liver fract 3 | fp316 |
| 8-11 | Fract 2 stomached exptl rat (140-141) livers | 316 |
| 8-12 | Fract 2 homogenised exptl rat livers (6) | 316 |
| 8-13 | Fract 3 homogenised exptl rat livers (6) | 316 |
| 8-14 | Fract 3 homog exptl rat livers (138,139,142,143) | 316 |
| 8-15 | Interface of discont grad of dog 38's lymph node | 316 |
| 8-16 | Interface of discont grad of exptl dog 38's lymph node | 316 |
| 8-17 | Fract 4 of dog 38's 1st lymph node prep | fp318 |
| 8-18 | Fract A of dog 38's 2nd lymph node prep | fp318 |
| 8-19 | Fract A of dog 38's 2nd lymph node prep | fp318 |
| 8-20 | Fract A of dog 38's 2nd lymph node prep | fp317 |

| | | Page |
|---|---|---|
| 8-21 | Fract D of dog 38's 2nd lymph node prep | fp317 |
| 9-1 | AGID expt 1 plates with dog J as Ag in outer ring | fp327 |
| 9-2 | AGID expt 1 plates with dog D as Ag in outer ring | fp327 |
| 9-3 | AGID plates of expt 2 with dog D's liver as Ag in outer ring | fp330 |
| 9-4 | PAP CACH exptl dog D's liver with normal rabbit serum 1:400 = NEGATIVE CONTROL | fp334 |
| 9-5 | PAP expt 3 CACH exptl dog D's liver with absorbed rabbit serum 1:500 | fp334 |
| 9-6 | PAP CACH exptl dog D's liver with absorbed rabbit serum 1:500 = POSITIVE CONTROL | fp335 |
| 9-7 | PAP CACH field case dog A's liver with absorbed rabbit serum 1:500 | fp335 |
| 9-8 | PAP CACH field case dog N's liver with absorbed rabbit serum 1:500 | fp336 |
| 9-9 | PAP CACH field case dog O's liver with absorbed rabbit serum 1:500 | fp336 |
| 9-10 | Liver of ICH field case dog S | fp337 |
| 9-11 | PAP normal control dog C liver with absorbed rabbit serum 1:500 | fp337 |
| 9-12 | PAP ICH "control" liver of dog S with absorbed rabbit serum 1:500 | fp338 |
| 9-13 | Exptl dog 38's lymph node prep fraction 4 + 1:2 immune serum | fp339 |
| 9-14 | Exptl dog 38's lymph node prep fract 4 + 1:2 immune serum | fp340 |
| 9-15 | Dog 38's lymph node prep fract 4 + 1:2 immune serum | fp340 |
| 9-16 | Dog 38's l.node prep fract 3+ 1:2 immune serum | fp340 |
| 9-17 | Dog 38's 2nd lymph node prep fract A + 1:10 serum | fp340 |
| 9-18 | Dog 38's 2nd lymph node prep fract A + 1:10 serum | fp340 |
| 9-19 | Dog 38's 2nd lymph node prep fract A + 1:10 serum | fp340 |

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## Author's Declaration

I hereby declare that the work presented in this thesis is original and was conducted under supervision by the author. I also certify that no part of this thesis has been submitted previously, for the award of a degree, to any university.

Any samples, used in these investigations, which were obtained from sources other than the author's own work are indicated in the text. In particular, material from dog's D, E, H, I and J was provided by Professor WFH Jarrett. These dogs had been part of his own experimental work (Jarrett and O'Neil, 1985; Jarrett et al., 1987), although the author was involved in the latter part of the extended study of dogs I and J (Jarrett et al., 1987).

The author was initially trained in and practiced all of the techniques used in this thesis. However, due to the large numbers of samples investigated, the author was grateful to have the benefit of the services of the appropriate routine laboratories of GUVS for biochemical and haematological analyses and the processing of histological specimens.

## Definitions

Canine acidophil cell hepatitis (CACH)
A liver disease characterised by the distribution of acidophilic dying hepatocytes, especially in the limiting plates of portal tracts, extending out with increasing severity.

Acidophil cell
The classic cell seen in CACH lesions. A polygonal cell, often with concave sides, with acidophilic cytoplasm and a shrunken condensed nucleus.

# CHAPTER 1 GENERAL INTRODUCTION

# 1.0 INTRODUCTION

This chapter deals first with normal liver (section 1.1). A brief outline of the liver's physiological role is followed by its general structure and ultrastructure. The next section (1.2) is concerned with general liver disease. Various causes of liver disease are given and the effects of the resultant disturbance of hepatic function are described. Some of the biochemical and histological features of liver disease and the ultrastructural changes revealed by electron microscopy are also given.

Viruses causing liver disease are considered in more detail in section 1.3. The causal agent of canine acidophil cell hepatitis (CACH) is thought to be a virus (Jarrett et al., 1987). Since its identity and therefore any relationship to other viruses are not yet known, both human and animal hepatitis viruses are discussed.

The general features of acute hepatitis are dealt with briefly in section 1.4. Chronic hepatitis is the subject of section 1.5. The differences between the classification of the disease in humans and dogs are discussed, before chronic hepatitis in the dog is considered in more detail.

Section 1.6 deals with cirrhosis and the last section (1.7) gives some suggestions for therapy for liver disease cases.

## 1.1 NORMAL LIVER

### 1.1.1 Physiology

The liver is involved in many important physiological processes including protein, carbohydrate and fat metabolism. Some plasma proteins, e.g. albumin and fibrinogen, are synthesised in the liver and urea is produced as a

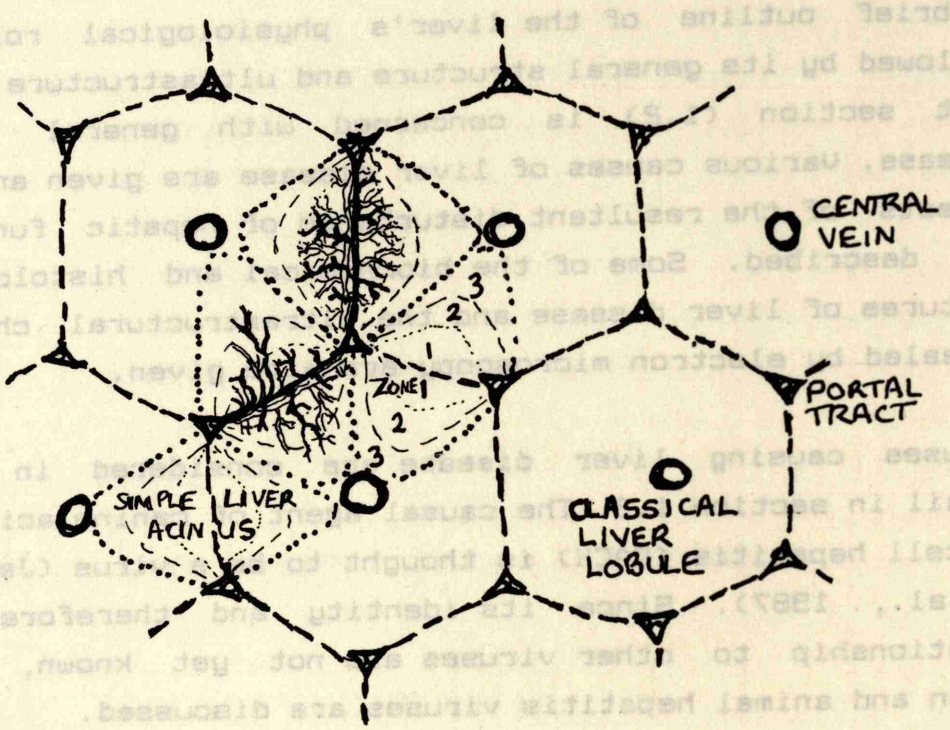


Fig 1-1 Structure of liver lobules and acini

protein metabolism by-product. The liver plays a role in the maintenance of the blood glucose concentration, by converting blood glucose into glycogen for storage until required. It is important in the maintenance of circulating lipid levels, incorporating lipid from various sources into lipoproteins. Liver cells detoxify endogenous waste products and exogenous toxins and drugs. The liver is responsible for bile formation and secretion, which involves bile salt secretion and bile pigment excretion. It is also involved in the synthesis of cholesterol and various enzymes, and in the storage of some vitamins and metals.

### 1.1.2 Histology

A thin connective tissue capsule surrounds the liver. The connective tissue invests the portal tracts, dividing the parenchyma into lobules. In most mammals, including the species used in these investigations, the small amounts of connective tissue present result in only indistinct lobulation, the hepatic parenchyma thus appearing continuous. Lobules can, however, be imagined using the central veins and portal tracts as landmarks, and the radiating pattern of hepatocyte cords and sinuses.

The classical liver lobule is the area of parenchyma around one draining central vein, with portal tracts at its periphery (see figure 1-1). It is primarily an anatomical, descriptive structural unit since its blood supply and bile drainage are both multiple and shared with other areas of parenchyma. However, it can sometimes be regarded as a functional unit. Two examples are the deposition of glycogen after a meal, in concentric zones from the periphery of the lobule towards its central vein, and the centrilobular necrosis seen in chlorinated hydrocarbon poisoning.

An alternative structural unit, the simple liver acinus

(figure 1-1) defined by Rappaport et al. (1954), constitutes the smallest functional unit of the liver. It is the parenchyma, situated between two central veins, which receives blood from its axial vessels (a terminal portal venule and hepatic arteriole) and passes bile into one bile ductule in the same portal tract. Each acinus can be subdivided functionally into three concentric zones. Periportal zone 1 hepatocytes receive blood richer in nutrients and oxygen and are more active metabolically than the more peripheral mid-zone 2 and perivenular zone 3. Cells of the last are the least metabolically active, the most susceptible to hypoxic damage and are at the microcirculatory periphery of the acinus. A complex acinus (figure 1-1) is formed by the three or more simple acini relating to the preterminal branches of a portal venule and a hepatic arteriole.

In cross section, classical lobules are seen to consist of anastomosing cords of hepatocytes (two cells wide) radiating from a central vein. Vascular sinusoids lie in the lacunae between the cords, separated from them by the perisinusoidal space of Disse; they empty into the central vein. The continous limiting plate of hepatocytes, around portal tracts, is fenestrated for passage of terminal branches of the hepatic artery, portal vein and bile duct. Within the lobules is a supportive network of reticular fibres which, at the periphery of the lobules, is continuous with the interlobular portal connective tissue.

The liver's blood supply is carried by the portal vein and the hepatic artery; these branch and enter the liver surrounded by connective tissue. Along with the branches of the hepatic duct they form portal triads, which are accompanied by lymph vessels. The terminal branches of the portal vein supply the sinusoids, while those of the hepatic artery mostly supply the peribiliary plexus or empty directly into sinusoids. The latter have thin cellular walls of fenestrated endothelial cells and fixed

macrophages (Kupffer cells). They empty through pores into the central vein (really a thin walled venule), which is surrounded by a thin layer of connective tissue. At the end of the lobule it opens perpendicularly into an intercalated vein. These coalesce to form anastomosing hepatic veins which run alone, surrounded by abundant connective tissue and eventually empty into the caudal vena cava.

Hepatocytes are large, polyhedral cells with large, spherical, centrally-located nuclei. The latter are rather light in appearance, with a few chromatin clumps and one or more distinct nucleoli. The fluctuating functional state of hepatocytes contributes to the considerable variety in appearance of their cytoplasm. Scattered basophilic clumps of ergastoplasm are usually seen. Inclusions of glycogen and fat appear as empty spaces in the cytoplasm, with routine histological preparation. They have an irregular (glycogen) or rounded (fat) shape. Lipid droplets normally occur in modest numbers. When fat accumulates it usually does so centrilobularly first; the small, spherical droplets enlarge by coalescence as well as further accumulation, until the cell may be distended by a single large drop. The amount of glycogen is variable, being greatest shortly after a meal when it is deposited from the periphery of the lobule inwards; it is later removed from the most central deposits first.

Bile canaliculi, situated centrally between two adjoining hepatocytes, form a three-dimensional network. Their walls are formed by the locally specialized surfaces of adjoining liver cells. The bile is carried by short canals (of Hering) to terminal bile ducts (bile ductules) which empty into the bile ducts of the portal tracts. The initially cuboidal bile duct epithelium increases in height, to columnar, with increasing duct diameter. It stains very lightly and is surrounded by a dense layer of connective tissue. These interlobular bile ducts anastomose and join, to eventually form the hepatic duct. It is joined by the

cystic duct, from the gall bladder (when present), to form the bile duct which opens into the duodenum.

Kupffer cells have plumper nuclei than the flattened sinusoidal endothelial cells. These fixed macrophages arise from blood monocytes and are part of the reticulo-endothelial system. They can contain remnants of phagocytosed erythrocytes.

In the liver capsule, superficial lymph vessels form plexuses with deep lymph vessels in the interlobular connective tissue. Lymph vessels accompany portal triads right to the terminal ramifications. Nerves also accompany the triads, supplying the smooth muscle of the arterial walls.

### 1.1.3 Ultrastructure

Electron microscopy reveals that the liver cell nucleus is surrounded by a double-layered nuclear membrane which is perforated by numerous nuclear pores. Heterochromatin appears as masses of dense particles, mostly at the periphery of the nucleus. The dense, usually eccentric nucleolus also has associated chromatin at its circumference.

Hepatocyte cytoplasm contains numerous mitochondria which tend to be large and ovoid, with only small numbers of cristae and conspicuous intramitochondrial granules. They are frequently associated with lipid inclusions. Rough endoplasmic reticulum (RER) is scattered throughout the cytoplasm, often in parallel arrays corresponding to the basophilic clumps of ergastoplasm visible with light microscopy. Free polyribosomes also occur in the cytoplasm. The smooth endoplasmic reticulum (SER) tends to be located along the cell membrane and near glycogen deposits. Golgi complexes are situated between the nucleus and bile canaliculi, often close to the latter. Small,

electron dense bodies are present in the cytoplasm. They are mainly lysosomes, which are very electron dense and finely granular, but there are also microbodies, which have a finely granular matrix and can have a dense or lamellar nucleus.

Various intracytoplasmic inclusions can be found in the cytoplasm of hepatocytes. Lipid inclusions can be of two types: homogeneous or sometimes reticulated fat droplets, and laminated myelin figures. Stored glycogen occurs as small homogeneous particles or rosettes of particles. Pigmentary inclusions can be multilobulated lipofuscin granules or bile pigment deposits in vacuoles. Iron-containing granules consist of ferritin particles scattered through the cytoplasm or concentrated in lysosomes; normally haemosiderin granules are very rare.

At the secretory surface of the liver cell, microvilli project into the bile canaliculus; while from the sinusoidal surface, numerous irregularly orientated microvilli project into the space of Disse. Bundles of collagenous fibrils are also found in the latter.

As well as the differences in metabolic activity and light microscopical appearance between hepatocytes in different parts of the classical lobule, variation can be seen at the ultrastructural level with the electron microscope. In normal human livers, Ma and Biempica (1971) noted intralobular variations in hepatocellular organelles. Centrilobular hepatocytes contained round/oval mitochondria and more SER and peroxisomes; peripheral hepatocytes contained more and larger, oval/oblong mitochondria and golgi complexes, more RER, larger lysosomes and wider bile canaliculi with more microvilli.

Two types of cells can be identified lining the hepatic sinusoids: endothelial cells and Kupffer cells (Ebe and Kobayashi, 1972; Johannessen, 1979a). The lining endothel-

ial cells are attenuated, with no marked projections on their free surface. They contain few organelles, but do have phagocytic potential, and extend processes to form loose junctions with one another. Intercellular gaps and cytoplasmic fenestrations (pores) mean that the sinusoidal wall is discontinuous. In contrast, the larger Kupffer cells bulge into the sinusoidal lumen and have numerous long cytoplasmic processes. Kupffer cells have a more heterogenous nucleus than hepatocytes and their cytoplasm contains less mitochondria and RER. Abundant lysosomes and well-developed Golgi complexes are indicative of their phagocytic ability, while the presence of small vesicles near their surface suggests pinocytotic activity.

Fat-storing cells of Ito, thought to be derived from fibroblastic cells (Ebe and Kobayashi, 1972), are occasionally seen in the space of Disse or between two hepatocytes. They have also been called fat-storing adventitial fibrocytes and perisinusoidal lipocytes (Johannessen, 1979a). These polygonal, process-bearing cells contain few organelles but several large cytoplasmic lipid droplets, in which most of the liver's vitamin A is stored. The absence of any indication of phagocytosis differentiates them from sinusoidal endothelial cells and Kupffer cells.

Bile duct epithelial cells are cuboidal and smaller than hepatocytes. Their low density nucleus is large in relation to cytoplasmic volume. A small nucleolus is rarely visible. These cells contain only a small number of organelles. The few mitochondria have a clear matrix and long cristae; they are often present in two groups, above and below the nucleus. There is scanty RER and the Golgi complex is always situated between the nucleus and the apical pole. Many short microvilli project from the cell surface into the bile duct lumen, at the apical pole.

## 1.2 GENERAL LIVER DISEASE

### 1.2.1 Aetiology

There are many different causes of liver disease. Viral, bacterial and fungal infections can all cause hepatitis. Hypoxic damage can occur in shock, venous congestion (e.g. in congestive cardiac failure), or anaemia. Primary liver tumours occur and the liver is a common site for secondary metastases. The liver is susceptible to damage by toxins and poisons, including some drugs. Metabolic disturbances can also cause liver disease; specific dietary deficiencies may lead to fatty liver and liver cell necrosis, specific enzyme deficiencies can result in hepatic storage diseases or failure of biliary secretion. Extrahepatic biliary obstructions and cholangitis (which is often the result of an ascending bacterial infection) cause biliary tract lesions. Prolonged biliary obstruction can lead to biliary cirrhosis.

### 1.2.2 Pathophysiology

The varied effects of the disturbance of hepatic function by liver disease can be considered in three main groups: hepatocellular failure, portal hypertension and biliary obstruction (MacSween, 1985).

HEPATOCELLULAR FAILURE occurs when there is inadequate total hepatocyte function to maintain the normal physiological state. This can be due to the loss of many cells (e.g. in severe viral hepatitis) and/or the impaired function of hepatocytes, often resulting from chronic interference with blood flow (e.g. in cirrhosis). The following conditions are some of the many and varied effects of hepatocellular failure.

Hepatic encephalopathy is a term for neurological disturbances due to the effect on the central nervous system

(CNS) of changes in nitrogen metabolism leading to increased blood levels of toxic nitrogenous compounds, such as bacterial metabolites from the gut. The factors contributing to hepatic encephalopathy and the signs of it are detailed more fully later in this section.

Jaundice results from failure of the removal of bilirubin from blood, its conjugation and excretion in bile. It is usual in acute hepatocellular failure, the severity reflecting the degree of damage. In chronic hepatocellular failure the degree and type of jaundice vary. An obstructive jaundice precedes hepatic dysfunction in secondary biliary cirrhosis. In other types of chronic failure it is a late, bad prognostic sign, usually only mild or moderate in degree and probably due to failure of hepatocytes to excrete bilirubin.

Hormonal disturbances can occur in hepatocellular failure e.g. failure of hepatic inactivation of oestrogens, or hyperadrenocorticism due to reduced metabolism and excretion of adrenal corticosteroids.

Circulatory disturbances are possible e.g. a hyperkinetic circulation, which is probably due to vasoactive substances or impaired sympathetic responsiveness. Coagulation defects result from the defective synthesis of coagulation factors.

Functional renal failure (the hepatorenal syndrome) can occur in acute and chronic hepatic failure and is possibly due to impairment of proximal tubular function by humoral factors, released in severe liver cell injury.

Ascites only occurs when portal hypertension is also present, as in cirrhosis. In chronic liver failure, reduced albumin production leads to a fall in plasma osmotic pressure; portal hypertension raises the hydrostatic pressure in the hepatic and intestinal microvasculature.

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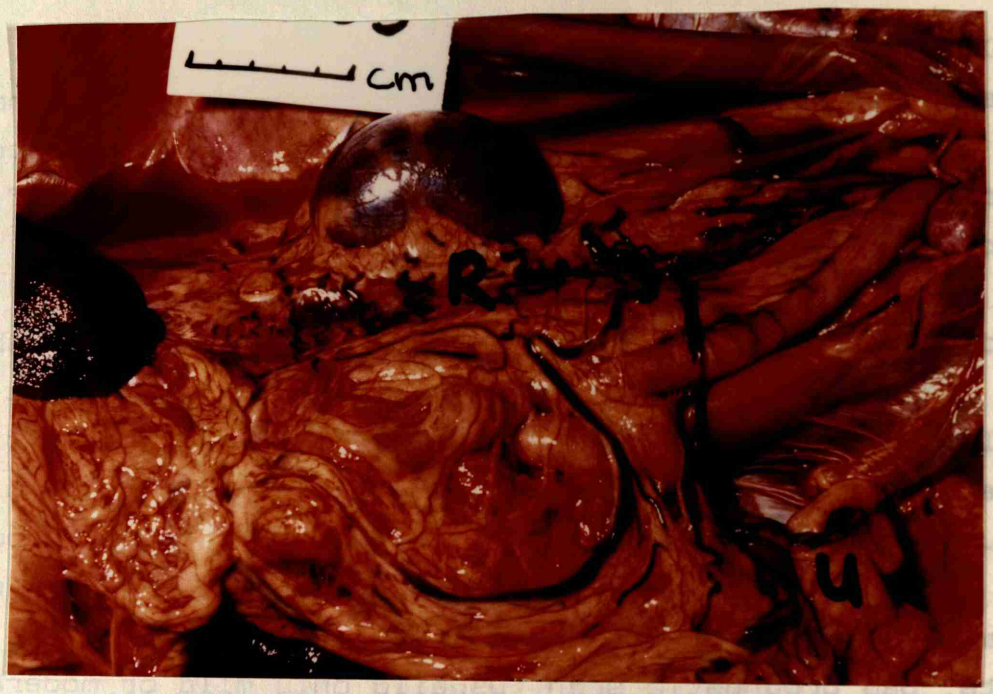


Fig 1-2 Example of an accessory portal circulation

Well developed APC with renal (R) and umbilical (U) anastomoses in 8-year-old M dog

Lymphatic compensation is overwhelmed (Grauer and Nichols, 1985) and there can also be lymph leakage due to outflow obstruction. Peripheral oedema is mainly the result of the fall in plasma osmotic pressure.

Fever is common in acute hepatocellular failure; hypothermia can occur in chronic failure. Other features of hepatocellular failure can include: general ill health with anorexia, wasting and vomiting. Bacteraemia can be a complication of hepatocellular failure.

PORTAL HYPERTENSION, due to extra or intrahepatic blood flow obstruction, can result in enlargement of multiple, frequently tortuous anastomoses shunting some portal blood directly into the systemic circulation, bypassing the liver (i.e. the opening up of pre-existing portosystemic collaterals). This accessory portal circulation increases the blood level of toxic compounds absorbed from the gut, aggravating the effect of hepatocellular failure on the CNS. Portal vessels can anastomose with the renal veins or empty directly into the posterior vena cava. Anastomoses may also develop in gastric, oesophageal, lower rectal and anal vessels, in the falciform ligament and at points of contact between abdominal viscera and the abdominal wall (see figure 1-2). The cause of such multiple aquired shunts in dogs with chronic hepatitis/ hepatic fibrosis or overt cirrhosis is intrahepatic blood flow obstruction. In contrast, the single, large portosystemic shunts which can also occur in dogs are usually congenital in origin (Hardy, 1989; Vulgamott, 1985).

BILIARY OBSTRUCTION can be due to obstruction of a major duct, or stagnation in bile canaliculi. It results in obstructive jaundice, due to reabsorption into the blood of conjugated bilirubin. Bile acids and cholesterol are also reabsorbed. A lack of bile salts reaching the intestine causes malabsorption of fat, hence steatorrhoea and malabsorption of fat-soluble vitamins, leading to

deficiencies of vitamins A, D, E and K. The harmful effect of retained bile upon hepatocytes can result in liver cell necrosis and eventually cirrhosis if cholestasis is prolonged. A secondary, bacterial ascending cholangitis can complicate major duct obstruction.

Jaundice can have a variety of aetiologies including: nonhepatic (haemolytic), with abnormal packed cell volume (PCV) and high unconjugated bilirubin level; hepatic; and extrahepatic biliary obstructive diseases. The serum enzyme pattern can be helpful when trying to differentiate the last two (see section 1.2.3). Functional changes in hepatocytes, canaliculi and bile ductules are now thought to dominate the pathogenesis of intrahepatic cholestasis (Teodori, 1975); the older concept of mechanical obstruction of intrahepatic ducts, is thought to be less important.

Hepatic encephalopathy is contributed to by three factors: hyperammonaemia; altered plasma concentrations of the amino acid precursors for neurotransmitter synthesis; and increased plasma concentrations of short-chain fatty acids (carbohydrate fermentation products) and mercaptans (methionine degradation products) which act synergistically, in the intestine, with ammonia to cause clinical signs (Zieve et al., 1974). A reduction in plasma concentrations of branched-chain amino acids and an increase in aromatic amino acids results in the production of less excitatory neurotransmitters and more inhibitory neurotransmitters. The varied signs of hepatic encephalopathy include: alterations in consciousness, personality changes, circling, head pressing, blindness, seizures and coma.

### 1.2.3 Biochemical Evaluation

Laboratory tests can provide information about three interrelated aspects of liver disease: whether there has been recent hepatocellular damage, whether there is

cholestasis and whether the liver is functioning normally (Bush, 1980). No single test can provide an adequate overall evaluation and many are not specific for liver dysfunction. Therefore several tests are usually performed when liver disease is suspected and the results are interpreted in conjunction with the history and clinical signs.

Increased serum activity of liver-specific enzymes, commonly used as an indicator of liver disease, can be due to either increased hepatocellular release or production.

The release of hepatocellular enzymes can result from increased membrane permeability or cellular necrosis. In the former case, affected hepatocytes may revert to normal or undergo necrosis. The size of the increase in serum enzyme activity parallels the number of damaged hepatocytes but cannot be correlated with reversibility of the damage. Liver function may be near-normal despite severe liver damage or may be seriously impaired despite normal serum enzyme levels (Cornelius, 1985a). The two most commonly measured "leakage" enzymes are alanine aminotransferase (ALT, formerly SGPT), which is essentially liver-specific in dogs, and aspartate aminotransferase (AST, formerly SGOT), which is also present in other tissues like muscle. The duration of increased serum activity depends not only on their leakage but also on the rate of disappearance from plasma. The plasma half-life of most enzymes is 2-4 days; however ALT has a short half-life of only 2½ hours. It is therefore possible to have high ALT concentrations that return to normal within 24 hours (Zinkl et al., 1971). In fact the ALT level can return to normal before morphologic evidence of necrosis disappears (Dixon et al., 1975). Although ALT is the main indicator of hepatocellular injury or necrosis, the increase in its activity in serum is roughly proportional to the number of cells injured and not to the degree of injury (Bush, 1980).

In contrast, alkaline phosphatase (AP) is one of the enzymes whose increased serum activity can result from increased cellular production without necrosis or cell membrane damage (Cornelius, 1985a). Sources of AP are hepatocytes, biliary epithelial cells, osteoblasts, intestine, placenta and some neoplasms. Although the AP activity in many tissues is at a much higher level than that in liver, the latter is now considered to be the main source of normal serum AP in adult dogs (Milne, 1985). This may be due to the very short half-life (less than 6 minutes) of placental, renal and intestinal AP isoenzymes, compared to the 3-day half-life of liver AP. The higher serum AP levels seen in young dogs are due to AP from osteoblasts in growing bone. Alkaline phosphatase released from damaged hepatocytes produces only a modest (2-3 times) increase in serum activity compared to the increases of up to 100 times, due to increased production, seen in cholestasis (Bush, 1980; Cornelius, 1985a). Raised AP levels are a nonspecific change reflecting bile duct epithelium proliferation, as in biliary obstruction and regeneration (Pekarthy et al., 1972), since large amounts are present in bile duct epithelium. Drug-induced increases should also be considered; glucocorticoids are reported to increase synthesis of hepatic AP (Milne, 1985).

As mentioned in section 1.2.2, the serum enzyme pattern can help differentiate whether a jaundiced case has hepatic disease or extrahepatic biliary obstructive disease (Schmidt, 1978). Elevation of ALT indicates parenchymal cell damage (disturbed hepatocellular permeability); while a raised AP level indicates biliary system disease, resulting in cholestasis.

Whether due to intra- or extrahepatic disease, cholestasis results in hyperbilirubinaemia and bilirubinuria which are accompanied by increased AP activity (Duncan and Prasse, 1977). Although the latter may be detected before the onset and in the absence of hyperbilirubinaemia.

In humans, plasma gamma glutamyl transferase (GGT) is a sensitive indicator of cholestatic disease due to hepatobiliary obstruction (Schmidt, 1978). The much lower GGT activity in canine liver compared to that in other species, may explain the lower serum levels of GGT in dogs (Shull and Hornbuckle, 1979). In experimental studies in dogs, plasma GGT activity was found to parallel that of plasma AP (Noonan and Meyer, 1979). As in humans, GGT may be helpful in differentiating the source of elevated serum AP without using complicated isoenzymology. However Shull and Hornbuckle (1979) among others suggest that the occasional benefit from knowledge of serum GGT in dogs does not merit its routine determination.

The condition of the liver's excretory function can be suggested by the serum bilirubin level and bromsulphthalein (BSP) or indocyanine green (ICG) clearance tests. The last two tend to be used when serum biochemistry is inconclusive. Whereas ALT, AST and AP indicate damage occurring at that time, the BSP clearance test is really an index of hepatic functional mass. The liver has a large spare functional capacity, so there has to be a major problem before the BSP clearance is markedly altered e.g. in a chronic hepatitis case with a lot of fibrosis rather than in an acute hepatitis. It is better not to perform the BSP clearnce test when serum bilirubin levels exceed 3mg/dl (Strombeck and Gribble, 1978).

Urea is synthesized from ammonia in the liver. Advanced liver disease cases have low blood urea nitrogen levels (BUN) and elevated blood ammonia (due to reduced hepatic function and/or portosystemic shunting of blood ammonia). However it should be remembered that fasting, not liver disease, is the most common cause of low BUN levels. Due to the liver's influence on BUN levels, serum creatinine should be used for evaluating renal function in canine liver disease cases.

Abnormal ammonia tolerance test results, like increased BSP retention, indicate changes in hepatocyte function and in hepatic circulation. However, the test now appears to be more specific for detection of hepatic circulatory disturbance than for reduced hepatic function (Conn, 1961; Hardy, 1983; Twedt, 1985). The liver's capacity to convert ammonia to urea is reduced by necrosis. The exchange of substances between hepatocytes and sinusoidal blood is adversely affected by hepatic architectural changes. Hepatocyte regeneration as double-cell plates, halves the perfused surface area. Collagen deposition both separates cells from their circulation and alters haemodynamics; vascular shunts in the fibrous septa bypass the sinusoidal circulation.

Serum bile acid (SBA) levels have been used increasingly in the diagnosis of canine hepatobiliary disease, since the validation of two methods for their measurement in dogs and cats (Bunch et al., 1984b; Center et al., 1984). They would also appear to be useful tests of hepatic excretory function in dogs (Matern and Gerok, 1979). Hauge and Abdelkader (1984) found the SBA concentration to be a very sensitive parameter for the detection of liver disease in dogs, including secondary liver involvement in other problems like diabetes or heart failure. Fasting SBA (FSBA) values were found to be increased in dogs with all forms of liver disease (Center et al., 1985). They are better used to detect severe hepatobiliary disease than mild and are not, on their own, of use in the differential diagnosis of liver diseases. In Jensen's (1991) evaluation of FSBA and 2-hour postprandial SBA (PSBA) levels he found that the latter tended to be superior in sensitivity, specificity and predictive values to the other hepatic tests. However they are still best used in conjunction with tests for liver enzyme activity (Center et al., 1991). Bile acids are synthesized in the liver from cholesterol, conjugated to the amino acids taurine or glycine, then transported in the bile to the intestines,

where their detergent action facilitates lipid digestion and absorption. Most of the bile acids are reabsorbed, by active transport, in the ileum. They are then carried, in the portal blood, to the liver where they are actively reabsorbed with very little spillover to the systemic circulation. Normal, efficient enterohepatic circulation results in only a small daily loss of bile acids in the faeces (Dowling, 1972), and only small amounts in the systemic circulation after a 12-hour fast. The small physiological requirement for bile acids and vast reserve capacity of hepatic synthesis means their levels are not limited by hepatic failure. The FSBA level is determined by the net rate of hepatic uptake from plasma, in relation to the rate of absorption from the intestine (LaRusso et al., 1978); so values rise with substantial hepatobiliary dysfunction or portohepatic circulatory insufficiency.

Serum levels of acute phase proteins (APP) change during most acute and chronic inflammatory processes in humans (Kushner, 1982). Their levels have been investigated in hepatitis (Courtoy et al., 1981) and various chronic liver diseases (Meliconi et al., 1988; Ozeki et al., 1988). Measurement of APP may be helpful in differential aetiological diagnosis.

Since hepatic function must be reduced by about 80% before hypoalbuminaemia is detectable, this usually signifies end-stage liver disease (Feldman, 1980). The circulating half-life of canine serum albumin is 7-10 days and therefore hypoalbuminaemia is not usually associated with acute liver failure and is only seen in subacute or chronic diffuse hepatic disease (Cornelius, 1985b). But apart from reduced synthesis, low albumin levels can also be due (by dilution) to plasma volume expansion and ascites.

Raised beta and gamma globulin levels occur in chronic liver disease, probably due to immune stimulation. The latter can result from impaired filtration of bacteria and

endotoxins from portal blood by the hepatic reticuloendothelial system, or can be an indication of hepatic inflammation.

Prothrombin and other coagulation factors are synthesized by the liver, so abnormal clotting times may be seen in cases with severe damage. These clotting factors have a much shorter half-life than serum albumin and so increased prothrombin time may be present in acute hepatic failure.

### 1.2.4 Histopathology

It must be remembered that the histological image obtained from postmortem liver samples is not necessarily that which was present in life. There are three main groups of phenomena involved in distortion of the original cytological picture: agonal changes, postmortem changes and technical artefacts (Majno, 1964). Various agonal changes may occur when death is other than instantaneous. There is disappearance of glycogen from hepatocytes, mainly due to anoxia and prolonged fasting. Liver parenchyma appears darker and more uniformly stained. Perisinusoidal spaces become wider. Postmortem changes may be less morphologically significant than these agonal changes, for a few hours at least. Technical artefacts can occur when liver is kept at room temperature for several hours, despite the remarkable degree of preservation of morphological detail, due to an increase in susceptibility to damage by certain fixation and embedding techniques.

It is also worth noting that liver cells may have been irreversibly damaged without it showing histologically, especially with the light microscope. For example, when ischaemia is induced in rats, by clamping of a lobe's blood supply (Majno, 1964), there is initially a period of reversible alterations, then a point of no return after which irreversible changes eventually lead to total destruction. The critical time range for rat and dog liver

appears to be between 30 and 60 minutes; after this most cells cannot recover. However, at this time virtually no morphological changes are apparent with the light microscope and cells remain normal looking for several more hours.

When liver cell necrosis occurs, it can be in a variety of patterns. Necrobiosis is when single, scattered liver cells die one by one, in the midst of living tissue. In piecemeal necrosis, individual hepatocytes are surrounded by fibrosis and die; this often occurs adjacent to portal tracts (e.g. see figures 4-135 and 4-181). Cells dying in small, irregularly distributed groups, at which macrophages and lymphocytes may accumulate, can be termed focal necrosis. Confluent necrosis involves large groups of dying hepatocytes, the most severe degree of this being massive hepatic necrosis.

There are many other possible features of liver disease which may be visualised with the light microscope. They include: hypertrophy or atrophy of hepatocytes; hepatocellular hyperplasia; intracellular inclusion bodies; excessive intracellular accumulation of substances e.g. fat vacuoles; cholestasis with bile plugs in canaliculi; congestion; haemorrhage; inflammatory or other cell infiltrates; fibrosis; structural abnormalities and some infectious agents e.g. fungi, leptospires and bacteria. As with necrosis, it is not only the presence of any of these features but also their degree and distribution which are significant.

### 1.2.5 Ultrastructural Changes

The acidophilic body of light microscopy, resulting from coagulation necrosis, is seen with the electron microscope to be a very dense, entire cell with loss of microvilli but recognisable endoplasmic reticulum and mitochondria. It is smaller than, and detached from, neighbouring

hepatocytes. Degenerating hepatocytes generally have shrunken nuclei, irregularly shaped mitochondria and fat droplets. They are often surrounded by macrophages, lymphocytes or fibroblasts.

The ballooned cells seen in some acute viral hepatitides are the result of functional impairment of the plasma membrane, leading to increased water content in the hepatocyte cytoplasm (Tanikawa, 1979). Changes in the cell membrane can follow injury to any organelle; they may be reversible or may result in liquifaction necrosis of the hepatocyte. The susceptibility to injury of the plasma membrane, in pathological conditions like hepatitis, may result in large blebs, or organelles free in the tissue spaces.

Mitochondrial swelling is a frequent, nonspecific, reversible phenomenon seen in a wide variety of conditions including fasting, acute hypoxia, viral hepatitis and cholestatis. The fact that normal mitochondria may be seen next to swollen ones, in the same cell, suggests that it is not simply a passive reaction. Abnormal large mitochondria can enclose many dense granules, as well as electron opaque material in parallel layers and in filaments in a regular paracrystalline arrangement - the "myelin-like" degeneration of the organelle (Rouiller and Jézéquel, 1963).

Hypertrophied nucleoli may be noticeable in hepatocytes in various conditions including hepatic regeneration and viral hepatitis (Rouiller and Jézéquel, 1963).

Bile ductules are often proliferated with fibre formation around them, in chronic hepatitis, cirrhosis and obstructive jaundice.

In cholestasis electron microscopy reveals changes in hepatocytes, bile canaliculi, and bile duct epithelial

cells (Tanikawa, 1979). There is hypoactive hypertrophy of hepatocyte SER (enzyme activity reduced) and enlarged mitochondria, occasionally with curled cristae or matrix inclusions. The golgi apparatus near the bile canaliculus is also distended and hypertrophied. Bile accumulates in hepatocytes as fine granular or fibrillar aggregates, with or without a membrane; in severe cholestasis it can occupy most of the cytoplasm of some hepatocytes. However, the most conspicuous morphological alterations in cholestasis occur in the bile canaliculi (Schaff and Lapis, 1979). Dilation of the lumen, with loss or stunting (bleb formation) of microvilli, is common to all clinlical types of cholestasis. Although the canalicular lumen may be empty, it often contains dense bile thrombi which can be finely granular, fibrillar, crystalline, lamellar or whorled. Bile materials may also be seen in, or between bile duct epithelial cells. Whorled, lamellar material occurs in the lysosomes.

In fibrosis the fat-storing cells of Ito, normally found in the space of Disse or between hepatocytes and thought to have fibre-forming potential, are hypertrophied and increased in number in the immature connective tissue surrounding degenerating hepatocytes. Their proliferated and dilated RER suggests active protein production, probably protocollagen (Tanikawa, 1975). But in cirrhotic nodules, with hepatocyte plates three or more cells thick, collagen synthesis also occurs in regions distant from sinusoids or limiting septa. It is thought to be partly due to condensation of the original reticular network, after necrosis and collapse of parenchyma, and partly due to new fibre production from precursors coming, via the maze of intercellular canals, from portal tracts and septa (Johannessen, 1979b). Portal tracts and proliferating portal bile ducts are the main centres of fibre genesis and the ductular proliferation may have an inducer role in fibrosis.

The mature connective tissue septa of cirrhosis consist of many interlaced bundles of collagen fibres and a few fibroblasts; they contain small blood vessels. In the parenchyma, dilation of intercellular spaces is a conspicuous ultrastructural feature of cirrhosis (Johannessen, 1979b) and many microvilli project into them. Capillarization of sinusoids with formation of a basement membrane can be another feature of cirrhosis (Schaffner and Popper, 1963); although this does not occur throughout the lobule, but mainly peripherally near the septa (Johannessen 1979b). This can lead to further injury, by inhibiting the supply of oxygen and nutrients to hepatocytes.

## 1.3 HEPATITIS VIRUSES AND THEIR DISEASES

Of the major causes of liver disease detailed in section 1.2.1 viral infections, resulting in hepatitis and sometimes cirrhosis, are of the most relevance to this study and so will be considered in more detail.

Human viral hepatitis has been more extensively studied than viral hepatitis in animals. The major human hepatitis viruses are hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), delta virus (HDV), and hepatitis E virus (HEV). They will be considered first, then other viruses causing human hepatitis, followed by some causing hepatitis in humans and animals and finally viruses causing hepatitis in dogs will be discussed.

Hepatitis A, hepatitis B and hepatitis C have similar clinical and biochemical features (MacSween, 1985). Early symptoms include nausea, anorexia, fat intolerance; and often retching, vomiting and fever. Arthralgia can occur and occasionally skin rashes. There is often epigastric pain with painful hepatomegaly. Jaundice tends to develop 3-9 days after the prodromal illness and peaks in 10 days, accompanied by pale faeces and dark urine. One third of

cases have palpable splenomegaly. Within 2-6 weeks these features subside but full clinical recovery may take much longer. An initial leucopenia in the pre-icteric stage is followed by lymphocytosis. ALT and AST levels are markedly raised early in the illness but fall rapidly with the onset of jaundice. The best single indicator of the severity of the hepatitis is the prolonged, one-stage prothrombin time. Most patients recover; but in severe cases, death results from fulminant liver failure due to massive hepatic necrosis. When jaundice is deeper and persistent the problem is termed cholestatic jaundice; this usually ends in eventual recovery.

### 1.3.1 Hepatitis A Virus

Hepatitis A (Infectious hepatitis) virus, an RNA picorna virus, is a naked spherical particle of 27-28nm diameter (Siegl, 1988). It has an oro-faecal transmission, being found in faeces and blood early in infection (although parenteral spread is possible). The incubation period is around 15-40 days (MacSween, 1985). An exceptional stability favours its distribution via faecal material and contaminated water and food. It tends to cause a mild disease of low mortality (1% or less) but it often has a high infectivity rate in susceptible populations. Only one serotype has been identified. In areas where it is endemic, infection often occurs in youth so there is a lower attack rate in adults.

There is now strong evidence for the development of cytotoxic T cells, capable of lysing HAV-infected target cells, which may play a role in hepatocyte destruction (Kurane et al., 1985; Vallbracht et al., 1986). HAV has been experimentally transmitted to marmosets and chimpanzees. It can now be propagated in vitro in a variety of cultures, including African green monkey kidney cells, human embryonic fibroblasts, MRC-5 and vero cells. However, its recovery from clinical samples and adaptation

of infectious virus to growth in vitro can still be difficult.

### 1.3.2 Hepatitis B Virus

Hepatitis B (serum hepatitis) virus is a DNA hepadnavirus, the complete infectious virion being the 42nm diameter "Dane particle". Excess coat material is seen as 20nm spherical particles and tubular structures in hepatocyte cytoplasm and in serum. Viral replication occurs in the nucleus, so core antigen (HBcAg) is mainly seen there but it may also be seen in the cytoplasm. Coat material surface antigen (HBsAg), is found in the SER. Hepatitis B virus is mainly spread parenterally but it can also be spread sexually, by intimate contact, oro-faecally and vertically. Any age can be affected and the incubation period is long, 50-180 days. It causes a clinically severe disease with a variable mortality up to 15%. Although most cases recover, with elimination of the virus, a small percentage (about 5%) develop chronic hepatitis then progress to cirrhosis; about another 5% become chronic carriers (MacSween, 1985). There is an association between hepatocellular carcinoma (HCC) and the chronic carrier state. HBV can be transmitted to anthropoid apes causing a less severe hepatitis. It is thought that the principal immune mechanism involved in hepatocyte damage, in acute and chronic HBV infection, is a cytotoxic T cell response to viral antigen on the surface of infected cells (Eddleston, 1988).

The animal hepadnaviruses, which are related to HBV, are discussed in section 1.3.8.

### 1.3.3 Hepatitis C Virus

Until recently non-A, non-B hepatitis (NANB) was a term used to cover what appeared to be at least two different diseases, caused by two entirely different viruses.

Parenteral (transfusion-associated) NANB hepatitis is now recognised as hepatitis C. Enteric (epidemic) non-A, non-B (ENANB) hepatitis is now termed hepatitis E (see section 1.3.5). Although, as Alter (1988) postulated, there may also be other less well defined disease entities in the old NANB group of diseases. The possible existence of another parenteral NANB hepatitis (which might be termed hepatitis F) has been suggested by a proportion of cases which are negative for HCV (Editorial, 1990).

In what was still classified at the time as parenteral NANB hepatitis, a cytoplasmic tubular-forming agent was visualised with the electron microscope. It was found to be less than 80nm in diameter by Bradley et al. (1985); while He et al. (1987) estimated its size to be between 30 and 60nm, by comparative filtration studies. This small, chloroform-sensitive, lipid-encapsulated agent was designated type 1. A nontubular-forming, chloroform-resistant agent was also visualised and designated type 2. In 1988 the biotechnology company Chiron announced it had isolated and partially characterised a virus responsible for a large proportion of blood-borne NANB hepatitis cases. The agent was said to be a single-stranded RNA virus which might be classed as a toga virus (Ezzell, 1988). Later named hepatitis C virus, it is now considered to be an RNA virus which has homology with the flaviviridae (Editorial, 1990).

Hepatitis C can be a significant, sometimes fatal, disease which can also result in chronic active hepatitis (CAH), cirrhosis and apparently hepatocellular carcinoma. Any age group can be affected and the incubation period is between 10-90 days. It can be transmitted experimentally to chimpanzees.

### 1.3.4 Hepatitis D Virus

Delta virus is a defective RNA virus which is dependent on

the helper functions of HBV for its replication. The 36nm particle consists of HBsAg encoating the RNA genome and a specific delta antigen (HDAg) which is a structural protein. Transmission modes are similar to HBV, except that vertical transmission is rare. Coinfection with HBV is commonest and has a low chronicity rate, about 2% (Caredda et al., 1987). Superinfection of HBV carriers, with HDV, has a high hepatitis D chronicity rate of 70-90% (De Cock et al., 1986; Caredda et al., 1987). HDV infection results in a more severe disease, than that due to HBV alone (although it may cause early repression of HBV), with more lobular inflammation and eosinophilic changes in hepatocytes resembling NANB hepatitis (Verme et al., 1986). Damage is thought to be due to cytotoxic cell effects (Popper, 1984).

### 1.3.5 Hepatitis E Virus

Hepatitis E was until recently called enteric (epidemic) non-A, non-B hepatitis. Probably an old disease, only recently discovered, it is important in the developing world, where sanitation is poor. Using immune electron microscopy, 27-30nm particles were found in acute phase faecal samples (Balayan et al., 1983) before the putative aetiological agent was classified as a possible calici virus (Bradley and Balayan, 1988), named, and the RNA genome cloned (Keyes et al., 1990). Serologic tests for hepatitis E now exist, but in practice diagnosis can be made by serologic exclusion of HAV and HBV, and demonstration of the epidemiologic characteristics of hepatitis E (high attack rate in adults and high mortality rate in pregnant women). Unlike hepatitis C, with its high chronicity rate, hepatitis E appears to be a self-limiting disease. There is focal necrosis with little inflammation and no lobular predilection. The modest inflammatory infiltrate consists of Kupffer cells and polymorphs with few lymphocytes. Cellular and lobular cholestasis are seen; also hepatocyte ballooning, focal cytolytic necrosis

with acidophilic body formation, and pseudoglandular alteration of hepatocyte plates.

### 1.3.6 Other Viruses Causing Hepatitis in Humans

Although hepatitis A, B, C, D and E account for the largest proportion of human liver diseases in the world, there are other viral agents which can infect the liver and cause hepatitis.

Hepatitis has been reported in up to 80% of measles infections in adults (Gavish et al., 1983). Philips et al., (1991) recently described a viral particle associated with and possibly the cause of syncytial giant-cell hepatitis. The ultrastructural appearance of the particle was suggestive of a paramyxovirus. Although measles could not be definitely excluded as the causal agent, staining for measles antibodies proved negative and the onset and clinical course of the disease were different from measles, suggesting that another paramyxovirus was the cause. It has been suggested that the disease might even become known as hepatitis G (Editorial, 1991).

Hepatitis can be an unusual complication in rubella or herpes simplex infections in children. Infectious mononucleosis, due to Epstein-Barr virus, can involve hepatitis, and cytomegalovirus hepatitis also occurs.

Hepatitis is seen in yellow fever; a disease due to a group B arbovirus, transmitted from monkeys to man by mosquitoes. Its severity can range from a mild, unsuspected, febrile illness to a fatal condition. Jaundice and a haemorrhagic tendancy are features of yellow fever. Histological lesions include midzonal necrosis with fatty infiltration, acidophilic necrosis with Councilman body formation, and proximal tubular necrosis in the kidneys.

Some of the arenaviruses usually found in animals can also

cause human disease (including hepatitis). The arenavirus group is discussed in more detail in section 1.3.7, because it was considered possible that the speculative agent of canine acidophil cell hepatitis might be an arenavirus.

### 1.3.7 Arenaviruses

Arenaviruses are round, oval or pleomorphic, enveloped, single-stranded RNA viruses. They range in size from 50-300nm (Pedersen, 1970) or 60-350nm (Casals, 1975) in diameter, with a mean size of 110-130nm (Lehmann-Grube, 1984; Pedersen, 1979; Rawls and Leung, 1979). The group contains some disease agents of animals (most can cause acute or persistent infections in rodents (Howard and Simpson, 1980)), but also the aetiological agents of some of the rare, exotic human diseases, like Lassa fever and severe haemorrhagic diseases. Arenaviruses have a unique restriction to a limited number of rodent species in nature; there is no evidence of arthropod transmission with any of them. The high degree of specificity may be because they are only capable of producing persistent infection in the appropriate host and in nature the virus is maintained by persistently infected animals. The age at infection is the major factor affecting persistence (Rawls and Leung, 1979). Congenital and neonatal infections frequently become chronic, while those aquired in adult life do not usually result in continued virus excretion.

The family is often divided into two groups on geographical occurrence: Old World and New World arenaviruses. Lymphocytic choriomeningitis (LCM), Lassa, Mopeia and Mobala viruses belong to the former group; although LCM virus occurs in America as well as Europe. The New World arenaviruses are all considered to be members of the Tacaribe complex and include Junin, Tacaribe, Machupo, Ampari, Tamiami, Parana, Latino, Pichinde and Flexal viruses. The arenavirus family share certain antigenic

components, although the degree of cross reactivity varies with different assay systems. Lassa, Mopeia and Mobala viruses are more serologically related to LCM virus than to the New World viruses. Tacaribe virus is serologically related to Junin virus and all the other New World viruses, to varying degrees.

LYMPHOCYTIC CHORIOMENINGITIS VIRUS (Armstrong and Lillie, 1934), is a human pathogen with a natural reservoir in common house mice. It is the only arenavirus pathogen known to persistently infect peridomestic rodents in North America and Western Europe. Human infections are not common; they can result from either natural exposure to LCM virus shed by infected rodents (including laboratory-bred and pet hamsters) or laboratory accidents. Although in humans the virus exhibits a neurotropism, it most commonly produces a subclinical infection or a mild, febrile, influenza-like (non-meningeal) illness which may go unrecognised. It can, however, produce aseptic meningitis or meningoencephalomyelitis (Oldstone and Peters, 1978).

LCM virus infection of adult mice results in a severe immune-mediated disease; whereas infection of immunologically immature animals results in carriers, which may remain healthy or later develop chronic illness (Lehmann-Grube, 1984). The infection in adult experimental mice is an example of a virus-induced immunopathological disease and immunosuppression can protect adults against fatal LCM infection. Intracerebral inoculation of adults or intraperitoneal inoculation with vicerotropic strains can induce the severe lethal disease, with myelitis and generalized lymphocytic infiltration of all the major organs (Howard, 1986 pp71-72). Liver involvement can result in severe hepatitis with necrotic foci. In contrast, subcutaneous or intranasal inoculation results in only slight disease with recovery to complete immunity.

The virus can be transmitted experimentally to both adult and neonate Golden Syrian hamsters and in the latter, chronic viraemia and viruria may persist a long time (Parker et al., 1976). Outbred guinea pigs have a variable response to LCM virus and most viral strains do not cause overt disease, although at least one is fatal. Monkeys have also been infected experimentally with LCM virus (Armstrong and Lillie, 1934).

LASSA VIRUS (Frame et al., 1970), found in only one type of mouse in West Africa, is also a human pathogen. Clinically Lassa fever in humans is notable for: liver involvement, pyrexia for longer than a week, pharyngitis, malaise, leucopenia unresponsive to antibiotic or antimalarial drugs, and a high mortality rate - although infection in children often follows an inapparent or milder course (Howard, 1986 p52). Other features include: abnormal bleeding, swelling of the face or neck, pleural effusion or ascites, rashes, and death on or after the 10th day of illness. Minor criteria can be: headaches, myalgia, abdominal or chest pain, vomiting, a cough, and lymphadenopathy. Lassa virus is hepatotropic in man, unlike the neurotropic LCM virus and haemorrhagic fever viruses. The notable liver involvement results in elevated serum transaminase levels in acute infections. Microscopy reveals severe liver cell damage (Winn et al., 1975) with individual hepatocytes showing particular cytoplasmic pathology, often progressing to almost complete destruction of the plasma membrane and cytoplasmic structures. Less severely damaged cells show vacuolation and pigment accumulation resulting in the formation of Councilman-like bodies (Edington and White, 1972). Some lipid accumulation may be seen. Nuclear changes are less marked, although nucleoli can be very prominent. Areas of focal degeneration are occasionally seen, but hepatocellular damage is mainly centrilobular with bridging necrosis. However, inflammation is minimal. In the areas of greatest cell damage, the few cells present are mainly monocytes and a

few polymorphs at the periphery and along sinusoids, although enlarged Kupffer cells are prominent. Another notable feature, also seen at the ultrastructural level, is that severely damaged hepatocytes may be adjacent to cells showing little or negligible signs of infection. Splenic necrosis occurs in the marginal zone of the periarteriolar lymphocytes and mononuclear cells (Walker et al., 1982b). In the adrenals, multifocal necrosis in the zona fasciculata is often accompanied by focal inflammation. Prominent acidophilic, 3-15μm cytoplasmic inclusions can occur in the adrenocortical cells at the junction of the zona reticularis and medulla. Electron microscopy may reveal virus particles in perisinusoidal and extracellular spaces and in bile canaliculi. Maturing virions may be seen at the surface of infected hepatocytes. Ultrastructural changes associated with viral replication are: chromatin margination, increased nucleolar density, dilation and disruption of RER (the hepatocyte's first response to any injury) with ribosome detachment and formation of small vesicles. Extreme cellular swelling (ballooned cells) is not a feature of Lassa hepatitis.

Lassa virus transmitted experimentally to squirrel monkeys, by intramuscular (IM) injection (Walker et al., 1975), exhibits a pantropism for the major organs, especially the liver, kidney and lymph nodes. There is a close correlation between the severity of the disease and the extent of virus replication. A marked hepatotropism is seen in experimentally-infected rhesus monkeys and clinical signs, similar to those seen in human Lassa fever, are produced (Jahrling et al., 1980). Early lethargy and pyrexia are followed by conjunctivitis, a skin rash and later death in some animals. Symptoms generally develop later in animals that survive; their pyrexia is intermittent and of variable duration. Hepatocellular necrosis is a consistent feature of acute Lassa virus infection in primates, in which the liver frequently contains the greatest amount of virus. Although in rhesus monkeys

viraemia coincides with elevated serum transaminase levels, indicative of hepatic damage (Callis et al., 1982), only a small proportion (no more than 10%) of hepatocytes appear to be affected (Walker et al., 1982a). Randomly distributed, multifocal necrotic foci are accompanied by only a slight mononuclear cell infiltration. The acidophilic cytoplasm of randomly distributed, individual hepatocytes contains a lot of viral antigen (Jahrling et al., 1980; Walker et al., 1982a). There is an increase in mononuclear cells in the spleen and areas of lymphocytic depletion in lymph nodes. Kidneys, adrenal glands and lungs are also often affected histologically. The parallel appearance of virus and serum transaminases in the circulation and the minimal inflammatory response suggest Lassa virus may be directly cytopathic.

Walker et al. (1975) found infection of the natural rodent host (Mastomys natalensis), at any age, to result in an asymptomatic infection. Histopathology of neonatally-infected animals is unremarkable, although immunofluorescence reveals the presence of virus in the liver and other organs. Meningoencephalitis is the only histological lesion found in infected adults, although virus is found in several organs (especially kidneys and lymph nodes). Some adults remain persistently infected and continue to shed Lassa virus in their urine. Neonatal outbred white mice are susceptible to intracranial inoculation of Lassa virus (Buckley and Casals, 1970). The outcome of experimental infection of guinea pigs is dependent of the strain of animals used. Jahrling et al. (1982) found a low dose to be fatal in some inbred strains, while outbred (Hartley strain) animals survived despite inapparent infections in many of them. Although the virus replicates in many organs, including the liver, spleen, kidneys, lymph nodes and especially the lungs, the extent does not correspond to the, only mild, degree of tissue damage seen in them. Lassa virus shows a similar tissue tropism in all susceptible host species. However, in the guinea pig lesions are

substantially less severe and the liver is not a major site of virus activity. Therefore although the species is useful for primary isolation of the virus from clinical specimens, it is less suitable for detailed pathogenesis studies of Lassa fever.

MOPEIA VIRUS (Wulff et al., 1977), found in Southeast Africa in the same large mouse as Lassa virus, does not cause significant human disease.

MOBALA VIRUS (Gonzalez et al., 1983) is found in a different rodent in Central Africa and does not cause significant human disease.

JUNIN VIRUS (Parodi et al., 1958), the cause of Argentinian haemorrhagic fever, is found in three types of rodents which do not frequent human dwellings. Human infection is therefore seen predominantly in agricultural workers. The pathological features (Elsner et al., 1973) are general haemorrhage, myocarditis, renal damage and focal hepatic necrosis with acidophilic bodies (areas of degenerated cytoplasmic membranes) similar to those found in yellow fever. Kupffer cells are hypertrophied and there is evidence of erythrophagocytosis. Monkeys have been infected experimentally, but Junin virus is only pathogenic in some species.

Experimental infection of guinea pigs with Junin virus results in weight loss, pyrexia, a reduction in the white blood cell (wbc) count and increased acid and alkaline phosphatase levels (Kierzembaum et al., 1970). Virulent strains of Junin virus are immunosuppressive in guinea pigs. However, when guinea pigs are immunosuppressed pharmacologically, a normally avirulent strain induces severe illness (Kenyon et al., 1985). Hamsters are susceptible to experimental infection with Junin virus, it causes extensive neuropathology but only a limited degree of hepatitis without elevated transaminase levels. Wistar

rats are also susceptible to Junin virus, the outcome of infection is dependent on the viral strain and the age of the animals at inoculation (Avila et al., 1981). Animals under 2 days of age tend to be resistant to a strain pathogenic for humans, susceptibility increases from 8-12 days, then declines rapidly so that adults are resistant. Susceptibility to a nonpathogenic strain is greater from birth to day 12.

TACARIBE VIRUS (Downs et al., 1963), found in bats (and some mosquitoes) in Trinidad, is the only one isolated from a non-rodent species and is not a human pathogen. Tacaribe virus is uniformly lethal to neonatal mice, although adults survive (Borden and Nathanson, 1974). This is in direct contrast to LCM virus, although both cause immune-mediated diseases. All members of the Tacaribe complex normally produce lethal infections in newborn hamsters (Howard, 1986 p97); the susceptibility of adults is strain-dependent.

MACHUPO VIRUS (Johnson et al., 1966) is transmitted from one type of mouse, via urine contamination of soil and food, to humans causing Bolivian haemorrhagic fever. Although extensive haemorrhage and congestion are seen in postmortem cases, Child et al. (1967) found the most prominent histological changes to be in the liver. There is an increase in the number and size of Kupffer cells with erythrophagocytosis, increased eosinophilic staining of hepatic cells, lymphocytic infiltration in periportal regions only and frequent mononuclear cells in sinusoids. Although focal necrosis is occasionally seen, hepatic architecture is usually preserved. Acidophilic bodies, 10-20μm in diameter, are seen in sinusoids and sometimes within Kupffer cells.

Webb et al. (1967) found marmosets to be susceptible to Machupo virus by subcutaneous (SC) inoculation. Virus passaged through hamsters, mice or cell culture can cause

infection. Other types of monkeys can also be infected and hepatic necrosis is a consistent feature in primates. Necrosis of the skin, gastrointestinal tract and adrenal glands can also be seen (Terrell et al., 1973).

Experimental infection of the natural host after 9 days of age, results in a split response; half become immune, half develop persistent viraemia. Inoculation of neonates invariably results in a chronic infection. It has also been transmitted experimentally to newborn hamsters which appear to be more sensitive to machupo virus than newborn mice.

AMPARI VIRUS (Pinheiro et al., 1966), isolated from rodents and mites in Brazil, is not a human pathogen. It has been transmitted experimentally to suckling mice.

TAMIAMI VIRUS (Calisher et al., 1970) is found in Florida marshlands but only in the cotton rat. Apart from LCM virus, introduced by man, it is the only arenavirus found to be indigenous to the North American rodent fauna. Isolation of Tamiami virus in suckling mice is frequently enhanced by dilution of tissue extracts, suggesting a high proportion of noninfectious, interfering particles in the organs of persistently infected animals. There is no evidence that Tamiami virus is a human pathogen. In experimental Tamiami infections, in the natural host, immunofluorescence reveals viral antigen in many organs; although no indication of viral cytopathology is detectable with light microscopy. In the liver the architecture is preserved and Murphy et al. (1976) never found more than 30% of hepatocytes to be infected.

PARANA VIRUS (Webb et al., 1970), isolated in Paraguay, is not a human pathogen.

LATINO VIRUS (Webb et al., 1973), isolated in Bolivia from the same rodent host as Machupo virus, is apparently not a

human pathogen. In contrast to all the other arenaviruses, it appears not to be transmissible to laboratory mice.

PICHINDE VIRUS (Trapido and Sanmartin, 1971), isolated from rodents and some ectoparasites in Columbia, may be able to cause asymptomatic human infection in the laboratory setting (Buchmeier et al., 1974).

The virus can be isolated experimentally in infant mice, hamsters and vero cell cultures. Jahrling et al. (1981) adapted Pichinde virus to guinea pigs as an alternative model for Lassa virus infection. They found a difference between passage in an inbred strain and outbred animals: infection was lethal by the eighth passage in the former, but still inapparent in the latter. Infected inbred animals develop severe leucopenia and raised transaminase levels just prior to death. Moderate to severe hepatocellular necrosis is seen in the livers of moribund animals, but minimal inflammatory infiltrate.

FLEXAL VIRUS (Pinheiro et al., 1977), isolated in Brazil, has only one rodent host species and is not a human pathogen.

### 1.3.8 Animal Hepadnaviruses

Several animal hepadnaviruses, related to human HBV, have been identified. They tend to be named after the animal from which they were first isolated, but some have been found in more than one species.

WOODCHUCK HEPATITIS VIRUS (WHV) found in America by Summers et al. (1978), has also been found in some chinese marmots (Kai et al., 1988).

GROUND SQUIRREL HEPATITIS VIRUS (GSHV) was isolated from Beechey ground squirrels in America (Marion et al., 1980).

DUCK HEPATITIS B VIRUS (DHBV) has been found in domestic Pekin ducks in America (Mason et al., 1980), China, Europe and Australia; as well as in wild mallards in France (Cova et al., 1986) and America (Lambert et al., 1988). It has been experimentally transmitted to domestic geese (Newbold and Cullen, 1988).

Similar particles have been seen in serum from tree squirrels (Feitelson et al., 1986), herons (Will et al., 1987) and snakes (Yang et al., 1984). These are less well characterised, but it may be that hepadnaviruses also infect these species.

Hepadnaviruses share a unique virion ultrastructure, and common genome size, structure and replication mechanism. The family can cause acute and chronic hepatitis and sometimes immune complex-mediated disease and HCC. They have a strong tropism for hepatocytes and can all commonly cause persistent infection, with complete and incomplete viral forms in blood and other body fluids for years. Although DNA viruses, they appear to utilise a reverse transcriptase step in genome replication, with an RNA intermediate copy of the genome (Summers and Mason, 1982). The family appears to be phylogenetically related to retroviruses (Robinson and Marion, 1988). There was no infected cell culture system available until Tuttleman et al. (1986) reported replication of DHBV in duck hepatocyte culture and it can be grown in mixed yolk sac cell culture (O'Connell and London, 1988). Coculture of infected duck hepatocytes with rat liver epithelial cells has been used to maintain the differentiated functions of infected hepatocytes and to help them survive longer (Fourel et al., 1988). Thēzē et al. (1987) maintained WHV in woodchuck hepatocyte primary culture.

### 1.3.9 Canine Adenovirus

Canine adenovirus type 1 (CAV-1) is a DNA virus, with only

one serotype defined by neutralisation assays. Adenoviruses range from 70 to 90nm in diameter. Canine adenovirus 1 is relatively heat-labile but acid-stable and can survive 3-11 days on solid fomites. It is highly contagious and is transmitted by direct contact (with saliva, respiratory tract secretions, urine or faeces) or by contact with contaminated items. The usual incubation period is 2-5 days. One factor in the spread of CAV-1 is that it can be excreted in the urine of recovered animals for as long as 6 months after infection (Poppensiek and Baker, 1951).

Canine adenovirus 1 is associated with systemic infection, characterized by damage to liver, lymphatic tissue and vascular endothelium, resulting in a clinical syndrome of varying severity termed infectious canine hepatitis (ICH). The disease was first described, in dogs, by Rubarth (1947), hence its old name of Rubarth's disease. More recently CAV-1 has been isolated from dogs with respiratory disease. Clinical signs appear to depend, to some extent, on the route of exposure to the virus (Wright et al., 1971). The oropharynx is a common entry point for natural infection; spread to regional lymph nodes is followed by viraemia and dissemination to, for example liver, kidney, lymph nodes and vascular endothelium with resultant clinical signs (Wright, 1973). Although there can be a high incidence of infection in unvaccinated populations, most cases are subclinical and inapparent. A mild case might exhibit anorexia, depression or fever; while more severe cases can show general depression, anorexia, vomiting, abdominal pain, tonsillar enlargement and occasionally generalized lymphadenopathy. A notable feature is the temperature, which tends to be higher than in most other infections, even approaching 41°C (Farrow and Love, 1983). Jaundice is rare in uncomplicated systemic CAV-1 infection. The most severe cases collapse and die after a short illness of 12-24 hours, with severe abdominal pain, marked hyperthermia, and occasionally haematemesis and dysentry. Laboratory findings early in

the disease include albuminuria and bilirubinuria, due to kidney and liver damage. If hepatic damage is sufficient, alkaline phosphatase, ALT and AST levels will be raised. Leucopenia in the febrile phase, increased red blood cell (rbc) sedimentation rate and prolonged clotting are additional features. Diagnosis can be made on demonstration of virus in the blood or in biopsy material, by fluorescence or isolation in cell culture. Detection of a rising antibody titre during recovery gives further confirmation of diagnosis. During recovery some dogs develop interstitial keratitis and corneal oedema, resulting in the characteristic "blue eye".

Postmortem findings in the fatal case include: bloody fluid (even pure blood) in the abdomen; an enlarged, mottled liver with rounded edges; marked gall bladder oedema; gastrointestinal haemorrhage; often congested kidneys; and frequently enlarged, haemorrhagic lymph nodes. Microscopy of the liver reveals extensive hepatocellular necrosis and haemorrhage, with large basophilic intranuclear inclusion bodies. The latter can also be found in kidney glomeruli and consist of viral particles in an amorphous matrix (Rakich et al., 1986). In less acute cases inclusions may occur in renal tubular epithelial cells, with focal interstitial nephritis. CAV-1 may have a role in the aetiology of chronic progressive hepatitis following infection of partially immune dogs (Gocke et al., 1970; see section 1.5.5). Various vaccines against CAV-1 are in common usage in dogs.

Wolves and foxes are susceptible to this virus and it has been isolated from skunks (Karstad et al., 1975).

### 1.3.10 Canine Herpesvirus

The herpesviruses are DNA viruses, which range from 120 to 150nm in diameter. Canine herpesvirus 1 (CHV-1) can be transmitted to pups from the bitch in utero or during

birth, by contact with infected littermates, or by inhalation or ingestion of infected material. Older dogs can be asymptomatic carriers of CHV-1. In pups more than 2 weeks old, exposure to the virus usually results in only an inapparent infection with virus remaining localized in the nasal cavity and oropharynx. However CHV is a cause of neonatal mortality in younger pups, due to their lower body temperature (Carmichael et al., 1969). In such animals there is a leucocyte-associated viraemia with secondary viral replication throughout the body, leading to disseminated focal necrosis, haemorrhages and death 5-12 days after infection (Carmichael, 1970). Histology reveals lesions, in all major organs, of necrotizing vasculitis with extension into surrounding parenchymal tissue (Cornwell and Wright, 1969). Eosinophilic intranuclear inclusion bodies occur especially in infected respiratory tract, liver and kidney cells, but they may be difficult to find.

### 1.3.11 Canine Acidophil Cell Hepatitis Agent

The aetiological agent of canine acidophil cell hepatitis (CACH) is believed to be a virus. It has been transmitted experimentally to dogs, by parenteral inoculation, with serum and/or homogenised liver from infected field cases (Jarrett and O'Neil, 1985). Material from both acute and chronic cases was found to be infective and second passages in experimental dogs were achieved by Professor Jarrett. His studies demonstrated the transmissibility of CACH and its apparently episodic nature with persistence of infectivity for most of the course.

Both natural and experimental infection can result in acute hepatitis and cytolytic necrosis, severe enough to kill; or a persistent infection which can develop into chronic hepatitis, hepatic fibrosis and cirrhosis. Clinical signs exhibited by CACH cases are not usually diagnostic, being typical of liver disease. The agent

appears to result in the death of cells, particularly in the limiting plate, giving rise to the characteristic distribution of acidophilic necrobiosis round portal tracts. The acidophil cells are usually shrunken with varying degrees of cytoplasmic acidophilia and often concave or angular surfaces. Their nuclei are often shrunken and condensed (see e.g. figures 4-50 and 4-137). In severe cases the acidophil cell pathology extends out from portal tracts to form bridging lesions (see e.g. figures 3-5 and 4-1). Where fibrosis is present it usually follows the distribution of acidophil cells, extending out from portal tracts, and piecemeal necrosis is a common feature. Infiltration by inflammatory cells is not a particular feature of the disease and is often minimal.

Since the experimentally-induced hepatitis appears able to persist in outwardly healthy looking dogs, CACH may occur as an unnoticed subclinical entity in the field.

### 1.3.12 Other Unidentified Agents

Tiong and Smirk (1978) reported finding viral particles in an electron microscopic study of a haematoxylin and eosin (H&E) stained section of dog liver which exhibited acidophilic crystalline inclusion bodies in both the nucleus and cytoplasm of hepatocytes. Numerous electron-dense particles (thought to be viral central cores) and a few coated viral particles were seen in enlarged nuclei. Coated particles were seen, in clumps, in the cytoplasm. Intact viral particles (65-70nm diameter), in both sites, exhibited hexagonal central cores (20-30nm) surrounded by 19nm thick capsids and many were trapped in the inclusions. It was suggested that the formation of inclusions was an intracellular immune process for the exclusion of parasitized virus of low cytocidal activity in a possibly new viral disease of dogs.

## 1.4 ACUTE HEPATITIS

Acute hepatitis can result in acute hepatic failure in dogs, although acute widespread hepatic necrosis causes it more consistently (Sherding, 1985). Of the many causes of acute hepatic disease in dogs the only well-recognised viral ones are CAV-1 and CHV. Although fulminant ICH, caused by CAV-1, can result in acute hepatic failure, it is now uncommon due to the widespread use of vaccines.

### 1.4.1 Clinical and Biochemical Features

The clinical findings in acute hepatic failure tend to be vague and nonspecific, reflecting general liver dysfunction rather than a specific diagnosis and many are not even specific for liver disease (Sherding, 1985). They include acute onset of anorexia, depression, vomiting and diarrhoea, as well as polyuria and polydipsia. Hepatic encephalopathy signs may be seen in severe cases; as may hyperbilirubinaemia and bilirubinuria, leading to jaundice and pigmented urine. Haemostasis abnormalities are less common but can result in gastrointestinal bleeding (with haematemesis or melaena), haematuria or cutaneous haemorrhage.

In severe acute hepatocellular injury or necrosis there is a substantial elevation of serum ALT levels. The less liver-specific AST tends to parallel ALT in acute hepatic failure. Serum alkaline phosphatase is usually also increased in acute hepatic injury where there is cholestasis. Other possible findings in severe hepatic failure include hyperbilirubinaemia, bilirubinuria, hypoglycaemia, abnormal BSP retention, hypokalaemia, respiratory and metabolic acidosis, reduced serum albumin and cholesterol.

As well as the clinical features of acute hepatic failure, common findings in acute ICH are fever, leucopenia, proteinuria and generalized bleeding diathesis.

### 1.4.2 Histopathology

The fulminating form of ICH is characterised by acute hepatic necrosis, widespread vasculitis and severe disseminated intravascular coagulation (DIC).

In classical acute hepatitis in humans there is diffuse involvement of the liver, usually worst in perivenular areas (acinar zone 3), with scattered degenerating hepatocytes and a mononuclear inflammatory infiltrate in portal tracts and in the parenchyma, intimate to the necrosis. The infiltrate consists of lymphocytes with very occasional plasma cells and a few polymorphs. Varying degrees of hepatocyte regeneration are seen and reactive hyperplasia of Kupffer cells contributes to the characteristic marked increase in cellularity. Both ballooning degeneration (enlarged cells with granular cytoplasm condensing around the nucleus) and acidophilic degeneration (shrunken cells with increased cytoplasmic eosinophilia and pyknosis) are seen. In the latter type, eventual nuclear extrusion can leave the acidophilic Councilman body. Intracytoplasmic bile pigment granules and occasional bile thrombi are seen.

The nonspecific ultrastructural changes include: irregular swelling of endoplasmic reticulum forming vesicles, ribosome detachment, and enlarged phagosomes containing altered mitochondria and debris.

Necrosis and lysis disrupt the hepatocyte plates but leave the reticlulum framework intact. Hepatocellular hypertrophy and hyperplasia occur, with the formation of bi- and multinucleate cells. Before clinical subsidence of an attack there is a reduction in necrosis and an increase in phagocytosis by Kupffer cells.

When confluent necrosis occurs (especially in acinar zone 3) it results in more extensive loss of hepatocytes

(bridging necrosis), leaving a loose meshwork or septum infiltrated by lymphocytes and macrophages. An unusual complication of viral hepatitis can be massive (panacinar) necrosis, causing death when extensive enough. Surviving cases can develop multinodular hyperplasia with postnecrotic scarring between nodules.

## 1.5 CHRONIC HEPATITIS

The classification of chronic hepatitis is rather more specific in humans than in dogs and some of the divisions identified in humans may not occur in dogs. The situation in humans is discussed first, since it may prove useful in the understanding of chronic hepatitis in dogs.

### 1.5.1 Human Classification

The term chronic hepatitis is used in humans to describe a group of liver diseases characterised by a clinically apparent necroinflammatory process of at least six months duration. It can be considered as two types: chronic persistent hepatitis (CPH), in which inflammation is confined to the portal tracts, and chronic active hepatitis (CAH), a more aggressive disease with portal and parenchymal involvement, in which progressive fibrosis leads to cirrhosis.

CHRONIC PERSISTENT HEPATITIS is a benign disorder, following subclinical or clinical acute viral hepatitis, or drug-induced hepatitis. It is usually asymptomatic and detected by biochemical screening; or patients may exhibit mild symptoms including fatigue, malaise, vague upper abdominal pain, sometimes dietary fat intolerance and possibly a tender, slightly enlarged liver. Serum aminotransferase levels are moderately raised. Biopsy is used to differentiate it from CAH. The mononuclear cell infiltrate is primarily confined to the portal tracts. There is

minimal hepatocellular necrosis, so lobular architecture is preserved with intact limiting plates. There is little or no increase in fibrosis and piecemeal necrosis is absent (or very mild). This disease does not progress to cirrhosis, although mild hepatocellular inflammation may persist for years before spontaneous resolution. There is no need for therapy, although follow-up biopsies may be used to differentiate the process from early CAH if there is any confusion.

CHRONIC ACTIVE HEPATITIS is an obvious clinical illness with biochemical abnormalities (a 5-10 x times increase in aminotransferase levels) for more than 6 months. It is a continuing progressive inflammation, with liver cell degeneration and necrosis accompanied by fibrosis. A proportion of cases progress to cirrhosis. A variety of aetiological factors can lead to CAH including: hepatitis B and C viruses, drugs, and autoimmune, cryptogenic or idiopathic problems. Autoimmune reactions against hepatocytes are involved in the pathogenesis of CAH due to any of these factors and there may be a degree of genetic predisposition (Hardy, 1985). The binding of drugs, drug metabolites or viruses to the cell surface results in antibodies and sensitised lymphocytes being directed against the altered hepatocyte membranes, eventually leading to death of the cell. The release of hepatocyte-specific antigens initiates additional immunological reactions leading to complement-mediated cytotoxic reactions which may be self-perpetuating. Although immunological injury is critical early in the disease, it may become less important in late phases, when vascular factors or changes in collagen formation probably become dominant factors (Hardy, 1985).

The onset of CAH can be acute or insidious with nonspecific symptoms of anorexia, tiredness, vague upper abdominal pain, hepatomegaly and often splenomegaly. The disease fluctuates in severity. Other features include: arthralg-

ia, skin rashes, pleural effusions, thrombocytopenia, leucopenia, and proteinuria (due to glomerular lesions). Chronic inflammatory bowel disease, chronic thyroiditis, Sjøgren's syndrome and autoimmune problems are also seen occasionally. Serum aminotransferase levels are raised and sometimes jaundice is present, usually with a moderately raised AP level. Immunoglobulin (Ig) G hyperglobulinaemia occurs, with a later hypoalbuminaemia and an increased prothrombin time.

In CAH portal inflammation extends into the parenchyma and progresses to piecemeal necrosis and periportal fibrosis. It frequently progresses to macronodular cirrhosis, hepatic insufficiency and death. Chronic active hepatitis can be divided into two types, really degrees of severity, which can both end in cirrhosis. The first type is CAH with predominately portal and periportal inflammation and piecemeal necrosis (destruction of hepatocytes at the interface of parenchyma and connective tissue). The infiltrate is mainly lymphocytes and plasma cells, but a variable number of neutrophils can be present. The expansion of the inflammation, out from portal tracts, destroys the limiting plate and cells infiltrate between hepatocytes showing feathery degeneration (swelling and cytoplasmic vacuolation) with eventual necrosis. There can also be: fatty change, bile duct proliferation, bile stasis and pseudoglandular rosette pattern formation by some regenerating liver cells surrounded by fibrous tissue. Figure 3-6 shows an example of rosette formation in a CACH field case. Eventually one can get fibrous septa linking portal tracts, lobular collapse and ultimately cirrhosis. The other type is CAH with bridging necrosis, in which bands of inflammatory cells and necrotic hepatocytes extend between adjacent portal tracts, and between portal tracts and central veins as the destruction progresses. This carries a more serious prognosis and often progresses to cirrhosis and death; although as stated earlier, both types of CAH can result in macronod-

ular cirrhosis. In active cirrhosis, fibrosis replaces the inflammatory cells in areas of bridging necrosis. Widespread regenerating nodules of hepatocytes cause marked architectural distortion. This can be seen grossly, the smooth liver surface becoming nodular.

In summary, a definitive diagnosis of chronic active hepatitis in humans requires clinical data of active hepatic disease for 6 months or more, multiple immunological abnormalities and biopsy confirmation.

### 1.5.2 Canine Classification

In dogs the designation of liver disease as chronic can be based on: history (often unreliable), repetitive biochemical evaluations (not usually obtained), or histopathological findings supporting chronicity (fibrosis) (Hardy, 1985). Designation of the disease as active requires evidence of continuing inflammation and/or necrosis; this is most easily determined by aminotransferase levels (see section 1.2.3).

Even in humans, the histopathologic changes in CAH are not pathognomonic and similarly in dogs care must be taken in diagnosis. There has been much debate, in human medicine, about whether the term chronic active hepatitis should be reserved for the multisystems disease which may progress to cirrhosis and which may be viral (HBV mainly) in origin (Whitcomb, 1979); or whether it should be regarded as describing a group of pathological changes with a variety of aetiologies, rather than one specific disease entity (Scheuer, 1977). Similar debate has taken place in canine medicine. Thornburg (1982) came to the conclusion that, there was a lack of evidence to suggest a disease, similar to CAH of man, occurred in the dog. He gave five criteria to be satisfied before designating a canine disease as CAH:

1) a duration of at least 6 months

2) persistently raised ALT levels throughout the disease
3) hypergammaglobulinaemia
4) relentless hepatocellular destruction with inflammation (piecemeal necrosis) in sequential liver biopsies
5) enhanced in vitro cytotoxicity toward canine hepatocytes by the patient's lymphocytes.

He felt that few of the published reports of CAH-like disease in dogs met the above criteria. Thornburg advocated that in dogs, as in man, the term CAH should be restricted to an immune-mediated disease directed against hepatocytes. However in this thesis, as in many of the reports discussed in this chapter, chronic active hepatitis is used, in dogs, as a very useful descriptive term for liver diseases exhibiting the typical histopathological picture, even if not strictly fulfilling all of Thornburg's criteria; rather than restricting its use to a canine version of the specific human entity, which has not yet been proved to exist in dogs.

### 1.5.3 Clinical and Biochemical Features

Nonspecific clinical signs of depression and anorexia are frequently seen; while jaundice, ascites, polyuria and polydipsia are the most consistent signs of liver disease. Periportal inflammation results in jaundice, more readily than that in other parts of the lobule (where more massive necrosis is required), because it obstructs bile flow in the bile ductules entering the portal tract. Ammonia toxicosis may contribute to the depression although there is no direct correlation in severity (Fischer and Baldessarini, 1976; Schenker et al., 1974). Signs of hepatic encephalopathy may be seen in severely affected cases; these and the factors contributing to hepatic encephalopathy are discussed in section 1.2.2. Polydipsia and polyuria may be contributed to by reduced metabolism and excretion of hormones like adrenal corticosteroids (section 1.2.2). The insidious nature of CAH contributes to its poor prognosis, since cases are often not presented

until late in the course of the disease, when the pathological process is already well developed.

No definite aminotransferase ranges have been established for chronic hepatitis in the dog; Strombeck and Gribble, (1978) suggested ALT levels averaged fifteen times normal in canine chronic active hepatitis, but cases of CAH with normal ALT levels have been seen. In man five to ten times normal ALT levels are considered compatible with CAH (section 1.5.1). A normal ALT level does not rule out the possibility of CAH (Strombeck and Gribble, 1978) because of ALT's short half-life (section 1.2.3): it can fall before morphological evidence of necrosis disappears. Although serum biochemistry results cannot be specific for CAH, raised ALT and alkaline phosphatase levels concurrent with increased BSP retention and a low albumin concentration are suggestive of progressive hepatic disease (see section 1.2.3). Ammonia tolerance test results may also be abnormal (section 1.2.3).

There is a lack of supportive data for an immune component in hepatic injury in dogs (Hardy, 1985), as very little measurement of antibodies is done. Although elevated levels of IgG have been noted in some cases of CAH, this is a nonspecific feature of many canine inflammatory liver diseases. Kupffer cells have an important role in antigen sequestration and degradation. There is evidence that their strategic interposition, in the liver, between the gastrointestinal tract and antibody-forming cells elsewhere (e.g. the spleen) may regulate sensitisation to intraluminal antigens. In humans it is postulated that, due to functional impairment or shunting of portal blood, Kupffer cells may fail to sequester antigen absorbed from the gut, resulting in hypergammaglobulinaemia due to increased access to antibody-forming organs (Triger and Wright, 1973). So hypergammaglobulinaemia can be due to: the infective agent (e.g. the hepatitis virus), release of autoantigens, or an unrelated antigenic stimulus (release

of sequestered antigen, persistent viral infection, or gut-associated bacteria).

### 1.5.4 Histopathology

The severity of hepatitis can vary between different areas of the same liver, so care must be taken in interpretation of biopsy specimens (especially if being used to monitor the response to therapy). There can be a mixed inflammatory infiltrate in portal tracts (usually plasma cells, monocytes and lymphocytes, although neutrophils are prominent in some cases) with disruption or destruction of limiting plates and a variable degree of piecemeal necrosis. The infiltrate can vary in degree between different areas of liver. Piecemeal necrosis is postulated to be a consequence of infiltration of mononuclear cells, including T and/or K lymphocytes, causing depletion of hepatocytes by apoptosis. Scattered, individual cells affected by apoptosis condense and bud to form membrane bound fragments, which may be taken up by surrounding cells (Kerr et al., 1979). Larger fragments have previously been called Councilman bodies. Although evidence of apoptosis (eosinophilic bodies) has been seen in association with CAH in dogs (Meyer et al., 1980; Strombeck and Gribble, 1978; Doige and Lester, 1981) the role of immune mechanisms in canine CAH is not clear. In some cases (Doige and Lester, 1981) individual necrotic hepatocytes were characterized by pyknosis or karyorrhexis and cytoplasmic eosinophilia. These circular cells, apparently undergoing coagulation necrosis, were often surrounded by viable hepatocytes and were not always associated with inflammatory cells. They were scattered throughout the lobule and were only occasionally found adjacent to portal tracts.

The liver's response to the necrosis is regeneration of hepatocytes and bile duct epithelial cells. If the limiting plate regenerates it reforms as two cells thick,

halving the surface area perfused by sinusoidal blood. Although bile duct hyperplasia is often seen, ducts can sometimes appear proliferated, when in fact their numbers are normal, due to marked hepatocyte loss and lobule collapse. Mild hepatic lipidosis is a feature of some cases.

Fibrosis usually follows the inflammatory process accompanying hepatocyte necrosis. It can be defined as an abnormal deposition of collagen, without disruption of hepatic architecture. Collagen deposition occurs adjacent to the hepatocytes at the portal edge of the lobule, and in the inflamed septa radiating from portal tracts to central veins. Experimentally it has been proven that fibrosis is not always irreversible (Rojkind and Kershenobich, 1976). The term cirrhosis defines a more diffuse, advanced process which involves distortion of normal architecture, it is dealt with in section 1.6.

The disease usually progresses, as can be seen if time elapses between a biopsy and necropsy. Portal infiltration persists, usually worsening. There usually appears to be an increase in: hepatocyte loss, fibrosis, parenchymatous nodules and intrahepatic venous stasis. The similar microscopic appearance of the liver's response to injury, by various aetiologic agents, can make it difficult to distinguish specific disease entities.

### 1.5.5 Aetiological Types

Various types of chronic hepatitis in dogs, with lesions compatible with a definition of CAH but probably not autoimmune in nature, are covered in this section.

COPPER-ASSOCIATED liver diseases are seen in several breeds of dog. Copper is a proven hepatotoxin in several species (Sternlieb, 1980). It is known to accumulate in periportal hepatocytes secondary to cholestasis (Johnson

et al., 1982; Goldfischer et al., 1980), due to interference with biliary excretion, which is the main route for elimination of copper from the liver, and via faeces, from the body. Although in 1985 Thornburg and Rottinghaus considered normal hepatic copper levels in dogs to range up to 400ppm dry weight (DW), in a later study with others of 623 healthy dogs they could not determine an uppermost limit for normal hepatic copper (Thornburg et al., 1990). They did determine that the threshold for histochemical determination of copper-containing granules, using rhodanine or rubeanic acid staining, was 400ppm; at which concentration they were localised in centrilobular (zone 3) hepatocytes. Copper-containing granules were also found in midzonal and periportal (zone 1) hepatocytes in livers with copper levels more than 1000ppm. Multifocal hepatitis lesions were found to be associated with liver copper levels of 2000ppm or more.

Chronic hepatitis in Bedlington terriers is an inherited autosomal recessive problem in which liver damage results from progressive accumulation of copper, initially in centrilobular hepatocytes then midzonal as deposits increase (Hardy et al., 1975; Ludwig et al., 1980; Owen Jr and Ludwig, 1982; Eriksson, 1983; Johnson et al., 1984; Thornburg et al., 1985b; Hultgren et al., 1986). This can present as acute fulminant hepatitis, chronic hepatitis, or cirrhosis. Hepatic injury increases with age and with the liver copper concentration, although the latter tends to decline after 6 years of age (Twedt et al., 1979). The very high incidence of the disease suggests that the majority of normal dogs may be heterozygous carriers.

Copper-associated hepatitis in West Highland white terriers is a hereditary problem in which centrilobular necrosis is associated with significant copper accumulation, again centrilobular towards midzonal. However in contrast to the Bedlington terrier disease, no age-related accumulation and no direct relationship between the liver

copper concentration and the severity of lesions were found in a study by Thornburg et al. (1986c).

Chronic hepatitis in Doberman pinschers is associated with copper accumulation within hepatocytes. Johnson et al. (1982) suggested there might be a hereditary basis to this problem, with an interplay of genetic and environmental factors. Females appear much more susceptible than males; 10 of Johnson's 11 cases were female, as were 25 of the 26 investigated by Crawford et al. (1985), Fiorito's case (1985), Cornelius's case (1989) and the 5 Doberman Pinschers in a mixed breed review of 14 dogs with CAH (Doige and Lester, 1981). It was not initially clear whether the accumulation of copper was the primary initiating cause of the disease; or whether it was secondary, due to prolonged cholestasis. Studies by Fuentealba et al. (1989) suggested the latter. Intrahepatic cholestasis, hyperbilirubinaemia and iron accumulation are other features of this disease. High liver copper levels have also been reported in 2 Doberman pinschers with subacute hepatitis (Thornburg et al., 1984).

Copper-associated liver disease, of an apparently hereditary nature, has been reported in other breeds including: cocker spaniels, Labrador retrievers, German shepherd dogs(GSDs), collies, wire-haired fox terriers, Pekingese, pit bulldogs, keeshonds, English bulldogs, schnauzers, Kerry blue terriers and Skye terriers (Thornburg et al., 1981; Thornburg et al., 1985a; Thornburg et al., 1986a; Haywood et al., 1988)). Although unusual, it has also been reported in some mixed-breed dogs including 2 Pekingese-poodle crosses, a cocker spaniel cross and a German shepherd-collie cross (Thornburg et al., 1986a; Thornburg et al., 1986b).

LOBULAR DISSECTING HEPATITIS, a chronic hepatitis of unknown aetiology, has been described by Bennett et al. (1983). The histopathology was characterised by a mixed

inflammatory infiltrate (neutrophils, lymphocytes, macrophages and occasional plasma cells) in the sinusoids, hepatocyte clusters and some portal tracts; although portal infiltrates were generally scanty. There was disruption of lobular architecture with an abnormal intralobular reticulin pattern. Reticulin and fine collagen fibres subdivided the lobular parenchyma into individual, and small groups of hepatocytes. The limiting plate was often disrupted but there was little or no increase in portal connective tissue. Hepatocellular appearance was variable; some cells were pale and swollen, some had irregular cytoplasmic clearing and others had eosinophilic granular cytoplasm. Although some hepatocytes contained lipid droplets, fatty change was not marked. There were occasional necrotic hepatocytes. Mitoses were rare, anisokaryosis was marked, and binucleate cells and rosettes of hepatocytes were common. Small, sublobular regenerative nodules were seen. Prominent vascular changes were a feature of this disease, including dilated portal venous radicles, sinusoidal distension and intralobular pooling of blood. Central veins were often inconspicous, while lymphatics were dilated. It was considered that the chronic hepatitis was responsible for the alteration in portal haemodynamics, resulting in the prominent aquired portosystemic shunts and ascites of all six cases. The disease was found in several young standard poodles as well as in other breeds.

Jensen amd Nielsen (1991) described a morphologically similar type of chronic hepatitis in three young, related standard poodles. The disease had some resemblance to lobular dissecting hepatitis, although it could not be concluded that that was actually the diagnosis.

LEPTOSPIROSIS-ASSOCIATED chronic active hepatitis was reported by Bishop et al. (1979) in five American fox-hounds. Leptospires had previously been known to cause multisystem disease, including hepatitis, during acute

phase infection but chronic leptospirosis had generally been believed to be limited to persistent renal infection. Spirochaetes were identified in the liver of four of the dogs, while a rising titre to Leptospira interrogans serovar grippotyphosa was found in the fifth; six of thirteen kennel mates also showed serological evidence of exposure. Although one of the commonest serotypes in the USA, it had previously been thought to have low pathogenicity in dogs. Histologically all five showed active inflammation, focal hepatocyte necrosis, and fibrosis in periportal areas with variable intralobular involvement. Lobular architecture was only distorted in the two most severely affected dogs. A variable amount of infiltrate distorted portal tract margins, disrupting limiting plates and dissecting into lobules. This consisted of lymphocytes, plasma cells, macrophages, a few neutrophils, infrequent eosinophils and mast cells. There was an increase in collagen and reticulin fibres at portal tracts, often enclosing individual hepatocytes at the limiting plate, with extension into lobules; sometimes even bridging portal tracts and fibrotic central veins. Both atrophic cords, compressed by sinusoidal congestion, and thickened, distorted hypertrophic cords were seen, although only one dog had true regenerative nodules. Only occasional lymphoid nodules were found, in or near portal tracts. Apart from periportal and lobular necrosis of individual hepatocytes, other degenerative changes seen included: reduced cytoplasmic basophilia; pale, vesicular nuclei; occasional binucleate cells; foci of swollen hepatocytes, with pyknotic or karyolytic nuclei and irregular cytoplasmic clearing, associated with extensive biliary hyperplasia or sinusoidal fibrosis. Also present were hepatocyte rosette formation, bile ductular hyperplasia, preportal intracanalicular bile stasis, distension of portal lymphatics and Kupffer cell hypertrophy and hyperplasia. Using Warthin-Starry stain spirochaetes could be seen singly or in small clusters, between liver cells, within hepatic cords. They were even less numerous in the

kidney, where they were found in the lumens or epithelium of proximal convoluted tubules of the outer cortex.

INFECTIOUS CANINE HEPATITIS VIRUS-ASSOCIATED chronic active hepatitis, with periportal mononuclear infiltrates, has been produced experimentally in dogs with low concentrations of antibody to CAV-1 (Gocke et al., 1967; Gocke et al., 1970). Virus was only identifiable in the very early stages of the experimental infection, so these partially immune dogs developed evidence of chronic persistent hepatic damage in the absence of demonstrable virus. Since persistence of virus in liver was thought to be essential for the induction and maintenance of chronic hepatocellular damage (Mackay, 1983) it was uncertain whether CAV-1 played a role in the pathogenesis of naturally occurring chronic hepatitis in dogs. However a later study using more sensitive immunohistochemical staining detected small amounts of CAV in livers of dogs with various types of hepatic disease, suggesting a possible relationship (Rakich et al., 1986).

CANINE ACIDOPHIL CELL HEPATITIS can manifest itself as chronic hepatitis, but the pathological complex also includes: acute hepatitis, cirrhosis with multilobular hyperplasia, and acute and subacute cytolytic necrosis (Jarrett and O'Neil, 1985; Jarrett et al., 1987). Evidence from biopsies, clinical histories and necropsies indicates that chronic canine acidophil cell hepatitis can develop after an episode of acute nonlethal hepatitis, or without an earlier identifiable clinical episode of disease. It is believed that some cases of chronic CACH can remain subclinical until the development of cirrhosis and liver failure.

As well as the presence of acidophil cells in the characteristic distribution (see section 1.3.11), the chronic condition can exhibit fibrosis of the bridging areas, pericellular fibrosis, fatty vacuolation and multilobular

hyperplasia. Professor Jarrett et al. (1987) found a striking lack of sustained inflammatory response, despite phasic elevations of ALT indicative of chronic active hepatitis.

DRUG-INDUCED chronic hepatitis and cirrhosis have been reported in dogs on long-term anticonvulsant therapy (Bunch et al., 1982 & 1984a; Poffenbarger and Hardy, 1985). Bunch et al. (1984a) estimated that up to 15% of dogs on such therapy (primidone or primidone and phenytoin) are at risk of suffering severe hepatotoxicity. Although the histopathology is not typical of CAH care must be taken in diagnosis since, despite the presence of long-term biochemical abnormalities and severe hepatic lesions, affected dogs require only a change in medication.

IDIOPATHIC chronic active hepatitis can be diagnosed in cases of active liver disease, established on clinical history and biochemical tests (especially ALT), where no identifiable cause can be found. Only supportive care is required if signs are mild or intermittent. If signs worsen, if biochemistry indicates a continuing necroinflammatory process, or if the disease is chronic a liver biopsy can be performed. Examination of this may permit diagnosis of CAH of undefined aetiology (possibly autoimmune). Steroid therapy has been tried in dogs with idiopathic CAH (section 1.7), although care is required since dogs are prone to steroid-induced hepatopathy (Fittschen and Bellamy, 1984) and steroid catabolism is slowed in the diseased liver.

An unusual HEPATITIS WITH FIBROSIS IN GERMAN SHEPHERD DOGS was noticed amongst the field case material investigated during this study. There were several cases of a morphologically similar liver disease, histologically distinct from canine acidophil cell hepatitis, including four sets of multiple cases. The main characteristic of the hepatit-

is was extensive very fine fibrosis, often extending throughout the parenchyma, dissecting between individual hepatocytes. None of the cases stained with rubeanic acid exhibited any acumulation of copper in the liver. The lesion appears to be different from that described for lobular dissecting hepatitis (Bennett et al., 1983), with less inflammatory infiltrate and without the dilated vascular channels.

The first multiple set occurred in the East of Scotland and involved an 8-month-old male German shepherd dog, euthanased after 3 months of intermittent vomiting and diarrhoea followed by ascites, which was found to have cirrhosis. The owner obtained his next dog, another male GSD, from the same kennels. The two were born 7 months apart and were never in contact, but there was no information about whether or not they were related. The second dog (dog M) also developed ascites, it had raised liver enzymes and was euthanased at 19 months of age. It exhibited macronodular cirrhosis with no evidence of copper accumulation. Material from dog M was used in the second immunodiffusion experiment (section 9.4). The next group of cases came from central England. An 8-month old female GSD with micronodular cirrhosis had exhibited vomiting, diarrhoea and ascites. Much later, material was received from her last remaining littermate, a 3½-year-old male with micronodular cirrhosis. The rest of the litter had all died of liver failure. The owners of the last dog also owned the dam which became ill and possibly ascitic 9 months later, at 6 years of age. Her blood biochemistry results were normal and the outcome of the illness is not known. The third set of multiple cases came from the Northeast of England. Material was first received from a 5-month-old female GSD with hepatitis, extensive fibrosis and early nodular hyperplasia; stains for copper accumulation proved negative. Material was then received from a male littermate (dog V), euthanased at 6 months of age by a different veterinary surgeon. It also had hepatic

fp76

Fig 1-3 Liver of dog U (Mass tri x56)
Diffuse hepatic fibrosis in 6-month-old M GSD

fibrosis without any accumulation of copper (see fig. 1-3) and had exhibited raised levels of ALT, AP and bilirubin. This animal had been a replacement to the owner for another littermate which had been euthanased at 4 months of age because of an apparently similar liver disease. Two others of the original litter of thirteen were still with the breeder; they had normal blood biochemistry results, although the vet did not measure ALT levels. The fourth group, a GSD bitch and her two pups, were all owned by someone in the north of England. The male pup became ill at 4 months of age with ascites, but then recovered. An exploratory laparotomy revealed the liver to be abnormal but not nodular. Ascites recurred at 7 months of age, with abnormal blood biochemistry results. The pup was euthanased and found to have active hepatitis and cirrhosis. The female pup was mildly ill at 4 and 6 months of age but had normal blood biochemistry results at the time of the brother's death. The dam had been ill and ascitic when the pups were 4 months of age, but improved only to worsen again a month later when, very ascitic, she died. Other individual cases of hepatitis with extensive fine fibrosis or cirrhosis in German shepherd dogs were obtained from practices in central and western Scotland. These cases may not all have had the same disease, despite similar histological appearances, and the aetiology or aetiologies were not established. However the frequency of multiple cases in related dogs and the severity of the process in often very young dogs is suggestive of a possible hereditary factor.

There have been reports of similar problems in German shepherd dogs. In Zimbabwe, Obwolo and French (1988) found cirrhosis in a 2-year-old GSD and a 3-year-old male GSD cross from different households; however they both exhibited minimal fibrosis. Savage (1987) described an apparently similar hepatitis and early cirrhosis in two young GSD littermates. Apparently there had been a similarly affected puppy in the previous litter from the

same sire and dam. Most recently, Rutgers et al. (1993) described a series of fifteen, mainly young, dogs with idiopathic hepatic fibrosis. Nine of the series were German shepherd dogs; an overrepresentation which they also felt suggested a genetic component.

## 1.6 CIRRHOSIS

### 1.6.1 Classification

Cirrhosis is the end stage of many types of chronic liver disease and is generally considered irreversible. Removal or control of the aetiological agent may slow progression of the disease, but its consequences usually result in death. The progression from chronic hepatitis to cirrhosis is a gradual one, with no specific anatomical transition point. Although some workers consider human cirrhosis a single entity, others subclassify the type in morphology or aetiology (Gall, 1960; Millward-Sadler and Wright, 1979). Twedt (1985) considers it better not to subdivide cirrhosis in veterinary medicine since different histological features can be present in different parts of the liver, different aetiologies tend not to result in distinctive morphological patterns and morphological forms may change during the course of the disease. Two basic forms can be differentiated on gross macroscopic appearance: macronodular and micronodular cirrhosis; where both are present, the term mixed cirrhosis may be used. The usefulness of this morphologic classification is not obvious since there is poor correlation with aetiological factors.

Macronodular cirrhosis (the commonest classification in dogs) is characterized by variable sized, irregular nodules ranging from 5mm to 5cm in diameter (Twedt, 1985). The liver is usually reduced in size. An example of the typical gross appearance of macronodular cirrhosis can be

fp48

Fig 1-4 Gross appearance of macronodular cirrhosis

seen in figure 1-4. Wide fibrous bands separate nodules and usually contain two or more portal tracts. This form can result from continued single-cell and piecemeal necrosis or from submassive necrosis with fibrous bridging of portal and central areas (Millward-Sadler and Wright, 1979).

Micronodular cirrhosis (not common in dogs) is characterized by small, similar sized, evenly distributed nodules, usually less than 5mm in diameter (Twedt, 1985). The liver tends to be firm, with a finely granular surface and may be increased or reduced in size. This pattern can follow zonal necrosis replaced by fine fibrous bands.

Biliary cirrhosis, the final stage in the progression of cholangiohepatitis (Garvey and Zawie, 1984), appears to be more common in cats than in dogs. Histological features include marked portal fibrosis, bile duct hyperplasia, nodular hyperplasia and a variable degree of chronic inflammation. In humans biliary cirrhosis can be divided into two types: primary and secondary (MacSween, 1985). Primary biliary cirrhosis is a nonsuppurative destructive process of unknown aetiology, affecting intrahepatic bile ducts. Secondary biliary cirrhosis, due to prolonged mechanical obstruction of the larger bile ducts, is often aggravated by ascending bacterial cholangitis.

### 1.6.2 Pathogenesis

Although a single acute massive necrosis may result in postnecrotic cirrhosis (Thornburg, 1983; Thornburg and Daniels, 1983; Thornburg et al., 1983a;b;c;d), most evidence suggests the damaging agent must be continually inflicted to result in fibrosis and nodular regeneration (Strombeck, 1979). In most naturally occurring cases of cirrhosis in the dog the aetiology is never determined, but the common denominator in pathogenesis is hepatocyte death (Twedt, 1985).

The long-continued loss of liver cells is accompanied by persistent inflammation, fibrosis and compensatory hyperplasia, with further changes altering hepatic circulation. Inflammation can be stimulated by the aetiological agent, an immunological process, or the release of breakdown products by injured hepatocytes. The last two factors may be a self-perpetuating process. The intensity of the mononuclear cell infiltrate is used to assess the degree of activity of inflammatory conditions, it is this that is then often treated with immunosuppressives. Fibrosis, the usual repair mechanism in inflammation, is due to increased collagen synthesis by fibroblasts and hepatocytes, and reduced hepatic collagenase activity (Rojkind and Kershenobich, 1976). The focal nature of hepatocyte regeneration and proliferation, in response to necrosis and reduced hepatic function, results in architectural disruption with portal tracts and hepatic veins spaced irregularly in nodules and embedded in fibrous septa. If cell death exceeds (rather than balances) regeneration, then rapid clinical deterioration occurs. Even when compensation is adequate, it can become self-limiting if expanding nodules compress the fibrous stroma, vascular channels and bile ducts. A vicious cycle of ischaemic hepatocellullar damage results from fibrosis and nodular regeneration increasing vascular resistance, reducing hepatic blood flow, and reducing hepatic uptake capacity. So the liver cell loss continues until hepatocellular failure and/or portal hypertension results in death. Apart from the blood supply being pushed to the periphery of regenerating nodules, other vascular changes may occur in cirrhosis. Vascular bridging from portal tracts to central veins bypasses sinusoids. Extrahepatic anastomosing collaterals develop between the portal and venous circulation, due to portal hypertension (see section 1.2.2 and figure 1-3). By preventing hepatic clearance, these intra and extrahepatic shunts may allow toxins and metabolites to enter the systemic circulation.

### 1.6.3 Clinical and Biochemical Features

The variability of clinical features in cirrhosis can make diagnosis difficult. Laboratory evidence of chronic liver disease is almost always evident before clinical presentation, which may occur at any time during the progression to cirrhosis, or not at all. In the latter situation, clinically latent or compensated cases may be diagnosed only incidentally at necropsy.

The three major clinical signs, which together suggest cirrhosis, are jaundice, hepatic encephalopathy and ascites. Jaundice from hepatic causes tends to indicate extensive damage of hepatocytes or cholestasis, since the liver has a large reserve capacity for bilirubin processing. Reduced hepatocellular function and portosystemic shunting of blood contribute to hepatic encephalopathy, the clinical manifestations of which are given in section 1.2.2. Ascites results from complex hepatic and renal metabolic changes and from portal hypertension, altering the equilibrium between hydrostatic and osmotic pressure (section 1.2.2).

Various other nonspecific signs can be present in cirrhotic cases. These include general deterioration with anorexia, weight loss, weakness and exercise intolerance. Gastrointestinal signs like vomiting, diarrhoea or melaena may occur. Polydipsia and polyuria are less common. Late clinical signs may include bleeding disorders and testicular atrophy or anoestrus.

Laboratory findings in cirrhosis are very variable. They are affected by various factors, such as whether or not the disease is active or inactive and the severity of hepatocellular damage. Both ALT and AP levels are usually abnormal, but need not be markedly raised e.g. the ALT level may be virtually normal in an inactive stage. Nodules and fibrosis stimulate a rise in AP. GGT, another

hepatobiliary-associated enzyme, is said to increase under similar conditions to AP (Feldman, 1980). Although significant hepatic damage is necessary before serum levels of bilirubin rise, many cirrhotic dogs have mild elevations. Increased BSP retention (slowed excretion) is expected in cirrhosis, it is said to occur once a 55% reduction in functional mass is reached (Cornelius, 1979). Indicators of hepatic function (e.g. urea, albumin, globulin and coagulation factors) tend to be abnormal only when damage is severe, in the end stages of liver disease (see section 1.2.3) and therefore may indicate a very poor prognosis e.g. bleeding abnormalities with cirrhosis. Serum levels of acute phase proteins and bile acids may be useful as additional aids in differential diagnosis (section 1.2.3).

The two most important effects of cirrhosis, portal hypertension and hepatocellular failure, are the cause of many of its other complications which frequently lead to death of the patient (see section 1.2.2). These include aquired portosystemic shunts, ascites, hepatic encephalopathy, gastrointestinal ulceration, bacterial infections, bleeding disorders, renal failure, and serum electrolyte and pH (acid-base) changes (including sodium retention, hypokalaemia, alkalosis or even acidosis).

### 1.6.4 Gross Pathology

Since the cirrhotic liver is usually small and may have an irregular surface, abdominal radiographs may aid diagnosis. Definitive diagnosis of cirrhosis requires a liver biopsy. However sampling errors, with regional variations and regenerative nodules, are even more significant than in hepatitis cases. Gross visual inspection is therefore a very useful complement to microscopic findings.

The gross appearance of the cirrhotic liver is variable. It may be normal or enlarged, in cases with fatty change or excessive hyperplasia; whereas it will be reduced in

size, when loss exceeds regeneration. Cirrhosis involves the entire liver, it has been defined in a World Health Organization bulletin as a diffuse process characterized by fibrosis and a conversion of normal architecture into structurally abnormal nodules (Anthony et al., 1977). The liver surface is irregular due to the division of parenchyma into rounded nodules by irregular branching and anastomosing fibrous tissue sheets (see figure 1-4). The colour can vary depending on the presence or absence of fatty change and cholestasis, and the degree of congestion. Nodules often appear paler, than surrounding parenchyma, due to the lipochrome deficiency of recently divided liver cells.

### 1.6.5 Histopathology

Microscopically there is loss of the normal architecture and the regular spacing of portal tracts and central veins. Foci of atrophy and loss, and foci of hypertrophy and hyperplasia are apparent, so both small cells and enlarged (even binucleate) cells are seen. Fine or dense collagen fibres develop in relation to damaged hepatocytes, to form septa whose vascularity depends on duration. These septa involve portal tracts and also hepatic veins, and often contain entrapped, individual and small groups of hepatocytes. Increased numbers of small bile duct elements are seen in them - sometimes termed ductular proliferation. Cholestasis, although usually not marked, can be focal and is seen terminally in hepatocellular failure. Infiltrates vary, with lymphocytes and, less often, plasma cells infiltrating connective tissue and sometimes parenchymal nodules. Feathery degeneration (section 1.5.1) may occur, especially at nodule margins and is indicative of an actively progressing cirrhosis, especially if accompanied by a heavy lymphocyte reaction.

## 1.7 THERAPY

The mainstay of therapy for hepatic failure remains supportive and symptomatic care; aimed at reducing the severity of clinical signs, improving the quality of life, and providing optimal conditions for hepatic repair and regeneration. Specific therapy to remove the aetiological agent, applicable in the few cases where this is identifiable, may slow or stop progression of the disease e.g. in certain drug toxicities or in inherited copper toxicity. D-penicillamine (a copper chelater) is the recommended therapy for the latter.

Dietary therapy is very important, especially the type and quantity of protein. The aim is to reduce blood ammonia and normalize the altered circulating amino acid ratios (branched:aromatic). Multiple small feeds of readily digestible protein (high in branched-chain amino acids and low in aromatic amino acids) can be used to allow assimilation to be completed in the small intestine, avoiding the ammonia-producing bacteria in the large intestine. Dogs in hepatic failure should get the majority of their calories from easily digestible carbohydrates to avoid dietary protein (and even body protein) being used to meet energy requirements, resulting in even more ammonia production. Also, readily digested, easily absorbed carbohydrate leaves less to ferment to volatile free fatty acids (FFA) in the colon. Small amounts of fats are required to supply essential fatty acids and fat soluble vitamins, and to improve palatability. Vitamin and mineral supplements are only required with home-made diets. Strombeck et al. (1983) published formulations for well-balanced, home-made diets for hepatic insufficiency. However, the various commercial reduced protein diets now available are easier for owners to use. More detailed discussions of dietary therapy are given by Hardy (1985; 1989). Lipotropic drugs, although used in hepatic disease cases to treat lipidosis, should be avoided in dogs with hepatic failure since, even

at therapeutic doses, methionine-containing ones can induce hepatic coma in such cases (Merino et al., 1975).

The aim with general therapy in cirrhotic cases is to maintain fluid and electrolyte balance, prevent hypoglycaemia, control infection and prevent/control secondary complications (Twedt, 1985). Half-strength (0.45%) saline with 2.5% dextrose is the fluid of choice (isotonic and low in sodium) for chronic liver disease (Twedt and Grauer, 1982) with 20mEq/l potassium supplementation. Nonabsorbable, enteric antibiotics like neomycin, recommended in hepatic encephalopathy treatment (Hoyumpa Jr et al., 1979) to reduce intestinal bacterial flora, may acutely cause release of lots of bacterial toxic products while the liver's ability to stop them entering the systemic circulation is impaired. The synthetic disaccharide lactulose is as effective in the management of acute and chronic hepatic encephalopathy (Conn et al., 1977; Atterbury, 1978), but it avoids the potential complication of bacterial toxin release, since it has no antibacterial properties.

In humans with moderate to severe autoimmune CAH, steroid therapy can improve the quality and length of life, and reduce the progression of early lesions to cirrhosis. Prednisone can be used alone or in combination with azothioprine (Schalm, 1982). Histological improvement may take months to years and complete cures are uncommon. However, there is no evidence that steroid therapy is useful if the hepatitis is asymptomatic and it is contraindicated in viral-induced hepatitis. Steroid therapy has been tried in dogs with idiopathic chronic hepatitis. Strombeck and Gribble (1978) recommended dosages of 0.5-1 mg/lb body weight (BW) daily (1-2mg/kg /day) of prednisolone until clinical remission. Dosage can then be reduced gradually to maintenance levels of 0.4 mg/kg/day (Hardy, 1985). Prednisone, its precursor, has also been used but is considered less suitable by some workers since it

requires metabolism, by the liver, to the active form (Powell and Axelsen, 1972). However, others found no therapeutic advantage of one drug over the other (Uribe et al., 1982). Blood biochemistry should be monitored regularly and treatment may be tapered until stopped if complete remission occurs (biochemical and histopathological recovery), but re-evalulation in case of relapse is required.

There is a shortage of comprehensive, controlled studies to prove whether or not specific anti-inflammatory agents (e.g. corticosteroids) and immunosuppressives (e.g. azothioprine) prolong survival in dogs with cirrhosis. The detrimental effects of corticosteroids in advanced liver disease sometimes outweigh the beneficial ones and result in deterioration (Twedt, 1985).

Some of the drugs tried experimentally in humans might be of use in dogs. The antifibrotic immunomodulatory agent d-penicillamine (Chen et al., 1979) may be used in dogs, at 10-15mg/kg twice daily, to attempt to reverse or delay hepatic fibrosis in CAH (Hardy, 1985). Polyunsaturated phosphatidylcholine (PPC) has been used experimentally in humans, to try to modify the immune injury in CAH; it may help protect hepatocytes from cytotoxic lymphocytes (Jenkins et al., 1982). The use of colchicine, to try to inhibit or reverse hepatic fibrosis in cirrhotic patients (Rojkind and Kershenobich, 1976; Kershenobich et al., 1979), resulted in clinical, biochemical and histological improvements in some patients. Experimentally colchicine interferes with procollagen synthesis and secretion by fibroblasts, and stimulates the production of a collagenase enzyme (Rojkind and Kershenobich, 1976; Ehrlich et al., 1974; Harris and Krane, 1971). So it may inhibit collagen production and increase collagenase-mediated removal of fibrous tissue already deposited. Colchicine has been tried experimentally in rats (Rojkind et al., 1973) and in dogs (Rojkind and Kershenobich, 1976) with apparent

success. Its use by Boer et al. (1984), in a dog with hepatic fibrosis (an uncontrolled case), apparently resulted in several months of clinical improvement without any of the adverse reactions associated with its use in man (Rojkind and Kershenobich, 1976; Flower et al., 1980). It is worth noting that, dietary management and corticosteroid therapy (where appropriate) can result in the disappearance of a moderate amount of fibrosis, without the use of antifibrotic agents (Strombeck and Gribble, 1978).

# CHAPTER 2 GENERAL MATERIALS AND METHODS

## 2.0 INTRODUCTION

This chapter covers the animals, materials, equipment and techniques used in most of the investigations undertaken. Variations, specific to individual experiments, are usually detailed in the chapters dealing with those experiments.

The usual sources of canine field case material and the procedures for dealing with it are explained at the start of this chapter (section 2.1).

The transmission experiments are dealt with in the next section (section 2.2). It covers: the sources, housing and clinical examination of experimental animals; the equipment and techniques used for the haematological and biochemical investigations; the preparation of immunosuppressive agents; and the postmortem protocol used.

Although the processing of tissue samples for light microscopy is covered in section 2.3, the details of the procedure and the recipes for the stains used are given in appendix 1. Section 2.4 details the processing of various types of samples for electron microscopy.

The techniques used to raise antiserum in rabbits and its application in agar gel immunodiffusion, enzyme immunocytochemistry and immune electron microscopy are described in section 2.5.

## 2.1 FIELD CASES

Canine field case material was obtained from many sources. Formalin-fixed tissues, from suspected liver disease cases, were received from veterinary practitioners throughout the country. These were usually postmortem samples but sometimes biopsies; some were accompanied by blood

samples. Postmortem material was also obtained from liver disease cases passing through the Glasgow University Veterinary School (GUVS) postmortem room, these originated from either GUVS hospital or from local practitioners. Blood samples were sometimes available from hospital cases suspected of having liver disease. Liver biopsies were obtained from some live hospital cases. Material, from some suspected canine acidophil cell hepatitis cases, was passed on from the histological diagnostic service operated by the Canine Infectious Disease Research Unit (CIDRU) in the veterinary pathology department of GUVS.

### 2.1.1 Clinical Details

There was great variation in the quantity of information available on field cases (history, clinical signs etc.), although attempts were always made to gain as many details as possible. With biopsy samples, when the dog was still alive, attempts were made to follow the progress of the disease. Unfortunately these often proved unsuccessful for a variety of reasons e.g. owners not returning to their vet with the animal.

### 2.1.2 Postmortem Examination

Postmortem room cases were subjected to a full postmortem examination with all organs being examined. Samples for histological investigation were usually collected, into 10% neutral buffered formalin (NBF), from the following: liver, kidney, spleen, mesenteric lymph node, tonsil, pancreas, thymus, adrenal gland, lung and any other tissues exhibiting lesions. These samples were then processed as described in section 2.3. If the carcass was fresh some liver was usually frozen in liquid nitrogen and stored at -70°C for later studies.

10% Neutral buffered formalin (NBF):

| | |
|---|---|
| Tap water | 900ml |
| Concentrated formaldehyde (40%) | 100ml |
| Sodium dihydrogen orthophosphate | 4.6g/l |
| Disodium hydrogen orthophosphate | 8.0g/l |

### 2.1.3 Haematological And Biochemical Examinations

When appropriate blood samples were received haematological and biochemical analyses were performed as described in sections 2.2.5 and 2.2.6. If whole, clotted blood was available, but no heparinised blood for plasma extraction, then serum was used for the biochemical investigations. Any serum left over was frozen and stored at -20°C for later studies.

## 2.2 TRANSMISSION EXPERIMENTS

### 2.2.1 Experimental Dogs

The dogs used in the first two transmission experiments were young, unvaccinated pups obtained from rural sources. They were border collies and border collie crosses. The implementation of the Animals (Scientific Procedures) Act 1986 prevented the use of any animals other than those obtained from registered breeders. The dogs used in the third experiment were therefore commercially-reared, vaccinated beagles purchased at around 6 months of age, from a closed breeding colony.

On arrival, the dogs were given a clinical examination and were sprayed for fleas with Nuvan Top (an aerosol of dichlorvos and fenitrothion, Ciba Geigy Agrochemicals, Cambridge). Later they were vaccinated and prophylactic anthelmintic regimes were instigated.

The groups of dogs were housed in isolation units from

arrival, except for the first group which initially spent some time in other accommodation (detailed in section 4.3.3). Two isolation units were used, each consisting of a work side and a dog accommodation side. The entrance door opened into a small area, separated from the rest of the work area by a low wall. Here workers changed into boiler suits, kept in the unit, and leaving outdoor footwear behind stepped over the wall into rubber boots on the other side. In the work area was a sink and a counter for equipment storage and food preparation. The dog accommodation side was fenced off from the work area, with access by a gate. Metal grill partitions divided the dog accommodation area into a bare run area and a straw-bedded living area with a raised wooden bed and a self-filling water bowl. The units were built of stone blocks and had concrete floors. They were equipped with screened ventilation fans and thermostatically controlled electric fan heaters. The isolation units were thoroughly cleaned and fumigated before and after use by each group of dogs. Two types of fumigation were used; either formaldehyde gas (produced by adding potassium permanganate to formalin), or an aerosol mist of Tegodor (Th. Goldschmidt Ltd, Middlesex).

The dogs were fed once daily on a mixture of commercial dog meal (Wilson's Dog Meal, Wilson & Sons, Dundee) and tinned dog food (Spillars Foods Ltd, Cambridge). The straw bedding was changed daily. They were taken into the work area for most procedures e.g. blood sampling and temperature measurement.

### 2.2.2 Experimental Laboratory Animals

The rats, guinea pigs, hamsters and mice used in this research were virtually all bred in-house. Only the rats used in rat experiment 5 (section 5.7) were not bred in-house, along with their mothers who were purchased while pregnant and later became part of rat experiment 6

(section 5.8). A commercial supplier was the source of the rabbits used. The laboratory animals were housed in suitable cages in various rooms of a controlled-environment animal house. They were fed ad lib on appropriate commercial, pelleted diets.

### 2.2.3 Inocula

Homogenised canine liver was the most commonly used inoculum for the transmission experiments, but rat liver and canine serum were among others used. The method of inoculum preparation also varied between experiments. Specific details of each inoculum are given in the appropriate experiment description.

All the inocula used were subjected to both bacteriological and virological examinations. Smears were prepared and inspected microscopically for the presence of bacteria. The culture of organisms was attempted aerobically in sheep blood and MacConkey's agars as well as anaerobically. Inocula were also checked for the presence of canine adenovirus by attempting its culture in both MDCK (Madin-Darby canine kidney) cells and GH (greyhound kidney) cells. The use of freshly thawed and prepared liver homogenates meant that the results of the bacteriological and virological checks were not available until after the inoculation of experimental animals. Although not ideal, the use of this procedure did not appear to result in any adverse reactions and it insured that the checks were performed on what the animal actually received. Both preparation of a small sample for checking, before preparation of the main inoculum, and freezing of prepared homogenates until tests could be performed would have allowed the possibility of variations between tested and inoculated samples. No evidence of canine adenovirus infection was ever found in any of the inocula used in experimental animals.

Inocula were always administered subcutaneously in the scruff of the neck and large volumes were split between two sites.

### 2.2.4 Clinical Investigations

Each dog was given a thorough clinical examination on arrival. They were monitored daily for any signs of ill health, attention being paid to features such as demeanour and appetite. Rectal temperatures were recorded daily. Blood samples were collected by jugular venipuncture, twice or thrice weekly for biochemical analysis and weekly or fortnightly for haematological examination. At regular intervals, extra blood was taken for serum collection. This was spun at about 1 500 rpm for 10 minutes in a bench centrifuge (Minor Centrifuge, MSE, England) to separate the clot from the serum, which was frozen and stored for later studies. Faecal samples were collected periodically from the dogs, to be checked for evidence of intestinal parasites. The McMaster's Technique (Dunn, 1978) was used to obtain faecal egg counts (FEC).

The laboratory animals were checked daily for deaths or signs of ill health. Prior to destruction blood samples were collected by cardiac puncture, under anaesthesia, for serum collection and sometimes for biochemical and haematological analyses.

### 2.2.5 Haematological Procedures

Blood samples, ½ml, for haematological examination were placed in Potassium EDTA-coated tubes (LIP. (Equipment & Services) Ltd, Middlesex) and gently mixed. A Coulter Counter S-PLUS IV (Coulter Electronics Ltd, Coatbridge) was used to test the following parameters: total white blood cell (wbc) count, total red blood cell (rbc) count, platelet count, haemoglobin concentration (Hb), haematocrit (HCT), mean rbc volume (MCV), mean rbc haemoglobin

content (MCH), and mean rbc haemoglobin concentration (MCHC). A differential wbc count was performed on a blood smear, made from each sample and stained with the May-Gruenwald Giemsa method. A standard 200 cell count was used, from which were calculated absolute numbers of neutrophils, lymphocytes, monocytes, basophils, eosinophils and normoblasts. When considered necessary, reticulocyte numbers were counted from another smear stained with New Methylene Blue, a supravital stain technique.

### 2.2.6 Biochemical Procedures

Two ml blood samples for biochemical analysis were placed in Lithium Heparin-coated tubes (LIP. (Equipment & Services) Ltd, Middlesex) and gently mixed. The tubes were spun in a bench centrifuge for 2 minutes at about 3 000 rpm to separate the blood cells from the plasma, which was then collected and analysed. A Cobas Mira analyser (Roche Diagnostica, Switzerland) was used to measure blood levels of alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, urea, bilirubin, cholesterol and gamma glutamyl transferase. A Technicon AutoAnalyser II (Technicon (Ireland) Ltd, Dublin) was used to measure the levels of total protein (method AA II-14) and albumin (bromocresol green method). The globulin level was calculated by subtracting the latter from the former. Sodium (Na) and potassium (K) levels were measured with a IL Flame Photometer, model 543 (Instrumentation Laboratories (UK) Ltd, Cheshire). An EEL 920 Chloride meter (Evans Electoslenium, Essex) was used to measure the blood Chloride (Cl) level.

### 2.2.7 Preparation of Azothioprine Solutions

The protocol for the production of azothioprine solutions was adapted from the manufacturers instructions (Burroughs Wellcome Co, London). Using the same ratios, the quantities were adjusted to provide enough solution for a few days

treatment. E.g. 50mg azothioprine powder suspended in 5ml normal saline (NS) then 0.181ml 1N sodium hydroxide (NaOH) added dropwise. After dilution with NS to 25ml, the 2mg/ml solution should have a pH of 8.5. Since solutions were prepared in advance and stored at 4°C, their stability had to be checked daily. The optical density (OD) of a sample diluted to 10μg/ml at pH 1 was measured in a spectrophotometer at 280 and 330μm. The normal ratio of the two ODs should be 3.3-3.6. Any samples with lower ratios, indicating breakdown to 6-mercaptopurine, were discarded.

2.2.8 Postmortem Examination

Full postmortem examinations were performed on all of the experimental dogs immediately after death, to minimise the effects of the postmortem changes and technical artefacts discussed in section 1.2.4. They were killed by overdosing with intravenous (IV) Pentobarbitone Sodium 20% (Euthatal, RMB Animal Health Ltd, Dagenham). After death, the dogs were bled out by venisection of the jugular vein. Blood was collected for serum separation and, into sodium citrate anticoagulant, for buffy coat and lymphocyte extraction. The abdomen was opened and sterile samples of liver were collected for bacteriological examination, virological testing for canine adenovirus (CAV), attempted novel agent extraction and dissociated cell preparations for electron microscopical examination. All organs were then examined.

Small samples of the following tissues were taken for electron microscopical examination of tissue blocks: liver (3 or 4 sites), kidney, spleen, mesenteric lymph node, tonsil, pancreas and bone marrow. Bone marrow was also taken for smear production. Tissue samples, frozen in liquid nitrogen, to be stored at -70°C for possible future studies included: liver, kidney, spleen, lymph node, tonsil, pancreas, bone marrow, thymus and adrenal gland. Urine was collected, directly from the bladder, for

biochemical examination and to be checked for cellular deposits. Samples for histological examination were collected, into 10% neutral buffered formalin, from the following: liver (each lobe), kidney, spleen, lymph nodes, tonsil, pancreas, bone marrow, thymus, adrenal gland, lung, groin skin, brain and any other tissues exhibiting lesions. The full length of the intestinal tract was checked for nematode parasites.

Laboratory animals were also given a full postmortem examination immediately after death. They were anaesthetised with intramuscular Pentobarbitone Sodium 6% (Sagatal, RMB Animal Health Ltd, Dagenham). As much blood as possible was collected, by cardiac puncture, for serum separation and sometimes biochemical and haematological examinations. The animal was then killed by cervical dislocation under anaesthesia and a postmortem was performed, all organs being examined.

Liver was occasionally taken from laboratory animals for electron microscopical examination. Whenever possible, samples of liver, kidney and mesenteric lymph node were frozen in liquid nitrogen for storage at -70°C. Tissues taken into 10% NBF for histological examination included: liver (from 3 lobes), kidney, spleen, mesenteric lymph node, adrenal gland, thymus, lung and any other tissues exhibiting lesions.

## 2.3 HISTOLOGICAL PROCEDURES

Small blocks of tissue were fixed in 10% neutral buffered formalin, usually for a minimum of 24 hours. Each block was then trimmed to a thickness of not more than 4mm and transferred to fresh 10% NBF to ensure complete fixation. Canine tissue blocks were post-fixed in corrosive formol for 24 hours, the smaller blocks from laboratory animals were post-fixed for around 12 hours. All blocks then went

through a 24-hour processing cycle (appendix 1) involving dehydration through a series of alcohols, clearing in xylene and embedding in paraffin wax. Sections were cut from the paraffin wax blocks at a thickness of 3μm and stained using Mayer's haematoxylin & eosin (H&E) (appendix 1). Other specialised stains used, especially on liver sections, included Masson's trichrome, Masson-orange G, acid fuchine, Gordon and Sweet's reticulin, rubeanic acid, Shikita's orcein, Victoria blue, PAS, Sudan black and oil red O (appendix 1).

Corrosive formol:

| | |
|---|---|
| Concentrated formaldehyde (40%) | 100ml |
| Mercuric chloride (sat. aqueous soln.) | 900ml |

## 2.4 ULTRASTRUCTURAL PROCEDURES

Transmission electron microscopy (TEM) was used for the ultrastructural examination of tissue blocks, preparations of dissociated cells, buffy coat, lymphoctes and purified, extracted preparations suspected of containing a causative agent. Material from both dogs and laboratory animals was examined. All investigations were performed with an EM801 transmission electron microscope (AEI Scientific Apparatus Division, Essex).

### 2.4.1 Tissue Blocks

Small pieces of tissue to be examined were removed immediately after death and placed in drops of chilled paraformaldehyde/glutaraldehyde (para./glut.) fixative, on blocks of dental wax. They were chopped into tiny blocks, transferred to glass phials containing chilled fixative and fixed at 4°C for a minimum of 4 hours. Each tissue was then rinsed overnight in cacodylate rinsing solution and post-fixed in osmium tetroxide for 1 hour. Fixed tissue

was dehydrated through an ascending series of 30%, 60%, 90% and absolute acetone. Tissue was then soaked for: 30 minutes in a 50:50 mixture of araldite and acetone, 30 minutes in a 75:25 araldite:acetone mixture and a minimum of 1 hour in 100% araldite mixture. Individual blocks were then embedded and the embedding resin polymerised at 60°C for 48 hours.

An LKB ultramicrotome (Ultrotome III, LKB Instruments Ltd, Surrey) with glass knives, was used to cut 1μm thick sections. These were mounted on glass slides and stained with methylene blue and azure II. A light microscope was used to select fields for ultramicroscopy and the original blocks were trimmed accordingly. Ultrathin sections (~50-70nm thickness) were cut on the ultramicrotome and mounted on uncoated Athene 400 copper specimen grids (Agar Scientific Ltd, Stansted). They were stained for 2 minutes with uranyl acetate, rinsed in methanol, then in 50% methanol, then in distilled water and dried on filter paper. Next followed 2 minutes staining with lead citrate, rinsing with 0.02N NaOH, then in distilled water and air drying on filter paper. Sections were then ready for examination in the electron microscope.

Paraformaldehyde/glutaraldehyde (para./glut.) fixative: A mixture of 1.3% paraformaldehyde and 1.6% glutaraldehyde in cacodylate buffer pH 7.2-7.4.

| | |
|---|---|
| Paraformaldehyde | 2g |
| Distilled water | 25ml |
| 1M Sodium hydroxide | 2-3 drops |
| 25% glutaraldehyde | 10ml |
| Cacodylate buffer | 115ml |
| Anhydrous calcium chloride | 25mg |

Cacodylate buffer: A 0.1M solution of sodium cacodylate in distilled water (21.4g/l) adjusted to pH 7.4-7.6 with a few drops of concentrated hydrochloric acid (HCl).

Cacodylate rinsing solution: A 0.1M solution of sucrose in cacodylate buffer (34.2g sucrose/l) adjusted to pH 7.2-7.4 with a few drops of concentrated HCl.

Osmium tetroxide: 1% osmic acid in Millonig's phosphate buffer pH 7.2-7.4. The latter consists of:

| | |
|---|---|
| Sodium dihydrogen phosphate (2.26%) | 83ml |
| Sodium hydroxide (2.52%) | 17ml |
| Distilled water | 10ml |
| Sucrose | 0.54g |

Stain for 1μm sections: Equal parts of the following were mixed in a coplin jar and warmed on a hotplate:

1% Methylene blue
1% Azure II
1% Borax

Slides were left in the warm stain for approximately 5 minutes, before being rinsed with water and dried on the hotplate.

Araldite mixture: Equal parts of the araldite resin and hardener (DDSA) were mixed, before the addition of the accelerator (BDMA). (Agar Scientific Ltd, Essex)

| | |
|---|---|
| Araldite CY212 | 10ml |
| DDSA (dodecyenyl succinic anhydride) | 10ml |
| BDMA (N-benzyldimethylamine) | 0.4ml |

Uranyl acetate: A 20% solution made up in 100% methanol.

Lead citrate: 1.33g lead nitrate and 1.75g sodium citrate, each dissolved in 15ml of distilled water, were mixed. The lead citrate precipitate was shaken for 1 minute, allowed to stand for 30 minutes with periodic agitation, then solubilised by the addition of 8ml of 1M NaOH. The solution was diluted to 50ml with distilled water, final pH 11.9-12.1.

## 2.4.2 Dissociated And Other Cell Preparations

Dissociated cell preparations were most commonly made from liver, the various techniques used are described in chapter 8 (section 8.2.1). The cell preparations were usually mixed 50:50 with chilled para./glut. fixative and pelleted in a bench centrifuge. The supernatant was removed, fresh fixative added, and the cell pellet left to fix at 4°C for 2-4 hours. It was then rinsed for the same amount of time in cacodylate rinsing solution. Subsequent processing of cell pellets for electron microscopical examination was the same as for tissue blocks.

Buffy coat was obtained by spinning blood, collected in sodium citrate or potassium EDTA anticoagulant, at around 3 500 rpm for 10 minutes to form three layers: packed rbcs, buffy coat (wbcs and platelets) and plasma. The latter was drawn off, and chilled para./glut. fixative was added over the buffy coat layer. After about 20 minutes this was firm enough to be extracted from the tube and transferred to fresh chilled fixative for 2-4 hours at 4°C. Further processing, before examination in the electron microscope, was the same as for dissociated cell preparations.

Preparations of lymphocytes were processed for electron microscopical examination in the same way as the dissociated cells. The ficoll-hypaque extraction method was used to obtain lymphocytes from blood samples collected in sodium citrate.

1) Using a syringe, 10ml of citrated blood was carefully transferred onto the surface of 10ml of ficoll-hypaque solution in a universal.
2) This was spun at 1 400 rpm for 20 minutes, without refridgeration.
3) Lymphocytes were found in the middle layer, which was removed and washed twice in phosphate-buffered saline (PBS). The first wash and spin (750 rpm for 30 minut-

es) removed platelets. After the second wash and spin (1 000 rpm for 10 minutes) the lymphocytes were resuspended in PBS.

Ficoll-hypaque solution: Stored at 4°C after being mixed for several hours.

9% ficoll in distilled water 288ml

33.9% hypaque in distilled water 120ml

(90.36ml 45% hypaque soln. + 29.64ml water)

Phosphate-buffered saline (PBS): 1 PBS tablet (Oxoid Ltd, England) dissolved in 100ml distilled water and solution autoclaved.

2.4.3 Purified preparations

Variations on two purification techniques (detailed in chapter 8) were used in attempts to extract a causative agent for CACH from various samples. Liver and mesenteric lymph node were the two main tissues studied. They were finely chopped before either homogenisation, with a Silverson Homogeniser (Silverson Machines Ltd, Chesham), or stomaching, with a Colworth Stomacher 80 (A.J. Seward, London), in phosphate-buffered saline or tris-buffered saline (TBS) pH 7.4. Preparations were then clarified to remove gross debris; this usually involved spinning at about 1 500 rpm for 10 minutes in a bench centrifuge, followed by spinning of the supernatant at 10 000 rpm for 10 minutes at 0°C in a Sorvall OTD-50 Ultra Centrifuge (Du Pont Instruments, Newtown) using an SW-41 rotor. The resultant supernatant was then subjected to either density gradient centrifugation in caesium chloride (CsCl) or centrifugation in sucrose gradients.

Preformed caesium chloride gradients were made in Ultra-Clear centrifuge tubes (Beckman Instruments) by adding solutions of CsCl of density ranges from either 1.1g/cc up to 1.4g/cc or 1.2g/cc up to 1.5g/cc. 1.5ml of the lowest

density CsCl solution, e.g. 1.1g/cc, were added to the centrifuge tube by means of 2mm tubing attached to a syringe, this was displaced upwards by 1.5ml of the 1.2g/cc density CsCl being added to the bottom of the tube. This procedure was repeated until the highest density CsCl solution, e.g. 1.4g/cc, was in place. The clarified sample was carefully layered on top of the gradient which was spun at about 35 000 rpm for around 36 hours in the ultracentrifuge. The bottom of the tube was pierced with a needle and fractions were collected. These were dialysed overnight against cold TBS, before negative staining for examination in the electron microscope. Dialysis was found to be more efficient at caesium removal than washing the sample on the specimen grid with ammonium acetate for 2 minutes before phosphotungstic acid (PTA) staining.

The sucrose gradient centrifugation procedure used was adapted from Pedersen (1973). The clarified sample was centrifuged in a discontinuous gradient, consisting of 5ml 65% sucrose and 5ml 15% sucrose in TBS pH 7.4, at around 23 000 rpm for about 1½ hours at 4°C in the SW-41 rotor. The band, hoped to contain virus, formed in the interface between the two sucrose layers was collected and bovine serum albumin added to a final concentration of 0.1%. The preparation was dialysed overnight against cold TBS pH 7.4. A gradient maker powered by a 2132 Microperpex Peristaltic Pump (LKB) was used to form a 15% to 65% continuous sucrose gradient in TBS pH 7.4, in an Ultra-Clear SW-41 tube. The sample was layered on top of this for centrifugation to equilibrium in the ultracentrifuge at 23 000-25 000 rpm for 16 hours at 4°C. When examined against direct, light various bands were visible. The bottom of the tube was pierced with a needle and the gradient was collected in fractions. The refractive indices of these were ascertained using an Abbe 60 refractometer (Bellingham and Stanley Ltd, Turnbridge Wells) and the density (D) of each fraction was calculated. The

fractions were dialysed overnight against cold TBS pH 7.4, before negative staining for examination in the electron microscope.

Phosphotungstic acid (PTA) negative staining was used on the fractions from both types of gradient.

1) One drop of the preparation for examination was dispensed onto a copper specimen grid, coated with a thin parlodion support film. This was left for 2 minutes at room temperature before most of the excess fluid was removed with filter paper.
2) Without allowing the grid to dry out, 1 drop of freshly prepared 2% PTA solution (pH 7.2) was added and left for 2 minutes at room temperature.
3) The excess fluid was removed with filter paper and once dry, the grid was ready for examination in the electron microscope.

Tris-buffered saline

TBS x10: The following were dissolved in distilled water and made up to 1 litre before autoclaving:

| | |
|---|---|
| Sodium chloride (NaCl) | 58.44g |
| Trizma base (Tris[hydroxymethyl]aminomethane) | 12.11g |
| Ethylenediaminetetraacetic acid (EDTA) | 3.72g |

TBS x1: 900ml distilled water was added to 100ml TBS x10 and the pH was adjusted to 7.4 with concentrated HCl.

Caesium chloride solutions

1.1g/cc: 1.33g CsCl dissolved in 10ml TBS

1.5g/cc: 6.718g CsCl dissolved in 10ml TBS

Sucrose solutions

15%: 15.89g sucrose dissolved in 100ml TBS pH 7.4

65%: 82.28g sucrose dissolved in 100ml TBS pH 7.4

Parlodion solution: A 3% stock solution of parlodion in amyl acetate was made by allowing 2 days to dissolve 0.3g parlodion pieces in 10ml amyl acetate. A 0.6% solution was

used to coat the copper grids i.e. 0.2ml of 3% stock solution diluted with 0.8ml amyl acetate.

## 2.5 IMMUNOLOGICAL PROCEDURES

### 2.5.1 Raising Antiserum

The aim of this work was to immunise rabbits to the causative agent of canine acidophil cell hepatitis, so that they would produce antiserum which could be used in various studies.

Two New Zealand White rabbits had already been put through an immunisation regime, 2½ years previously. Dog D (dog 8) from an earlier CACH transmission experiment (Jarrett and O'Neil, 1985), had been the source of antigenic material used. This animal's details and history are given in section 5.7.2. A 10% weight:volume (wt:vol) homogenate of liver in PBS had been sonicated, then clarified in a bench centrifuge. The supernatant had been emulsified with an equal volume of Freund's complete adjuvant (FCA) (ICN, ImmunoBiologicals). The emulsion had been administered to the rabbits by an intramuscular injection of 1.5ml into each hind leg. One week later the procedure had been repeated, except that Freund's incomplete adjuvant (FIA) (ICN, ImmunoBiologicals) had been used to make the emulsion. That process had been repeated 1 week after that. Ten days after the third inoculation the rabbits had been bled, by venisection of the marginal ear vein, and serum collected.

It was decided to boost these two rabbits, with slightly more purified material from dog D again, to obtain antiserum for the current study. First they were bled and a pre-boost serum sample was collected. For each rabbit, a 5% wt:vol homogenate of liver in TBS was prepared and clarified for 10 minutes in the MSE bench centrifuge. The

supernatant was then clarified, at 10 000 rpm for 10 minutes at 1°C, in the Sorvall OTD-50 ultracentrifuge. To concentrate the preparation; the supernatant was spun in the ultracentrifuge, at 35 000 rpm for 75 minutes at 4°C, and the resultant pellet was then resuspended in 1.5ml of TBS, using a rotamixer (Hook & Tucker Ltd) and sonicater (Millipore). An equal volume of FIA was added and the mixture emulsified using the rotamixer. The emulsion was divided into two doses of just over 1ml, given intramuscularly in each hind leg. Eleven days later the rabbits were bled and the immune serum collected.

Before inoculation, the integrity of the emulsion was always determined by allowing a drop of the suspension to fall onto the surface of cold water. Only when the droplet remained intact, indicating that the aqueous phase containing the antigen was entirely closed within the oil, was the suspension deemed fit to use.

Seventeen months later it was decided, due to their age, to boost and bleed the rabbits again, before humane destruction. A 10% wt:vol homogenate of dog D's liver in TBS was prepared and then clarified twice, as before. The resultant supernatant was spun in the ultracentrifuge, at 35 000 rpm for 90 minutes at 4°C, to form a pellet which was resuspended in TBS by rotamixing and sonication. This was emulsified with an equal volume of FIA. The dose of emulsion, 1.25ml for each rabbit, was split between the two hind legs and given intramuscularly. Twelve days later the rabbits were bled out, by cardiac puncture under anaesthesia, and then destroyed. The immune serum was collected.

### 2.5.2 Absorption of Antiserum

Several different powdered preparations of canine liver were used, to remove any antibodies to normal canine liver components, in the production of absorbed sera for the

serological investigations.

Two preparations of dried dog liver were already available at the start of the study (liver powders 1 and 2) and they were used to absorb the rabbit sera for the first immunodiffusion experiment (section 9.3). Since they had been prepared previously, the exact method used was not known.

To produce absorbed serum, 1ml of tissue powder dampened with PBS was mixed with 1ml of serum and incubated at 37°C for 30 minutes. Then followed a 10 minute spin at 3 000 rpm in a bench centrifuge and a further 10 minute spin of the supernatant, at 10 000 rpm at 4°C in an ultracentrifuge. The resultant supernatant was put through a 0.45µm Acrodisc disposable filter (Gelman Sciences UK), moistened with PBS, to give the absorbed serum.

The freeze-dried, normal dog liver powders (3 and 4) used for absorbing sera in the second immunodiffusion experiment (section 9.4) were prepared by the following method. Liver was chopped, then homogenised in an equal volume of TBS, before spinning in a bench centrifuge at 3 000 rpm for 30 minutes. The supernatant was discarded and the pelleted liver resuspended in PBS to wash out the haemoglobin. The preparation was spun again for 10 minutes and the supernatant discarded. This rinsing procedure was repeated (usually twice) until the supernatant appeared free of haemoglobin. The precipitate was then resuspended and the preparation lyophilised in a freeze-drier (Edwards High Vacuum, Crawley). The freeze-dried flakes were ground to a powder with a mortar and pestle and then stored at 4°C.

The protocol for the production of absorbed serum for the second immunodiffusion experiment was also slightly different. After 900µl of serum had been incubated with 0.45g of liver powder, an equal volume of PBS was added, before clarification, to increase the volume of absorbed

serum. This produced a 1 in 2 dilution of absorbed serum.

2.5.3 Agar Gel Immunodiffusion (AGID)

The Ochterlony double diffusion technique was used to test whether the rabbits had been immunised with the dog liver inoculum, and whether any of the antibodies produced were specific for CACH infection. Antigen-antibody reactions between both unabsorbed and absorbed antisera and liver preparations from various dogs were investigated. The theory behind the technique is given in chapter 9 (section 9.2).

Dog livers for testing were thawed (from storage at -70°C) and homogenised in TBS, using the Silverson homogeniser. The homogenate was clarified in a bench centrifuge at 1 500 rpm for 10 minutes, then the supernatant was further clarified at 10 000 rpm for 10 minutes at 4°C in the ultracentrifuge.

The medium used for the immunodiffusion tests was Ochterlony gel, prepared using Ochterlony buffer. Before use the gel was warmed to melt it, then it was pipetted onto glass microscope slides (Blue Star, Chance Propper Ltd, Warley) on a levelling table - 2ml/slide. Once set, the slides were stored in moisture chambers at 4°C and usually kept until the next day before two 7-well rosettes were cut out with a punch and they could be used. Rabbit serum (antibody) was always put in the central well of each rosette. The dilutions of dog liver preparations were put in the surrounding ring of six wells, working clockwise from neat in the "1 o'clock" position round in two-fold dilutions to 1/32. Half-strength Ochterlony buffer was used to make the dilutions.

Slides were kept in moisture chambers at 4°C for several days, to allow diffusion of reactants and the formation of precipitin lines, before staining. A rubber band was wound

round one end of each slide to prevent the gel from floating off when slides were washed in PBS for 6-8 hours and then soaked overnight in distilled water. They were then stained with Coomassie blue for 25 minutes before being put in destain for 5-15 minutes.

Ochterlony buffer: 41ml of A. mixed with 9ml of B.

A. 1.78g disodium hydrogen phosphate dissolved in 50ml distilled water.

B. 0.21g citric acid dissolved in 10ml water.

Ochterlony gel: 1.5g Ionagar No.2 dissolved, slowly over a bunsen, in 50ml water. Then 50ml Ochterlony buffer added (prewarmed in 56°C water bath to prevent it from setting the gel). 0.2g Sodium azide ($NaN_3$) added and mixture dispensed into universals for storage at 4°C.

Coomassie blue stain:

| | |
|---|---|
| Coomassie blue | 1g |
| Ethanol | 450ml |
| Acetic acid | 100ml |
| Distilled water to | 1000ml |

Destaining solution:

| | |
|---|---|
| Acetic acid | 100ml |
| Ethanol | 250ml |
| Distilled water to | 650ml |

### 2.5.4 Enzyme Immunocytochemistry

Tissues, fixed in formalin and corrosive formol then embedded in paraffin wax, were sectioned and mounted on poly-L-lysine-coated glass slides. The latter were prepared by immersing clean slides in poly-L-lysine solution (0.1g/100ml distilled water) for 5-10 minutes, then drying them in an oven. The immunoperoxidase technique employed was the following peroxidase-antiperoxidase (PAP) method, adapted from Sternberger (1974).

1) Sections were deparaffinised in Histo-Clear (National Diagnostics, New Jersey) for 3 minutes, then rehydrated through two baths of alcohol and one of 95% methylated spirits (meth. spirit).
2) They were washed in running tap water then immersed in Lugol's iodine for 3-5 minutes.
3) Another wash in water was followed by a short wash in 5% sodium thiosulphate, to remove mercuric chloride artefact, then another wash in water.
4) Endogenous peroxidase was inhibited by treating with freshly prepared 0.5% hydrogen peroxide ($H_2O_2$) in methanol for 30 minutes.
5) Sections were washed well in distilled water, then the temperature of the slides was equilibrated in distilled water at 37°C for 10 minutes.
6) Sections were treated with 0.1% trypsin and 0.1% calcium chloride ($CaCl_2$) in TBS (adjusted to pH 7.8 with N/10 NaOH) for 30 minutes at 37°C.
7) They were transferred to cold distilled water, with agitation for 2-3 minutes.
8) To reduce the staining of nonspecific tissue binding sites, the sections were washed for 5-10 minutes in each of two baths of tris-buffered saline pH 7.6 containing 1% normal swine serum (TBS/NSS).
9) The primary antiserum (rabbit), absorbed with freeze-dried tissue powder, at optimal dilutions was applied to each section after the rest of the slide had been dried. The area of the section was never allowed to dry out. The diluent used was TBS containing 0.1% bovine serum albumin (BSA) and 0.01% sodium azide; it prevented the proteins from adhering to the glass. The primary antiserum was left on for either 1 hour, or overnight for higher dilutions.
10) Sections were washed for 10 minutes in each of two baths of TBS/NSS.
11) The bridging antibody, swine anti-rabbit IgG (Dakopatts a/s, Denmark), at 1/20 dilution in TBS was applied for 1 hour.

12) Sections were washed in two 10-minute TBS/NSS baths.
13) The peroxidase-rabbit antiperoxidase (Dakopatts a/s) was applied at 1/20 dilution in TBS for 30 minutes.
14) Sections were washed in two 10-minute baths of TBS.
15) The peroxidase reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) for up to 5 minutes (Graham and Karnovsky, 1966).
16) Sections were washed in TBS, then in distilled water.
17) The sections were counterstained with Mayer's haematoxylin and differentiated in 1% HCl in alcohol before blueing in Scott's tap water substitute (STWS).
18) They were dehydrated through 95% meth. spirit and alcohol before clearing in Histo-Clear and mounting with a resinous medium.

Peroxidase activity stained brown. The following controls were routinely employed:
1) Known positive control tissue was included, once found, to eliminate technical error.
2) Primary antibody was omitted, PBS used in its place, to check endogenous peroxidase activity and the nonspecific binding of the secondary antibody.
3) Primary antibody was replaced with normal rabbit serum.

Lugol's iodine:

| | |
|---|---|
| Iodine | 1g |
| Potassium iodide | 2g |
| Distilled water | 100ml |

DAB developer: 25mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB) dissolved in 50ml TBS with 0.15ml 3% $H_2O_2$ added immediately before use.

Mayer's haematoxylin: The following were dissolved in 1 litre of distilled water.

| | |
|---|---|
| Haematoxylin | 1g |
| Sodium iodate | 0.2g |

| | |
|---|---|
| Potassium alum | 50g |
| Citric acid | 1g |
| Chloral hydrate | 50g |

Scott's tap water substitute (STWS): The following were dissolved in 1 litre of distilled water.

| | |
|---|---|
| Sodium bicarbonate | 3.5g |
| Magnesium sulphate | 20g |

2.5.5 Immune Electron Microscopy

The method employed was adapted from Almeida and Waterson (1969). It involved mixing a suspected virus suspension (antigen) with immune serum (antibody) with the aim of forming antigen-antibody complexes, more easily visualised with the electron microscope than individual virions. Purified preparations from the tissues of an experimentally-infected dog were the sources of antigen used. The antibody source was the same rabbit antiserum, absorbed with normal dog liver, that was used in the immunoperoxidase technique, two dilutions of this were tested.

1) 40µl aliquots of antigen preparation were mixed with 20µl absorbed antiserum diluted 1:2 in PBS or with 20µl absorbed antiserum diluted 1:10 in PBS.
2) To each was added 140µl TBS (pH 7.4) to make two mixtures of 0.2ml each.
3) Samples were incubated at room temperature for 1 hour, then overnight at 4°C. This optimum approach should be used for a system where the concentration of neither antigen nor antiserum is known and maximum clumping of immune aggregates is needed.
4) Samples were pelleted by spinning at 10 000-14 000 rpm for about 20 minutes in a microcentrifuge (Eppendorf centrifuge 5415). The supernatant was collected and the pellet resuspended in a small volume of sterile distilled water (SDW) before negative staining with PTA (as described in section 2.4.3) and examination in the electron microscope.

5) The supernatant collected in 4) above was spun at 14 000 rpm for 60-90 minutes in the microcentrifuge. The resultant pellet was resuspended in a small volume of SDW and, after negative staining, was examined in the electron microscope.

It is important that the time between suspension of the pellet in distilled water and placement of the grid in the electron microscope is kept as short as possible, because conditions are nonphysiological at this time and dissociation or other changes will almost certainly take place.

# CHAPTER 3 CANINE FIELD CASES

## 3.0 INTRODUCTION

This chapter deals with naturally occurring cases of canine acidophil cell hepatitis, which were identified histologically during the study. The main objective of the study was the experimental transmission of CACH; the investigation of field cases, although of interest, was not a major part of this study. To avoid a deluge of material, no request for suspected cases was made to general practitioners and comprehensive screening of all the cases passing through Glasgow University Veterinary School hospital and pathology department was not attempted. Adequate numbers of suspected CACH cases for investigation were received from interested practitioners and vet school staff. Not all of these cases turned out to be CACH and only those which did are described in this chapter.

The amount of information available about field cases was very variable. The pathological findings for each dog in section 3.2 are accompanied, whenever possible, by details of the case history and clinical findings. Some of the important features of the CACH field cases are summarized in table 3-1 and discussed in the conclusion (section 3.3).

A few of the dogs discussed in this chapter were used as sources of CACH infection for the dog and laboratory animal transmission experiments described in chapters 4 to 7. Material from CACH field cases was also used in some of the serological studies described in chapter 9.

## 3.1 MATERIALS AND METHODS

Field cases of canine acidophil cell hepatitis were diagnosed on the histopathological appearance of liver lesions. As explained in section 2.1, cases were obtained from various sources. Some veterinary practitioners sent

in material from suspected cases, of their own volition, after reading about the disease in the Veterinary Record (Jarrett and O'Neil, 1985; Jarrett et al., 1987). Workers in GUVS hospital, postmortem room and Canine Infectious Disease Research Unit (CIDRU) were kind enough to draw attention to other suspected cases of CACH. The CIDRU run a national diagnostic pathology service for practitioners. The postmortem room receive cases from the veterinary hospital and the pathology department, as well as monitoring cases from a disposal service performed for some local practitioners.

When it was possible to perform a postmortem, the protocol given in section 2.1.2 was followed. When formalin-fixed tissues were received, they were processed for histological examination as described in section 2.3. The haematological and biochemical analyses, performed on any blood samples received, are described in sections 2.2.5 and 2.2.6. As explained in section 2.1.1, it often proved difficult to follow the progress of the living cases identified from biopsy samples.

## 3.2 CASE HISTORIES

The first three CACH field cases described in this section (dogs A, F and G) died before the start of this study, but were included because of their role in the study. The field cases of CACH in this chapter are numbered in the chronological order of their biopsies or necropsies.

### 3.2.1 Field Case 1 / Dog A

Dog FC1, known as dog A, was used as the source case for the first rat, mouse and guinea pig transmission experiments and the second hamster experiment (sections 5.3, 6.2, 6.3 and 7.4 respectively). Its history, clinical details and gross pathology are described in this section,

but the description of dog A's liver histology is given in chapter 5 (section 5.3.2).

Dog A was an 8-month-old, male German shepherd dog, from the West of Scotland, with a 4-month history of occasional vomiting, intermittent diarrhoea, slight dullness and weight loss as well as ascites and polydipsia later. As a result of diuretic therapy, the dog was not ascitic when admitted to GUVS hospital but fluid then began to reform. On admission dog A had raised blood levels of ALT and AST (71 and 63 IU/l respectively), a very high BSP retention (52.5% at 30 minutes) and a reduced blood albumin level (23g/l). Its alkaline phosphatase level was 501 IU/l, the blood bilirubin level was 12μmol/l and there was bilirubin in the urine. Dog A had a normal blood ammonia level (67μmol/l) and normal haematology results apart from a low platelet count and the presence of target cells. A splenoportogram, performed to check for a congenital portacaval shunt, indicated instead an aquired shunt to the renal veins and capsules i.e. an accessory portal circulation.

When a laparotomy was performed, 6 days after admission, dog A's liver was found to be fibrous and covered with pale spots (up to 2mm in diameter). The entire liver was affected and it bled freely when a wedge biopsy was taken. Splenomegaly was present and more than 2 litres of ascitic fluid were removed from the abdomen. Some of the biopsy was processed for histology and the remainder was frozen in liquid nitrogen, for storage at -70°C along with some serum from the case.

Microscopy of dog A's liver biopsy revealed acidophil cell hepatitis with extensive fibrosis, dividing up the parenchyma, piecemeal necrosis and fatty vacuolation (see figures 5-1 and 5-2). A full description of the histological appearance is given in chapter 5 (section 5.3.2).

By 2 days after the operation dog A's wbc count had risen

to 22.8 $\times 10^9$/l, due to neutrophilia, but its ALT level had fallen to 47 IU/l (AST=64 IU/l, AP=561 IU/l and bilirubin 13$\mu$mol/l). The dog was discharged the following day, but the ascites later returned and by 1 week after the laparotomy dog A was dull, vomiting and exhibiting pica. It was immediately readmitted to the hospital and found to also be ataxic and pyrexic (40.5°C). It had a very high wbc count (36.2 $\times 10^9$/l) due to neutrophilia; markedly raised ALT, AST and creatinine levels (173 IU/l, 457 IU/l and 309 $\mu$mol/l respectively) and a raised blood urea (18.9mmol/l). Blood ammonia and bilirubin levels were normal (50 and 3$\mu$mol/l respectively). The morning after admission dog A collapsed, with fits, pyrexia (41.8°C) and a raised blood ammonia level (122$\mu$mol/l), before dying. A postmortem revealed a postoperative, suppurative peritonitis in addition to the chronic hepatitis already diagnosed. There was no ascites but the liver was small, firm and granular with an accessory portal circulation. The spleen was nodular with fibrous bands. There was also oesophagitis and evidence of inhalation of vomit.

Histological diagnosis: CACH and fibrosis, death was later precipitated by postoperative complications.

### 3.2.2 Field Case 2 / Dog F

Dog FC2, known as dog F, was the field case of CACH used by Jarrett and O'Neil (1985) to infect dog D. Experimental dog D was used in this study as the source case for the fifth rat and first hamster transmission experiments (sections 5.7 and 7.3 respectively).

Dog F was a 13-month-old, male cocker spaniel from the West of Scotland. During the 6 months after its vaccinations the dog had been presented to the veterinary surgeon with sarcoptic mange, a brief bout of diarrhoea, conjunctivitis with periorbital eczema and eczematous otitis. It was then presented as a 1-year-old for treatment of

to 22.8 $\times 10^9$/l, due to neutrophilia, but its ALT level had fallen to 47 IU/l (AST=64 IU/l, AP=561 IU/l and bilirubin 13μmol/l). The dog was discharged the following day, but the ascites later returned and by 1 week after the laparotomy dog A was dull, vomiting and exhibiting pica. It was immediately readmitted to the hospital and found to also be ataxic and pyrexic (40.5°C). It had a very high wbc count (36.2 $\times 10^9$/l) due to neutrophilia; markedly raised ALT, AST and creatinine levels (173 IU/l, 457 IU/l and 309 μmol/l respectively) and a raised blood urea (18.9mmol/l). Blood ammonia and bilirubin levels were normal (50 and 3μmol/l respectively). The morning after admission dog A collapsed, with fits, pyrexia (41.8°C) and a raised blood ammonia level (122μmol/l), before dying. A postmortem revealed a postoperative, suppurative peritonitis in addition to the chronic hepatitis already diagnosed. There was no ascites but the liver was small, firm and granular with an accessory portal circulation. The spleen was nodular with fibrous bands. There was also oesophagitis and evidence of inhalation of vomit.

Histological diagnosis: CACH and fibrosis, death was later precipitated by postoperative complications.

### 3.2.2 Field Case 2 / Dog F

Dog FC2, known as dog F, was the field case of CACH used by Jarrett and O'Neil (1985) to infect dog D. Experimental dog D was used in this study as the source case for the fifth rat and first hamster transmission experiments (sections 5.7 and 7.3 respectively).

Dog F was a 13-month-old, male cocker spaniel from the West of Scotland. During the 6 months after its vaccinations the dog had been presented to the veterinary surgeon with sarcoptic mange, a brief bout of diarrhoea, conjunctivitis with periorbital eczema and eczematous otitis. It was then presented as a 1-year-old for treatment of

abscessed anal glands. By the following day the dog was shivering, anorexic and pyrexic (40°C). It appeared to respond to antibiotic therapy, but within a fortnight it was back at the vet with abdominal distention, anorexia, a temperature of 39.5°C and an apparently enlarged liver. Since obvious ascites developed over a few days and dog F remained anorexic and began to cough, it was referred to GUVS hospital.

When admitted, dog F had been ill for 4 weeks. It exhibited abdominal enlargement, weight loss, occasional coughing, a reduced appetite and behavioural changes (lethargy with occasional aggression), but had a normal water intake. Blood tests revealed a raised wbc count (25 $x10^9/l$) due to neutrophilia, numerous target cells, a low blood albumin level (17g/l), a high BSP retention (35.5%) and ALT, AST and AP levels of 53, 87 and 481 IU/l respectively. Blood urea and bilirubin levels were within normal ranges. Two and a half litres of clear fluid, with a low albumin content (4g/l), were removed from the abdomen but dog F did not improve and was destroyed. At postmortem the liver was found to be small and firm with several hyperplastic nodules and an accessory portal circulation.

Histological examination of dog F's liver revealed diffuse chronic hepatitis with extensive fibrosis throughout the parenchyma, as can be seen in figure 5-20. There was fine dissecting fibrosis between individual cells and around small groups of hepatocytes, as well as fibrous linking of portal tracts. Acidophil cells were present in varying numbers around portal tracts, although the latter were often difficult to locate due to the degree of fibrosis. Piecemeal necrosis and vacuolation were prominent features and there was a little scattered cell infiltrate. Three of five liver sections examined had one hyperplastic nodule each.

Histological diagnosis: CACH and fibrosis, progressing to early macronodular cirrhosis.

### 3.2.3 Field Case 3 / Dog G

Dog FC3, known as dog G, was the CACH field case used by Jarrett and O'Neil (1985) to infect dog E. Experimental dog E was used as the source case for the second dog transmission experiment of this study (section 4.4).

Dog G was a 19-month-old, female German shepherd dog from the West of Scotland. It was presented to the veterinary surgeon with a 1-month history of abdominal swelling, polydipsia, weight loss and an increased frequency of vomiting; although still bright and active. The animal had vomited infrequently before, but had started to vomit about every other day usually 10-30 minutes after eating. The vet operated on dog G, identifying an accessory portal circulation and removing 9 litres of clear fluid from the abdomen.

When dog G was younger there had been an episode of complete anorexia for one week, accompanied by vomiting of fluid and water, abdominal pain and slight diarrhoea; but the animal had improved after intravenous fluid therapy.

Dog G was admitted to GUVS hospital the day after its operation and was found to have raised blood levels of ALT and AST (177 and 173 IU/l respectively) and a low albumin level of 16g/l. Target cells, seen in a blood film, were also indicative of liver disease. The abdominal fluid reformed and dog G was euthanased. At postmortem there was marked ascites and a pronounced accessory portal circulation. The liver was bronze in colour, very reduced in size, but soft to cut. There were only two or three nodules visible on the liver. Many small urinary calculi were found in the bladder and there was one in the left kidney.

Histological examination of dog G's liver revealed acidophil cell hepatitis (see figure 4-50) with varying numbers of acidophil cells at most portal tracts. There was a slight increase in the amount of fibrous tissue in a few portal tracts, with very fine fibrous processes extending out from them, and a little fatty vacuolation. Eosinophilic intranuclear inclusion bodies were present, some of them were found in the limiting plate hepatocytes of portal tracts exhibiting an acidophil cell reaction.

Histological diagnosis: CACH

### 3.2.4 Field Case 4

Dog FC4 was an adult, female German shepherd dog from the West of Scotland. At postmortem the veterinary surgeon found ascites and an accessory portal circulation.

Microscopy, of the sample of FC4's liver received, revealed a marked acidophil cell reaction. Numerous acidophilic hepatocytes were seen in the limiting plates of many portal tracts and the majority of portal tracts were affected to some extent. There was an increase in the amount of fibrous tissue in portal tracts, with small fibrous processes extending out from some. In other areas little fibrous networks were seen in the parenchyma. Piecemeal necrosis was evident and there was marked fatty vacuolation, mainly in central zones. Occasional neutrophils were scattered in the sinusoids, but cellular infiltration was not a feature of this lesion.

Histological diagnosis: CACH

### 3.2.5 Field Case 5

Dog FC5 was a 4-year-old male cocker spaniel, from the West of Scotland. It had a history of vomiting after food for 2 days, followed by anorexia and a cough. When

presented at GUVS hospital the dog was thin, ascitic, dull and had an abnormal gait due to weakness. On admission dog FC5 was found to have a markedly increased BSP retention (49.5%); very high AP, AST and ALT blood levels (1504, 125 and 154 IU/l respectively); a raised bililrubin level (19μmol/l) and a low albumin concentration (17g/l). Numerous target cells seen in a blood film were also indicative of a liver lesion. Bilirubin was found in the urine. Despite the removal of 2¼ litres of clear yellow fluid from the abdomen the ascites recurred. Analysis of the ascitic fluid revealed it to be a transudate, low in protein (4g/l). The blood ammonia level was slightly raised (96μmol/l) when measured a week after admission. During 12 days of hospitalisation the dog was diarrhoeic twice and vomited once, it was also polydipsic. Dog FC5 was then destroyed and at postmortem another 2 litres of abdominal fluid were collected. The animal exhibited marked portal hypertension with an accessory portal circulation. Its liver was reduced in size and mottled yellow in colour. Small nodules (2-3cm in diameter) protruded from the surface of all of the liver lobes except the right lateral lobe.

Microscopy of dog FC5's liver revealed fibrosis of portal tracts with processes extending out to form fine pericellular fibrous networks in places. There were also bands, of loose fibrous tissue and vacuolated hepatocytes, dividing up the parenchyma and some nodular hyperplasia. Piecemeal necrosis and biliary thrombi were obvious and megalocytes were also seen. Acidophil cells were present in the limiting plates of many portal tracts, in hyperplastic nodules and scattered in the fatty fibrous tracts. The marked fatty vacuolation was in a bridging pattern between portal tracts. Many of the affected cells were filled by single large droplets. A scattered cellular infiltrate, mainly neutrophils, was present especially in the fatty fibrous tracts. There were also occasional foci of mononuclear cells.

Histological diagnosis: CACH and cirrhosis

### 3.2.6 Field Case 6

Dog FC6, a 4-month-old standard poodle from the West of Scotland, had been bred by its owner from her own bitch. At 5 days of age a littermate had developed diarrhoea and become dehydrated. The problem had spread rapidly to the rest of the litter and two of the pups had died. Faecal bacteriology at the time had revealed a mixed culture of E. coli, streptococci and ß toxin-producing staphylococci. Dog FC6 was well and gaining weight when vaccinated, at 12 and 14 weeks of age. It was then presented to the veterinary surgeon at 16 weeks of age because it was off colour, with a reduced appetite and increased thirst. At that time, FC6 was smaller and thinner than a littermate. The vet found the dog to be ascitic, with normal blood biochemistry except for raised AST and alkaline phosphatase levels (88 and 2178 IU/l respectively) and a reduced albumin level of 25g/l (ALT=31 IU/l).

When there was no improvement in its condition, despite diuretic and antibiotic therapy, the dog was referred to GUVS hospital. On admission, at 18 weeks of age, dog FC6 was dull and still exhibiting the same clinical signs. Its blood biochemistry results were by then indicative of liver disease: markedly raised ALT and AST levels (169 and 125 IU/l respectively), low blood albumin (17g/l), slightly increased bilirubin level (12µmol/l) and increased BSP retention (31.7%). The dog had dilute urine and was diarrhoeic on 2 of its 3 days in the hospital. At postmortem, 500ml of clear fluid was found in the abdomen. The liver was slightly shrunken, with numerous nodules (2mm to 2cm in diameter) protruding from the surface.

There was a marked variation in the histological appearance of sections from different sites in FC6's liver. Some sections exhibited extensive fibrosis and hepatocyte loss:

with areas of dense fibrous tissue around individual hepatocytes, as well as areas of lacy pericellular fibrous networks and a few hyperplastic nodules which were the only easily recognisable parenchyma. Acidophil cells were present in the nodules, around structures like bile ducts, as well as in the fibrosed areas. Other liver sections exhibited only a little fibrosis but a more prominent acidophil cell reaction. In these, most portal tracts were affected and in places the acidophil cells extended out to bridge between portal tracts. Fine fibrous processes extended out from some portal tracts and there were areas of fibrous tissue full of bile ducts and groups of hepatocytes. Both types of FC6's liver sections had a lot of intracellular glycogen, except in the regenerative nodules.

Histological diagnosis: CACH and cirrhosis

### 3.2.7 Field Case 7

Dog FC7 was a 14-month-old, female greyhound, which had been imported from Ireland to an English racing kennel. She exhibited a sudden onset of jaundice and died, despite antibiotic therapy, within 1 week of arrival. A necropsy was performed and tissue samples sent to the CIDRU.

Microscopy of dog FC7's liver sample revealed hepatitis, with no evidence of ICH or leptospirosis. Small numbers of acidophil cells were seen around some portal tracts. There was a slight increase in the amount of fibrous tissue at some portal tracts, with a few tiny fibrous processes extending out from them. Some piecemeal necrosis was present and a little vacuolation of hepatocytes, but no obvious cellular infiltration. The liver lesions were strongly suggestive of CACH, but were not of sufficient severity to permit a definite diagnosis of CACH.

Another greyhound bitch, imported along with dog FC7, also

exhibited jaundice but recovered. A blood sample was collected from the surviving bitch 1½ weeks after FC7's death, by which time the animal appeared to be fit and healthy, having lost all trace of the jaundice. However, biochemical analysis of the blood sample revealed a very high ALT level of 372 IU/l, with normal AST and bilirubin levels (41 IU/l and 7μmols/l). This indicated that, despite an apparent clinical recovery, there was still a degree of liver damage present in the surviving bitch.

Histological diagnosis: Acute hepatitis, probably CACH

3.2.8 Field Case 8

Dog FC8 was a 7-year-old retriever from the South of England. It had a sudden onset of illness and was presented to the local veterinary surgeon with obvious pallor of the mucous membranes and slight jaundice, but no other clinical signs. It was the only dog in the household and there were no instances of canine or human illness in the immediate neighbourhood. The dog was found to be anaemic (PCV=18%) with a prolonged clotting time and a neutrophilia with left shift. It had very high blood levels of alkaline phosphatase and ALT, with a normal blood urea level. Dog FC8 died shortly afterwards and the practitioner performed a postmortem. Haemorrhages were found throughout the body cavities and the liver was swollen, pale and firm to cut. No other lesions were found.

Histological examination of FC8's liver sample, received from the practitioner, revealed diffuse widespread lesions of acidophil cell hepatitis. There were small numbers of acidophil cells round some portal tracts and moderate numbers round many other portal tracts. Eosinophilic, intranuclear inclusion bodies were a noticeable feature in the liver section. There was only a very slight increase in fibrous tissue and very occasional evidence of piecemeal necrosis.

Another CACH field case (FC9) was later received from FC8's veterinary practice (see section 3.2.9).

Histological diagnosis: Acute CACH

### 3.2.9 Field Case 9

Dog FC9 was a 6-year-old, male crossbreed. This animal presented, to the same South of England veterinary practice as dog FC8, with emesis and diarrhoea. Its condition declined rapidly, despite antibiotic therapy, and it was euthanased 2 days after presentation (within a month of FC8's death). The veterinary surgeon who performed the postmortem described the liver as "grossly abnormal". Although this animal attended the same practice as dog FC8, it did not live in the same neighbourhood.

The most noticeable histological feature of FC9's liver was extreme fatty vacuolation of most hepatocytes. Many of the affected cells had a foamy appearance due to the presence of several small vacuoles; in others, these had coalesced to form large vacuoles. The least affected hepatocytes were often those around portal tracts, some of which exhibited an acidophilic reaction. The severity of the vacuolation made it difficult to assess the extent of the hepatitis, but although there were usually only small numbers of acidophil cells in the limiting plates, the majority of portal tracts appeared to be affected. There was a little increase in the fibrous tissue content of the liver and a few small bile plugs. Eosinophilic intranuclear inclusion bodies were found throughout the liver section and there was a little scattered cell infiltrate.

Histological diagnosis: CACH and fatty liver

### 3.2.10 Field Case 10 / Dog B

Dog FC10, known as dog B, was a 9-year-old, neutered

female German shepherd dog. It was used as the source case for the first dog and second rat transmission experiments of this study (sections 4.3 and 5.4). Dog B's pathology is described fully in chapter 4 (section 4.3.2) along with a brief summary of its clinical history.

Dog B was first referred to GUVS hospital, at around 4 years of age, exhibiting anorexia, weight loss, dullness, vomiting (usually 2-3 hours after food), watery diarrhoea, ascites and scaly skin. On admission the dog was also found to be halitotic, proteinuric and anaemic (PCV=24.3%), with a neutrophilic leucocytosis and a low platelet count. It had very high blood levels of ALT and AST (140 and 195 IU/l respectively), a low blood albumin level (17g/l) and an alkaline phosphatase level of 343 IU/l. Radiography indicated a reduction in liver size. A large volume of ascitic fluid, with a low protein content (4g/l), was drained from the dog's abdomen. Despite a clinical and haematological improvement, with treatment during hospitalisation, the dog's blood biochemistry results worsened; especially the AP and ALT levels which rose dramatically. Three days after admission dog B was found to have a high BSP retention of 22.8% and AP, ALT and AST levels of 568, 162 and 57 IU/l respectively. After 10 days in hospital dog B's BSP retention (10.3%) was improved, although still abnormal; but its AP, ALT and AST levels had risen to 1320, 313 and 140 IU/l respectively. It also had slightly raised blood levels of urea, bilirubin and creatinine (10mmol/l, 12μmol/l and 159μmol/l respectively). A clinical diagnosis of cirrhosis was made and a poor prognosis was given.

Ascites recurred within a month and dog B was readmitted to GUVS hospital for abdominal drainage. Although it was polydipsic and still had an abnormal BSP retention (12.5%), some of its blood biochemistry and haematology results had improved a little (AP=355 IU/l, ALT=28 IU/l and AST=97 IU/l). It has a raised blood sugar level

(8.3mmol/l) and low phosphate level (0.98mmol/l). At a checkup a fortnight after the second hospitalisation AP, ALT and AST blood levels were 422, 50 and 44 IU/l respectively. After this episode the dog remained reasonably well for 2 years, apart from gynaecological problems which necessitated neutering.

At 6 years of age dog B was readmitted to the veterinary hospital because of restlessness, vomiting and continual wandering around. It was also pyrexic (39.8°C), breathless, panting, polydipsic, diarrhoeic and appeared to be exhibiting mental aberrations. The dog was found to have markedly raised blood levels of ALT, AST and ammonia (553 IU/l, 1914 IU/l and 384µmol/l respectively), a high BSP retention of 22.5%, low blood albumin (19g/l) and phosphate levels and an AP level of 244 IU/l. Haematology results were normal apart from rbc morphology; target cells were present. Despite a clinical diagnosis of terminal hepatic failure, the owners were determined to maintain the dog using dietary and supportive therapy. At a checkup, 4 months later, dog B was bright with normal blood biochemistry apart from raised aminotransferase levels (ALT=110 and AST=62 IU/l) and a low blood albumin level. When checked after another year the dog was found to be anaemic (PCV=28.8%) with leucocytosis. It had an increased BSP retention of 25.5%, ALT and AST levels of 62 and 46 IU/l and low blood phosphate and albumin levels.

Dog B was finally presented for destruction at around 9 years of age, nearly 11 months after its last checkup, with a leaking lesion on its oedematous right hind limb and nervous signs which included extreme dullness and being unaware of its surroundings. The dog had a high BSP retention of 25.4%, a slightly raised bilirubin level (16µmol/l), low blood phosphate and albumin levels, but unremarkable aminotransferase levels (ALT=47 and AST=38 IU/l).

At postmortem dog B's liver was found to be firm and shrunken with one cystic nodule, but there was no ascites. Microscopy of the liver revealed a marked acidophil cell reaction, including occasional bridging lesions between portal tracts (see figure 4-1). The degree of fibrosis varied between sections from different sites in the liver. Nodular hyperplasia was only seen in the sections with the most fibrosis. Piecemeal necrosis and hepatocellular vacuolation were also features of the liver pathology. A more detailed description of dog B's histopathology is given in chapter 4 (section 4.3.2).

Histological diagnosis: Chronic CACH with cirrhosis

3.2.11 Field Case 11

Dog FC11 was a 6-year-old, male Irish wolfhound from Southeast England. The dog presented with sudden onset pyrexia (41.1°C), anorexia, vomiting, mild lameness, congested mucous membranes and a purulent nasal discharge. A urine sample was found to contain high levels of bilirubin and blood. Despite antibiotics and intravenous fluid therapy, the animal did not improve and was euthanased by the veterinary surgeon 4 days after presentation. Distemper or adenovirus infection was suspected and blood and tissue samples were sent to the CIDRU for investigation. Blood titres were found to measure 128 for CDV and 16 for CAV.

Microscopy of a postmortem liver sample from FC11 failed to reveal any evidence of infection with CDV, CAV or leptospires. However, many portal tracts had numerous acidophil cells in their limiting plates. There was a little increase in the amount of fibrous tissue in many portal tracts, some small areas of delicate fibrous networks in the parenchyma and fibrosis of some of the small and medium-sized veins, which had fibrous processes extending out from them. The small amount of cellular

infiltrate present (monocuclear cells and occasional neutrophils) was mainly confined to areas of fibrosis. Piecemeal necrosis was seen at some portal tracts and in the little fibrous networks. Fatty vacuolation occurred in foci and in individual scattered cells. Eosinophilic intranuclear inclusion bodies were also seen.

Histological diagnosis: CACH

3.2.12 Field Case 12

Dog FC12 was a beagle from a large commercial establishment in Scotland.

The only material available for inspection, from this animal, was a histological section of liver, which had been processed by another laboratory. Microscopy of this revealed acidophil cell hepatitis, with varying numbers of acidophil cells grouped around most portal tracts and some scattered in the parenchyma. Cell infiltration of some portal tracts and central veins, with neutrophils and mononuclear cells, was a noticeable feature in the section examined. The extent of fibrosis varied: with a little increase in fibrous tissue at some portal tracts, but a general increase in other areas disrupting the architecture. A little piecemeal necrosis was also seen.

Histological diagnosis: CACH

3.2.13 Field Case 13

Dog FC13 was a 20-month-old, male Doberman pinscher from the North of England. The dog had a 5-month history of being in poor condition, with occasional vomiting and persistent soft motions. The animal's coat was thin, but it had had skin problems from an early age. Laboratory tests for malabsorption and pancreatic problems had proved negative. Blood biochemical analysis revealed raised ALT

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Fig 3-1 Liver biopsy of CACH Field Case 13
(Mass tri x175) 20-month-old M Doberman Pin.
ACs lining fibrous process extending out from PT


Fig 3-2 Liver biopsy of cirrhotic CACH Field Case 14
(Mass tri x87.5) 4-year-old F cocker spaniel
ACs at portal tracts and some fibrous septa

and GGT levels, an alkaline phosphatase level of 188 IU/l, and normal levels of blood urea and protein. Haematology parameters were normal apart from a raised eosinophil count of 11.1 $\times 10^9$/l (17% of wbc count).

The practitioner performed a laparotomy, for the collection of a wedge liver biopsy, during which the liver was seen to have an exaggerated "nutmeg" pattern and was thought to possibly be reduced in size. After the operation the dog was put on a restricted protein diet and initially appeared to be doing quite well and maintaining weight. Unfortunately, attempts to follow the progress of dog FC13 further were unsuccessful.

The liver biopsy sample, sent to the CIDRU, was very small; histological examination of it revealed mild hepatitis lesions compatible with a diagnosis of CACH. Although there were only small portal tracts present in the three small sections examined, several of them had small numbers of acidophil cells round them and one or two per section were surrounded by larger numbers of acidophil cells. Figure 3-1 shows a few acidophil cells at one little portal tract, with more extending out from it along a fine fibrous process. However, fibrosis was not an obvious feature of this case; there was only a slight increase in fibrous tissue in some portal tracts.

Histological diagnosis: CACH

### 3.2.14 Field Case 14

Dog FC14 was a 4-year-old, female cocker spaniel from South Wales. The local veterinary surgeon had diagnosed liver failure and performed a laparotomy. The liver was found to be pale, nodular and slightly enlarged. The dog was anaemic and mildly jaundiced. A wedge liver biopsy was sent to the CIDRU to obtain a prognosis. There had been no history of illness in this dog's family, nor had there

been any outbreaks of disease in the neighbourhood or any human hepatitis.

The daughter of dog FC14, a 2-year-old cocker spaniel living in the same house, appeared to be healthy. Biochemical analysis of a blood sample, from the younger animal, produced normal results apart from a high AST level (114 IU/l), which may have been the result of haemolysis of the sample.

Histological examination of FC14's liver biopsy confirmed the presence of severe progressive liver disease. There was extensive fibrosis, loss of hepatocytes, reactive nodular hyperplasia, a marked acidophil cell reaction, piecemeal necrosis, vacuolar degeneration of hepatocytes and some inflammatory infiltrate (neutrophils and lymphocytes) which was mainly in some of the fibrous bands. The histological features of this case, including the acidophil cell reaction around portal tracts and some fibrous bands, can be seen in figure 3-2.

A poor prognosis of imminent terminal liver failure was given.

Histological diagnosis: CACH and cirrhosis

3.2.15 Field Case 15

Dog FC15 was a 7-year-old male German shepherd dog from Northern Ireland which presented to the veterinary surgeon with ascites. A laparotomy revealed peritonitis as well as free abdominal fluid; a liver biopsy was taken and sent to the CIDRU. This animal had never been boarded at a commercial kennel and there had been no disease outbreaks in the neighbourhood, nor was there any history of human hepatitis in its contacts.

Microscopy of dog FC15's liver biopsy revealed a marked

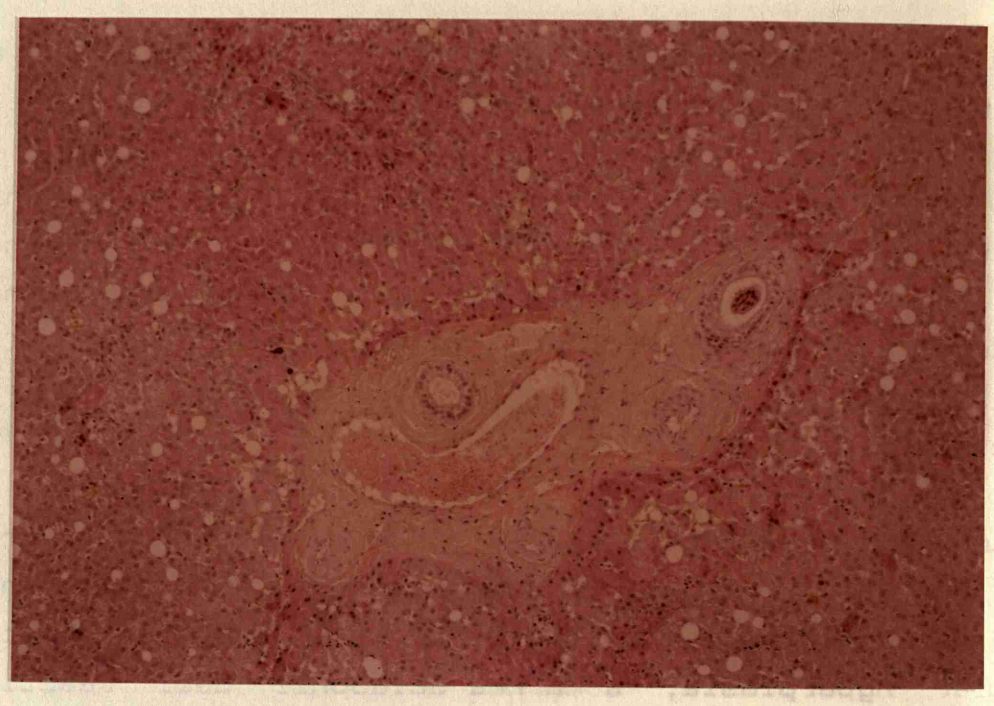

Fig 3-3 Liver biopsy of CACH Field Case 15 (H&E x87.5)
7-year-old M GSD ACs round a portal tract

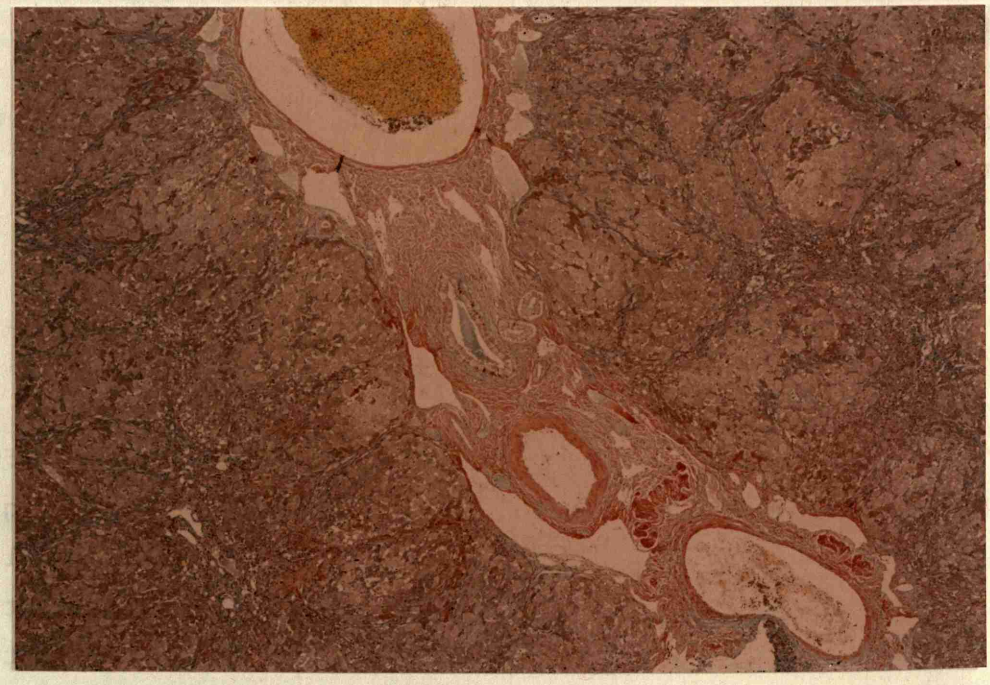

Fig 3-4 Liver of CACH Field Case 16 (Mass tri x35)
11-month-old M GSD ACs at PIs and fibrous septa

acidophil cell reaction in the limiting plates of many portal tracts. Figure 3-3 shows a typical portal tract with acidophil cells forming most of its limiting plate. The surface of the liver was irregular, indicating collapse of the parenchyma. The fibrous capsule was thickened in places and there was subcapsular inflammatory cell infiltration. Eosinophilic intranuclear inclusion bodies were present and some hepatocellular vacuolation, but only as scattered individual cells. Shikita's orcein and rubeanic acid stains of liver sections, for hepatitis B and copper respectively, proved negative.

One month after the biopsy, dog FC15 still had gross ascites, with considerable loss of weight and muscle bulk. Unfortunately it was not possible to follow the progress of this case further since the vet did not see the dog again. Material from two other CACH field cases (FC18 and FC19) was later received from FC15's veterinary practice.

Histological diagnosis: CACH

### 3.2.16 Field Case 16

Dog FC16, a male German shepherd dog, was presented to a veterinary practice in Central England, at 9 months of age, with severe ascites. The owners believed that the swelling had developed within 1 week. The animal was also off its food, pyrexic (39.4°C), polydipsic and had congested mucous membranes. After 4 days of antibiotic and diuretic therapy its temperature was down to 38.8°C. A large volume (2-3 litres) of clear proteinaceous fluid was drained from the abdomen and prednisolone was added to the treatment regime. After a further week of therapy the dog was lively and eating well, although it still had some ascites. Blood samples taken at that time contained raised levels of alkaline phosphatase, ALT and AST (446, 99 and 90 IU/l respectively) but normal levels of bilirubin, GGT (12 IU/l), cholesterol and salts. Haematology results were

normal, apart from a slightly low platelet count. Within 4 weeks of the first presentation, the ascites had gone and dog FC16 was progressing well. However, during the following month the animal's condition steadily deteriorated until, by 8 weeks after the initial presentation, it was in an extremely depressed state. The dog was then euthanased, after an exploratory laparotomy, at 11 months of age and a sample of liver was collected.

Microscopical investigation, of the sample of FC16's liver received, suggested an active hepatitis which had progressed into cirrhosis. There was fibrosis of portal tracts with processes extending out from them, creating fibrous linking in places. These linking bands of loose fibrous tissue contained hepatocytes, bile ducts and cell infiltrate. Fibrosis had also resulted in fine fibrous networks in some areas of parenchyma. The fibrous tracts and nodular hepatocellular hyperplasia disrupted the hepatic architecture. Acidophilic hepatocytes were arranged around many portal tracts and along many of the fibrous tracts, as well as scattered in the parencyhma (see figure 3-4). Cholestasis and lymphatic dilatation were also evident and there was an inflammatory cell infiltrate (of neutrophils with some mononuclear cells) especially in the fibrous tracts.

Histological diagnosis: CACH and cirrhosis

### 3.2.17 Field Case 17

Dog FC17, a 2-year-old male Doberman pinscher, came from the same county in Southeast England as dog FC11. However, it attended a different practice, in a different town, 20 months after FC11 was euthanased. It presented with weight loss to the point of emaciation and polyphagia. Very high blood levels of alkaline phosphatase and bilirubin were found (31649 IU/l and 120μmol/l respectively), but the aminotransferase levels were not recorded. A liver biopsy

was performed by the veterinary surgeon and the sample was sent to the CIDRU.

Microscopy of FC17's liver revealed canine acidophil cell hepatitis with developing fibrosis. Acidophilic, shrunken hepatocytes were found around both portal tracts and fibrous areas. In places the liver surface was indented by areas of collapse and fibrosis. At other points on the surface, similar shaped areas of acidophilic hepatocytes were suggestive of an earlier stage in the same progressive process. There was some fibrous bridging between portal tracts and veins and piecemeal necrosis was evident. Fatty vacuolation was not a feature of this case, but there was microvesiculation of some hepatocytes caught in the fibrous networks. A little, mainly mononuclear, cellular infiltrate was found in the fibrous areas.

Histological diagnosis: CACH and fibrosis

3.2.18 Field Case 18

Dog FC18, a 12-year-old male Labrador, attended the same veterinary practice in Northern Ireland as dog FC15, about 9 months after the latter was biopsied. It was polydipsic, stiff and in pain. Biochemical analysis of a blood sample revealed an ALT level of 45 IU/l. A liver biopsy was performed, but the dog died within hours of the operation. A year before this presentation, dog FC18 had been castrated to remove interstitial cell tumours of both testes.

Microscopy of the tiny sample of FC18's liver supplied to the CIDRU, revealed acidophil cell hepatitis and fibrosis. Increased fibrous tissue was seen at portal tracts and there was evidence of early fibrous bridging lesions. Included in the tiny section were a few small areas of fibrous tissue, with bile ducts and hepatocellular rosette formation. There was a little piecemeal necrosis and a

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Fig 3-5 Liver biopsy of CACH Field Case 19 (Mass-OG X56)
12-year-old F Dalmatian (↖)acidophilic bridging

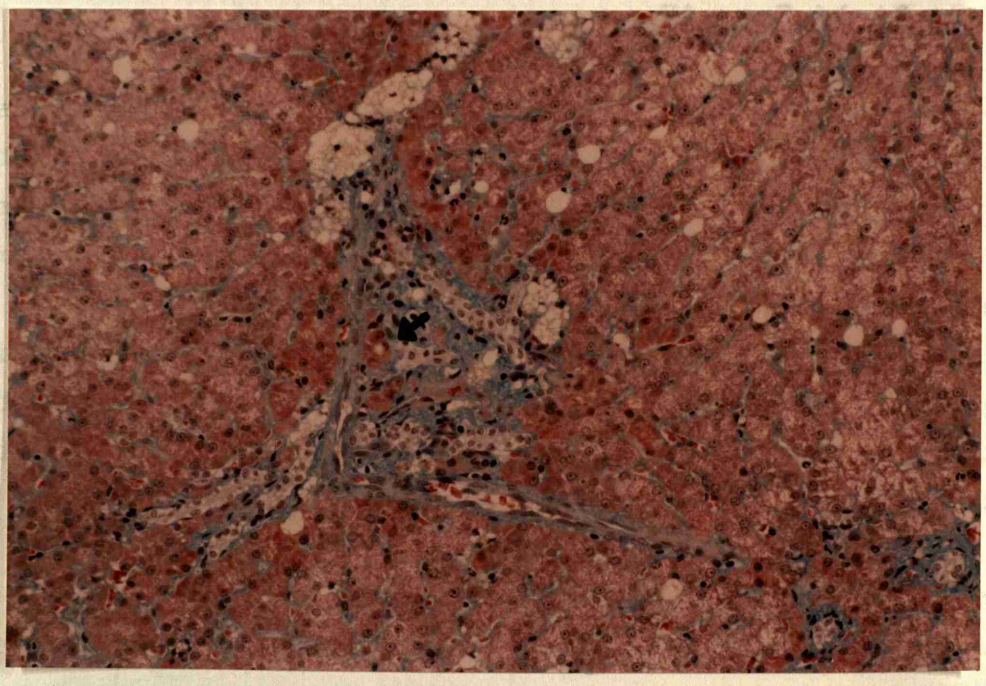

Fig 3-6 Liver biopsy of CACH Field Case 19
(Mass tri x175) (↙) Hepatocyte rosette formation

little scattered infiltrate, which included neutrophils. The foci of vacuolation present were often in the areas of fibrosis.

A third CACH field case (FC19) was later received from the same veterinary practice.

Histological diagnosis: CACH and fibrosis

3.2.19 Field Case 19

Dog FC19, a 12-year-old female Dalmatian, attended the same Northern Irish veterinary practice as dogs FC15 and FC18. It presented with a 2-week history of poor appetite, progressing to anorexia. Since a liver disorder was suspected a biopsy was performed (1 month after FC18's liver biopsy), during which the liver was seen to have a fatty degenerate appearance.

Microscopy of FC19's small liver biopsy sample, sent to the CIDRU, revealed numerous acidophil cells at many portal tracts, with acidophilic bridging between some portal tracts (see figure 3-5). Foci of vacuolation were also seen and they often had acidophil cells at them. There was a little increase in the amount of fibrous tissue at portal tracts. Figure 3-6 shows hepatocytes, encorporated in fibrous tissue, taking up a ductular appearance i.e. hepatocyte rosette formation. Occasional eosinophilic intranuclear inclusion bodies were seen.

A guarded prognosis was given, but attempts to follow the progress of this case were unsuccessful.

Histological diagnosis: CACH

### 3.2.20 Field Case 20

Dog FC20, a 1-year-old male Doberman pinscher, came from Central England. It was originally presented to the veterinary surgeon with anorexia and excessive salivation, which had persisted after a bout of vomiting and diarrhoea had cleared without treatment. Nearly 8 weeks later the animal was presented again, exhibiting polydipsia, weight loss, abdominal pain and pyrexia (39.4°C). Urine tested showed a high bilirubin level and moderate proteinuria. Antibiotics and B vitamins were prescribed, but the dog started vomiting the following day. It was found to have a raised wbc count with a neutrophilia, elevated blood levels of liver enzymes and a massively raised CPK level. Despite an initial response to treatment, after 3 days it began to show vague nervous signs, became unaware of its surroundings and later destructive. Within a week of the second presentation, dog FC20 was presented in extremis and euthanased. The vet performed a postmortem and found the liver to be enlarged and knobbly with rounded margins.

Dog FC20's owner had another 1-year-old male dog; it had had a severe bout of vomiting and diarrhoea, about a month previously, but had responded to intensive therapy. Around the same time the veterinary surgeon had seen a few cases of similar acute liver problems in other dogs. One in particular, a 3-year-old neutered female Shetland sheep-dog, was a near neighbour of dog FC20. It was exhibiting intermittent signs of liver disease including: dullness, polydipsia, pyrexia and increased blood levels of liver enzymes.

Microscopy of dog FC20's liver revealed numerous acidophil cells around some portal tracts and varying numbers around many others. There was an increase in the amount of fibrous tissue at portal tracts and central veins as well as some early fibrous bridging lesions. Piecemeal necrosis was obvious at portal tracts, areas of fibrosis, central

| DOG No. | Breed | Age | Sex | Outcome | AC Score | Fibrosis Score | Cirrh. | Piece-Nec | Vacuol | Infiltration | Cholestasis | IN IBs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FC1 | GSD | 8m | M | b/d | +++ + | ++ ++ | - | ✓ | ✓ | ✓ | - | - |
| FC2 | CocSp | 13m | M | euth | +++ | ++ ++ | ✓ | ✓ | ✓ | ✓ | - | - |
| FC3 | GSD | 19m | F | euth | +++ + | + | - | ✓ | ✓ | - | - | - |
| FC4 | GSD | adult | F | died | +++ ++ | ++ | - | ✓ | ✓ | - | - | - |
| FC5 | CocSp | 4y | M | euth | +++ + | ++ ++ | ✓ | ✓ | ✓ | ✓ | ✓ | - |
| FC6 | Pood | 4m | F | euth | +++ + | ++ ++ | ✓ | ✓ | ✓ | ✓ | - | - |
| FC7 | GreyH | 14m | F | euth | ++ | + | - | ✓ | ✓ | - | - | - |
| FC8 | Retr | 7y | U | euth | +++ | + | - | ✓ | - | - | - | ✓ |
| FC9 | X | 6y | M | euth | +++ | + | - | - | ✓ | ✓ | ✓ | ✓ |
| FC10 | GSD | 9y | FN | euth | +++ ++ | ++ + | ✓ | ✓ | ✓ | - | - | - |
| FC11 | IWH | 6y | M | euth | +++ + | ++ | - | ✓ | ✓ | ✓ | - | ✓ |
| FC12 | Beagl | U | U | died | +++ + | ++ | - | ✓ | - | ✓ | - | - |
| FC13 | DobP | 20m | M | biop | +++ + | + | - | - | - | - | - | ✓ |
| FC14 | CocSp | 4y | F | biop | +++ + | ++ ++ | ✓ | ✓ | ✓ | ✓ | - | - |
| FC15 | GSD | 7y | M | biop | +++ + | + | - | ✓ | ✓ | - | - | ✓ |
| FC16 | GSD | 11m | M | euth | +++ + | ++ ++ | ✓ | ✓ | ✓ | ✓ | ✓ | - |
| FC17 | DobP | 2y | M | biop | +++ + | ++ + | - | ✓ | - | ✓ | - | - |
| FC18 | Lab | 12y | MN | b/d | +++ + | ++ | - | ✓ | ✓ | ✓ | - | - |
| FC19 | Dalm | 12y | F | biop | +++ ++ | + | - | ✓ | ✓ | - | - | ✓ |
| FC20 | DobP | 1y | M | euth | +++ ++ | ++ | - | ✓ | - | - | - | ✓ |

Table 3-1 Summary of histological features of CACH field cases

KEY:
Acidophil Cell Score: - to ++++++, +++ or more considered positive CACH case
Fibrosis Score: - = normal + = slight increase
++ = increased, processes extending out from portal tracts
+++ = increased further with some fibrous bridging
++++ = severe fibrosis dividing up parenchyma
U = unknown - = absent ✓ = present
Outcome: euthanased, died or biopsy, b/d= died after biopsy
Piec.Nec = piecemealnecrosis Vacuol = fatty vacuolation
IN IBs = eosinophilic intranuclear inclusion bodies

veins and larger veins. Eosinophilic intranuclear inclusion bodies were also easily identified. A reticulin stain revealed some distortion of hepatic architecture, with an increase in the reticulin content of the liver. There was shrinkage or compression of hepatic cords in some places, as well as what appeared to be a nodule suggesting the start of a hyperplastic response by hepatocytes.

Histological diagnosis: CACH

## 3.3 CONCLUSION

Some of the main features of the cases described in this chapter are collated, for comparison, in table 3-1. An explanation of the acidophil cell scoring system is given in appendix 2.

Field cases of canine acidophil cell hepatitis were received from Scotland, England, Ireland and Wales. A wide age range of dogs were affected, from 4 months to 12 years of age. Nearly half of the cases were young animals under, or only, 2 years of age. A variety of breeds were represented (ten) and there was one mongrel. Three breeds (German shepherd dogs, Doberman pinschers and cocker spaniels) accounted for more than half of the cases in this chapter, but that could have been a reflection of their popularity as pets.

Clinical histories were not available for a quarter of the CACH field cases described in this chapter. Clinical signs which were each exhibited by around half of those with histories, at some stage in their illness, included: intermittent vomiting, occasional diarrhoea, appetite loss, weight loss and polydipsia. Biochemical evidence of liver injury was common in the field cases, especially elevated blood levels of ALT. However, normal blood levels of ALT were also found at times in some of the CACH field

cases tested. This might be expected from the episodic nature of the ALT peaks found in experimentally-infected dogs, in both this study (chapter 4) and studies by Jarrett (1985 and 1987).

More than half of all the CACH cases in this chapter were ascitic, at some point during their illness, and an accessory portal circulation was identified in a quarter of the field cases. Neither of these features were restricted to the cirrhotic cases and not all of those exhibited one or both of them.

Both acute forms of canine acidophil cell hepatitis and chronic forms with varying degrees of fibrosis or even cirrhosis were identified. The stage of the disease present was not necessarily related to the age of the case: there were some young cirrhotic cases and old dogs with hepatitis but very little fibrosis.

Only one suspected case of CACH, with insufficient lesions for a definite diagnosis, was included in this chapter (FC7); but a great many more were examined. As well as cases with mild acidophil cell reactions, there were many cirrhotic cases with acidophil cell lesions which were not sufficient in number to definitely indicate that CACH was the initiating factor for the cirrhosis. Most of the severely cirrhotic cases examined had so much fibrosis and hepatocyte loss that it was impossible to identify the original aetiology. The problems in the identification of very mild and severely cirrhotic cases highlight the need for a definitive test for canine acidophil cell hepatitis.

# CHAPTER 4 DOG TRANSMISSION EXPERIMENTS

## 4.0 INTRODUCTION

This chapter covers three canine acidophil cell hepatitis transmission experiments performed with dogs. Some of the materials and methods used in the investigations are mentioned first, before a short discussion about the choice of normal ranges for some of the parameters measured.

The first experiment involved three pairs of dogs (section 4.3). Two controls were not given anything, two dogs received liver (which had been frozen) from a canine field case of acidophil cell hepatitis and the other two were given human immunoglobulin, thought to possibly be capable of causing an unidentified hepatitis in dogs. Both of the inoculated groups were also given a faecal extract, thought to be necessary for the transmission of the unidentified hepatitis.

In the second experiment, frozen liver from an experimentally-infected dog was used to attempt a second passage of CACH, in two groups of dogs (section 4.4). Two dogs were given clarified inoculum and another three received the same material after it had also been filtered, to check that filtration could not remove the infectious agent. One from each group was kept for a long-term study. This experiment overlapped the end of the previous one; the inoculations took place 2½ months after those of experiment 1.

The third experiment attempted the transmission, back to dogs, of CACH infection which had been passaged in another species - the rat (section 4.5). A third experimental passage of the disease, since the rats used had been inoculated with material from an experimentally-infected dog. Another variation investigated was the use of fresh rather than frozen liver for the inoculum. The inoculation of three dogs, 3 years after the experiment 1 inoculat-

ions, overlapped the end of experiment 2 by 1½ months.

## 4.1 MATERIALS AND METHODS

Experimental dogs were obtained from various sources, described in chapter 2 (section 2.2.1). Their general wellbeing was checked daily along with their rectal temperatures, which were always measured before they were fed. Blood samples were collected, as described in section 2.2.4, for biochemical and haematological analysis and, less frequently, for serum extraction. Faecal samples were collected periodically, frequently from the young pups, less often from older animals. Faecal egg counts, obtained from these samples, were used to monitor intestinal parasitism and the anthelmintic dosage regimes were adapted in accordance with the results.

The majority of the results of the biochemical and haematological analyses are graphed and discussed in this chapter. Other variables were monitored, but only mentioned if abnormal results occurred. Factors in the latter category, which were monitored in every haematology sample, included: the numbers of monocytes, eosinophils, basophils, normoblasts and platelets, as well as the MCV of the rbcs. Urea levels, usually only measured once per week, were not graphed. Other substances, measured less frequently, which were not graphed include: total protein, albumin, globulin, bilirubin and cholesterol.

Inocula were subjected to the bacteriological and virological examinations described in section 2.2.3. No viruses were isolated from any of the inocula used in dogs. The bacteriology results for the different preparations are given in the appropriate sections. Inoculation was always performed subcutaneously, in the scruff of the neck.

The postmortem protocol followed is given in section

2.2.8. Tissue samples, collected for histological examination, were processed as described in section 2.3. The processing of samples for ultrastructural examination is detailed in section 2.4.

## 4.2 NORMAL VALUES

A noticeable degree of variation in "normal" clinical results, both between individuals in the same experiment and between the animals of different experiments, became apparent as the experiments progressed. The variations seen between experiments were probably mainly due to differences in the types and ages of the dogs used. The variations seen occasionally between dogs in the same experiment, often littermates, mainly appeared to be the result of normal individual variation. Although, it is possible that a few of the dogs involved may have had permanently abnormal results.

The application of one set of strictly-defined normal ranges, across all of the experiments, was therefore not always appropriate. The use of normal ranges, wide enough to apply to all of the dogs used, might mask abnormalities in the animals with the lowest norms. Even results which fall within the normal range defined for one experiment, but are marked departures from the norm of an individual animal, may still be worthy of note in that animal.

The generally-accepted normal ranges (section 1.2.3) were used as guidelines for the analysis of the experimental dogs' clinical results. However, the results of preinoculation monitoring were also taken into account when the normal levels, for individual animals and each experiment, were defined. Allowance was made for the fact that the preinoculation results were not always normal. Age was another important factor, in the definition of the normal levels, for some of the variables measured. Details of any

variations in the defined normal ranges are given in the appropriate sections.

The high levels of alkaline phosphatase seen in puppies and the general trend of a gradual reduction, with age, to adult levels made it especially difficult to pick a specific range of normal levels for the pups in the first two experiments. The level of AP in individual plasma samples was therefore mainly compared to the range of levels found in that dog around that time.

Young puppies tend to have low red blood cell counts which increase to normal adult levels within a few months, they also tend to have higher numbers of lymphocytes than adults. The following haematology reference ranges were used for all three dog experiments:

| | | |
|---|---|---|
| rbc | (adult) | 5.0-8.5 $\times 10^{12}$/l |
| | (pup) | lower levels acceptable |
| Hb | (adult) | 11-18g/dl |
| | (pup) | lower levels acceptable |
| Hct | (adult) | 35-55% |
| | (pup) | lower levels acceptable |
| wbc | (adult) | 6-18 $\times 10^{9}$/l |
| | (pup) | 6-20 $\times 10^{9}$/l |
| Neutrophils | | 3.5-11.5 $\times 10^{9}$/l |
| Lymphocytes | (adult) | 0.7-5.0 $\times 10^{9}$/l |
| | (pup) | 0.7-7.0 $\times 10^{9}$/l |

## 4.3 DOG EXPERIMENT ONE

### 4.3.1 Experiment Design

This experiment actually consisted of two different transmission studies. The aim of one part of the experiment was to attempt transmission of canine acidophil cell hepatitis by inoculation with a liver preparation from a field case, dog B (described in section 4.3.2). The aim of

the other part was to try to produce hepatitis in dogs by inoculation with a sample of contaminated, pooled human immunoglobulin (Ig) and, at the same time, a faecal extract (FE) from marmosets with hepatitis. This was part of a collaboration with Professor Thomas of London.

Some of the marmoset faecal extract was also given to the dogs in the CACH transmission study, in case it could assist the transmission of CACH.

The batch of pooled human Ig had been withdrawn from use in humans because it was contaminated with non-A, non-B hepatitis virus. Hepatitis had been produced in marmosets by inoculating them simultaneously with contaminated, pooled human Ig and a human faecal extract (Professor Thomas, personal communication). Marmoset to marmoset passage of the disease had also required simultaneous inoculation with faecal extract from infected marmosets. It was thought that the disease infecting the marmosets might also be transmissible to dogs because batches of human Factor VIII, withdrawn from use in humans due to contamination with NANB hepatitis virus and human immunodeficiency virus (HIV), had apparently caused hepatitis in a few experimental dogs (Professor Thomas, personal communication). However, that diagnosis had been made on clinical features and blood biochemistry alone, without any histology.

Six dogs were used in this experiment: five border collie littermates (three males and two females) and one male border collie cross. The latter (dog 30) was thought to be about 8 weeks of age when obtained and the five littermates (dogs 31-35) were approximately 6 weeks of age. The two females were used as controls for the condition of the dogs at the start of the experiment. One of them (dog 34) was killed 7½ weeks after arrival, at 13½ weeks of age; the other (dog 35) was 16 weeks of age when killed, on the day the males were inoculated, 10 weeks after arrival.

Frozen liver from dog B was thawed, chopped and homogenised in sterile phosphate-buffered saline to form a 20% weight:volume homogenate. Mixed cultures of streptococci and coliforms, isolated from this homogenate, were found to be sensitive in vitro to oxytetracycline and trimethoprim/sulphonamide. Frozen aliquots of the pooled human immunoglobulin and marmoset faecal extract, supplied by Professor Thomas of London, were also thawed. Two of the males (dogs 30 and 31) were each inoculated with 1ml of the marmoset faecal extract, in the scruff at midline, and 12ml of dog B's liver preparation split between 2 sites, on either side of the scruff. The other two males (dogs 32 and 33) were each inoculated with 1ml of the marmoset faecal extract on one side of the scruff and 5 ml of the pooled human Ig on the other side. At the time of inoculation dog 30, the crossbreed was 18 weeks of age and the three border collie littermates were 16 weeks of age. One of each of the two groups (dogs 31 and 33) were killed 13½ weeks after inoculation, 1½ weeks later dogs 30 and 32 were killed.

### 4.3.2 Source Case

The source of liver, dog B (FC10), was a 9-year-old, neutered female German shepherd dog with a 4½-year history of liver disease. A more detailed clinical history is given in chapter 3 (section 3.2.10).

Dog B was about 4 years of age when first referred to GUVS hospital exhibiting anorexia, weight loss, dullness, vomiting, ascites and biochemical evidence of liver disease. A clinical diagnosis of cirrhosis was made and a poor prognosis given. Ascites recurred after abdominal drainage, but after a second hospitalisation the dog remained reasonably well for 2 years. It was then readmitted at 6 years of age, exhibiting behavioural changes, vomiting pyrexia, polydipsia and biochemical evidence of liver disease. Terminal hepatic failure was diagnosed but

fp146

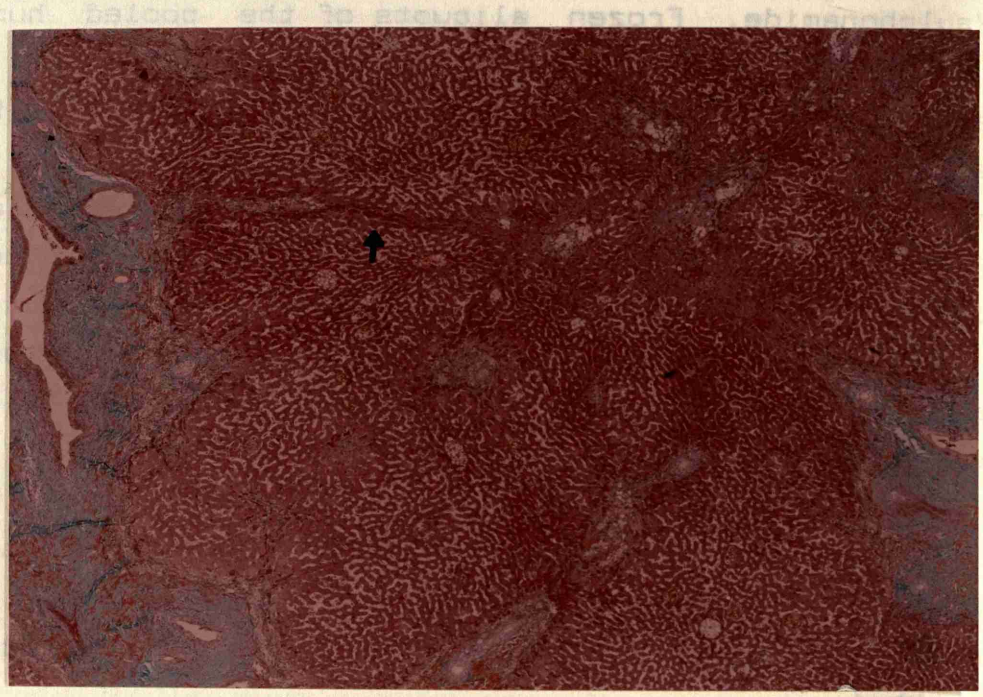

Fig 4-1 Liver of source dog B (FC10) (Mass tri x35)
9-year-old FN GSD Pronounced AC reaction with some acidophilic bridging lesions (↑)

the owners were determined to maintain dog B and succeeded in doing so for another 3 years. When it was finally presented for destruction with neurological signs, its aminotransferase levels were unremarkable; but it still had a high BSP retention indicating loss of liver function.

At postmortem there was no evidence of ascites or jaundice. The liver was small and firm, with one dark, cystic nodule 3cm in diameter. There were numerous slight renal scars. No cause was found for a leaking hind limb lesion. Samples were taken from various organs for histology. Liver was frozen in liquid nitrogen and stored at -70°C. Blood was also collected and the serum frozen to be stored at -70°C.

Histological examination of the liver revealed chronic CACH with cirrhosis. There was an increase in the amount of fibrous tissue in most portal tracts and some large bands of fibrosis. A marked acidophil cell reaction was a prominent feature of all the sections examined, with lots of acidophil cells around some portal tracts and some around most of the other portal tracts. Occasionally the acidophil cells formed bridging lesions between adjacent portal tracts, as can be seen in figure 4-1. Piecemeal necrosis was obvious at the limiting plates of many portal tracts. Although the hepatic architecture was disrupted by fibrosis, in and around portal tracts and branching out from some of them, most of the sections examined showed very little fibrous portal-portal linking. There was no fibrous dissection of the parenchyma into nodules. However, fibrous portal-portal bridging lesions were evident in two of the sections examined. The only evidence of nodular hyperplasia was also found in these, the most severely affected areas. Another feature of this case was numerous small, round foci of vacuolated hepatocytes scattered throughout the parenchyma. Vacuolated cells were also prominent in some of the fibrotic

portal tracts. Vacuolation of bile duct epithelial cells was evident in some portal tracts. The apparent increase in cellularity in many portal tracts was partly due to a combination of bile duct hyperplasia and rosette formation by entrapped hepatocytes. There was some neutrophil infiltration, but only in a very small number of portal tracts.

Histological examination of the kidneys revealed evidence of chronic nephritis. The scars visible at postmortem were areas of tubule loss, fibrosis and cellular infiltration of the interstitium. They consisted of fibrous tissue with a sparse infiltrate, mainly of plasma cells, surrounding glomeruli and some dilated tubules. In the renal pelvis there was heavy subepithelial infiltration by plasma cells and lymphocytes, forming occasional lymphoid follicles with germinal centres. Special stains failed to reveal any evidence of amyloid deposition, but a few protein casts were seen in tubules at the corticomedullary junction. The lung sections showed foci of chronic inhalation pneumonia, with a cellular and fibrous reaction apparently to an amorphous material in the alveoli.

### 4.3.3 General Clinical History

Two days after the dogs' arrival (67 days before inoculation), they were vaccinated against distemper, parvovirus enteritis, infectious canine hepatitis and leptospirosis. The vaccines used were the live, attenuated Kavak D and the inactivated Kavak Parvo and Kavak i-HL (Duphar Veterinary Ltd, Southampton). They were vaccinated again, with the same products, 6 weeks later (26 days before inoculation).

The dogs' antibody titres were measured before vaccination, 18 days after their first dose of vaccine and 12 days after their second dose. The serology results are given in table 4-1. Two days after their arrival, before vaccinat-

| TIMING of blood sampling | 30's age wks | Others' age wks | Dog No. 30 | 31 | 32 | 33 | 34 | 35 |
|---|---|---|---|---|---|---|---|---|
| | Antibody TITRES to DISTEMPER virus | | | | | | | |
| 2 dys post arrival.Pre vaccination | 8 | 6 | ⩽16 | ⩽16 | ⩽16 | ⩽16 | ⩽16 | <16 |
| 18 dys post vaccination | 11 | 9 | 256 | 2884 | 48 | 1024 | 512 | ⩾32768 |
| 12 dys post 2nd vaccin. | 16 | 14 | 512 | 8192 | 1024 | 2884 | —— | 23240 |
| | Antibody TITRES to PARVOVIRUS | | | | | | | |
| 2 dys post arrival.Pre vaccination | 8 | 6 | <4 | <4 | 4-8 | 4-8 | 4 | 4 |
| 18 dys post vaccination | 11 | 9 | 4-8 | 256 | 256 | 64 | 64 | 128 |
| 12 dys post 2nd vaccin. | 16 | 14 | >4096 | 2048 | >4096 | >4096 | —— | >4096 |
| | Antibody TITRES to ADENOVIRUS | | | | | | | |
| 2 dys post arrival.Pre vaccination | 8 | 6 | ⩽24 | 32 | ⩽24 | ⩽34 | ⩽24 | ⩽16 |
| 18 dys post vaccination | 11 | 9 | ⩾16384 | 256 | 128 | 90 | 32-64 | ~24 |
| 12 dys post 2nd vaccin. | 16 | 14 | ⩾16384 | ⩾16384 | ⩾16384 | 11620 | —— | ⩾16384 |

Table 4-1 Dog experiment 1 antibody titres

ion, all six dogs were found to have low titres to distemper, parvo and adeno viruses. They all responded, to a variable degree, to the first distemper vaccine and after the second dose had high antibody titres, within the range expected for this live vaccine. Only dog 30, the oldest pup, failed to respond to the first dose of parvovirus vaccine and all six dogs had high antibody titres after the second dose. The five littermates all responded, to a variable degree, to the first adenovirus vaccine. Dog 30 however, had a much higher antibody level than would be expected for this inactivated vaccine (Cornwell HJC, personal communication). It was within the range expected for natural infection. When tested after the second dose of vaccine, the other remaining dogs had similarly high titres. These results suggest that dog 30 may have brought in a naturally acquired adenovirus infection. It is not clear whether or not vaccination of the other dogs was able to protect them to any degree. Their high titres may have been the result of adenovirus infection caught from dog 30, or they may have been the stimulated response of immune animals to exposure to adenovirus shed by dog 30.

The dogs' faeces were examined regularly for evidence of intestinal parasites. Faecal egg counts were obtained from samples collected weekly for the first 3 months and then fortnightly after that. The first few FEC results and the anthelmintics used during that period are given in table 4-2.

The pups were wormed with piperazine adipate tablets (Head-To-Tail Veterinary Roundworm Tablets, Coopers Animal Health Ltd, Crewe) just after arrival. The first FECs were done 2 weeks after arrival, these and the following week's tests were all negative. By 4 weeks after arrival three of the dogs (31, 32 and 35) had Toxocara eggs in their faeces. The pups were each given a single dose of fenbendazole (Panacur 22% Granules, Hoechst Animal Health, Milton

| Dog No. | TIME after arrival (weeks) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 3 | 4 | | 5 | 6 | | 7 | | 8 | | 9 |
| 30 | piperazine | 0 | 0 | 0 | fenbendazole | 0 | 0 | fenbendazole | 0 | piperazine | 0 | fenbendazole | 0 |
| 31 | | 0 | 0 | 50 | | 0 | 500 | | 0 | | 50 | | 0 |
| 32 | | 0 | 0 | 50 | | 150 | 800 | | 500 | | 200 | | 0 |
| 33 | | 0 | 0 | 0 | | 0 | 0 | | 0 | | 100 | | 0 |
| 34 | | 0 | 0 | 0 | | 50 | 0 | | 0 | | - | | - |
| 35 | | 0 | 0 | 250 | | 0 | 0 | | 0 | | 100 | | 0 |

Table 4-2 Dog experiment 1 early faecal egg counts and anthelmintic regime (Toxocara)

Keynes). The following week's tests revealed only two positive faecal samples, those of dogs 34 and 32. The latter had even more eggs than before. The fenbendazole treatment was repeated 2 weeks after the first dose and resulted in negative FECs in all except dog 32. A week later, despite another dose of piperazine, four of the five remaining dogs had Toxocara eggs in their faeces. It took a third dose of fenbendazole to clear all the dogs of intestinal parasites. Although they remained free of parasites for the remainder of the experiment, a regular anthelmintic program was followed as a prophylactic measure.

The pups were initially housed in a pen in the dog room of the animal house, as it was the only dog accommodation available at that time. However, it could not have accommodated them as they grew and it was required for other users. They were therefore moved, after 2 weeks, into accommodation belonging to another department. This was a small isolation building, with one room for food preparation and other work and another with several large kennel cages. The pups were housed in pairs in these for 1½ weeks. The four male pups were then moved to a newly prepared isolation unit, described in section 2.2.1. The two females, which were to be used as controls, remained where they were. The periods spent in the different premises are depicted in figure 4-2, along with the timing of some of the other events described in this section.

In the second accommodation in which the dogs were kept they were caged in pairs as follows: dogs 30 and 31, dogs 32 and 35, and dogs 33 and 34. Three days after moving to this accommodation dogs 31, 33 and 34 became diarrhoeic. Faecal samples and rectal swabs were taken from each of the dogs. By the next day there was diarrhoea in all three cages, but none was seen the following day. The faecal samples were checked for the presence of parvovirus, using a haemagglutination test, and were found to be negative.

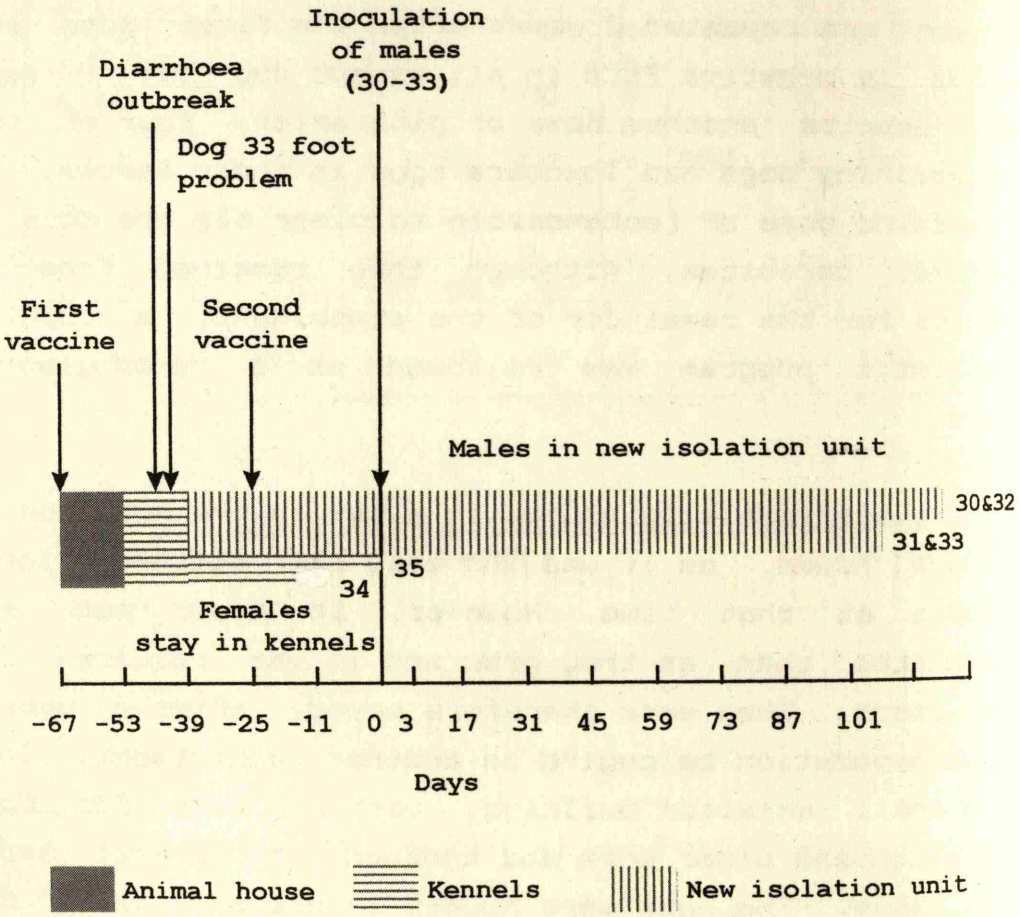


**Figure 4-2** **Time sequence of early events and premises used**

Bacteriological examination of the rectal swabs revealed Campylobacter infections in dogs 31 to 35, the organism was sensitive in vitro to neomycin. Campylobacter infection in dogs is normally only treated if they are diarrhoeic. In this case, by the time the diagnosis had been made, the diarrhoea had cleared up without treatment. Another set of rectal swabs, taken 1 week after the first, revealed that all six dogs were by then infected with Campylobacter. Two days later dog 34 became diarrhoeic again, so all six dogs were put on a 5-day course of Kaobiotic tablets (Upjohn Ltd, Crawley) which contain neomycin, several sulphonamides, kaolin and pectin. The following day the four male pups were moved to the new isolation accommodation. Although no diarrhoea was seen in the new accommodation and the bitches were only diarrhoeic on two more occasions, rectal swabs from all six dogs were still positive for Campylobacter 7 weeks after the infection was first diagnosed. The dogs were then considered to be immune carriers.

Another minor problem also developed during the short period in the second accommodation. After 5 days in the kennels, bedded on newspaper, dog 33 exhibited superficial abrasions of the footpads due to urine scalding. Daily washing and drying of all the pups' feet and changing the newspaper bedding twice daily prevented any of the others developing the problem, although dog 34 did exhibit a very slight reddening of some footpads for a couple of days. Dog 33's pads, however, did not heal completely until the males were moved into the third accommodation, where they were bedded on straw in a large pen with a raised bed.

The day after the males had been moved out, dog 34 developed a clear occular discharge; 5 days later she became pyrexic and by the next day the occular discharge had become purulent. On the following day dog 35 also had a little dried occular discharge, enlarged submandibular lymph nodes, a small pea-sized mass below her right eye

(presumed to be reactive lymphoid tissue) and was making snoring noises as she breathed. Bacteriological examination of nasal swabs failed to isolate any Bordetella, Pasturella or other pathogens. Conjunctival smears were taken and stained with H&E and with PTI but histological examination of these failed to reveal any abnormalities and no distemper inclusion bodies were seen. Both dogs were put on a 9-day oral course of trimethoprim/sulphonamide (Tribrissen 20 trimethoprim and sulphadiazine tablets, Coopers Animal Health Ltd, Crewe). Although dog 35 exhibited sneezing for a short time, no cough could be elicited and the lung sounds of both dogs remained normal on auscultation. Within 5 days the clinical signs had virtually cleared up and the dogs were bright. During this period the male dogs remained healthy in their new accommodation.

The two dogs given the liver preparation (dogs 30 and 31) exhibited a localised, adverse reaction to it. Two days after inoculation they developed firm swellings where the liver preparation had been injected. On the third day after inoculation the swelling on the right side of dog 30's neck ruptured, releasing some bloodstained discharge. By the next day the other side had also ruptured, as had one of dog 31's swellings. The discharge from the latter looked like the actual liver preparation. The wounds were kept clean and treated topically with oxytetracycline (Alamycin Aerosol, Norbrook Laboratories Ltd, London). Both dogs were also given a 6-day oral course of oxytetracycline (Oxytetracycline 250mg Tablets, Animalcare Ltd), since the bacteriology report on the liver preparation had indicated an in vitro sensitivity to oxytetracycline (section 4.3.1). Within 3 days the swellings had reduced dramatically and the dogs were bright and happy again, although the wounds took another fortnight to heal over completely.

A few minor scuffles over the dominance hierarchy occurred

around 7 and 13 weeks after inoculation, during these dog 33 received a few slight abrasions.

### 4.3.4 Rectal Temperatures

During the period between arrival and inoculation the dogs' body temperatures were only checked on most weekdays and not at weekends. From the day of inoculation temperatures were monitored daily, before feeding.

In the preinoculation period the rectal temperatures of the four males were mainly in the range of 38.4-39.5°C (figures 4-3 to 4-6), while those of the two females were mainly in the range of 38.0-39.5°C (figures 4-7 and 4-8). Rectal temperatures of 39.8°C and above were considered to be abnormally raised, in this experiment. The possibility, that some of the results just below this level were also abnormal, cannot be discounted. Dogs 30, 31, 32 and 34 each had only one preinoculation temperature peak greater than 39.7°C and dog 33 had three. Dog 33's rectal temperatures tended generally to be higher than those of the other dogs, both before and after inoculation. Although this may simply have been a result of individual variation, there may have been some unidentified reason. The following abnormalities, occurring at the time of some of the temperature peaks, are not known to have been responsible for them; they are only offered as possible causes or contributing factors. When dog 30's temperature was raised the only finding was a very small amount of dried matter, of unknown significance, at the corners of his eyes. Dog 31's bout of pyrexia was during the outbreak of diarrhoea, described in section 4.3.3, although he himself was not diarrhoeic at that point. The bout of pyrexia seen in dog 34 coincided with her short episode of purulent occular discharge, described in section 4.3.3.

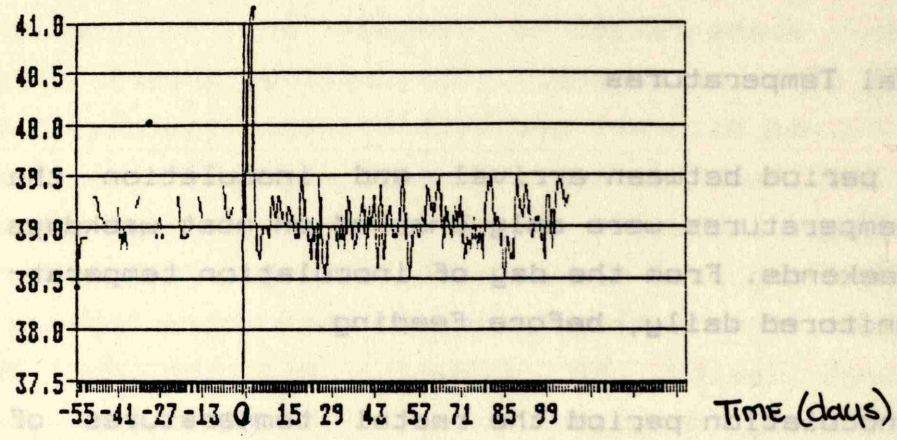


Fig 4-3 Dog 30's rectal temperature (°C)
day 0 = inoculation day

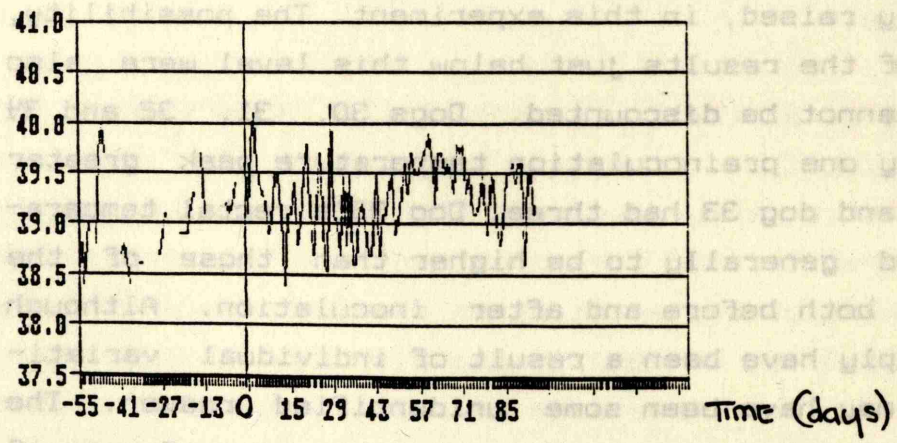


Fig 4-4 Dog 31's rectal temperature (°C)

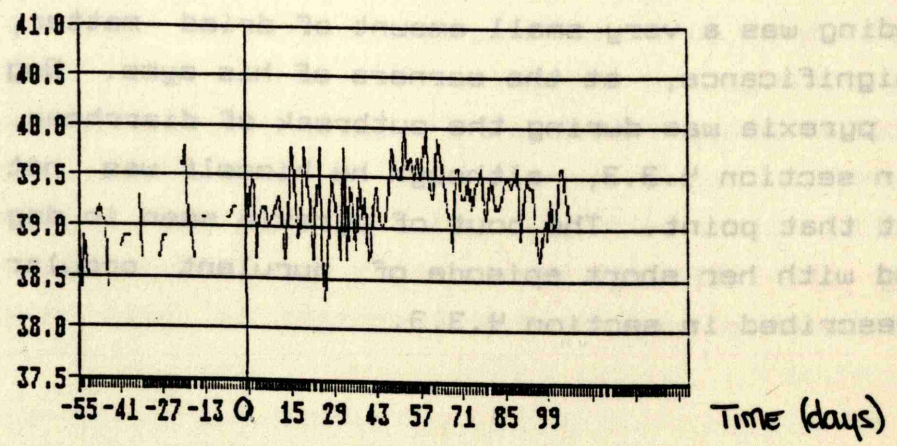


Fig 4-5 Dog 32's rectal temperature (°C)

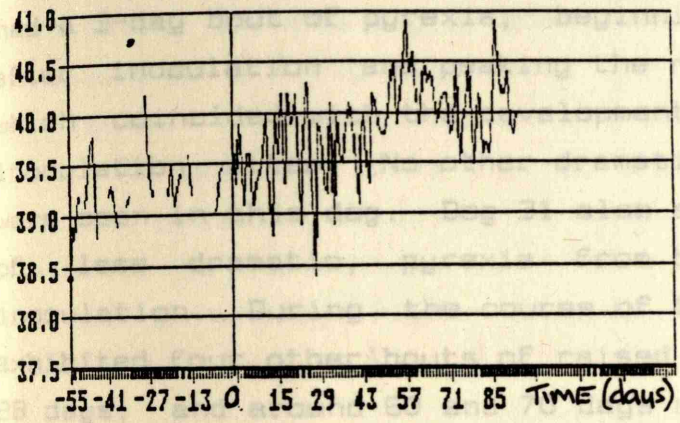


Fig 4-6 Dog 33's rectal temperature (°C)

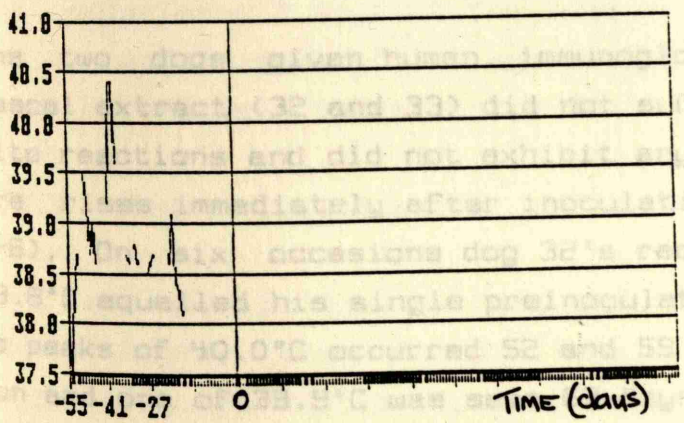


Fig 4-7 Dog 34's rectal temperature (°C)

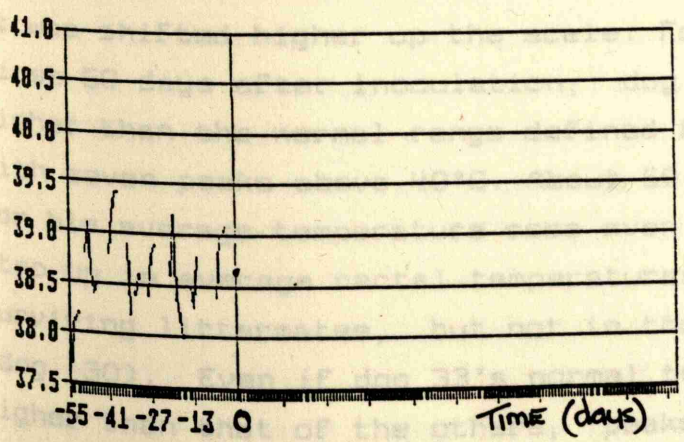


Fig 4-8 Dog 35's rectal temperature (°C)

Figures 4-3 and 4-4 also show the rectal temperatures of dogs 30 and 31 after inoculation with dog liver. Dog 30 had a 3-day bout of pyrexia, beginning on the second day after inoculation and peaking the next day at 41.1°C, which coincided with the development of swellings at the inoculation sites. No other dramatic temperature peaks were seen in this dog. Dog 31 also exhibited a 3-day bout of, less dramatic, pyrexia from the second day after inoculation. During the course of the experiment dog 31 exhibited four other bouts of raised temperatures: 20 and 28 days, and around 60 and 70 days after inoculation. No abnormalities were seen in the biochemistry and haematology results at those times. Only the peaks immediately and 28 days after inoculation exceeded 40°C, measuring 40.1°C and 40.2°C respectively.

The two dogs given human immunoglobulin and marmoset faecal extract (32 and 33) did not suffer any inoculation site reactions and did not exhibit any dramatic temperature rises immediately after inoculation (figures 4-5 and 4-6). On six occasions dog 32's rectal temperature of 38.8°C equalled his single preinoculation peak. Temperature peaks of 40.0°C occurred 52 and 59 days after inoculation and one of 39.9°C was seen 63 days after inoculation. None of them were accompanied by any other abnormalities. As mentioned earlier, dog 33's temperature results were quite different from the rest of the dogs. Although the general pattern was similar in shape to his littermates, it was shifted higher up the scale. For almost half of the first 50 days after inoculation, dog 33's temperature was higher than the normal range defined for this experiment, with seven peaks above 40°C. About 50 days after inoculation his average temperature rose even higher. A similar step up in average rectal temperatures was seen in his two surviving littermates, but not in the unrelated crossbred (dog 30). Even if dog 33's normal temperature range was higher than that of the others, peaks were still distinguishable. One of 40.3°C occurred 24 days after inoculat-

ion, but the two most prominent peaks, of 40.9°C, were seen 57 and 86 days after inoculation. From 50 days after inoculation onwards dog 33 sometimes exhibited minor skin abrasions or a dirty damp coat. In the presence of humans he tended to bark boldly at the other dogs and pester them. He appeared to be trying, unsuccessfully, to challenge the established dominance order and was being put in his place by the others. Whether or not this contributed to his generally higher rectal temperatures was not established.

The step up in average rectal temperatures, seen in dogs 31 to 33, occurred in early summer (the end of May) and may have been related to a rise in the ambient temperature. Similar step-like changes were later seen in two of the experiment 3 dogs at the same time of year (section 4.5.4) and in some of the experiment 2 dogs at different times of the year (section 4.4.5).

### 4.3.5 Biochemical Results

Five of the six dogs in this experiment never had ALT levels higher than 40 IU/l during the preinoculation period (figures 4-9,12,15,18,21 & 24). Only dog 35 exceeded that level with two consecutive results of 62 and 45 IU/l. Forty-five IU/l was taken to be the top of the normal ALT range for this experiment.

In the period before inoculation the AST levels of all of the dogs, except dog 35, never exceeded 36 IU/l (figures 4-10,13,16,19,22 & 25). To allow a margin for error, 40 IU/l was taken as the upper end of the normal range of AST levels in these experimental dogs. Using this criterion, dog 35 had raised AST levels in three samples (fig. 4-25). The first two were collected consecutively, 49 and 42 days before the males were inoculated, and coincided with raised ALT levels. The cause of the raised levels of transferase enzymes was not known. They occurred during

her outbreak of diarrhoea, but the enzyme levels of the other diarrhoeic dogs were not similarly affected.

The pups of this experiment exhibited more variation in their preinoculation AP levels (figures 4-11,14,17,20,23 & 26), and often higher results, than the similarly-aged pups of experiment 2. Although dog 30 had the highest preinoculation AP levels, with only one result below 450 IU/l, they were reasonably consistent. Both 49 and 35 days before inoculation dog 31 had an AP level of 448 IU/l, noticeably higher than surrounding levels. Throughout the experiment dog 32's range of AP levels tended to be lower than both dogs 30 and 31. Preinoculation AP levels in dog 33 were similar to those of dog 32, apart from two noticeably higher results 42 and 35 days before inoculation. The AP levels seen in dogs 34 and 35 were quite variable, the highest level in both occurred in the sample collected 42 days before the others were inoculated.

Both of the dogs inoculated with the dog liver preparation showed dramatically raised levels of ALT in the first two plasma samples after inoculation (at 2 and 4 days). Dog 30's ALT level reached 199 IU/l and dog 31's reached 232 IU/l. The latter's level was still raised in the third sample, 7 days after inoculation. Some of the discharge from the adverse reactions, described in section 4.4.3, resembled the liver preparation inoculated. It was thought that the high plasma ALT levels may have been largely exogenous ALT, released into the circulation during breakdown of the inoculated liver cells, rather than being endogenous enzyme from the dogs' own livers. The only other noticeable peak in dog 30's ALT levels, of 63 IU/l, occurred 39 days after inoculation (figure 4-9). Dog 31's ALT levels, shown in figure 4-12, remained unremarkable after the initial postinoculation peak.

After inoculation dog 30 had two samples with slightly raised AST levels: 42 IU/l at 67 days and 43 IU/l at 81

days respectively (figure 4-10). The ALT and AP levels in those samples were unremarkable. Dog 31's only raised AST level of 66 IU/l occurred 44 days after inoculation (figure 4-13) and was also accompanied by normal ALT and AP levels.

Dog 30's AP levels began to reduce after inoculation, but there was a slight peak of 541 IU/l at 32 days and another lower one, 57 days after inoculation (figure 4-11). There were no dramatic peaks in dog 31's AP levels after inoculation, but they did tend to rise and fall repeatedly (figure 4-14).

No dramatic peaks were seen in the ALT levels of the two dogs given the human immunoglobulin. Dog 32 exhibited one low peak of 48 IU/l, 39 days after inoculation (figure 4-15). Another smaller peak, 65 days after inoculation, did not exceed the normal range of ALT levels. The only raised ALT level found in dog 33, 42 days after inoculation, was 48 IU/l (figure 4-18).

None of dog 32's plasma samples had raised levels of AST as can be seen in figure 4-16. The single high AST result of 55 IU/l seen in dog 33, 53 days after inoculation (figure 4-19), occurred at a time of normal ALT and AP levels.

Although dog 32 had a low range of AP levels, there were occasional samples with AP levels higher than those surrounding them. The highest of these, 310, 356 and 316 IU/l, were found 21, 70 and 106 days after inoculation respectively (figure 4-17). Dog 33's AP levels remained unremarkable after inoculation (figure 4-20), their general range appeared to be closer to that of dog 32, than those of dogs 30 and 31.

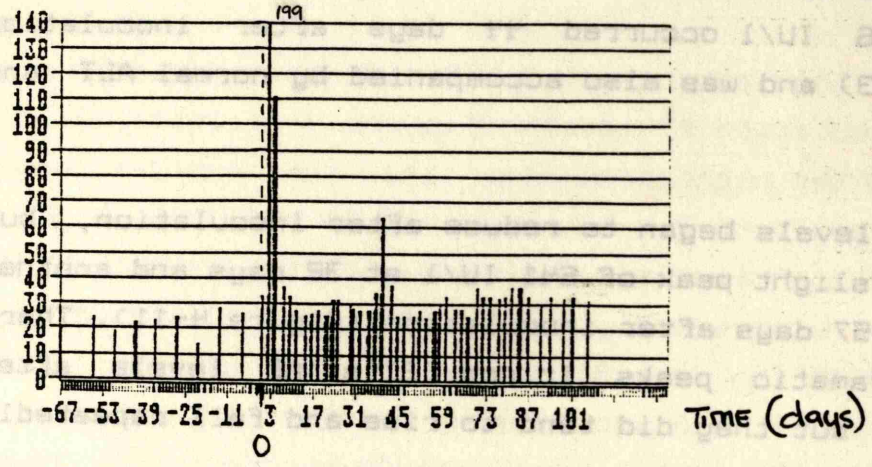


Fig 4-9 Dog 30's ALT levels (IU/l)

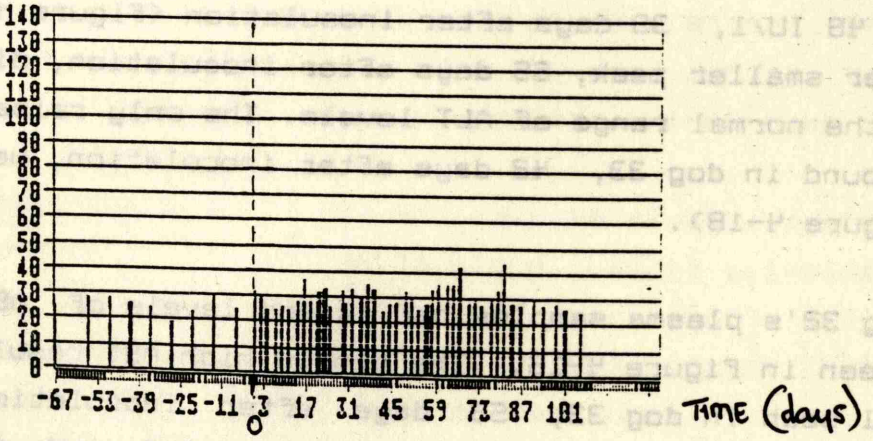


Fig 4-10 Dog 30's AST levels (IU/l)

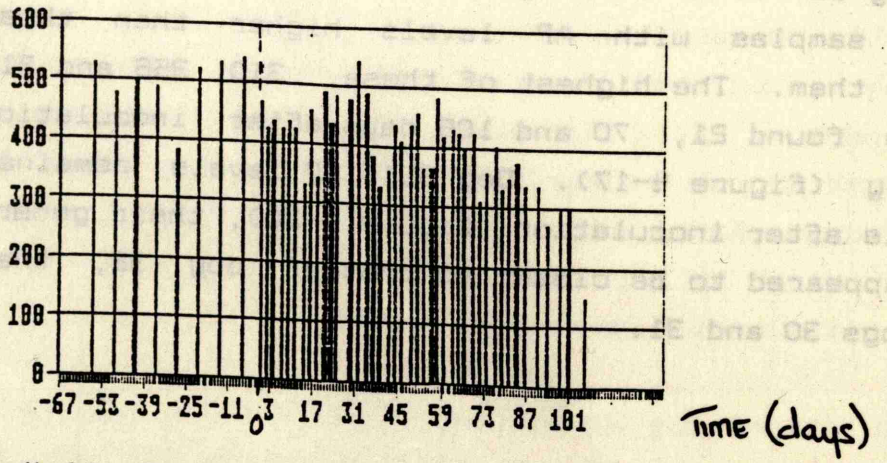


Fig 4-11 Dog 30's alkaline phosphatase levels (IU/l)

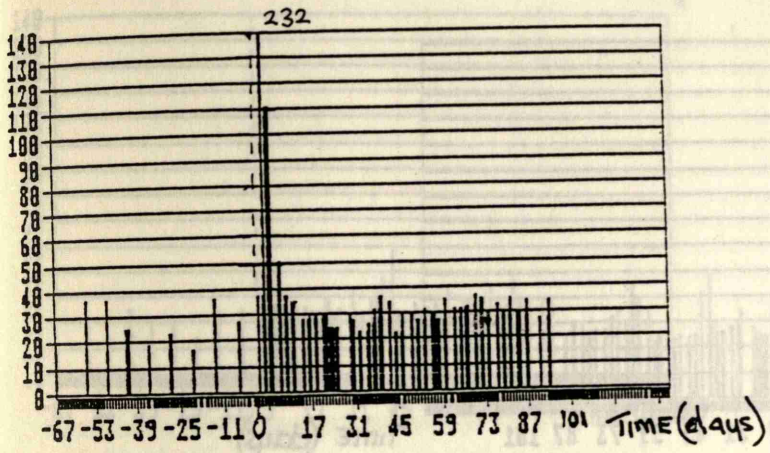


Fig 4-12 Dog 31's ALT levels (IU/l)

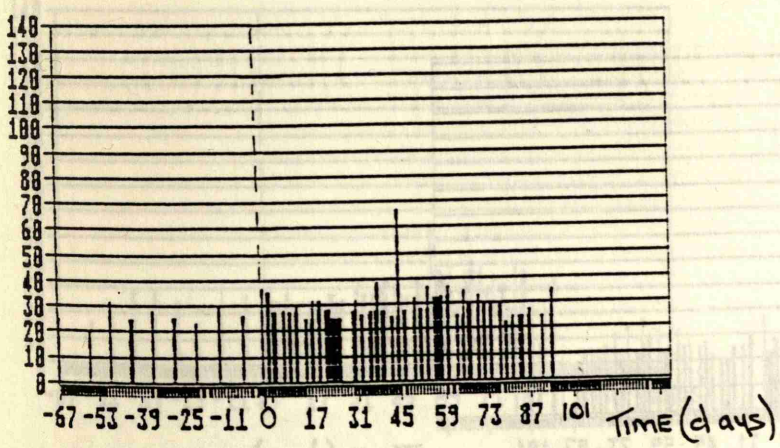


Fig 4-13 Dog 31's AST levels (IU/l)

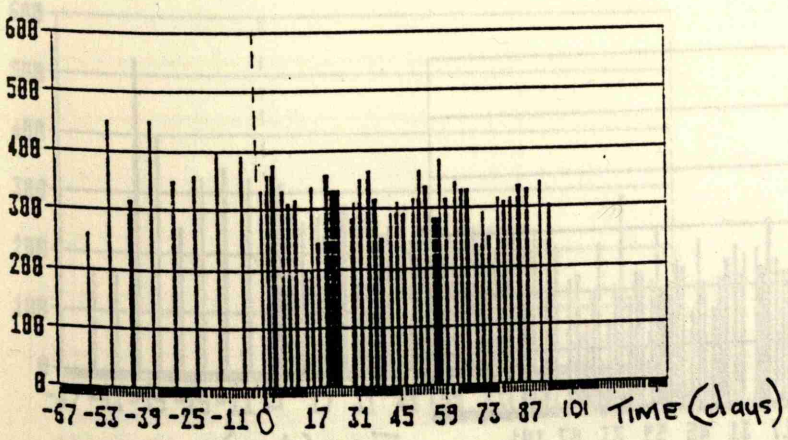


Fig 4-14 Dog 31's alkaline phosphatase levels (IU/l)

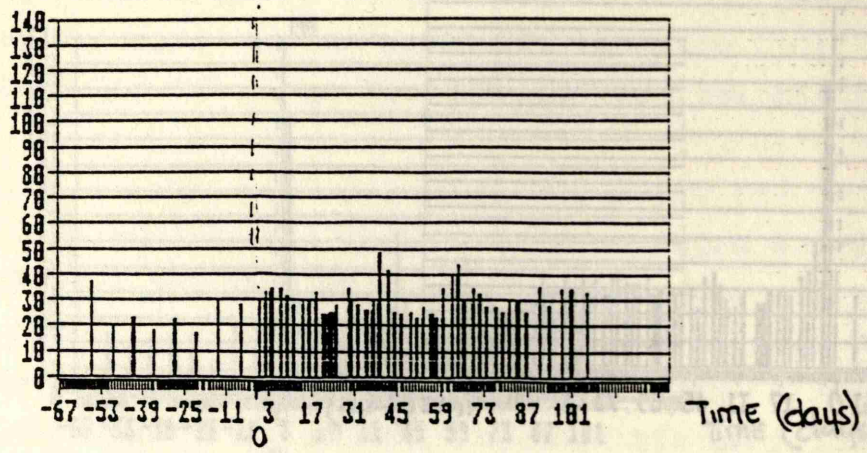


Fig 4-15 Dog 32's ALT levels (IU/l)
day 0 = inoculation day

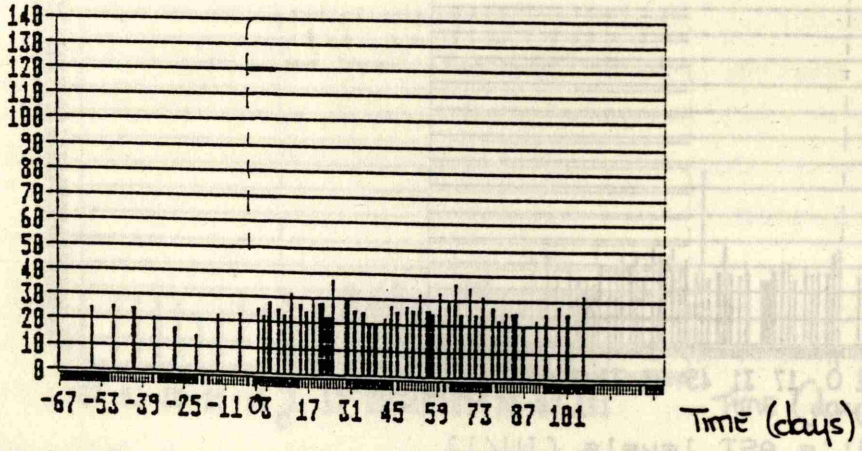


Fig 4-16 Dog 32's AST levels (IU/l)

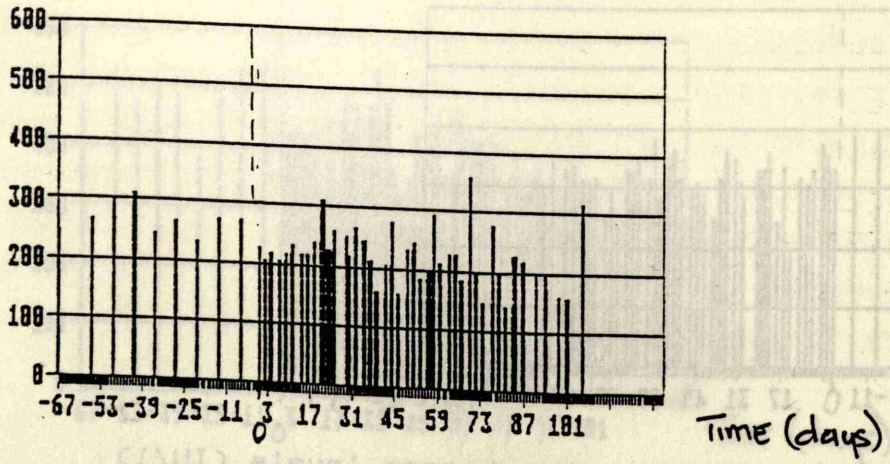


Fig 4-17 Dog 32's alkaline phosphatase levels (IU/l)

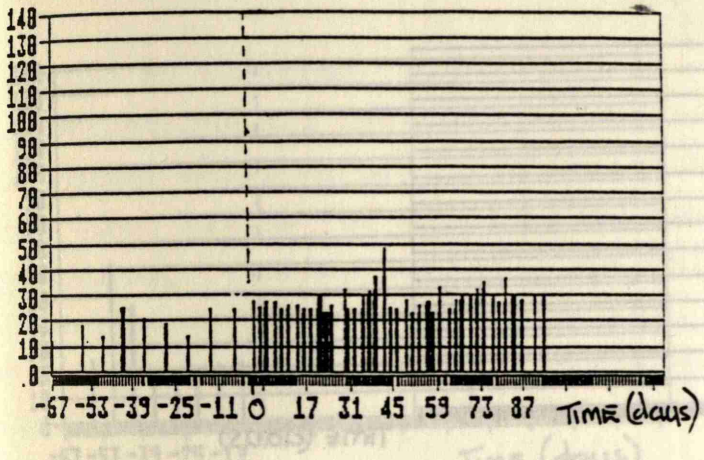


Fig 4-18 Dog 33's ALT levels (IU/l)

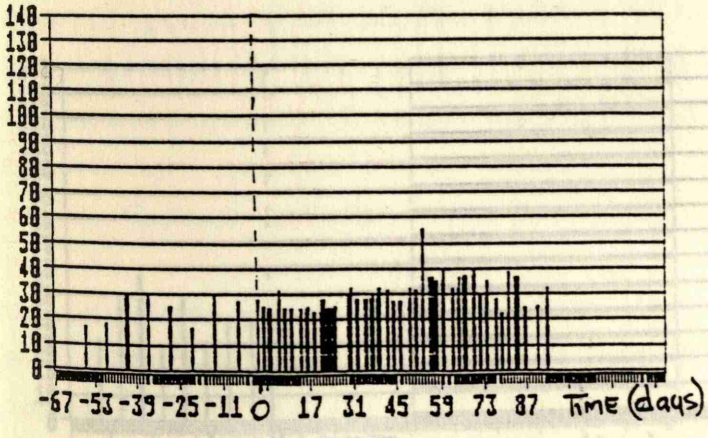


Fig 4-19 Dog 33's AST levels (IU/l)

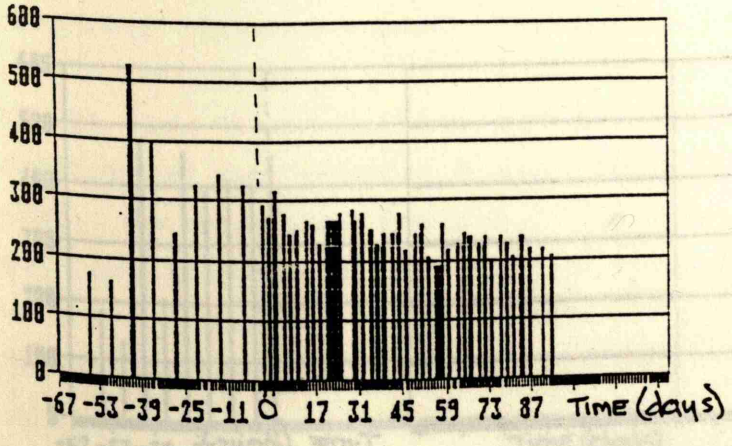


Fig 4-20 Dog 33's alkaline phosphatase levels (IU/l)

fp160

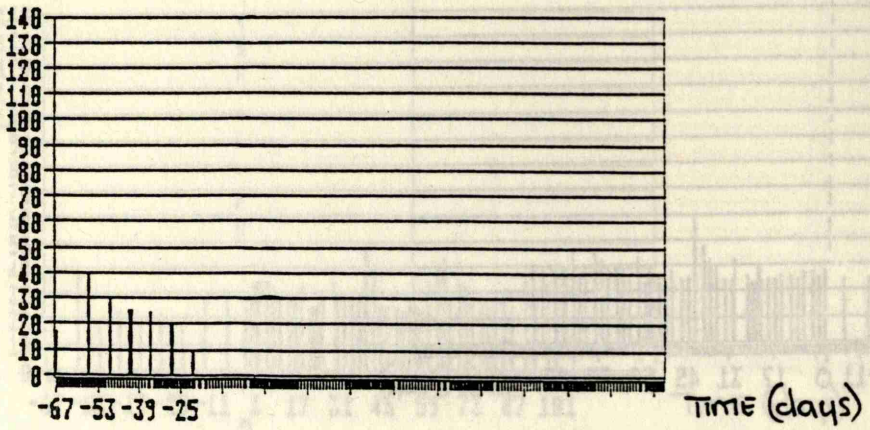


Fig 4-21 Control dog 34's ALT levels (IU/l)

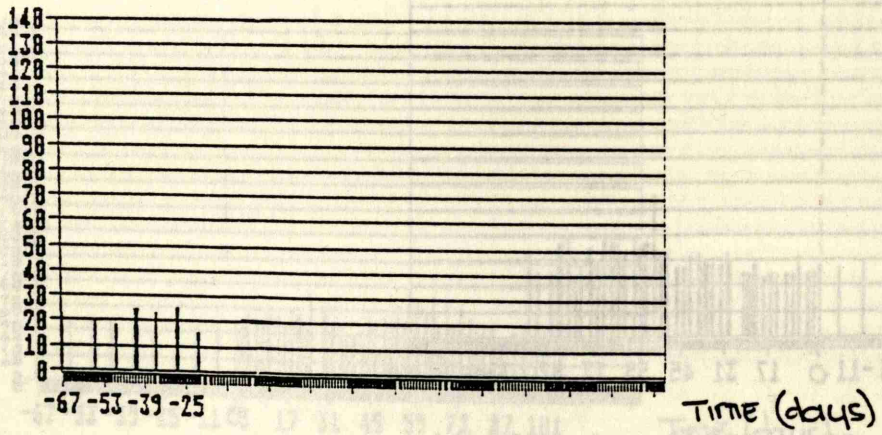


Fig 4-22 Control dog 34's AST levels (IU/l)

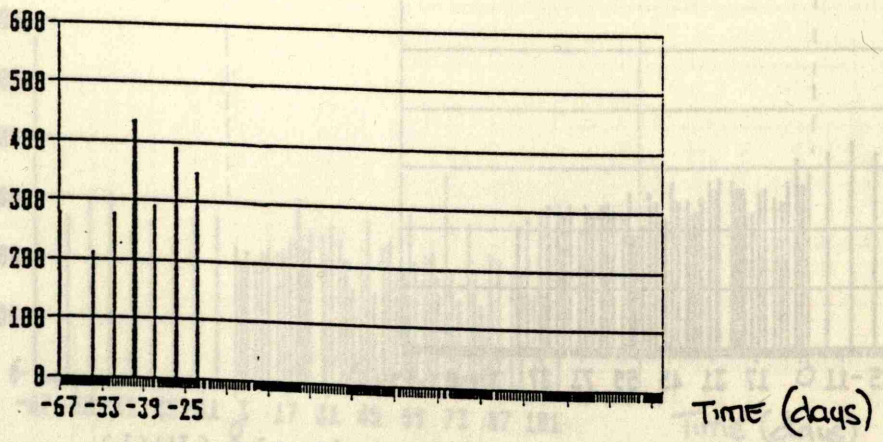


Fig 4-23 Control dog 34's alk. phosphatase levels (IU/l)

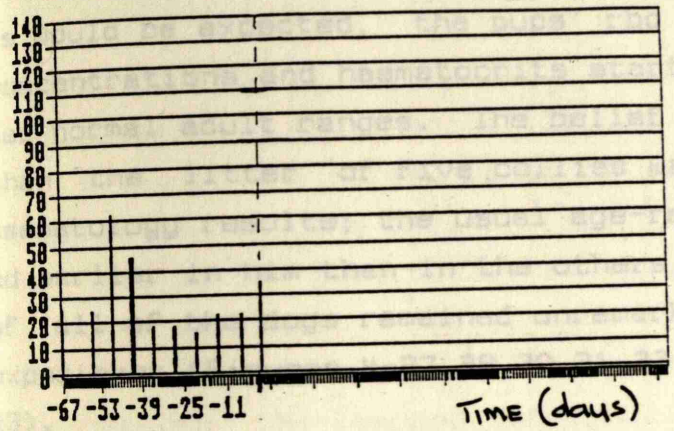


Fig 4-24 Control dog 35's ALT levels (IU/l)

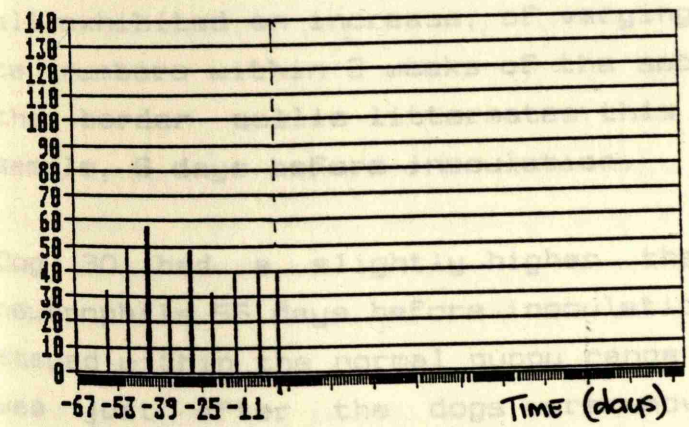


Fig 4-25 Control dog 35's AST levels (IU/l)

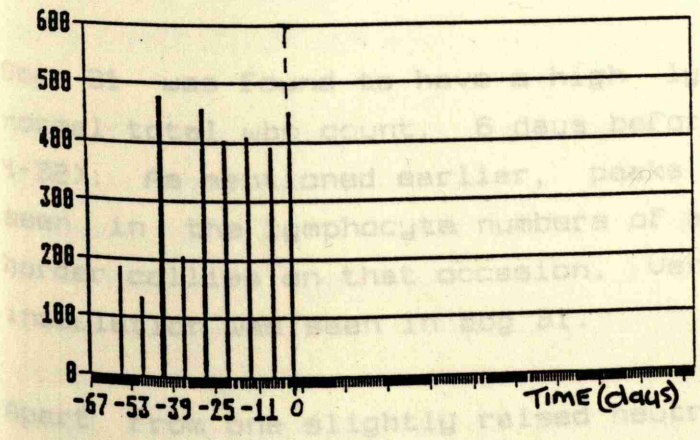


Fig 4-26 Control dog 35's alk. phosphatase levels (IU/l)

4.3.6 Haematological Results

As would be expected, the pups' rbc counts, haemoglobin concentrations and haematocrits started below the recognised normal adult ranges. The belief that dog 30 was older than the litter of five collies was supported by the haematology results; the usual age-related changes occurred earlier in him than in the others. The rbc parameters of all of the dogs remained unremarkable throughout the experiment (figures 4-27,28,30,31,33,34,36,37,39,40,42 and 43).

All of the dogs, apart from dog 33, showed an increase in lymphocyte numbers within a fortnight of the first vaccination (figures 4-29,32,35,38,41 & 44). The surviving dogs all exhibited an increase, of varying degree, in lymphocyte numbers within 3 weeks of the second vaccination. In the border collie littermates this peaked in the third sample, 6 days before inoculation.

Dog 30 had a slightly higher than normal number of neutrophils 56 days before inoculation, but his wbc count stayed within the normal puppy range (figure 4-29). This was just after the dogs were moved into the second accommodation and just before the outbreak of diarrhoea. Although dog 30's neutrophil numbers and wbc count increased after inoculation, they did not exceed the recognised normal ranges.

Dog 31 was found to have a high lymphocyte count, but normal total wbc count, 6 days before inoculation (figure 4-32). As mentioned earlier, peaks of various sizes were seen in the lymphocyte numbers of all of the surviving border collies on that occasion. Very little reaction to inoculation was seen in dog 31.

Apart from one slightly raised neutrophil count with a normal total wbc count, 3 weeks before inoculation, dog

32's leucocytology results remained within the recognised normal ranges for the whole of the experiment (fig. 4-35).

Before inoculation dog 33 had two wbc count peaks, due to high numbers of neutrophils (figure 4-38). Only the first one exceeded the normal range of wbc counts in pups; it occurred during the period, described in section 4.3.3, when dog 33 had superficial abrasion of the footpads due to urine scalding. A later rise in lymphocyte numbers, peaking 6 days before inoculation, may have been part of a response to the second vaccination. Two weeks after inoculation dog 33's neutrophil count rose; there then followed a 4-week period in which three of the four blood samples collected had high wbc counts, due to raised numbers of neutrophils. The cause was not known and it occurred before dog 33's period of highest rectal temperatures.

The initial increase in lymphocyte numbers, mentioned earlier, was most marked in dog 34 (figure 4-41). She exhibited another, very high, peak in lymphocyte numbers 3 weeks later, towards the end of the short episode of pyrexia and occular discharge described in section 4.3.3. Between the two peaks, dog 34 also had a peak in neutrophil numbers which took the total wbc count to the upper part of the normal range in pups. This virtually coincided with the 2-day episode of very slight reddening of her footpads, described in section 4.3.3, which may or may not have contributed to it.

Dog 35 had a very high wbc count, due mainly to a high peak in neutrophil numbers, in her second blood sample (figure 4-44). It was collected on the day before the outbreak of diarrhoea, but the two are not definitely known to have been connected. In dog 35's last two blood samples, lymphocyte numbers were above the normal adult range but within the range for pups.

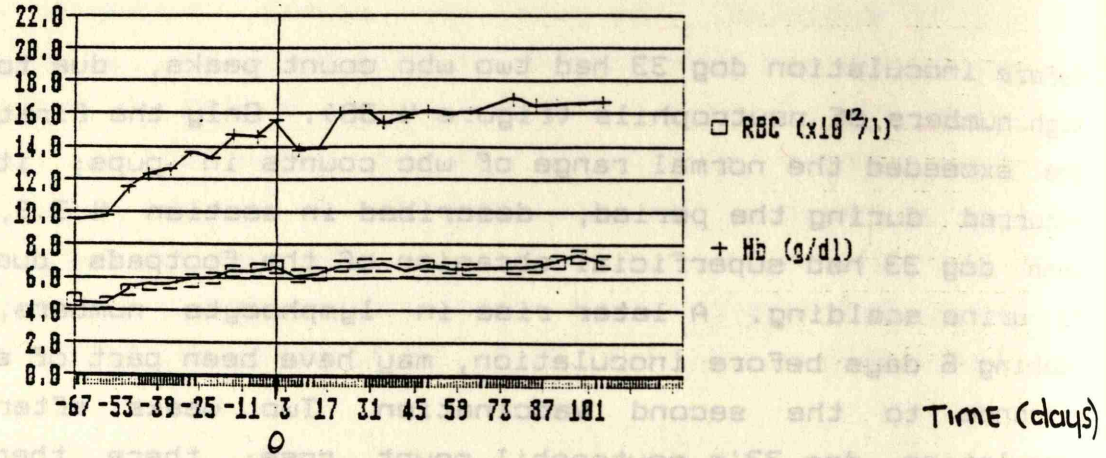


Fig 4-27 Dog 30's rbc counts and haemoglobin levels
day 0 = inoculation day

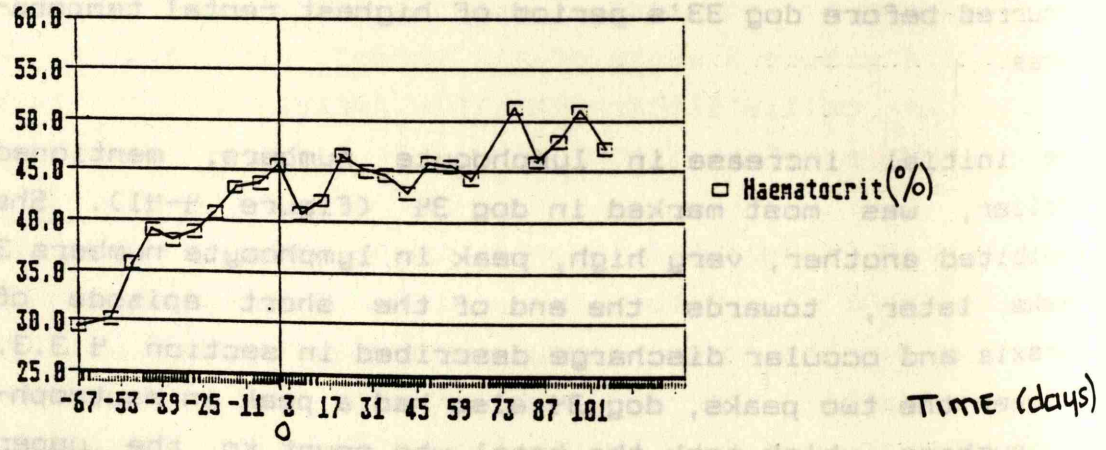


Fig 4-28 Dog 30's haematocrits (%)

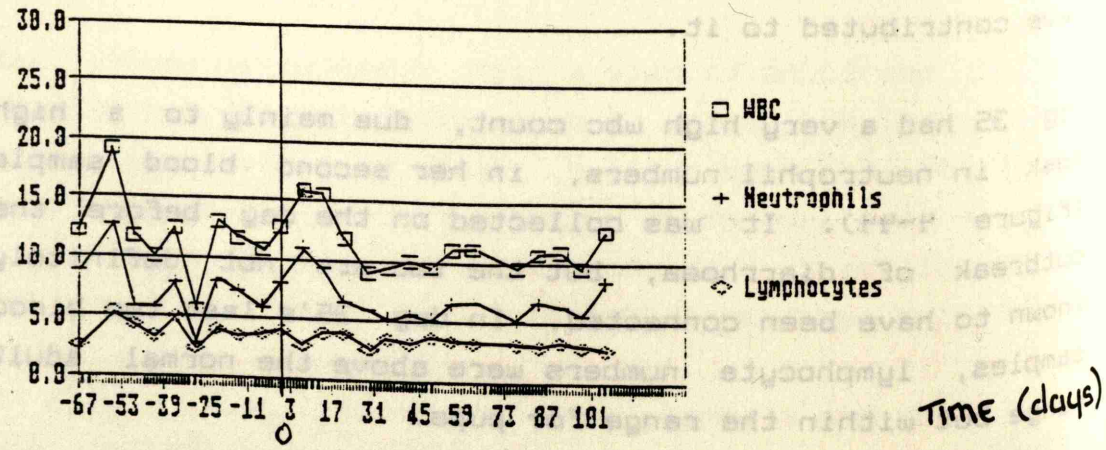


Fig 4-29 Dog 30's leucocytology results (x10^9/l)

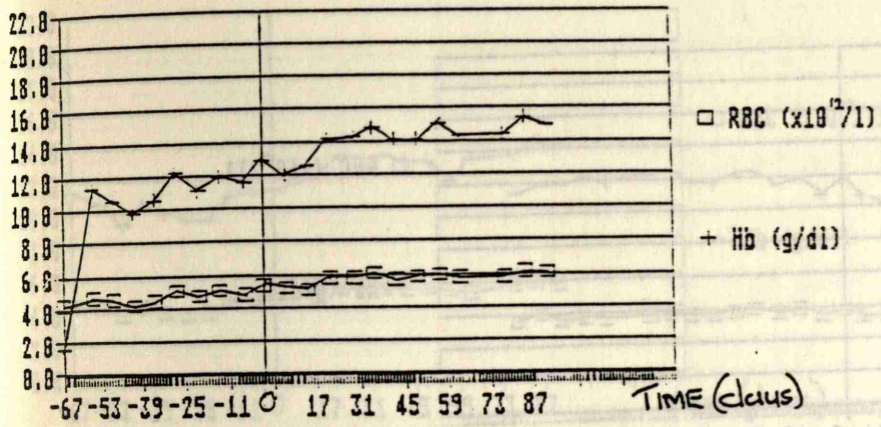


Fig 4-30 Dog 31's rbc counts and haemoglobin levels

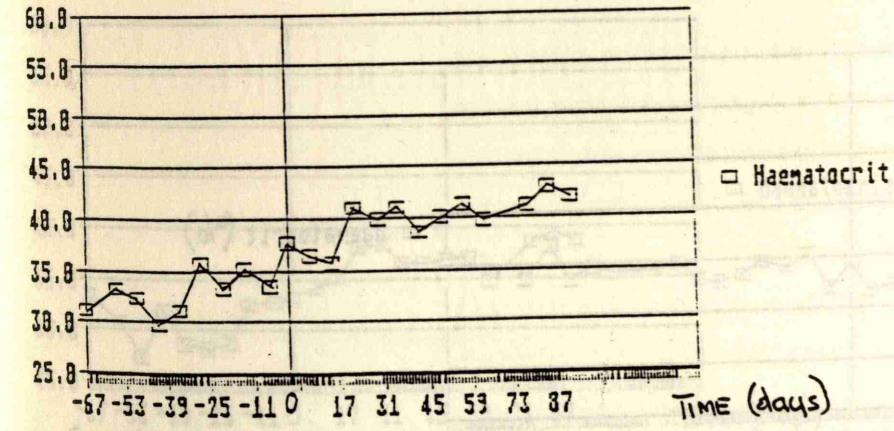


Fig 4-31 Dog 31's Haematocrits (%)

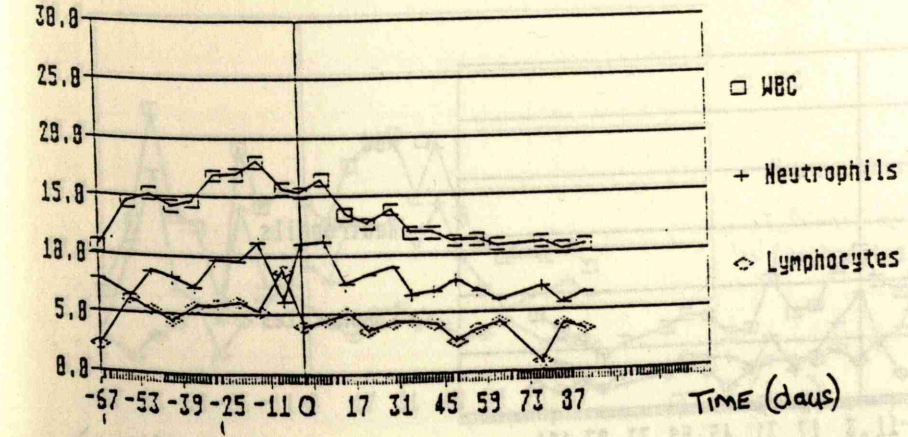


Fig 4-32 Dog 31's leucocytology results (x10⁹/l)

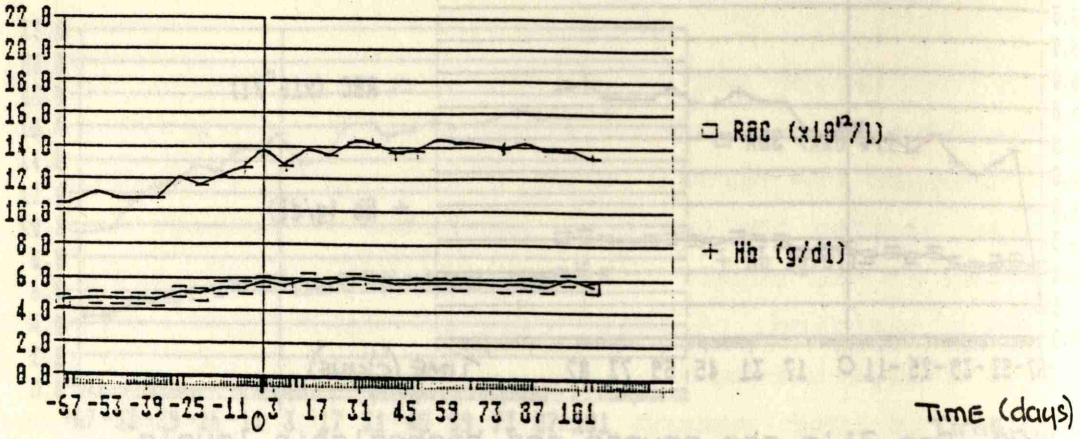


Fig 4-33 Dog 32's rbc counts and haemoglobin levels

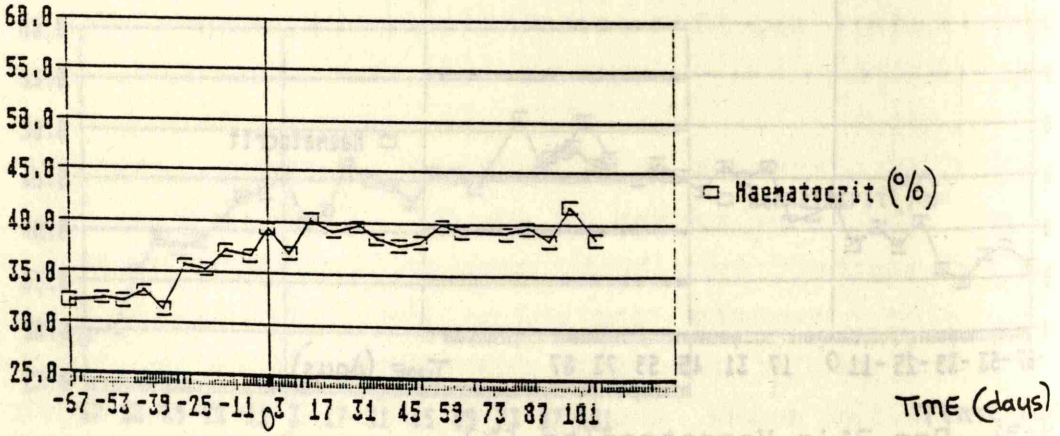


Fig 4-34 Dog 32's haematocrits (%)

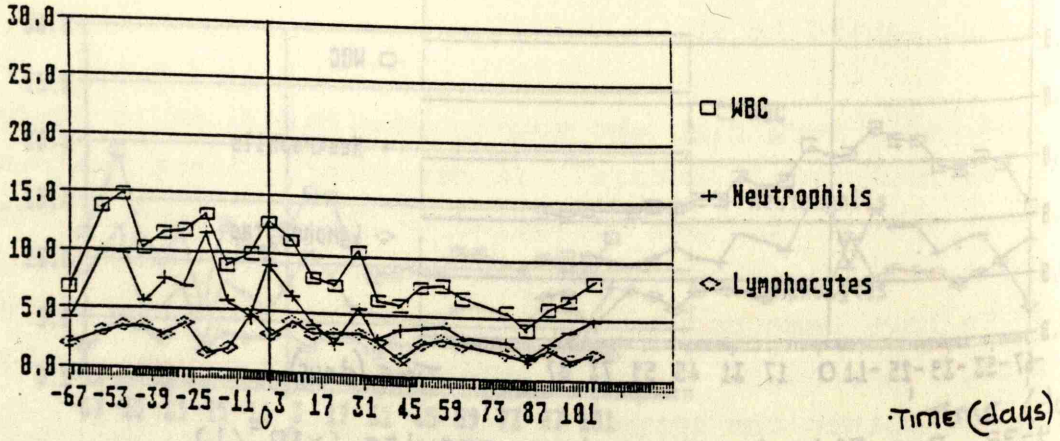


Fig 4-35 Dog 32's leucocytology results (x10⁹/l)

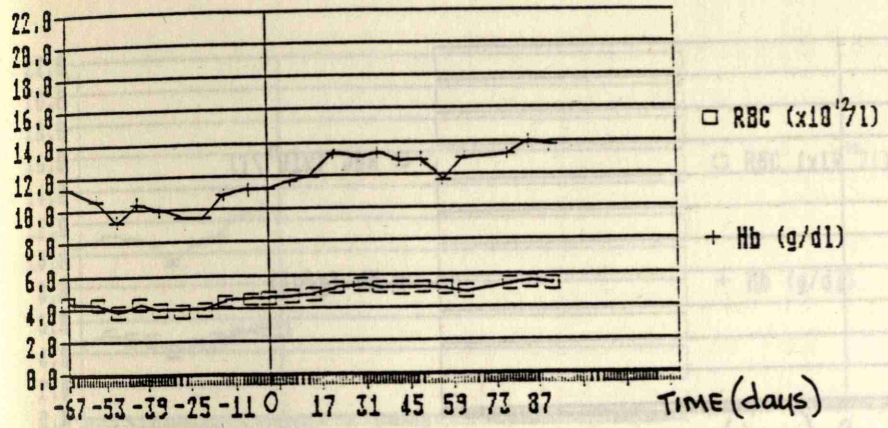


Fig 4-36 Dog 33's rbc counts and haemoglobin levels

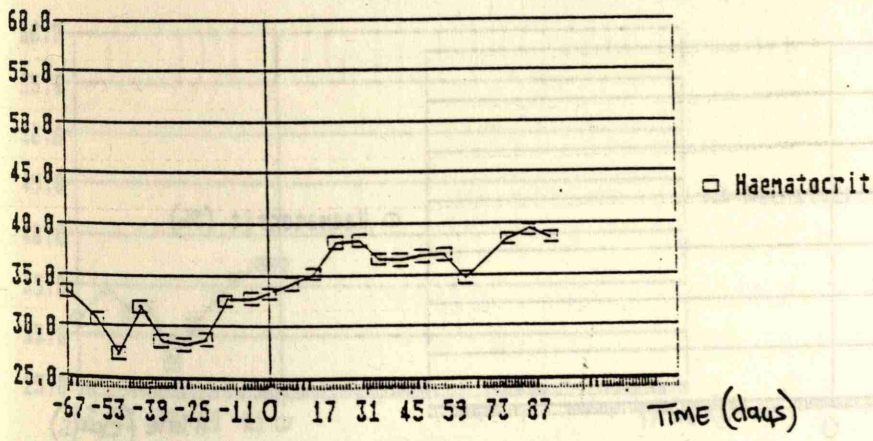


Fig 4-37 Dog 33's haematocrits (%)

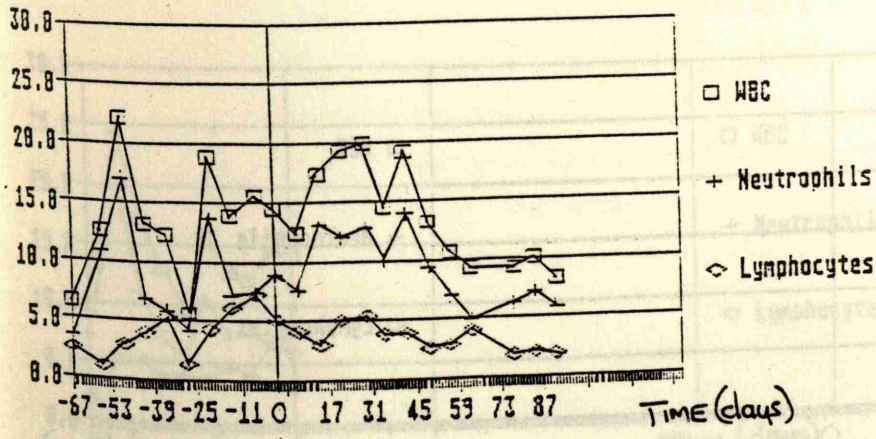


Fig 4-38 Dog 33's leucocytology results (x10^9/l)

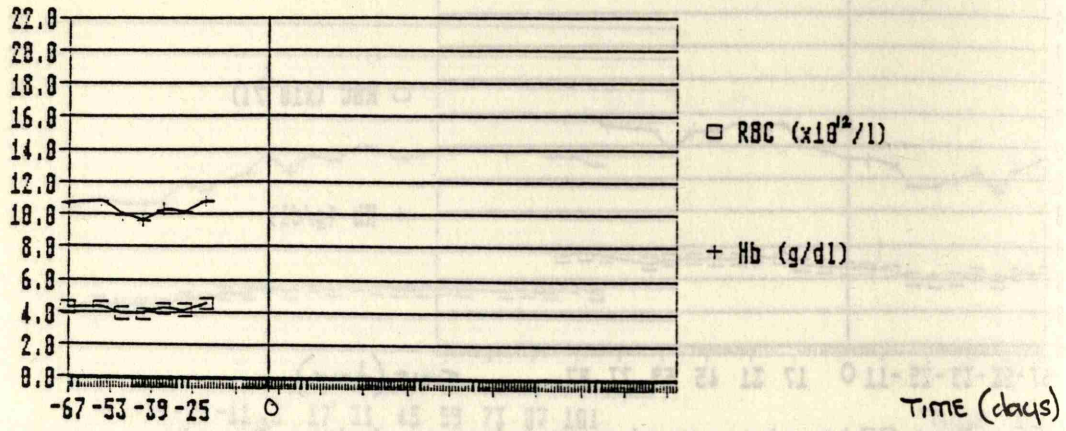


Fig 4-39 Control dog 34's rbc counts and Hb levels

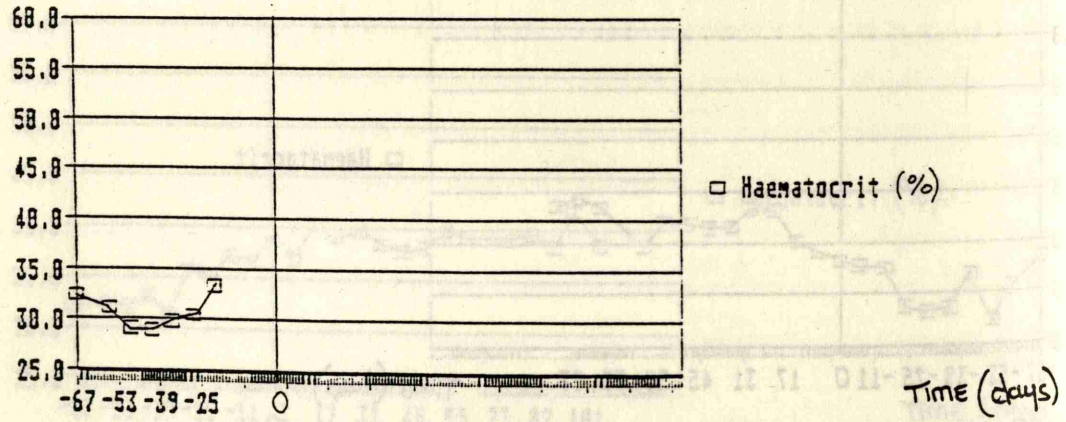


Fig 4-40 Control dog 34's haematocrits (%)

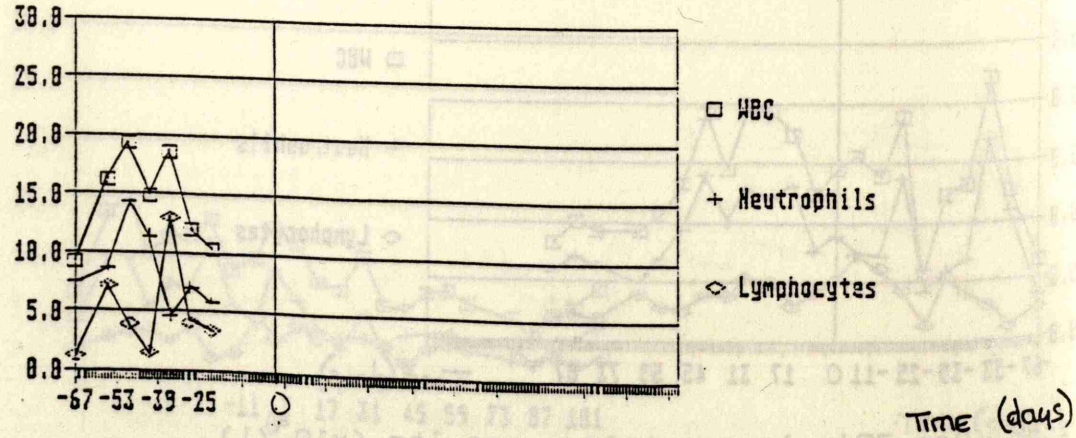


Fig 4-41 Control dog 34's leucocytology results (x10⁹/l)

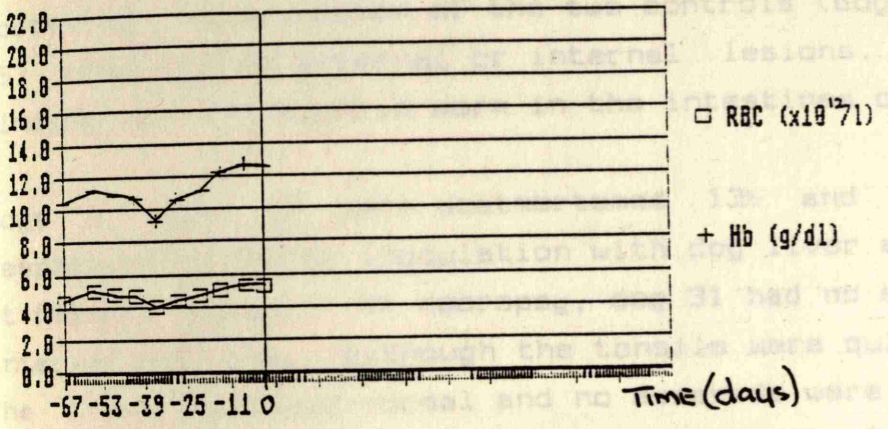


Fig 4-42 Control dog 35's rbc counts and Hb levels

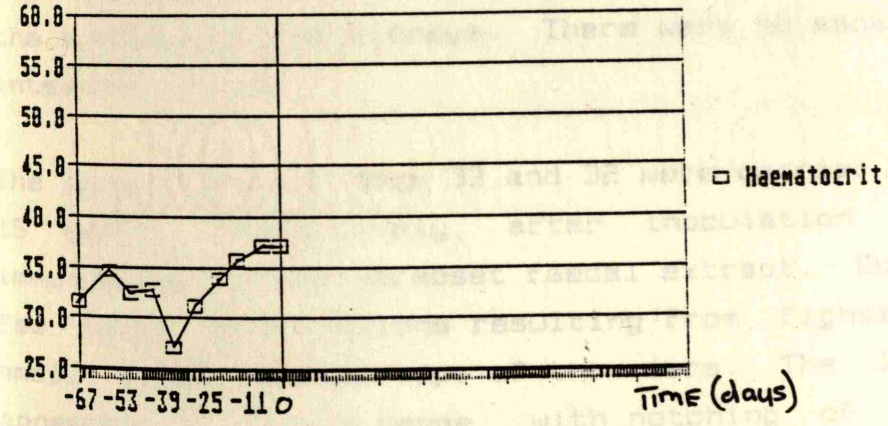


Fig 4-43 Control dog 35's haematocrits (%)

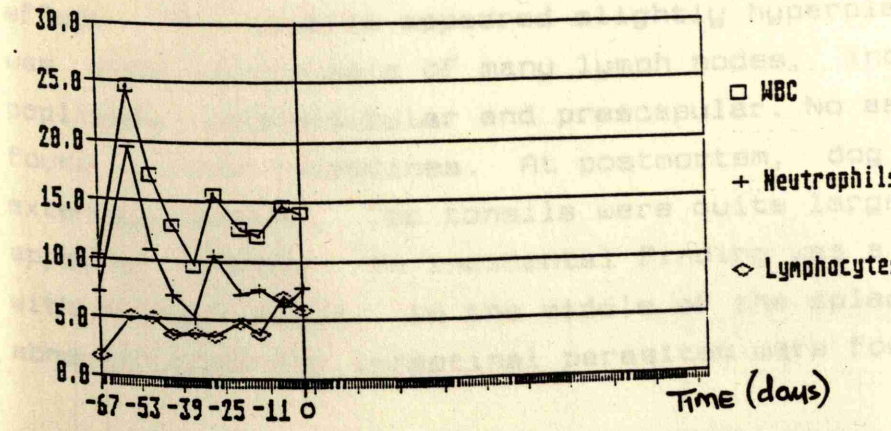


Fig 4-44 Control dog 35's leucocytology results (x10⁹/l)

4.3.7 Postmortem Findings

Postmortem examination of the two controls (dogs 34 and 35) revealed no external or internal lesions. The only finding was one ascarid worm in the intestines of dog 34.

Dogs 31 and 30 were postmortemed 13½ and 15 weeks, respectively, after inoculation with dog liver and marmoset faecal extract. At necropsy, dog 31 had no external or internal lesions, although the tonsils were quite large. The liver appeared normal and no ascarids were found in the intestinal tract. Postmortem examination of dog 30 did not reveal any external lesions, but the left medial hepatic lobe had a slightly more rounded and notched edge than normal. A few pale, indistinct foci were visible on the surface of the kidneys. There were no ascarids in the intestinal tract.

The postmortems of dogs 33 and 32 were carried out 13½ and 15 weeks, respectively, after inoculation with human immunoglobulin and marmoset faecal extract. Dog 33 had a few minor skin lesions resulting from fighting: a few small scabs on the tips of the ears. The liver lobes appeared a little tense, with notching of the edges. Several pale foci were visible on the surface of both kidneys. When cut into, these were found to be pale streaks running through the cortex into the medulla. At least one distorted the surface, producing a scalloped effect. The tonsils appeared slightly hyperplastic. There was also hyperplasia of many lymph nodes, including the popliteal, submandibular and prescapular. No ascarids were found in the intestines. At postmortem, dog 32 had no external lesions. The tonsils were quite large. The liver appeared normal. An incidental finding was a 2cm slit, with rounded edges, in the middle of the spleen. No other abnormalities nor intestinal parasites were found.

Urine samples were collected at postmortem from the

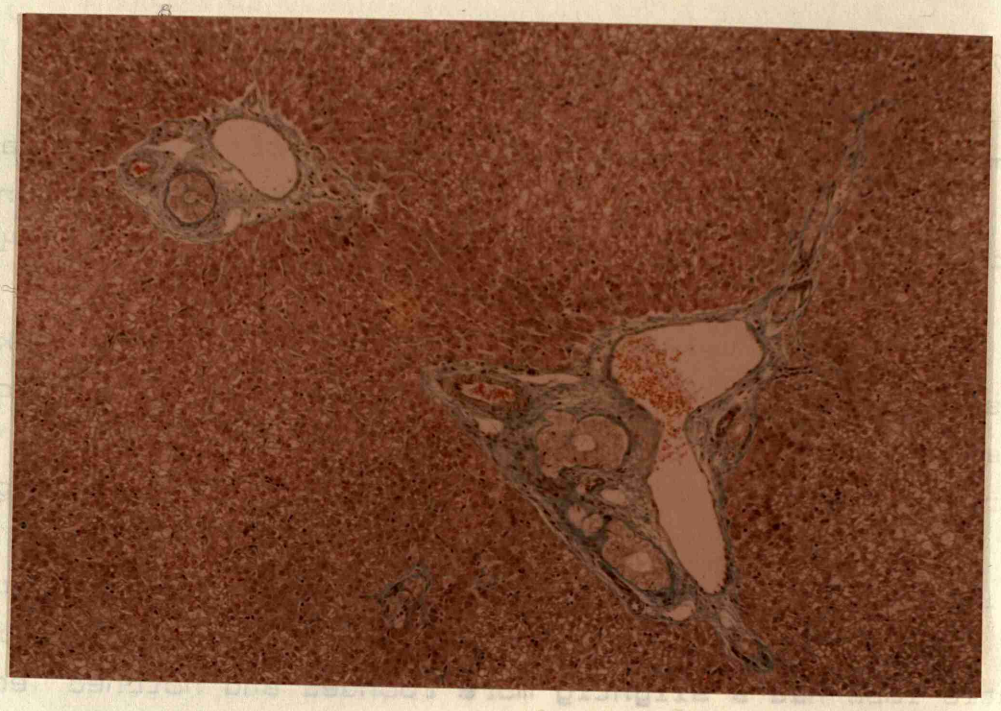

Fig 4-45 Liver of control dog 34 (Mass tri ×87.5)

Fig 4-46 Liver of control dog 35 (Mass tri ×35)

bladders of all the dogs except dog 34, whose bladder was empty. The results of the biochemical analysis of these, given in table 4-3, were mainly within recognised normal ranges. The urine of dog 33 was slightly alkaline. Dog 35's urine had a low urea level and contained a trace amount of protein.

| Dog No | Protein mg/100ml | Urea mmol/l | pH | Specific Gravity |
|---|---|---|---|---|
| 30 | 0 | 455 | 7.0 | 1.034 |
| 31 | 0 | 295 | 5.8 | 1.020 |
| 32 | 0 | 330 | 7.0 | 1.024 |
| 33 | 0 | 605 | 8.0 | 1.031 |
| 35 | 2 | 28 | 5.8 | 1.021 |

Table 4-3 Dog experiment 1 urine biochemistry results

4.3.8 Histological Results

Table 4-4 gives the acidophil cell scores of the dogs in this experiment. In this section the descriptions of the histological findings in the control pair are given first, followed by those of the pair inoculated with dog liver and then those of the pair given human immunoglobulin. Within each pair, the findings are described in the order in which the postmortems were performed.

Histological examination of dog 34's liver (figure 4-45) revealed very few acidophilic cells, mainly in ones and twos at portal tracts, in some sections and none in other sections. Small foci of Kupffer cells and occasional small foci of lymphoid cells were present in the liver. Several small foci of lymphoid cells, including plasma cells, were seen in the cortex and medulla of both kidneys. Some of the medullary foci consisted almost totally of plasma cells. Most of the follicles in the tonsils and lymph nodes had germinal centres. No abnormalities were found in

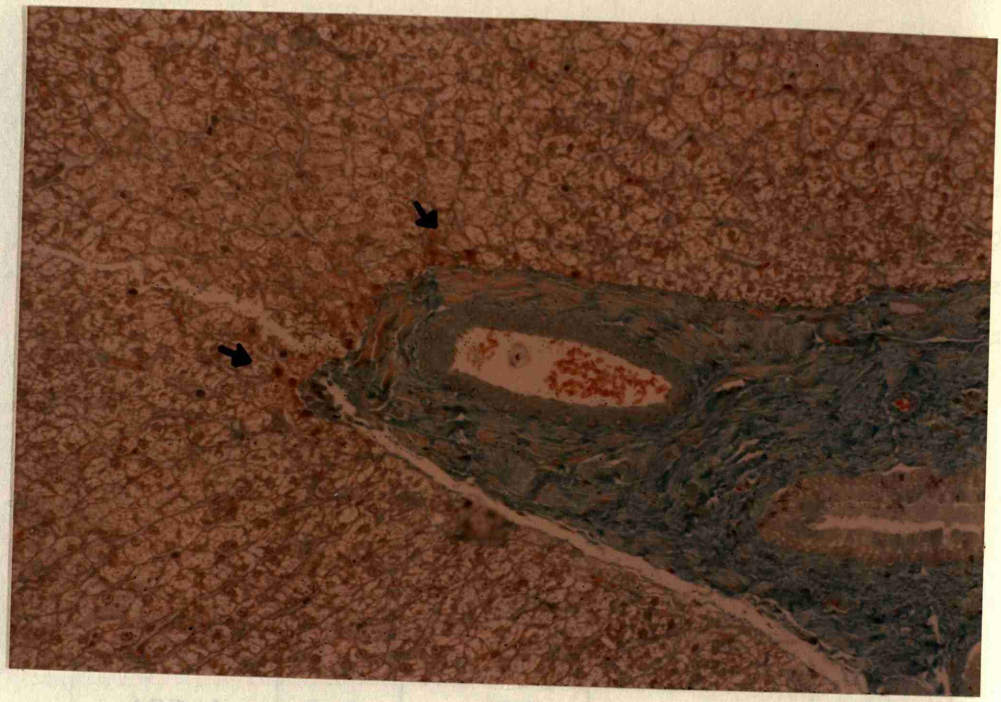

Fig 4-47 Liver of dog 31 (Mass tri x175)
acidophil cells (↘)

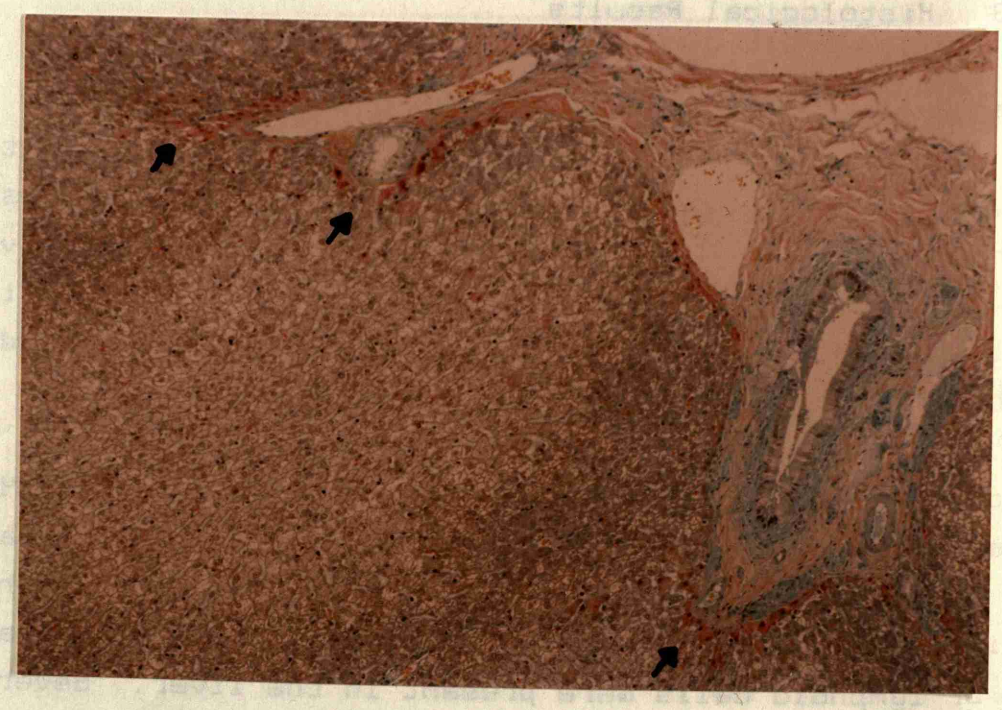

Fig 4-48 Liver of dog 30 (Mass tri x87.5)
acidophil cells (↗)

any of the other tissues.

Dog 35 had virtually no acidophil cells in its liver sections (figure 4-46). A few small foci of lymphoid cells were found in the kidney cortex and medulla. A few of the medullary foci consisted mainly of plasma cells and two of them formed streaks between the tubules. In one of the two lung sections there was one very small area of lymphoid infiltration. No other abnormalities were detected in the other tissues examined.

Some of dog 31's liver sections did not contain any acidophil cells, others had one or two here and there. Figure 4-47 shows one of the more affected areas. A small area of light, subepithelial lymphoid cell infiltration was seen in the calyx in one kidney section. Very little lymphoid reaction was apparent in the other kidney sections examined. The tonsils and mesenteric lymph node had quite large germinal centres. A couple of foci of lymphoid cells were seen in the lung sections. The other tissues examined had no abnormalities.

Microscopy of dog 30's liver sections revealed quite a few acidophil cells, in small groups, in the limiting plates of quite a few portal tracts (figure 4-48). The dog also had a degree of pyelitis, with a subepithelial lymphoid reaction in the renal calyxes. A few follicles had formed and there was a little infiltration of the epithelium in places. The tonsils contained large germinal follicles. There were a few foci of lymphoid cells at airways in one of the lung sections. This dog's other tissues appeared normal.

Histological examination of dog 33's liver sections revealed either no acidophil cells or hardly any. The kidney lesions, visible grossly at postmortem as pale streaks, were found to consist of localised areas of interstitial lymphoid cell infiltration, including plasma

fp169

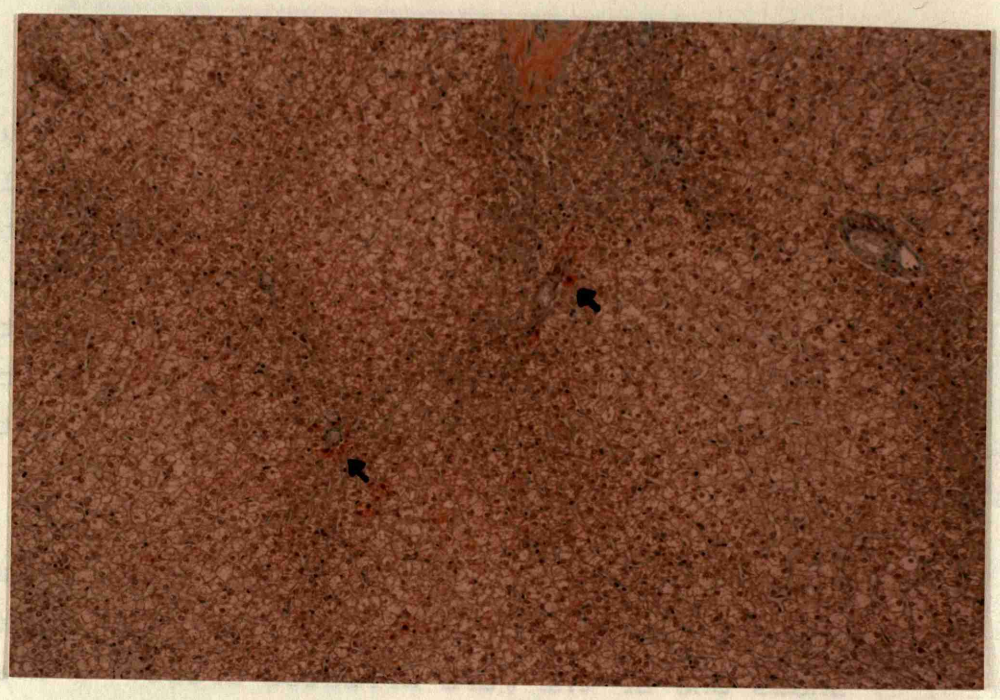

Fig 4-49 Liver of dog 32 (Mass tri ×87.5)
acidophil cells (↖)

cells. This focal reaction was somewhat reminiscent of an old adenovirus reaction; but even if it was, it would be too late to expect any liver lesions. There was some loss of tubules in the most heavily infiltrated streak, in other areas there was only sparse infiltration between tubules. Some subepithelial lymphoid infiltration was present in the calyx. The tonsils had quite large germinal follicles. Only a few small foci of lymphoid cells were seen in two of the four lung sections. No abnormalities were found in any of the other tissues examined.

Dog 32's liver sections contained either no acidophil cells, very few or a few, mainly in ones and twos. Figure 4-49 shows the largest numbers of acidophil cells seen at any portal tract. Only one of the four lung sections had one or two foci of lymphoid cells. Large germinal centres were seen in the tonsils. This dog's other tissues all appeared normal.

4.3.9 Conclusion

The inoculation of dogs 32 and 33 with pooled human Ig and marmoset faecal extract appeared not to have resulted in hepatitis.

Dog 31, culled 13½ weeks after inoculation with dog B's liver and marmoset faecal extract, did not show histological evidence of CACH. The liver of dog 30, killed 15 weeks after the same inoculations, did have a suggestion of development of CACH although the lesions were not numerous enough to be considered a definite positive reaction.

The rectal temperature peaks seen in dogs 30 and 31, just after inoculation with dog liver, were considered to be due to the localised adverse reaction at the inoculation site rather than any CACH infection. No other dramatic peaks were exhibited by dog 30 and dog 31 had only one other peak. The rectal temperature of dog 32 never rose

| Dog No. | Inoculum used | Time post inoc.(wks) | Age (wks) | AC score | Result |
|---|---|---|---|---|---|
| 31 | dog B liver & faecal extract | 13½ | 29½ | - | — |
| 30 | dog B liver & faecal extract | 15 | 33 | ++ | — |
| 33 | human immunoglob & faecal extract | 13½ | 29½ | - | — |
| 32 | human immunoglob & faecal extract | 15 | 31 | + | — |
| 34 | none = CONTROL | n/a | 13½ | - | — |
| 35 | none = CONTROL | n/a | 16 | - | — |

Table 4-4 Dog experiment 1 liver histology results - acidophil cell scores

Liver lesions were graded on a scale of - to ++++++, which cannot be compared directly to the scales used in other species. A positive acidophil cell reaction was taken as +++ or more. The acidophil cell scoring system is explained in appendix 2.

above 40°C. Dog 33's temperature range always appeared to be different from those of the others for no obvious reason, although fighting may have contributed to some of the later peaks. Examination of the rectal temperatures does little to help clarify the results of this experiment.

The dramatic ALT peaks seen in dogs 30 and 31, immediately after inoculation with the dog liver, were probably mainly exogenous enzyme. Dog 30 was the only one of the pair to exhibit another ALT peak; it measured 63 IU/l and occurred 39 days (5½ weeks) after inoculation. This could be considered to help strengthen the possibility that dog 30 was developing CACH, but it cannot be taken as conclusive proof since the two dogs (32 and 33) given human material each had one lower peak, just above the normal ALT range. Analysis of the AST and alkaline phosphatase levels did not help clarify the situation any.

The haematological analyses performed during this experiment revealed unremarkable results for red blood cell parameters. The degree of variation, in the leucocytology results of most of the dogs before inoculation, made it difficult to attribute very much significance to any of the changes seen after inoculation.

The results of CACH part of this experiment were inconclusive, especially considering the added complication of dog 30's probable adenovirus infection and its possible spread to the other dogs. The experiment did, however, highlight several things to be avoided in future experiments including: the mixing of dogs from more than one source, the use of the first two accommodation facilities and the use of unrefined liver homogenate as an inoculum.

## 4.4 DOG EXPERIMENT TWO

### 4.4.1 Experiment Design

In this experiment a second passage of CACH was attempted by inoculation of dogs with material from an experimentally-infected dog. The liver preparation was clarified before use, in the hope of avoiding the adverse reaction seen in experiment 1 (section 4.3.3). The experiment was also designed to test whether filtration of the clarified preparation affected transmission of the disease i.e. whether it removed the infectious agent.

The six border collie crosses used in this experiment were all littermates; four were male (dogs 36 to 39) and two were female (dogs 40 and 41). They were obtained at 7 weeks of age and were delivered directly to one of the freshly prepared isolation units described in section 2.2.1. They therefore avoided the temporary accommodation which the experiment 1 dogs had to use initially.

Frozen liver from dog E was thawed, chopped and stomached in sterile PBS to form a 20% weight:volume preparation. This was clarified in a bench centrifuge at 2 000 rpm for 10 minutes, the supernatant was further clarified in an ultracentrifuge at 10 000 rpm for 10 minutes at 4°C. Half of the resultant supernatant was passed through a 0.65μm filter under negative pressure.

One of the males (dog 39) was euthanased at 4½ months of age, as a control for liver condition, on the day the others were inoculated. He was picked because he was being bullyed by his littermates. One male and one female (dogs 36 and 40 respectively) were inoculated with 6ml each of the clarified liver preparation, split between two sites in the scruff. The remaining two males (dogs 37 and 38) and the other female (dog 41) were each inoculated with 6ml of the clarified and filtered preparation, split

fp172

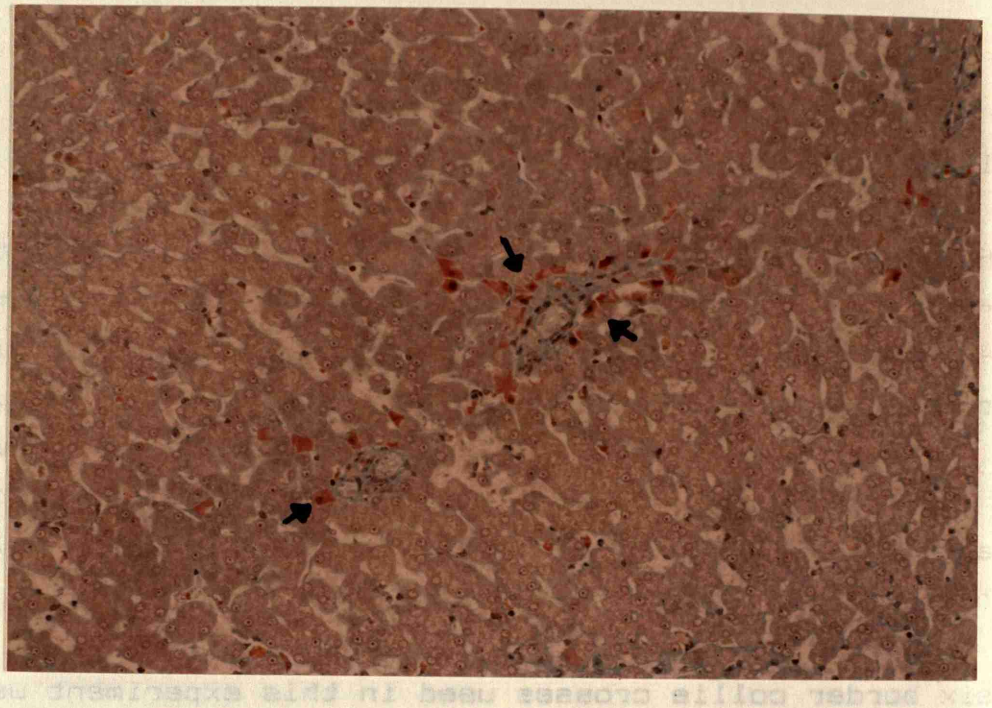

Fig 4-50 Liver of CACH field dog G/FC3 (Mass tri x175)
19-month-old F GSD classic shrunken, polygonal acidophil cells with condensed nuclei (↗)

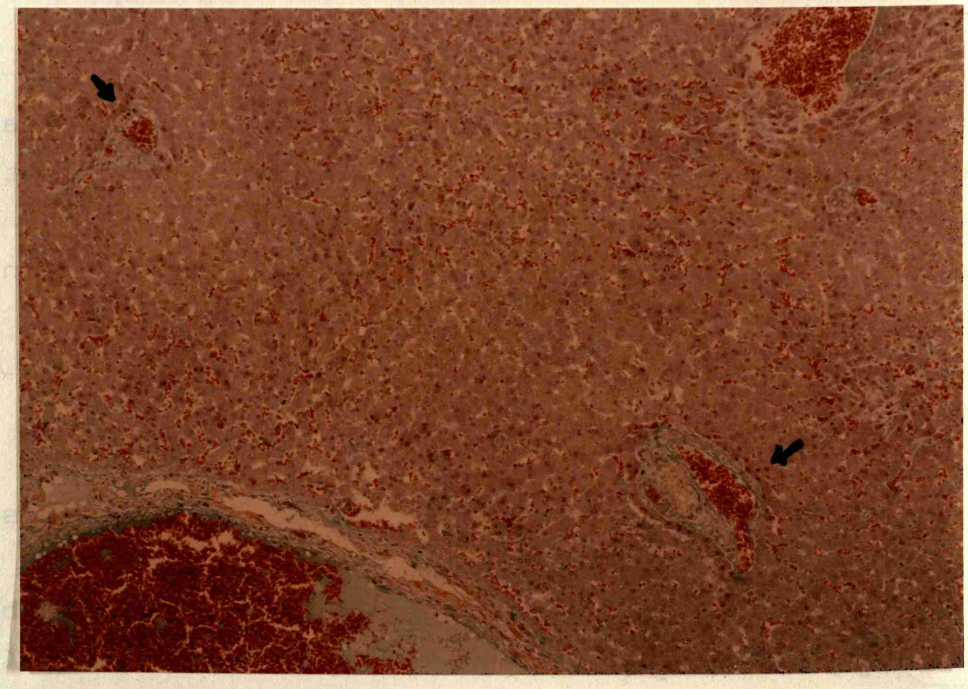

Fig 4-51 Liver of CACH exptl dog E (Mass tri x87.5)
15-week-old F cross ACs in PT limiting plates

between two scruff sites. Dogs 36, 37 and 38 were killed 4, 5½ and 7½ months after inoculation respectively. The two females were kept for a long-term study, during which immunosuppression was attempted. Dog 41 was killed 26 months after inoculation but it was 36 months after inoculation before dog 40 was killed. All five of the inoculated dogs appeared healthy and active throughout the experiment.

During the course of the experiment it became clear that, although there was biochemical and histological evidence of liver disease, the dogs were not affected severely enough to become obviously ill. To test the effect of immunosuppression on the course of CACH, attempts were then made to immunosuppress the two long-term study females. It was hoped that immunosuppression might allow a more severe form of the disease to develop. The different pharmacological means tried are given in section 4.4.4.

### 4.4.2 Source Case

The source of liver, dog E, was a female, crossbred experimental dog [dog 9 in Jarrett and O'Neil (1985)]. This had been inoculated at 14 weeks of age with serum and a liver suspension from dog G (FC3), a 19-month-old, female German shepherd dog. The latter had had a 1-month history of ascites and vomiting. At postmortem it had marked ascites, an accessory portal circulation and a nodular liver. Histologically dog G (FC3) exhibited a diffuse, severe acidophil cell hepatitis, with varying numbers of acidophil cells at most portal tracts (figure 4-50). Dog G's clinical history and pathology are discussed in more detail in chapter 3 (section 3.2.3).

Experimental dog E had become moribund 9 days after inoculation at which time its ALT level had been 107 IU/l. Histological examination of dog E's liver also revealed acidophil cell hepatitis (figure 4-51). In the most

| TIMING of blood sampling | AGE wks | Antibody TITRES to DISTEMPER | | | | | | Antibody TITRES to PARVOVIRUS | | | | | | Antibody TITRES to ADENOVIRUS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 36 | 37 | 38 | 39 | 40 | 41 | 36 | 37 | 38 | 39 | 40 | 41 | 36 | 37 | 38 | 39 | 40 | 41 |
| Pups still with dam | 5½ | ⩽8 | | ⩽8 | ⩽8 | ⩽8 | ⩽8 | 32 | | 32 | 32 | 16 | 32 | <8 | | <8 | <8 | <8 | 16 |
| 4 dys post arrival | 7½ | | ⩽8 | | | | | <4 | 8 | 16 | 16 | <4 | 8 | | <8 | | | | |
| Pre vaccination | 9½ | ⩽8 | ⩽16 | ⩽8 | ⩽8 | ⩽8 | 12 | <4 | 8 | 16 | 32 | 32 | 16 | ⩽8 | ⩽16 | ⩽16 | ⩽8 | ⩽8 | ⩽8 |
| 12 dys post vaccination | 11½ | | | | | | | 128 | 16 | 16 | 8 | 16 | 32 | | | | | | |
| 20 dys post 2nd vaccin. | 16½ | 5813 | 8192 | 5813 | 5813 | 16384 | 2884 | 2048 | 2048 | 1024 | 2048 | 512 | 2048 | 1024 | 4096 | 1024 | 734 | 2884 | 367 |
| 5 mths post 2nd vaccin. | 35 | 23240 | | | — | | | 128 | | | — | | | 1453 | 1453 | 2048 | — | ⩾32768 | 1453 |
| 6½ mth post 2nd vaccin. | 41½ | — | 16384 | | — | | | — | 1024 | | — | | | — | 2048 | | — | | |
| 8½ mth post 2nd vaccin. | 50½ | — | — | 2048 | — | | | — | — | 32 | — | | | — | — | 1453 | — | | |
| 9½ mth post 2nd vaccin. | 55½ | — | — | — | — | 11620 | 4096 | — | — | — | — | 256 | 512 | — | — | — | — | 8192 | 2048 |
| 20 mth post 2nd vaccin. | 2yr | — | — | — | — | 11620 | 2048 | — | — | — | — | 128 | 128 | — | — | — | — | 16384 | 1453 |

Table 4-5 Dog experiment 2 antibody titres

When the pups were 5½ weeks of age their dam's titres were: distemper 16,
parvo 1024 and adeno 16.

severely affected areas necrobiotic acidophil cells formed a bridging pattern. There was a very slight increase in the amount of fibrous tissue in the liver but no hepatocellular vacuolation.

### 4.4.3 General Clinical History

Three weeks after their arrival, the dogs were vaccinated against distemper, parvovirus enteritis, infectious canine hepatitis and leptospirosis with the same Kavak products as were used in experiment 1 (section 4.3.3). The resultant immune response to the parvovirus part was poor in five of the dogs, so different products were used for the second vaccination. The pups were given the live, attenuated Nobivac DH2 and Nobivac Parvo-c, along with the inactivated Nobivac L (Intervet Laboratories Ltd, Cambridge) at 13½ weeks of age.

Blood samples for serology had been obtained from the dam and five of the six pups at 5½ weeks of age, before they were weaned and brought to the veterinary school. The pups' antibody titres were also measured 4 days after arrival, 2½ weeks after arrival (just before the first vaccination), 12 days after the first vaccination and 20 days after the second vaccination. The titres of dogs 36 to 38 were checked before each was killed. Dogs 40 and 41 had their titres measured twice when they were older. These results are all given in table 4-5.

After the second vaccination, the dogs all had good protective titres against distemper, parvo and adeno viruses. In general, reasonable antibody levels were maintained throughout life. Dog 40's very high adeno titre, 5 months after the second vaccination, was not considered suggestive of infection since the animal was already immune to adenovirus.

Faecal egg counts were performed on samples collected

| TIME re arrival | -2½ | -1½ | ARRIVAL =0 | ½ | 1 | 1½ | 2 | 2½ | 2½-3 | 3½ |
|---|---|---|---|---|---|---|---|---|---|---|
| AGE of dogs (weeks) | 4½ | 5½ | 7 | 7½ | 8 | 8½ | 9 | 9½ | 9½-10 | 10½ |
| Unidentif. faeces | | | 0 | 4x0 | | | | | | |
| Dog 36 | piperazine | piperazine | piperazine | | piperazine | 0 | piperazine | 0 | *fenbendazole | 0 |
| Dog 37 | | | | | | 600 | | 400 | | 0 |
| Dog 38 | | | | 0 | | 50 | | | | 0 |
| Dog 39 | | | | | | 0 | | 0 | | 0 |
| Dog 40 | | | 0 | | | 0 | | 0 | | 0 |
| Dog 41 | | | | | | 0 | | 0 | | 0 |

Table 4-6 Dog experiment 2 early faecal egg counts and anthelmintic regime (Toxocara)

The unidentified faecal samples were collected from individual stools on the floor.
* = a four day divided dose course

regularly from the dogs, at lengthening intervals. For 2 months after arrival samples were collected weekly, then fortnightly for 2 months, then every third week for about 5 months, then every second month and eventually every third month. The results of the first few parasitological examinations are given in table 4-6.

A regime of anthelmintic treatment was started while the pups were still with the dam, before their arrival at the vet school. At 4½ weeks of age they and their dam were wormed with piperazine adipate tablets (Head-To-Tail Veterinary Roundworm Tablets). The pups were wormed again, with the same product, at 5½ weeks of age. At the same time they were sprayed with Nuvan Top (dichlorvos and fenitrothion aerosol) since they were scratching a lot.

On arrival the pups were too small for the collection of rectal faecal samples, however one was observed defaecating and another fresh, unidentified sample was collected from the pen floor. Similarly, a few days later another dog was observed defaecating and a sample was collected along with four different fresh, unidentified samples from the pen floor. By the following week the pups were large enough to enable rectal samples to be collected and two of them had evidence of Toxocara infection, despite having had two doses of piperazine since their arrival. After another dose failed to clear the parasites, the pups were given fenbendazole (Panacur 22% granules) in a divided-dose course, over 4 days. Such a regime is considered more effective than giving the full dose at one time. The dogs all had negative FECs after the fenbendazole and from then on. To avoid any further outbreaks of parasitism a regular anthelmintic regime, with a reducing frequency of dosage, was followed throughout the experiment.

When the dogs were about 4 months of age dog 37 sustained some minor superficial abrasions, presumably in an episode of bullying. However 2 days later in a more serious

episode dog 39, the smallest male, was badly bitten. He sustained many superficial abrasions and puncture wounds, including a 3cm diameter abrasion on the anterior ventral abdomen, and he developed a little submandibular and ventral oedema. The dog was separated from its littermates, given an intramuscular diuretic injection (Lasix 5% solution, Hoechst Animal Health, Milton Keynes) and put on an oral course of ampicillin (one Amfipen 125mg tablet bid, Mycofarm UK Ltd, Cambridge). The wounds were cleaned and treated topically with oxytetracycline (Alamycin aerosol). The oedema had resolved by the following day and the dog's demenour had improved, but the sternal and anterior abdominal region had became swollen and firm. Later the circular abraided area of skin began to slough. Although the wounds would probably have healed with time, dog 39 would have had to be kept apart from the others for a long time and could not have been used in the experiment until fully recovered. Since the experiment was scheduled to begin it was decided that, in the circumstances, it would be kinder to euthanase dog 39. This was done 4 days after the injury, on the day the others were inoculated.

### 4.4.4 Immunosuppressive Regimes

Seven months (212 days) after inoculation, dog 40 was put on an oral course of azothioprine in solution. The preparation of the azothioprine solution is described in chapter 2 (section 2.2.7). The daily dose of 1mg/kg was mixed into the food on weekdays only, i.e. 5 consecutive days per week. The dose was increased after 2 weeks, since there was no noticeable effect on haematological parameters, to 1½mg/kg/day weekdays. After a further 3 weeks the dose was increased to 2mg/kg, but this was only administered for 3 days until the first day's biochemistry results were available. The ALT level had been markedly raised in the blood sample taken just prior to administration of the first 2mg/kg dose. Azathioprine administration was resumed, over 3 weeks later, at a dose of 1mg/kg/day weekdays.

After 4 weeks without any noticeable effect on wbc counts, the dose was increased to 1½mg/kg for a further 2 weeks. Administration ceased when dog 40's ALT level rose again and it was not recommenced since it appeared to have failed to induce clinical immunosuppression.

Prednisolone was tried in dog 41 as an alternative immunosuppressant. Ten months (307 days) after inoculation this dog was put on an oral course of 0.5mg/kg prednisolone every second day. The alternate-day regime is designed to reduce the occurrence of some of the adverse side effects of therapeutic corticosteroid administration, by lessening the suppression of the patient's natural corticosteroid production. After 5 weeks without a noticeable effect on the haematological profile, the dose was gradually reduced to nothing, over a 10 day period. Since it was thought that the alternate-day regime might have reduced the induction of immunosuppression along with the undesired side effects, daily administration of 0.25mg/kg was begun 3 days later. After 2 weeks the dose was increased to an alternating regime of 0.5mg/kg one day and 0.25mg/kg the next. This was increased, after another week, to 0.5mg/kg daily for 2 weeks. Then 0.75mg/kg was administered daily for 5 days. The dose of prednisolone was reduced to 0.5mg/kg, because of a raised ALT level, for 4 days before returning to 0.75mg/kg for another 2½ weeks. No clinical immunosuppression had been produced, so the attempt was abandoned. Prednisolone administration was reduced gradually, over a 3-week period, to avoid the problems encountered with sudden withdrawl after prolonged administration.

### 4.4.5 Rectal Temperatures

In this experiment the dogs' rectal temperatures were only checked twice weekly during the period between arrival and inoculation. From the day of inoculation onwards the temperatures were monitored daily, as in the previous

experiment.

During the preinoculation period the dogs' rectal temperatures were mainly in the range of 38.3-39.5°C, as can be seen in figures 4-52 to 4-56 and 4-60. Dogs 36, 38 and 41 did not have any higher preinoculation results. Eighteen days before inoculation dogs 37, 39 and 40 all had a rectal temperature of 39.7°C. That was the only occasion when dog 37's preinoculation rectal temperature exceeded 39.5°C. Dog 39 was pyrexic on the day he was injured, but his temperature had returned to normal by the next day. Dog 40 had had one other result of 39.7°C, 21 days before inoculation, and two later temperatures of 39.6°C. As in experiment 1, 39.7°C was taken to be the top of the normal rectal temperature range; 39.8°C was considered to be an abnormally raised result. Therefore, during the preinoculation period, only the injured dog 39 exceeded the normal temperature range with a spike of 40.0°C

Dog 36 exhibited two low temperature peaks of 39.8°C at 38 and 104 days after inoculation (figure 4-52). A smaller peak of 39.7°C at 73 days, although within the normal range, may have been significant since dog 36's preinoculation temperatures had never exceeded 39.4°C.

Dog 37 exhibited two bouts of mild pyrexia, during a period when his average temperature was higher than usual (figure 4-53). The step up in his average temperature appeared to start about 100 days after inoculation and lasted for around 3 weeks. A similar phenomenon was also seen in dogs 40 and 41. The first peak of 39.9°C occurred 103 days after inoculation. It was followed by a reading of 39.7°C the next day, when dog 36's temperature was also raised. The second peak of 39.8°C occurred 111 days after inoculation.

Several bouts of mild to moderate pyrexia were seen in dog 38 after inoculation (figure 4-54). The first and highest

peak of 40.1°C occurred 38 days after inoculation, when dog 36's temperature was also raised. Temperature peaks of 39.9°C were detected on days 125, 132, 158 and 162. Dog 38's temperature also just exceeded the normal range 70, 87, 130, 157 and 159 days after inoculation.

Dog 40 also had several bouts of mild pyrexia during her much longer lifetime (figures 4-56 to 4-59). Temperature peaks of 39.8°C were seen: 25 days after inoculation, from day 103 to day 105, from day 107 to day 108, and 118 days after inoculation. There appeared to be a step up in the usual range of dog 40's temperature, similar to that seen in dogs 37 and 41, around 100 days after inoculation which lasted about 3 weeks. Dog 40's highest temperature peak of 39.9°C occurred 193 days after inoculation. Just over 7 months (around 225 days) after inoculation, dog 40's usual rectal temperature range appeared to lower to 38.1-39.3°C. Within the original normal range occasional peaks, relative to the dog's general rectal temperatures of the time, were still noticeable (figures 4-57 to 4-59). A rectal temperature of 39.4°C was recorded 288, 374, 424 and 870 days after inoculation and on day 531 dog 40 had a temperature spike of 39.5°C. Twenty-nine months (around 880 days) after inoculation, dog 40's usual rectal temperature range fell again to 37.7-38.9°C. From then until the end of the experiment her temperature only exceeded this range twice, peaking at 39.0°C 920 and 1008 days after inoculation.

Dog 41 was pyrexic on the third day after inoculation, with a temperature of 39.9°C (figure 4-60). As in dogs 37 and 40, there appeared to be a step up in dog 41's average rectal temperature about 100 days after inoculation, which lasted 3 weeks. However there were no dramatic peaks during that period. Her next bout of pyrexia occurred just over 6 months after inoculation, with temperatures of 39.9°C and 39.8°C on days 193 and 195 respectively (figure 4-60). The former coincided with a temperature peak in dog

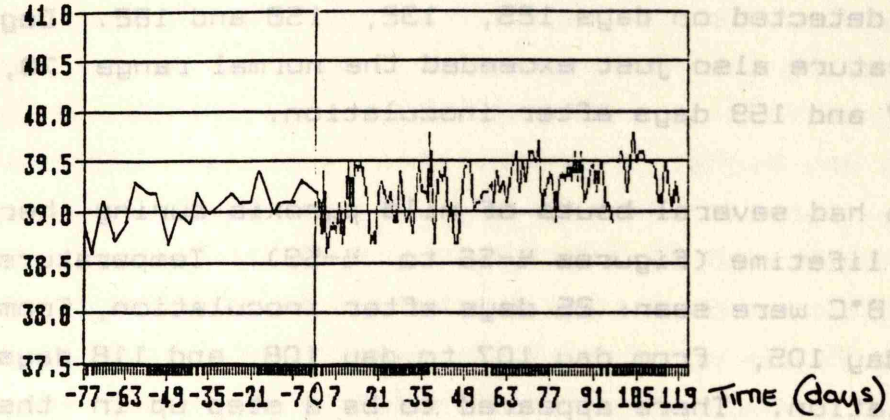


Fig 4-52 Dog 36's rectal temperature (°C)

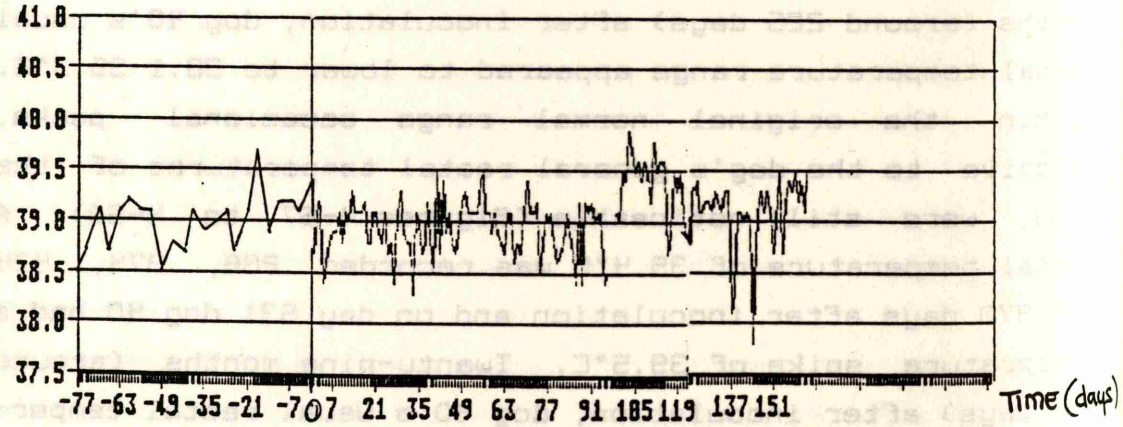


Fig 4-53 Dog 37's rectal temperature (°C)

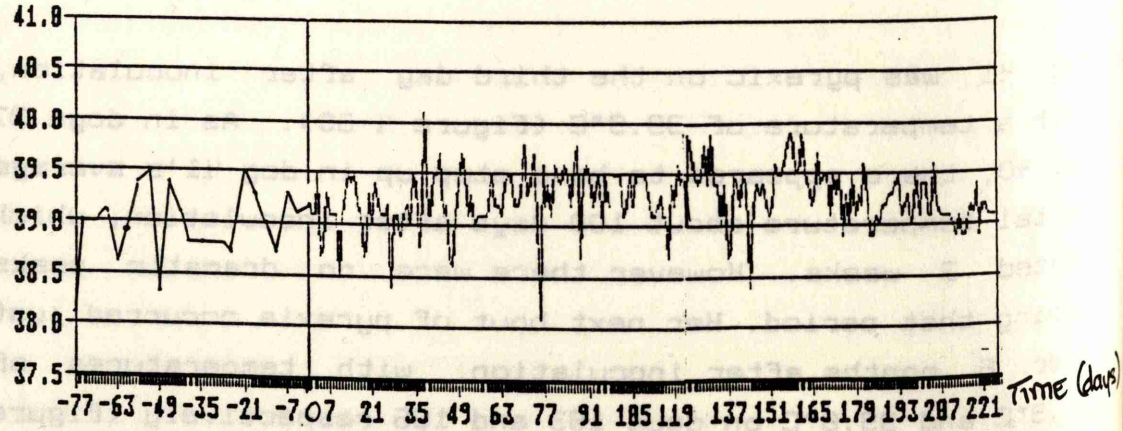


Fig 4-54 Dog 38's rectal temperature (°C)

40. A slight lowering of dog 41's usual temperature range to 38.3-39.3°C occurred just after the bout of pyrexia, slightly earlier than the similar phenomenon in dog 40. This accentuated the peak of 39.9°C seen 237 days after inoculation. About 16 months after inoculation there were two spells of slightly raised temperatures in dog 41. On three of the days between 477 and 480 days after inoculation her temperature reached 39.4°C (figure 4-61). The second bout started a week later at 39.4°C, 488 days after inoculation. Relative peaks of 39.5°C followed on days 490 and 493, then another reading of 39.4°C occurred 496 days after inoculation. Dog 41's rectal temperature only exceeded the described range on one other occasion, reaching 39.5°C 646 days after inoculation (figure 4-62).

The step up in the usual temperature ranges of dogs 37, 40 and 41 occurred in the autumn (early October), unlike the early summer changes seen in some of the dogs of experiments 1 and 3 (sections 4.3.4 and 4.5.4).

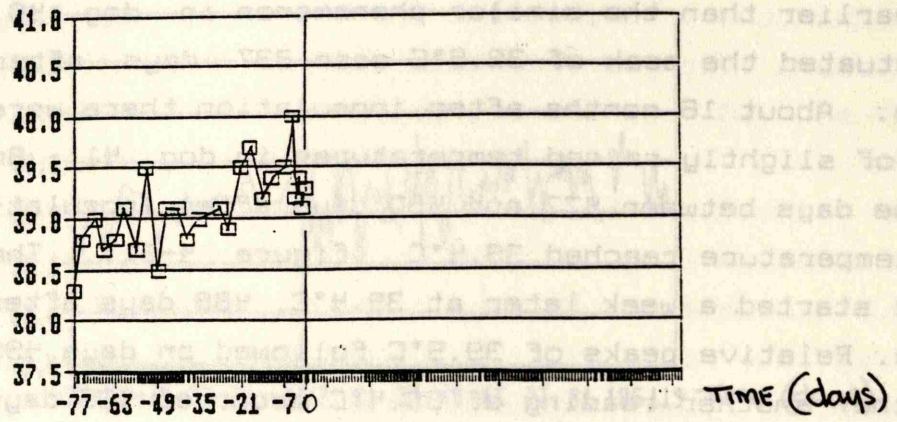


Fig 4-55 Dog 39's rectal temperature (°C)

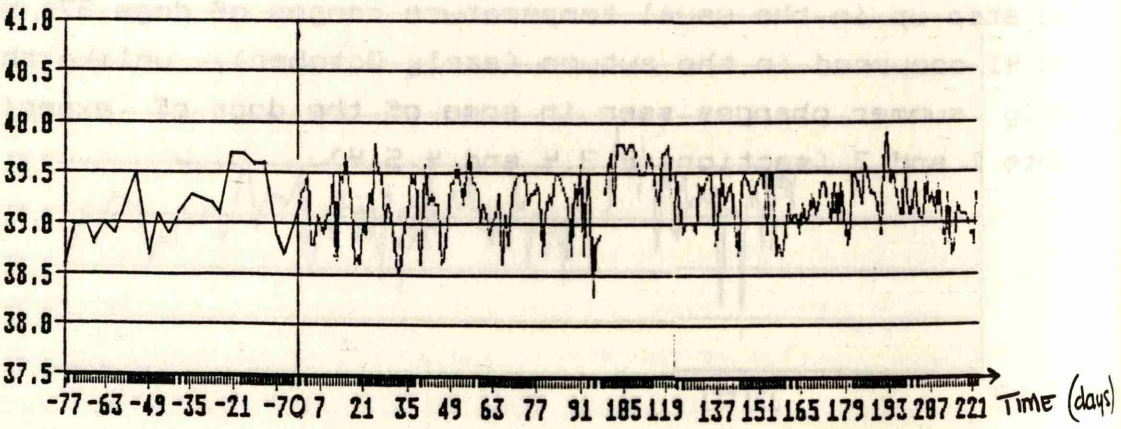


Fig 4-56 Dog 40's rectal temperature - graph 1 (°C)

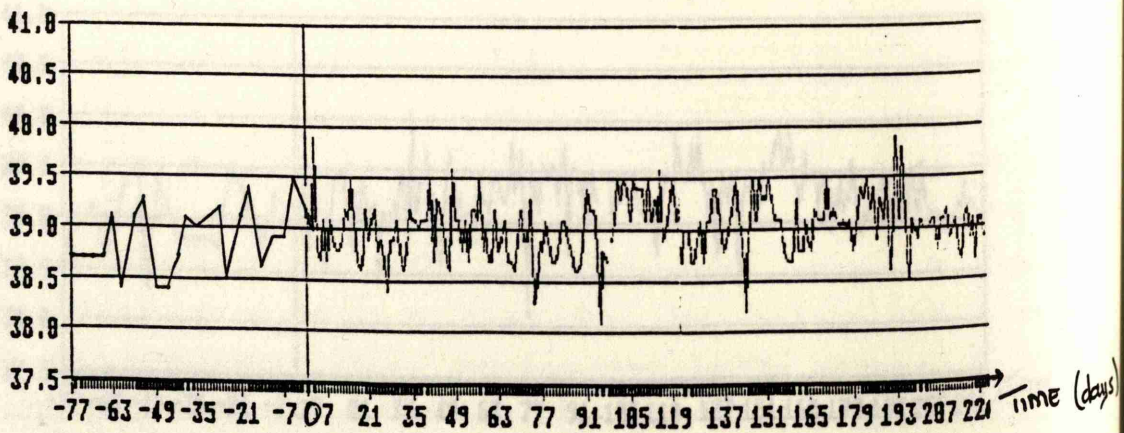


Fig 4-60 Dog 41's rectal temperature - 1 (°C)

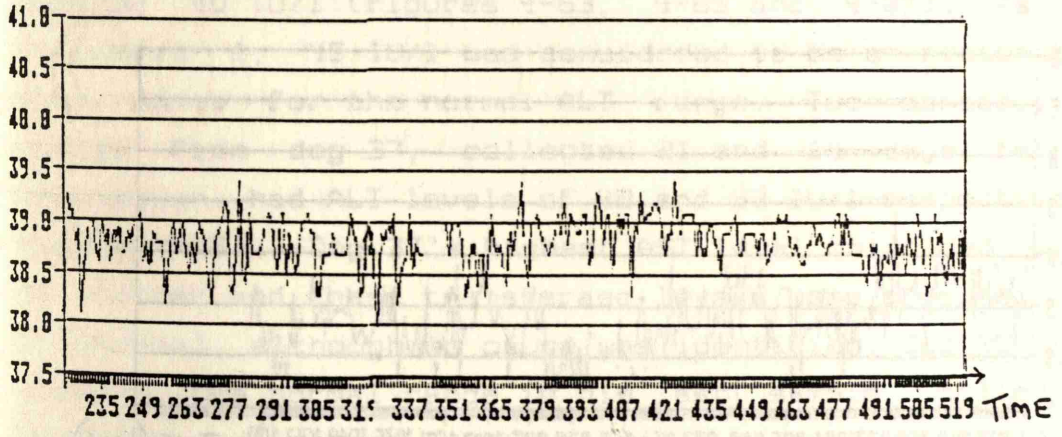


Fig 4-57 Dog 40's rectal temperature (°C) - 2

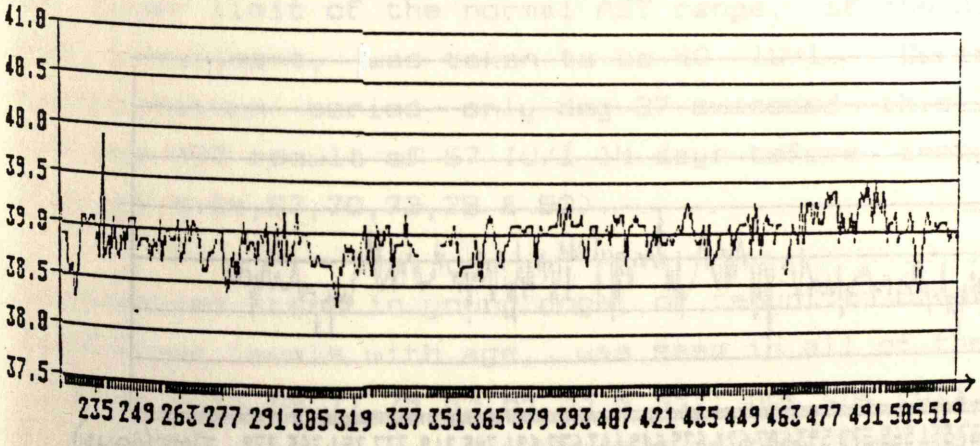


Fig 4-61 Dog 41's rectal temperature (°C) - 2

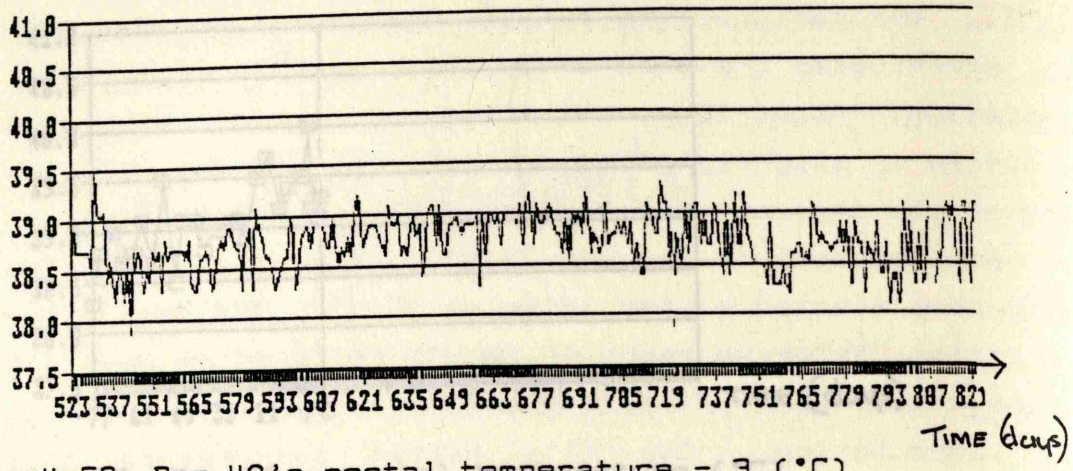


Fig 4-58 Dog 40's rectal temperature - 3 (°C)

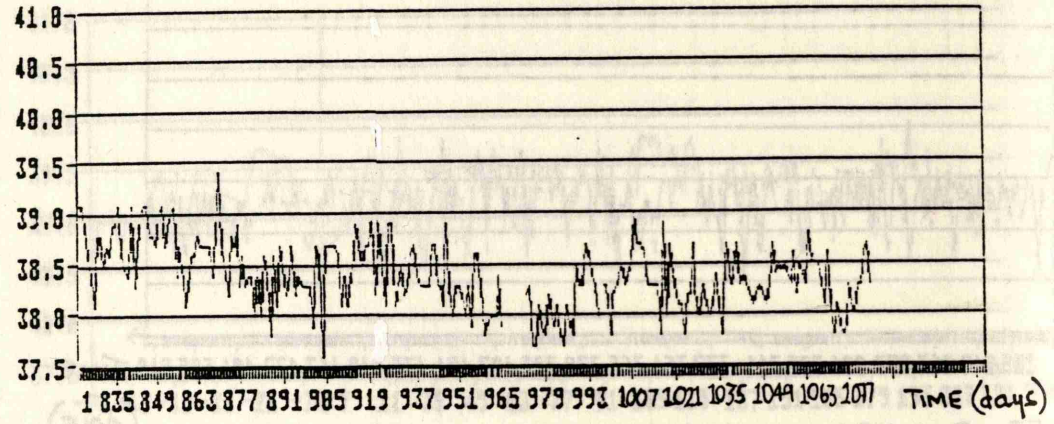


Fig 4-59 Dog 40's rectal temperature - 4 (°C)

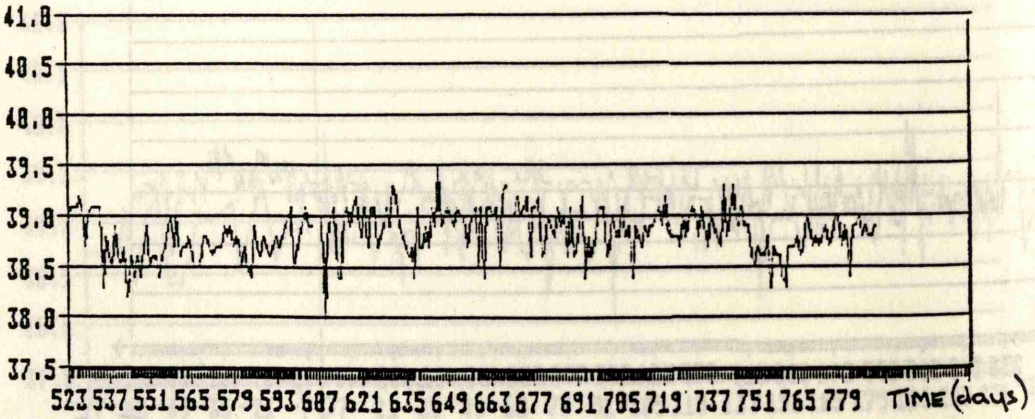


Fig 2-62 Dog 41's rectal temperature - 3 (°C)

### 4.4.6 Biochemical Results

In this experiment blood was collected weekly before inoculation, for biochemical and haematological analyses. For the first 5 weeks after inoculation plasma samples for biochemical analyses were collected twice weekly; thrice weekly collection was then introduced to reduce the chance of missing any peaks in transaminase levels. The measurement of gamma glutamyl transferase levels did not begin until late in the experiment, when only dogs 40 and 41 remained.

During the preinoculation period of this experiment the ALT levels of three of the dogs (36, 38 and 41) never exceeded 40 IU/l (figures 4-63, 4-69 and 4-87). As in experiment 1, 45 IU/l was considered to be a reasonable upper limit for the normal ALT range. Two consecutive samples from dog 37, collected 21 and 14 days before inoculation, had ALT levels of 48 and 53 IU/l respectively (figure 4-66). Dog 37's highest AST level coincided with the latter and these transferase levels were presumed to be abnormal, although no cause was identified. Dog 39 only exceeded the normal range in his last sample, collected after he was injured, with a high ALT level of 151 IU/l (figure 4-72). All of dog 40's preinoculation ALT levels were within the normal range (figure 4-75).

The upper limit of the normal AST range, of the dogs in this experiment, was taken to be 40 IU/l. During the preinoculation period only dog 37 exceeded this level, with one AST result of 57 IU/l 14 days before inoculation (figures 4-64,67,70,73,79 & 90).

The expected trend in young dogs, of reduction of alkaline phosphatase levels with age, was seen in all of the dogs (figures 4-65,68,71,74,83,84,93 & 94); but the full extent of the phenomenon was most obvious in the two long-term study animals. As explained in section 4.2, in the adoles-

cent dogs any increased AP levels tended to be assessed by comparison with other results from the same animal around that time. The general range of preinoculation AP levels found in the dogs of this experiment appeared more uniform than that of the experiment 1 dogs. Dog 40's AP level was always less than 250 IU/l before inoculation. The preinoculation AP levels in dogs 36 and 38 never exceeded 300 IU/l and neither did dog 41's, apart from one result of 330 IU/l. The highest preinoculation AP level seen in dogs 37 and 39 was 316 IU/l. These levels were all less than many of those taken as normal in the experiment 1 dogs and they were considered to be within the normal range for pups of their age.

Dog 36 had ALT levels just above the normal range 17 and 71 days after inoculation and a higher level of 61 IU/l 77 days after inoculation (figure 4-63). A dramatic peak of 132 IU/l occurred 98 days after inoculation and dog 36's ALT level remained elevated from then until he was euthanased, 118 days after inoculation.

The AST level in dog 36 just exceeded the normal range 17 days after inoculation; but higher peaks, of 90 and 57 IU/L, occurred 108 and 113 days after inoculation (figure 4-64). There were no obviously abnormal peaks in dog 36's AP levels, but low peaks, in comparison with surrounding samples, were seen 34, 76, 85 and 118 days after inoculation (figure 4-65). The second of these immediately preceded a sample with a raised ALT level and the last one, which was well within the normal range, coincided with a raised ALT level.

As mentioned earlier, dog 37's slightly raised ALT levels 21 and 14 days before inoculation were presumed to be abnormal. However his early postinoculation ALT levels tended to be a little higher than those of the other dogs around that time (figure 4-66). To make allowance for the possibility that this was a genuine case of individual

variation, 50 IU/l was taken as a more conservative upper limit for dog 37's normal ALT range. Even using this criterion, elevated levels of ALT were still found on several occasions. An initial low spike of 57 IU/l was seen 3 days after inoculation. Dog 37's ALT level was over 50 IU/l in the eight samples collected between 73 and 90 days after inoculation; the sample at 85 days had the highest level of 83 IU/l. Slightly raised ALT levels were seen in two consecutive samples, 98 and 101 days after inoculation. The next bout of seven elevated ALT results occurred between 113 and 128 days after inoculation and peaked at 69 IU/l in the last sample. The same level was attained 153 days after inoculation, in dog 37's last ALT peak.

After inoculation dog 37's AST level was slightly raised on only three occasions: 64, 120 and 153 days after inoculation (figure 4-67). The last of them, with the highest level of 49 IU/l, coincided with the last ALT peak. In dog 37 low peaks in alkaline phosphatase levels were seen 85 and 118 days after inoculation (figure 4-68), during bouts of elevated ALT levels. A marked peak of 587 IU/l, 146 days (21 weeks) after inoculation, clearly exceeded dog 37's normal AP range.

Slight increases in dog 38's ALT level 3 days and 62 days after inoculation did not exceed the normal range (figure 4-69). It was first exceeded 85 days after inoculation, by a result of 51 IU/l. A dramatic peak, of 132 IU/l, 98 days after inoculation was followed by elevated ALT levels, in the next nine samples, up to 120 days after inoculation. There were then only intermittent, slight ALT elevations until the next prolonged bout of high results started, 204 days (29 weeks) after inoculation. Dog 38's ALT level remained elevated until the end of the experiment, but the highest result (243 IU/l) occurred 216 days after inoculation.

In dog 38 raised AST levels of 53 and 58 IU/l were found 59 and 64 days after inoculation respectively (fig. 4-70). Then, during the first of the prolonged bouts of high ALT levels, three elevated AST levels were seen 106, 108 and 113 days after inoculation. The first and highest was 110 IU /l, the other two were both 68 IU/l. Apart from a very slight rise 128 days after inoculation, dog 38 only exceeded the normal AST range on one other occasion; an AST level of 47 IU/l was found in the sample with the highest ALT level, 216 days after inoculation. Alkaline phosphatase levels in dog 38 (figure 4-71) remained within the normal range apart from a peak, of 369 IU/l 118 days after inoculation, which occurred towards the end of the first of the two prolonged bouts of very high ALT levels.

Dog 39 had normal levels of ALT, AST and AP until his last sample, collected on the day that he was euthanased because of his injuries (figures 4-72 to 4-74). It had an ALT level of 151 IU/l.

The first sample collected from dog 40 after inoculation had an elevated ALT level of 55 IU/l (figure 4-75). A dramatic peak of 276 IU/l was found 24 days after inoculation, along with an AST level of 221 IU/l. This was followed by two more high ALT results, but normal AST levels. The next bout of elevated ALT levels started, with a peak of 74 IU/l, 90 days after inoculation and lasted over a week. From 18 to 30 weeks (125 to 211 days) after inoculation there were several bouts of, slightly to moderately, increased ALT levels. The administration of azothioprine commenced 212 days after inoculation. Then only a few very slightly raised ALT results were seen until a dramatic peak of 162 IU/l, 36 weeks after inoculation, which caused a temporary halt in azothioprine administration (figure 4-76). Dog 40's ALT level remained elevated for over a fortnight. Azothioprine administration recommenced and ALT levels continued to exhibit occasional, mainly slight, increases until 45 weeks after

inoculation when there was another bout of very high levels and azothioprine administration was stopped permanently. There then followed more than a year of periodic, low to moderate, peaks in ALT levels (figures 4-76 and 4-77). Dog 40's ALT levels rose markedly again 27½ months after inoculation, peaking at 850 days at 85 IU/l (figure 4-78). Although dog 40's ALT level fell after that peak, it tended to remain just above the normal range until the next prolonged bout of high results, between 126 and 133 weeks after inoculation. The highest levels seen during that period were 106 IU/l and 121 IU/l, at 884 and 920 days after inoculation respectively. Dog 40's ALT level remained slightly elevated for most of the next 7 weeks and showed two moderate peaks. An ALT peak of 77 IU/l, just over 142 weeks after inoculation, was the last one seen in dog 40; her ALT level then remained within the normal range until the end of the experiment, 1084 days (155 weeks) after inoculation.

As mentioned earlier, dog 40's first and highest AST spike of 221 IU/l, 24 days after inoculation (figure 4-79), coincided with the highest ALT peak. Moderately elevated AST levels were seen 57 and 59 days after inoculation. Six AST levels just above the normal range were found between 18 and 36 weeks after inoculation, they either coincided with or immediately preceded elevated ALT levels (figures 4-79 and 4-80). Dog 40's AST level then remained within the normal range for a year until a spike of 51 IU/l and a similar two sample peak, 88 and 91 weeks after inoculation respectively (figure 4-81). Only the last of those results coincided with a raised ALT level; as did the next spike (51 IU/l) 115 weeks after inoculation. There was a bout of slightly raised AST levels, between 126½ and 129 weeks after inoculation, during part of the prolonged ALT elevation (figure 4-82). A later AST spike of 54 IU/l, which occurred during the same period, accompanied a moderately raised ALT level. Dog 40's AST level slightly exceeded the normal range on two further occasions, around

139 and 145 weeks after inoculation, only the first of which coincided with an elevated ALT level).

Dog 40's range of alkaline phosphatase levels was generally lower than those of the other dogs, during the early part of this experiment (figure 4-83). A low peak 43 days after inoculation was within the normal range. A single high AP level of 435 IU/l was found, 164 days after inoculation, at the end of a bout of slightly raised ALT levels. There was another single high result of 488 IU/l 230 days after inoculation (figure 4-84). The only prolonged bout of high AP levels in dog 40 started, with a peak of 349 IU/l, 36 weeks after inoculation and coincided with the first of the two high ALT peaks which occurred during the administration of azothioprine. A low peak of 158 IU/l, well within the normal range, coincided with the second of them. By the time dog 40 was 15 months of age, 11 months after inoculation, her AP level was consistently below 100 IU/l. It remained so apart from one result, of 111 IU/l 895 days (128 weeks) after inoculation, during the prolonged bout of high ALT levels (figures 4-85 and 4-86).

Dog 41's ALT level was slightly elevated 3, 62 and 98 days after inoculation (figure 4-87). The first bout of moderately elevated ALT levels, from 19 to 21 weeks after inoculation, peaked at 58 IU/l 134 days after inoculation. The normal ALT range was just exceeded 164 days after inoculation. All but one of dog 41's ALT levels, from 27 to 31 weeks after inoculation, were above the normal range; the peak (75 IU/l) occurred in the sample collected 204 days after inoculation. Dog 41's ALT level was above normal for a week from 33 weeks after inoculation (figure 4-88). There were three more results just above the normal range and a low peak of 57 IU/l, before prednisolone administration began 307 days after inoculation. During the 4½ months of various prednisolone dosage regimes there were two short episodes of elevated ALT levels, around 47

and 57 weeks after inoculation. Dog 41's ALT level remained within the normal range for 7 months after the second episode. Then, between 88 and 91½ weeks after inoculation, there were three low ALT peaks (figure 4-89). After a peak of 71 IU/l at 94 weeks, dog 41's ALT level did not return to normal for a week. The next peak of 75 IU/l , 97½ weeks after inoculation (682 days), was followed by three raised results. A lower peak of 59 IU/l, 101 weeks after inoculation, was followed by a single raised level a fortnight later. The next, 3-sample, bout of raised ALT levels ended with dog 41's highest peak of 134 IU/l, 25½ months (110½ weeks) after inoculation. Her ALT level then returned to normal for 2 weeks, but was slightly elevated at 50 IU/l in the last sample collected.

Dog 41's AST level was raised much less frequently than her ALT level. Her first result to just exceed the normal AST range was 91 days after inoculation; the next was 195 days after inoculation (figure 4-90). The latter and the first moderate spike, of 54 IU/l 204 days after inoculation, coincided with elevated ALT levels. Dog 41's AST level remained normal for over a year until 618 days (88 weeks) after inoculation when, at 69 IU/l, it exceeded the also elevated ALT level (figures 4-91 and 4-92). Apart from just exceeding the normal range 95 weeks (667 days) after inoculation, just after a bout of elevated ALT levels, dog 41's AST level remained normal until the sample with the highest ALT level. This was collected 110½ weeks after inoculation and also had the last raised AST level of 58 IU/l).

The alkaline phosphatase level of 250 IU/l, seen in dog 41 104 days after inoculation, was slightly higher than surrounding results (figure 4-93). Another higher level, of 264 IU/l, occurred 125 days after inoculation. The expected decline of AP levels with age, appeared to halt in dog 41 during the period of oral prednisolone administration (figure 4-94). At that time her AP levels,

although not abnormally high, remained slightly higher than expected from comparsions with her other results and dog 40's corresponding levels. Dog 41's AP level did not therefore drop to consistently below 100 IU/l until she was 19 months of age, 14½ months after inoculation, just as the prednisolone dosage regime tailed off. That level was then only slightly exceeded on two occasions: AP levels of 106 IU/l and 114 IU/l were found 608 and 643 days (87 and 92 weeks) after inoculation respectively (figure 4-95).

The gamma glutamyl transferase levels found in dogs 40 and 41 were very variable and often above the laboratory's usual normal range of 0-10 IU/l (figures 4-96 to 4-98). Peaks of over 15 IU/l were taken as abnormal in this experiment. Several such peaks were seen in both dogs, sometimes they coincided with peaks in ALT levels but peaks of each also occurred alone.

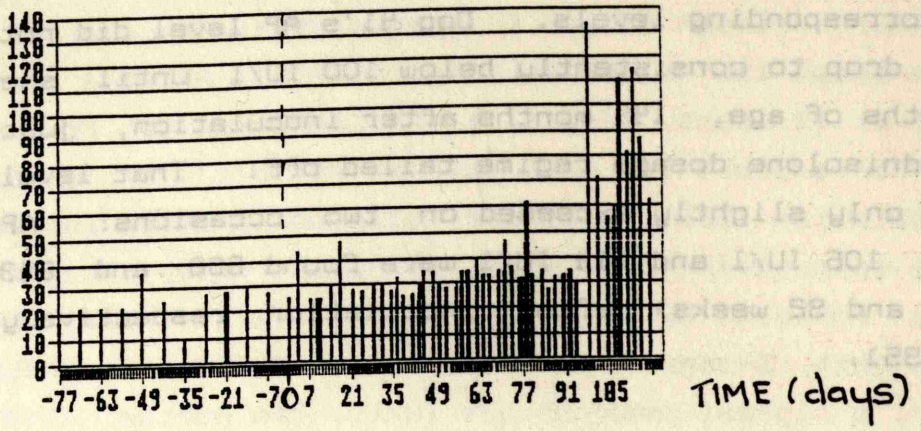


Fig 4-63 Dog 36's ALT levels (IU/l)

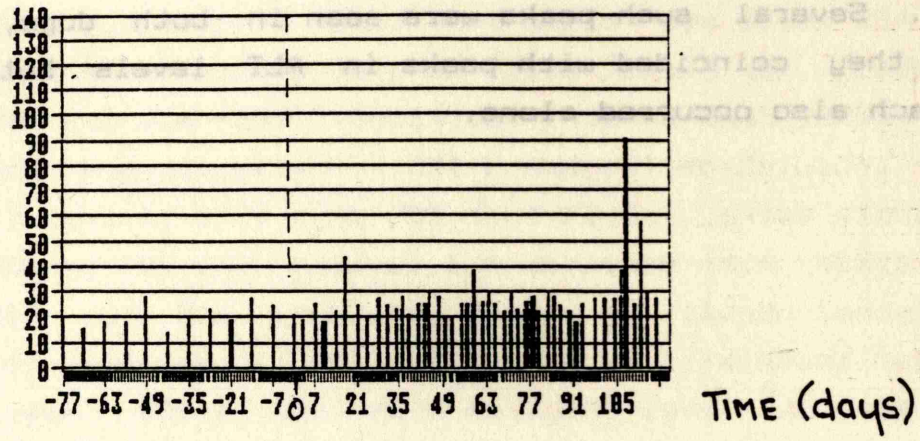


Fig 4-64 Dog 36's AST levels (IU/l)

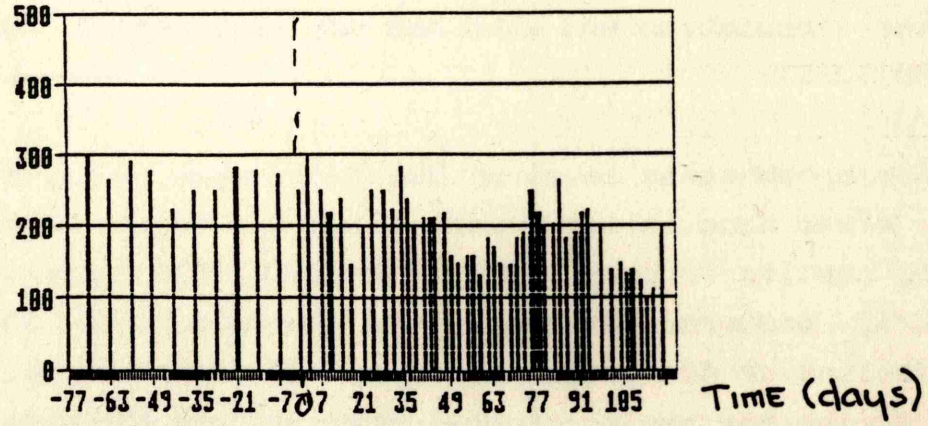


Fig 4-65 Dog 36's alkaline phosphatase levels (IU/l)

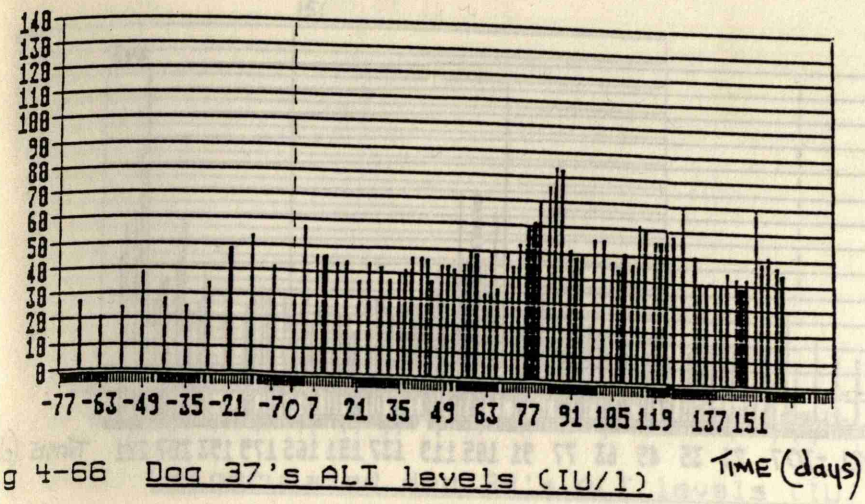


Fig 4-66 Dog 37's ALT levels (IU/l)

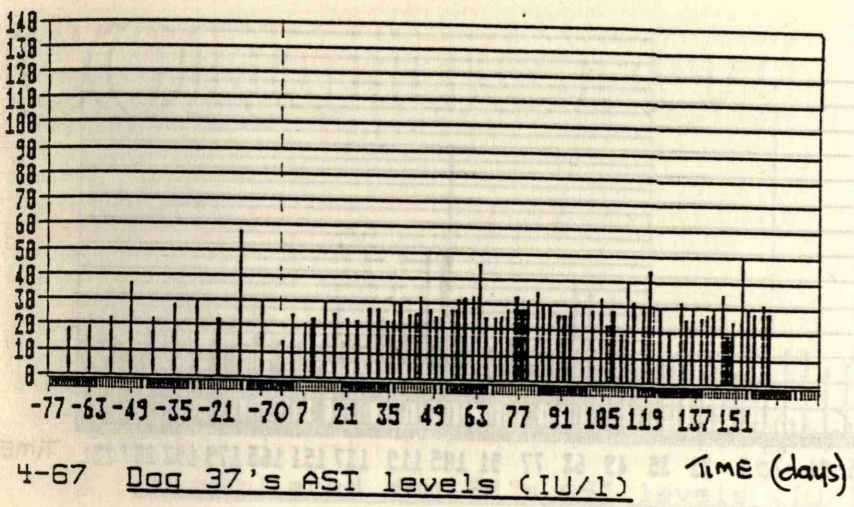


Fig 4-67 Dog 37's AST levels (IU/l)

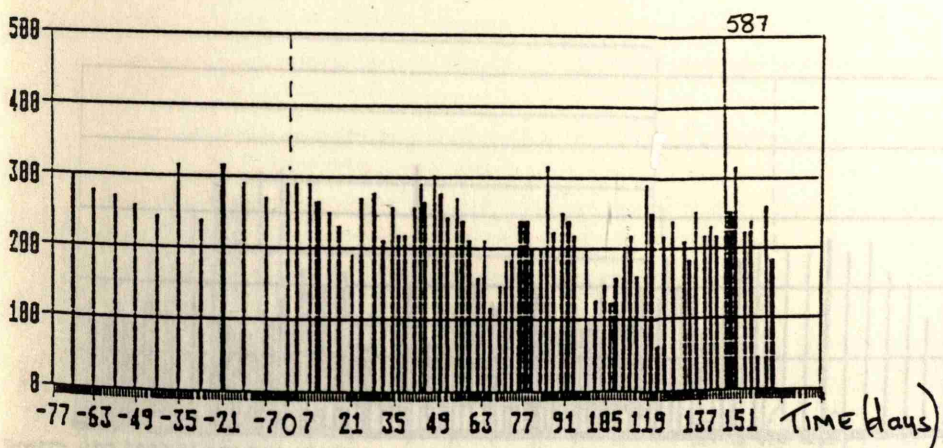


Fig 4-68 Dog 37's alkaline phosphatase levels (IU/l)

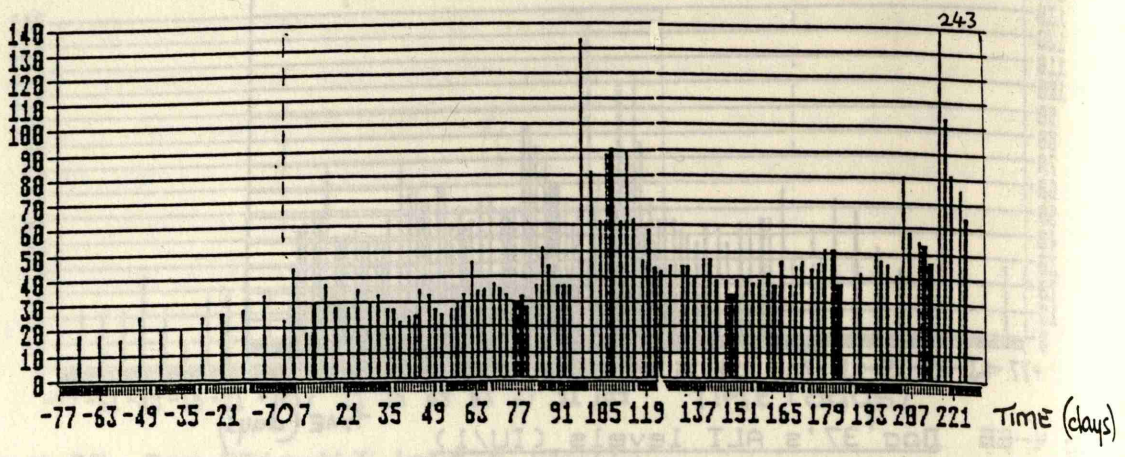


Fig 4-69 Dog 38's ALT levels (IU/l)

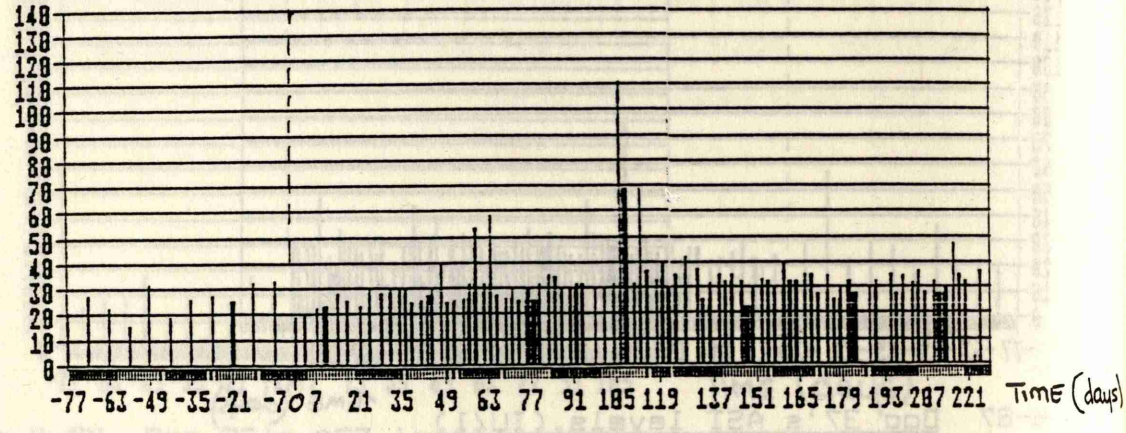


Fig 4-70 Dog 38's AST levels (IU/l)

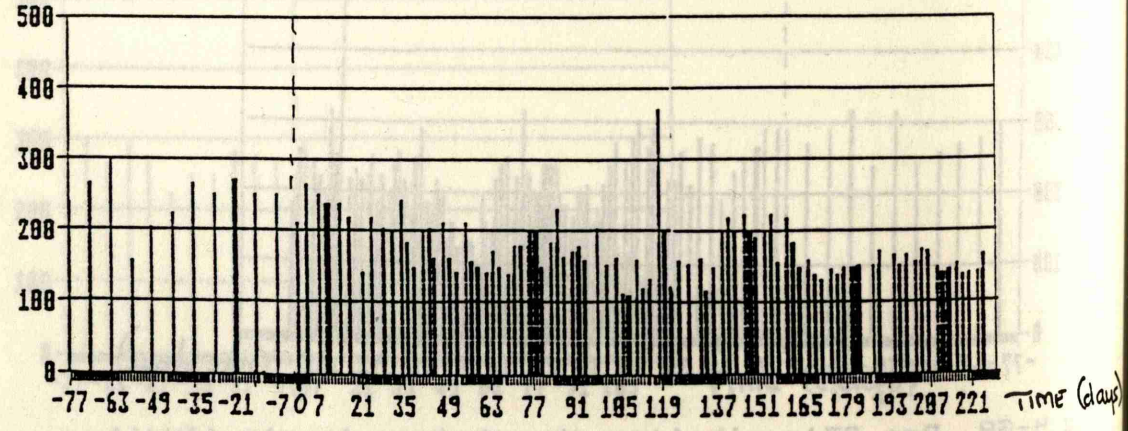


Fig 4-71 Dog 38's alkaline phosphatase levels (IU/l)

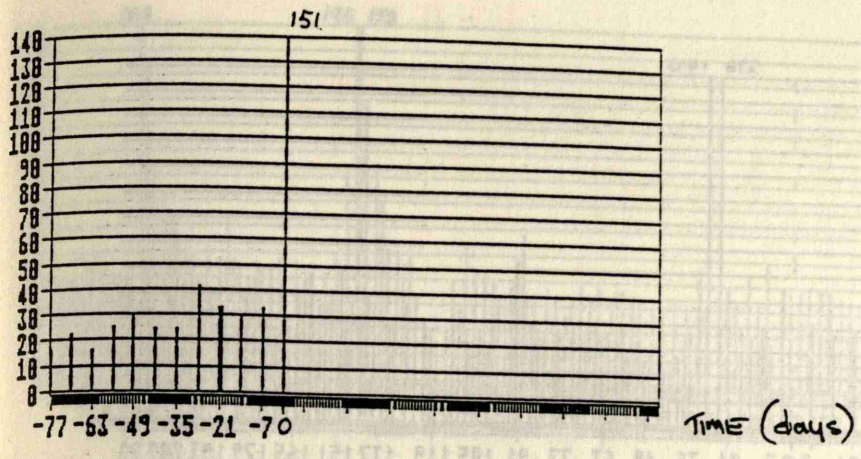


Fig 4-72 Uninoculated dog 39's ALT levels (IU/l)

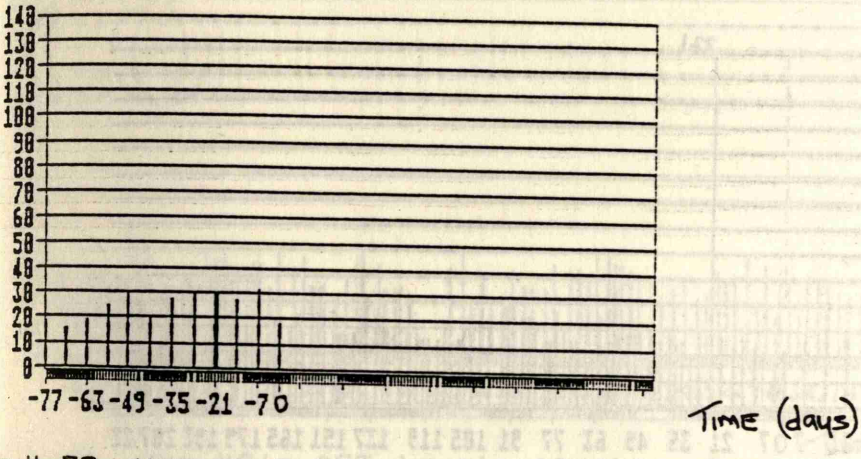


Fig 4-73 Uninoculated dog 39's AST levels (IU/l)

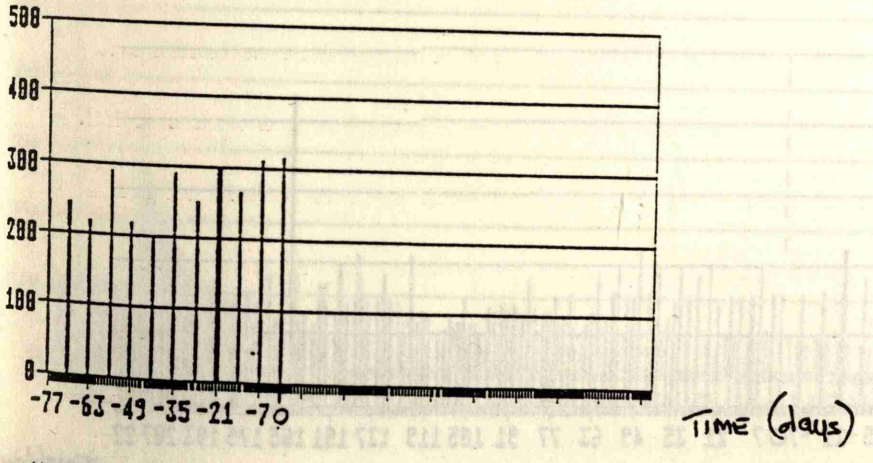


Fig 4-74 Uninoculated dog 39's alk. phosph. levels (IU/l)

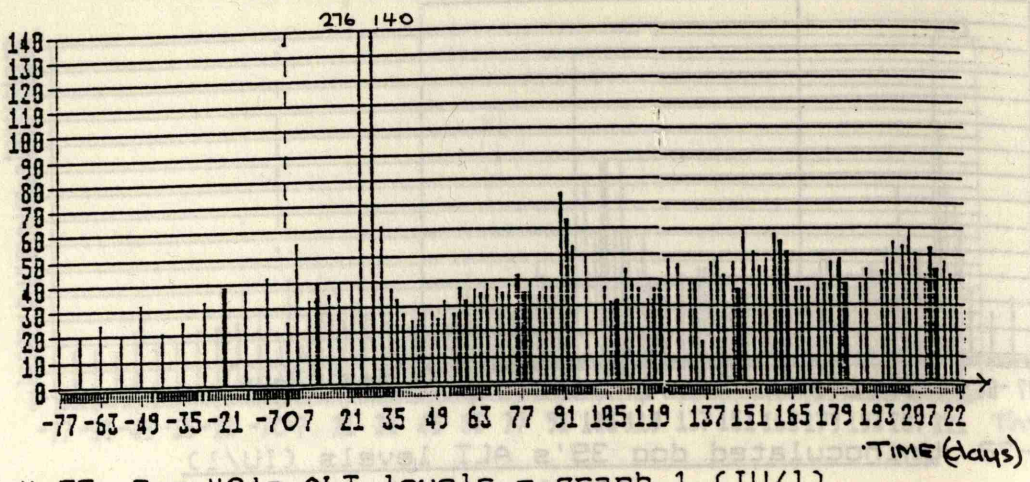


Fig 4-75 Dog 40's ALT levels - graph 1 (IU/l)

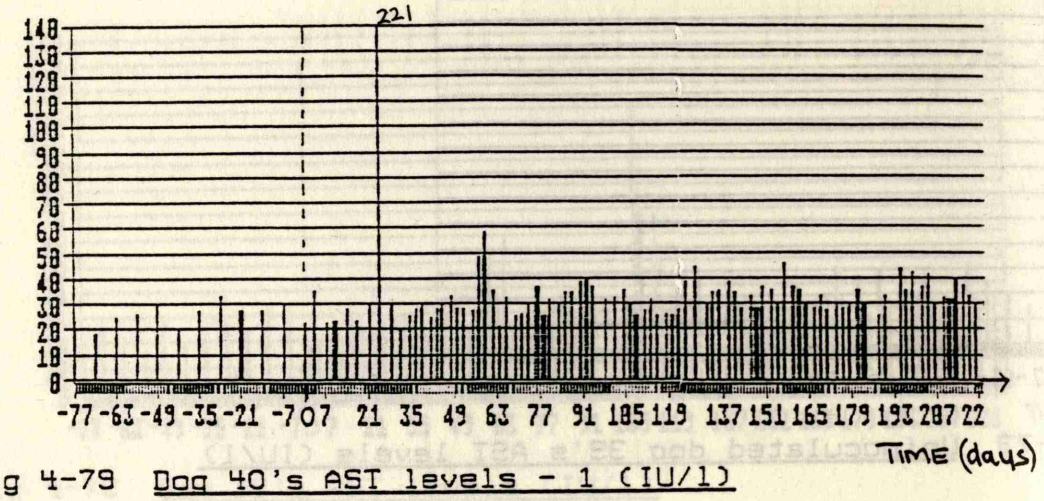


Fig 4-79 Dog 40's AST levels - 1 (IU/l)

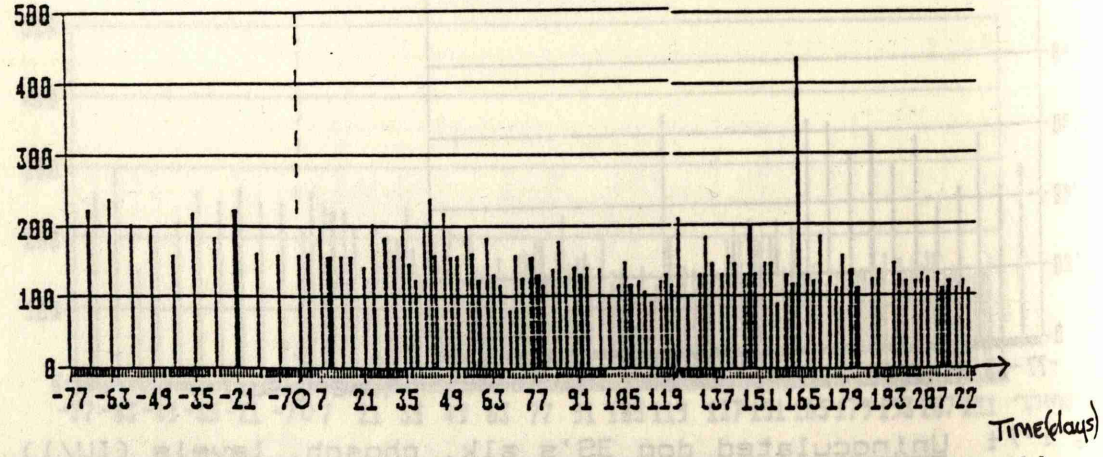


Fig 4-83 Dog 40's alkaline phosphatase levels - 1 (IU/l)

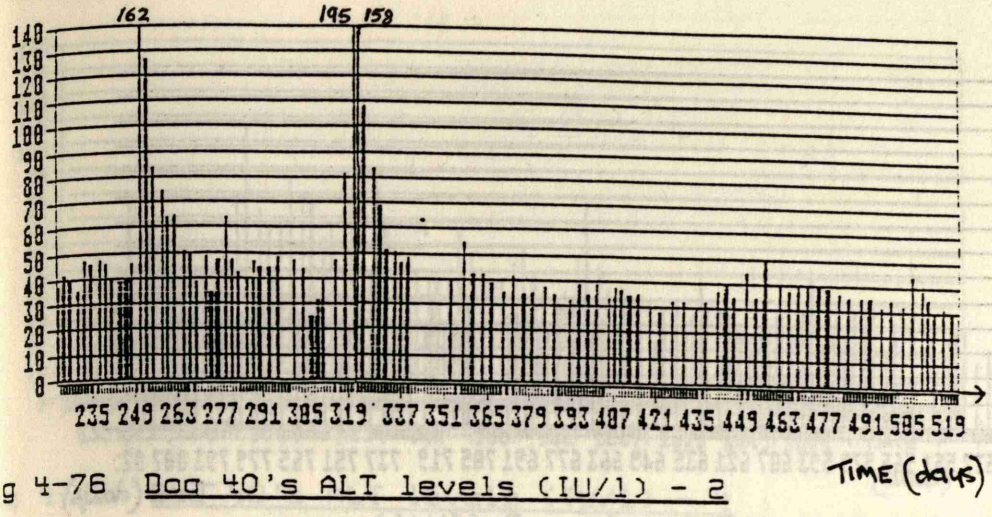


Fig 4-76 Dog 40's ALT levels (IU/l) - 2

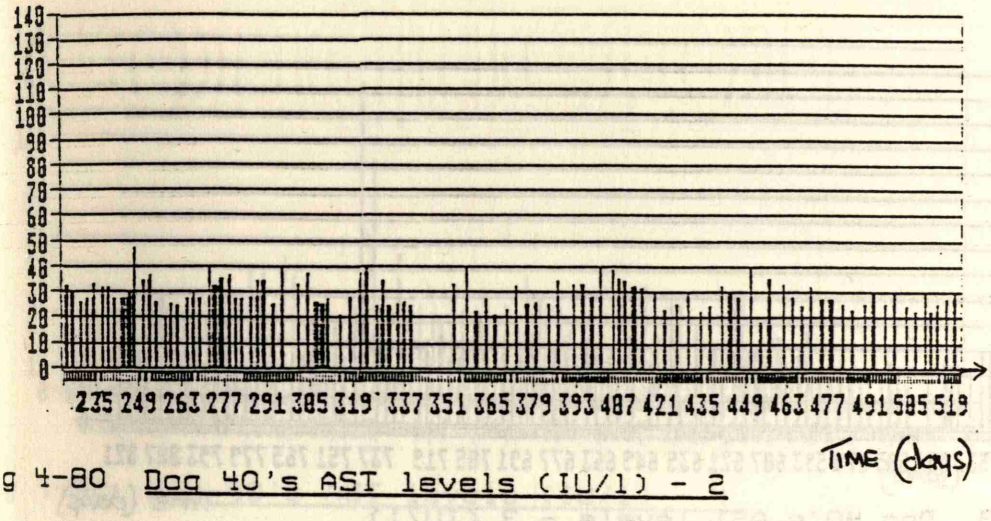


Fig 4-80 Dog 40's AST levels (IU/l) - 2

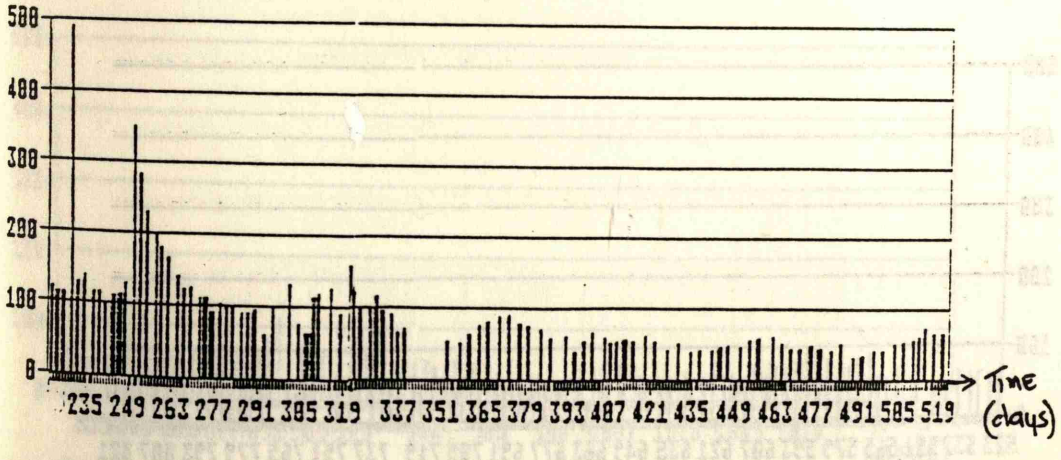


Fig 4-84 Dog 40's alkaline phosphatase levels (IU/l) - 2

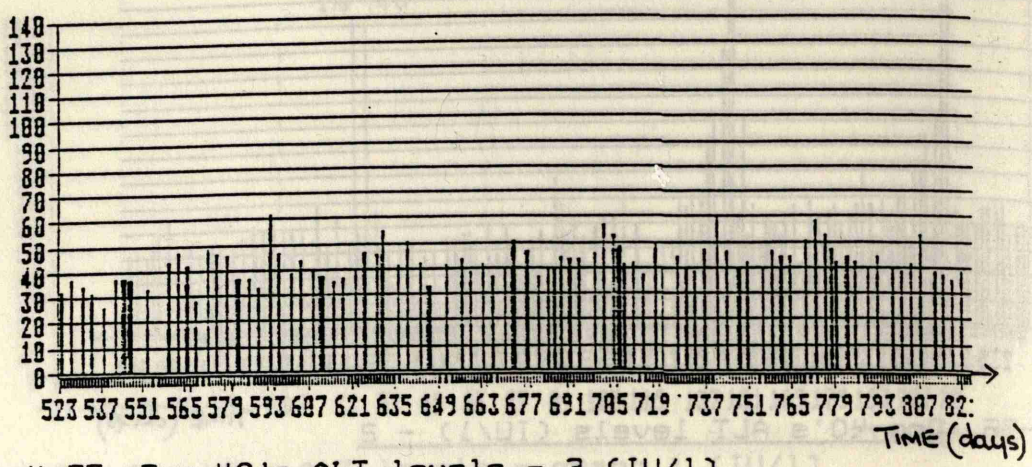


Fig 4-77 Dog 40's ALT levels - 3 (IU/l)

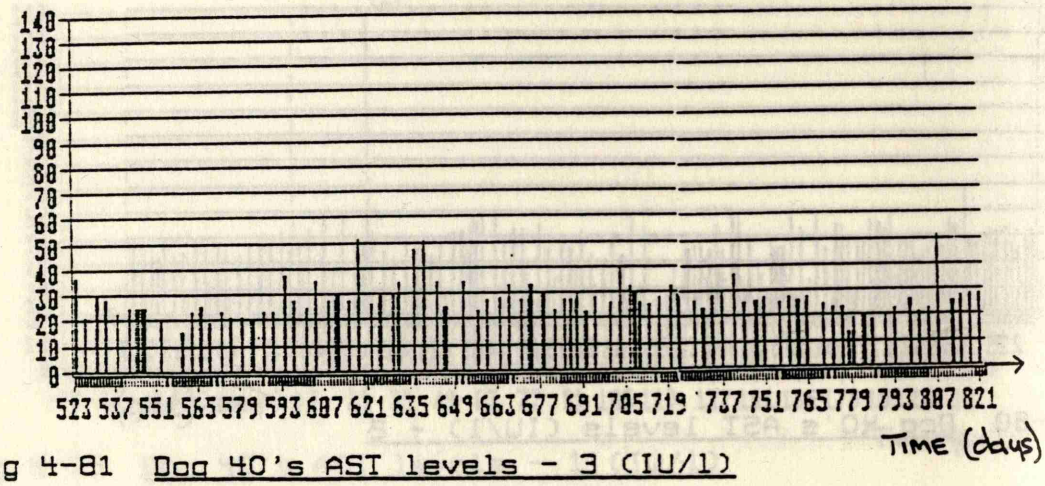


Fig 4-81 Dog 40's AST levels - 3 (IU/l)

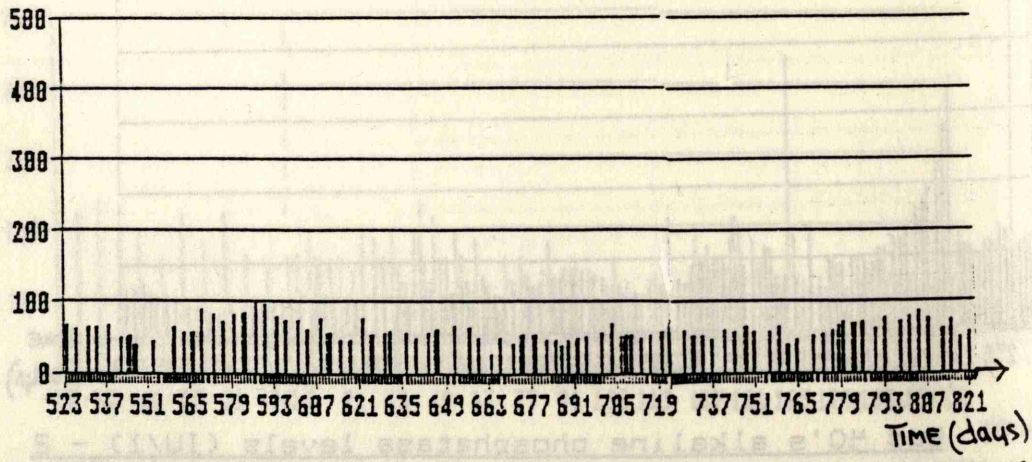


Fig 4-85 Dog 40's alkaline phosphatase levels - 3 (IU/l)

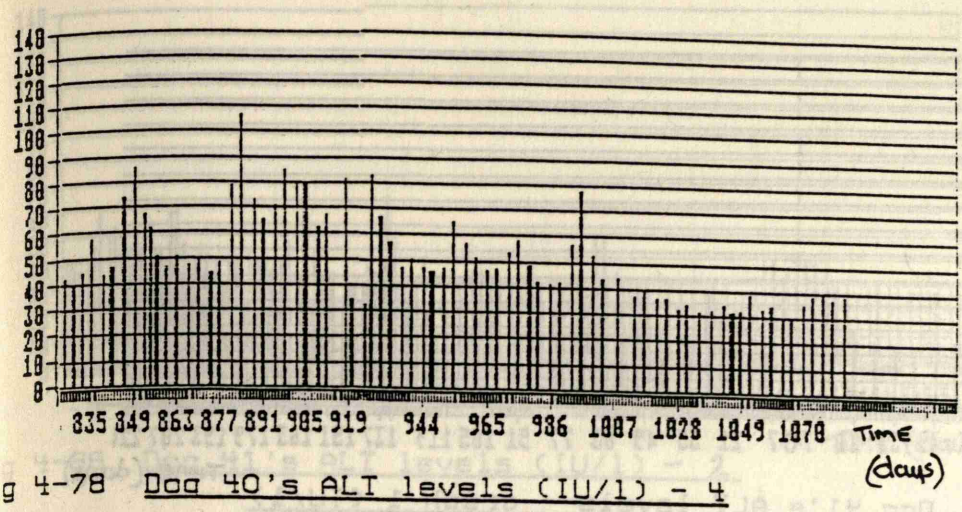


Fig 4-78 Dog 40's ALT levels (IU/l) - 4

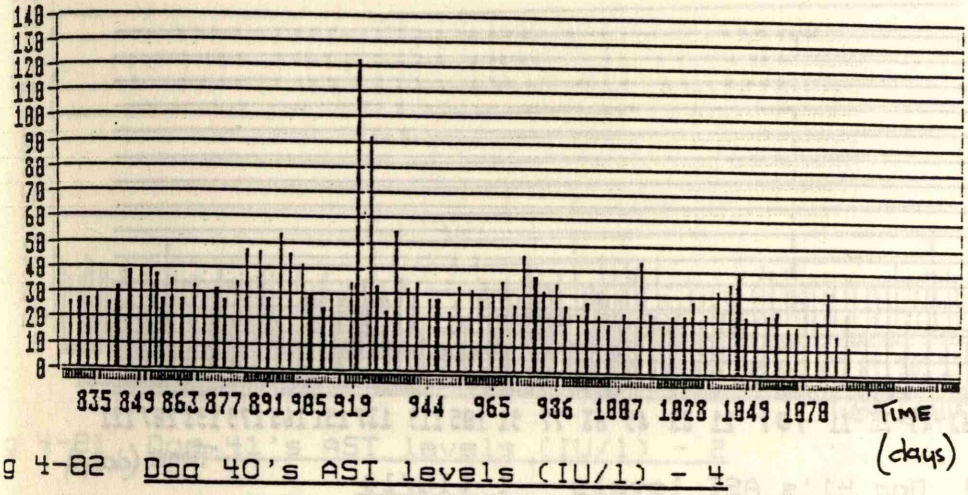


Fig 4-82 Dog 40's AST levels (IU/l) - 4

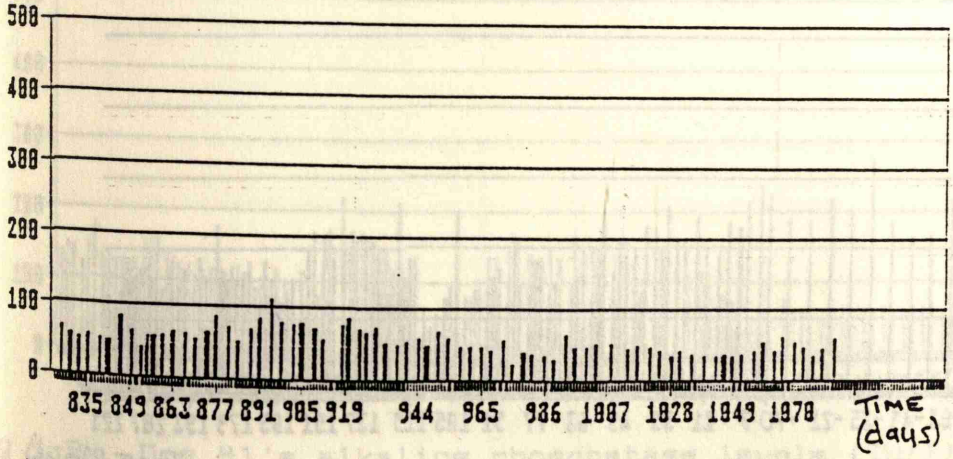


Fig 4-86 Dog 40's alkaline phosphatase levels (IU/l) - 4

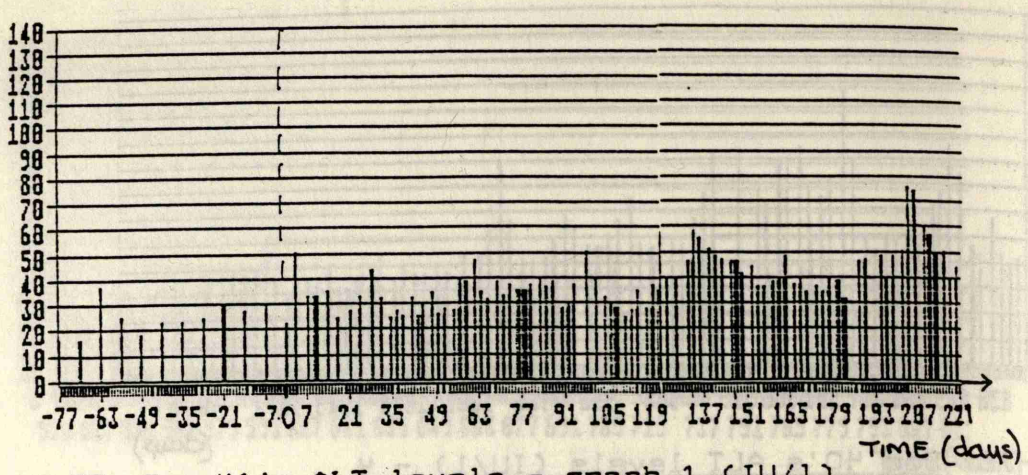


Fig 4-87 Dog 41's ALT levels - graph 1 (IU/l)

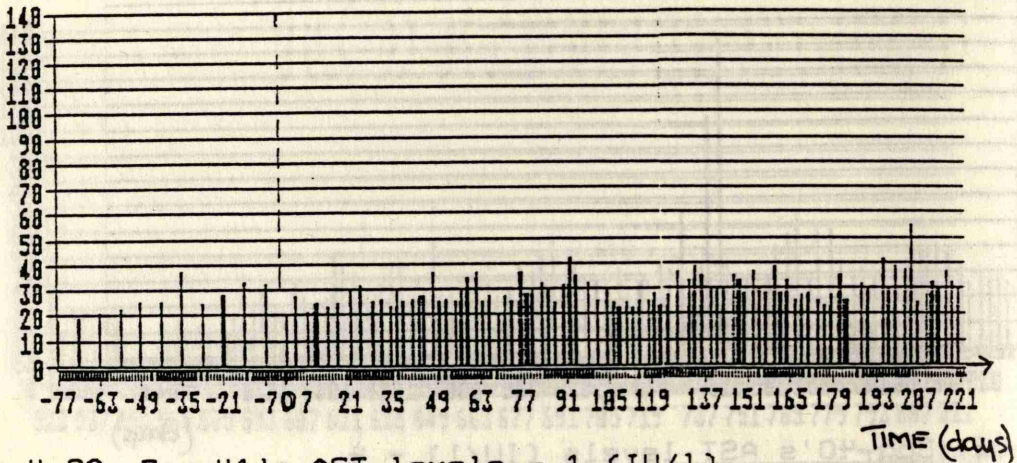


Fig 4-90 Dog 41's AST levels - 1 (IU/l)

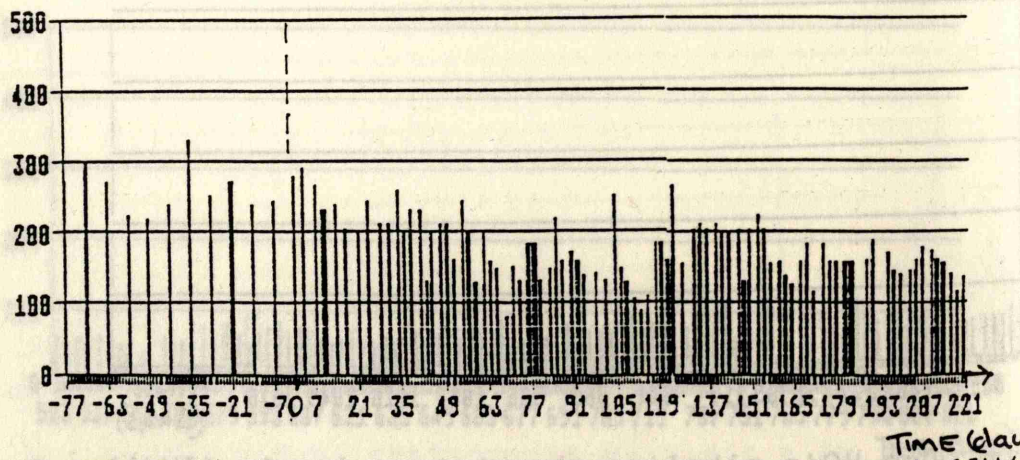


Fig 4-93 Dog 41's alkaline phosphatase levels - 1 (IU/l)

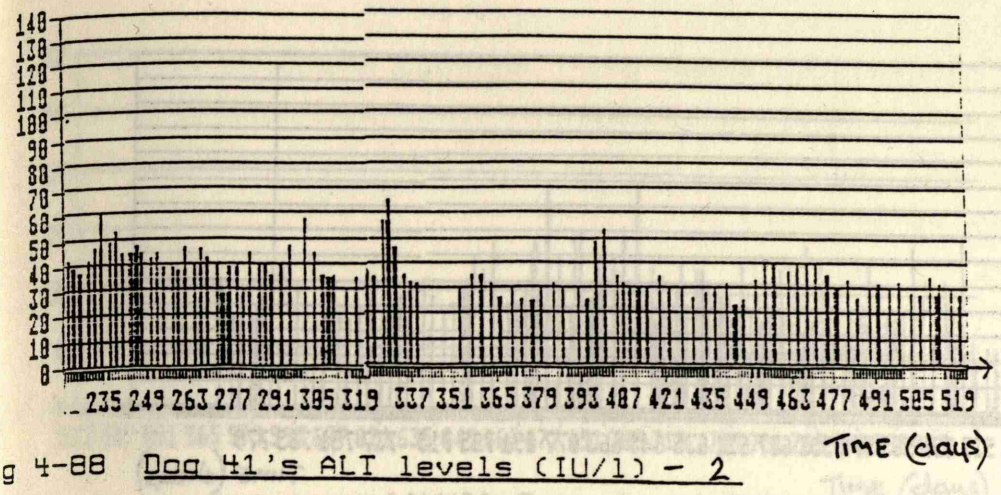


Fig 4-88 Dog 41's ALT levels (IU/l) - 2

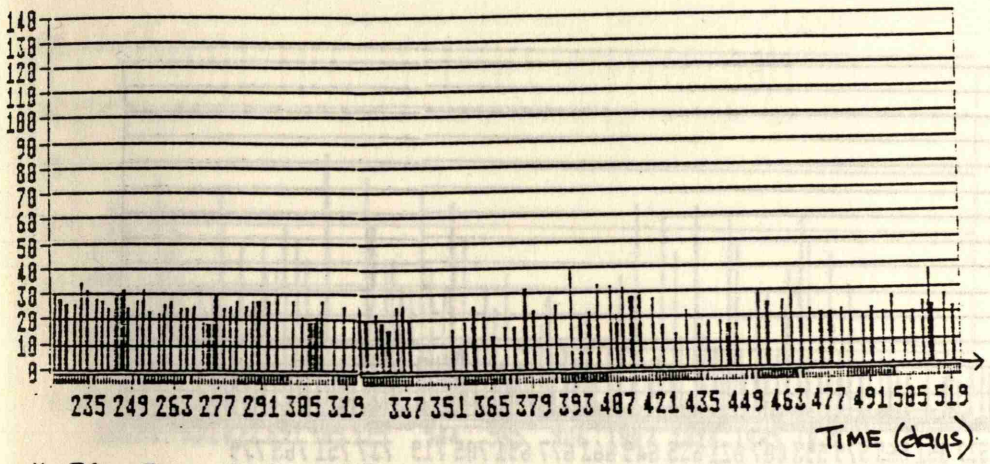


Fig 4-91 Dog 41's AST levels (IU/l) - 2

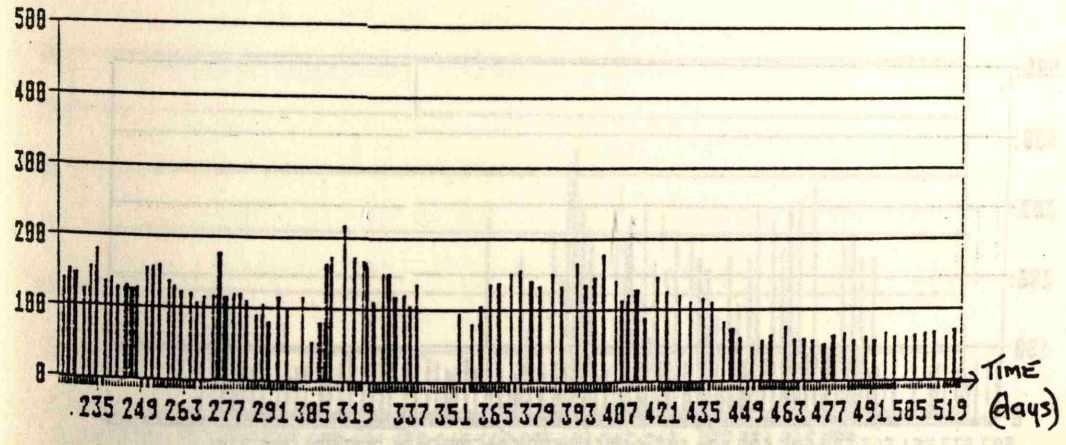


Fig 4-94 Dog 41's alkaline phosphatase levels (IU/l) - 2

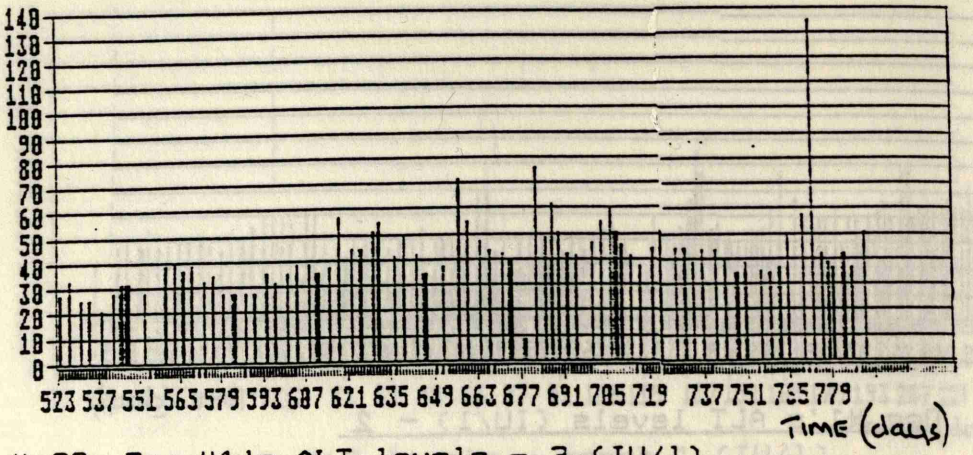


Fig 4-89 Dog 41's ALT levels - 3 (IU/l)

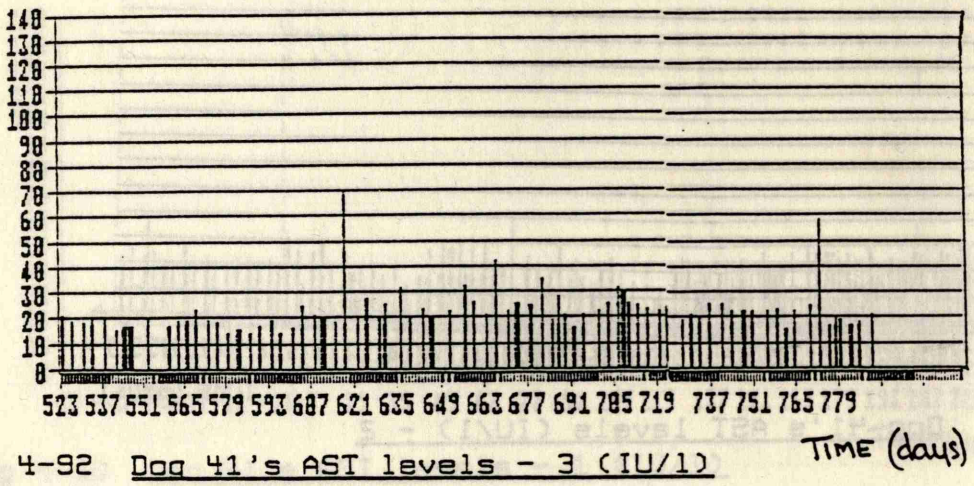


Fig 4-92 Dog 41's AST levels - 3 (IU/l)

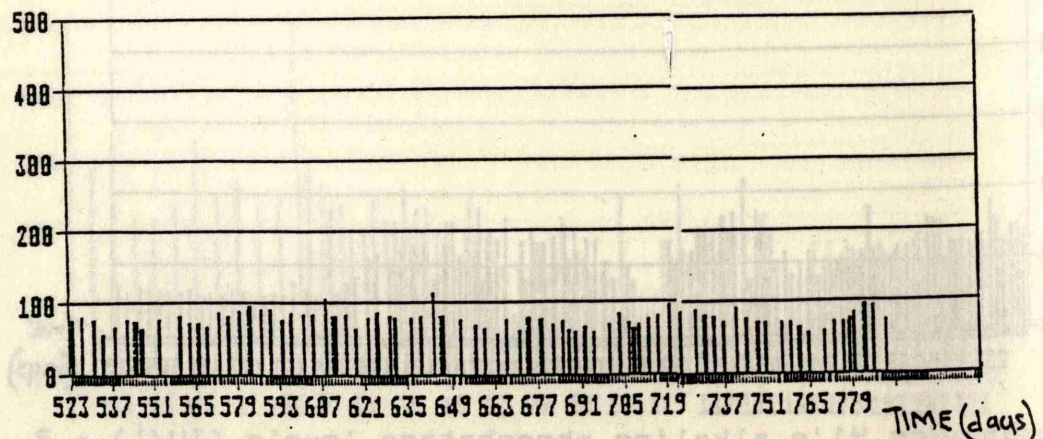


Fig 4-95 Dog 41's alkaline phosphatase levels - 3 (IU/l)

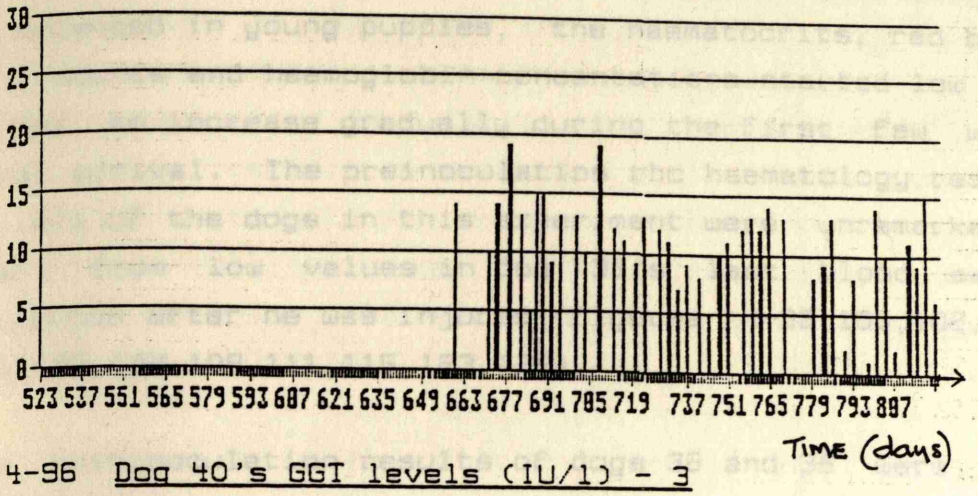


Fig 4-96 Dog 40's GGT levels (IU/l) - 3

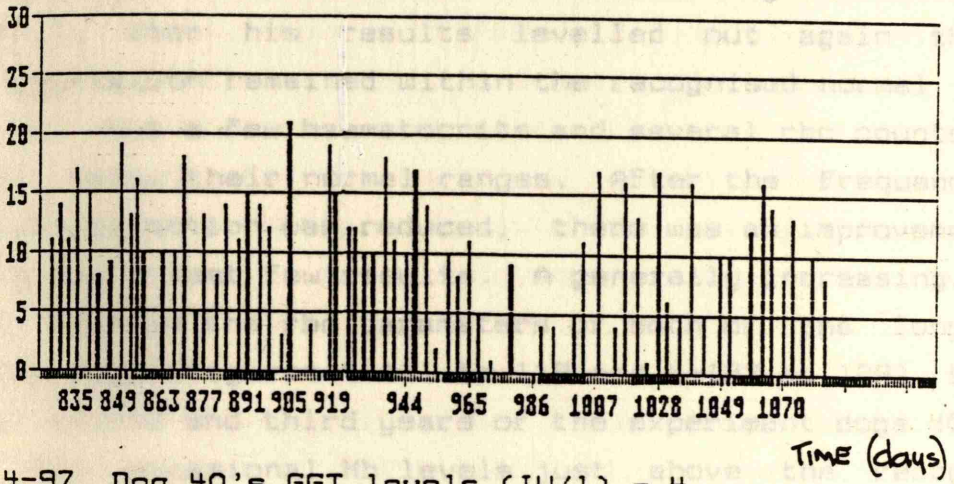


Fig 4-97 Dog 40's GGT levels (IU/l) - 4

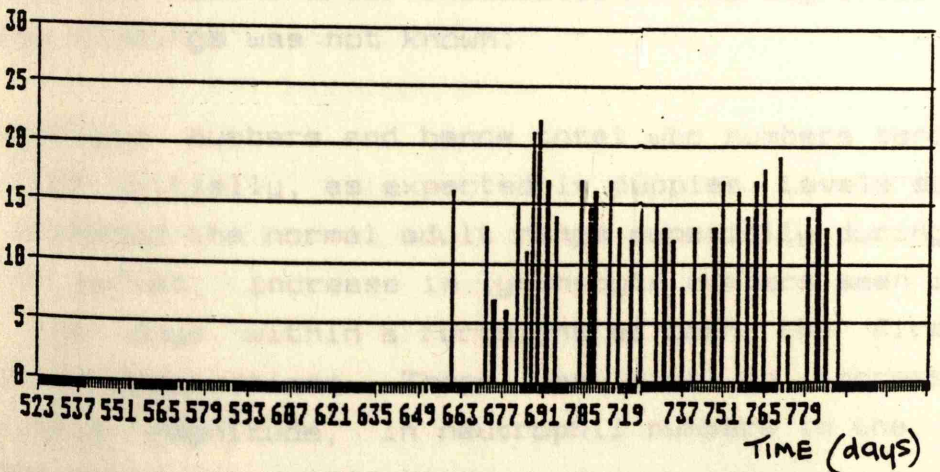


Fig 4-98 Dog 41's GGT levels (IU/l) - 3

4.4.7 Haematological Results

As expected in young puppies, the haematocrits, red blood cell counts and haemoglobin concentations started low and tended to increase gradually during the first few weeks after arrival. The preinoculation rbc haematology results of all of the dogs in this experiment were unremarkable, apart from low values in dog 39's last blood sample collected after he was injured (figures 4-99,100,102,103, 105,106,108,109,111,115,123,126).

The postinoculation results of dogs 36 and 38 were also unremarkable (figures 4-99,100,105 & 106). A short downward trend in dog 37's Hct, rbc and Hb levels started after the third postinoculation sample (figures 4-102 and 4-103). When his results levelled out again the Hb concentration remained within the recognised normal adult range, but a few haematocrits and several rbc counts were just below their normal ranges. After the frequency of serum collection was reduced, there was an improvement in this dog's last few results. A generally increasing trend was seen in the rbc parameters of both of the long-term study dogs (figures 4-111 to 118 and 4-123 to 128). During the second and third years of the experiment dogs 40 and 41 had occasional Hb levels just above the recognised normal range. Dog 40 also exhibited more variation than her littermates in the levels of rbc parameters during the first few months after inoculation. The significance of these findings was not known.

Lymphocyte numbers and hence total wbc numbers tended to be high initially, as expected in puppies. Levels sometimes exceeded the normal adult range especially during the, often marked, increase in lymphocyte numbers seen in all of the dogs within a fortnight of both the first and second vaccinations. There was also an increase, of variable magnitude, in neutrophil numbers in the sample after the first vaccination.

One week after inoculation dog 36 exhibited a peak in lymphocyte numbers of 7.12 $x10^9/l$ and levels were slightly raised 28 and 34 days after inoculation (figure 4-101). Although neutrophil numbers increased gradually for the first 3 weeks after inoculation they remained well within their normal range, as did total wbc numbers. Dog 36's leucocytology results all remained normal for the rest of the experiment; although, in comparison to surrounding levels, a very low peak was seen in all three variables 98 days after inoculation.

There were high levels of lymphocytes in dog 37's second and third samples after vaccination, but his total wbc count remained within the normal range (figure 4-104). Lymphocyte numbers in dog 37 were a little raised at the time of inoculation and afterwards decreased slightly, along with both neutrophil and total wbc numbers. Although lymphocyte numbers remained a little above the normal adult range, for several weeks after inoculation, the levels were probably acceptable in a pup of that age. A peak in lymphocyte numbers occurred 98 days after inoculation. A small rise in the lymphocyte count 125 days after inoculation was within the normal range. Neutrophil numbers were always within the normal range; although, in comparison to surrounding levels, there was a low peak 104 days after inoculation. Dog 37's total wbc numbers also remained within the normal range throughout the experiment, although the consecutive lymphocyte and neutrophil peaks did increase total wbc numbers, in the samples collected 98 and 104 days after inoculation.

Dog 38 has the most extreme of the postvaccination increases in lymphocyte numbers with a peak of 10.1 $x10^9/l$, 49 days before inoculation, which took the wbc count to the top of the normal puppy range (figure 4-107). Apart from that, dog 38's leucocytology results were always within their normal ranges. Very little reaction to inoculation was seen in the lymphocyte numbers, a slight

fall before a small increase, and there was only a gradual increase in neutrophil numbers in dog 38's first two samples after inoculation. In comparison with surrounding results, very low peaks in neutrophil numbers and usually also total wbc numbers occurred between 98 and 111 days, as well as 132, 160 and 195 days after inoculation.

Apart from the postvaccination lymphocyte increases, dog 39's leucocytology results were all within normal ranges (figure 4-110). However, the neutrophil increase in the first sample after vaccination was more prominent in this dog than in most of the others.

Despite increases in neutrophil and lymphocyte numbers in dog 40's first sample after vaccination her wbc count remained within the normal puppy range (figure 4-119). In the first two samples after inoculation there was a small, gradual increase in neutrophil and total wbc numbers, but very little lymphocyte reaction. Low peaks in neutrophil numbers, 34 and 48 days after inoculation, were well within the normal range. As was a very small rise in lymphocyte numbers, 98 days after inoculation. Every so often there were other similar very low peaks in lymphocyte numbers, in comparison with surrounding levels e.g. at 125-132, 174, 209, 301, 392 and 580 days after inoculation (figures 4-119 to 4-121). The only occasion that dog 40's lymphocyte numbers actually exceeded the normal range was a low peak of 5.43 $x10^9/l$, 1043 days (149 weeks) after inoculation, near the end of the experiment (fig. 4-122). Marked peaks in neutrophil and wbc numbers were also seen in that sample. During the experiment, neutrophil numbers had exhibited occasional low peaks, mostly within the normal range. The highest of these occurred 251, 279, 307 (during oestrus), 392, 482, 531 and 1043 days after inoculation. Only the peak of 11.68 $x10^9/l$ at 531 days (during oestrus) and the last peak of 13.67 $x10^9/l$ exceeded the normal range of neutrophil numbers. Dog 40's wbc count only exceeded the normal range once, with a result

of 20.1 $\times 10^9$/l in the sample collected 1043 days (149 weeks) after inoculation. However, low peaks in wbc counts, in comparison to surrounding levels, were seen 48, 251, 301 and 531 days after inoculation. The last of these was the highest, at 16.0 $\times 10^9$/l, and occurred during dog 40's second oestrus.

After the postvaccination increase, dog 41's lymphocyte numbers remained within the normal range for the rest of the experiment (figures 4-129 to 4-131). Very little initial response to inoculation was seen in dog 41, there was only a slight increase in lymphocyte numbers in the second sample, collected a fortnight after inoculation. As in the other dogs, there were occasional small peaks in lymphocyte numbers, in comparison to surrounding results. The highest of these were seen 55, 98, 265 and 573 days after inoculation. Although usually within the normal range, dog 41's neutrophil numbers also peaked intermittently; the highest results occurred 251, 307 (during oestrus), 318, 377, 409, 482, 538 and 770 days after inoculation. The only two results which actually exceeded the normal range were 11.914 $\times 10^9$/l at 409 days and 11.692 $\times 10^9$/l at 538 days (during oestrus). Other lower peaks, which coincided with ones in other dogs, were seen on days 104 and 392. Dog 41's wbc count never exceeded the normal range. However, comparative peaks were seen 251, 265, 307 (during oestrus), 318, 377, 392, 409, 538 and 770 days after inoculation. The highest, at 15.8 $\times 10^9$/l, was the second last peak; it occurred during dog 41's second oestrus.

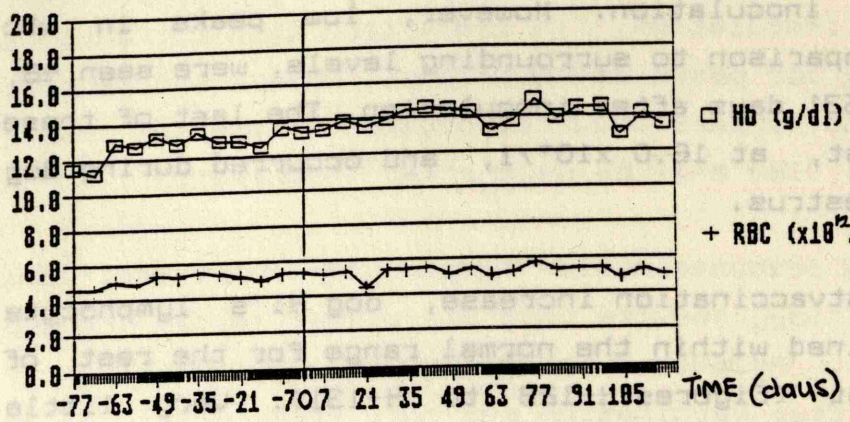


Fig 4-99 Dog 36's rbc counts and haemoglobin levels

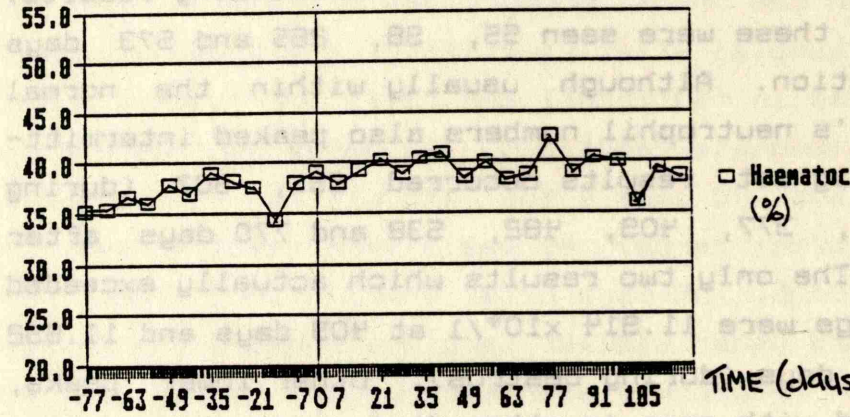


Fig 4-100 Dog 36's haematocrits (%)

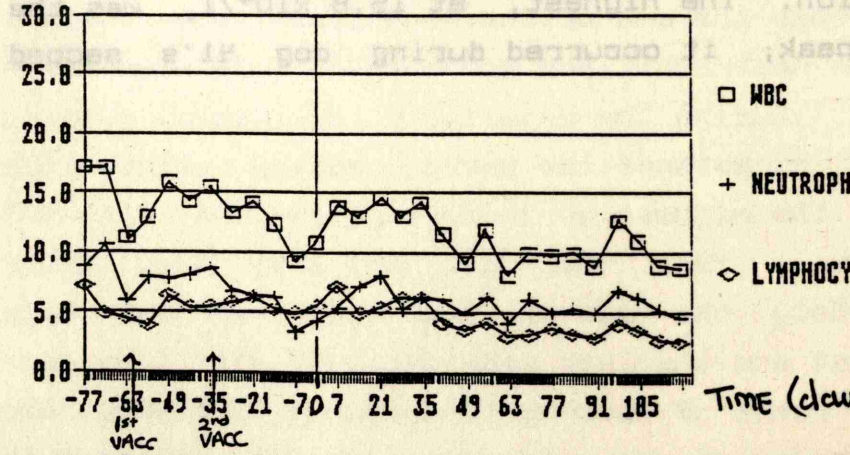


Fig 4-101 Dog 36's leucocytology results (x10^9/l)

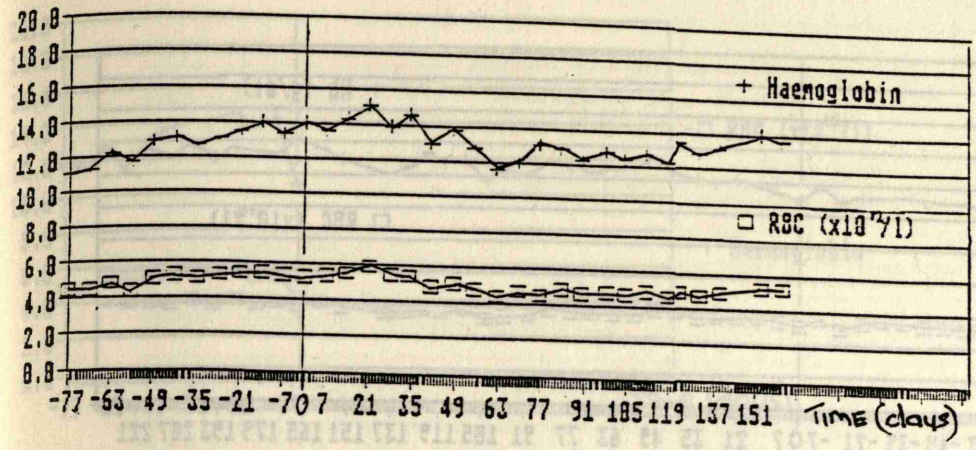


Fig 4-102 Dog 37's rbc counts and haemoglobin levels

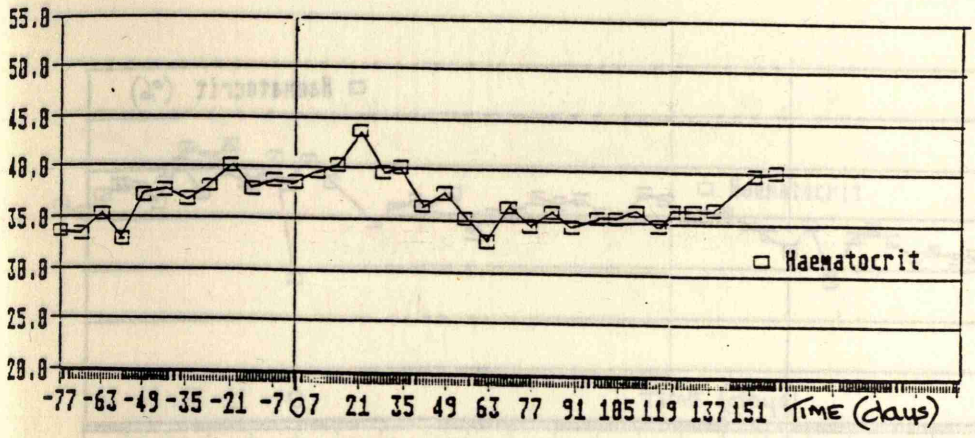


Fig 4-103 Dog 37's haematocrits (%)

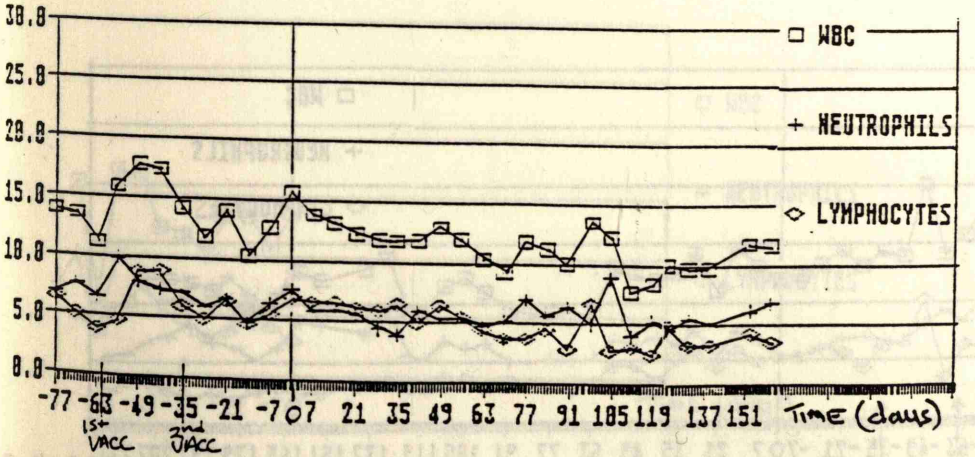


Fig 4-104 Dog 37's leucocytology results ($x10^9/l$)

fp200

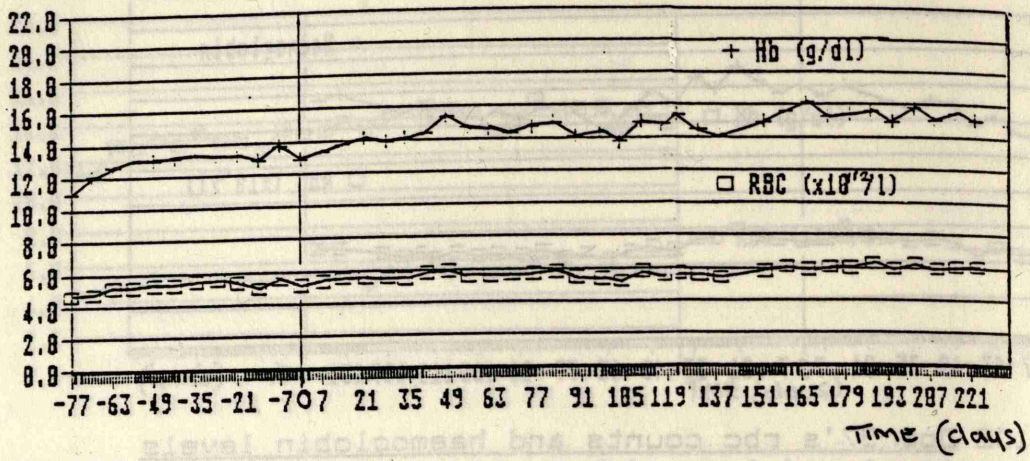


Fig 4-105 Dog 38's rbc counts and haemoglobin levels

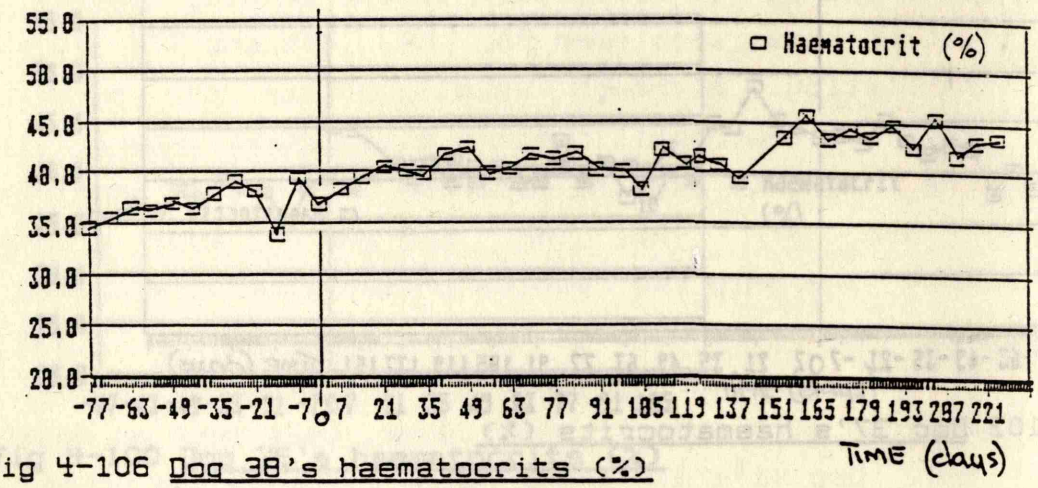


Fig 4-106 Dog 38's haematocrits (%)

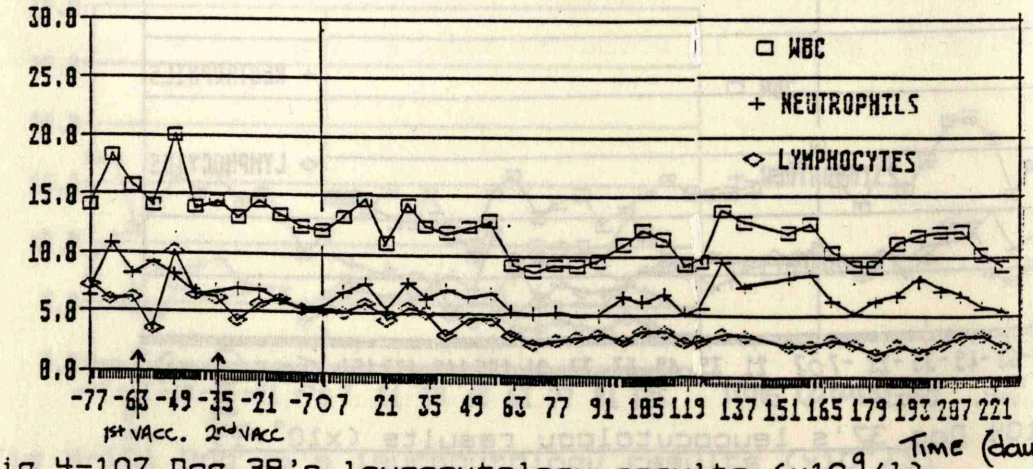


Fig 4-107 Dog 38's leucocytology results ($x10^9/l$)

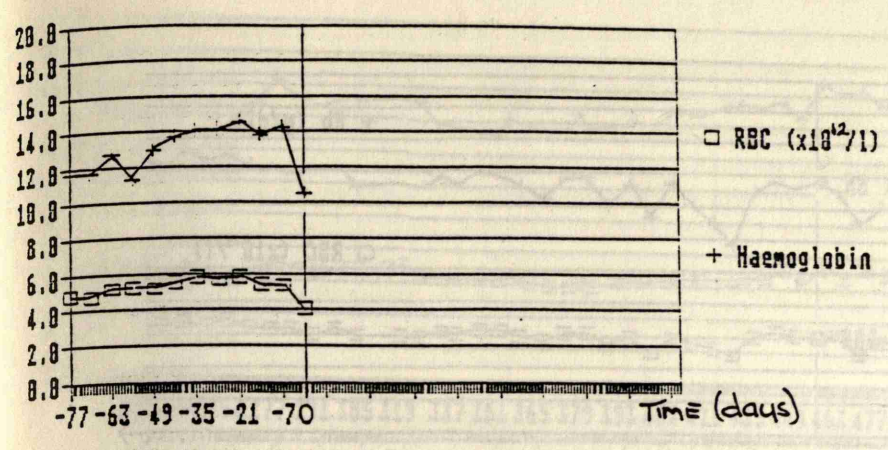


Fig 4-108 Uninoculated dog 39's rbc counts and Hb levels

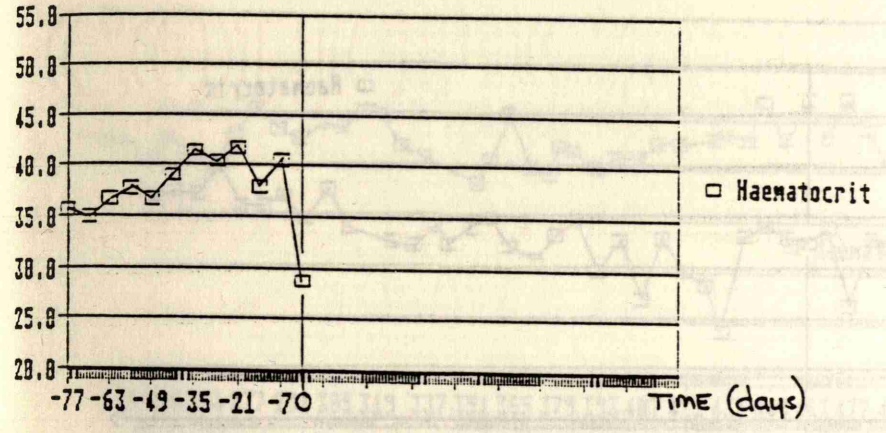


Fig 4-109 Uninoculated dog 39's haematocrit (%)

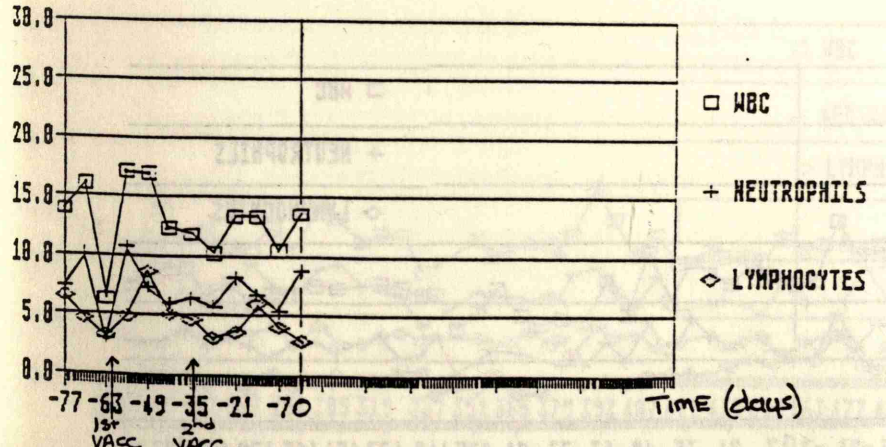


Fig 4-110 Dog 39's leucocytology results (x10^9/l)

fpz01

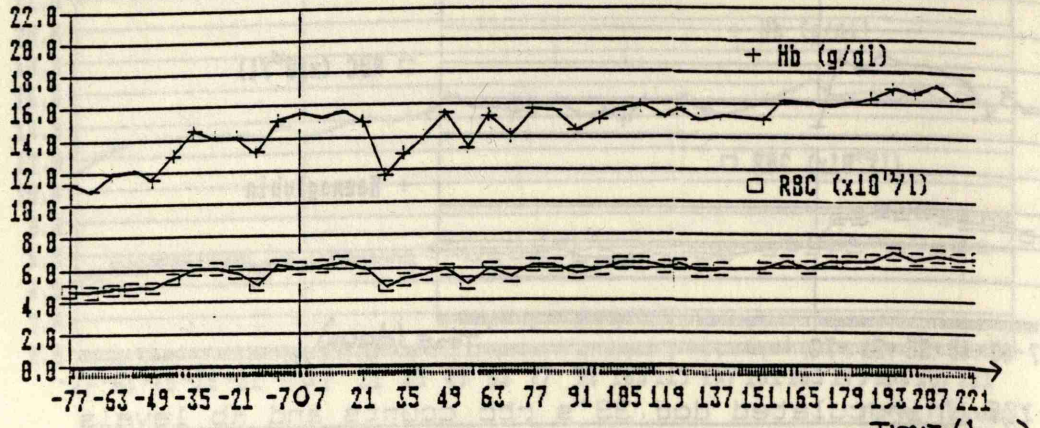


Fig 4-111 Dog 40's rbc counts and Hb levels - graph 1

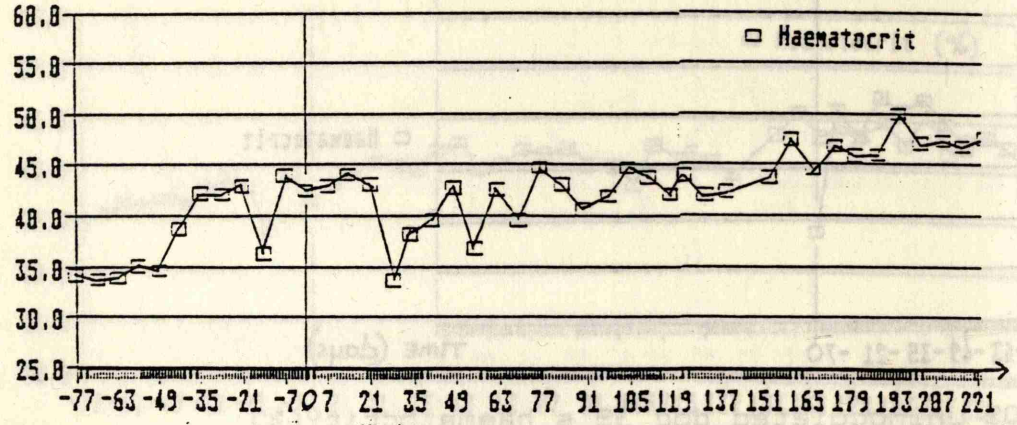


Fig 4-115 Dog 40's haematocrits - 1 (%)

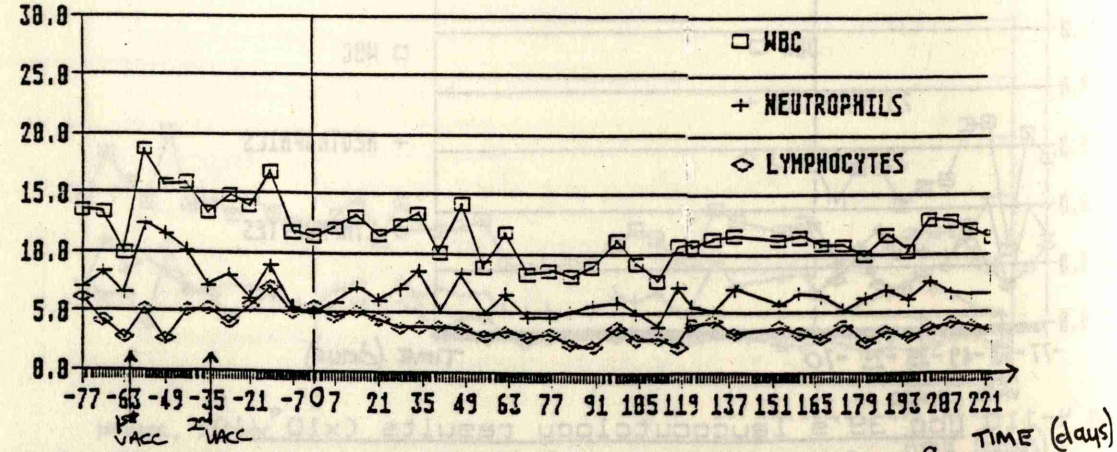


Fig 4-119 Dog 40's leucocytology results - 1 ($x10^9/l$)

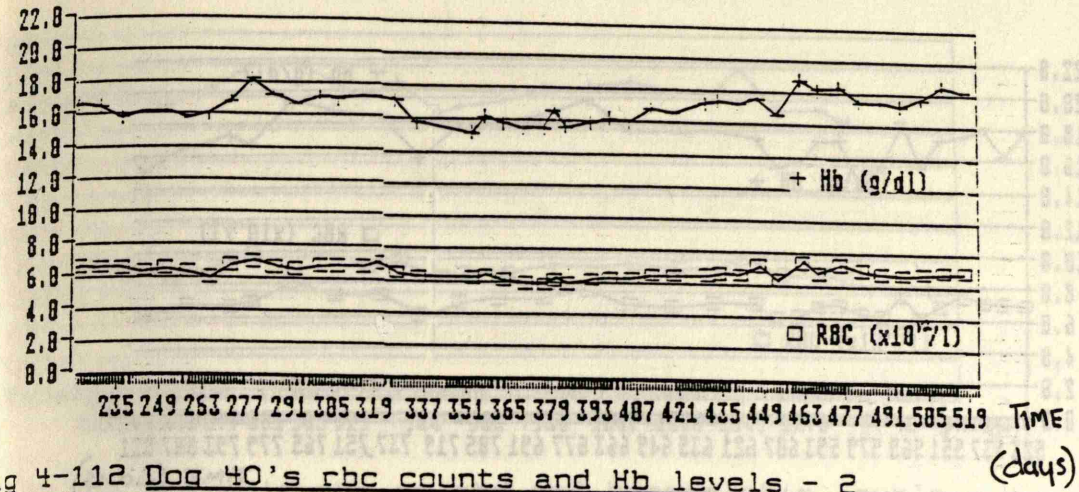


Fig 4-112 Dog 40's rbc counts and Hb levels - 2

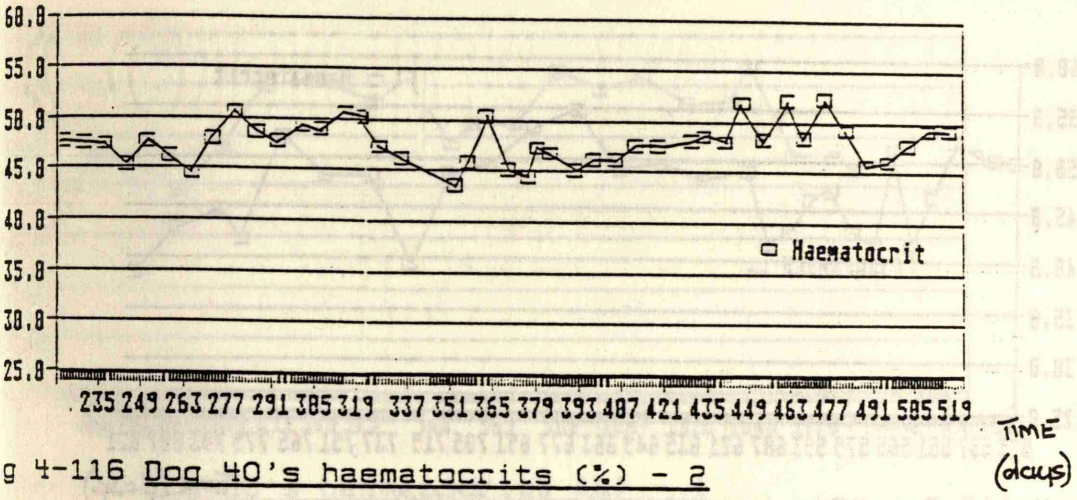


Fig 4-116 Dog 40's haematocrits (%) - 2

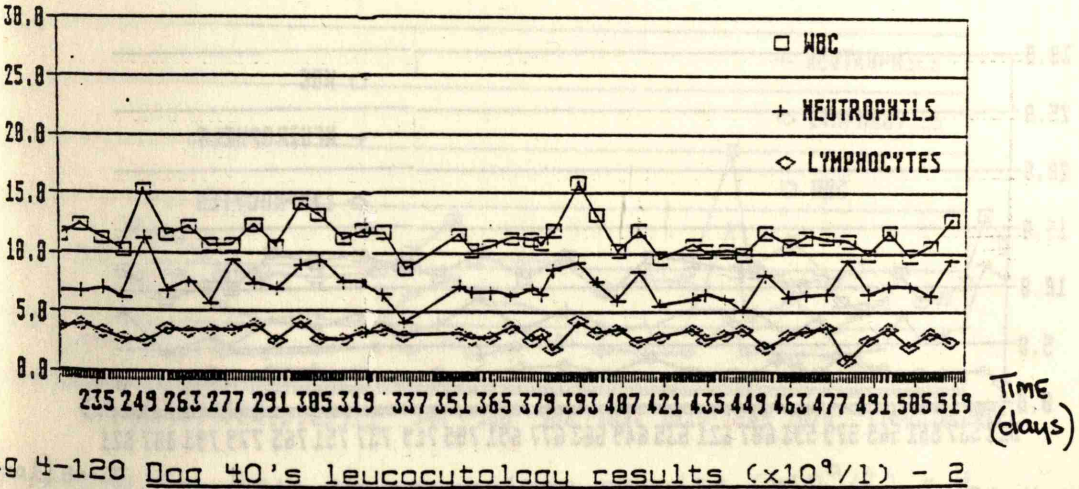


Fig 4-120 Dog 40's leucocytology results ($x10^9/l$) - 2

fp2102

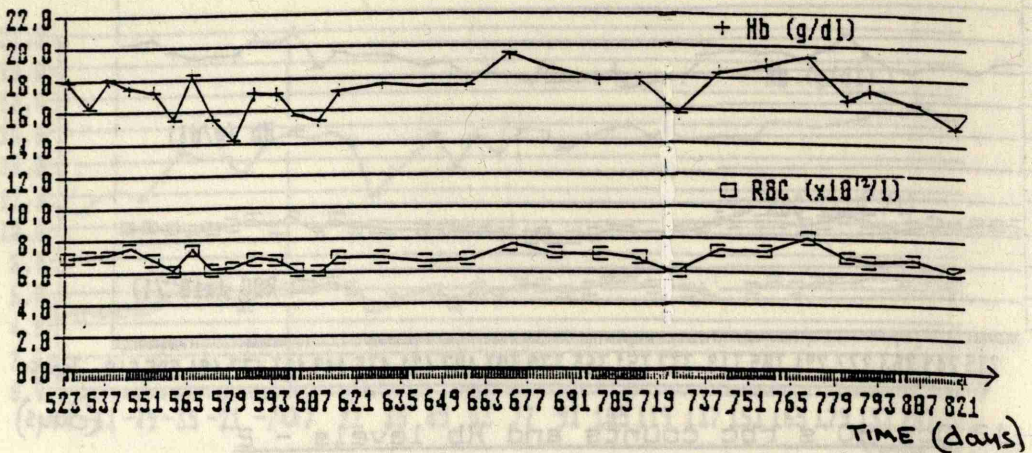


Fig 4-113 Dog 40's rbc counts and haemoglobin levels - 3

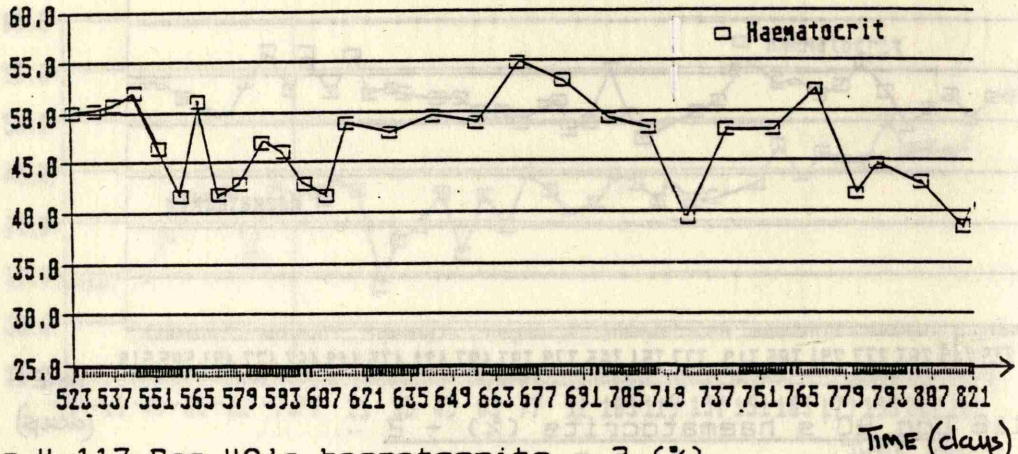


Fig 4-117 Dog 40's haematocrits - 3 (%)

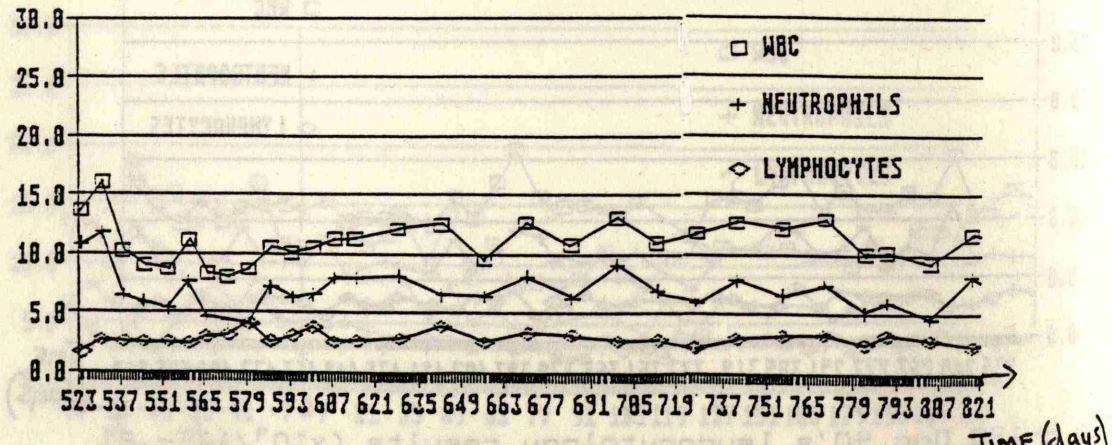


Fig 4-121 Dog 40's leucocytology results - 3 ($x10^9/l$)

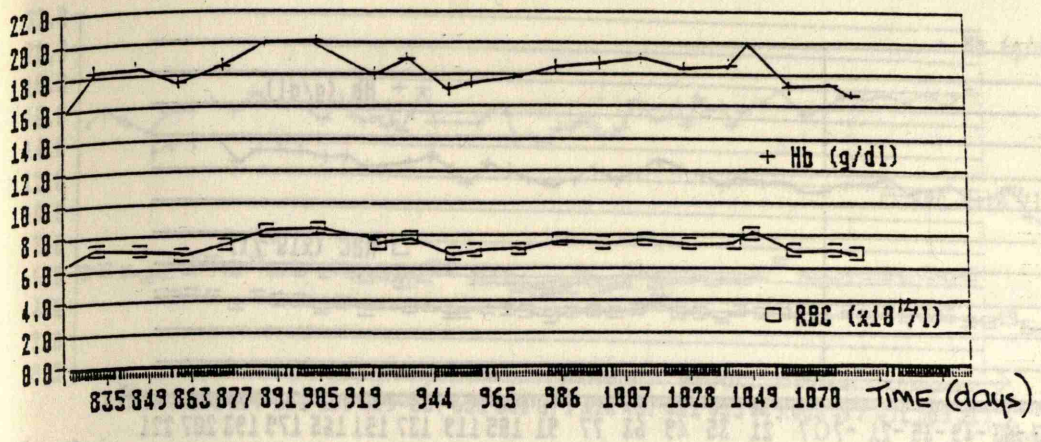


Fig 4-114 Dog 40's rbc counts and haemoglobin levels - 4

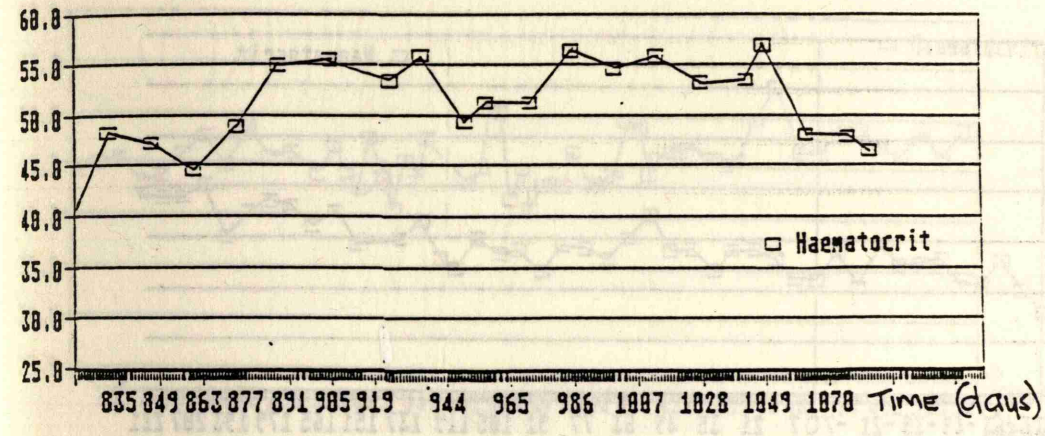


Fig 4-118 Dog 40's haematocrits (%) - 4

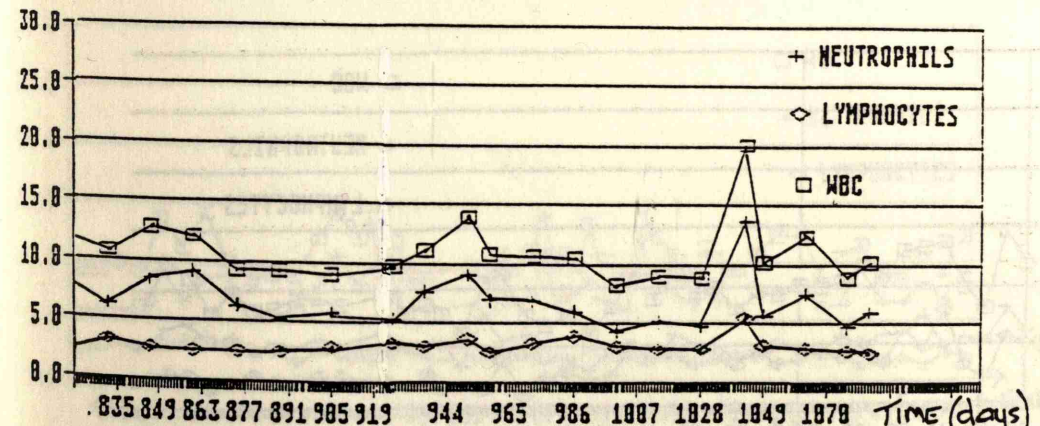


Fig 4-122 Dog 40's leucocytology results ($x10^9/l$) - 4

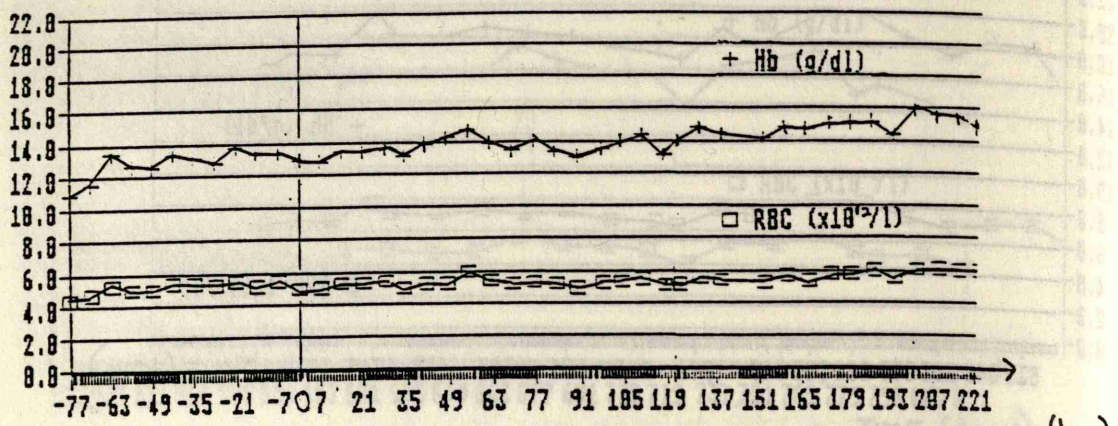


Fig 4-123 Dog 41's rbc counts and Hb levels - graph 1

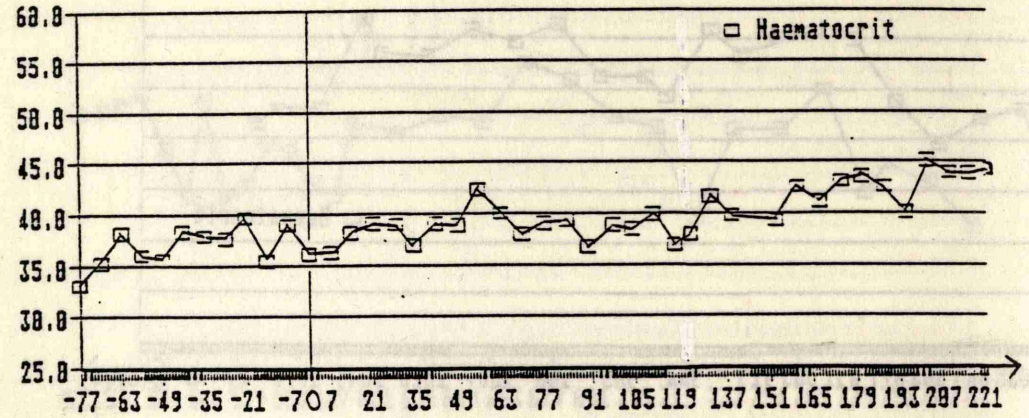


Fig 4-126 Dog 41's haematocrits - 1 (%)

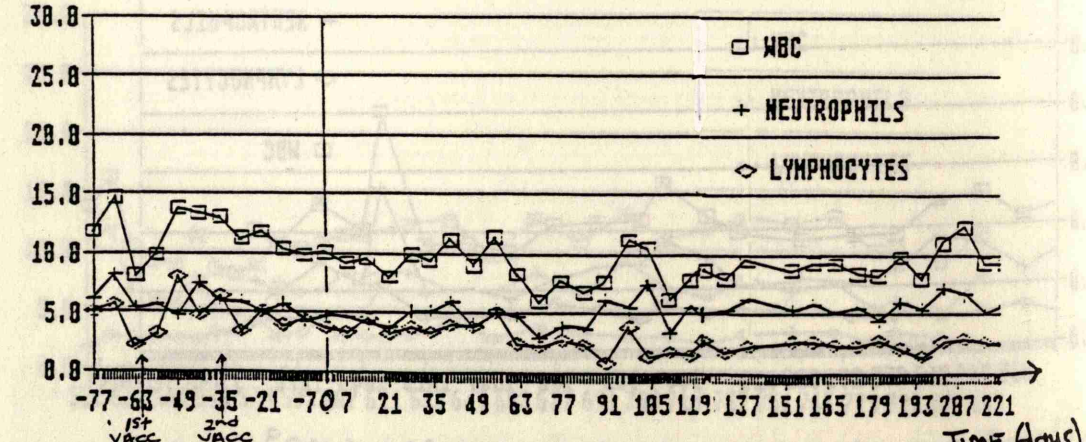


Fig 4-129 Dog 41's leucocytology results - 1 ($x10^9/l$)

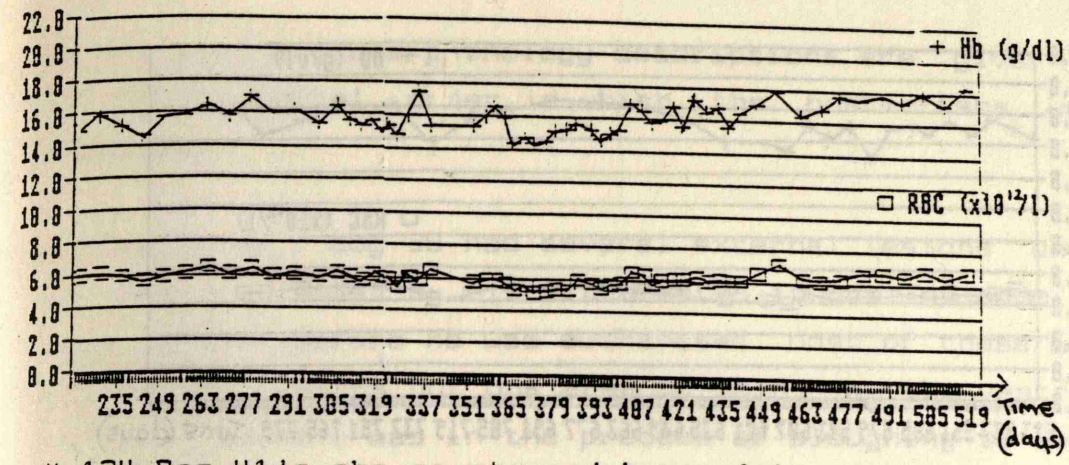


Fig 4-124 Dog 41's rbc counts and haemoglobin levels - 2

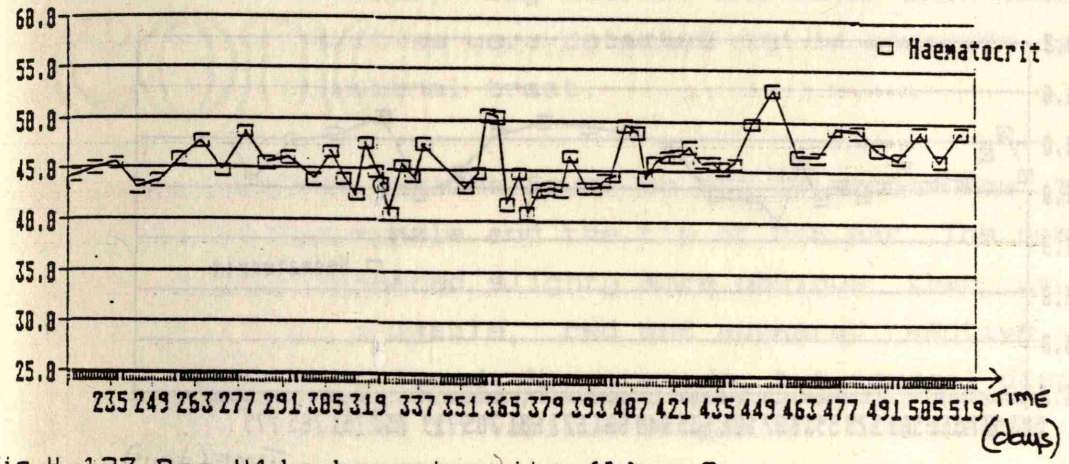


Fig 4-127 Dog 41's haematocrits (%) - 2

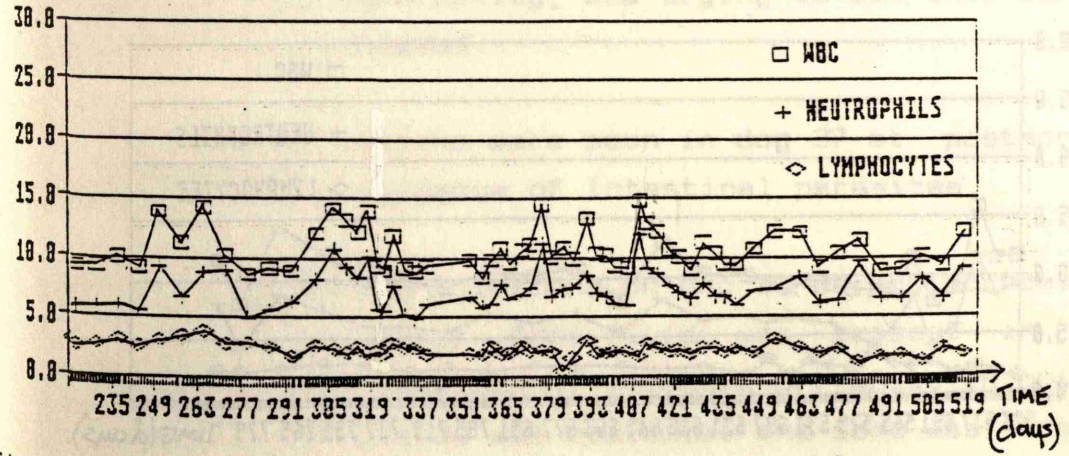


Fig 4-130 Dog 41's leucocytology results ($x10^9/l$) - 2

fp204

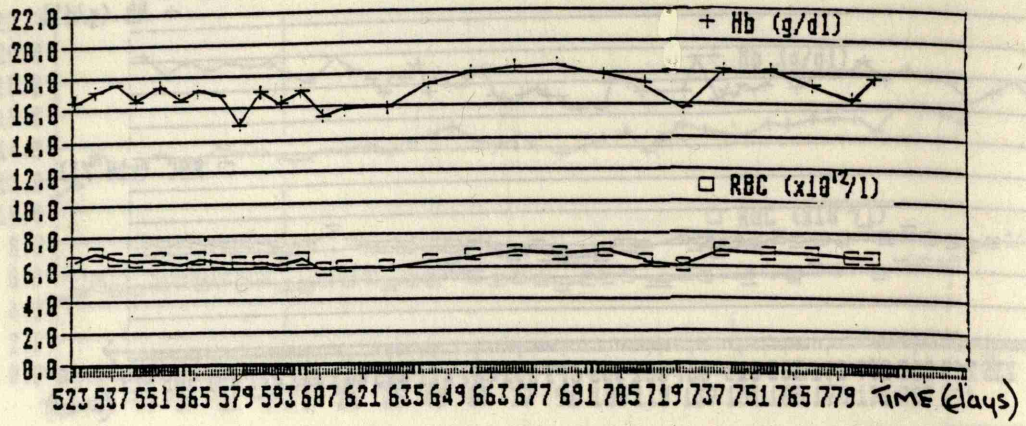


Fig 4-125 Dog 41's rbc counts and haemoglobin levels - 3

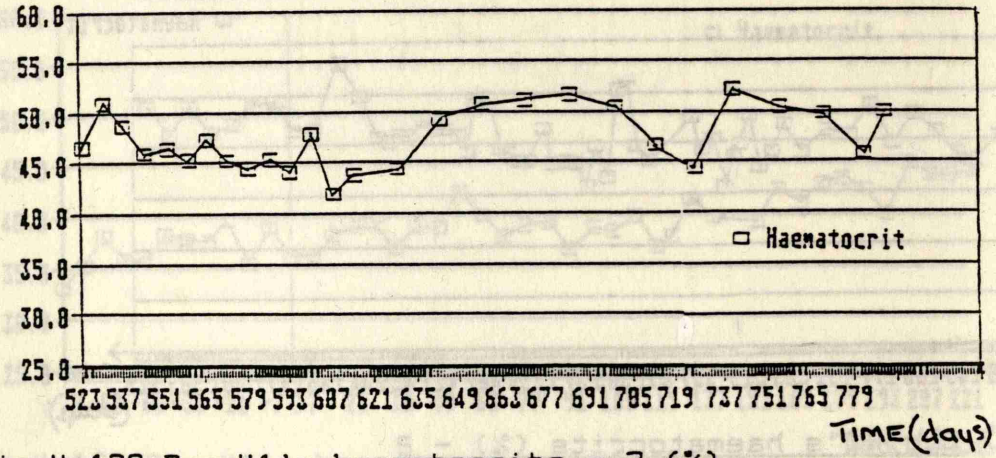


Fig 4-128 Dog 41's haematocrits - 3 (%)

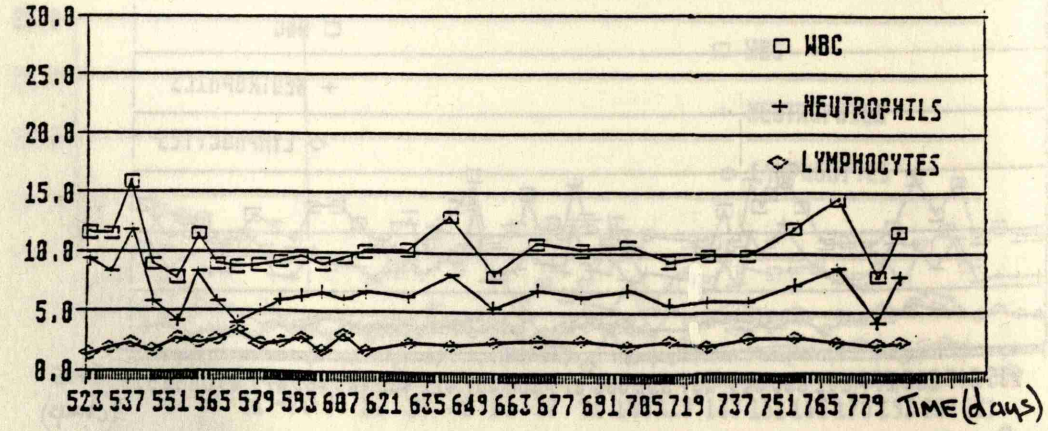


Fig 4-131 Dog 41's leucocytology results - 3 (x10⁹/l)

4.4.8 Postmortem Findings

The following gross pathology descriptions are given in the chronological order in which the postmortems were performed.

At postmortem, dog 39 had several external lesions which had been inflicted by his littermates in one episode of bullying just before he was euthanased. Most of these were only tiny abrasions but one area of skin, on the ventral, anterior abdomen, was in the process of sloughing to leave an open wound. The superficial tissues around this wound were swollen and firm. Pale yellow foci were seen in several of the liver lobes, but especially in the area of liver which, in life, lay nearest the major skin lesion. No other abnormalities were detected and no ascarids were found in the intestinal tract.

The only external lesions found on dog 36 were a few tiny abrasions on the muzzle and the tip of one ear. The normal liver pattern appeared slighty more obvious than usual. The tonsils were sizeable, red and appeared reactive. No other lesions were found. No ascarids, but several disposable plastic gloves in various stages of disintegration, were found in the stomach and intestines. They appeared not to have caused any functional or pathological problems. This dog had had a habit of stealing plastic gloves, at any opportunity, and trying to eat them before they could be retrieved.

No significant lesions were seen in dog 37 at postmortem and there was no evidence of intestinal parasites.

There were no external lesions on dog 38 at postmortem. A very small volume of free fluid was present in the abdomen; it was translucent, slightly off-white in colour and viscous. A little was collected and some was frozen for later investigations, described in chapter 8 (sections

fp205

Fig 4-132 Liver of uninoculated dog 39 (Mass OG x35)

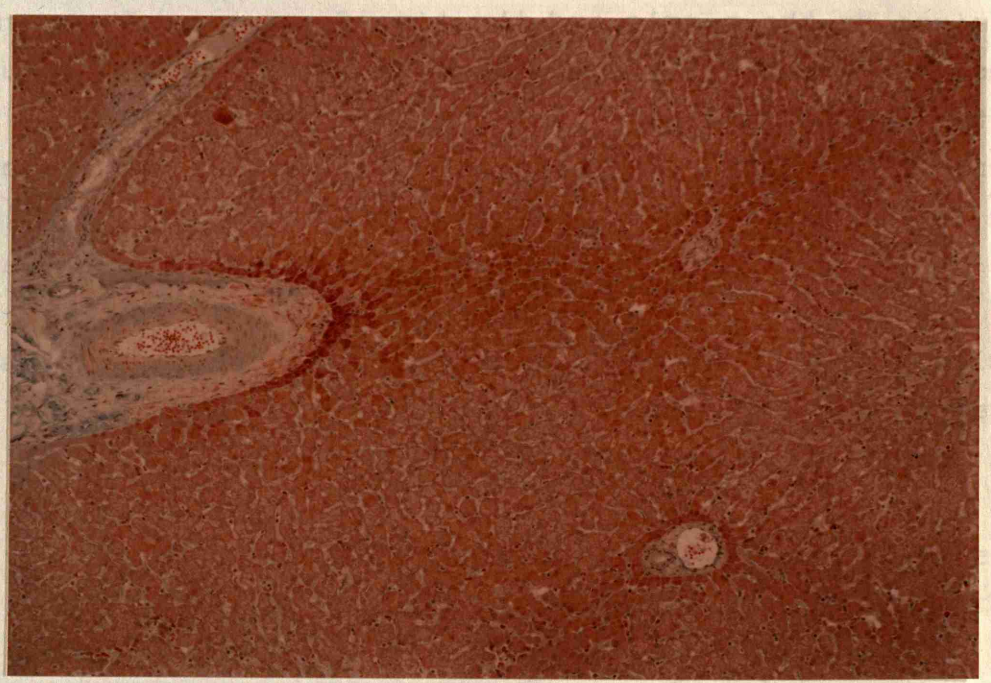

Fig 4-133 Liver of dog 36 (Mass tri x87.5)
ACs in limiting plates of portal tracts

8.2.4 and 8.3.4). The liver appeared reasonably normal. A small dark lesion of multiple tiny nodules protruded from the wall of the right atrium, just above the atrioventricular valve. No other lesions were found and there were no ascarids in the intestines.

At postmortem dog 41 had no significant lesions and no evidence of intestinal parasites. An incidental finding of a small slit was seen near one end of the spleen, at right angles to its long axis.

Dog 40 had no external lesions at postmortem. The liver was lighter in colour than expected. There were a few bits of straw in both tonsillar crypts and at least one piece appeared to be embedded in the tonsillar substance. No ascarids were found in the intestines.

The results of biochemical analysis of the urine samples, collected at postmortem, are given in table 4-7. They were relatively unremarkable. Dogs 37, 38 and 39 had alkaline urine and the urine of dog 38 contained a trace of protein.

| Dog No | Protein mg/100ml | Urea mmol/l | pH | Specific Gravity |
|---|---|---|---|---|
| 36 | 0 | 325 | 6.0 | 1.020 |
| 37 | 0 | 230 | 8.0 | 1.018 |
| 38 | 3 | 605 | 8.3 | 1.032 |
| 39 | 0 | 495 | 9.0 | 1.031 |
| 40 | 0 | 327 | 6.0 | 1.017 |
| 41 | 0 | 620 | 5.5 | 1.025 |

Table 4-7 Dog experiment 2 urine biochemistry results

4.4.9 Histological Results

The histological findings are described in the chronologi-

fp206

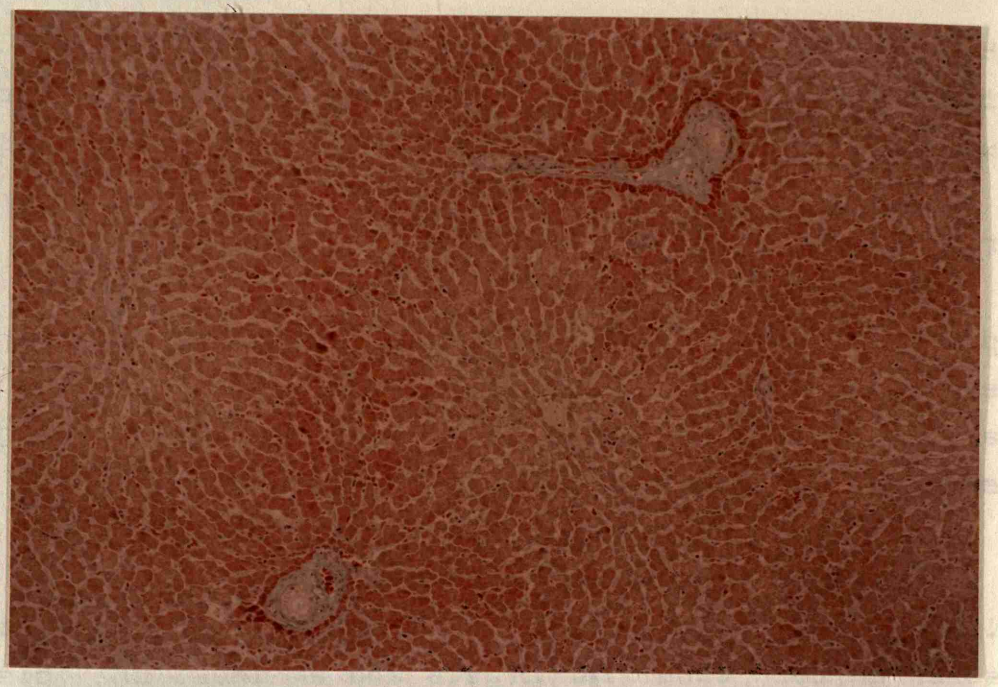

Fig 4-134 Liver of dog 37 (Mass tri x87.5)
Acidophil cells in portal tract limiting plates

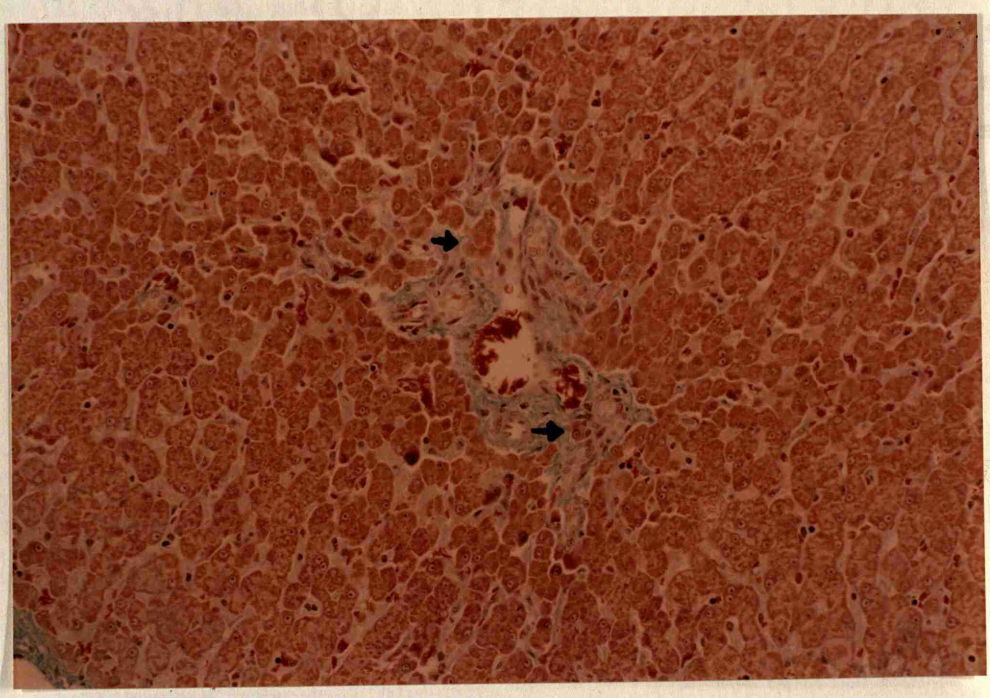

Fig 4-135 Liver of dog 37 (Mass tri x175)
Piecemeal necrosis (→)
Individual and small groups of hepatocytes being encorporated into fibrous tissue at PT edge

cal order in which the dogs' postmortems were performed. The acidophil cell scores for the dogs in this experiment are given in table 4-8.

Microscopy of dog 39's liver revealed only very few acidophil cells which were mainly in ones and twos at portal tracts. Figure 4-132 demonstrates the paucity of acidophil cells. One of two lung sections had two very small foci of lymphoid cells beside airways. No abnormalities were found in any of the other organs examined.

Liver sections from dog 36 contained many acidophil cells, arranged in moderate numbers in the limiting plates of many portal tracts (figure 4-133). Many acidophilic, crystalline, intranuclear inclusion bodies were also seen in the liver. One of two lung sections contained only one focus of lymphoid cells beside a large airway, the other section had a few such foci. The tonsils were quite reactive, with lots of very large secondary follicles with very large germinal centres.

Histological examination of dog 37's liver revealed a similar degree of acidophil cell reaction to that of dog 36 (figure 4-134). However, there was also a slight degree of fibrosis, with very fine fibrous processes extending out, between the hepatocyte plates, from some portal tracts (figure 4-135). Two, of three, lung sections each had one small focus of lymphoid cells beside a small airway.

Dog 38 also exhibited a positive acidophil cell reaction in its liver (figure 4-136) and a very slight degree of fine fibrosis, extending out from a few portal tracts. Acidophilic, intranuclear inclusion bodies were also seen (figure 4-137). One lung section had only one tiny focus of lymphoid cells, two others had two foci each. The tonsils were quite reactive, packed with large secondary follicles with very large germinal centres.

fp207

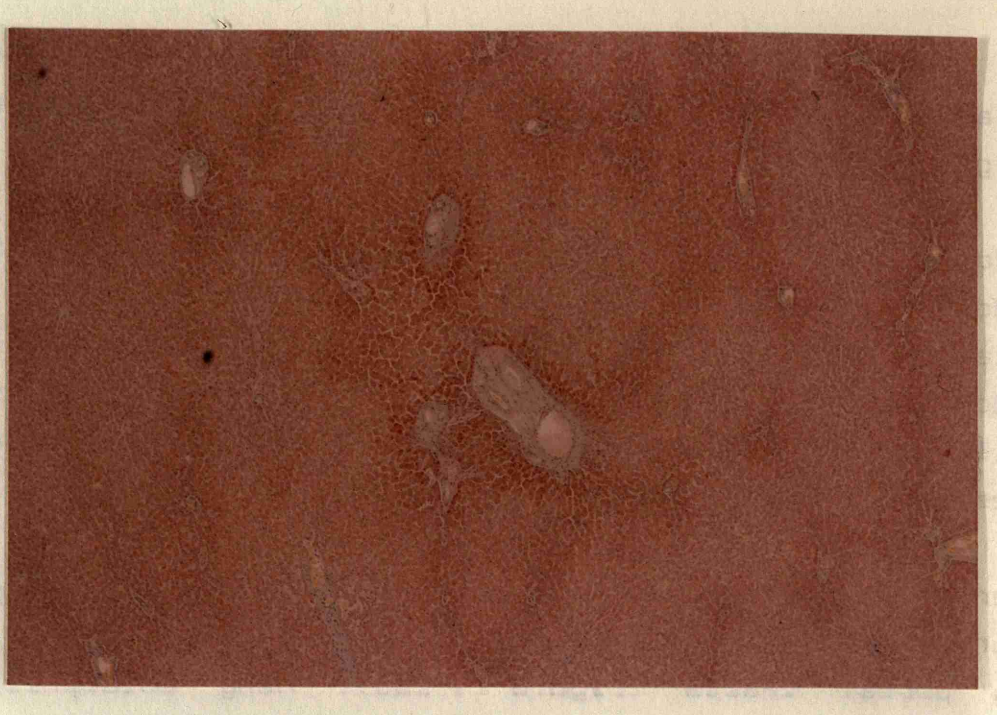

Fig 4-136 Liver of dog 38 (Mass-OG x35)
Acidophil cell reaction around portal tracts

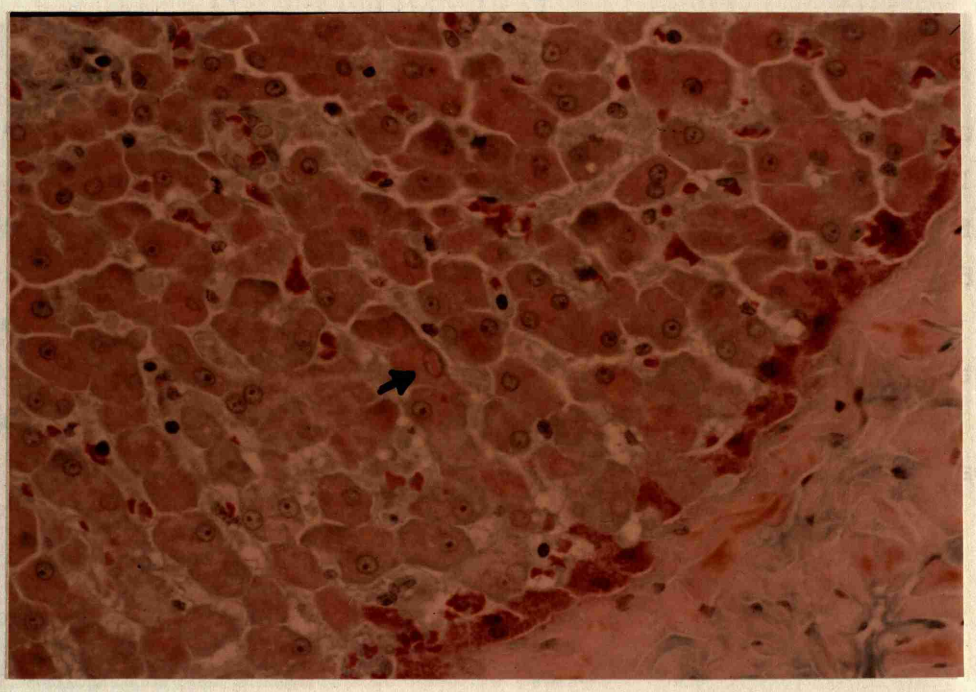

Fig 4-137 Incl. body in liver of dog 38 (Mass tri x350)
(↗) Longitudinal section of eosinophilic intranuclear inclusion body, near ACs in limiting plate

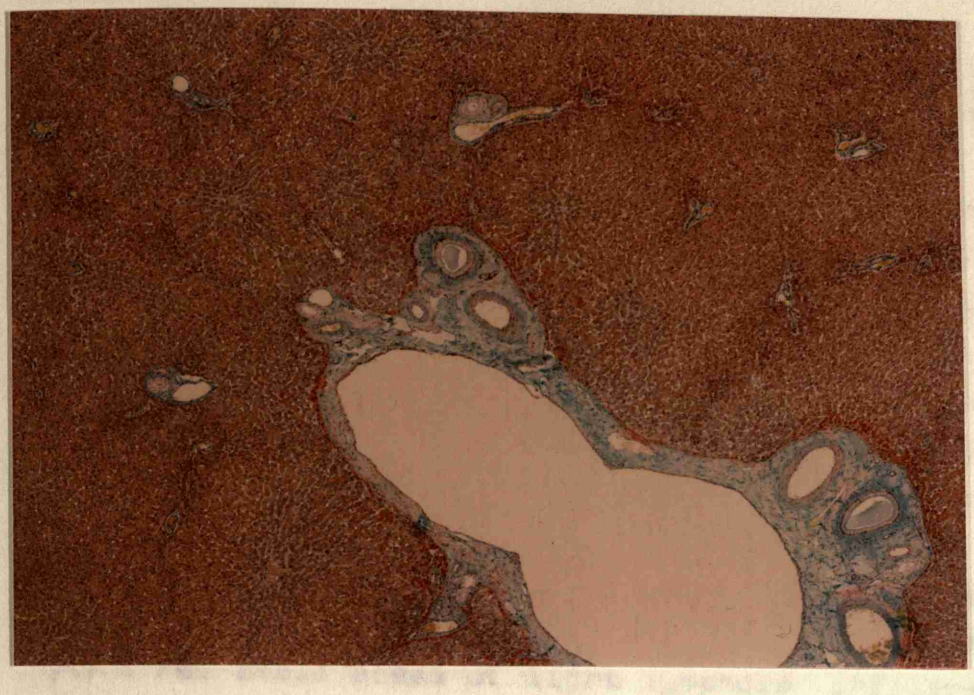

Fig 4-138 Liver of dog 41 (Mass-OG x35)

Fig 4-139 Liver of dog 41 (Mass tri x87.5)

fp208

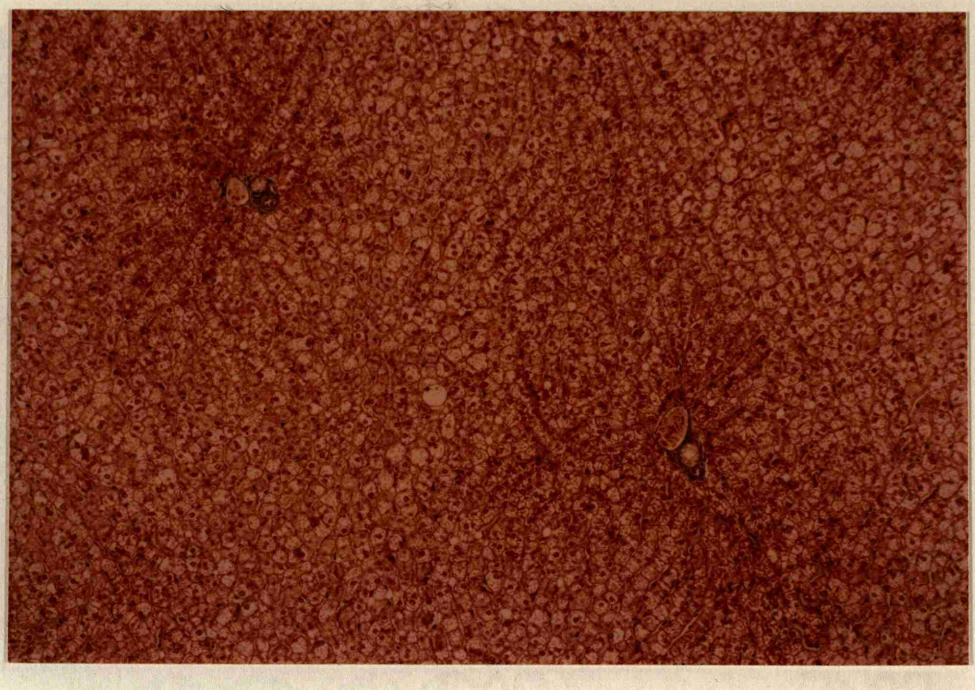

Fig 4-140 Liver of dog 40 (Mass tri ×87.5)
acidophil cells at small portal tracts

Although dog 41 was over 2½ years of age when killed its liver still exhibited a positive acidophil cell reaction (figure 4-138), with small numbers of acidophil cells in the limiting plates of several portal tracts. There was a degree of fibrosis in the liver, with fine fibrous processes extending out from some portal tracts (figure 4-139). Intranuclear inclusion bodies were also a feature of the liver histology. In two, of five, liver sections a few portal tracts contained a few mixed cells, but infiltration was not really a feature of this case. Histological examination of the slit in the spleen indicated that it was lined on both sides by splenic capsule and there was no indication of any abnormalities in the parenchyma. There were a few small areas of light lymphoid infiltration in the lungs: two foci in one section, a few in another.

Dog 40, culled 3 years after inoculation, was found to exhibit a mild acidophil cell reaction with small numbers of acidophil cells in the limiting plates of several portal tracts (figure 4-140). Fibrosis was not much of a feature of this dog's liver. There were many acidophilic, intranuclear inclusion bodies in the liver parenchyma. The lungs contained a few small foci of lymphoid cells. One tonsil had a small cavity, open to the surface, lined by stratified squamous epithelium. If this was a straw-penetration wound, then it was an old healed one and the straw seen protruding from the tonsil, at postmortem, had not been causing any reaction.

### 4.4.10 Conclusion

Canine acidophil cell hepatitis was produced in all five of the inoculated dogs. Neither clarification nor subsequent filtration of the liver preparation prevented transmission of the disease, although the adverse reaction to unclarified inoculum, seen in experiment 1 (section 4.3.3), was avoided.

| Dog No. | Inoculum used | Time post inoculation wks | months | Age | AC score | Result |
|---|---|---|---|---|---|---|
| 36 | clarified liver preparation | 17 | 4 | 8½m | ++++ | + |
| 37 | clarified & filtered prep. | 23 | 5½ | 10m | ++++ | + |
| 38 | clarified & filtered prep. | 32 | 7½ | 1yr | +++ | + |
| 41 | clarified & filtered prep. | 113 | 26 | 2½yr | +++ | + |
| 40 | clarified liver preparation | 155 | 36 | 3½yr | +++ | + |
| 39 | none | n\a | | 4½m | + | — |

Table 4-8 Dog experiment 2 liver histology results - acidophil cell scores

Liver lesions were graded on a scale of - to ++++++. A positive acidophil cell reaction was taken as +++ or more. The acidophil cell scoring system is explained in appendix 2.

There appeared to be a little progression in the histological changes seen in some of the dogs' livers. Dog 36, killed 4 months after inoculation with the clarified preparation, had acidophil cell hepatitis lesions without a noticeable fibrous reaction. Five and a half months after inoculation, with the filtered preparation, dog 37 was found to have a similar degree of acidophilic hepatocyte lesions but also a little, very fine fibrosis. Dog 38 had CACH when killed, 7½ months after inoculation with the filtered preparation. He had slightly more fibrous reaction around his portal tracts, although it appeared that a smaller proportion were affected. Twenty-six months after inoculation, with the filtered preparation, dog 41 still exhibited acidophil cell hepatitis; with slightly fewer acidophilic hepatocytes and slightly more fibrosis than the earlier dogs. However, fibrosis was not really a feature of dog 40's CACH lesions when she was postmortemed, 36 months after inoculation with the clarified preparation.

The dogs all had a slight increase in their ALT level in the first sample after inoculation (collected on the third day). These low ALT spikes only exceeded the normal range in dogs 37, 40 and 41. Whether the spikes were purely endogenous enzyme, or whether exogenous enzyme from the inoculum had contributed to them, was not known. Abnormally high levels of ALT were seen periodically in all of the inoculated dogs. Raised ALT levels in different dogs often coincided or overlapped, as can be seen in figure 4-141. Peaks in AST levels were also seen periodically in all of the inoculated dogs. They often coincided with ALT peaks, but were usually lower than them. A few of the AST peaks occurred when ALT levels were normal and several of the ALT peaks were not accompanied by abnormal AST levels. The few peaks seen in the dogs' alkaline phosphatase levels were mostly low and the majority occurred during, or near to, ALT peaks. GGT levels were only measured in dogs 40 and 41 late in the experiment. They appeared very

fpz10

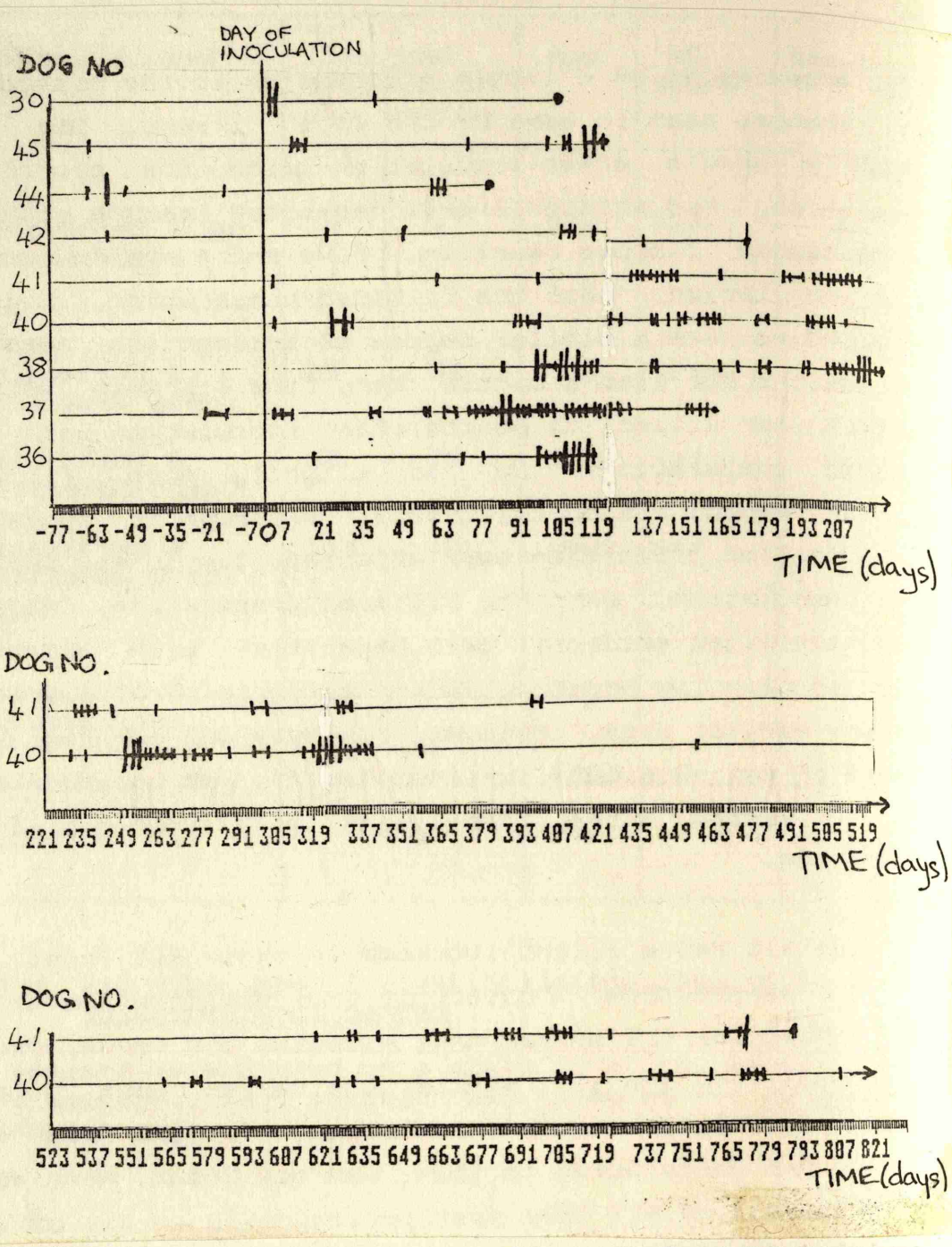


Fig 4-141 Comparison of enzyme peaks (ALT)

Only raised ALT levels are marked on the time lines, consecutive results are joined ——

• = last sample collected

DOGS 30–41 + = ALT > 45 IU/l — = ALT > 80 IU/l

DOGS 42+45 + = ALT > 55 IU/l — = ALT > 90 IU/l

DOG 44 + = ALT > 65 IU/l — = ALT > 100 IU/l

variable, although elevated levels were seen in both dogs.

Some of the peaks seen in the inoculated dogs' rectal temperatures occurred during, or near to, ALT peaks. Even when this happened, the top of the temperature peak often did not coincide with the highest of the ALT levels in a peak. Other temperature peaks occurred during times of normal biochemistry results and normal temperatures were often found during peaks in enzyme levels.

The dogs' rbc haematology results appeared not to be affected by the CACH infection. After inoculation the dogs' leucocytology results rarely exceeded normal ranges, although occasional low peaks were distinguishable from surrounding levels. They sometimes coincided in two or more dogs and only some of them occurred during, or near, peaks in the biochemistry results. The significance of these findings and any correlation with CACH infection is not clear.

## 4.5 DOG EXPERIMENT THREE

### 4.5.1 Experiment Design

The aim of this experiment was to attempt the transmission to dogs of CACH which had been experimentally passaged in rats inoculated with canine material. The experiment was also designed to test the effect of using fresh liver as a source of infection instead of the frozen liver used in all previous experiments.

Four female, 6-month-old beagles were obtained, from a registered breeder, for this experiment (dogs 42 to 45). They were housed in one of the two isolation units described in section 2.2.1. This had previously housed the inoculated dogs of experiment 1, but had been thoroughly cleaned and fumigated after its use. The unit was fumigat-

ed again, before this experiment, after lying empty for some time.

Equal weights of liver from four freshly killed experimental rats were pooled, chopped finely in a few drops of tris-buffered saline and homogenised in TBS to form a 40% weight:volume preparation. This was diluted with TBS to a 20% wt:vol homogenate and clarified in a bench centrifuge for 10 minutes. The supernatant was then clarified further by spinning at 10 000 rpm in an ultracentrifuge for 10 minutes at 4°C. Bacteriological investigation of the 40% wt:vol liver preparation resulted in the culture of only one individual colony which was considered unimportant. A piece of liver from one animal (rat 142) was also checked, but no bacteria were seen in a smear of it and none were isolated from it. Checks for adenovirus and other pathogenic canine viruses failed to isolate anything from the pooled liver preparation or from rat 142's liver sample.

Dogs 42, 44 and 45 were each inoculated subcutaneously with 10ml of the 20% wt:vol pooled liver preparation, split between two sites in the scruff. Dog 43 was not inoculated at the same time as the others, because she had been unwell (see section 4.5.3). She was to have been inoculated once fully recovered, but due to indications of liver damage it was considered pointless to do so; any lesions found at postmortem could never have been unequivocally attributed to inoculation with the liver preparation. The dog was therefore euthanased, just over a week after the others were inoculated. Dog 44 was euthanased 11 weeks after inoculation because, on the previous day, she had suddenly developed a mass in the right submandibular area. Eighteen weeks after inoculation dog 45 was culled and dog 42 was culled 25 weeks after inoculation.

### 4.5.2 Source Cases

Pooled fresh liver from rats 138, 139, 142 and 143 of rat experiment 5 was used as the source of infection. The four rats were killed, just over 6 weeks after inoculation with liver from experimentally-infected dog D (section 5.7), specifically to provide liver for use in dog experiment 3.

The rat killed 6 weeks after inoculation had been the first in rat experiment 5 to have a positive acidophil cell reaction in its liver (see figure 5-22). Since the aim of this experiment was to use fresh liver it was not possible to have liver samples histologically processed, stained and inspected before they were inoculated. For the early culls of rat experiment 5, a rapid processing technique was used to permit histological examination of two liver sections the day after the postmortem. This was simply a speeded up version of the routine process described in section 2.3. The poorer cell detail obtained with cryostat sections was considered inadequate for the requirements of this experiment and so although the technique could have produced faster results it was not used. It had been decided that as soon as a good positive acidophil cell reaction was found in the fortnightly culls of rat experiment 5, a number of other rats would be culled and their livers pooled for preparation of the inoculum. It was hoped that the pattern seen in the other rat experiments, of the acidophil cell reaction taking 4-6 weeks to develop and then most of the rats remaining positive, would be repeated.

Liver from several rats was pooled to give adequate amounts and to allow for the possibility of one or two less affected or even unaffected individuals. When liver sections, from the four rats used to provide the inoculum, were examined it was found that only three of the rats actually exhibited definite acidophil cell reactions. Although acidophil cells were seen in rat 138's liver,

fp213

Fig 4-142 Liver of experimtal rat (139) (Mass-OG x87.5)
acidophil cells in limiting plates

there were insufficient to be considered a positive reaction (see table 5-4). An example of rat 139's liver histology can be seen in figure 4-142.

### 4.5.3 General Clinical History

The beagles had been routinely vaccinated as pups in the breeding establishment. Their titres against distemper, parvo and adeno viruses were checked 6 weeks after arrival, the results are given in table 4-9. All four dogs were found to have good levels of immunity.

| Dog No | Distemper virus | Parvo virus | Adeno virus |
|---|---|---|---|
| 42 | 1453 | 356 | 4096 |
| 43 | 734 | 512 | 1453 |
| 44 | 734 | 256 | 5813 |
| 45 | 1024 | 256 | 512 |

Table 4-9 Dog experiment 3 antibody titres

The beagles all tended to defaecate, in the mornings, before they had been examined. This prevented the collection of rectal faecal samples for their first 8 weeks on the premises. During that time unidentified faecal samples had to be collected, from the pen floor, for parasitological examination. The first few faecal egg count results are given in table 4-10 along with the anthelmintic regime used. The first unidentified sample, collected 5 days after their arrival, showed evidence of both Toxocara (roundworm) and Uncinaria (hookworm) infestations. Even before the result was obtained the dogs had been put on a split-dose, 5-day oral course of fenbendazole (Panacur 22% granules) as a precautionary measure. As soon as the result was known the pen was swept clean, scrubbed with disinfectant and rinsed to remove any sticky Uncinaria eggs and prevent any invasion of the dogs' feet. Two other

| TIME after arrival | | 5d | 5-9d | 7d | 8d | 3wk | 4wk | 6w | 6½w | 8w | 8½w | 13w |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Unident. faecal samples | Toxoc. | 250 | ▼fenbendazole | 550 | <50 | 4x0 | 4x 0 | *fenbendazole | 3x 0 | | piperazine | |
| | Uncin. | 200 | | 100 | 0 | 4x0 | 3x 0<br>1x50 | | 1x50<br>4x 0 | | | |
| Dog 42 | | | | | | | | | | 0 | | 0 |
| Dog 43 | | | | | | | | | | 0 | | 0 |
| Dog 44 | | | | | | | | | | 0 | | 0 |
| Dog 45 | | | | | | | | | | | | |

Table 4-10 Dog experiment 3 early faecal egg counts and anthelmintic regime (Toxocara and Uncinaria)

The unidentified faecal samples were collected from individual stools on the floor.
▼ = a five day divided dose course
* = a three day divided dose course

unidentified samples, collected during the anthelmintic course, also contained parasite eggs. Three weeks after arrival four unidentified samples, collected at the one time, were found to be clear of parasite eggs; there was no guarantee that all four dogs were represented by the samples tested. One of another four unidentified samples, tested a week later, was found to contain some Uncinaria eggs. Although the dogs had already been given piperazine adipate (Head-To-Tail veterinary roundworm tablets) before the result was known, they were then given a 3-day, divided-dose course of fenbendazole. Toxocara eggs were seen in one of four unidentified samples collected on the third day of this course. They had probably been released earlier by adult worms which were dead by the time the eggs had passed through the intestines and out in the faeces to be collected. The first set of rectal faecal samples, collected 8 weeks after the dogs' arrival were free of parasite eggs. The FECs of all four dogs remained negative for the rest of their lives.

From arrival, dog 44's left eye always watered, wetting the coat below the medial canthus. Opthalmic investigations revealed that this was a simple overflow problem, due to restricted drainage and was not a matter for concern.

About 4½ weeks after arrival the dogs, especially 42 and 45, were found to have mild otitis externa. Their ear canals were all cleaned and treated with a course of GAC ear drops (Arnolds Veterinary Products, Shrewsbury). Ten weeks after arrival, they were given a 10-day course of Oterna ear drops (Pitman-Moore Ltd, Uxbridge) to deal with a mild recurrence of the problem.

Dog 43 began to come into season after 11 weeks on the premises. Within a week dog 42 was also coming into season, closely followed by dog 44. These three appeared to have synchronised oestrus cycles, as bitches kept together sometimes do. However, dog 45 showed no evidence

of impending oestrus. This was a very dainty dog which did not grow as much as the others, always remaining smaller than them.

Within a week of showing signs of coming into season, dog 42 developed ventral pitting oedema in the caudal mammary region and a soft cough. It was thought that the dog might have had a subclinical heart problem which it had been able to compensate for, until oestrus had put extra strain on the heart. By the following day the cough had disappeared. Extensive cardiological examinations suggested a possible slight problem with the right side of the heart, in the region of the pulmonic valve. The absence of a detectable heart murmur suggested that this was not a routine pulmonic stenosis case. There may have been a little stenosis slightly higher than usual and echocardiography indicated turbulence in the blood flow in that area. No stenosis could be visualised with ultrasonography of the heart, but although the pulmonic valve was clearly visualised the area beyond it was near the limit of the probe's range of clear demonstration. The oedema cleared up quickly as the dog's season finished.

On the same day that dog 42 had developed the oedema, a mass was seen protruding from dog 44's vulva. Further examinations revealed this to be an area of hypertrophied vaginal wall. It was advised that this should simply be kept clean and that it would regress after oestrus, although the condition tends to recur at subsequent oestruses. As expected the mass regressed within a few days.

Although the dogs were fed from individual bowls and not permitted to steal each others food, dogs 42 and 43 tended to gulp their food down as fast as possible. Dog 45, with a smaller appetite than the others, had to be encouraged to finish her bowlful and even then often left some. Seventeen weeks after arrival, there was an unexpected problem with dog 43. Within 5 minutes of gulping down her

food she exhibited sudden onset abdominal pain, assuming the classic abdominal pain position: standing stiffly, reluctant to move, legs apart and head and neck extended. There were no faeces palpable in the rectum. A few hours later the dog was still obviously uncomfortable. Faeces were present in the rectum by then and defaecation followed anal stimulation. Radiography failed to reveal any evidence of a torsion and a barium swallow appeared normal, but it did reveal delayed gastric emptying; there was a lot more food in the stomach than should have been there by that time. A diagnosis of ileus was made. It was thought that the gastrointestinal tract had gone into stasis as a result of the large volume of food, gulped down, upsetting the stomach. The dog was treated with SC injections of Metoclopramide hydrochloride bid (Emequell, Beecham Animal Health, Brentford) to encourage gastric emptying and promote normal peristaltic action. Liquid paraffin was given orally twice daily. By the evening dog 43 had improved slightly; she was willing to run a bit and was barking and wagging her tail, although her abdomen was still distended. Food was withheld the day after the incident, because she was still exhibiting abdominal discomfort. It was 3 days before dog 43 appeared normal again. At that point in the experiment, before inoculation, the dogs were only bled once weekly for biochemical and haematological evaluations. Dog 43 was given a weeks course of ampicillin bid (Amfipen 125mg tablets) because she had a high wbc count, mainly neutrophils. Despite an apparent clinical recovery, alkaline phosphatase and ALT levels were markedly raised and the AST level was high. Although the latter quickly returned to normal, the AP and ALT levels reduced only gradually and were still raised when dog 43 was euthanased.

There were virtually no adverse reactions to inoculation; only dog 45 had a slight, soft swelling on one side, by the following day. The areas around the injection sites were massaged well, after inoculation and twice daily for

2 days afterwards to help avoid any problems.

One morming, 11 weeks after inoculation, dog 44 was found to have a swelling around the right side of her lower jaw. She had appeared perfectly normal the previous morning, even when held for the collection of a blood sample from her left jugular vein. A soft mass, in the right submandibular region, was discernable amidst the generalised swelling in the area. There were no visible external lesions to explain the dramatic appearance of the mass, which did not appear to be painful. A scheduled cull was imminent so it was brought forward to the following day and dog 44 was designated, to save her any discomfort and to avoid the need for any medical intervention.

### 4.5.4 Rectal Temperatures

In this experiment the dogs' rectal temperatures were only monitored irregularly before inoculation, afterwards they were checked daily as in previous experiments (figs. 4-143 to 4-146). The few results obtained in the preinoculation period were mainly in the range of 38.7-39.5°C; apart from the first result of both dogs 43 and 44 (39.7°C) and a lower result of 38.2°C in dog 45, 48 days before inoculation. For the dogs in this experiment 39.5°C was considered to be the top of their normal range of temperatures. Dog 45's temperature range was noticeably lower (below 39.0°C) than those of the others. None of the three inoculated dogs exhibited any temperature peaks immediately after inoculation.

During the first few weeks just after inoculation dog 42's range of rectal temperatures was lower than her usual range; the step back up occurred 4 weeks after inoculation (figure 4-143). Dog 42's rectal temperature only exceeded 39.5°C on five occasions. Two peaks of 39.6°C occurred 35 and 68 days after inoculation and two peaks of 39.7°C were seen 45 and 71 days after inoculation. The highest peak of

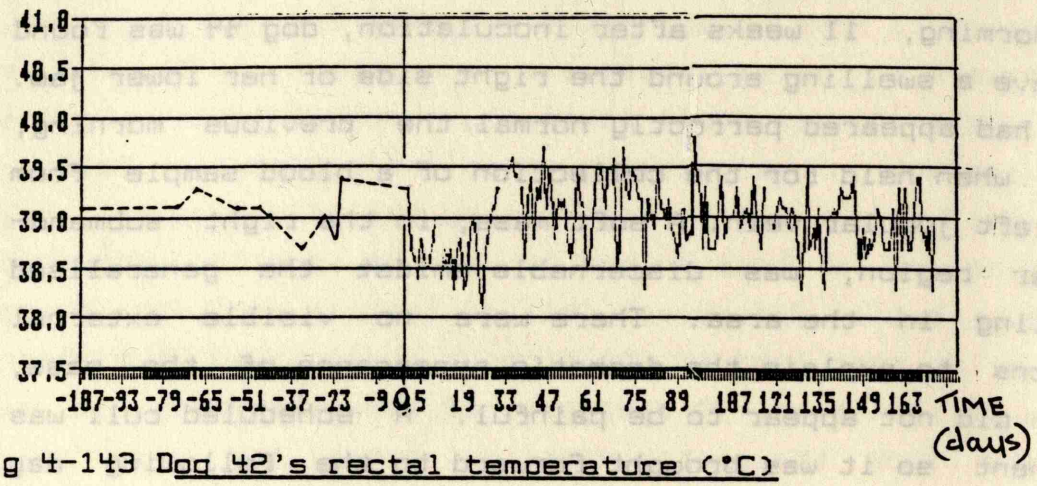


Fig 4-143 Dog 42's rectal temperature (°C)

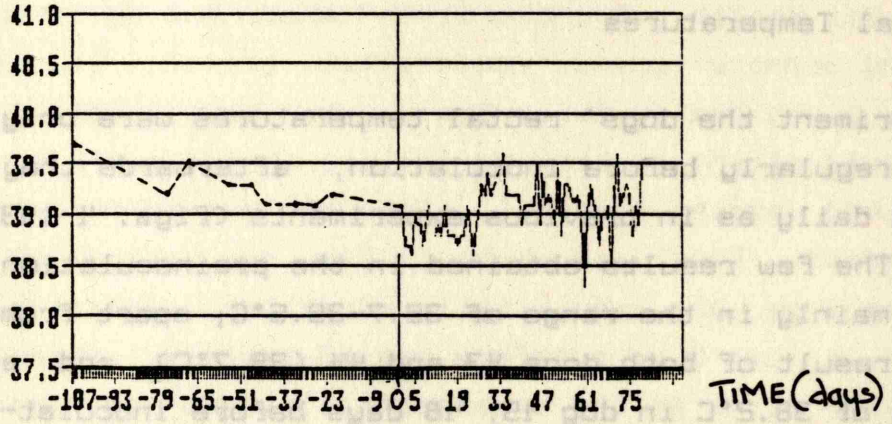


Fig 4-145 Dog 44's rectal temperature (°C)

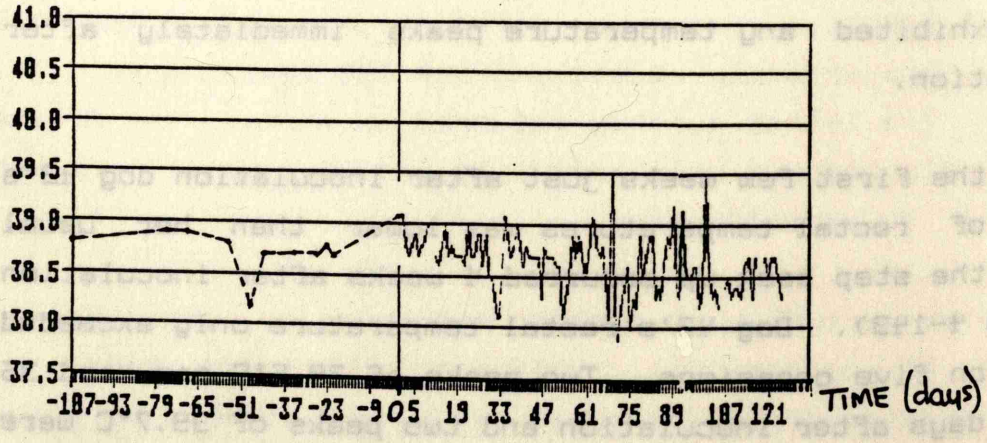


Fig 4-146 Dog 45's rectal temperature (°C)

39.8°C occurred 94 days after inoculation. Several other lower peaks, within the normal range, were discernable in dog 42.

Apart from her first result, dog 43's rectal temperature never exceeded the normal range (figure 4-144).

As in dog 42, there was a period just after inoculation when dog 44's rectal temperature range appeared to be lower than usual (figure 4-145). The step back up occurred just under 4 weeks after inoculation. Two temperature peaks of 39.6°C were seen in dog 44, 34 and 71 days after inoculation. Although these were the only ones to exceed the normal range, three lower peaks of 39.5°C were also distinguishable from surrounding readings. The last of these was the last temperature recorded in dog 44, the day after the sudden appearance of the submandibular mass (described in section 4.5.3).

After inoculation, the rectal temperature range of dog 45 continued to be lower than that of the other three dogs (figure 4-146). Her temperature was usually in the range of 38.1-39.0°C. Any peaks above this range, although within the normal range for the experiment, might be significant in this dog. On three occasions, 1, 34 and 62 days after inoculation, dog 45 had a temperature of 39.1°C. Temperatures of 39.2°C and 39.3°C were recorded 89 and 93 days after inoculation respectively. The two highest spikes, both of 39.5°C, occurred 69 and 101 days after inoculation. Although within the general normal range, they were considered to be abnormally high levels for dog 45 when compared to her own usual range.

The step up in average rectal temperatures seen in dogs 42 and 44, about 4 weeks after inoculation, occurred in early summer (late May). Similar step-like changes were seen at the same time of year in some of the experiment 1 dogs (section 4.3.4) and at other times of year in some of the

fp219

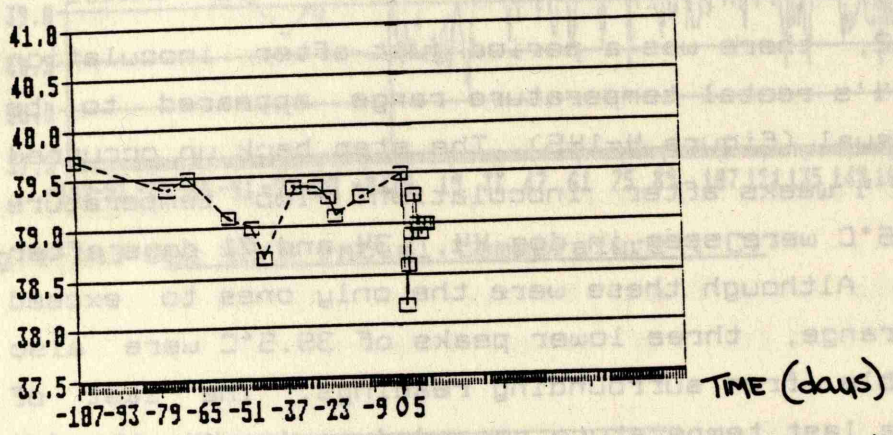


Fig 4-144 Uninoculated dog 43's rectal temperature (°C)

experiment 2 dogs (section 4.4.5).

4.5.5 Biochemical Results

The usual ALT levels seen in the beagles in this experiment generally tended to be slightly higher than those seen in the dogs of experiments 1 and 2. To compensate for this, a higher upper limit of 55 IU/l was chosen for their normal ALT range. During the preinoculation period dog 42 only exceeded this limit twice, with an ALT spike of 84 IU/l 99 days before inoculation and a very slightly raised result 57 days before inoculation (figure 4-147). Dog 43 had a raised ALT level of 72 IU/l, 85 days before the others were inoculated (figure 4-150). Her ALT level was raised again in the 3 weeks before the episode of abdominal pain, after which there was a dramatic peak of 622 IU/l. Despite an apparent clinical recovery, dog 43's ALT level remained elevated. She was not inoculated because there was no way of predicting the aftereffects of her illness and it would not have been possible to differentiate them from any future reactions to inoculation. The usual ALT level seen in dog 44 appeared to be even higher than those of the other dogs; all but three of her preinoculation results were over 55 IU/l (figure 4-153). Two spikes stood out from the rest of dog 44's preinoculation ALT levels: 127 IU/l and 84 IU/l at 57 and 14 days before inoculation respectively. In dog 45 an ALT result just above the normal range, preceded the only preinoculation peak of 69 IU/l at 64 days (figure 4-156).

The general preinoculation range of AST levels seen in this experiment was a little higher than those of most of the dogs in experiments 1 and 2, whose preinoculation results were nearly always below 30 IU/l. Most of the preinoculation AST results of dogs 42 to 45 were below 40 IU/l and 45 IU/l was taken as the upper limit of the normal AST range for this experiment. Dog 42 only had two preinoculation AST results above the normal range: 51 and

53 IU/l at 99 and 92 days before inoculation (fig. 4-148), the first of these coincided with a high ALT level. Only normal levels of AST were found in dog 43 until she became ill and her AST level shot up to 164 IU/l (figure 4-151). Her AST level fell much sooner than her ALT level; by the next blood sample, 3 days later, it was down to 45 IU/l. Before inoculation dog 44 had only one AST level just above the normal range (figure 4-154), which coincided with a raised ALT level 99 days before inoculation. Dog 45's AST level remained normal during the whole of the preinoculation period; it never exceeded 40 IU/l (figure 4-157).

The exact ages of the dogs used in this experiment were not known, but their early alkaline phosphatase levels did not contradict the estimate of around 6 months of age. Although generally lower than those of very young puppies, their levels were still in the process of dropping towards the lower levels expected in adults. The general range of AP levels in dogs 42 and 45, at the start of the experiment, was a little lower than that of most of the experiment 1 and 2 dogs at around 6 months of age. Even though dog 43's early range of AP levels was higher than those of the rest of the beagles, it was not dissimilar to the range seen in dogs 31 and 32 around 6 months of age. Dog 44's early AP range was similar to that of many of the dogs in earlier experiments at 6 months of age.

Before inoculation dog 42's AP results were unremarkable and never exceeded 150 IU/l (figure 4-149). Dog 43's higher AP levels may have meant that she was younger than the other beagles. Three of her early results, which were noticeably higher than the rest, were 420, 389 and 399 IU/l found 111, 92 and 85 days respectively before the others were inoculated (figure 4-152). Only the last one coincided with an elevated ALT level. After the onset of abdominal pain, dog 43's AP level rocketed into the thousands for three samples and, like her ALT level,

remained high until she was euthanased. Dog 44's preinoculation AP level was always below 200 IU/l, except for a two-sample peak of 269 and 296 IU/l 57 and 50 days before inoculation (figure 4-155). The first of these results was in the sample with the high ALT level. During the preinoculation period dog 45's AP level only exceeded 150 IU/l on two occasions (figure 4-158). Levels of 183 and 196 IU/l, seen 57 and 50 days before inoculation, may have been within the normal range for a dog of her age.

Dog 42 had slight peaks in her ALT level 21 and 49 days (3 and 7 weeks) after inoculation (figure 4-147). A peak of 86 IU/l 107 days (just over 15 weeks) after inoculation was the first of three high ALT results. ALT spikes of 64 and 75 IU/l were seen 119 and 133 days (17 and 19 weeks) after inoculation respectively. Dog 42's highest ALT level of 166 IU/l occurred in her last sample, 173 days (more than 25 weeks) after inoculation.

There was a low AST spike to the top of the normal range in dog 42, 47 days after inoculation, in a sample with a normal ALT level (figure 4-148). Her AST level only exceeded the normal range twice, with spikes of 54 and 61 IU/l 107 and 119 days after inoculation. On those occasions dog 42 also had a high ALT level however, two intervening and two later samples with elevated ALT levels had normal AST levels.

After inoculation dog 42's AP level remained reasonably constant apart from a cluster of results over 100 IU/l between 15 and 18 weeks after inoculation (figure 4-149). The three highest of these were 161, 154 and 172 IU/l on days 107, 112 and 119 respectively. They coincided with some of the elevated ALT results of that period and two of them were in the samples with the raised AST levels.

fp222

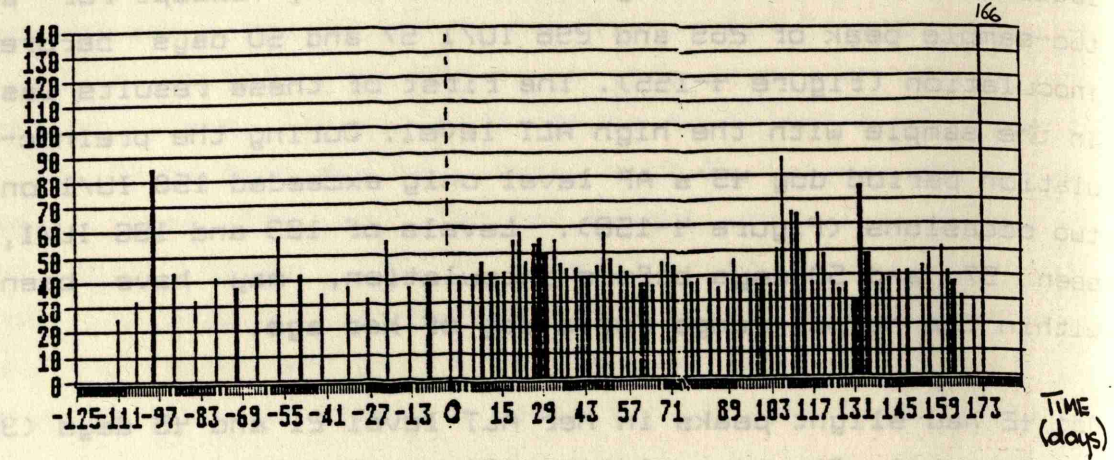


Fig 4-147 Dog 42's ALT levels (IU/l)

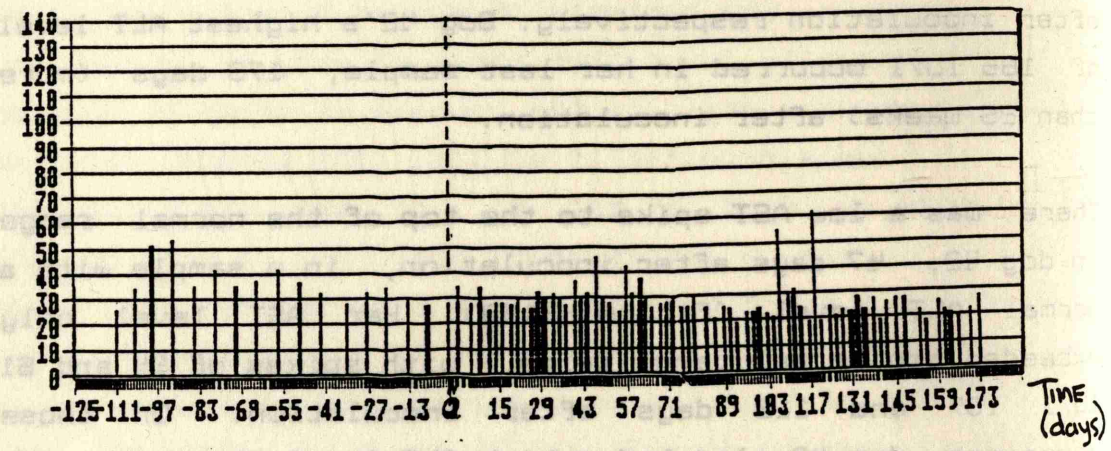


Fig 4-148 Dog 42's AST levels (IU/l)

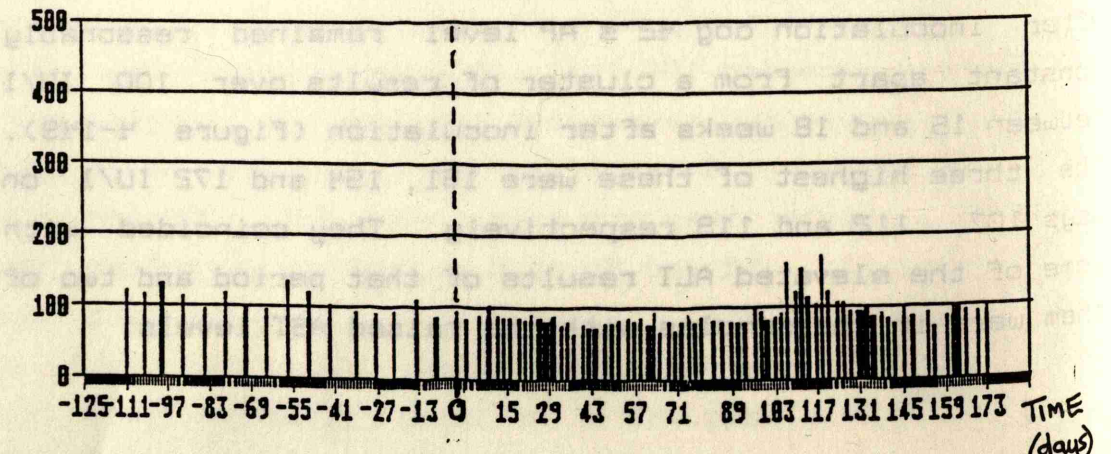


Fig 4-149 Dog 42's alkaline phosphatase levels (IU/l)

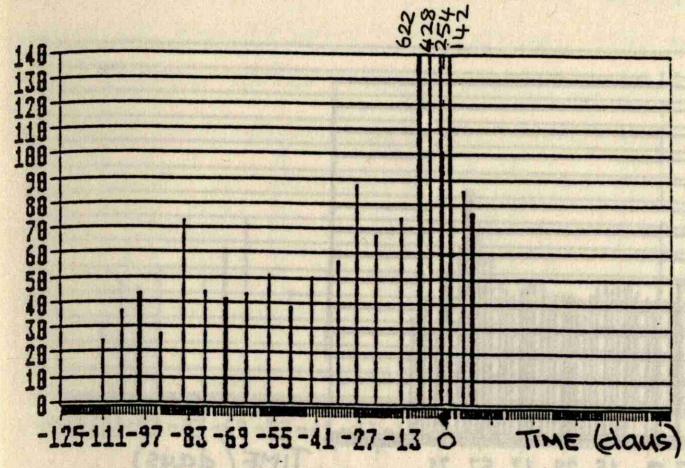


Fig 4-150 Uninoculated dog 43's ALT levels (IU/1)

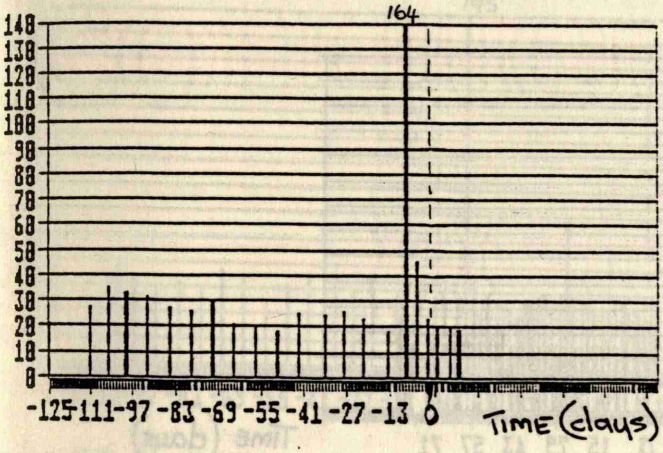


Fig 4-151 Uninoculated dog 43's AST levels (IU/1)

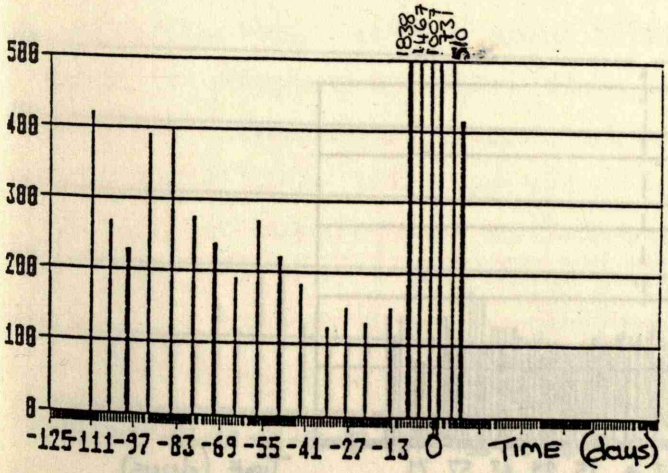


Fig 4-152 Uninoculated dog 43's alk. phos. levels (IU/1)

fp223

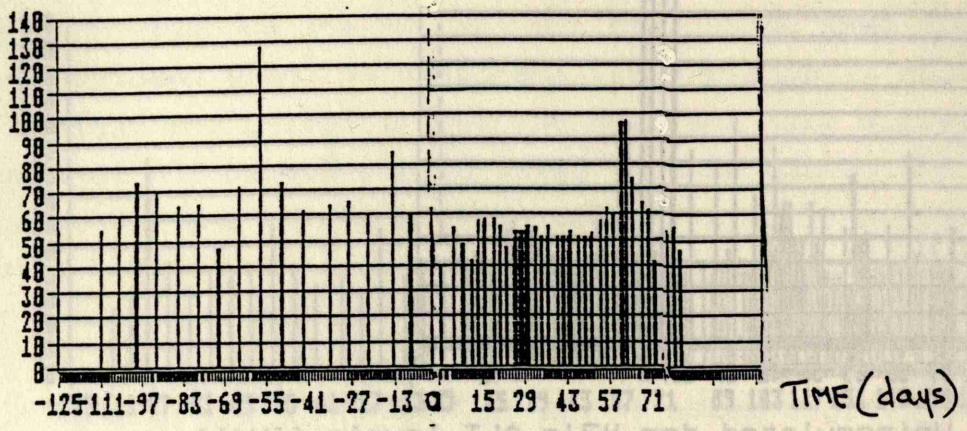


Fig 4-153 Dog 44's ALT levels (IU/l)

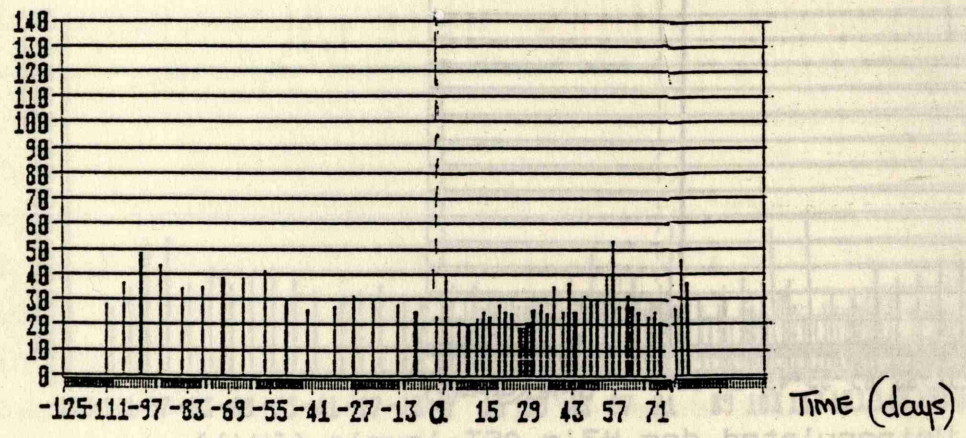


Fig 4-154 Dog 44's AST levels (IU/l)

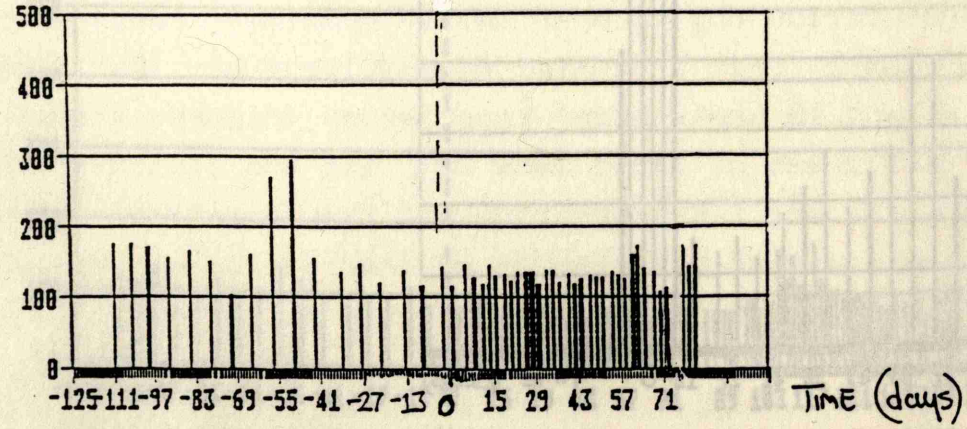


Fig 4-155 Dog 44's alkaline phosphatase levels (IU/l)

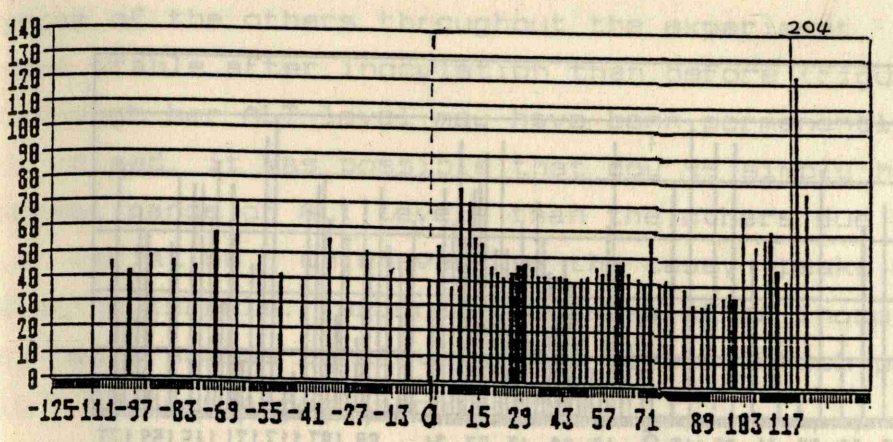


Fig 4-156 Dog 45's ALT levels (IU/l)

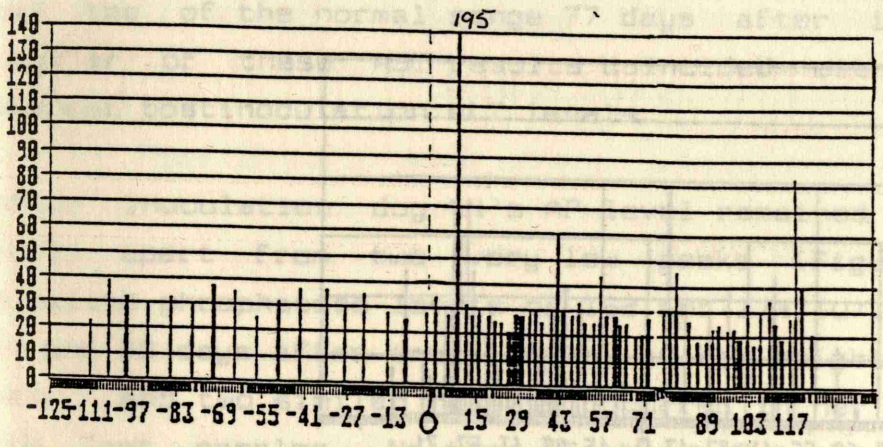


Fig 4-157 Dog 45's AST levels (IU/l)

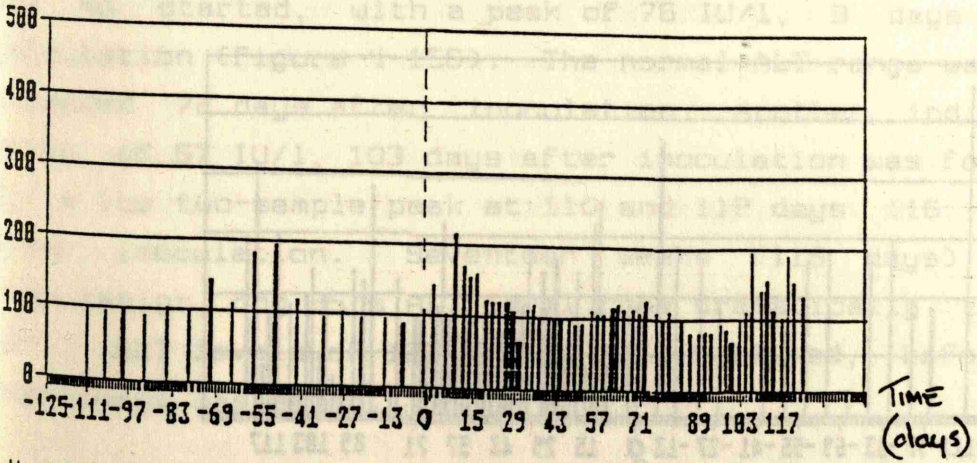


Fig 4-158 Dog 45's alkaline phosphatase levels (IU/l)

fp224

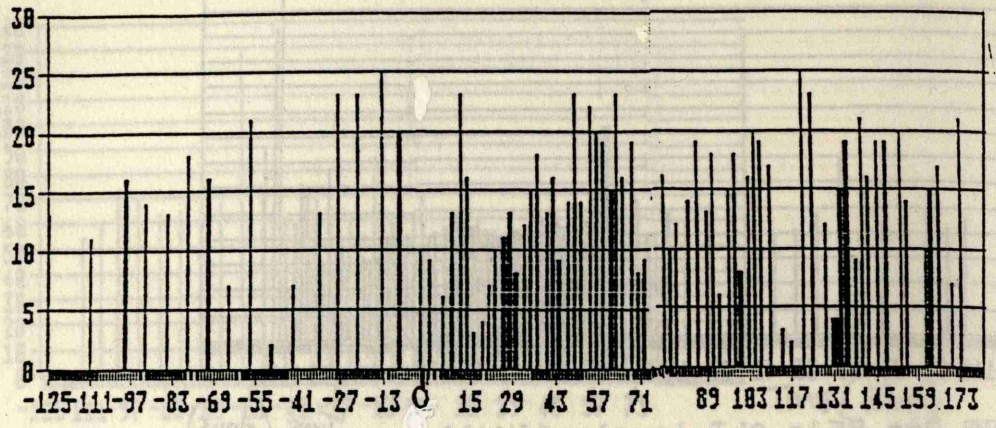


Fig 4-159 Dog 42's GGT levels (IU/l)

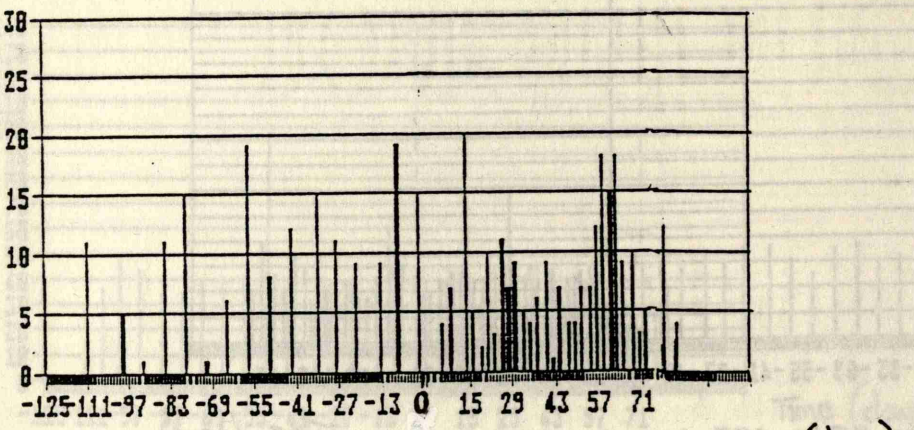


Fig 4-161 Dog 44's GGT levels (IU/l)

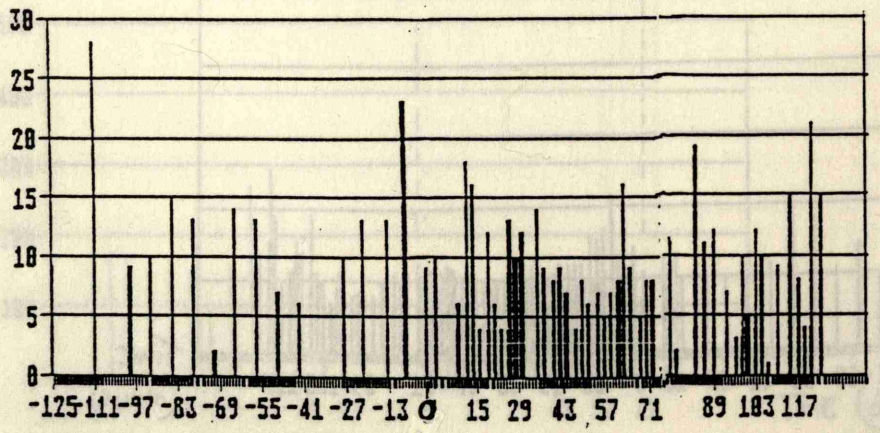


Fig 4-162 Dog 45's GGT levels (IU/l)

Dog 44's ALT level generally tended to be higher than those of the others throughout the experiment, but it was more stable after inoculation than before (figure 4-153). Although her ALT level may have been permanently abnormally raised, it was possible that dog 44 simply had a higher normal range of ALT levels than the others due to individual variation. Whichever was the case, peaks were still distinguishable. Around nine weeks after inoculation dog 44 had a series of elevated ALT levels, which peaked at 97 IU/l 63 days after inoculation.

Dog 44's had only one postinoculation AST peak which exceeded the normal range: a result of 53 IU/l, 56 days after inoculation (figure 4-154). Her AST level reached the top of the normal range 77 days after inoculation. Neither of these AST results coincided with dog 44's highest postinoculation ALT levels.

After inoculation dog 44's AP level remained below 150 IU/l, apart from two very low peaks (figure 4-155). Alkaline phosphatase levels of 162 and 174 IU/l were found 61 and 63 days after inoculation, at the height of the ALT peak, and two similar results occurred in her third last and last samples. Neither of these AP peaks were high enough to be considered as definitely abnormal.

The first postinoculation bout of elevated ALT levels in dog 45 started, with a peak of 76 IU/l, 9 days after inoculation (figure 4-156). The normal ALT range was just exceeded 72 days after inoculation. Another individual spike, of 67 IU/l, 103 days after inoculation was followed by a low two-sample peak at 110 and 112 days (16 weeks) after inoculation. Seventeen weeks (119 days) after inoculation dog 45's ALT level rose dramatically to 204 IU/l. ALT levels of 122 and 76 IU/l followed, before the experiment was ended.

In dog 45 a dramatic AST spike of 195 IU/l occurred 9 days

fp225

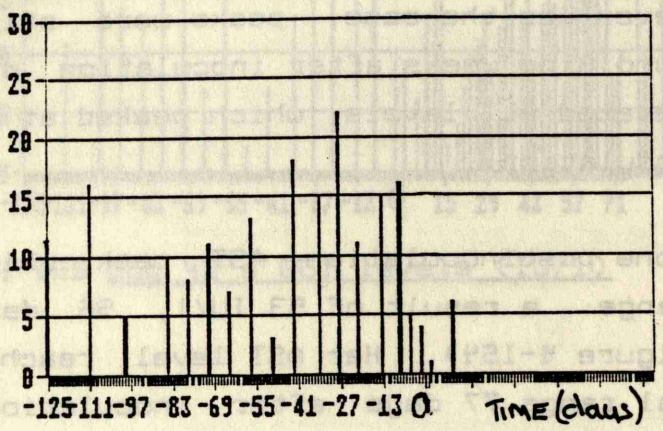


Fig 4-160 Dog 43's GGT levels (IU/l)

after inoculation (figure 4-157), along with the ALT peak. The normal AST level was exceeded by only the first of the next three spikes: a result of 60 IU/l, found 42 days after inoculation with a normal ALT level. Dog 45's AST level rose to 82 IU/l, in the sample with her highest ALT level, 119 days (17 weeks) after inoculation.

The highest AP peak in dog 45 (215 IU/l) started, 9 days after inoculation, in the sample with the highest AST level and an elevated ALT level (figure 4-158). Dog 45's AP levels then remained unremarkable until two small peaks 107 and 119 days after inoculation, the latter coincided with the highest ALT peak and an elevated AST level.

The GGT levels of all the dogs were usually less than 15 IU/l, only occasionally over 20 IU/l and never greater than 25 IU/l. Of the four, dog 42 had the most results greater than 15 IU/l. None of them showed more abnormalities after than before inoculation and the majority of high results did not coincide with raised ALT levels.

### 4.5.6 Haematological Results

The dogs used in this experiment were older on arrival than those used in the two previous experiments; their rbc parameters therefore tended to be in the adult range (figures 4-163,164,166,167,169,170,172 & 173), higher than the values found in the pups at the start of experiments 1 and 2. In fact the experiment 3 dogs' rbc haematology results were all normal apart from low values for all three parameters in one of dog 42's preinoculation samples (figures 4-163 and 4-164) and low haematocrits in dog 45's last two blood samples (figure 4-173).

The dogs' leucocytology results appeared a little abnormal initially and tended to improve after a couple of months (figures 4-165,168,171 & 174). The irregularities may have been a reaction to the change in environment and their

exposure to new things, or it may have been due more to the various parasitic infestations they brought with them (see section 4.5.3).

Dog 42 had an elevated wbc count in three of the early blood samples (18, 15 and 11 weeks before inoculation), slightly increased neutrophil numbers in four and slightly increased lymphocyte numbers in six preinoculation samples (figure 4-165). By 57 days before inoculation dog 42's total wbc and neutrophil numbers had settled down to well within the normal ranges, where they remained for the duration of the experiment. Lymphocyte numbers rose to just above normal levels in the first sample after inoculation and were slighty raised 40 and 47 days after inoculation.

Abnormal leucocytology results were seen in dog 43 for much of her stay (figure 4-168). Her total wbc counts were high in all but four samples and her neutrophil numbers were high in all but three. There was a particularly dramatic peak in both total wbc and neutrophil numbers (39.2 $x10^9$/l & 29.79 $x10^9$/l respectively) 13 weeks before inoculation. Lymphocytes numbers were above the normal adult range in two thirds of dog 43's samples. Most of these abnormal findings occurred before the episode of abdominal pain and no explanation for them was identified.

Two of dog 44's early wbc counts, 15 and 13 weeks before inoculation, were just above the normal range (figure 4-171). The highest wbc count (20.6 $x10^9$/l) occurred during oestrus, 6 weeks before inoculation, at the time of the vaginal hypertrophy problem (section 4.5.3). Before inoculation dog 44's wbc count had fallen to well within the normal range and it remained there for the rest of the experiment. Neutrophil numbers were often a little elevated until after her season. They then remained within the normal range until a peak in dog 44's last sample, collected the day after the submandibular mass appeared

(section 4.5.3). Dog 44's lymphocyte numbers tended to be in the upper part of the normal range, occasionally just above it, until about 2 months before inoculation; they then remained within the normal range for the rest of the experiment.

Apart from two wbc counts just above the normal level, due to abnormal peaks in neutrophil numbers 15 and 9 weeks before inoculation, dog 45's total wbc and neutrophil results were all normal (figure 4-174). There were raised numbers of lymphocytes in several of her early blood samples; levels then remained normal, apart from one sample, until inoculation. After inoculation dog 45's lymphocyte numbers rose and fell repeatedly, forming six low peaks which just exceeded the normal range.

fp228

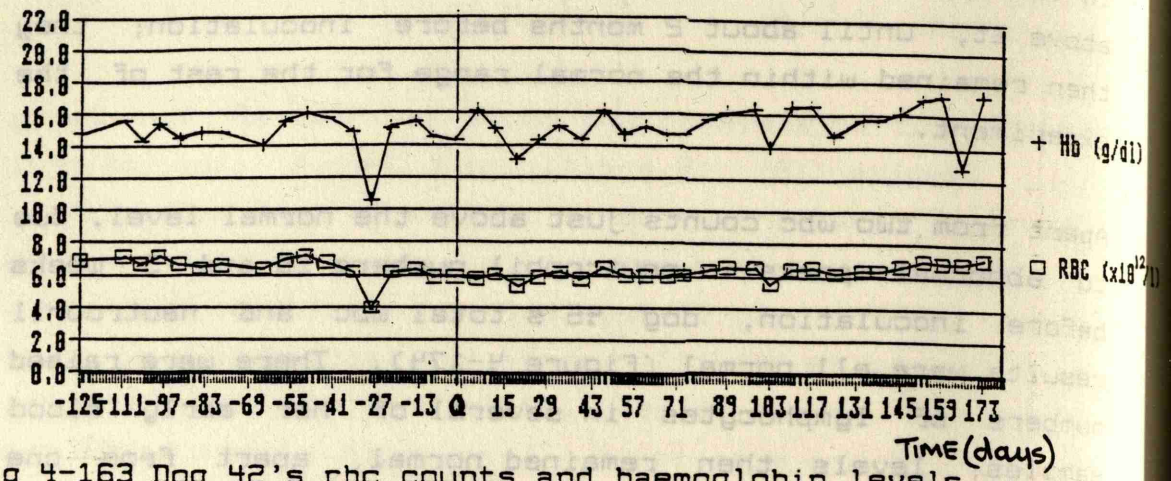


Fig 4-163 Dog 42's rbc counts and haemoglobin levels

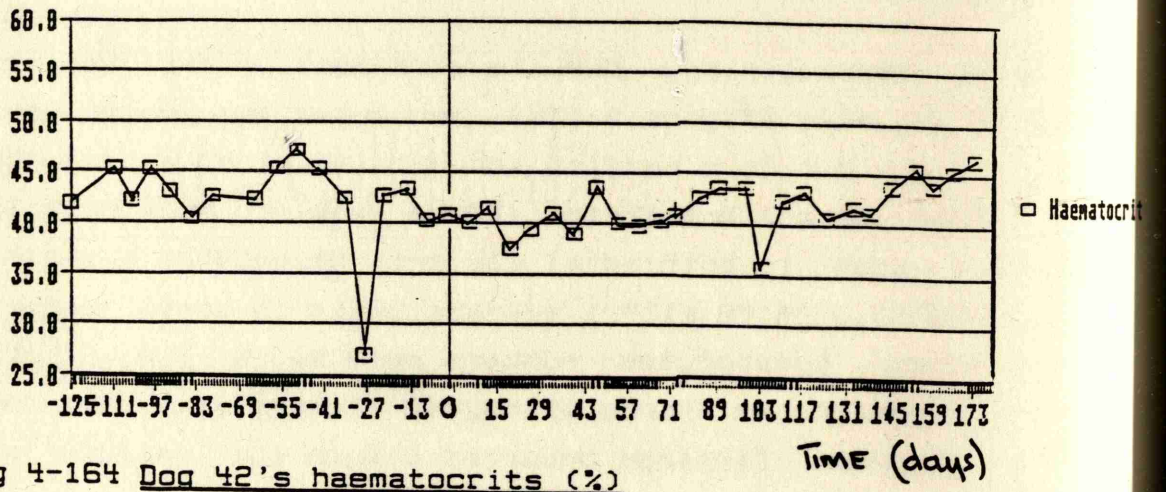


Fig 4-164 Dog 42's haematocrits (%)

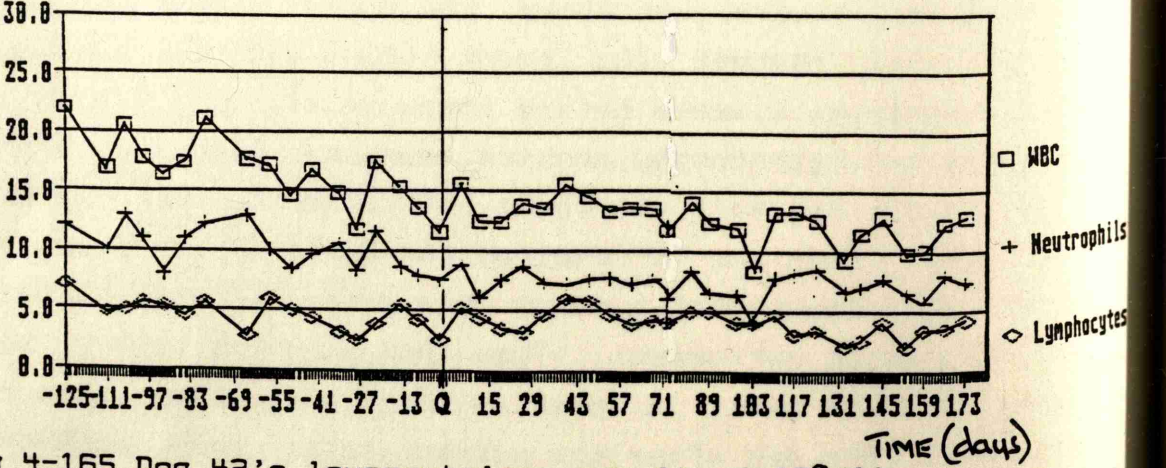


Fig 4-165 Dog 42's leucocytology results (x10⁹/l)

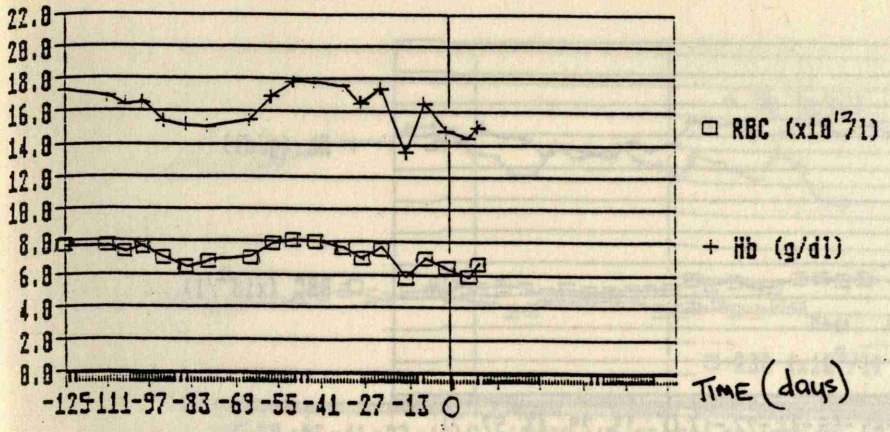


Fig 4-166 Uninoculated dog 43's rbc counts and Hb levels

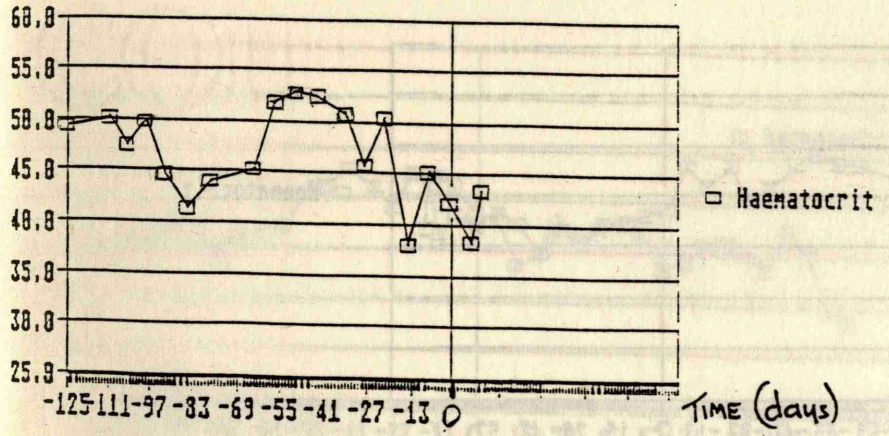


Fig 4-167 Uninoculated dog 43's haematocrits (%)

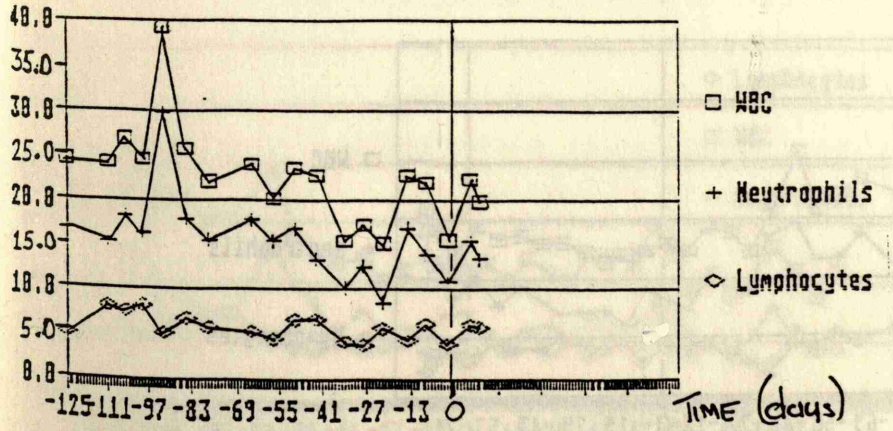


Fig 4-168 Dog 43's leucocytology results ($x10^9/l$)

fp229

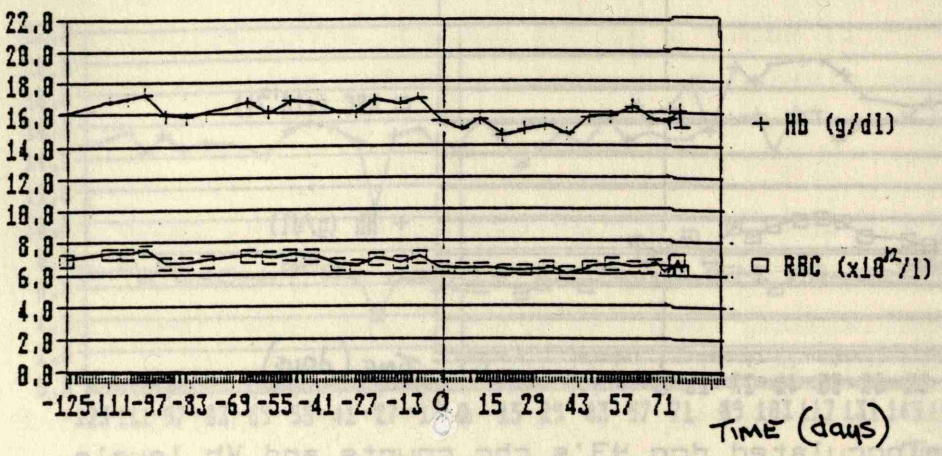


Fig 4-169 Dog 44's rbc counts and haemoglobin levels

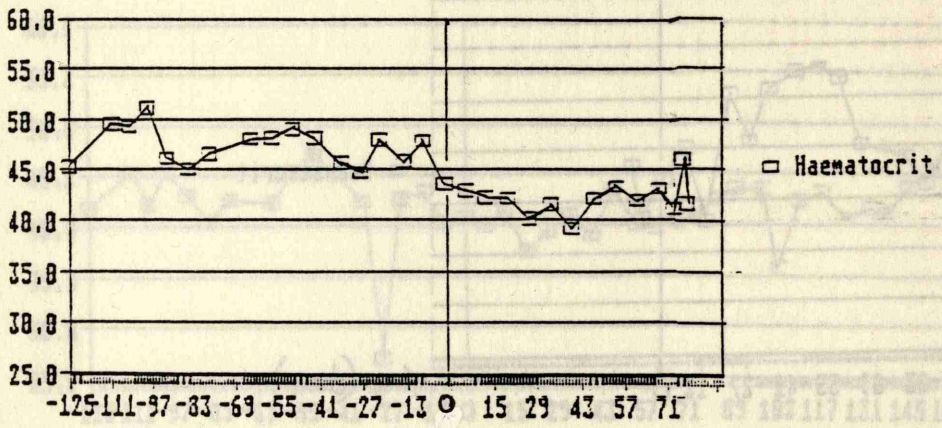


Fig 4-170 Dog 44's haematocrits (%)

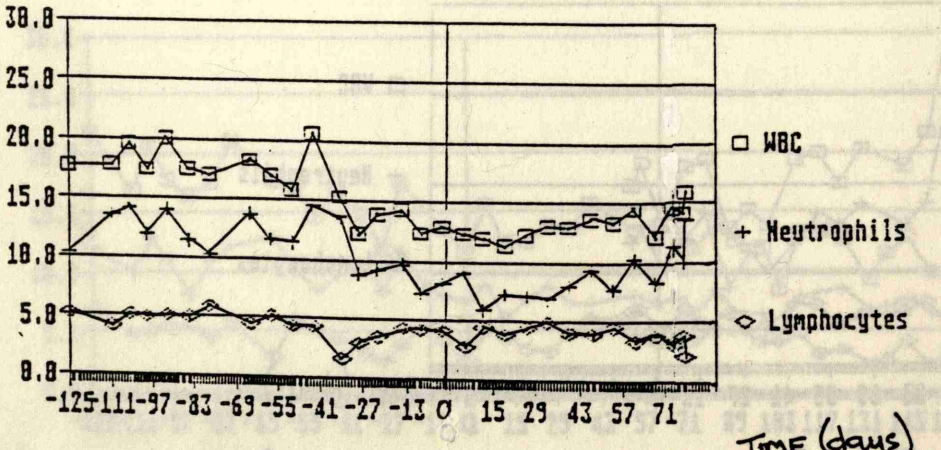


Fig 4-171 Dog 44's leucocytology results (x10$^9$/l)

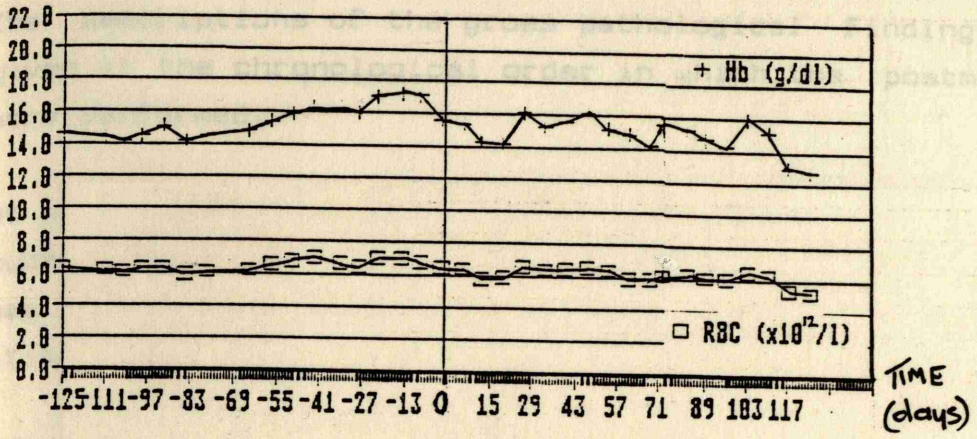


Fig 4-172 Dog 45's rbc counts and haemoglobin levels

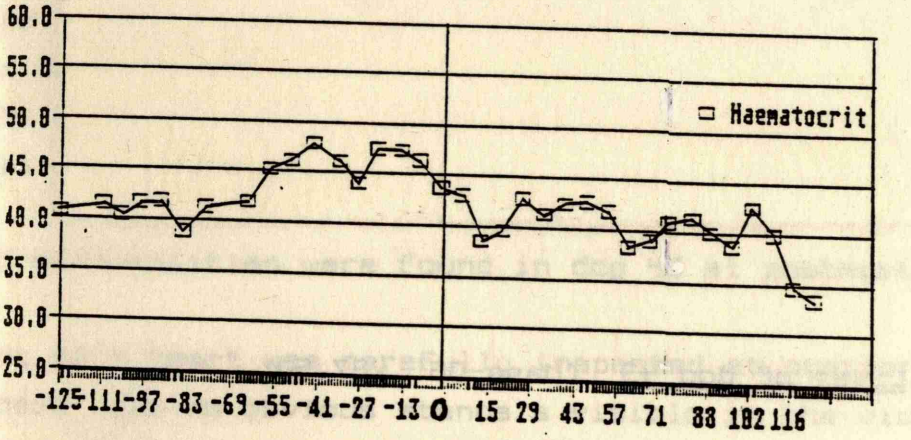


Fig 4-173 Dog 45's haematocrits (%)

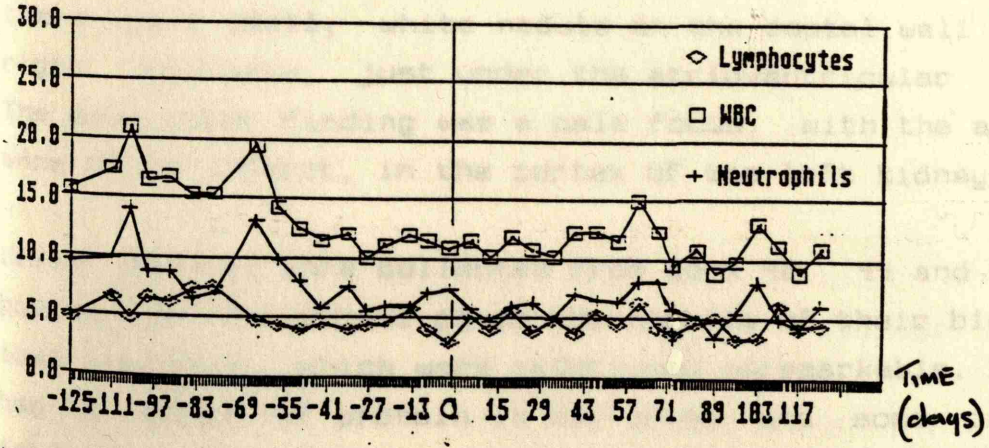


Fig 4-174 Dog 45's leucocytology results (x10⁹/l)

fp230

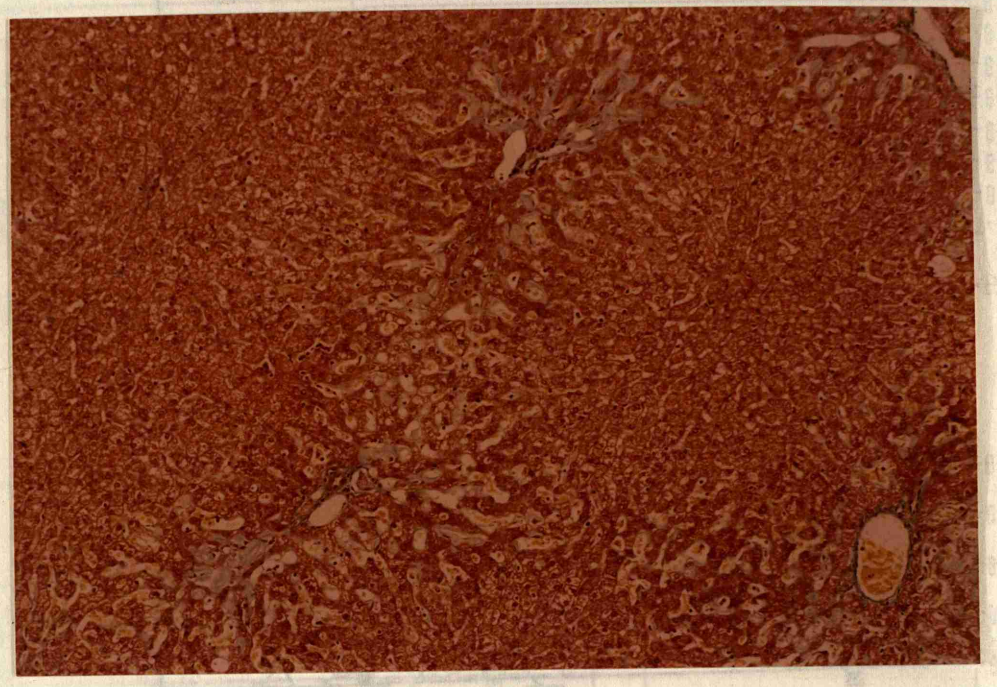

Fig 4-175 Liver of dog 43 (Mass-OG x87.5)

### 4.5.7 Postmortem Findings

The descriptions of the gross pathological findings are given in the chronological order in which the postmortems were performed.

At postmortem there was a slight reddening on part of the outer surface of dog 43's stomach. An incidental finding was a small erosion on the soft palate. No other abnormalities could be found.

When dog 44 was postmortemed no wounds could be found in the skin over the submandibular mass. The fleshy mass, about 3cm in diameter, was neither cystic nor very firm and it did not appear to be encapsulated. On dissection, the submandibular lymph nodes of that side were visible amidst pinkish tissue which constituted the majority of the mass. The gross appearance of the reproductive tract was normal.

No abnormalities were found in dog 45 at postmortem.

Dog 42's heart was carefully inspected at postmortem, but there was no obvious stenosis visible in the vicinity of the pulmonic valve. There was only a small focus of calcification on the luminal surface of the wall of the first branch of the pulmonic vessel. Two small, white foci were seen on the inside of the right ventricle wall and there was a small, white nodule on the septal wall of the right ventricle, just under the atrioventricular valve. The only other finding was a pale focus, with the appearance of an infarct, in the cortex of the left kidney.

Urine samples were collected from dogs 42, 44 and 45 at postmortem. Table 4-11 gives the results of their biochemical analysis, which were relatively unremarkable. Dog 42 had a trace of protein in her urine and some crystal formation of unknown significance. Dog 44's urine was

fpz31

Fig 4-176 Liver of dog 44 (Mass-OG x35) ACs in limiting plates of many small PIs & many around large PI

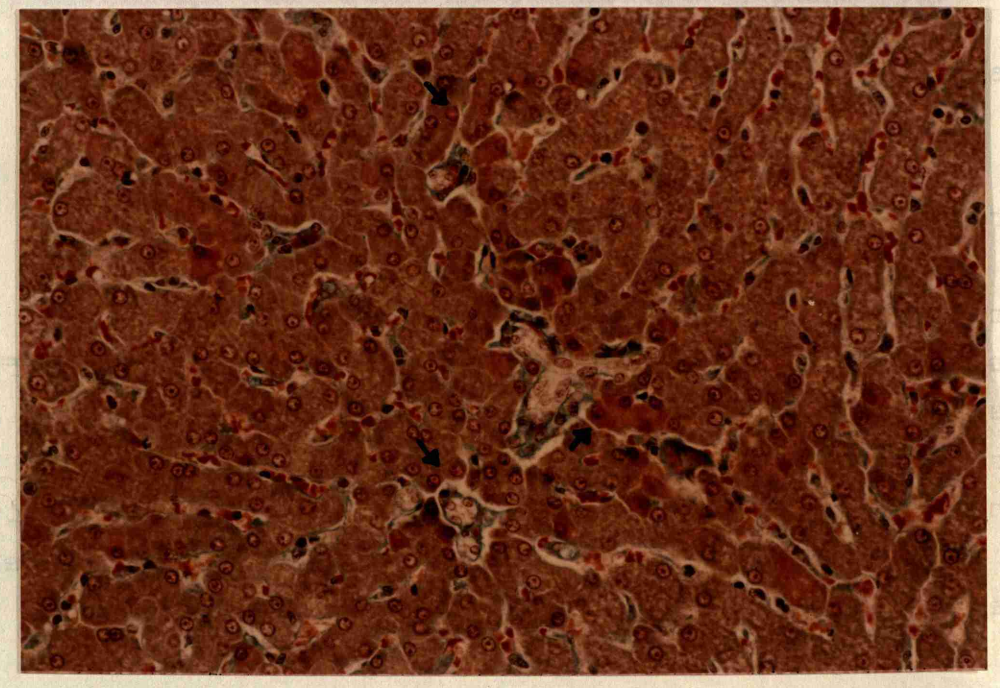

Fig 4-177 Liver of dog 44 (Mass tri x280) several intranuclear inclusion bodies (↗)

slightly alkaline.

| Dog No | Protein mg/100ml | Urea mmol/l | pH | Specific Gravity |
|---|---|---|---|---|
| 42 | 3 | 992 | 7.0 | 1.036 |
| 44 | 0 | 850 | 7.5 | 1.022 |
| 45 | 0 | 250 | 6.5 | 1.020 |

Table 4-11 Dog experiment 3 urine biochemistry results

4.5.8 Histological Results

The descriptions, of the histological findings in the dogs of this experiment, are given in the chronological order of the postmortems. The dogs' acidophil cell scores are given in table 4-12.

Microscopical examination of dog 43's liver revealed virtually no acidophil cells. There did appear to be a degree of atrophy of the hepatic cords radiating out from some portal tracts (figure 4-175). The hepatocytes in these had less cytoplasm than normal, causing the cords to appear thinner. In these areas there was an increase in the amount of fine fibrous strands lining the sinusoids. The pattern of fibrosis was different from that seen in some of the CACH cases, in which fibrous strands extend out from the portal tracts, disrupting the limiting plates of hepatocytes. There was a minimal degree of portal tract infiltration, with a very few mixed cells in some portal tracts. Occasional acidophilic, intranuclear inclusion bodies were seen in the liver, but much fewer than in the other three dogs. The reactive tonsils were packed with big lymphoid follicles with very large germinal centres. The only finding in the gastrointestinal tract was plasma cell infiltration in the tips of the duodenal villi. In the soft palate lesion there was a loss of epithelium with bits of necrotic material and a heavy neutrophil infiltra-

fp232

Fig 4-178 Liver of dog 45 (Mass tri x175) Hepatocyte (↘) with IN inclusion body being encorporated into fibrous tissue, at PT with ACs in limiting plate


Fig 4-179 Liver of dog 45 (Mass tri x87.5) small areas of fibrosis (F) & fine processess extending out from PTs

tion filling the gap. There was a little granulation tissue and a scattered infiltration of neutrophils in the dermis.

Dog 44 exhibited canine acidophil cell hepatitis with lots of acidophil cells, arranged in varying numbers, around many of the portal tracts in its liver (figure 4-176). Acidophilic, crystalline, intranuclear inclusion bodies were also numerous (figure 4-177). Little fibrotic foci were seen in some of the liver sections. The mass of reactive tissue surrounding the submandibular lymph nodes consisted of very loose connective tissue and loose early granulation tissue with lots of little capillaries. Neutrophils were scattered throughout this and in some areas formed large, dense collections. There were also areas of congestion and haemorrhage. The absence of a detectable fibrous capsule, fitted in with the sudden development of the lesion. One of the kidney sections had a small lymphoid follicle in the cortex and a very few tiny foci of plasma cells between tubules in the medulla. A section of vaginal wall had a normal histological appearance.

A positive acidophil cell reaction was also found in dog 45's liver, with plenty of acidophil cells in small numbers around some portal tracts (figure 4-178). There was a very slight increase in fibrous tissue at some portal tracts, with a few slim fibrous processes extending out between hepatocytes. This and a little area of fibrosis are demonstrated in figure 4-179. Intranuclear, acidophilic inclusion bodies were also a feature of this case. This dog's tonsils were quite reactive; packed with big follicles with very large germinal centres.

All but one of dog 42's liver sections contained some acidophil cells, mainly in ones and twos at portal tracts, but not sufficient to be termed a definite positive acidophil cell reaction. Although the other (one of seven

fpz33


Fig 4-180 Liver of dog 42 (H&E x87.5)
ACs at portal tract and at fibrous area (F)


Fig 4-181 Liver of dog 42 (Mass tri x140) (↗) Piecemeal necrosis, hepatocytes being encorporated into tiny PT and at fibrous area

liver sections examined) contained many more acidophil cells, the classification of dog 42's liver histology was taken from the majority of less-affected sections. Figure 4-180 shows acidophil cells at a portal tract as well as some at a small fibrosed area. Liver fibrosis was an obvious feature of this case. Individual and small groups of hepatocytes were enclosed in fibrous tissue at the edges of portal tracts and fibrous processes extended out from many portal tracts (figure 4-181). In some liver sections this had progressed to early bridging fibrosis, especially near lobe edges. There were a few small fibrotic areas containing virtually no hepatocytes, only several small bile ducts. Intranuclear inclusion bodies were also seen in dog 42's liver. Microscopy revealed that the nodule under the right atrioventricular valve was a small granuloma, surrounded by lymphocytes, neutrophils and plasma cells, interposed between the cardiac muscle and the endocardium. Despite attempts to include the little white foci in the areas of cardiac muscle sampled, no evidence of them was found in the resultant histological sections. The lesion in the kidney cortex, which had looked grossly like an infarct, was found histologically to be a small granuloma surrounded by mainly lymphoid and plasma cells. A section from the other kidney contained a similar sized focus of lymphoid cells and a few neutrophils in the cortex. There were also scattered tiny groups of lymphoid cells elsewhere in the cortex and a small focus at the top of a calyx.

4.5.9 Conclusion

Canine acidophil cell hepatitis was produced in at least two of the three dogs inoculated with rat material (dogs 44 and 45). Even after the infection had been passaged once in rats, it was still capable of causing hepatitis in dogs.

Dog 44, killed 2½ months (11 weeks) after inoculation with

| Dog No. | Inoculum used | Time post inoculat. wks | Time post inoculat. mths | Age mths | AC score | Result |
|---|---|---|---|---|---|---|
| 44 | clarified, pooled rat liver prep. | 11 | 2½ | 13 | ++++ | + |
| 45 | clarified, pooled rat liver prep. | 18 | 4 | 14½ | +++ | + |
| 42 | clarified, pooled rat liver prep. | 25 | 5½ | 16 | ++ | — |
| 43 | none | n\a | | 10½ | - | — |

Table 4-12 Dog experiment 3 liver histology results - acidophil cell scores

Liver lesions were graded on a scale of - to ++++++. A positive acidophil cell reaction was taken as +++ or more. The acidophil cell scoring system is explained in appendix 2.

the rat liver preparation, had plenty of the acidophilic hepatocyte lesions but no noticeable fibrous reaction. Four months (18 weeks) after inoculation, with the rat material, dog 45 also exhibited CACH. She had slightly fewer acidophilic hepatocytes than dog 44, but a slight fibrous reaction was evident in some of her portal tracts. Dog 42, killed 25 weeks (about 5½ months) after inoculation, had some acidophil cells in her liver but not enough to be considered a definite positive reaction; she did however have hepatic fibrosis. There was a trend, in the three dogs, of increasing fibrosis and reducing numbers of acidophil cells with increasing length of time between inoculation and postmortem. It may have been that dog 42 had more obvious CACH earlier, but fibrous replacement followed the death of the necrobiotic cells, so that by the time of the postmortem the histological picture was that of a more chronic lesion. In the absence of methods of identifying cases of CACH other than the presence of the characteristic pattern of acidophilic, necrobiotic hepatocytes, this hypothesis cannot by proven. It may be that some of the cirrhotic cases of unknown aetiology seen in the field are a result of earlier, possibly subclinical, CACH.

Dog 43's clinical signs, the abnormal appearance of the parenchyma around portal tracts, the relative absence of cell infiltration in the liver and the very high levels of serum enzymes were all suggestive of a possible toxic damage effect, related to gastrointestinal stasis during the episode of ileus. However, there were also some raised ALT levels before the incident and a lot of abnormal leucocytology results.

The submandibular mass around dog 44's lymph node appeared to be the result of a localised bacterial infection. The dramatic speed of development was supported by the histological evidence and by the late onset of the temperature rise and increase in neutrophil numbers. Although no

external lesion was found, the reaction could have resulted from a fine puncture wound, perhaps by a tooth.

The preinoculation ALT levels and leucocytology results of the dogs in this experiment were more irregular than those of most of the other experimental dogs. The beagles were older and had more incidental problems than the dogs used in earlier experiments; they appeared to have been exposed to more undesirable factors. They were less satisfactory as experimental animals than the freshly-weaned pups of experiment 2.

The postinoculation ALT peaks, seen in all three of the inoculated dogs, were usually more pronounced than most of the raised ALT levels occasionally seen before inoculation. Some of the elevated postinoculation ALT levels coincided in dogs 42 and 45, as can be seen in fig. 4-141. They also occurred about the same length of time after inoculation as some of the elevated ALT levels in dogs 36 to 38 of experiment 1. A few raised AST levels were seen after inoculation. Most, but not all, of them coincided with elevated ALT levels and a few ALT peaks occurred when AST levels were normal. There were even fewer peaks in alkaline phosphatase levels, but most of the major postinoculation ALT peaks were accompanied by low peaks in AP levels.

None of the temperature peaks, seen after inoculation, coincided with ALT peaks; but two of dog 42's and one of dog 44's occurred a few days before ALT peaks.

The dogs' rbc haematology results were normal for most of the experiment. Their leucocytology results were generally more normal after inoculation than before. Some of the low peaks, seen after inoculation, coincided with elevated ALT levels and others did not. No obvious specific relationship was identified.

# CHAPTER 5 RAT TRANSMISSION EXPERIMENTS

## 5.0 INTRODUCTION

The experimental transmission of canine acidophil cell hepatitis to rats was investigated in six rat experiments, which are described in chronological order in this chapter.

The first experiment attempted the transmission of CACH to young suckling rats from a field case with chronic active disease (section 5.3). A group of eleven, from two litters, were given an inoculum made from frozen liver. A control group of nine, from two litters, were not given anything. This 10-week study commenced at the same time as the first mouse and guinea pig experiments (sections 6.2 and 6.3).

More rats were used in the second experiment to permit a longer study (section 5.4). It involved four groups of young weaned rats. Sixteen were inoculated with liver from a chronic CACH field case with cirrhosis. Another group of thirteen were inoculated with serum from the same dog, to investigate whether the disease was also transmissible by serum. A blank control group of eight were inoculated with some of the sterile phosphate-buffered saline which was used in the liver preparation. A nil control group of eight were not given anything.

The third rat experiment used the dams of the experiment 1 litters and later litters from two of these dams, born during the course of experiment 1, to investigate whether there was any evidence of horizontal transmission during normal contact (section 5.5). The two dams of the experiment 1 control litters were used as controls for the two in-contact dams of the inoculated litters of experiment 1. The next litter from one of the control dams was used as a control for the next litter from one of the in-contact dams, who gave birth while some of her previous litter of inoculated rats were still living in her cage.

Rat experiment 4 was a two-part investigation (section 5.6). It was designed to attempt rat to rat transmission of CACH (a second experimental passage) and to show that the hepatitis produced in rat experiments 1 and 2 was not a reaction to any normal component of dog liver. This experiment therefore also has important implications for the dog transmission experiments (chapter 4). If the hepatitis produced in rats, given material from CACH-infected dogs, can be proved not to be a reaction to any normal liver component then it strengthens the theory that the hepatitis produced in experimentally-infected dogs is infectious rather than autoimmune in nature. The two parts of the experiment were only performed at the same time to reduce the number of control rats required. For the rat to rat transmission, frozen liver from two experimental rats was used to inoculate two groups of seven young rats each. To investigate the effects of inoculation with normal dog liver, fourteen rats were given a liver preparation from an uninoculated experimental control dog. A blank control group (seven rats given PBS) and a nil control group (seven rats not given anything) were used as controls for both the rat-inoculated and normal dog-inoculated groups. The collection of rat blood samples for biochemical analysis began in this experiment.

The fifth rat experiment (section 5.7) attempted the transmission of CACH from an experimentally-infected dog to thirty-four neonatal rats. The aim of this second experimental passage was to produce infected rat liver for use as a fresh inoculum, in dog experiment 3 (section 4.5), for the transmission back to dogs of CACH already passaged in rats. At the time of rat experiment 5 there were no other rats of the same strain available for use as controls, but age-matched neonates of the same strain were investigated later, in rat experiment 6.

Rat experiment 6 (section 5.8) investigated the possibility of horizontal transmission of CACH to rats which had

been in contact with the inoculated rats of experiment 5 or their dams, since such animals could not be returned to the general breeding stock. This experiment also served to produce the only available approximation to age-matched controls for the inoculated rats of experiment 5.

## 5.1 MATERIALS AND METHODS

Experimental rats were mainly bred in-house, as detailed in section 2.2.2, and were used as young as was possible. Each litter was caged with its dam, at least initially, since the baby rats were still suckling. This affected the numbers used in different experimental groups; different litter sizes often prevented exact matching of the numbers of controls and inoculates. The three strains of white rats used, depending on availability, were Wistar, Lou and Sprague Dawley.

All the inocula used were subjected to the bacteriological and virological examinations described in section 2.2.3. Inoculation was always by the subcutaneous route, in the scruff of the neck.

The blood samples collected, as described in section 2.2.4, for serum extraction were also subjected to biochemical analysis when volumes permitted.

The histological processing of postmortem tissue samples for both light and electron microscopy is described in sections 2.3 and 2.4.1 respectively. Mayer's haematoxylin and eosin stain was used on sections of all the tissues collected at postmortem. Masson's trichrome stain and usually also a Masson-orange G were used on at least one of the three (or four) liver sections taken from different lobes.

The process of cell death resulting in acidophilic, necro-

biotic hepatocytes is not confined to canine acidophil cell hepatitis cases. Occasional, scattered acidophilic hepatocytes may be seen in normal dog and laboratory animal liver. These cells can also occur in other liver diseases. It is their number and characteristic distribution in portal tract limiting plates, along with some other pathological features, that is considered pathognomonic for CACH (Jarrett and O'Neil, 1985).

## 5.2 RAT CLINICAL BIOCHEMISTRY

There are many variables affecting biochemical determinations in rats, including sex, age, strain and methods used. Meeks (1989) therefore recommended assessing results by comparing differences between control and test populations rather than between test populations and historical reference ranges.

Alanine aminotransferase is liver-specific in rats (Boyd, 1983) and increases with hepatocellular damage. Aspartate aminotransferase is widely distributed in rat tissues and is therefore of less diagnostic use. Alkaline phosphatase is also found in several rat tissues and elevated levels cannot be atributed to any one site, without measurement of isoenzymes. Male rats tend to have higher serum levels of AP than females, but sex and age are said not to affect ALT and AST levels (Meeks, 1989). Serum levels of GGT are only slightly increased in hepatocellular necrosis and its measurement is not of diagnostic use in the rat.

ALT levels ranging from 61-91 IU/l in one strain of rats and 19.8-39.5 IU/l in another strain were quoted by Kohn and Barthold (1984), with apparent variations with age and between sexes. They quoted AP levels of 40-95.9 IU/l in the latter strain and a range of 16-22 IU/l in a third strain. Other sources quoted mean ALT levels of 111-153 IU/l and mean AP levels of 133-405 IU/l in the last

strain, with variable differences between ages and sexes for both. Mean AST levels of 192-262 IU/l also appeared to vary between ages and sexes. Such variable results illustrate the difficulty in trying to define a range of normal levels for experimental rats. In these experiments levels in inoculated groups were compared to those in control groups of the same strain, as recommended by Meeks (1989).

## 5.3 RAT EXPERIMENT ONE

### 5.3.1 Experiment Design

Transmission of CACH to rats was attempted by inoculation with a liver preparation from a canine field case (dog A/FC1), whose clinical history and pathological details are given in sections 3.2.1 and 5.3.2 respectively.

Frozen liver from this dog's biopsy sample was thawed, diced and homogenised in PBS to form a 20% weight:volume suspension. Bacteriological checks proved negative and virological tests failed to find any adenovirus in the homogenate. Eleven young rats were each inoculated with 0.4ml of this preparation. They consisted of two litters; six Wistar rats (three males and three females) were 9 days old when inoculated, five Lou rats (three males and two females) were 8 days old when inoculated. Nine young rats, which were not inoculated, were used as controls and kept in a different room from the inoculated rats. These controls were also made up of two litters; four Wistar rats (two males and two females) were 10 days old at the start of the experiment, five Lou rats (two males and three females) were 8 days old at the start of the experiment (i.e. when the other group was inoculated).

During the short course of the experiment, the four litters were kept with their respective mothers. All the rats appeared clinically normal throughout. Unexpectedly

fp242

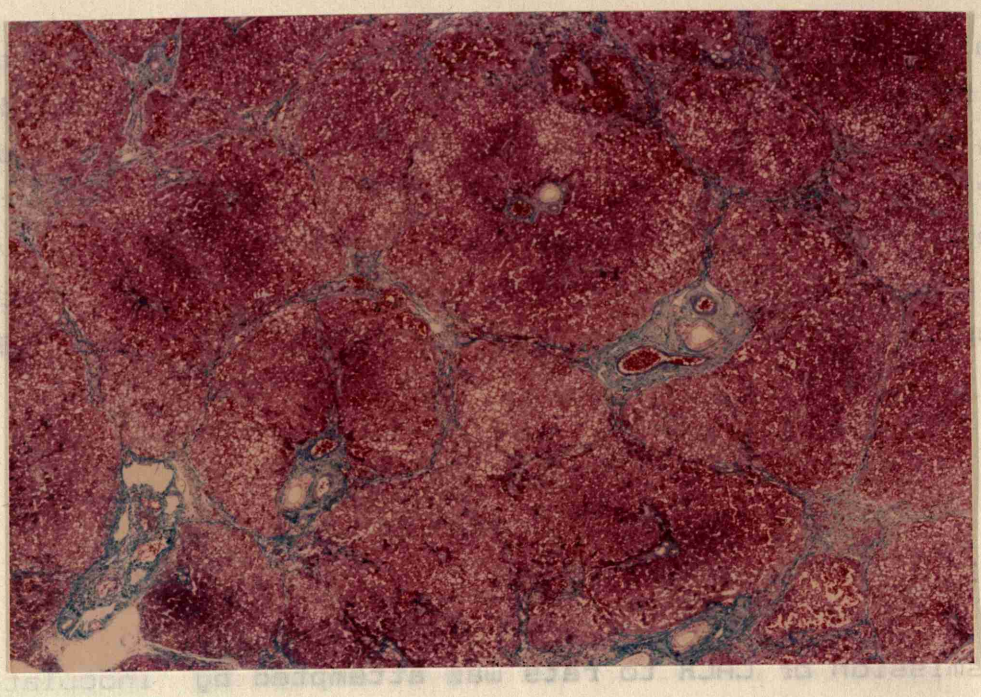

Fig 5-1 Liver of source dog A/FC1 (Mass acid fuch x35) acidophil cells lining many portal tracts and septa

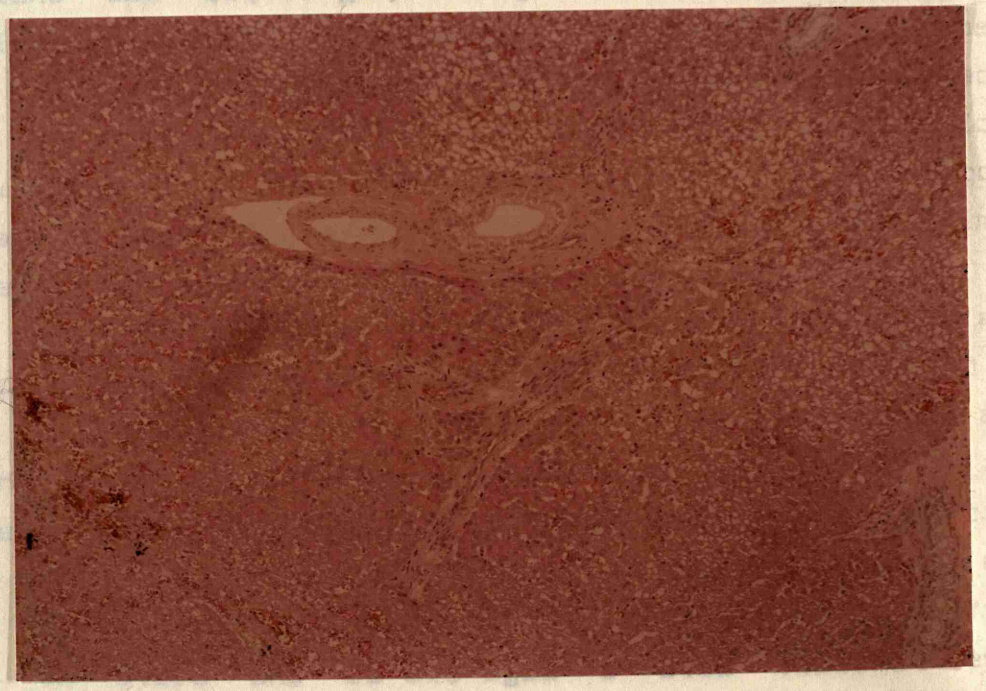

Fig 5-2 Liver of source dog A/FC1 (H&E x87.5) acidophil cells in limiting plates of PIs

the four dams each gave birth to another litter during the experiment. Three of these second litters, born 2½ weeks into the experiment, were healthy. Two of them were used in experiment 3 (section 5.5). The dam of the inoculated Wistars littered down 4 weeks into the experiment, but she rejected and neglected the neonates, which died within a few days.

Rats were culled weekly, using the postmortem protocol detailed in section 2.2.8, from 1 week after inoculation. The first two culls each consisted of one inoculated and one control rat. Three weeks after inoculation two inoculated rats and one control were culled. Two rats from each group were culled 4, 5 and 6 weeks into the experiment. The last of the inoculated rats was kept until 10 weeks after inoculation and had no control.

### 5.3.2 Source Case

The source of liver (dog A/FC1) was an 8-month-old, male German shepherd dog, biopsied after a 4-month illness. Full details of its clinical history are given in chapter 3 (section 3.2.1). It had exhibited vomiting, diarrhoea, dullness, weight loss, polydipsia, ascites, biochemical evidence of liver disease and an accessory portal circulation. Both liver and serum were collected and stored at -70°C.

Microscopy of the liver biopsy revealed chronic active hepatitis with fibrosis. The granularity of the gross appearance was due to complete dissection of the parenchyma, into nodules, by fibrous septa extending out from and linking portal tracts (see figure 5-1). Acidophilic, necrobiotic hepatocytes were apparent in the limiting plates of portal tracts, along fibrous septa and caught up in the latter (figures 5-1 and 5-2). There was much piecemeal necrosis, with individual and small groups of hepatocytes being incorporated into the fibrous bands. The

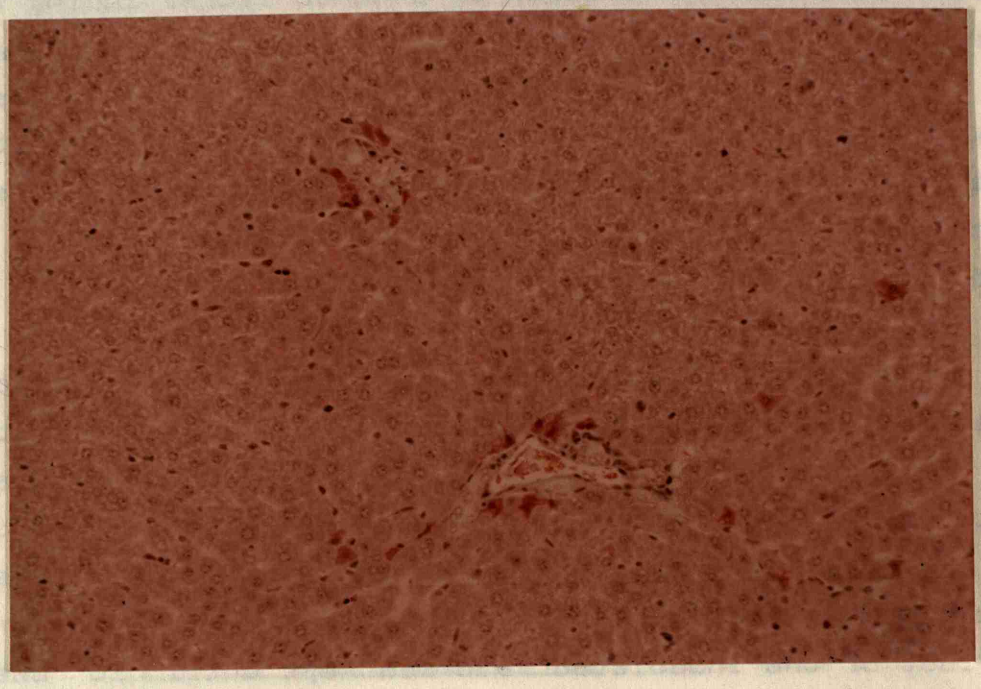

Fig 5-3 Liver of rat 19, 4 weeks post dog A's liver inoculum (Mass x175) ACs at small PIs

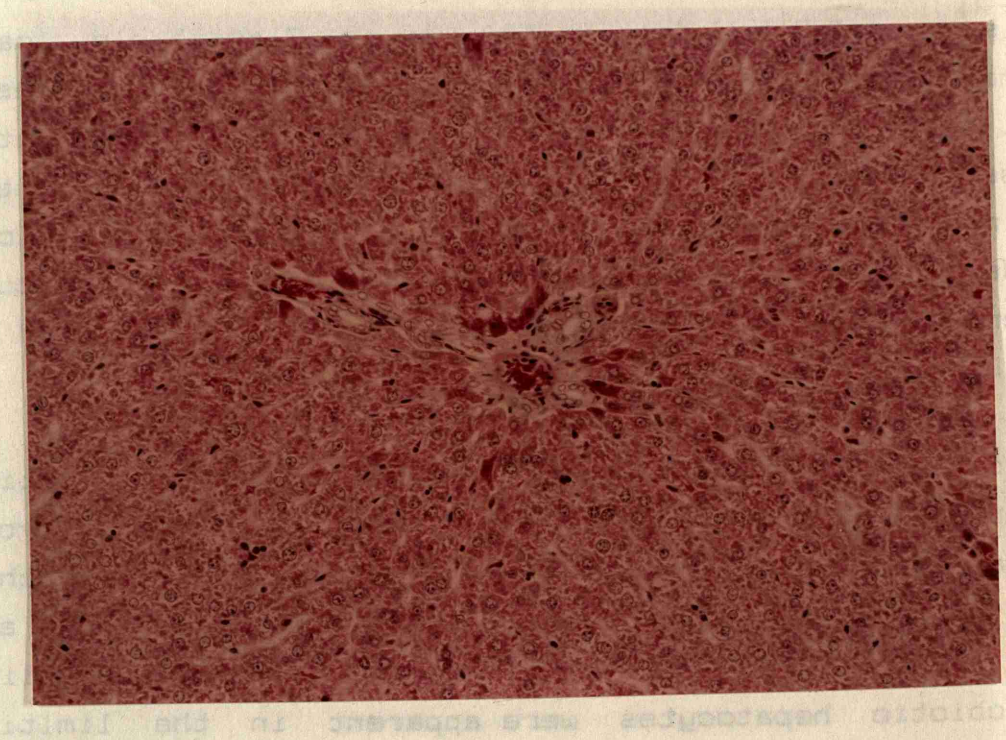

Fig 5-4 Liver of rat 23, 6 weeks post dog A's liver (Mass acid fuch x175) ACs at small PI

latter also contained many bile ducts and tiny vessels. Lymphatic dilatation was present in portal tracts and fibrous septa. Fatty vacuolation of many hepatocytes was also a feature. Masson's trichrome staining clearly demonstrated the increase in fibrous tissue in the thickened capsule and in virtually all of the portal tracts, many of which were linked by their radiating fibrous septa. Gordon and Sweet's reticulin stain showed complete disruption of the normal hepatic architecture by the fine septa and some much thicker ones. There was suprisingly little disruption of the microarchitecture in the small areas of parenchyma between the septa, with no indication of hypertrophic nodules. Victoria blue stain revealed that almost all the septa consisted of fine fibrous networks, interlaced around individual and small groups of hepatocytes, rather than dense fibrous tissue.

### 5.3.3 Results

Histological examination of the livers of the rats in this experiment revealed no differences between the inoculated and control groups at 1, 2 and 3 weeks after inoculation. Either no acidophil cells were detectable, or only one to a few were seen at one or two portal tracts. From 4 weeks after inoculation onwards, all but one of the inoculated rats' livers contained more acidophil cells than those of their time-matched controls. The odd one out was one of the 6-week cull (rat 22) which only surpassed one of its two controls, having a similar number of acidophil cells to its other control (rat 33). However, only one of each pair of inoculated rats killed from 4 weeks onwards exhibited sufficient numbers of characteristic acidophil cell lesions to be considered as definite positive acidophil cell reactions (rats 19,21 and 23). Figures 5-3 and 5-4 show typical examples of the acidophil cell lesions frequently seen in these three rats. Figure 5-5 shows one of the occasional portal tracts affected in rat 33, the control with the most acidophil cells in its liver.

fp244

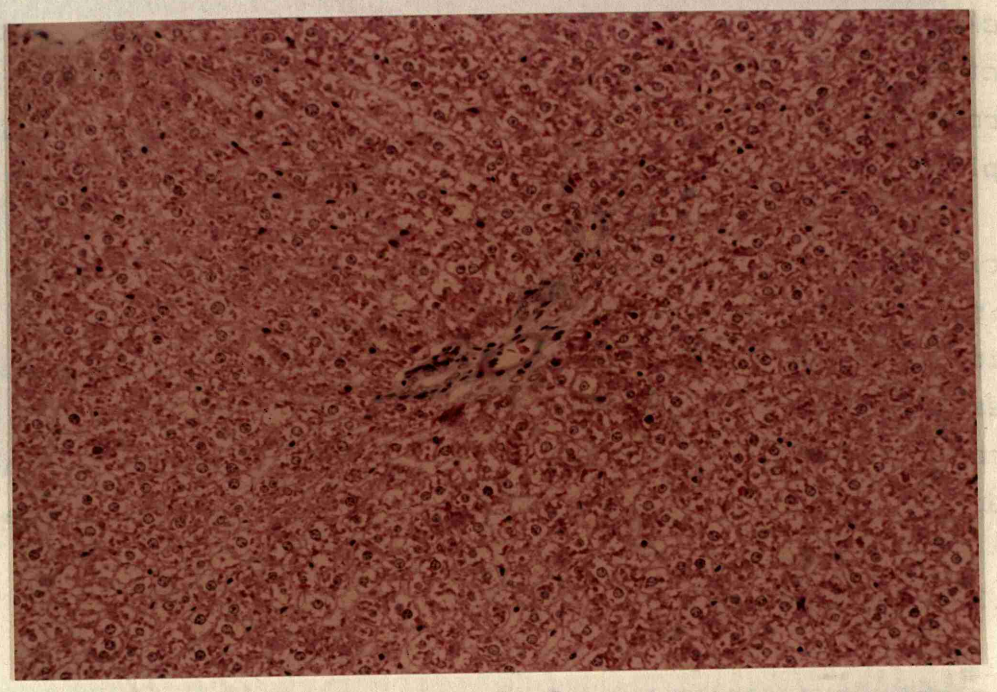

Fig 5-5 Liver of 6-week nil control rat 33 (Mass acid fuch x175) one of the few portal tracts with occasional ACs in the control with the most lesions

Although there was no time-matched control for the rat culled 10 weeks after inoculation, it had a distinct acidophil cell reaction in comparison to earlier controls. None of the control rats' livers contained more than a few acidophil cells. A possible tendency towards a very slight increase in the numbers of acidophil cells per liver section, with time, was probably simply a reflection of the increasing liver size in the young growing rats. The results of this experiment are tabulated in table 5-1.

Microscopy of lung sections, at least two sections per rat from different lobes, revealed evidence of chronic murine pneumonia. This is an insidious onset disease, with a slow protracted course, which can have a very high morbidity in a conventional breeding colony (Innes et al., 1967). The aetiology remained unknown for many years, but the causal relationship of Mycoplasma pulmonis has now been well established (Kohn and Kirk, 1969; Lindsey et al., 1971; Whittlestone et al., 1972) and the name murine respiratory mycoplasmosis (MRM) has become the accepted term for this disease. Although M. pulmonis is the primary aetiological agent, other viruses and bacteria can accompany the infection. MRM is by far the most common disease of laboratory rats (Holmes, 1984a). They are usually infected postpartum by direct contact with infected dams, although vertical transmission can occur when the genital tract is infected. This extremely contagious disease can also be transmitted sexually and by respiratory aerosol. There appears to be no significant age-related resistance to either infection or disease (Kohn and Barthold, 1984). Young animals tend to be only mildly affected and clinical signs are usually not evident until an advanced stage of disease is reached. Early lesions consist of peribronchial lymphoid hyperplasia, with bronchial tree involvement starting proximally and spreading distally with age. The lymphoid tissue can implicate all layers of a bronchus and can be of a massive kind with formation of primary (1°) follicles. As the disease progresses mucin and neutrophil exudation may be

| Weeks post inoculat. | LIVER INOCULATED | | | CONTROL | | |
|---|---|---|---|---|---|---|
| | Rat No. | AC score | Result | Rat No. | AC score | Result |
| 1 | 14 | - | — | 25 | - | — |
| 2 | 15 | - | — | 26 | - | — |
| 3 | 16 | - | — | 27 | - | — |
| | 17 | - | — | | | |
| 4 | 18 | + | — | 28 | - | — |
| | 19 | ++ | + | 29 | - | — |
| 5 | 20 | + | — | 30 | + | — |
| | 21 | ++ | + | 31 | - | — |
| 6 | 22 | + | — | 32 | - | — |
| | 23 | ++ | + | 33 | + | — |
| 10 | 24 | ++ | + | | | |

Table 5-1 Rat experiment 1 liver histology results

Liver lesions were graded on a scale of - to ++++, which cannot be compared directly to the scales used in other species. A positive acidophil cell reaction was taken as ++ or more. The acidophil cell scoring system is explained in appendix 2.

seen in the lumina of bronchi and bronchioles. Elimination of infection from large populations of infected rats is, for all practical purposes, impossible (Harkness & Wagner, 1989c) and treatment with antibiotics seldom influences the disease course of MRM in a colony situation.

Lung lesions in the rats in this experiment followed the recognised pattern. Lung sections from the first cull of rats, 1 week into the experiment (at 2½ weeks of age), contained only one or no foci of lymphoid cells at the larger bronchi. In general, the numbers of lymphoid foci increased with age and there was also some increase in the size of foci. Occasional large areas of lymphoid cells and even some complete cuffing of large bronchi were seen in the older rats, along with a little parenchymal involvement. The lesions in the inoculated and control groups followed similar patterns to each other and were of roughly similar severity.

No differences between the inoculated and control groups, were detected in the other tissues examined.

### 5.3.4 Conclusion

Mild acidophil cell lesions characteristic of CACH were produced in a small number of the rats inoculated with a liver preparation from a dog with chronic CACH. Control rats did not exhibit these lesions. The disease remained subclinical during the short course of this experiment. These results suggest that CACH is transmissible experimentally from a dog to rats.

Chronic murine pneumonia would appear to be present in the rat colony used in this experiment. Since the lung lesions were characteristic of chronic murine pneumonia and were of similar severity in the inoculated and control groups, they were considered to be unrelated to any CACH infection.

## 5.4 RAT EXPERIMENT TWO

### 5.4.1 Experiment Design

The aim of this experiment was to attempt transmission of CACH from another canine field case (dog B/FC10) to a larger number of rats, permitting a longer study. It was also designed to compare the effect of inoculation with serum with that of inoculation with a liver preparation and to check that the effect produced by the latter was a result of the liver component and neither the PBS used in the preparation nor the physical process of inoculation.

Frozen liver from dog B (FC10) was thawed, diced and homogenised in PBS to form a 20% wt:vol suspension. Some of the stored serum was also thawed. The forty-five Wistar rats used were 26 days old at the start of the experiment. Sixteen rats (ten males and six females) were each inoculated with 0.5ml of the liver preparation. Thirteen rats (seven males and six females) were inoculated with 0.5ml of serum each. Eight rats (four males and four females) were inoculated with 0.5ml of the sterile PBS used to prepare the liver suspension. This blank-inoculated group was to be a precise control for the liver-inoculated group. Eight rats (three males and five females) were not inoculated and formed the nil control group. Both control groups were kept in a different room from the rats given dog material.

All the rats remained clinically normal throughout the 16-week course of the experiment. Rats were killed fortnightly from 2 weeks after inoculation, using the postmortem protocol in section 2.2.8. Due to the numbers available, the first three culls each consisted of two liver-inoculated rats, one serum-inoculated rat, one PBS-inoculated rat and one uninoculated rat. From 8 weeks after inoculation onwards the culls consisted of two rats from each of the two groups inoculated with dog material

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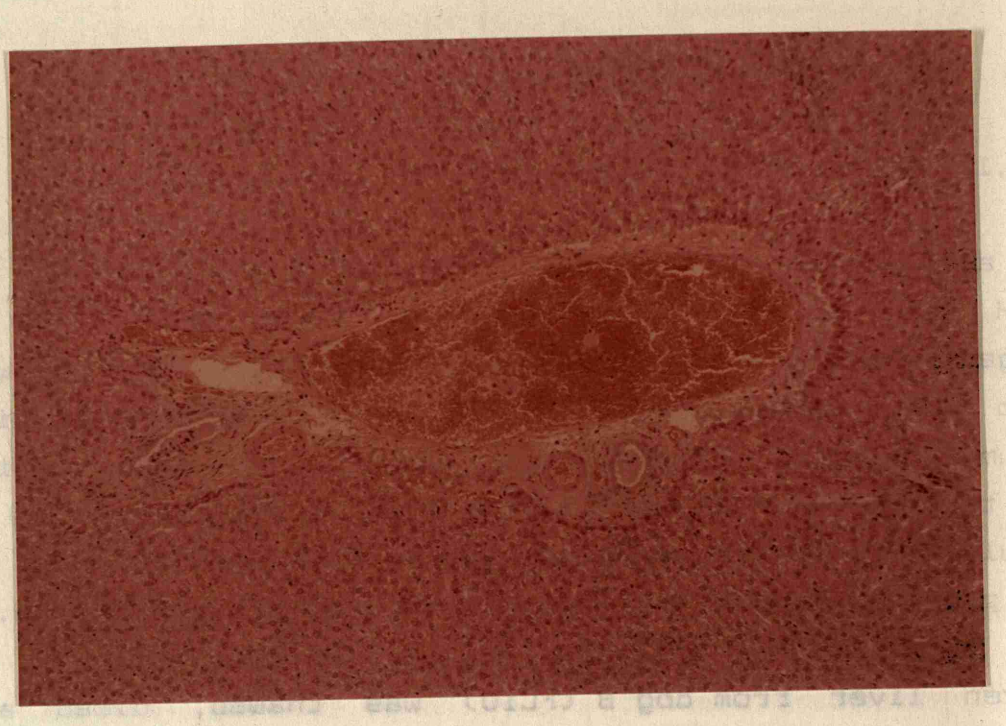

Fig 5-6 Liver of rat 40, 8 weeks post dog B's liver inoculum (H&E x87.5) acidophil cell reaction involving much of the limiting plate

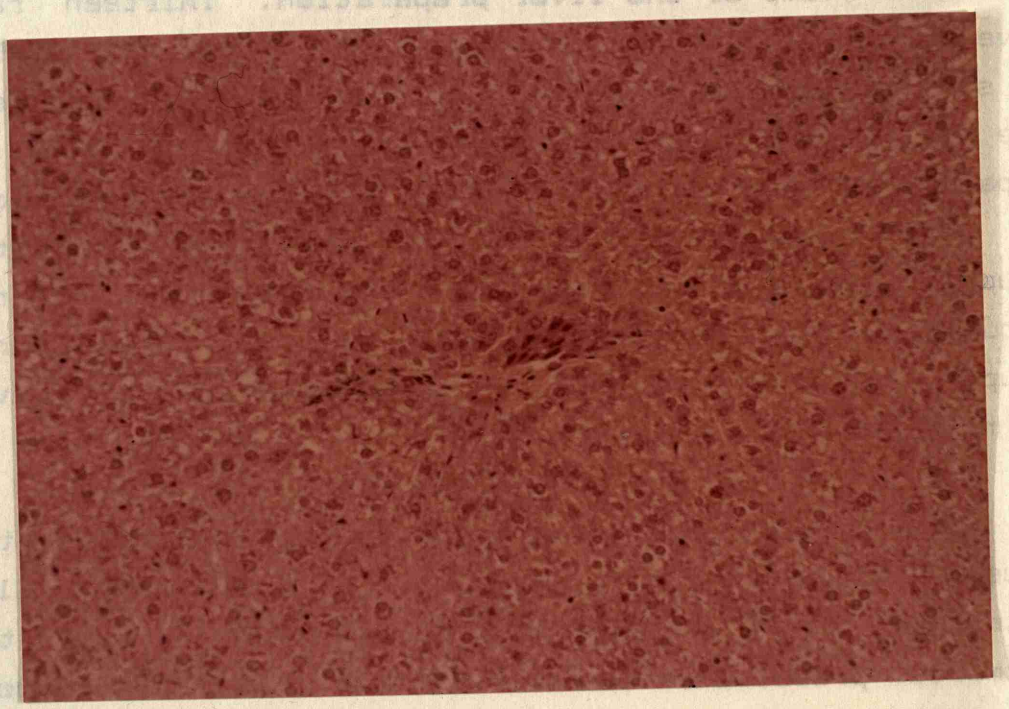

Fig 5-7 Liver of rat 53, 8 weeks post dog B's serum inoculum (H&E x175) even the tiniest PIs could be affected

and one rat from each of the two control groups.

### 5.4.2 Source Case

The source of liver and serum was dog B (FC10), a 9-year-old, neutered female German shepherd dog with a 4½-year history of liver disease. At postmortem it was found to have chronic CACH with cirrhosis. This dog was also the source case used for dog experiment 1 and its gross pathology and histology are detailed in chapter 4 (section 4.3.2). Figure 4-1 shows an example of the histological appearance of its liver. Full details of its clinical history are given in chapter 3 (section 3.2.10).

### 5.4.3 Results

Histological examination of the livers of the rats culled 2 weeks into the experiment revealed no differences between the four groups. None of them exhibited an acidophil cell reaction, they each had only one to a few acidophil cells at one or two portal tracts. By 4 weeks into the experiment the livers of the three rats inoculated with liver or serum all contained more acidophil cells than their two controls. The lesions were very mild and in one of the liver-inoculated pair they were insufficient to be called a positive acidophil cell reaction. The three rat livers, examined 6 weeks after inoculation with liver or serum, contained many more acidophil cells than their two controls, sufficient to be considered positive acidophil cell reactions. The 8-week cull revealed positive acidophil cell reactions in all four of the CACH-inoculated rats. The livers of three of these, contained more acidophil cells than those of the two controls. Both large and small portal tracts were affected, as can be seen in figures 5-6 and 5-7. However, the acidophil cell reaction in one of the liver-inoculated rats (rat 41) was not distinctly greater than that exhibited by the nil control (rat 74). In the 10-week cull, all four of the

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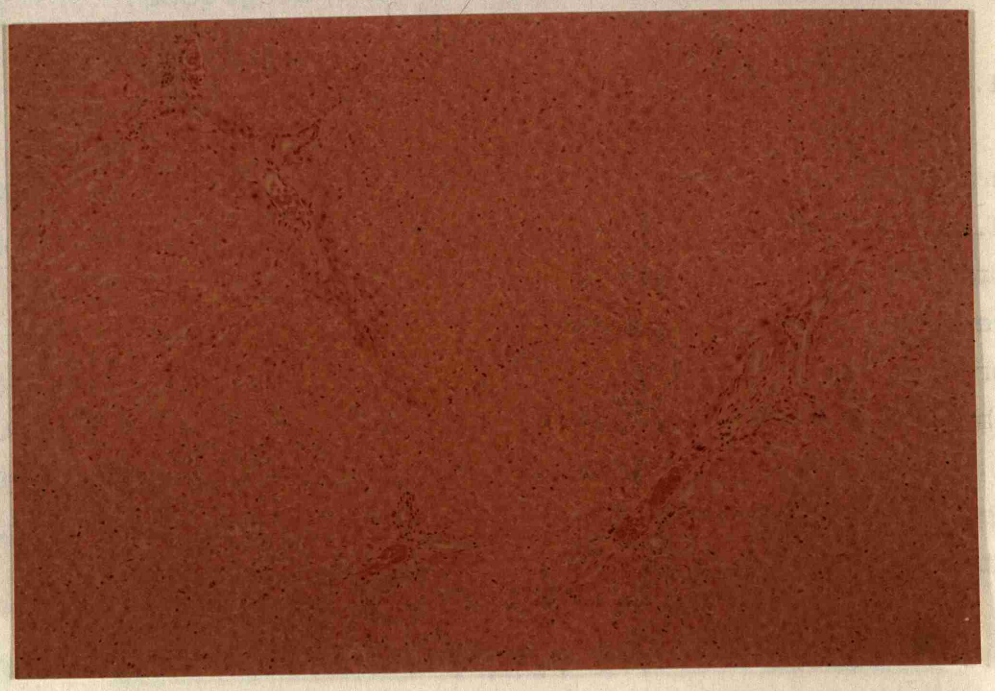

Fig 5-8 Liver of rat 55, 10 weeks post dog B's liver (H&E x87.5) ACs in limiting plates of PTs

Fig 5-9 Liver of 10-week PBS-control rat 67 (H&E x87.5)

rats inoculated with dog material had obvious acidophil cell reactions. Figure 5-8 shows an example of serum-inoculated rat 55's histology. The nil control did not exhibit an acidophil cell reaction, but the blank control of this cull (rat 67) did. Figure 5-9 shows small affected portal tracts. The four rats culled 12 weeks after inoculation with dog material also had obvious acidophil cell reactions. The livers of three of these contained markedly more acidophil cells than those of the two control rats (see figures 5-10 and 5-11). However the lesions in one of the serum-inoculated pair (rat 57) were not more severe than those in the nil control (rat 76) which also exhibited an acidophil cell reaction (figure 5-12). By 14 weeks after inoculation only the four rats given dog material had acidophil cell reactions in their livers, both controls being negative. None of the six rats in the final, 16-week, cull showed distinct acidophil cell reactions. Table 5-2 shows these results in another form.

As in experiment 1, the two control groups appeared to show a slight increase with age initially, in the numbers of acidophil cells per liver section, presumably due to growth. This first became evident in the nil control group 4 weeks into the experiment (at 7½ weeks of age) and in the blank control group 6 weeks into the experiment (at 9½ weeks of age). Despite this slight increase, seven of the eight rats inoculated with PBS had only very few, or a few acidophil cells in their livers. Only one rat (67), culled 10 weeks after inoculation, appeared to exhibit an acidophil cell reaction. Two rats (74 and 76) from the uninoculated control group, culled 8 and 12 weeks into the experiment, also appeared to have acidophil cell reactions in their livers (see figure 5-12). From 14 weeks into the experiment onwards, the numbers of acidophil cells in the livers of both control groups, were reduced to levels similar to those in rats culled early in the experiment.

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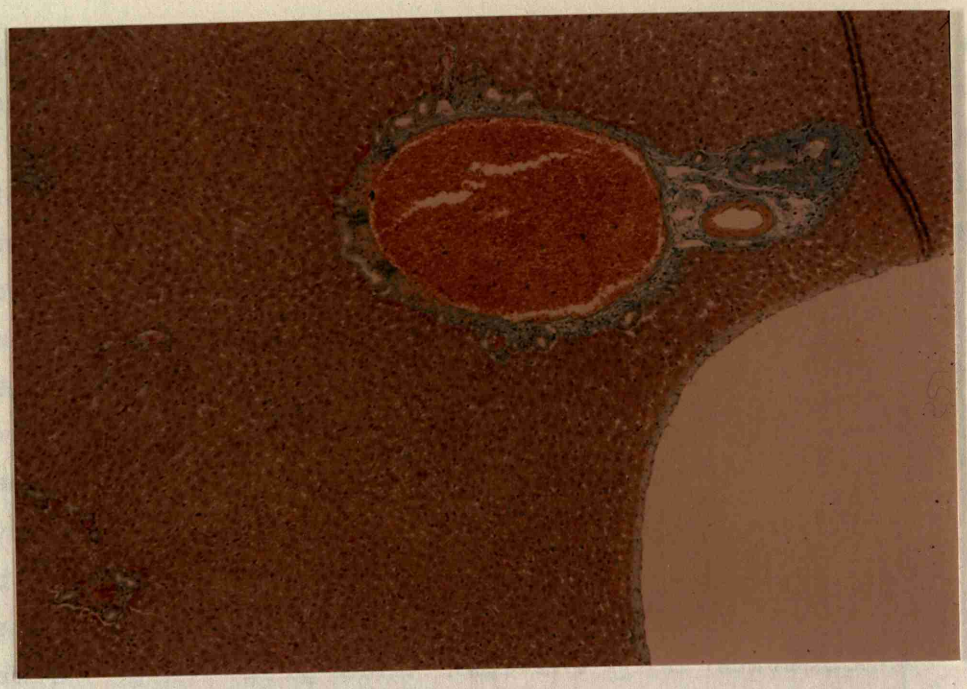

Fig 5-10 Liver of rat 44 (source rat A), 12 weeks post dog B's liver inoculum (Mass tri x70)
ACs in limiting plates of small & large PIs

Fig 5-11 Liver of rat 45 (source rat B), 12 weeks post dog B's liver inoculum (H&E x35)

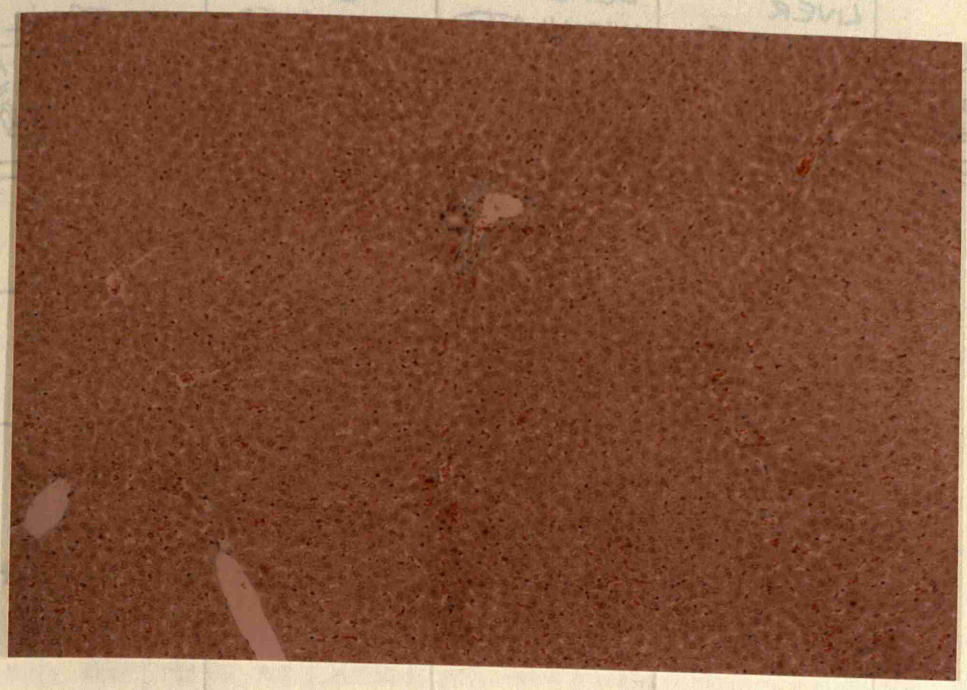

Fig 5-12 Liver of 12-wk control rat 76 (Mass tri x87.5)

Rats in all the groups of this experiment showed histological evidence of chronic murine pneumonia. The lesions were mildest in the youngest rats, with only a few lymphoid foci or none per lung section, and tended in general to increase in severity with age. Some older rats exhibited bronchial cuffing, occasionally with primary follicles or more rarely secondary (2°) follicles, with germinal centres, in the bands of lymphoid cells. Some of the heaviest-cuffed airways contained exudates of mainly neutrophils. Spread of the process to involve areas of parenchyma was also a feature in some of the older rats. The timescale and severity of lesions was similar in all the groups of rats in this experiment.

### 5.4.4 Conclusion

Although acidophil cell lesions characteristic of CACH were again produced in rats inoculated with material from a dog with chronic CACH and cirrhosis, three of the control rats also exhibited lesions. Seventeen of the twenty-two rats culled from 4 to 14 weeks after inoculat-

| Wks post inoc. | LIVER INOCULATED | | | SERUM INOCULATED | | | PBS INOCULATED | | | NIL CONTROL | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Rat No. | AC score | Result | Rat No. | AC score | Result | Rat No. | AC score | Result | Rat No. | AC score | Result |
| 2 | 34 | - | — | 50 | - | — | 63 | - | — | 71 | - | — |
| | 35 | - | — | | | | | | | | | |
| 4 | 36 | + | — | 51 | ++ | + | 64 | - | — | 72 | + | — |
| | 37 | ++ | + | | | | | | | | | |
| 6 | 38 | ++ | + | 52 | ++ | + | 65 | + | — | 73 | + | — |
| | 39 | ++ | + | | | | | | | | | |
| 8 | 40 | +++ | + | 53 | +++ | + | 66 | + | — | 74 | ++ | + |
| | 41 | ++ | + | 54 | ++ | + | | | | | | |
| 10 | 42 | ++ | + | 55 | +++ | + | 67 | ++ | + | 75 | + | — |
| | 43 | ++ | + | 56 | ++ | + | | | | | | |
| 12 | 44 | +++ | + | 57 | ++ | + | 68 | + | — | 76 | ++ | + |
| | 45 | ++ | + | 58 | +++ | + | | | | | | |
| 14 | 46 | ++ | + | 59 | ++ | + | 69 | - | — | 77 | - | — |
| | 47 | ++ | + | 60 | ++ | + | | | | | | |
| 16 | 48 | + | — | 61 | + | — | 70 | + | — | 78 | + | — |
| | 49 | + | — | 62 | + | — | | | | | | |

Table 5-2 Rat experiment 2 liver histology results

Liver lesions were graded on a scale of - to ++++. A positive acidophil cell reaction was taken as ++ or more. The acidophil cell scoring system is explained in appendix 2.

ion with dog material, exhibited more severe lesions than both of their respective controls. The remaining five exhibited more severe acidophil cell lesions than only one of their two timed controls, being similar to the other. For three of these it was the degree of reaction in their blank control (rat 67), culled at 10 weeks, that was similar. The other two were similar to their respective nil controls (rats 74 and 76), culled 8 and 12 weeks into the experiment.

As in experiment 1, CACH would appear to be transmissible experimentally from infected dogs to rats. The disease remained subclinical in the rats thoughout the 4-month course of the experiment. A pathological difference, between infected and control rats, again became apparent around 4 weeks after inoculation. The acidophil cell reaction in the infected rats, although initially very mild, increased in severity to be marked from 8 to 14 weeks. By 16 weeks after inoculation the damage appeared to have subsided. Since this was the last cull of the experiment there was no indication of whether the infection had run its course, or would lead to recurring bouts as in some dogs. Inoculation with serum appeared to be as effective in transmitting CACH as inoculation with liver suspension; the pattern of lesion production followed a similar time course. The lack of acidophil cell reaction in all but one of the rats inoculated with PBS alone, suggests that it is indeed the liver component of the liver suspension which results in production of lesions.

The respiratory lesions, characteristic of chronic murine pneumonia, followed a similar pattern in both the control groups and in the groups given dog liver and serum. They were therefore considered to be unrelated to inoculation with dog material.

## 5.5 RAT EXPERIMENT THREE

### 5.5.1 Experiment Design

This experiment was designed to investigate whether or not rats caged with those of experiment 1 were affected in any way by this contact. Evidence of horizontal transmission between rats was looked for.

As mentioned in section 5.3.1, the four dams of the experiment 1 rats all littered down again during that experiment. They must have mated at postpartum oestrus, before they were caged alone with their litters, since the normal 4-5 day oestrus cycle (Holmes, 1984d; Kohn and Barthold, 1984) does not resume until the end of lactation (Austin and Rowlands, 1978). In the rat the mean duration of gestation is 20-22 days (Short and Woodnott, 1978; Holmes, 1984a). Progestation (the period between fertilization and implantation of the blastocyst) usually only 4-6 days in small rodents, may be doubled when conception results from a postpartum mating due to the inhibiting effect of lactation on implantation (Short and Woodnott, 1978). This phenomenon was probably responsible for the delay between the birth of the experiment 1 litters, with subsequent mating at postpartum oestrus, and the birth of the second litters; an interval of 25 days for the two Lou dams, 27 days for the dam of the Wistar controls and 36 days for the dam of the inoculated Wistar rats. Since the second litter born in the cage of inoculated Wistars was neglected and died, the second litter born in the cage of Wistar controls was not required for experiment 3. Only the second litters born in the two experiment 1 Lou rat cages were investigated in experiment 3, along with all four of the dams.

The second litter in the inoculated Lou cage consisted of four rats (two males and two females) born 2½ weeks (17 days) after the inoculation of the older litter with a

suspension of liver from dog A. At the time of their birth there were four of this inoculated litter remaining in the cage. One of the latter was culled within a few days, then another each week until they were all gone by which time the in-contact litter was 3½ weeks of age. The second litter in the control Lou cage consisted of five rats (one male and four females) also born 2½ weeks (17 days) after the start of experiment 1, while four of the original control litter remained in the cage. The latter were removed on the same timescale as the inoculated rats. This cage of control rats was kept in a different room from the cage of inoculated and in-contact rats.

All of the in-contact and control litters and the four dams remained clinically normal throughout the course of this experiment. The young rats were killed fortnightly, from 5 to 11 weeks of age, using the postmortem protocol in section 2.2.8. Each cull consisted of one in-contact and one control rat, except for the last cull which involved one in-contact and two control rats due to the numbers available.

The dam of the experiment 1 inoculated Wistar litter was killed 10 weeks after their inoculation with liver suspension from dog A, at the same time as the last of them. Although not inoculated herself she had been in contact with them for the whole 10 weeks. The dam of the experiment 1 Wistar control litter was killed at the same time, to be used as a control for her. The dam of the experiment 1 inoculated Lou litter was killed 14 weeks after inoculation of that litter with liver suspension from dog A. However, since the last of them was culled 6 weeks after inoculation, she had only been in contact with inoculated rats for 6 weeks. Her second litter, which had been in contact with the inoculated rats for the first 3½ weeks of their lives, was also in the cage with her. The last of that litter was removed only days before her own death. The dam of the experiments 1 and 3 Lou control

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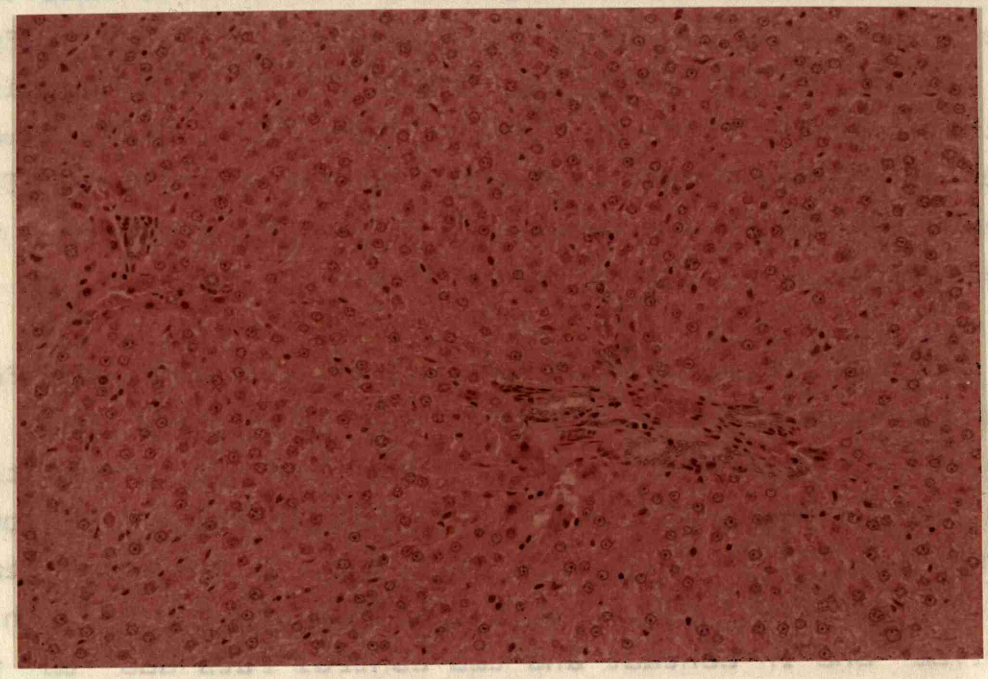

Fig 5-13 Liver of rat 90, an in-contact dam (H&E x175) a few acidophil cells

litters was killed at the same time, as a control.

5.5.2 Results

Histological examination of the livers of the experiment 3 control rats, both the young Lou litter and the Lou and Wistar dams, did not reveal any positive acidophil cell reactions. Only very small numbers of acidophil cells were present, mainly in ones and twos. There did not appear to be any obvious increase with age, in the numbers of acidophil cells in the livers of the small number of rats investigated.

Microscopy of the livers of the in-contact Lou litter and the two in-contact dams revealed more acidophil cells than in the livers of each of their timed controls, but most of the lesions were very mild and not of sufficient severity to be called definite acidophil cell reactions (see table 5-3). Figure 5-13 shows small numbers of acidophil cells in two portal tracts of rat 90, an incontact dam. Only the last young in-contact rat (82), killed at 11 weeks of age, appeared to exhibit a positive acidophil cell reaction (see table 5-3).

Once again, histological examination of two lung sections per rat, from different lobes, revealed evidence of chronic murine pneumonia. The youngest rats had the mildest lesions. The 5-week-old rats of the first cull exhibited only one or two small lymphoid foci or none, per lung section. The size and number of foci increased with age and a few 2° follicles with germinal centres were seen in some of the larger areas of lymphoid cells. The development of lesions in the control litter was similar to that in the in-contact litter, although at each cull the severity was very slighty greater in the former. The last two young control rats had quite extensive lymphoid infiltration in at least one of their two lung sections. The two control dams and the two in-contact dams also had

| Age (weeks) | IN-CONTACTS of expt 1 liver inoculated rats | | | IN-CONTACTS of expt 1 control rats | | |
|---|---|---|---|---|---|---|
| | Rat No. | AC score | Result | Rat No. | AC score | Result |
| 5 | 79 | - | — | 83 | - | — |
| 7 | 80 | + | — | 84 | - | — |
| 9 | 81 | + | — | 85 | - | — |
| 11 | 82 | ++ | + | 86 | + | — |
| | | | | 87 | - | — |
| Wks post inoc of their expt 1 offspring | IN-CONTACT DAMS of expt 1 liver inoculated rats | | | CONTROL DAMS of expt 1 control rats | | |
| 10 | 88 | + | — | 89 | - | — |
| 14 | 90 | + | — | 91 | - | — |

Table 5-3 Rat experiment 3 liver histology results

Liver lesions were graded on a scale of - to ++++. A positive acidophil cell score was taken as ++ or more. The acidophil cell scoring system is explained in appendix 2.

extensive lymphoid infiltration in their lung sections, with neutrophil exudates in some bronchi.

### 5.5.3 Conclusion

Although a mild difference between in-contact and control rats was detectable in every case, only the last of the in-contact litter exhibited enough lesions to be considered a positive acidophil cell reaction. Since this experiment involved only a very small number of rats it would be unwise to draw any definite conclusions from one positive result, however horizontal transmission of CACH between in-contact rats may be possible and warrants further investigation. The Lou dam had been in contact with decreasing numbers of her inoculated litter for the first 6 weeks after their inoculation. The Wistar dam had been in contact with decreasing numbers of her inoculated litter for 10 weeks after their inoculation. The young rats had only 3½ weeks of contact with a small, decreasing number of inoculated rats, from 2½ to 6 weeks after inoculation. The period of peak infectivity, if any, in inoculated rats is not known and further studies involving longer periods of contact with larger numbers of rats might be worthwhile.

As in experiments 1 and 2, the respiratory lesions, characteristic of chronic murine pneumonia, seen in both in-contact and control rats were considered to be unrelated to any possible CACH infection.

## 5.6 RAT EXPERIMENT FOUR

### 5.6.1 Experiment Design

In this experiment rat to rat transmission of CACH was attempted, by inoculation with liver preparations from two experimentally-infected rats (A and B). The experiment was

also designed to investigate the effect on rats of inoculation with a liver preparation from a normal healthy dog (dog C), to check that the lesions produced in experiments 1 and 2 were not reactions to normal canine liver components.

Samples of frozen liver from the three cases were individually thawed, diced and homogenised in PBS to form 20% wt:vol suspensions. The forty-two Wistar rats used were all about 7 weeks of age at the start of the experiment. Seven male rats were each inoculated with 0.5ml of rat A's liver preparation. Seven female rats were each inoculated with 0.5ml of rat B's liver preparation. Fourteen rats (seven males and seven females) were inoculated with 0.5ml each of dog C's liver preparation. Seven rats (five males and two females) formed the blank control group, they were each inoculated with 0.5ml of the sterile PBS used to prepare the liver suspensions. Seven rats (one male and six females) were not inoculated at all and formed the nil control group. Both control groups were kept in a different room from the rats given liver preparations.

The rats all remained clinically normal during the 14-week course of the experiment. They were killed fortnightly from 2 weeks after inoculation, using the postmortem protocol in section 2.2.8. Each cull consisted of six rats: one inoculated with rat A's liver, one inoculated with rat B's liver, two inoculated with dog liver, one PBS-inoculated rat and one of the nil control group. The blood samples collected for serum separation were also subjected to biochemical analysis, when volumes permitted.

### 5.6.2 Source Cases

The sources of rat liver were two 15½-week-old Wistar rats from experiment 2 (section 5.4): rat A (rat 44) was female, rat B (rat 45) was male. They had been killed 12 weeks after inoculation with a liver preparation from dog

| Wks post inoc. | RAT 44 LIVER inoculated | | | RAT 45 LIVER inoculated | | | NORMAL DOG LIVER inocul. | | | PBS inoculated | | | NIL CONTROL | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Rat No. | AC Score | Result | Rat No. | AC Score | Result | Rat No. | AC Score | Result | Rat No. | AC Score | Result | Rat No. | AC Score | Result |
| 2 | 92 | + | — | 99 | + | — | 106<br>107 | +<br>+ | —<br>— | 120 | + | — | 127 | – | — |
| 4 | 93 | – | — | 100 | + | — | 108<br>109 | –<br>– | —<br>— | 121 | – | — | 128 | – | — |
| 6 | 94 | + | — | 101 | – | — | 110<br>111 | –<br>+ | —<br>— | 122 | – | — | 129 | – | — |
| 8 | 95 | + | — | 102 | + | — | 112<br>113 | +<br>– | —<br>— | 123 | – | — | 130 | – | — |
| 10 | 96 | + | — | 103 | + | — | 114<br>115 | –<br>+ | —<br>— | 124 | – | — | 131 | – | — |
| 12 | 97 | + | — | 104 | + | — | 116<br>117 | –<br>+ | —<br>— | 125 | – | — | 132 | – | — |
| 14 | 98 | ++ | + | 105 | ++ | + | 118<br>119 | +<br>+ | —<br>— | 126 | + | — | 133 | – | — |

Table 5-4 Rat experiment 4 liver histology results

Liver lesions were graded on a scale of – to ++++. A positive acidophil cell reaction was taken as ++ or more. The acidophil cell scoring system is explained in appendix 2.

B. Although they had appeared clinically normal, at postmortem their livers both exhibited obvious acidophil cell lesions (see figures 5-10 and 5-11).

The source of normal dog liver was control dog 35 (dog C) from dog experiment 1 (section 4.3), a 4-month-old, female border collie. An example of its liver histology can be seen in figure 4-46.

### 5.6.3 Histological Results

Histological examination of the livers of both the nil and blank control groups revealed no positive acidophil cell reactions (see table 5-4). Only very few or a few acidophil cells were present, mainly in ones and twos. Figures 5-14 and 5-15 show some of the few portal tracts with any acidophil cells. There was no obvious variation in acidophil cell numbers with age in either of the control groups.

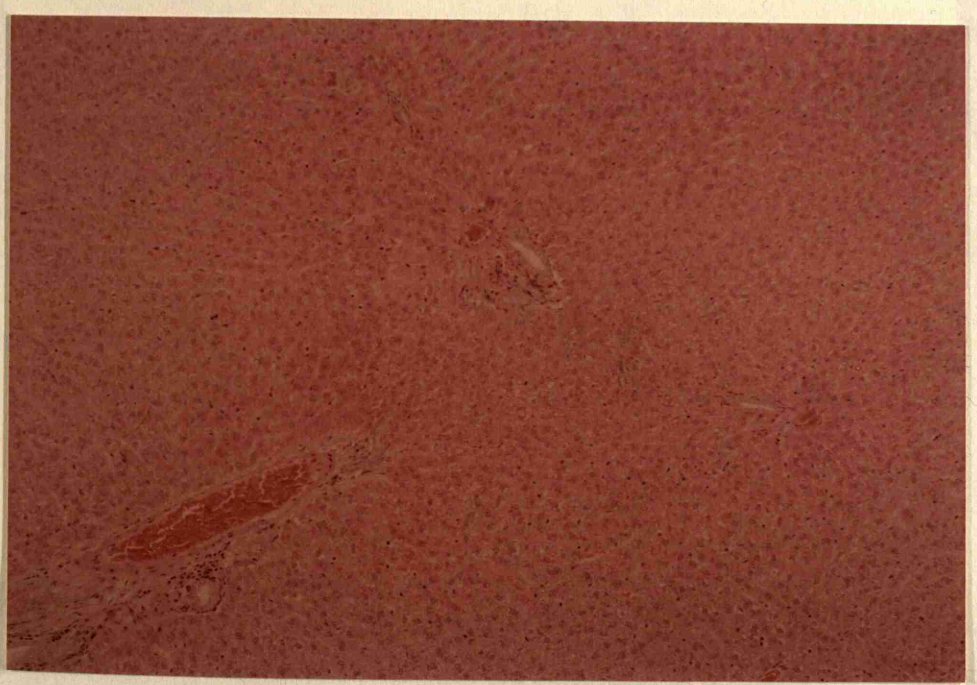

Fig 5-14 Liver of 14-week PBS-control rat 126 (H&E x87.5)

fp257

Fig 5-15 Liver of 14wk nil control rat 133 (H&E x87.5)

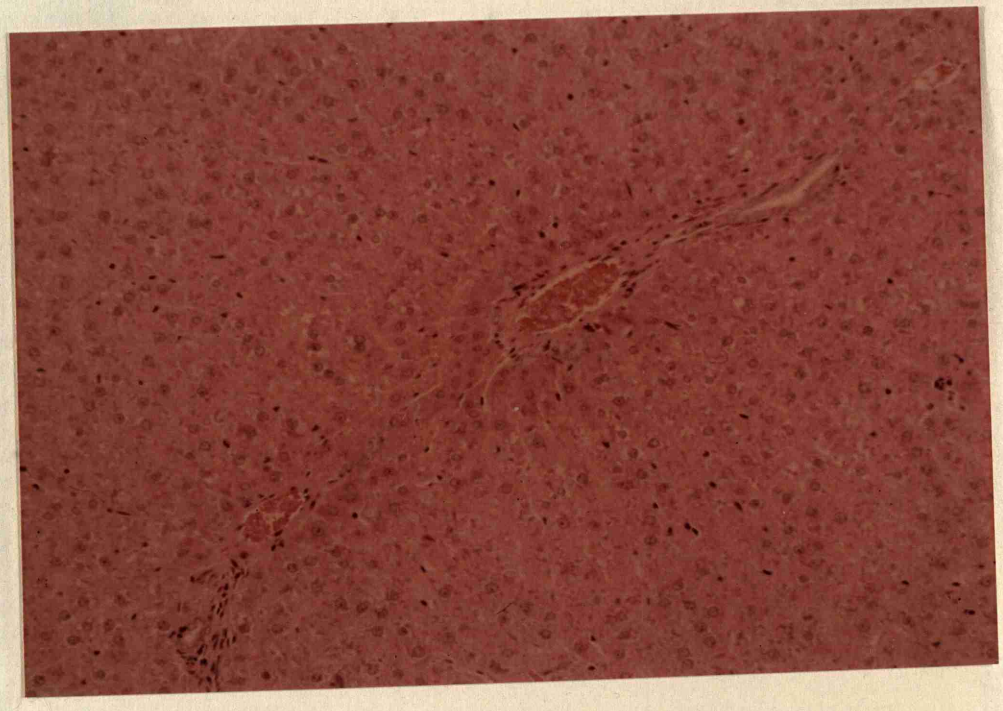

Fig 5-16 Liver of rat 112, 8 weeks postinoculation with normal dog liver (H&E x175)

Microscopy of the livers of the rats inoculated with normal dog liver also failed to reveal any positive acidophil cell reactions (see table 5-4). As in the two control groups, only small numbers of acidophil cells were present and these were mainly single or in small groups. Figures 5-16 and 5-17 show examples of the very few portal tracts with occasional acidophil cells. There was no obvious effect of age on the numbers of acidophil cells in the livers of these rats.

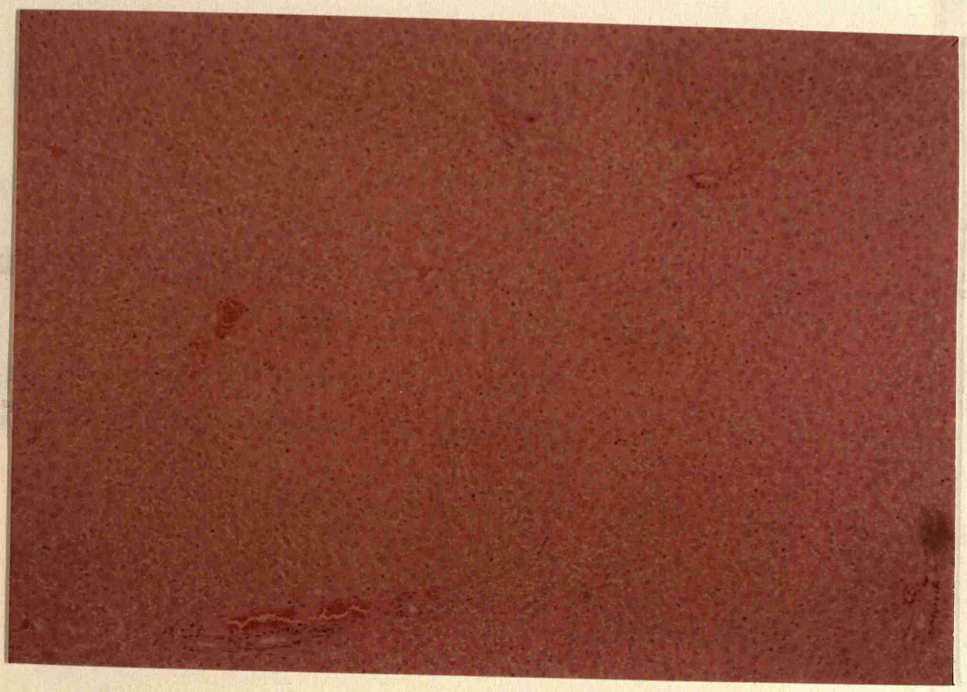

Fig 5-17 Liver of rat 118 14 weeks post-normal dog liver (H&E x87.5)

Histological examination of the livers of the rats culled 2, 4 and 6 weeks after inoculation with rat A's liver revealed only small numbers of acidophil cells. From 8 weeks after inoculation onwards, each rat's liver contained distinctly more acidophil cells than those of its two time-matched controls. However, acidophil cell numbers were still relatively small and insufficient to be considered positive acidophil cell reactions at 8, 10 and 12 weeks after inoculation (see table 5-4). Only the liver of

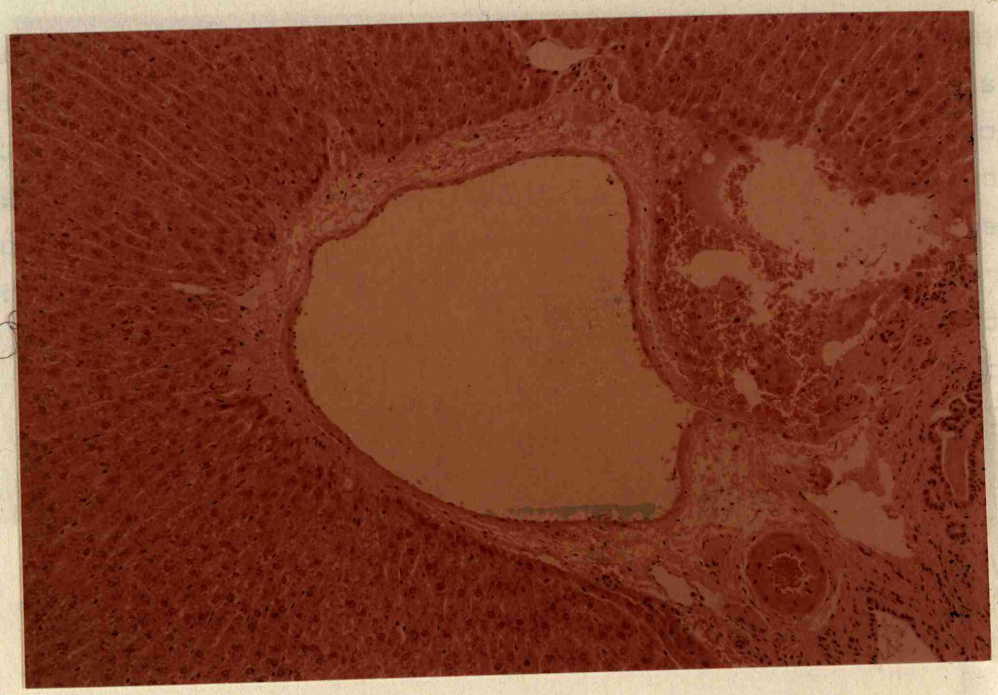

Fig 5-18 Liver of rat 98, 14 weeks post rat A's liver inoculum (H&E x115.5)

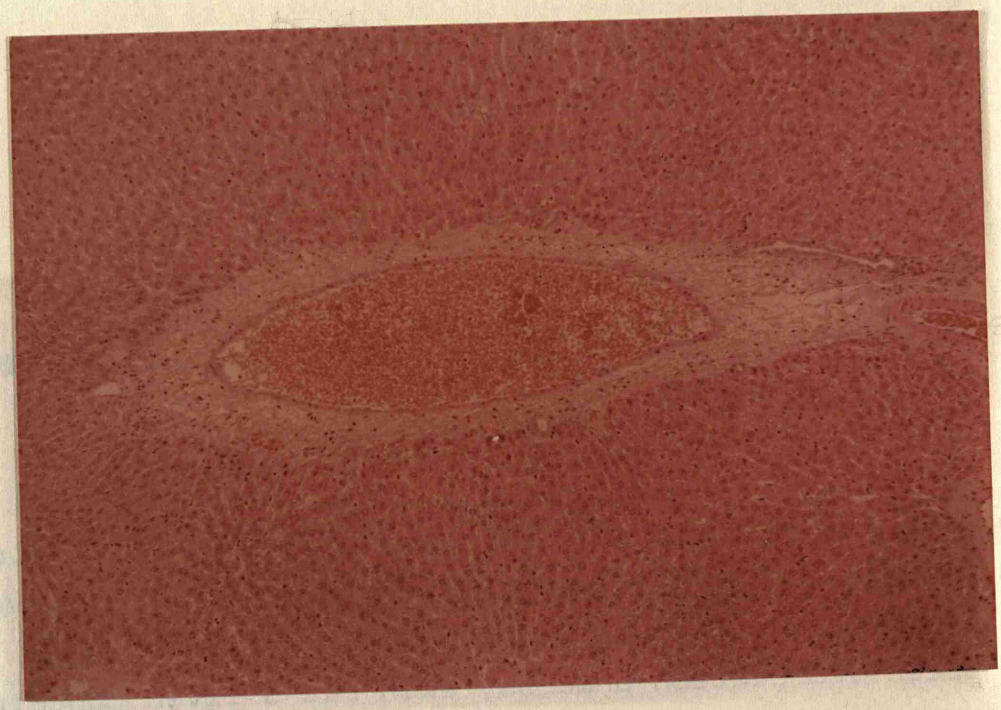

Fig 5-19 Liver of rat 105, 14 weeks post rat B's liver (H&E x87.5)

the rat culled 14 weeks into the experiment (rat 98) contained enough acidophil cells lesions to be considered a positive reaction (figure 5-18).

In the group given rat B's liver, microscopy again revealed only small numbers of acidophil cells in the livers of rats culled 2, 4 and 6 weeks into the experiment. Once again from 8 weeks after inoculation onwards, each rat's liver contained more acidophil cells than those of its two time-matched controls. However, only the last rat (105), culled 14 weeks after inoculation, exhibited enough lesions to be considered a positive acidophil cell reaction (see figure 5-19).

As in earlier experiments, histological evidence of chronic murine pneumonia was found in all of the rats of this experiment. Although the lung lesions followed the general trend of increasing in severity with age, this was not as marked a feature as in previous experiments. The earliest culled rats had slightly more severe lesions than those of experiments 1, 2 and 3, with more lymphoid foci and some parenchymal involvement. This was probably because they were older than those in earlier experiments, 9 weeks of age at the first cull. Lymphoid cuffing of bronchi was seen in some of the older rats. The severity of lung lesions was roughly similar in all five groups of rats in this experiment.

### 5.6.4 Biochemical Results

The blood levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and urea were assayed for most of the rats in this experiment.

The alanine aminotransferase levels were all between 15 and 40 IU/l except in three samples. Rat 94 had a level of 52 IU/l, 6 weeks after inoculation with rat A's liver. Eight weeks after inoculation with rat B's liver, rat

102's ALT level was 46 IU/l and the last rat (119) of the group given normal dog liver had the same level of ALT.

The aspartate aminotransferase levels appeared to be unremarkable (between 50 and 130 IU/l), apart from a high result of 284 IU/l for rat 102, killed 8 weeks after inoculation with rat B's liver.

Alkaline phosphatase levels tended to reduce with age in all four groups of rats, as happens in the young of many species. Apart from four results, their AP levels were all between 120 and 400 IU/l. Rats 93 and 94, killed 4 and 6 weeks after inoculation with rat A's liver, had AP levels of 462 and 448 IU/l respectively. The last rat (105) of the group given rat B's liver had an AP level of 462 IU/l. Unfortunately, no result was available for the last rat of the group given rat A's liver. Rat 115, killed 10 weeks after inoculation with normal dog liver, had the highest AP level of 528 IU/l.

The blood levels of urea appeared to be unremarkable.

### 5.6.5 Conclusion

Mild acidophil cell reactions characteristic of CACH were seen in two rats, 14 weeks after inoculation with liver from two other rats (A and B) which had been experimentally infected by inoculation with liver from a dog with chronic CACH and cirrhosis (section 5.4). Although no other positive results were obtained, from 8 weeks after inoculation onwards the rats of the two rat liver-inoculated groups did exhibit more acidophil cells than their respective time-matched controls. The results of this experiment suggest that CACH may indeed be transmissible experimentally from rats to rats as a very mild, subclinical disease. Since the peak period of infectivity in rats is unknown, further investigations, using liver preparations from rats at various times after inoculation

and perhaps more concentrated doses, are required to determine whether more severe lesions can be produced or whether rat to rat passaged disease is always mild.

The lack of acidophil cell reactions in the group of rats inoculated with normal dog liver suggests that the effects, of inoculation with liver from dogs with CACH, seen in rat experiments 1 and 2 were not due to any reaction to dog liver itself, but to the CACH infection.

The fact that no acidophil cell reactions were produced in the blank control group, inoculated with PBS, reinforces the similar findings in rat experiment 2.

Since characteristic lesions of chronic murine pneumonia were found in the lungs of both blank and nil control rats, as well as those inoculated with liver, they were considered to be unrelated to the inoculation of the animal material.

The biochemical results are interesting, but inconclusive.

## 5.7 RAT EXPERIMENT FIVE

### 5.7.1 Experiment Design

The aim of this experiment was to repeat the transmission of CACH from dogs to rats, but to use an experimentally-infected dog as the source of infective material (dog D). The experiment was also designed to produce fresh rat liver for immediate use in the third dog experiment (section 4.5.1) to attempt rat to dog transmission of CACH. Four pregnant female rats had to be purchased, in order to obtain sufficient numbers of newborn rats at the required time.

Frozen liver from dog D was thawed, diced and stomached in

tris-buffered saline before a short homogenisation to form a 30% wt:vol suspension. Thirty-four, 2- to 4-day-old, Sprague Dawley rats from four litters were each inoculated with 0.1ml of the liver preparation. The age and size of these baby rats determined the smaller than usual inoculum volume; a more concentrated suspension was therefore used to partially offset this reduction. Since the rats in this experiment were inoculated at a much younger age than those in previous experiments, the expected survival rate for the procedure and speed of onset of any lesions were not known. In an attempt to ensure the production of an adequate amount of material for the dog experiment despite these uncertainties, all of the available rats were inoculated. Unfortunately, this left none for use as controls. The four litters were caged with their respective mothers until weaning, just over 5 weeks after inoculation, when they were mixed and split into single-sex cages. The four dams were then caged together, with and an adult male Sprague Dawley rat in an attempt to breed more litters.

By about 1 week after inoculation three small, weak runts had been lost from one litter and two from another. Whether they died before being eaten by their dams, or were killed first, could not be determined. The only remaining runt, in the litter from which two had been lost, was killed and postmortemed 1½ weeks after inoculation in an attempt to investigate the problem. At that time the remaining twenty-eight rats all appeared to be growing well. From 2 weeks after inoculation onwards, rats were culled fortnightly. The rat culled at 2 weeks was chosen because it had a small, 6mm diameter, subcutaneous mass on its back. The rest of the rats remained clinically normal throughout the course of the experiment. A single rat was killed at each of the 4- and 6-week culls. When the latter was found to have a positive acidophil cell reaction in its liver, six additional rats were killed (6 weeks and 2 days after inoculation) to provide fresh liver for use in dog experiment 3 and other investigations. The usual

fp262

Fig 5-20 Liver of CACH field case dog F (FC2)
(Mass acid fuch x70)

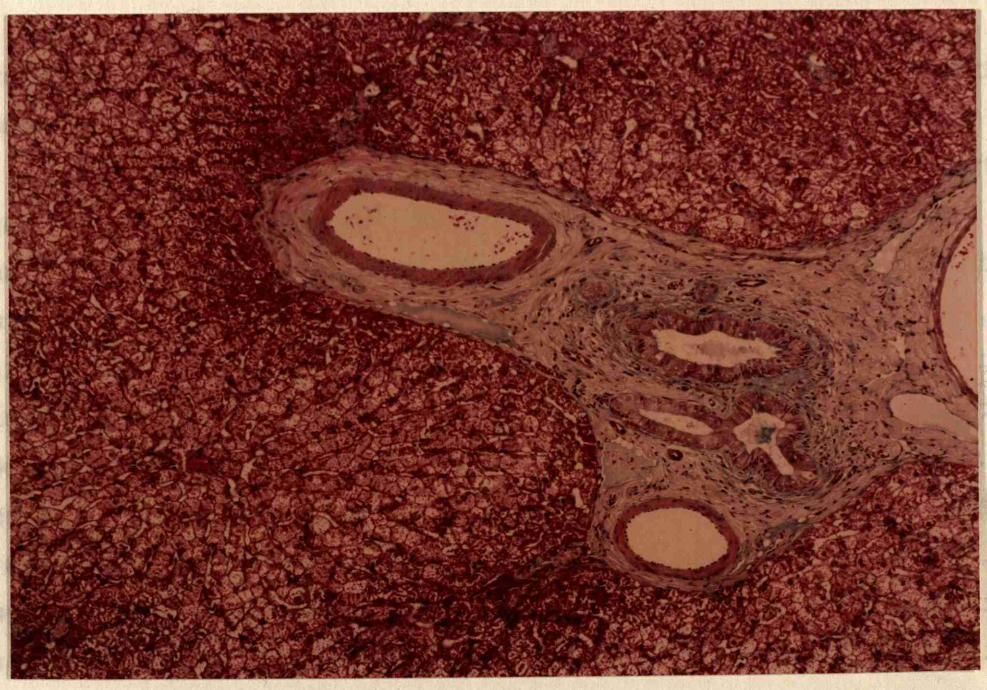

Fig 5-21 Liver of (source case) experimental dog D
(Mass acid fuch x87.5)

samples were collected for histology from only one of these rats; one liver section was all that was taken from each of the other five rats. From 8 weeks into the experiment onwards, the fortnightly culls each consisted of two rats; until the last remaining rat was killed 26 weeks after inoculation. Biochemical analysis of the blood samples, collected for serum separation, was performed whenever volumes permitted.

### 5.7.2 Source Case

The source of liver, dog D, was an 18-week-old, male crossbreed [experimental dog 8 in Jarrett and O'Neil (1985)]. Dog D had been inoculated, at 12 weeks of age, with serum and liver homogenate from dog F (FC2) a 13-month-old, male cocker spaniel with advanced canine acidophil cell hepatitis. The field case had a 3-month history of illness, it exhibited weight loss, lethargy, ascites, vomiting and a high BSP retention. Full details of its history and pathology are given in chapter 3 (section 3.2.2). Histologically dog F had exhibited diffuse chronic hepatitis with extensive delicate fibrosis (figure 5-20). Fatty vacuolation and a little scattered cell infiltrate were also present. Dog D had become moribund 6 weeks after inoculation. Although depression and collapse were the only clinical signs apparent, its previously normal ALT level was raised to 77 IU/l at that time. At necropsy dog D's liver had been enlarged and tense, with rounded edges and highly delineated lobulation. Microscopy had revealed severe acute hepatitis (figure 5-21) with almost complete acidophil change in peripheral areas of some lobules and acidophilic cells in the peritrabecular limiting plates around hepatic veins; in places this had advanced to complete cell necrosis. Foci of proliferated Kupffer cells were seen in sinusoids as well as a few small foci of lymphocytes.

fp263

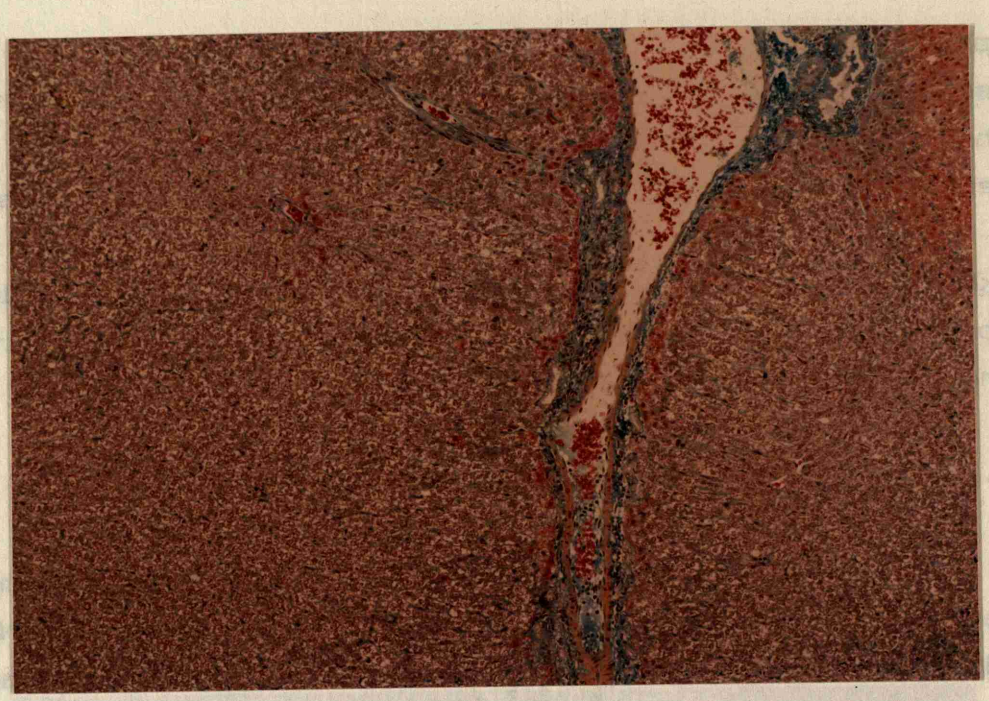

Fig 5-22 Liver of rat 137, 6 weeks post dog D's liver inoculum (Mass tri x87.5)

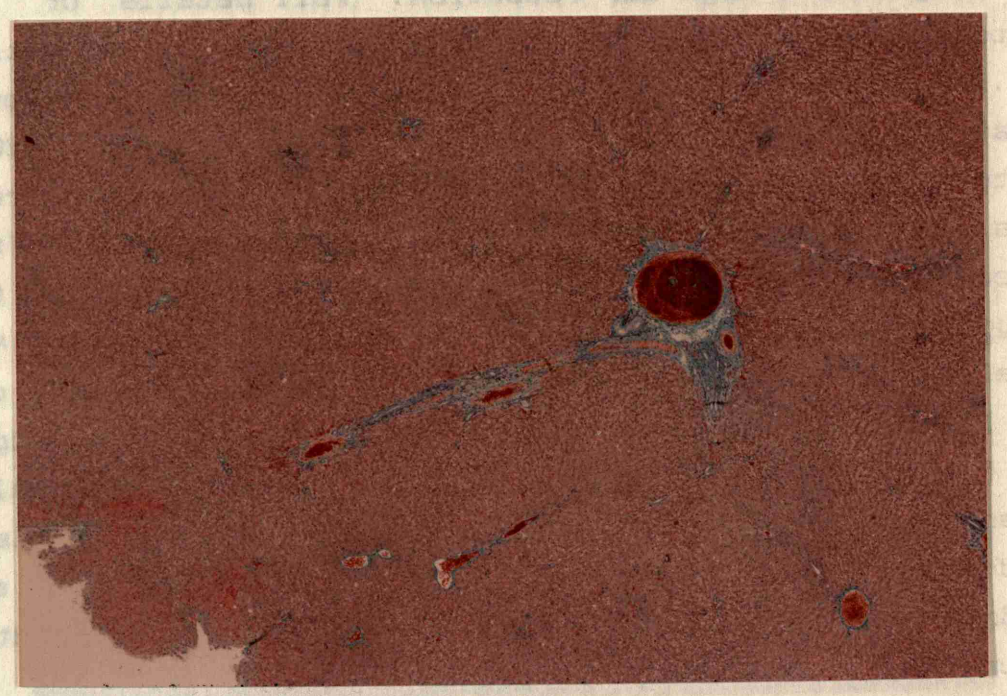

Fig 5-23 Liver of rat 156, 18 weeks post dog D's liver inoculum (Mass tri x35)

### 5.7.3 Histological Results

Histological examination of the organs of the rat killed 1½ weeks after inoculation failed to reveal any abnormalities to explain its small size. Its liver contained only very few acidophil cells in ones and twos. The mass on the back of the rat killed 2 weeks after inoculation was found, at postmortem, to be full of pale inspissated pus of putty-like consistency. Histology revealed a necrotic centre surrounded by neutrophils and other inflammatory cells, encased in a fibrous capsule. It was presumably an injection site abscess. The liver of this rat contained only small numbers of acidophil cells, but there were also small foci of inflammatory cells including neutrophils in some portal tracts, possibly related to the suppurative process on the back. Only small numbers of acidophil cells, in ones, twos and small groups, were seen in the liver of the rat culled 4 weeks into the experiment. The liver of rat 137, killed 6 weeks after inoculation, contained many acidophil cells and was the first positive acidophil cell reaction of this experiment (figure 5-22). Of the six rats killed 2 days later: five exhibited mild acidophil cell reactions (rats 139-143), the other one (rat 138) had only a few acidophil cells. An example of rat 139's liver histology can be seen in figure 4-142. From 8 weeks after inoculation onwards, all but two of the rats killed exhibited at least mild positive acidophil cell reactions. Liver lesions from one of the most affected, rat 156, are shown in figure 5-23. The two rats with insufficient acidophil cells in their livers to be considered positive results, came from the 12- and 24-week culls (see table 5-5).

Histological examination of the lungs of the inoculated rats revealed no abnormalities at 1½ and 2 weeks into the experiment and only one small focus of lymphoid cells in the lung section of each of the 4- and 6-week culls. The single lung section taken from one of the six rats killed

| Weeks post inoc. | Rat Experiment 5 | | | Rat experiment 6 | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | DOG D LIVER inoculated rats | | | Age matched UNINOCULATED rats | | | UNINOCULATED PARENT rats | | |
| | Rat No. | AC score | Result | Rat No. | AC score | Result | Rat No. | AC score | Result |
| 1½ | 134 | - | — | 169 | - | — | | | |
| 2 | 135 | + | — | 170 | - | — | | | |
| 3 | | | | 171 | - | — | | | |
| 4 | 136 | + | — | 172 | + | — | | | |
| 6 | 137 | +++ | + | 173 | - | — | | | |
| | | | | 174 | - | — | | | |
| 6wk 2dy | 138 | + | — | | | | | | |
| | 139 to 143 all 5 had | ++ | + | | | | | | |
| 8 | 144 | ++ | + | 175 | - | — | | | |
| | 145 | ++ | + | | | | | | |
| 10 | 146 | ++ | + | 176 | + | — | | | |
| | 147 | +++ | + | | | | | | |
| 12 | 148 | + | — | 177 | + | — | | | |
| | 149 | ++ | + | | | | | | |
| 14 | 150 | ++ | + | 178 | - | — | | | |
| | 151 | ++ | + | | | | | | |
| 15 | | | | | | | 152♂ | + | — |
| 16 | 153 | ++ | + | | | | | | |
| | 154 | ++ | + | | | | 164 | + | — |
| 18 | 155 | ++ | + | | | | | | |
| | 156 | +++ | + | | | | | | |
| 20 | 157 | ++ | + | | | | | | |
| | 158 | ++ | + | | | | | | |
| 22 | 159 | ++ | + | | | | | | |
| | 160 | ++ | + | | | | | | |
| 23 | | | | | | | 165 | ++ | + |
| | | | | | | | 168♂ | + | — |
| 24 | 161 | ++ | + | | | | | | |
| | 162 | + | — | | | | | | |
| 26 | 163 | ++ | + | | | | 166 | + | — |
| 29 | | | | | | | 167 | + | — |
| 31 | | | | | | | | | |

Table 5-5 Rat experiments 5 and 6 liver histology results

Liver lesions were graded on a scale of - to ++++. A positive acidophil cell reaction was taken as ++ or more. The acidophil cell scoring system is explained in appendix 2.

just over 6 weeks into the experiment showed no abnormalities. From 8 weeks after inoculation onwards, all of the rats showed at least some evidence of chronic murine pneumonia, mainly foci of lymphoid cells at the larger airways. Larger areas of lymphoid cells and lymphoid cuffing of bronchi were not common and were only seen in some of the older rats, from 20 weeks after inoculation.

5.7.4 Biochemical Results

In this experiment the blood levels of alkaline phosphatase, AST, ALT, urea, bilirubin, total protein, albumin, globulin and cholesterol were assayed for most of the rats.

The range of ALT and AST levels of the Sprague Dawley rats, of experiments 5 and 6, were noticeably higher than those of the experiment 4 Wistar rats.

The ALT levels were within the range of 45-90 IU/l except for a lower level of 17 IU/l in dog 135 and three slightly higher ones (106, 101 and 99 IU/l) in rats 136, 143 and 144 at 4, just over 6 and 8 weeks postinoculation respectively. However, these were all exceeded by the ALT levels of three of the experiment 6 control rats (section 5.8.3).

In this experiment the AST results were between 135 and 240 IU/l, except for the levels in rat 136 (309 IU/l), rats 139-143, rat 154 (293 IU/l) and rat 163 (288 IU/l) at 4, just over 6, 16 and 26 weeks postinoculation respectively. But of these, only rats 139-141, killed just over 6 weeks after inoculation, had higher levels (384, 405 and 353 IU/l) than the 4-week control rat of experiment 6 (see section 5.8.3).

As in rat experiment 4 (section 5.6.4), the AP levels were high in the youngest animals and tended to decrease with age. Although the early levels (568, 769 and 603 IU/l at

2, 4 and 6 weeks postinoculation) in these Sprague Dawley rats were much higher than those of the experiment 4 Wistar rats, the uninoculated Sprague Dawley rats of experiment 6 were later found to have even higher early levels (see section 5.8.3). The differences may therefore be strain-related. From 10 weeks into the experiment the AP levels remained between 120 and 365 IU/l, not so different from those of experiment 4.

### 5.7.5 Conclusion

Characteristic lesions of CACH were produced in rats from 6 weeks after inoculation with a liver preparation from an experimentally-infected dog. It would appear that infection which had already been passaged once in dogs was still transmissible to rats. Although five of the smallest baby rats disappeared shortly after inoculation, the remaining runt did not exhibit an acidophil cell reaction when killed at that time. In this experiment rats were inoculated at a very early age, when they were much smaller then those in previous experiments and the losses may have been due to other growth-stunting problems, maternal attack, or the trauma of the inoculation procedure. One young rat developed an injection site abscess. In the rest of the rats CACH appeared to be a mild, subclinical infection.

The mild, chronic murine pneumonia apparent in most of the rats was considered to be unrelated to the inoculation with dog liver since similar lesions were found in the uninoculated young rats of experiment 6 (section 5.8.2). The initial lack of lesions was presumably due to the extreme youth of the rats.

Once again, the biochemical results were inconclusive.

## 5.8 RAT EXPERIMENT SIX

### 5.8.1 Experiment Design

The aim of this experiment was to investigate whether there was any evidence of horizontal transmission of CACH from the rats of experiment 5 (section 5.7) to their dams. Two later litters from these females were also investigated, as they were the only young Sprague Dawley rats available for comparison with the rats of experiment 5, as rough controls. Two adult male rats which had lived with the dams were also postmortemed.

Unfortunately the first male rat caged with the four dams, of the experiment 1 rats, failed to sire any offspring and so was removed after about 9 weeks of contact. A second adult, male Sprague Dawley was then put into the breeding cage. One of the dams was later found dead, but a few days later two of the remaining three littered down together, 18½ weeks after the start of rat experiment 5. The ten rats from these two litters, of six and four, were not given anything and were culled at the same ages as the inoculated rats of experiment 5 had been. Individual rats were culled for comparison to the 1½-, 2- and 4-week culls of inoculated rats. There was also one rat culled at 3 weeks. Two rats were culled as 6-week controls, after which the fortnightly culls all consisted of only one rat. The last one was culled for comparison to the experiment 5 rat culled 14 weeks after inoculation. All these young rats remained clinically normal during the experiment.

The four adult females had been in contact with their inoculated litters until weaning, just over 5 weeks after the inoculation of their offspring (see section 5.7.1). The adult male, which failed to breed with them, had been in contact with the females for about 9 weeks when he was killed, 15 weeks after experiment 5 started. He had never been in contact with their inoculated litters. As mention-

fp267

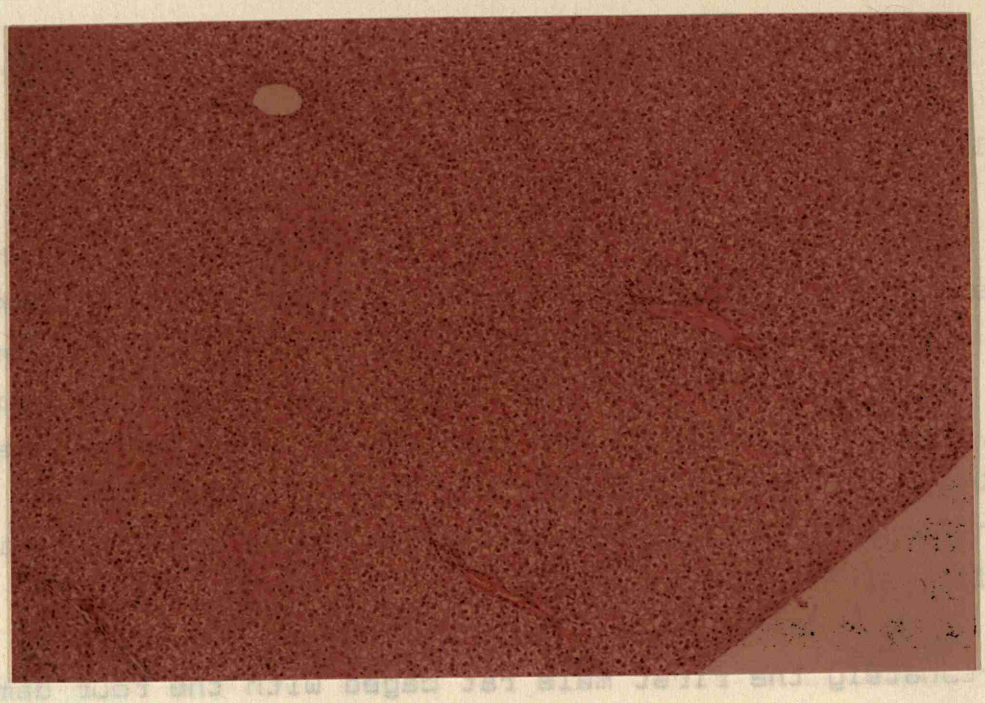

Fig 5-24 Liver of 6-week control rat 173

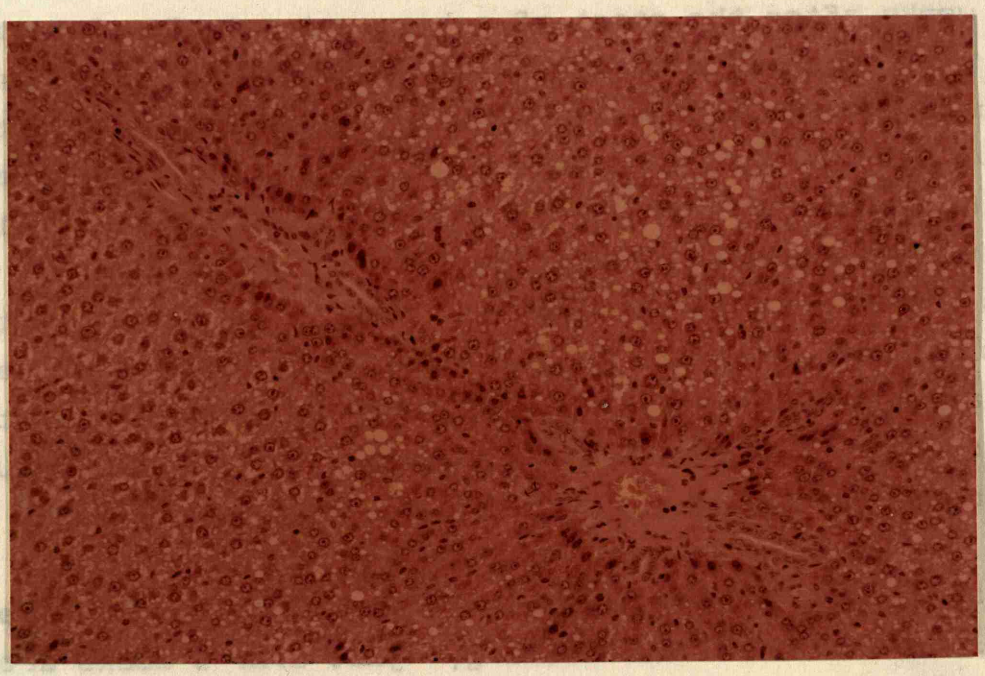

Fig 5-25 Liver of rat 165, an in-contact dam

ed earlier, one of the females was found dead just over 18 weeks from the start of experiment 5. The other female which failed to breed again was euthanased due to illness, just over 23 weeks after the inoculation of her experiment 5 litter. She was thin, hunched and in respiratory distress, with a dirty rear end, a red occular discharge, internal haemorrhage in the left eye and ulceration of the right eye. The sire of the two litters used in experiment 6 was killed on the same day, after just over 8 weeks of contact with the females. The two remaining females, which produced the litters used in this experiment, were killed 29 and 31 weeks after the inoculation of their earlier litters in experiment 5.

Blood samples, collected for serum separation, were also subjected to biochemical analysis whenever volumes permitted.

### 5.8.2 Histological Results

None of the young rats in this experiment exhibited positive acidophil cell reactions. They had only very few, or small numbers of acidophil cells in their livers, mainly in ones, twos or in small groups. Figure 5-24 shows a typical example. These results are given in table 5-5.

The small numbers of acidophil cells found in the livers of the two adult male rats were not sufficient to constitute positive acidophil cell reactions. Only small numbers of acidophil cells were found in the livers of three of the adult females. However rat 165, culled 23 weeks after the inoculation of her litter, exhibited enough acidophil cell lesions to be considered a positive reaction as can be seen in figure 5-25 (see table 5-5).

The lung sections of the young rats all showed evidence of chronic murine pneumonia, apart from the 3- and 4-week culls. Lesions were very mild, with only a slight increase

in severity in the later culls.

Both of the adult male rats showed evidence of chronic murine pneumonia. The first adult female, postmortemed 19 weeks after arrival in the colony, had only a few scattered lymphocytes in her lung sections. The next one, killed 24 weeks after purchase, had a few small foci of lymphoid cells. Lung lesions were considerably increased in the last two adult females, killed 29½ and 31½ weeks after arrival.

5.8.3 Biochemical Results

The blood levels of alkaline phosphatase, AST, ALT, urea, bilirubin, total protein, albumin, globulin and cholesterol were measured in most of the rats. No blood samples were collected from the first adult female and male rats to be postmortemed.

As in experiment 5 (section 5.7.4), alkaline phosphatase levels in the young rats were generally high initially, reducing with age. Although the highest levels (823, 833 and 717 IU/l) were in rats 172-174 culled 4 and 6 weeks into the experiment. Two of the three adult females sampled had AP levels below 200 IU/l, as did the second adult male. The female which had been ill when killed (rat 165) had a much higher AP level of 597 IU/l.

Rat 172, culled 4 weeks into the experiment, had a much higher AST level (340 IU/l) than the rest of the young rats and the four adults sampled. One of the latter, rat 165 euthanased due to ill health, had a low level of 55 IU/l. The rest had levels between 145 and 240 IU/l.

Three of the young rats, culled between 3 and 6 weeks into the experiment, had higher ALT levels (112, 132 and 151 IU/l) than any of the experiment 5 inoculated rats (section 5.7.4). The ALT levels of the other young rats and the

adults' levels were between 19 and 102 IU/l.

Blood levels of the other parameters measured were unremarkable.

5.8.4 Conclusion

The fact that no acidophil cell reactions were seen in the young, uninoculated Sprague Dawley rats, suggests that the characteristic lesions produced in experiment 5 were the result of the inoculation with dog liver.

Although one positive acidophil cell reaction was seen in the dams of the experiment 5 litters, the number of rats involved was too small to draw definite conclusions from. It does, however, give a little support to the single positive result of experiment 3 (section 5.5.2) and again suggests that CACH may possibly be transmissible horizontally and that this merits further investigation.

The evidence of chronic murine pneumonia in the young rats and the adult male rats, supports the theory that the disease is endemic in the colony and that its presence in the experiment 1 rats was unrelated to their inoculation with dog liver. The fact that the lung lesions in the bought-in females increased in severity the longer they had been in the colony, suggests that they may only have been exposed to infection when introduced to the colony.

The biochemical results of this experiment suggest that much of the generally higher levels of experiment 5 Sprague Dawley rats compared to those of experiment 4 Wistars, may be due to differences between rat strains. The results are otherwise inconclusive.

# CHAPTER 6 MOUSE AND GUINEA PIG TRANSMISSION EXPERIMENTS

## 6.0 INTRODUCTION

This chapter covers three canine acidophil cell hepatitis transmission experiments: one performed in mice and two performed in guinea pigs. The mouse experiment and the first of the guinea pig experiments commenced at the same time as rat experiment 1 (section 5.3) and all three used the same canine source case to attempt transmission of CACH to laboratory animals.

Three groups, each of ten juvenile mice, were involved in the mouse experiment (section 6.2). One group was inoculated with a preparation of thawed liver from the dog with chronic CACH, another group was given thawed serum and a control group was not given anything.

There were only two groups of animals in the first guinea pig experiment (section 6.3). Four adults were given some of the liver inoculum used in the first mouse and rat experiments. Two adult controls were not given anything..

The second guinea pig experiment (section 6.4) consisted of two parts. The first part was an attempt to transmit CACH from rats to guinea pigs. This would be a third experimental passage of the infection since the rats had been infected with material from an experimentally-infected dog, in rat experiment 5 (section 5.7).Four neonatal littermates were inoculated with a thawed preparation of pooled rat liver, this had previously been used fresh in dog experiment 3 (section 4.5). Four age-matched controls, from two different litters, were not given anything. In the second part of the experiment, evidence of horizontal transmission to the in-contact dam, of the inoculated litter, was looked for. The dam of one of the control litters was used as a control.

## 6.1 MATERIALS AND METHODS

The mice and guinea pigs used in these experiments were all bred in-house.

The inocula used were subjected to the same bacteriological and virological examinations as the inocula used in the other species of experimental animals (section 2.2.3). Inoculation was always performed subcutaneously, in the scruff of the neck.

Blood samples were collected for serum extraction, as described in section 2.2.4. The postmortem protocol followed is given in section 2.2.8 and tissue samples were processed for light microscopy as described in section 2.3.

## 6.2 MOUSE EXPERIMENT ONE

### 6.2.1 Experiment Design

The same canine liver preparation as was used in rat experiment 1 (section 5.3), was inoculated into mice in an attempt to transmit canine acidophil cell hepatitis from dogs to mice. The source of this liver was dog A/FC1, a field case, whose clinical and histological details were given in sections 3.2.1 and 5.3.2 respectively.

The thirty male, albino Balb/c mice used, were all 6½ weeks of age at the start of the experiment. Ten mice were inoculated with 0.4ml of the 20% weight:volume suspension of liver in phosphate-buffered saline. Ten mice were inoculated with 0.4ml of thawed serum from dog A. Ten control mice, not given anything, were housed in a different room from the inoculated mice.

All of the mice appeared clinically normal throughout the

fp273

Fig 6-1 Liver of 4-week nil control mouse 49 (H&E x87.5)

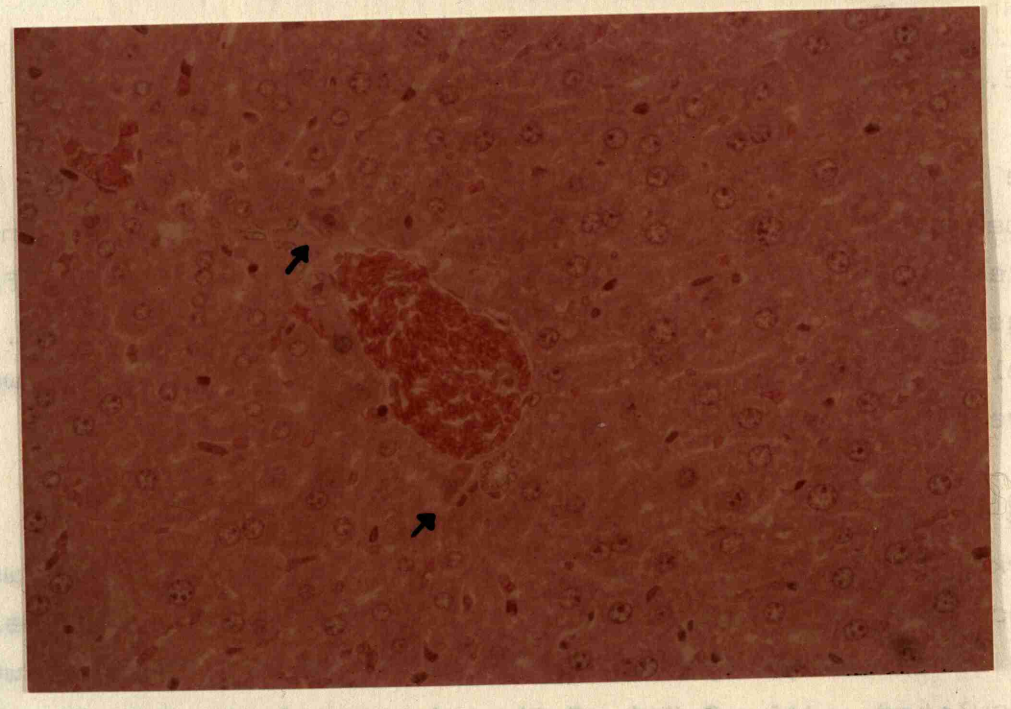

Fig 6-2 Liver of 4-week liver-inoculated mouse 30 (H&E x350) (↗) individual ACs at tiny portal tract

6-week course of the experiment. They were culled weekly from 1 week after inoculation. The first two culls consisted of one mouse from each group. Later culls consisted of two mice from each group.

### 6.2.2 Results

Histological examination of the control group failed to reveal any acidophil cell reactions in their livers. Half of them, including one of each of the double culls, did not exhibit any acidophil cells at all. The others had virtually none: only one to four acidophil cells in one or two of their three liver sections. Figure 6-1 shows an example of the liver histology of one of the 4-week controls. The control mice therefore differed from the control rats, in which very few to a few acidophil cells per liver section were commonly seen.

Microscopy of the liver-inoculated group revealed no definite acidophil cell reactions. Mice culled between 1 and 3 weeks after inoculation were similar to controls, with either no acidophil cells or only one to four in some of their liver sections. The two mice culled 4 weeks into the experiment had more acidophil cells in their livers than their two time-matched controls, although they were still in only very small numbers. Figure 6-2 shows a very mildly affected portal tract in one of them. The mice culled 5 weeks after inoculation had barely more acidophil cells than their time-matched controls. Six weeks into the experiment the livers of the liver-inoculated rats were similar to those of the control group.

The livers of the mice killed 1 and 2 weeks after inoculation with serum were similar to those of their controls. One of the mice culled 3 weeks into the experiment had a few more acidophil cells in its liver than either of its two time-matched controls. Both of the mice culled 4 weeks after inoculation exhibited a lot more

fp274

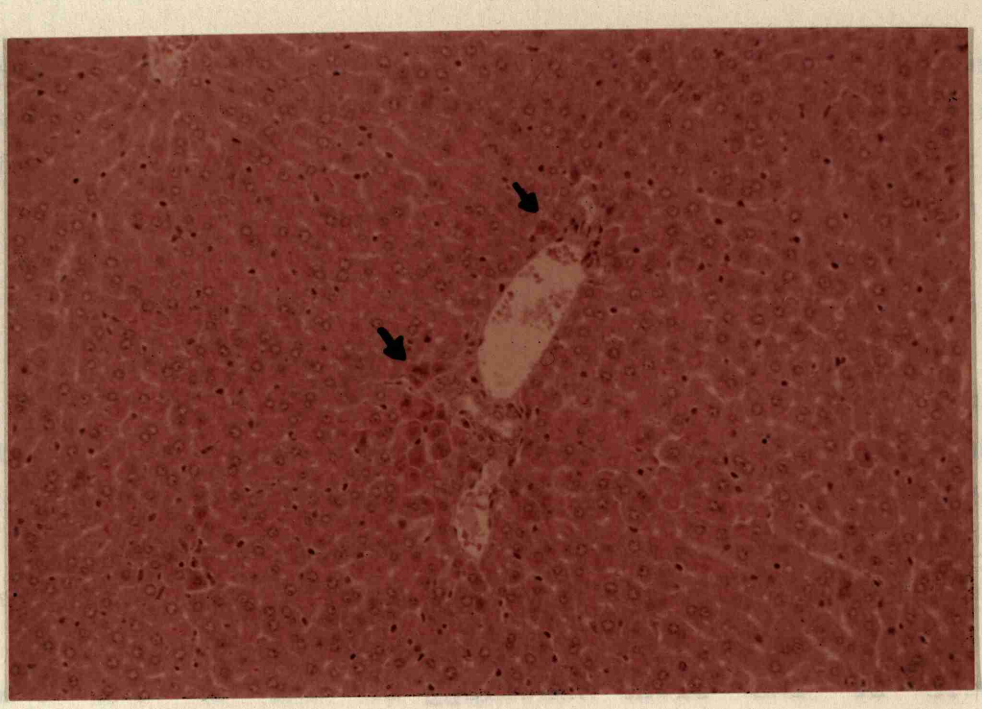

Fig 6-3 Liver of 4-week, serum-inoculated mouse 40 (H&E x175) (↘) ACs

lesions than control mice, having small numbers of acidophil cells around some portal tracts (see fig. 6-3). The 5-week culls had more acidophil cells in their livers than their time-matched controls, but less than the serum-inoculated mice culled at 4 weeks. By 6 weeks after inoculation the mice still had more acidophil cells than their controls, but less than the 4- and 5-week serum-inoculated mice. These results are displayed in table 6-1.

Microscopical examination of at least two lung sections, from different lobes, per mouse revealed no lesions in the 1-, 2- and 3-week controls and in one of the 4-week pair. The other control mouse in the 4-week cull and both of those culled 5 weeks into the experiment, had only a tiny group of lymphoid cells in one lung section each. One of the 6-week controls had a few small areas of loose lymphoid cell infiltration; the other had no lung lesions.

In the group given the liver preparation, the 1- and 4-week culls and one each of the 3- and 5-week culls had no lung lesions. The mouse culled 2 weeks after inoculation with liver and one each of the 3-, 5- and 6-week culls had only one or two tiny groups of lymphoid cells in one lung section each. The other mouse of the 6-week cull had only a few very small areas of lymphoid infiltration in both lung sections.

Histology of the lungs of the serum-inoculated mice failed to reveal any lesions in the 2-, 3- and 5-week culls. Only one or two very small areas of lymphoid infiltration were seen in the 1- and 4-week culls and one of the 6-week pair. The other mouse culled 6 weeks after inoculation with serum had a few small lung lesions.

No other abnormalities were detected in any of the other organs of the mice in this experiment.

| Wks post inoc. | LIVER inoculated | | | SERUM inoculated | | | CONTROLS | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mouse No. | AC score | Result | Mouse No. | AC score | Result | Mouse No. | AC score | Result |
| 1 | 25 | - | — | 35 | - | — | 45 | - | — |
| 2 | 26 | - | — | 36 | - | — | 46 | - | — |
| 3 | 27 | - | — | 37 | + | — | 47 | - | — |
| | 28 | - | — | 38 | - | — | 48 | - | — |
| 4 | 29 | - | — | 39 | +++ | + | 49 | - | — |
| | 30 | + | — | 40 | +++ | + | 50 | - | — |
| 5 | 31 | - | — | 41 | + | — | 51 | - | — |
| | 32 | - | — | 42 | ++ | — | 52 | - | — |
| 6 | 33 | - | — | 43 | + | — | 53 | - | — |
| | 34 | - | — | 44 | + | — | 54 | - | — |

Table 6-1 Mouse experiment 1 liver histology results

Liver lesions were graded on a scale of - to +++++, which cannot be compared directly to the scales used in other species. A positive acidophil cell reaction was taken as +++ or more. The acidophil cell scoring system is explained in appendix 2.

### 6.2.3 Conclusion

Extremely mild acidophil cell lesions, characteristic of CACH were produced in a very small number of mice inoculated with serum from a dog with chronic CACH. Control mice did not exhibit these lesions. Liver inoculation failed to produce any definite acidophil cell reactions, although two of the mice did have a few more acidophil cells in their livers than controls. The liver lesions produced in the serum-inoculated mice were of much lesser magnitude than those seen in the liver-inoculated rats of rat experiment 1 (section 5.3), but the control mice also had much fewer acidophil cells in their livers than control rats. It would appear that whereas normal rats often have a few acidophil cells in their livers, normal mice tend to have none or virtually none. The mouse liver lesions were considered as positive or negative results by comparison to the mouse controls. They were not on the same severity scale as the rat lesions. The results of this experiment suggest that CACH may possibly have been transmitted experimentally, with serum, from dog A to mice. However, the numbers involved were too small to draw definite conclusions from.

A few of the mice of each group exhibited a few very small areas of lymphoid infiltration in their lungs. Due to the similarity between groups, these exceedingly mild lesions were considered to be unrelated to any CACH infection. Chronic murine pneumonia would appear to be present in the resident rat colony (section 5.3.3) and the mild lung lesions may be related to this, since mice are known to be much less susceptible to infection with Mycoplasma pulmonis than rats (Holmes, 1984a).

## 6.3 GUINEA PIG EXPERIMENT ONE

### 6.3.1 Experiment Design

The canine liver preparation used in the first rat and mouse experiments (sections 5.3 and 6.2 respectively) was also inoculated into adult guinea pigs in an attempt to transmit canine acidophil cell hepatitis to them. The clinical and histological details of dog A/FC1, the source of the liver, were given in sections 3.2.1 and 5.3.2 respectively.

Four guinea pigs were inoculated with 2.5ml of 20% wt:vol suspension of liver in PBS. Two guinea pigs were not given anything and were kept in a different cage as controls.

The next day, two of the inoculated guinea pigs were very dull and holding their heads to one side. Firm, subcutaneous swellings could be palpated in their scruffs. The other two appeared slightly dull. One of the inoculated animals was found dead 2 days after inoculation; two more were dead by the following day. Postmortems were performed on all three animals and tissue samples were collected for histology. Within a week of inoculation the only surviving guinea pig was again bright but had a firm, brazil nut-sized, subcutaneous mass in its scruff. It was initially sensitive to palpation of the mass which gradually reduced in size and defined into two very small, firm, painless masses. By 3 weeks after inoculation this guinea pig also had a pair of 3cm diameter, spherical masses in its anterior abdomen. It appeared to be slightly dull and losing weight, but its body temperature had remained within the recognised normal range of 37.2-39.5°C (Manning et al., 1984; Harkness and Wagner, 1989a). This sole survivor of the inoculated guinea pigs and one of the controls were killed 4 weeks into the experiment. The other control guinea pig was not killed. At that time the inoculated animal was noticeably smaller than both

fp277

Fig 6-4 Liver of 4-week liver inoculated guinea pig 9

Fig 6-5 Liver of 4-week control guinea pig 10 (Mass tri
x87.5) numerous ACs at small portal tracts

controls; it weighed 582g while they weighed 772g and 794g.

### 6.3.2 Results

Histological examination of the livers of the three early fatalities revealed small numbers of acidophil cells at a few portal tracts. After comparison with later guinea pigs these were not considered to be exhibiting positive acidophil cell reactions. The liver of the last of the inoculated guinea pigs showed substantial numbers of acidophil cell lesions; several portal tracts had small numbers of acidophil cells in their limiting plates and a few had large numbers (see figure 6-4). This appeared to be a positive acidophil cell reaction when compared with the guinea pigs which died at the start of the experiment. These results are given in table 6-2. The guinea pig that died 2 days after inoculation and one of the next days fatalities both had a few small granulomas in their livers. The latter also had several granulomas in its mesenteric lymph node.

The control guinea pig, culled 4 weeks into the experiment, was also found to have substantial numbers of acidophil cell liver lesions, although slightly less than the inoculated animal (see figure 6-5). These could also be termed a positive acidophil cell reaction. A few small granulomas were seen in one of the liver sections.

Two lung sections per guinea pig, from different lobes, were examined. One or two foci of lymphoid cells were seen in the lungs of all of the guinea pigs in this experiment, except for one of those which died 3 days after inoculation. However, the latter had a few small granulomas in its lung sections, this was not the animal which had granulomas in its liver sections. The guinea pig culled 4 weeks after inoculation and its control also had a few granulomas in their lung sections.

| Time post inoculat. | LIVER inoculated | | | CONTROL | | |
|---|---|---|---|---|---|---|
| | Guinea pig No. | AC score | Result | Guinea pig No. | AC score | Result |
| 2 days | 6 | ++ | − | | | |
| 3 days | 7 | ++ | − | | | |
| | 8 | + | − | | | |
| 4 weeks | 9 | ++++ | + | 10 | ++++ | + |

Table 6-2 Guinea pig experiment 1 liver histology results

Liver lesions were graded on a scale of - to +++++, which cannot be compared directly to the scales used in other species. A positive acidophil cell reaction was taken as +++ or more. The acidophil cell scoring system is explained in appendix 2.

The abdominal masses in the last of the inoculated guinea pigs were found, at postmortem, to be abscesses with a strong capsule enclosing thick creamy green pus. A mixed culture containing Pasturella multocida was isolated from the latter. There was an adhesion between part of the omentum and the left mass. The right one had adhesions to two bits of intestine and one liver lobe, it was firmly connected to the dorsal abdominal wall. The uterus appeared to run up to the masses and the oviducts were stretched over their surfaces. No ovaries were detectable. Each kidney was partially adherent to its respective mass. Microscopy of the abscesses revealed a dense fibrous capsule around granulation tissue and some compressed remnants of ovarian tissue, surrounding lots of neutrophils and necrotic material. The animal also exhibited cystic dilation of the tubular endometrial glands of the uterus. Abscesses are relatively common in guinea pigs (Harkness and Wagner, 1989b) and cystic ovaries are known to occur (Wagner, 1976). The lesions in this guinea pig may have been the result of suppuration superimposed on the latter condition. The control had an apparently normal reproductive tract.

The 4-week cull had a small, irregular cellular area in its subcutaneous fat, at the site of inoculation. It consisted almost entirely of foamy macrophages, full of phagocytosed material, and irregular fibrous bands, with no evidence of suppuration.

None of the guinea pigs had any other abnormalities in any of the tissues examined.

### 6.3.3 Conclusion

All of the guinea pigs in this experiment, including the control, had granulomas in either their lungs or livers or both. The degree of organisation of these, suggested that they had been present for longer than the duration of the

experiment. It would therefore appear that these adult guinea pigs had some subclinical infection at the start of the experiment. The early fatalities may have been due to an exacerbation of this by the reaction at the site of inoculation; they were not thought to be due to CACH infection. Guinea pigs are highly susceptible to environmental factors which lower their resistance to infection, including experimental procedures (Holmes, 1984c). Alternatively the guinea pigs may have been more susceptible, to a low level of bacterial contamination of the inoculum, than the rats and mice inoculated with the same material. Although one guinea pig survived the initial illness, it was already losing weight when culled 4 weeks after inoculation and a suppurative process appeared to have taken over the ovaries. Whether this was also ongoing at the start of the experiment, or whether it was related to the inoculum is not known.

Many more acidophil cell lesions were present in the liver of this guinea pig than in those of the early fatalities, but no conclusions can be drawn about any CACH infection due to the roughly similar degree of acidophil cell pathology in the liver of the control guinea pig and the unknown health status of the animals at the start of the experiment.

Perivascular lymphoid nodules are a frequent finding in guinea pig lungs and appear to enlarge with age (Wagner, 1976). The occasional foci of lymphoid cells seen in the lungs of the inoculated guinea pigs, as well as the control, were therefore considered to be unrelated to the inoculation of dog liver. Evidence of chronic murine pneumonia has been seen in the experimental rats and mice, but although guinea pigs may occasionally carry Mycoplasma pulmonis, the causative agent, they are not clinically affected (Harkness and Wagner, 1989c).

## 6.4 GUINEA PIG EXPERIMENT TWO

### 6.4.1 Experiment Design

A preparation of pooled rat livers was inoculated into neonate guinea pigs in an attempt to transmit canine acidophil cell hepatitis from rats to guinea pigs. The same preparation had been used in dog experiment 3 (section 4.5). The in-contact dam of the inoculated litter and a control were also investigated, for any evidence of horizontal transmission between guinea pigs. Albino Dunkin-Hartley guinea pigs were used in this experiment.

Liver from four rats from experiment 5 (rats 138, 139, 142 and 143), killed just over 6 weeks after inoculation with liver from experimentally-infected dog D (section 5.7), had been pooled, homogenised in tris-buffered saline to form a 40% wt:vol preparation, frozen and stored at -20°C for less than a month. Some of this was thawed and diluted with TBS to a 20% wt:vol suspension, which was then clarified in a bench centrifuge. The supernatant was used for inoculation.

Four 1-day-old guinea pigs were each inoculated with 0.5ml of the clarified liver preparation, split between two sites in the scruff. Four control guinea pigs were not given anything and were culled at the same ages as the inoculated ones had been, although not at the same time. Guinea pigs tend to have much smaller litters than rats and mice. The in-house breeding program could not supply sufficient numbers of neonates, at one time, to allow the groups to run simultaneously. The two control litters, of two guinea pigs each, were born at different times after the inoculated litter.

The day after inoculation each guinea pig had a slight thickening in its scruff. These were massaged daily and although initially the thickenings increased slightly,

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Fig 6-6 Liver of 8-week control guinea pig 19
(Mass-OG ×87.5)

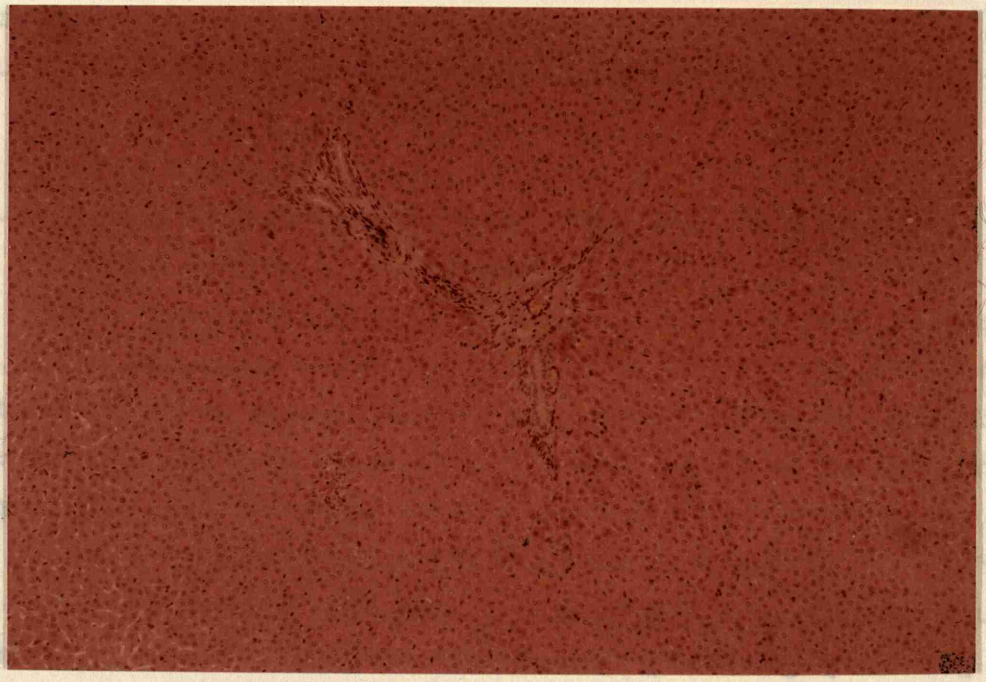

Fig 6-7 Liver of 8 week liver-inoculated guinea pig 14
(H&E ×87.5)

they soon resolved. The guinea pigs then remained clinically normal for the rest of the experiment. All of the controls and the two dams appeared clinically normal throughout the experiment. Individual guinea pigs were culled fortnightly from 4 to 10 weeks after inoculation. Although culled later, there was one age-matched control for each of the inoculated guinea pigs. The dam of the inoculated litter was culled 12 weeks after the start of the experiment, a fortnight after the last of her litter was culled. The dam of the first of the two control litters was culled, as a control for her, at the same length of time postpartum. The blood samples collected for serum separation, were also subjected to haematological and biochemical analyses.

### 6.4.2 Histological Results

Histology of the livers of the control guinea pigs failed to reveal any positive acidophil cell reactions. There were only small numbers of acidophil cells, mainly in ones, twos and small groups in a few limiting plates (see figure 6-6).

The livers of the guinea pigs culled 4, 6 and 10 weeks after inoculation with rat liver had only very few to a few acidophil cell lesions, mainly as ones and twos. The guinea pig culled 8 weeks after inoculation had slightly more acidophil cell lesions in its liver, mainly in small groups in limiting plates (figure 6-7); sufficient to be considered a postive acidophil cell reaction in comparison with the others and the controls (table 6-3).

Although the in-contact dam had slightly more acidophil cells in her liver (figure 6-8) than the control dam, these were insufficient to be termed a definite acidophil cell reaction (table 6-3).

| Weeks post inoculat. | LIVER inoculated | | | CONTROL | | |
|---|---|---|---|---|---|---|
| | Guinea pig No. | AC score | Result | Guinea pig No. | AC score | Result |
| 4 | 12 | + | — | 18 | + | — |
| 6 | 13 | - | — | 16 | ++ | — |
| 8 | 14 | +++ | + | 19 | ++ | — |
| 10 | 15 | + | — | 17 | + | — |
| | IN-CONTACT DAM | | | CONTROL DAM | | |
| 12 | 20 | ++ | — | 21 | - | — |

Table 6-3 Guinea pig experiment 2 liver histology results

Liver lesions were graded on a scale of - to +++++. A positive acidophil cell reaction was taken as +++ or more. The acidophil cell scoring system is explained in appendix 2.

Fig 6-8 Liver of in-contact dam, guinea pig 20 (Mass-06 x87.5)

Histological examination of one lung section per guinea pig, revealed no lymphoid foci in the animal culled 4 weeks after inoculation and only one each in the 6- and 8-week culls. The control guinea pigs all had a few small foci of lymphoid cells in their lung sections. Both dams had numerous small lymphoid foci scattered through their lungs.

### 6.4.3 Haematological and Biochemical Results

All the guinea pigs in this experiment had normal haematological profiles.

In guinea pigs alanine aminotransferase is an extremely insensitive and nonspecific marker of hepatocellular injury (White and Lang, 1989). This is due partly to its low total activity in guinea pig hepatocytes and partly to its low cytoplasmic and higher mitochondrial distribution (Clampitt and Hart, 1978). Serum alkaline phosphatase decreases with increasing body weight in guinea pigs,

comparable to the decrease with maturity observed in other species (White and Lang, 1989). During clotting, their blood cellular elements release gamma glutamyl transferase into the serum (White and Lang, 1989).

Altough raised ALT levels were detected in the last two inoculated guinea pigs culled, similar levels were also found in some of the controls. Blood sample volumes permitted the biochemical analyses to be performed on both serum and plasma for comparison. Variations in levels of biochemical parameters were usually small, with serum tending to give higher results. Only AST levels tended to show marked differences, with serum levels often much higher than those in plasma. Levels of GGT were not elevated and serum did not usually give noticeably higher results than plasma, despite White and Lang's statements (1989).

### 6.4.4 Conclusion

Only one acidophil cell reaction was seen, 8 weeks after inoculation with pooled, experimental rat livers. No definite conclusion can be drawn from such a small group of animals, but CACH may possibly be transmissible from rats to guinea pigs. The infection had already been experimentally passaged once in dogs, before transmission to the rats.

The lymphoid foci in the lungs, which were more numerous in the controls, were considered to be unrelated to the inoculation with rat livers.

The use of a clarified preparation and smaller inoculum volumes may have helped reduce the reaction problems at the inoculation site.

# CHAPTER 7 HAMSTER TRANSMISSION EXPERIMENTS

## 7.0 INTRODUCTION

This chapter covers attempts to transmit canine acidophil cell hepatitis from dogs to hamsters. Of the five experiments described, only the first two actually involved inoculation of hamsters with material from infected dogs. Those two experiments and experiment 3, their control, ran in conjunction with each other; neonates were incorporated into the three studies as they became available from the breeding program (described in section 7.1). Although the three studies could have been considered as three parts of the same experiment, they were described individually in an effort to clarify the rather complex situation. A brief discussion of the clinical biochemistry of hamsters (section 7.2) is given before the sections on the transmission experiments.

In hamster experiment 1 (section 7.3) transmission of CACH to sixteen neonates was attempted by inoculation with liver preparations from an experimentally-infected dog, which was also the source case for rat experiment 5 (section 5.7). During the same period, transmission of CACH from a canine field case to twenty-six neonatal hamsters was attempted in experiment 2 (section 7.4). This source case had already been used for the first rat, mouse and guinea pig experiments (sections 5.3, 6.2 and 6.3 respectively). Hamster experiment 3 (section 7.5) ran at the same time as experiments 1 and 2 and produced fifteen age-matched controls for the animals in them.

As in the experiments in other species, it was considered inadvisable to return to the general breeding stock animals which had been in contact with inoculated animals. The in-contact parents of the hamsters of experiments 1 and 2 were therefore investigated in experiment 4 (section 7.6) for any evidence of horizontal transmission of CACH. There were eight adults in the in-contact group. A control group was formed from the two parents of the

control litters of experiment 3 and six adults which had failed to breed and had not been in contact with any of the other hamsters.

The fifth experiment (section 7.7) investigated the possibility of horizontal and vertical transmission of CACH between hamsters. It made use of three groups of hamsters. One group consisted of seven later offspring of the in-contact parents of the experiment 1 and 2 hamsters. Only one of the seven had also been in contact with the inoculated animals, it had been born while some of the previous, inoculated litter were still in the parental cage. Two later offspring of the parents of the experiment 3 control litters, born after the end of that experiment, were used as a control group. They and the seven in the previous group were all part of the first generation from the breeding program. The remaining group consisted of four second generation hamsters. One was the offspring of an experiment 1 inoculated hamster, it had also been in contact with some of its dam's inoculated siblings. The other three were offspring of the later offspring of the in-contact parents of inoculated animals; neither they nor their parents had had any direct contact with inoculated hamsters, only their grandparents had been in contact with the hamsters of experiments 1 or 2.

## 7.1 MATERIALS AND METHODS

A breeding program was set up to produce neonatal Syrian hamsters for use in the transmission experiments. It involved eight breeding cages: seven of which contained one male and one female each, the other cage contained two females and a male. These three and one of the pairs were all in-house hamsters; the other six pairs had been bought in. They were all young adults. Hamsters are considered to be useful experimental animals for various reasons, including their relative freedom from spontaneous diseases

and their susceptibility to many introduced pathogenic agents (Van Hoosier Jr and Ladiges, 1984). They are also far more shock resistant than guinea pigs (Harkness and Wagner, 1989a). Neonate hamsters are a useful experimental animal system for arenavirus studies (Howard, 1986 p5).

The same bacteriological and virological examinations as were done on the inocula used in the other species (section 2.2.3), were performed on those used in the hamsters. The bacteriology results for the different preparations are given in the appropriate sections. Attempted culture of canine adenovirus and other recognised canine viral pathogens always failed; no viruses were isolated from any of the liver preparations used in the hamster experiments. Inoculation was always subcutaneous, in the scruff of the neck.

The technique used for blood collection, for serum extraction, was described in section 2.2.4. Biochemical analyses were performed on the serum samples when volumes permitted. Occasionally there was not enough serum for some or all of the usual analyses to be performed. Obviously no blood samples were available from the few hamsters which were found dead. The postmortem protocol in section 2.2.8 was followed and tissues were processed for light microscopy as described in section 2.3.

The breeding program produced litters of variable sizes at different times. To accumulate adequate numbers of hamsters for culling at a reasonable number of intervals after inoculation, several successive litters had to be inoculated with liver preparations from the same dog. Since the inoculated litters were still suckling and in contact with their parents, the inoculum used in the first litter of each cage determined what further litters from that cage were given. In practice, two breeding cages became dedicated to one source dog and two cages were dedicated to another. One cage was designated as a source

of uninoculated controls. The remaining three pairs failed to produce any offspring, even after new pairings were tried with two of them.

Weaned hamsters were removed to single sex cages whenever space permitted, sometimes they had to be mixed with other litters, which had been inoculated with material from the same dog as themselves. Offspring from the eight cages of breeding hamsters were termed first generation hamsters. Any offspring of first generation females were termed second generation hamsters, regardless of whether they were the result of father/daughter matings or sibling matings.

The adult hamsters were prone to killing and eating their offspring when stressed and occasionally even when left undisturbed. Litter abandonment and cannibalism of the young are common with hamsters, especially during the first week postpartum (Harkness and Wagner, 1989a). To reduce the risk of this happening, inoculation was usually not carried out until the second or third day after birth, although occasionally 1-day old or 4-day-old hamsters were inoculated. Despite this, some litters were still killed and eaten by their parents immediately after inoculation; details of these are not given. Occasionally individual animals went missing further into experiments, there was no way of knowing whether these animals had been victims of aggression which were subsequently eaten, or whether they had died of other causes before being eaten.

## 7.2 HAMSTER CLINICAL BIOCHEMISTRY

Studies on hamster blood chemistry have received little attention in the literature. Normal values have been published for a number of hamster blood constituents but the effects on these of disease or varying physiologic states are not well documented (Wardrop and Van Hoosier

Jr, 1989).

As in several other species, serum levels of alkaline phosphatase show age-related changes in hamsters; young animals have two to three times the levels of older animals (Dent, 1977; Eugster et al., 1966; Thomas et al., 1979). In one study which used hamsters as a model for virus-induced hepatitis, AP appeared to be a more sensitive indicator of liver damage than bilirubin or ALT (South and Jeffay, 1958).

Alanine aminotransferase levels have been shown to rise in hamsters in association with both acetaminophen-induced and viral-induced hepatic necrosis (Rollinson and White, 1983; El-Hage et al., 1983).

Aspartate aminotransferase is found in highest concentration in hamsters in skeletal muscle, heart muscle and liver. The level in serum has been shown to increase with neoplastic involvement of the liver and may also become elevated with hepatic metabolic change (Eugster et al., 1966).

Bilirubin can be a useful indicator of liver function in hamsters (Wardrop and Van Hoosier Jr, 1989).

The blood urea level is a commonly used indicator of renal function in most laboratory animals, including the hamster.

## 7.3 HAMSTER EXPERIMENT ONE

### 7.3.1 Experiment Design

The aim of this experiment was to attempt transmission of CACH from dogs to hamsters, by inoculation with liver preparations from an experimentally-infected dog. The

source of the liver used was dog D [dog 8 in Jarrett and O'Neil (1985)]. It was later also used as the source for rat experiment 5 (section 5.7) and its clinical and histological details are given in section 5.7.2.

Some of dog D's liver had been thawed and homogenised, then clarified by spinning for 10 minutes in a bench centrifuge before spinning the supernatant for 10 minutes at 10 000 rpm in an ultracentrifuge at 4°C. The preparation had then been frozen and stored at -20°C. Eight months after preparation some of it was thawed and three 2-day-old hamsters were each inoculated with 0.05ml, split between two sites in the scruff. They were culled individually 4, 6 and 10 weeks after inoculation.

Six weeks after the start of that experiment, a litter of two hamsters was born in the same breeding cage. They were both inoculated, at 4 days of age, with 0.02ml of a freshly prepared, stomached and clarified 20% weight: volume preparation of dog D's liver in tris-buffered saline. The remainder was frozen and stored at -20°C. No bacteria were seen in smears of this preparation and only a single colony of bacteria was isolated after prolonged incubation. One of these two hamsters was eaten during the second week of the experiment. The other one was culled 18 weeks after inoculation.

A week after its preparation, an aliquot of the new liver inoculum was thawed and six 3-day-old hamsters were each inoculated with 0.02ml of it. One of these was eaten by its parents 3½ weeks after inoculation and another disappeared 12½ weeks into the experiment. The remainder were culled individually 4, 6, 10 and 14 weeks after inoculation.

Another aliquot of the same liver preparation was thawed 5 weeks after it was made and three 4-day-old hamsters were inoculated with 0.02ml each. They were culled 6, 10 and

14 weeks after inoculation.

Seven weeks after its preparation, yet another aliquot of the same liver inoculum was thawed and two 2-day-old hamsters were each given 0.02ml of it. One of these disappeared 3 weeks into the experiment, the other was culled 4 weeks after inoculation. These two hamsters were in contact with the last two hamsters, 35 (who was later eaten) and 36, of an earlier inoculated litter which were still living in the breeding cage.

Of the sixteen neonatal hamsters, from five litters, inoculated with dog D's liver twelve survived to be culled for the experiment. Three were lost within a few weeks of inoculation and one was lost much later.

### 7.3.2 Histological Results

Histological examination of three liver sections per hamster, failed to reveal any acidophil cell reactions. Only one or two acidophil cells were seen at one or two portal tracts in most sections, some sections contained none at all. These results are given in table 7-1.

Examination of one lung section per hamster failed to reveal any abnormalities, in all but one case. Hamster 31, the 4-week cull of the third litter to be inoculated, exhibited a mild degree of subclinical pneumonia with areas of fluid-filled or collapsed alveoli, neutrophil infiltration and foamy macrophages. Like guinea pigs, hamsters may occasionally carry Mycoplasma pulmonis but are not usually clinically affected (Harkness and Wagner, 1989c).

Microscopy of the other tissues of the hamsters in this experiment did not reveal any abnormalities.

### 7.3.3 Biochemical Results

As expected, the alkaline phosphatase levels were highest in the youngest animals, generally dropping with age. All of the 4-, 10- and 14-week culls, for which results were obtained, had higher levels of AP than their age-matched control hamsters of experiment 3 (section 7.5.3). Of the three hamsters culled 6 weeks after inoculation, only one had a higher level than the two 6-week controls; the other two surpassed only one of their two controls. The hamster culled 18 weeks into the experiment only had a higher AP level than one of its two controls.

Only one of the hamsters, culled 10 weeks after inoculation with dog D's liver, had an ALT level outside the range of the majority of the experiment 3 controls (section 7.5.3). At 124 IU/l, its level was however lower than the single high result of the 26-week control.

Two of the hamsters in this experiment had AST levels higher than those of the experiment 3 control hamsters. Hamsters 3 and 23, culled 10 and 18 weeks after inoculation, had AST levels of 208 IU/l and 283 IU/l respectively.

The bilirubin, gamma glutamyl transpeptidase and blood urea levels in the hamsters of this experiment were all unremarkable.

### 7.3.4 Conclusion

No evidence of transmission of CACH from experimental dog D to hamsters, by inoculation with liver preparation, was found.

## 7.4 HAMSTER EXPERIMENT TWO

### 7.4.1 Experiment Design

In this experiment transmission of CACH from dogs to hamsters was again attempted, but by inoculation with liver preparations from a canine field case. The source of liver was dog A, the 8-month-old, male German shepherd dog used in the first rat, mouse and guinea pig experiments (sections 5.3, 6.2 and 6.3 respectively). Those experiments all commenced on the same day and used the same preparation of liver. The hamster experiments started two years later and involved inoculation with different preparations of dog A's liver. The clinical and histological details of dog A are given in sections 3.2.1 and 5.3.2 respectively.

Five grams of dog A's liver had been thawed and homogenised in 10ml of TBS to form a 50% wt:vol preparation. After 10 minutes clarification in a bench centrifuge at a medium setting, the supernatant had been further clarified by 10 minutes centrifugation at 10 000 rpm in an ultracentrifuge at 4°C. The resulting supernatant had been frozen and stored at -20°C for 6 months. An aliquot of this was thawed and six 2-day-old hamsters, from one litter, were inoculated with 0.02ml each. No bacteria could be isolated from this preparation. One hamster disappeared around 3 weeks after inoculation. The remainder were culled individually 6, 10, 14, 18 and 22 weeks after inoculation.

A fortnight after the inoculation of that litter, another aliquot of the same liver preparation was thawed and seven 3-day-old hamsters were each inoculated with 0.02ml of it. One of them disappeared during the first week, the remainder were culled individually 4, 6, 10, 14, 18 and 22 weeks after inoculation.

A new preparation of dog A's liver was made for use in the rest of this experiment. One gram of liver was thawed, chopped in a drop of TBS and then stomached in 5ml of TBS to form a 20% wt:vol preparation. This was clarified for

fp294

Fig 7-1 Liver of hamster 24, 4 weeks post dog A's liver (H&E tr. x175) prominent AC reaction at many portal tracts

10 minutes in a bench centrifuge at a medium setting and then the supernatant was spun for a further 10 minutes at 10 000 rpm in an ultracentrifuge at 4°C. Nine 2-day-old hamsters were inoculated with 0.02ml each of the resulting supernatant. The remainder was frozen and stored at -20 °C. Only a very sparse culture of staphylococci were isolated from this preparation. One of the baby hamsters disappeared around 3 days after inoculation, the rest were culled individually 1, 2, 4, 6, 10, 14, 18 and 22 weeks after inoculation.

A fortnight after its preparation, the 20% wt:vol liver suspension was thawed and four 1-day-old hamsters were inoculated with 0.02ml each. These second generation hamsters were culled individually at 2, 4, 6 and 10 weeks of age. This litter had been born in a cage of weaned hamsters. The dam was experimental hamster 19, one of the first litter inoculated with liver from dog A. She must have mated with her father just before she was removed from the breeding cage at 7 weeks of age.

A total of twenty-six hamsters, from four litters, were inoculated with liver preparations from dog A: twenty-three were culled during the experiment, two were lost within a few days of inoculation and one within a few weeks.

### 7.4.2 Histological Results

Microscopy of the livers of the hamsters in this experiment failed to reveal any positive acidophil cell reactions. Very few or no acidophil cells were seen in most of the sections. One hamster each from the 4- and 6-week culls had slightly more acidophil cells than the others but still only very small numbers. See figure 7-1 for an example of one of the 4-week rats. The results are tabulated in table 7-1.

| Weeks post inoc. | Expt. ONE | | | Expt. TWO | | | Expt. THREE | | |
|---|---|---|---|---|---|---|---|---|---|
| | DOG D LIVER inoculated | | | DOG A LIVER inoculated | | | CONTROL | | |
| | Ham. No. | AC score | Result | Ham. No. | AC score | Result | Ham. No. | AC score | Result |
| 1 | | | | 49 | - | — | 66 | - | — |
| 2 | | | | 50 | - | — | 67 | - | — |
| | | | | 62 | - | — | 68 | - | — |
| 4 | 1 | - | — | 24 | + | — | 37 | - | — |
| | 31 | - | — | 51 | - | — | 69 | - | — |
| | 61 | - | — | 63 | - | — | | | |
| 6 | 2 | - | — | 16 | + | — | 38 | - | — |
| | 33 | - | — | 26 | - | — | 70 | - | — |
| | 45 | - | — | 52 | - | — | | | |
| 10 | 3 | - | — | 18 | - | — | 71 | - | — |
| | 34 | - | — | 27 | - | — | 72 | - | — |
| | 46 | - | — | 53 | - | — | | | |
| | | | | 65 | - | — | | | |
| 14 | 36 | - | — | 19 | - | — | 39 | - | — |
| | 47 | - | — | 28 | - | — | 40 | - | — |
| | | | | 54 | - | — | | | |
| 18 | 18 | - | — | 20 | - | — | 41 | - | — |
| | | | | 29 | - | — | 73 | - | — |
| | | | | 55 | - | — | | | |
| 22 | | | | 21 | - | — | 42 | + | — |
| | | | | 30 | - | — | | | |
| | | | | 56 | - | — | | | |
| 26 | | | | | | | 43 | + | — |

Table 7-1 Hamster experiments 1, 2 and 3 liver histology Results

Liver lesions were graded on a scale of - to +++++, which cannot be compared directly to the scales used in other species. A positive acidophil cell reaction was taken as +++ or more. The acidophil cell scoring system is explained in appendix 2.

No abnormalities were detected in the lung sections of the hamsters in this experiment.

At postmortem an abnormality was found in hamster 26, the 6-week cull of the second litter to be inoculated. There were abdominal adhesions in the right side of the dorsal abdomen involving parts of the intestines, two liver lobes, the right kidney, abdominal muscles and subcutaneous tissues. Microscopy confirmed that rupture of the abdominal muscles had apparently occurred some time previously allowing adhesions to develop between some abdominal organs and the underside of the skin; there were some fibrous scars in the surrounding muscles . This did not appear to have affected the hamster clinically and was considered to be the result of a traumatic injury, unrelated to inoculation.

No other abnormalities were detected in the tissue sections from the hamsters in this experiment.

### 7.4.3 Biochemical Results

As in experiment 1, the alkaline phosphatase levels tended to be highest in the younger animals. All of the hamsters culled 10 and 14 weeks after inoculation, for which results were obtained, had higher AP levels than their age-matched controls of experiment 3 (section 7.5.3). Two of the three hamsters culled 4 weeks after inoculation, half of the 2-week culls and only one of the three hamsters culled at 22 weeks surpassed the AP levels of their time-matched controls. Two of the three hamsters culled 18 weeks into the experiment and only one of the four culled at 6 weeks had higher AP levels than one of their two controls.

Four of the hamsters inoculated with dog A's liver had ALT levels higher than the range of the majority of the experiment 3 controls (section 7.5.3). Two of these,

culled 4 and 6 weeks after inoculation, were surpassed by the unusually high ALT level of the 26-week control. The other two, who surpassed all of the controls, were a hamster culled at 10 weeks with an ALT level of 217 IU/l and one culled 14 weeks into the experiment with an ALT level of 243 IU/l.

Seven of the hamsters in this experiment had AST levels greater than those of the experiment 3 control hamsters. These were the two hamsters culled 2 weeks after inoculation, two of the hamsters culled 6 weeks after inoculation and one each of those culled 10, 14 and 18 weeks into the experiment.

The bilirubin levels were all between 0 and 2μmol/l, apart from hamster 18, culled 10 weeks after inoculation, which had 19μmol/l. This hamster had also had raised ALT and AST levels.

Apart from one, the GGT levels of the hamsters in this experiment were within the same range as those of all the other hamsters tested (0-6g/l). Hamster 20, culled 18 weeks after inoculation, had a slightly higher GGT level of 11g/l.

The blood urea, bilirubin and GGT levels were unremarkable.

### 7.4.4 Conclusion

There was no histological evidence of transmission of CACH to hamsters, by inoculation with liver from field case dog A. Although the biochemistry results were suggestive of a liver problem in some of the inoculated hamsters, they could not be considered conclusive evidence of transmission of any disease.

## 7.5 HAMSTER EXPERIMENT THREE

### 7.5.1 Experiment Design

This experiment was designed to provide age-matched controls for the hamsters inoculated in experiments 1 and 2 (sections 7.3 and 7.4 respectively). Two consecutive litters from the same parents provided fifteen neonates which were not given any inoculations.

The first litter of seven control hamsters were 2 days old at the start of the experiment and were culled 4, 6, 14, 18, 22 and 26 weeks into the experiment. The 14-week cull consisted of two hamsters, the rest were single culls.

A second litter of eight hamsters were 4 days of age at the start of their experiment and were culled 1, 2, 4, 6, 10 and 18 weeks into the experiment. The 2- and 10-week culls both consisted of two hamsters, the rest were single culls.

### 7.5.2 Histological Results

Histological examination of the livers of most of the control hamsters revealed only a very few acidophil cells or none. The two oldest hamsters had small numbers of lesions but not enough to be termed positive reactions. These results are displayed in table 7-1.

No abnormalities were detected in the lung sections of most of the hamsters in this experiment. Hamster 41, the 18-week cull of the first control litter, had one small focus of proliferating, large epithelialised cells. A very few small foci of neutrophil infiltration were seen in the lung section of hamster 42, the 22-week cull of the same litter.

The only lesions detected in any of the other tissue

sections examined were uterine abnormalities in hamsters 41 and 43, the 18- and 26-week culls of the first control litter. At postmortem hamster 41 was found to have a small brown mass on the medial side of the left uterine horn, near the ovarian end, to which the omentum was adhesed. Microscopy revealed a lot of fibrinous necrotic material surrounded by granulation tissue and a fibrous capsule. This appeared to be the result of an infective process coming from outside the uterus, inwards. Several small, purple, irregular masses were visible grossly on the luminal surface of hamster 43's uterus. Histological examination indicated that these were haemangiomas.

### 7.5.3 Biochemical Results

The alkaline phosphatase levels were generally highest in the younger animals, apart from the 1-week control, and tended to fall with age. However, hamster 41 an 18-week control had a higher than expected AP level which surpassed all of the other 18-week culls and even the 14-week culls.

In this experiment, apart from one hamster, the ALT levels were all within the range of 40 to 110 IU/l. The 26-week cull had an ALT level of 184 IU/l.

The AST levels of the control hamsters were all within the range of 79-196 IU/l.

In the control hamsters the bilirubin, GGT and blood urea levels were unremarkable

### 7.5.4 Conclusion

The similarity in the numbers of acidophil cells seen in the controls to the numbers seen in the hamsters of experiments 1 and 2, supported the conclusion that there was no histological evidence of CACH transmission in those

| IN-CONTACT PARENTS of dog D inoculated litters | | | IN-CONTACT PARENTS of dog A inoculated litters | | | IN-CONTACT PARENTS of CONTROL litters | | |
|---|---|---|---|---|---|---|---|---|
| Ham. No. | AC score | Result | Ham. No. | AC score | Result | Ham. No. | AC score | Result |
| 75♂ | - | — | 81 | - | — | 84* | ++ | — |
| | | | 86♂ | - | — | 85♂ | - | — |
| | | | 87 | - | — | | | |
| 94♂ | + | — | 90♂ | - | — | Adults that failed to breed | | |
| 95 | - | — | 91 | - | — | | | |
| | | | | | | 79 | - | — |
| | | | | | | 80♂ | - | — |
| | | | | | | 88♂ | - | — |
| | | | | | | 89 | - | — |
| | | | | | | 92♂ | + | — |
| | | | | | | 93 | | |

Table 7-2 Hamster experiment 4 liver histology results

Liver lesions were graded on a scale of - to +++++. A positive acidophil cell score was taken as +++ or more. The acidophil cell scoring system is explained in appendix 2.

♂ = male *= found dead

two experiments. The incidental findings of uterine and lung abnormalities probably contributed to the abnormal biochemistry results of hamsters 41 and 43.

## 7.6 HAMSTER EXPERIMENT FOUR

### 7.6.1 Experiment Design

The aim of this experiment was to investigate whether there was any evidence of horizontal transmission of CACH from the inoculated litters to their in-contact parents. The parents of the control litters and the adult pairs which failed to breed were used as controls.

Occasionally a young inoculated hamster would disappear from its cage, this happened in all four of the breeding cages supplying hamsters for experiments 1 and 2 (sections 7.3 and 7.4). Whether they died or were killed, they were presumably then eaten by the parents. So as well as being in contact with their inoculated litters, the adults may have ingested possibly infective material. The adults in one breeding cage (hamsters 81, 86 and 87) killed and cannibalised three whole litters of newly-inoculated offspring.

### 7.6.2 Histological Results

None of the adult hamsters had acidophil cell reactions in their livers. The liver histology results are given in table 7-2.

Incidental findings in one of the liver sections from hamster 95, the dam of two of the litters inoculated with dog D's liver, were two cystic structures. These were spaces lined by flattened cells, separated from surrounding hepatocytes by a thin fibrous capsule. Polycystic disease is often seen in hamsters over one year of age

(Holmes, 1984b). In one study it was observed in 76% of such animals (Gleiser et al., 1970). The liver is a common site but cysts may also be seen in the epididymis, seminal vesicle, pancreas, renal pelvis, uterus and ovary. They appear to be developmental defects of normal ductal structures, for example bile ducts in the case of hepatic cysts, and are not known to be associated with any clinical signs. Thin-walled hepatic cysts of varying size (½ to 3cm) are typical necropsy findings. This hamster's mate, hamster 94, had liver lesions of a different kind. It had some mixed cell infiltrate in most portal tracts, mainly neutrophils, lymphoid cells and a few plasma cells. Examination of a section of intestine revealed a heavy cellular infiltrate, in the deep lamina propria, of lymphoid cells and neutrophils; while the ends of the villi were distended by large numbers of plasma cells. The liver reaction was therefore probably an ascending cholangitis.

The sire of the control litters, hamster 85, exhibited hepatic fibrosis. There was an increase in fibrous tissue at many portal tracts, with some extension into the parenchyma and delicate fibrous strands linking some portal tracts. Hepatocytes were being incorporated into areas of fibrosis in places. There were a few areas of more extensive fibrosis and hepatocyte loss, at the edges of these the parenchyma was divided up by strong fibrous portal-portal bridging. The distribution of these lesions was not uniform throughout the liver; one of the three liver sections examined had only a little fibrosis at some portal tracts and no bridging.

The dam of the control litters, hamster 84, had a little increase in fibrosis at a few portal tracts, a little mononuclear cell infiltrate at a few and slightly more acidophil cells than the other hamsters. The liver was congested since the animal had been found dead.

One of the adults which never bred, hamster 80, had an episode of diarrhoea 7 months before being culled. This had cleared up on an oral course of oxytetracycline (Terramycin soluble powder, Pfizer Ltd, Kent) administered in the drinking water. At postmortem this male was found to be much thinner than its mate. It had slightly shrunken kidneys with dimpled surfaces. Microscopy of two of its liver sections revealed fibrosis at many portal tracts, with some extension out into the parenchyma and a little portal-portal fibrous linking. In the other section there was less portal tract fibrosis and only a little extension into the parenchyma. The kidney cortex had several areas of collapse, with tubule loss and a little fibrosis. Protein-filled tubules were also evident in places. This hamster appeared to be suffering from a chronic protein-losing nephropathy.

No abnormalities were found in any of the other tissue sections of the hamsters in this experiment.

### 7.6.3 Biochemical Results

Two of the three in-contact parents of dog D-inoculated litters had higher AP levels than most of the other adults, hamster 94 with ascending cholangitis was the higher of these. Only two of the adults which had never bred had levels similar to the higher of these. One was hamster 80, which had kidney and liver lesions; the other, hamster 92, had been apparently healthy with no obvious reason for a raised AP level.

The levels of ALT found in the in-contact and control parents appeared to be similar. They were all roughly within the range of levels found in the experiment 3 control litters (section 7.5.3).

The AST levels of the in-contact parents of the inoculated litters were lower than the levels of five of the six

other adults for which results were available.

In this experiment the bilirubin, GGT and blood urea levels were unremarkable.

Apart from two of the hamsters which had never bred, the blood urea levels were within the range of levels found in all the other hamsters (4.7-9.7mmol/l). Only hamsters 79 and 80 had slightly higher levels of 11.6 and 10.6mmol/l respectively.

### 7.6.4 Conclusion

There was no evidence of horizontal transmission of CACH from the inoculated litters to their in-contact parents.

## 7.7 EXPERIMENT FIVE

### 7.7.1 Experiment Design

This experiment looked for evidence of vertical and/or horizontal transmission of CACH to the offspring of an inoculated hamster, the offspring of the in-contact parents of earlier inoculated litters and the second generation offspring of these. The later offspring of the parents of the control litters were used as controls. Only a small number of hamsters were investigated since earlier experiments had not shown evidence of CACH transmission.

One offspring of experimental hamster 47, the product of an unplanned father/daughter mating, was postmortemed at 6 months of age (hamster 82). This second generation hamster had had 7 weeks of contact with its dam as well as 2 weeks of contact with one of its dam's littermates (hamster 46) and 5 weeks of contact with one of her older siblings (hamster 23). Hamsters 23, 46 and 47 had all

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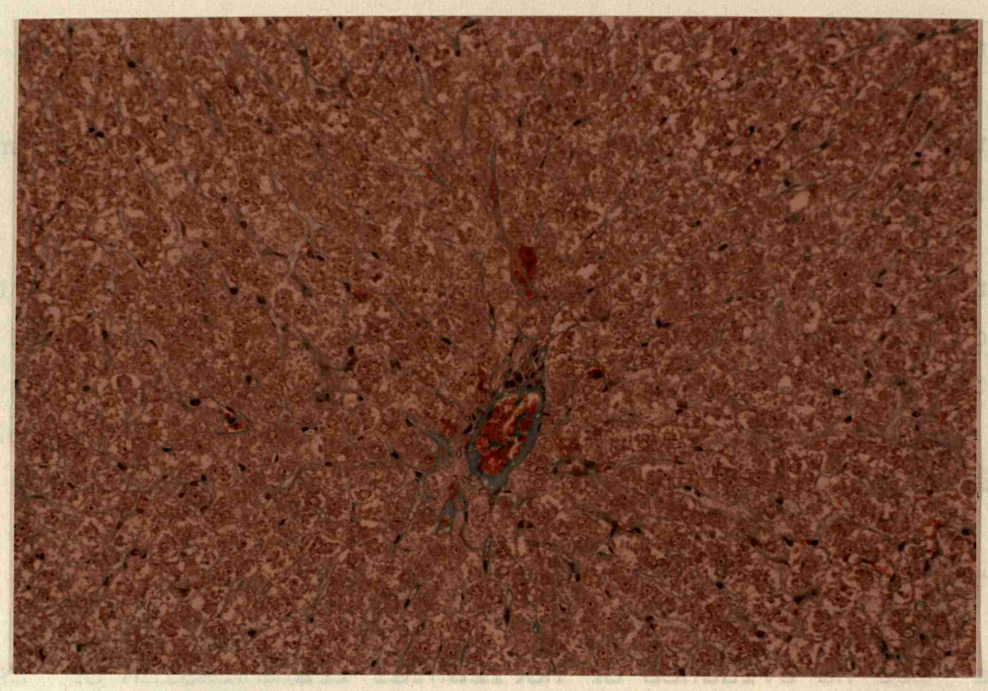

Fig 7-2 Liver of in-contact hamster 76 (Mass tri x175)

been inoculated as neonates with material from experimental dog D as part of experiment 1 (section 7.3).

Seven later offspring of the in-contact parents of the inoculated litters were investigated. Hamster 76 was the only one of these which had also been in contact with an inoculated animal. During its first week of life it had been in contact with hamster 36, which had been inoculated with a liver preparation from dog D in experiment 1 (section 7.3). Hamster 76 had 3½ months of contact with its parents (hamsters 94 and 95) before being moved, with its littermates, to a new cage. It was killed and postmortemed at 4½ months of age (see figure 7-2). Two hamsters from a different litter (hamsters 96 and 97) were culled and postmortemed at 8½ and 9 months of age respectively. Earlier litters of their parents had also been inoculated with material from dog D in experiment 1 (section 7.3) but hamsters 96 and 97 had not been in contact with any of those. They had only had 4½ weeks of contact with their parents when their dam was found dead and partially eaten. Their sire (hamster 75) was culled and postmortemed the next day. The remaining four of the seven were all offspring of one breeding pair (hamsters 90 and 91) but came from three different litters. Earlier litters of this breeding pair had been inoculated with material from dog A, as part of experiment 2 (section 7.4). Hamsters 99 and 100 had been in contact with the parents for 2 months and were killed and postmortemed at 10½ months of age. Hamster 98 had 2½ months of contact with the parents and was 7 months of age when culled. The fourth hamster (103) was culled at 2 months of age, it had only 5 weeks of contact with its sire and 7 weeks with its dam before they were killed as part of experiment 4 (section 7.6).

Three second generation hamsters were also investigated. Their grandparents were the breeding pairs, described in section 7.1, which had been in contact with the inoculated

| | | In-contacts of parents of expt 1 dog D inoculates | | | In-contacts of parents of expt 2 dog A inoculates | | | In-contacts of parents of expt 3 CONTROLS | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Ham. No. | AC score | Result | Ham. No. | AC score | Result | Ham. No. | AC score | Result |
| FIRST GENERATION | Offspring of expt 1 parents IN-CONTACT with inoc. hamsters | 76 | + | — | | | | | | |
| | Offspring of expt 1, 2 or 3 parents NEVER in-contact with inoc. hamsters | 96<br>97 | -<br>- | —<br>— | 98<br>99<br>100<br>103 | -<br>-<br>-<br>- | —<br>—<br>—<br>— | 77<br>78 | -<br>- | —<br>— |
| SECOND GENERATION | | In-contacts of offspring of expt 1 parents | | | In-contacts of offspring of expt 2 parents | | | | | |
| | Offspring of expt 1 inoculated hamster | 82 | - | — | | | | | | |
| | Offspring of uninoculated later litters | 83 | - | — | 101<br>102 | -<br>- | —<br>— | | | |

Table 7-3 Hamster experiment 5 liver histology results

Liver lesions were graded on a scale of - to +++++. A positive acidophil cell reaction was taken as +++ or more. The acidophil cell scoring system is explained in appendix 2.

litters of experiments 1 and 2 (sections 7.3 and 7.4). However, the parents of these second generation hamsters had not been in contact with any inoculated animals. Hamster 83, a runt, was killed and postmortemed as a neonate to investigate whether its lack of growth was due to a liver problem. Its grandparents' earlier litters had been inoculated with material from dog D in experiment 1 (section 7.3). The grandparents of hamsters 101 and 102 had been in contact with their dog A-inoculated offspring which were part of experiment 2 (section 7.4). These two littermates were killed and postmortemed at 4½ months of age.

Two offspring of the control breeding pair, whose earlier litters were used in experiment 3 (section 7.5), were killed and postmortemed at 2½ months of age.

### 7.7.2 Histological Results

None of the hamsters investigated exhibited positive acidophil cell reactions in their livers. They had only very few acidophil cells, mostly in ones and twos, or none per section. The liver histology results are given in table 7.3. Figure 7-2 shows an example of hamster 76's liver histology.

Histological examination of the other tissue sections of the hamsters in this experiment revealed no abnormalities. No cause for hamster 83's lack of growth was identified.

### 7.7.3 Biochemical Results

The alkaline phosphatase levels of the hamsters in this experiment were unremarkable for their ages.

Most of the ALT levels, were roughly within the range of levels found in the control hamsters of experiment 3 (section 7.5.3). Only hamster 103 had a raised ALT level

of 222 IU/l.

In this experiment only hamsters 82 and 103 had higher AST levels (204 and 340 IU/l respectively) than those of the experiment 3 controls.

The levels of bilirubin, GGT and blood urea found in this experiment were unremarkable.

### 7.7.4 Conclusion

There appeared to be no evidence of vertical or horizontal transmission of CACH in any of the hamsters investigated in this experiment. Hamster 103's raised enzyme levels were not accompanied by any histological lesions.

# CHAPTER 8 ELECTRON MICROSCOPICAL INVESTIGATIONS

## 8.0 INTRODUCTION

This chapter deals with the ultrastructural investigations of material by transmission electron microscopy. The microscope used and many of the techniques employed are described in chapter 2 (section 2.4).

The various tissues processed intact for electron microscopy, along with the findings of the examinations are discussed in section 8.1.

Dissociated cells and other free cell preparations which were processed as blocks, from which sections could be cut and examined, are covered in section 8.2. As are some of the pelleted preparations, from fluid samples, which were also embedded as blocks for sectioning.

The examination of liquid preparations, using negative staining technique, is dealt with in section 8.3. Most of this material was fractions obtained by applying various purification techniques to tissues, free cell and fluid samples.

Several investigations using immune electron microscopy are described in chapter 9 (section 9.7), with the other studies involving antibodies.

## 8.1 TISSUE SECTIONS

The processing of tissues for examination with the electron microscope is detailed in chapter 2 (section 2.4.1). Usually several blocks from each diced sample of tissue were embedded individually.

The objectives of the investigations were to attempt to visualise the aetiological agent of canine acidophil cell hepatitis and any structural changes related to the

infection. Tissue sections were therefore examined in the electron microscope at both low and high magnifications, for cellular ultrastructure and the causative agent respectively.

### 8.1.1 Tissues Collected and Processed for Electron Microscopy

Most of the tissues collected for electron microscopical investigations came from the animals of the transmission experiments, since only extremely fresh material was suitable. Material was collected from only three field dogs which, light microscopy later revealed, were not obvious CACH cases.

Blocks of liver and mesenteric lymph node were collected from the six rats culled 14 weeks into the second rat experiment (section 5.4). Only liver was collected from the six rats culled 16 weeks into the experiment. In rat experiment 4 (section 5.6) blocks of liver, from six rats killed in each of the 4, 6, 8, 10, 12 and 14-week culls, were processed for electron microscopy. Pieces of liver were collected for electron microscopical examination from the two rats killed in each of the 14, 18 and 20-week culls of the fifth rat experiment (section 5.7).

In the second guinea pig transmission experiment (section 6.4) liver was collected, for electron microscopical examination, from the animals killed 8 and 10 weeks after inoculation with pooled rat liver.

Pieces of liver, spleen, tonsil and kidney were collected from dog 33 in the first dog transmission experiment (section 4.3). Only liver (from 2 sites), spleen and tonsil were collected for electron microscopy from the other three inoculated dogs. The full list, of tissues for electron microscopical examination, given in section 2.2.8 were collected from dogs 36, 37, 38, 40 and 41 of the

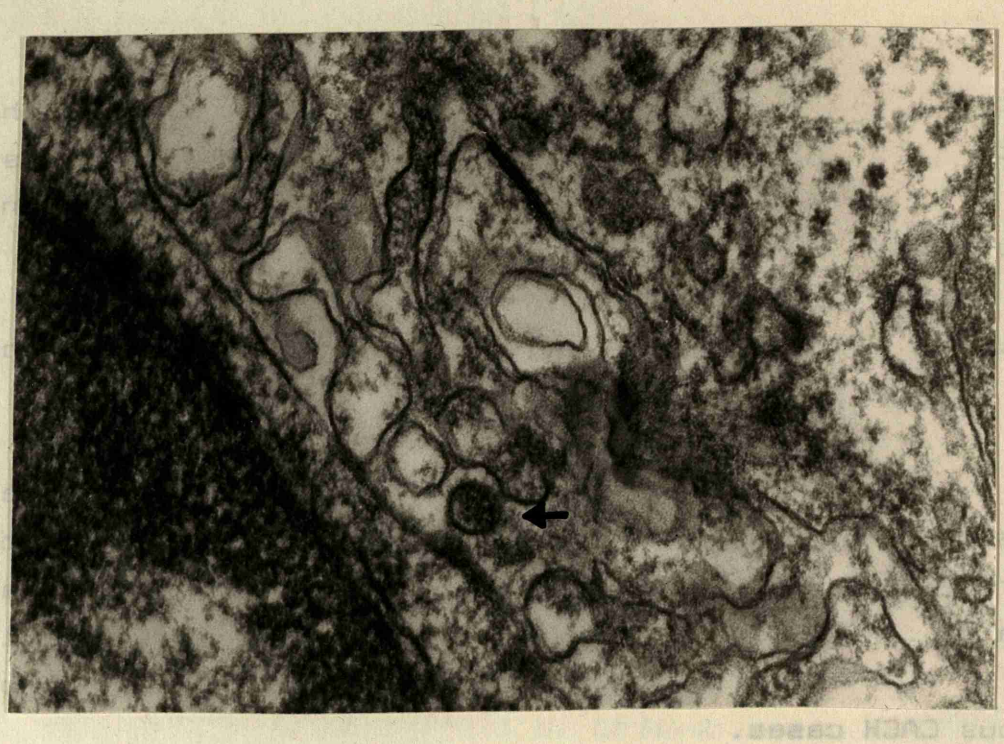

Fig 8-1 Liver of dog 38 of experiment 2 (×80 000)

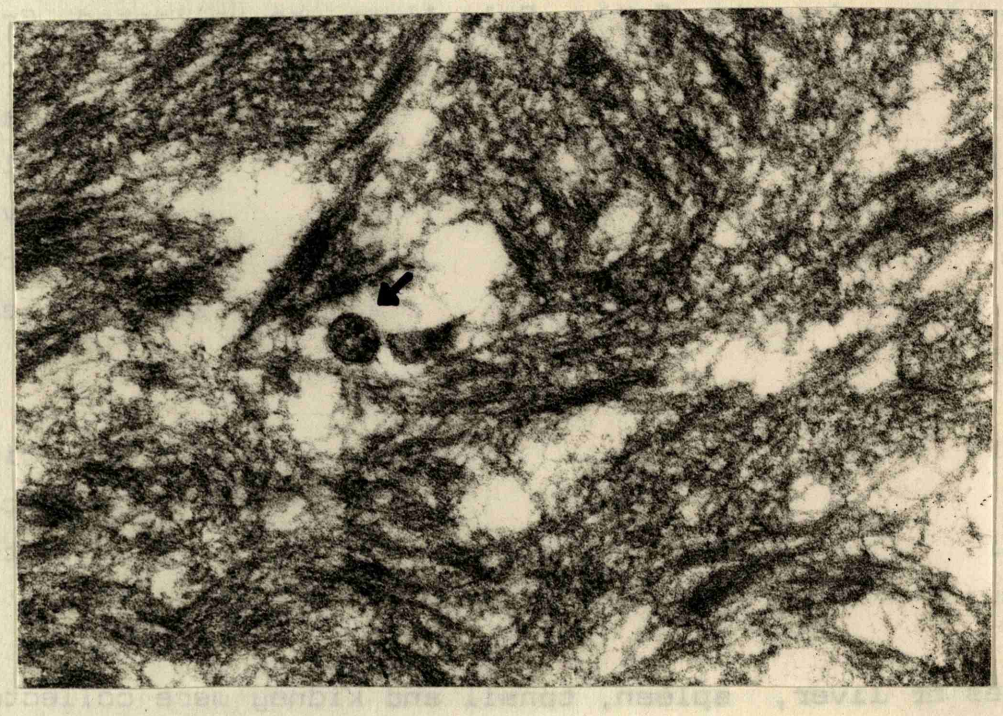

Fig 8-4 Pelleted peritoneal fluid preparation of dog 38 (×80 000)

second dog experiment (section 4.4) and dogs 42, 44 and 45 of the third experiment (section 4.5). In addition, a little of one of dog 38's adrenal glands was collected and processed.

### 8.1.2 Examination of Tissue Blocks

Unfortunately only a small sample of the tissue blocks collected and processed were examined in the electron microscope, due to the time consuming nature of the work. However, all the material processed remains available for any future investigations.

Very little of the experimental laboratory animals' tissues were studied with the electron microscope. Several sections, from more than one site, of dog 38's and 44's livers were examined and photographed; as were spleen sections from dogs 37 and 38. Mesenteric lymph node and tonsil from dog 36 were also inspected and photographed.

Although a great many photomicrographs were taken of suspicious structures, no viruses or virus-like particles were indisputeably demonstrated. Figure 8-1 shows a suspicious particle (reminiscent of some arenaviruses) in dog 38's liver, similar in appearance to ones seen in its pelleted peritoneal fluid (figure 8-4).

The hepatocellular intranuclear inclusion bodies, seen in many of the dogs with CACH, were revealed to be regular arrays of crystalline material without any obvious virions (see figures 8-2 and 8-3).

It proved difficult to locate individual acidophil cells, even when the initial thick tissue sections were searched for portal tracts to trim up for thin sectioning. Those that were found had a similar shrunken condensed appearance as seen with light microscopy. No obvious accumulations of virions were found in them. There sometimes appeared to

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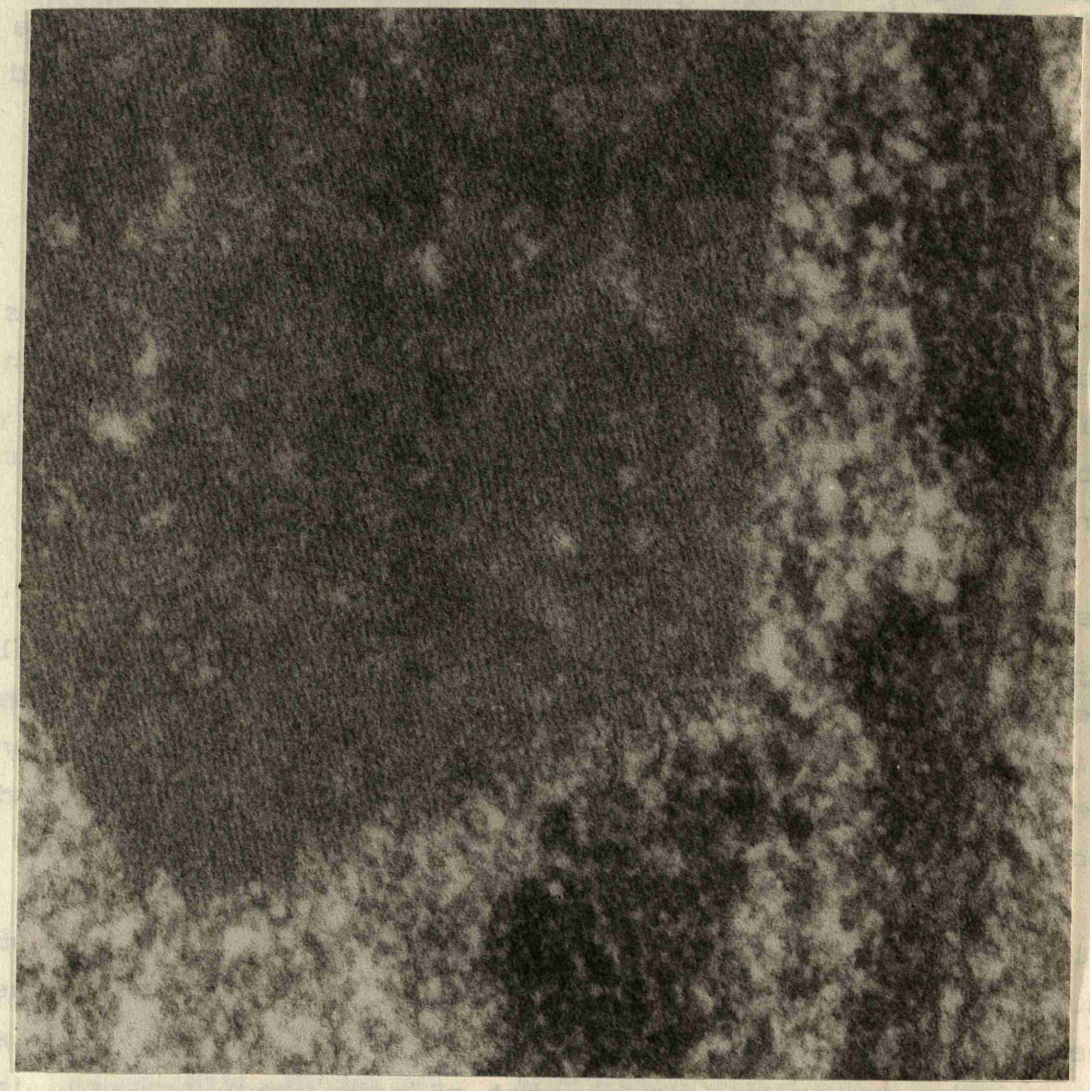

Fig 8-2 Crystalline intranuclear inclusion body in hepatocyte nucleus of CACH field case Dog G (FC 3) (x 160 000)

be less microvilli in some bile ducts in affected cases.

## 8.2 DISSOCIATED CELLS AND OTHER PREPARATIONS PROCESSED AS SOLIDS

Various cell and other preparations were processed for embedding (as described in section 2.4.2) to enable sections to be cut for examination in the electron microscope. The main object of this study was to search for the aetiological agent of CACH.

### 8.2.1 Dissociated Cells

The difficulty in locating any speculative aetiological agents in sections of solid tissue led to the inspection of preparations of dissociated cells. It was thought that if any viral budding was occurring at cell surfaces it might be more easily visualised with loosely packed dissociated cells. Various methods of dissociation and fixation of cells were tried since the most suitable, for the causative agent of CACH, were not known. Investigation under a range of conditions was designed to increase the chance of finding the agent or relevant pathology.

### 8.2.2 Buffy Coat

Samples of buffy coat were collected, as described in section 2.4.2, from the five inoculated dogs (36, 37, 38, 40 and 41) of the second dog transmission experiment and the three inoculated animals (dogs 42, 44 and 45) of the third. Several variations were tried, in an attempt to find the best method of obtaining a fixed, representative sample of buffy coat with as few red blood cells as possible.

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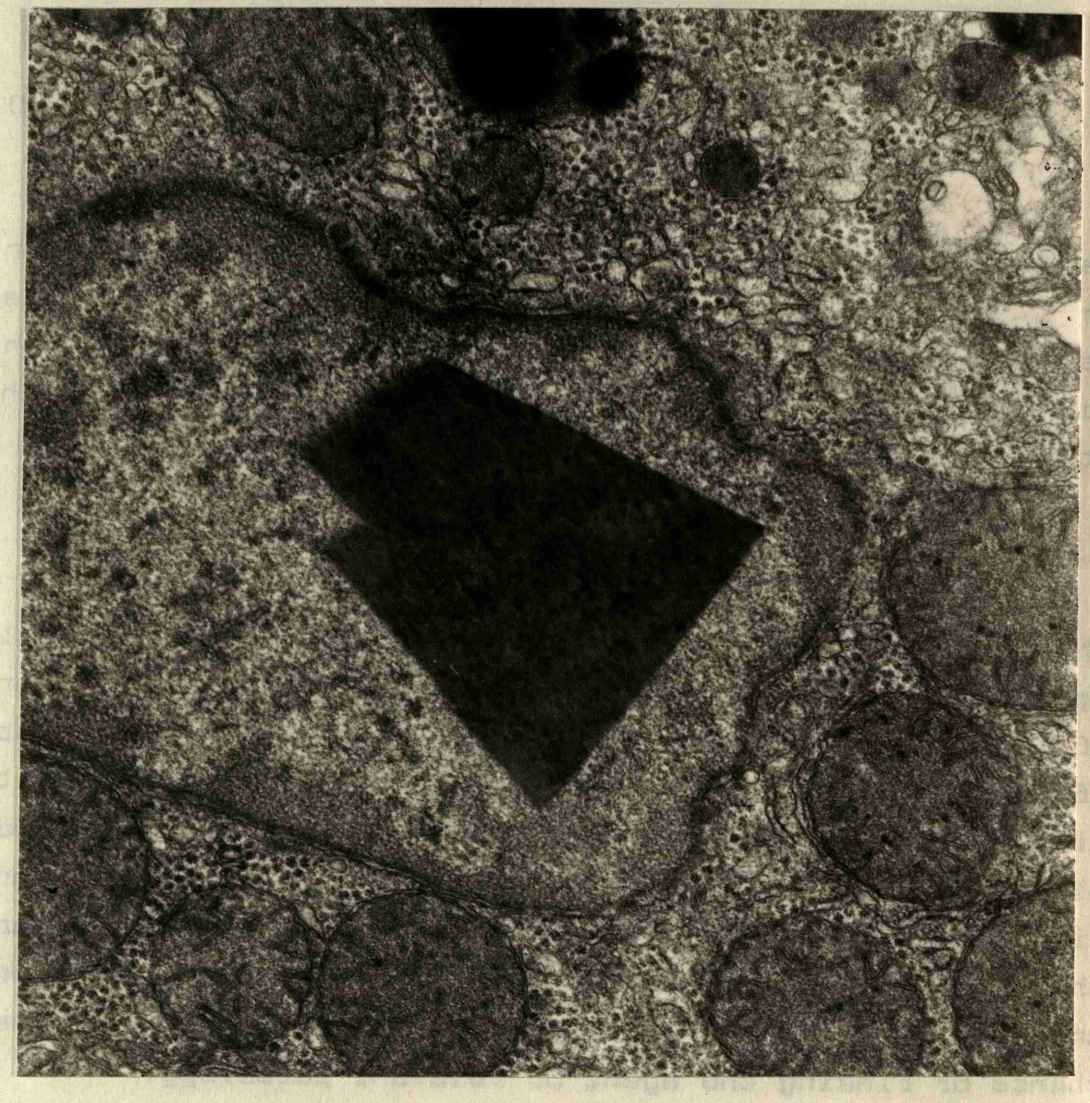

Fig 8-3 Crystalline intranuclear inclusion body in hepatocyte nucleus of experimental CACH case, Dog J (dog 18 of Jarrett & O'Neil, '85) (x30 000)

### 8.2.3 Lymphocytes

In this study the ficoll-hypaque method was used to extract lymphocytes from blood samples, it is described in section 2.4.2.

During the course of the second dog transmission experiment, lymphocyte preparations were obtained from blood samples collected from the four remaining animals (dogs 37, 38, 40 and 41), 21 weeks after their inoculation. Lymphocytes were also extracted from blood samples, collected just before death, from dogs 37, 38, 41 and 45). Smears of extracted lypmphocytes were usually made for examination by light microscopy, before samples were processed for electron microscopy in a similar way to the dissociated cell preparations.

### 8.2.4 Abdominal Fluid Preparations

The only experimental dog from which free abdominal fluid was collected at postmortem was dog 38 (see section 4.4.8). When a sample of the fluid was thawed, after being frozen for a week at -20°C, a protein clot formed. It remained free-floating when the sample was spun at 10 000 rpm for 10 minutes in the ultracentrifuge and hardly any pelleted material was visible in the base of the centrifuge tube. Half of the sample was then pipetted into a second centrifuge tube (tube 2), leaving the protein clot behind in the remainder, with any pelleted material, in tube 1. Both tubes were topped up with TBS and spun at 35 000 rpm for 90 minutes in the ultracentrifuge, resulting in the formation of obvious pellets in each. Both supernatants were collected and purified further for negative stain studies, along with the pellet from tube 2 (see section 8.3). The pellet from tube 1, which included the protein clot, was fixed in paraformaldehyde/glutaraldehyde fixative before being processed as a block, in the same way as the dissociated cell pellets (section 2.4.2), for

examination in the electron microscope. Figure 8-4 shows one of several similar suspicious particles found in the sectioned block. These were not unlike the one in dog 38's liver section (figure 8-1).

8.2.5 Cell Culture

On the day that the first rat, mouse and guinea pig experiments commenced some of the liver homogenate and serum used to inoculate the animals was also inoculated into four different cell culture lines (C127, BHK, Vero and 3T3 cells). Multiwell plates were used for each cell line and ten-fold dilutions of either dog A's liver homogenate or serum were added. Nothing was added to control wells. Unfortunately, bacterial contamination of the inocula resulted in bacterial overgrowth in the cell cultures, but samples of the three sets of each of the four cell lines were pelleted and fixed for electron microscopy 2½ weeks after inoculation.

Later attempts to culture cells from lymph nodes of experimentally-infected hamster 74 and dog 41 resulted in two, different stage, samples from each being processed for examination with the electron microscope. The idea was that if the lymphocytes were infected by the aetiological agent of CACH and they could be cultured, the agent might multiply to an identifiable level. The technique for enzymic dissociation of lymphoid tissue, adapted from Klaus (1986, p75), involved digesting diced lymph node with collagenase, neutral protease, DNAse and PHA. Dissociated cells were then plated out in RPMI cell culture medium with added foetal calf serum, glutamine, PHA and antibiotic.

## 8.3 NEGATIVE STAINS OF LIQUID PREPARATIONS

Phosphotungstic acid negative staining was applied to various liquid preparations to facilitate the examination of them, in the electron microscope, for the presence of the aetiological agent of CACH. The technique for negative staining with PTA is given in chapter 2 (section 2.4.3). Negative staining delineates particles, like viruses, by outlining their components with electron-dense material. For visualisation in the electron microscope, large numbers of virions (at least $10^6$/ml) must be present (Morgan-Capner and Pattison, 1985). When there are only small numbers of virions in a specimen, the chances of finding them with the electron microscope can be increased by concentrating them, using ultracentrifugation, or by aggregating them, with specific antibody. The latter option, termed immune electron microscopy, is dealt with in chapter 9 (section 9.7).

### 8.3.1 Isopycnic Centrifugation

Isopycnic centrifugation was the method chosen for attempts to purify the aetiological agent of CACH from tissues and body fluids of infected animals.

Unlike the separation techniques of differential pelleting and rate-zonal centrifugation, in which particle size is an important factor, isopycnic centrifugation relies solely on the densities of particles for their separation (Rickwood, 1984). During isopycnic centrifugation particles move down through a density gradient until their density matches that of the surrounding medium. Once the equilibrium point is reached the separation is not affected by prolonging centrifugation.

For isopycnic separation the maximum density of the gradient medium must not be greater than that of the particles. Of the wide range of possible media, ionic ones

(e.g. caesium salts) are generally only used for viruses, proteins and nucleic acids, while nonionic sucrose is used mainly for organelles and viruses. Both sucrose and caesium chloride gradients were used in this study; since the identity of the aetiological agent of CACH was not known, neither was the most suitable medium for its purification.

The discontinous sucrose gradient, used first in the double gradient purification method (section 2.4.3), was used mainly to concentrate the preparation to be separated. The refractive indices of collected fractions, measured before dialysis, were used to work out their densities.

### 8.3.2 Fractions from Liver Preparations

Negative staining for electron microscopy was first used, in this study, in the second dog transmission experiment. A little of dog 36's liver, collected at postmortem, was homogenised (20% wt:vol) and clarified, then PTA was used to stain the neat supernatant as well as several dilutions of it. The amount of cells and debris in the preparations made the search for virus-like particles very difficult. It was therefore decided to fractionate liver preparations, to obtain more purified extracts of suitably sized material for examination.

Density gradient centrifugation, using a 1.1 - 1.4g/cc caesium chloride gradient (as described in section 2.4.3), was used to purify some of dog 36's liver homogenate. Six areas were identifiable in the gradient and were collected as individual fractions. Electron microscopy revealed that washing with ammonium acetate had failed to remove all of the caesium, so the fractions were then dialysed overnight against PBS before another set of negative stains were made and the remainder of each fraction was frozen.

Two of the dialysed fractions, originally from the top of the gradient, were of the densities most likely to contain viruses. They were a light-scattering band and the clear top fraction. These were later thawed and purified further on caesium chloride gradients of density 1.2 - 1.5 g/cc.

Some of the 20% wt:vol liver homogenate prepared on the day of dog 36's postmortem, which had been kept at 4°C for 2 days before being frozen at -70°C, was thawed after 1 month. It was then fractionated using the discontinuous and continuous sucrose gradients method, described in section 2.4.3, for examination with the electron microscope.

Stomached preparations of liver in TBS from dogs 37, 38 and 40 (of dog experiment 2) and dogs 42 and 45 (of dog experiment 3) were also fractionated using the double sucrose gradient technique. Dialysed fractions of appropriate densities were then examined, in the electron microscope, for virus-like particles. Figures 8-5 to 8-10 show some examples of the types of particles found in material from dogs 42 and 45. The same techniques were applied to both stomached (see figure 8-11) and homogenised preparations of pooled rat liver from rats 140 and 141, as well as the pooled liver homogenate (from rats 138, 139, 142 and 143) used to inoculate the experimental animals of the third dog and second guinea pig transmission experiments (see figures 8-12 to 8-14).

A caesium chloride gradient (1.1 - 1.4g/cc) was used to fractionate liver, from dog 41, which had been stomached in TBS. Continuous caesium chloride gradients were also used to fractionate liver preparations from normal dog C (control dog 35 of dog experiment 1) and a CACH field case (FC6, described in section 3.2.6). Fractions from the latter were also pelleted to concentrate them for a second examination in the electron microscope.

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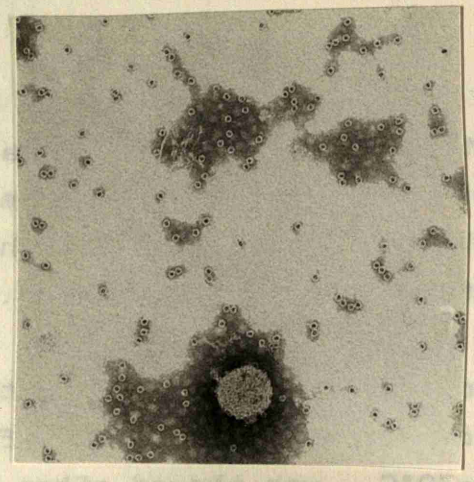

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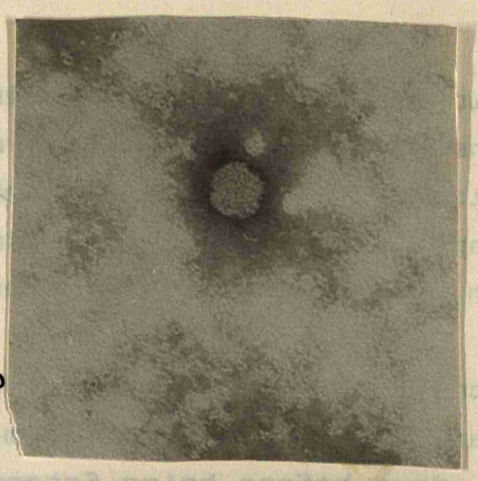

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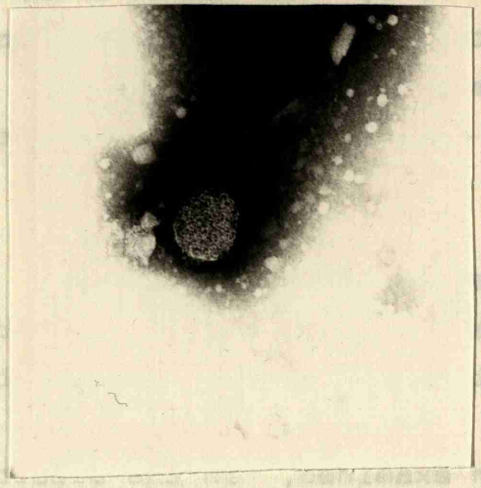

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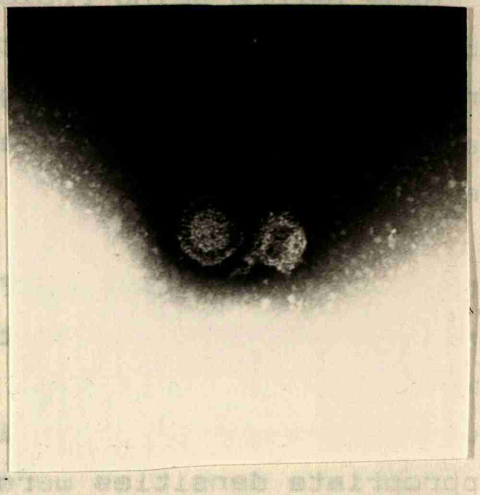

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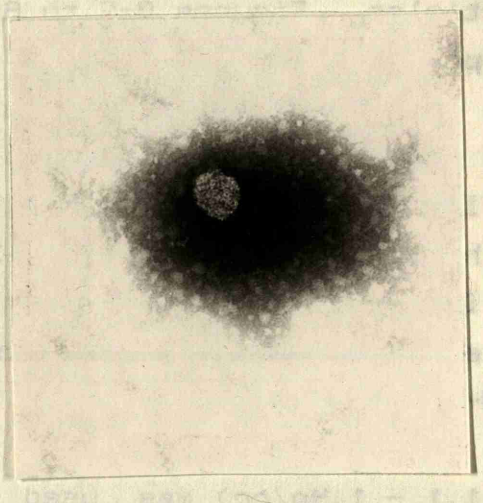

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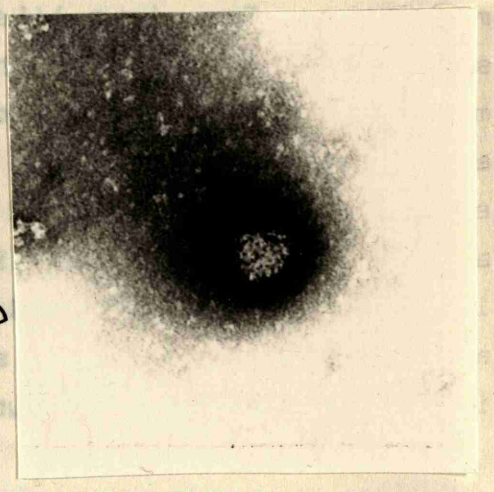

Fig 8-5 Exptl. CACH dog 42 Liver fract 3 (x80 000)
Fig 8-6 Experimental CACH dog 42 fract 4 Liver (x 126000)
Fig 8-7 Exptl dog 45 Liver fract. 2 (x80 000)
Fig 8-8 Dog 45 Liver fract. 2 (x 80 000)
Fig 8-9 Dog 45 Liver fraction 2 ( x80000)
Fig 8-10 Dog 45 Liver fract. 3 (x126 000)

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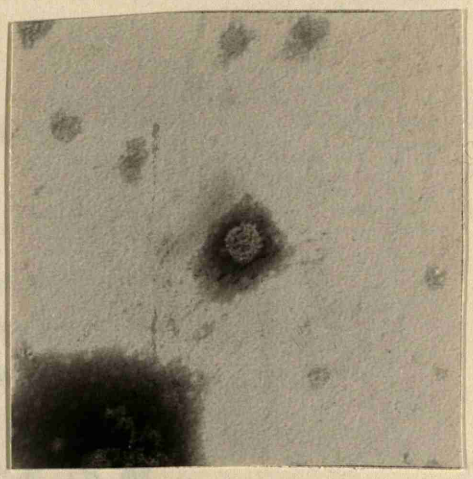

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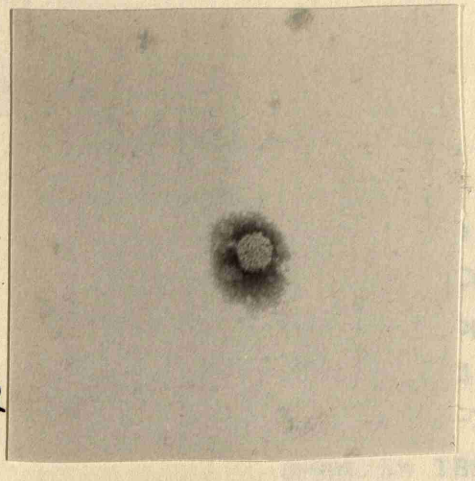

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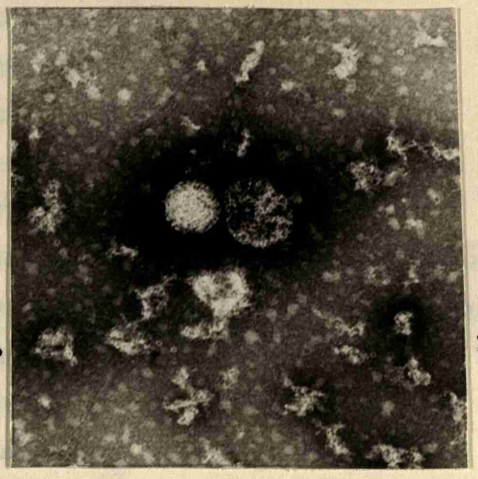

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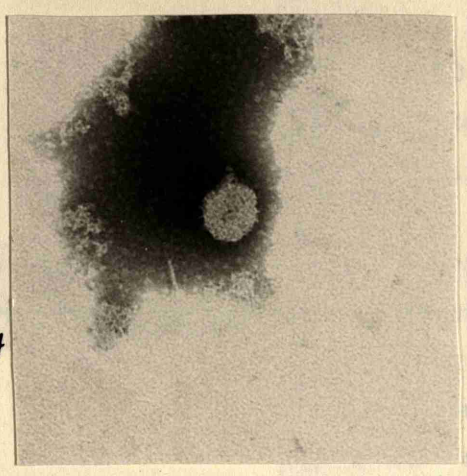

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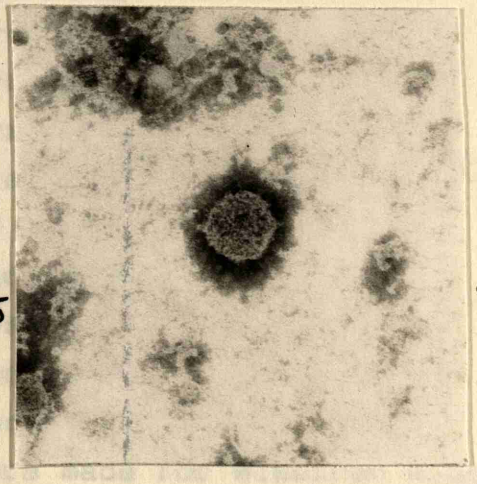

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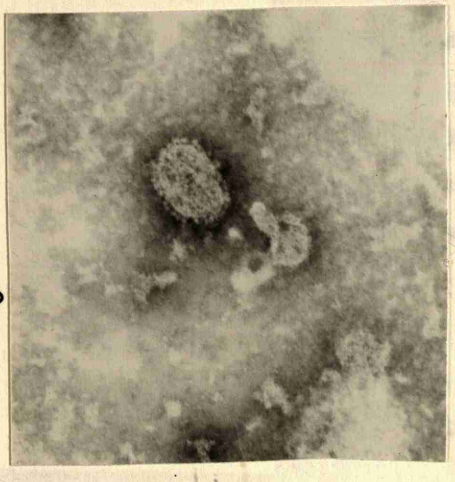
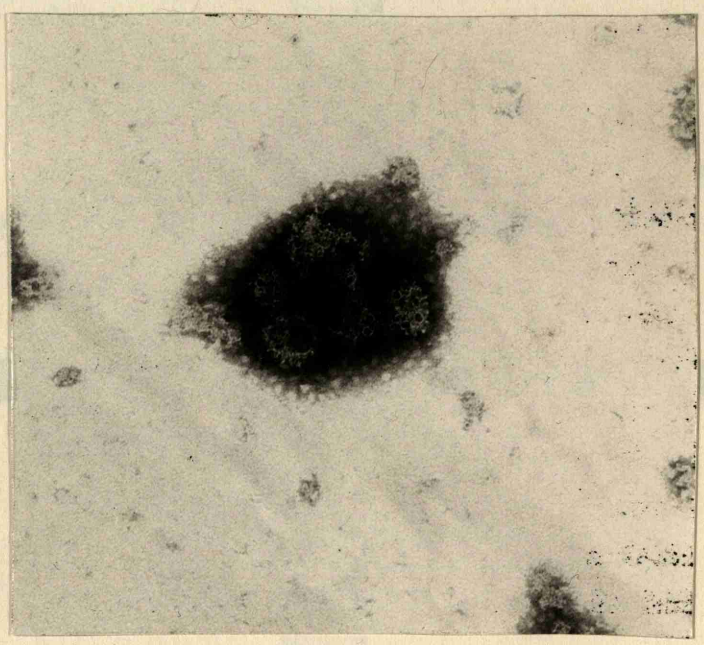


Fig 8-11 Fract 2 Stomached expt. rat (140+141) Livers
Fig 8-12 Fract 2 Homogenised expt rat livers (6)
Fig 8-13 Fract 3 Homogenised expt rat livers (6)
Fig 8-14 Fract 3 Homog. expt rat. livers (138, 139, 142 + 143)
Fig 8-15 Interface of discontin. grad of dog 38.'s lymph node
Fig 8-16 Interface of discont. grad. of expt dog 38's lymph node

(All x 80 000)

fp 317



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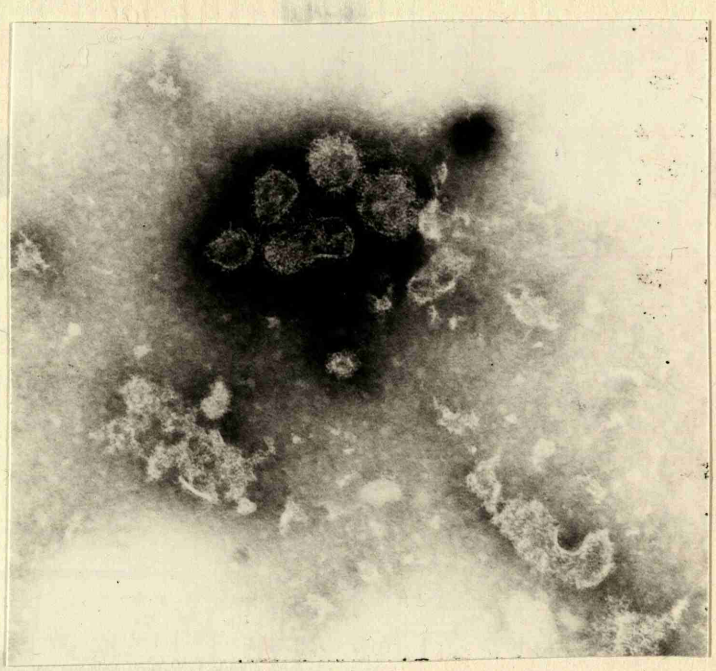


8-21

Fig 8-20 Fract A of Dog 38's 2nd lymph node prep (x 80 000)

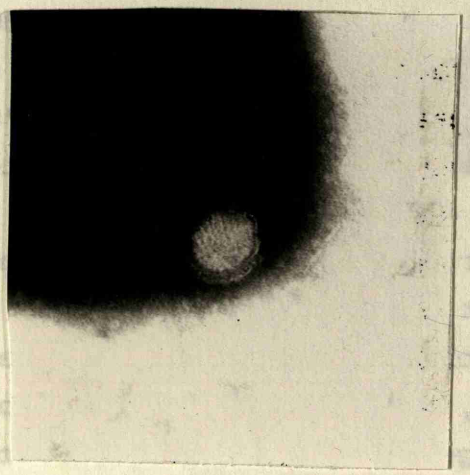
Fig 8-21 Fract D of Dog 38's 2nd lymph node prep (x 80 000)

### 8.3.3 Fractions from Lymph Node Preparations

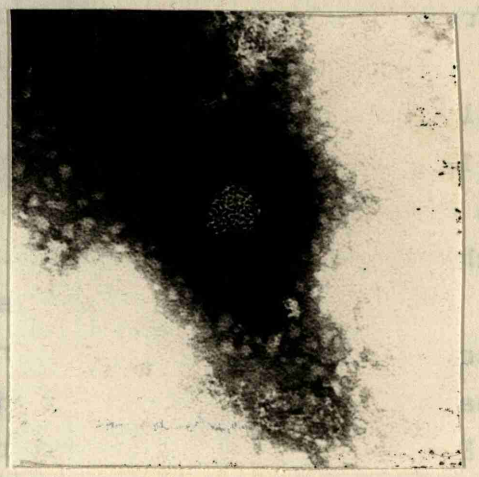
It was decided to investigate lymph node preparations as an alternative source of the aetiological agent of CACH, by fractionating in sucrose gradients. At the time, dog 38 was the last experimental dog to have been killed. A little of its lymph node, which had been frozen at -70°C for 15 months, was thawed, chopped and stomached in TBS to form a 10% wt:vol suspension. It was clarified, first in a bench centrifuge then in the ultracentrifuge at 10 000 rpm for 10 minutes. The supernatant was then layered onto a discontinuous sucrose gradient, of the type used for liver preparations (see section 2.4.3), and spun at 23 000 rpm for 2 hours in the ultracentrifuge at 4°C. The interface was collected as a fraction and dialysed. A negative stain was made, from a little of it, for examination in the electron microscope and the rest was fractionated on a continuous sucrose gradient. Figures 8-15 and 8-16 show examples of two types of particles found in the interface fraction from the discontinous gradient. The eight fractions produced by centrifugation in the continous gradient were dialysed and then sampled for negative staining. Then the two fractions which had been at the top of the continous gradient were combined and the two which had been at the bottom were combined. The resulting six fractions were concentrated, by pelleting at 35 000 rpm for 1 hour in the ultracentrifuge then resupending in a very small volume, before another set of negative stains were made for electron microscopy.

Concentrated fraction 4, a floccular band fourth from the base of the eight original fractions, was used for the first immune electron microscopy study (see section 9.7), it is also the subject of figure 8-17. The fractions from either side of this band were also investigated using immune techniques.

Two months later another sample of dog 38's lymph node was



8-17



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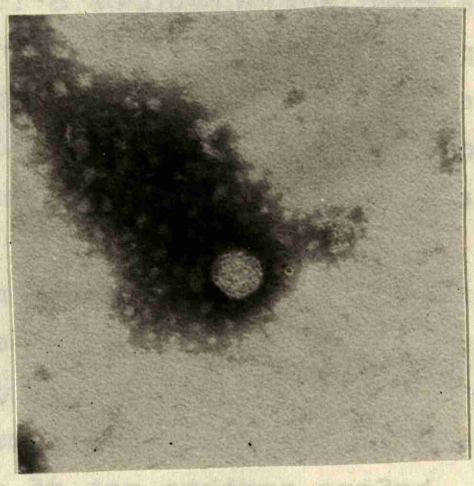


8-19

Fig 8-17 Fract 4 of Dog 38's 1st lymph node prep (x125000)

Fig 8-18 Fract A of Dog 38's 2nd lymph node prep (x126000)

Fig 8-19 Fract A of Dog 38's 2nd lymph node prep (x126000)

thawed, chopped and stomached in TBS to form a 20% wt:vol suspension. The double sucrose gradient technique was used again, but the final gradient was collected in twelve smaller fractions to get a sharper range of densities. Some fractions with similar densities were combined, so there were only eight dialysed fractions for examination. One of these (fraction A) was used for a repeat of the immune electron microscopy study (see section 9.7). Figures 8-18 to 8-20 show particles from fraction A, its density was similar to that of fraction 4 of the earlier study. Figure 21 shows particles, of a different appearance, from fraction D which were a little like those in the discontinous gradient shown in figure 8-16. Fraction D, although well removed from fraction A in the original gradient, had a similar density to it (out of step with its adjacent fractions) perhaps indicating mixing during collection.

### 8.3.4 Abdominal Fluid Preparations

A sample of abdominal fluid from dog 38 had been centrifuged in two tubes (1 and 2) as described in section 8.2.4. After removal of most of the supernatant from tube 2, the pellet of material in the base of it was resuspended by drawing it up and down a pipette several times. A little of the mixture was then taken for negative staining (labelled 2a); the remainder was sonicated, to mix it further, before some was stained with PTA (sample 2b).

The supernatants from the two tubes were kept separate and pipetted onto two dicontinuous sucrose gradients, of the type described in sections 2.4.3. After spinning, at 23 000 rpm for 16½ hours at 4°C, in the ultracentrifuge no visible bands had formed in the gradients. They were fractionated into small volumes and, using the refractometer, the density of each was calculated. The fractions with a density closest to the bouyant density of arenaviruses, along with those on either side, were dialysed

overnight in TBS before PTA staining.

### 8.3.5 Cell Culture Medium

After the first time dog 41's lymph node cell culture was harvested and centrifuged (see section 8.2.5). The supernatant, equivalent to the culture medium, was collected. It was then spun at 35 000 rpm for 1 hour at 4°C to sediment out any virions or other very small particles. The resultant pellet was resuspended in a small volume and negatively stained.

The supernatant (cell culture medium) collected from the centrifuged first sample of hamster 74's lymph node cell culture, was also spun at 35 000 rpm to pellet virions. No virions were identified.

## 8.4 CONCLUSION

No obvious arrays or collection of unmistakeable virions were seen in the sections of tissue blocks and other solid preparations examined. Inumerable varied, interesting "virus-like" particles were seen, most of which were probably only normal cellular components. Figures 8-1 and 8-4 show examples of the most uniform particles which were seen in some numbers, but scattered individually. They tended to be around 87-90nm in diameter.

Similarly the negative stains provided many possible candidates for an aetiological agent for CACH, but no large numbers of identical particles. However, not all viruses are perfectly uniform in size and shape and the possibility that an aetiological agent was among the particles visualised cannot be completely ruled out. Arenaviruses in particular can be very pleomorphic and exhibit a wide size range (see section 1.3.7).

Various groups of particles of similar size, density and appearance appeared to be present in different animals and sites. When the very large number of photomicrographs taken were sorted by particle size and fraction density, several clusters of similar particles became apparent. As might be expected, large particles of 90-120nm diameter were more numerous at a density of 1.24 (e.g. figures 8-7 and 8-14), with some at 1.21 (figure 8-5), but very few at density 1.18 (figure 8-15). Particles of 75-85nm were mainly found in fractions with density 1.24 (figure 8-9) but also around density 1.2-1.21 (figure 8-13). A lot of 60-69nm diameter particles were found at a density of 1.18 (the density of arenaviruses) e.g. those in figures 8-17 and 8-18, with only some of density 1.24 (figure 8-8). There were also a lot of 50-57nm particles with a density of 1.18 (e.g. figure 8-19) and some with a density of 1.24 (e.g. figures 8-11 and 8-12); these appeared to be two distinct population as there were not many of intermediate densities. A lot of the 40-45nm particles were found at or around density 1.18, but there were only a few of density 1.24 or more. There were very few particles of less than 40nm in diameter, most had a density of around 1.18.

# CHAPTER NINE SEROLOGICAL STUDIES

## 9.0 INTRODUCTION

This chapter covers some rudimentary serological studies of canine acidophil cell hepatitis, which were instigated to investigate the possibility of developing a serological diagnostic test.

The first aim was to stimulate the production of antibodies to CACH in rabbits, by inoculation with material from an experimentally-infected dog (section 9.1). The rabbit serum was then tested, for the presence of antibodies, by two agar gel immunodiffusion (AGID) experiments (sections 9.3 and 9.4). A brief explanation of the theory behind the AGID technique is given in section 9.2.

Immunocytochemistry was also used to investigate the antiserum, in five experiments described in section 9.6. A brief summary of the theory and application of the peroxidase-antiperoxidase (PAP) technique used is given in section 9.5. The first three experiments tested whether unabsorbed and absorbed serum from one of the rabbits would react with fixed antigens, in tissue sections from the dog used to immunise it, and investigated the optimum dilution of the serum. Two further PAP experiments investigated the specificity of the reactions obtained with the absorbed serum, using liver sections from field cases of CACH and other dogs.

## 9.1 ANTIBODIES FOR IMMUNOLOGICAL INVESTIGATIONS

### 9.1.1 Raising Antiserum in Rabbits

The techniques used in attempts to produce antibodies to the causative agent of CACH, in two New Zealand White rabbits (rabbits A and B), are detailed in chapter 2 (section 2.5.1).

Both rabbits had previously been put through a three-phase immunisation regime using material from experimentally-infected dog D [dog 8 in Jarrett and O'Neil (1985)]. They were boosted again for this study and then bled, 11 days later. The serum collected was used for all of the serological investigations described in this chapter.

At a later date, the two rabbits were boosted again and further serum samples were collected from them.

### 9.1.2 Absorbed Serum

The rabbits might have produced antibodies to anything in the inoculum which they recognised as foreign, including normal constituents of canine liver. The aim of this study was to find antibodies directed against antigens related to CACH infection. To eliminate any antibodies to normal dog liver components, the serum was incubated with powdered normal dog liver to allow antigen-antibody binding. After centrifugation, to remove insoluble material, the supernatant ("absorbed serum") would still contain any rabbit antibodies, present originally, directed against antigens not normally present in dog liver.

Full details of the method used to produce absorbed serum can be found in chapter 2 (section 2.5.2).

## 9.2 AGAR GEL IMMUNODIFFUSION THEORY AND METHOD

The AGID technique employs the precipitin reaction of antigen-antibody interactions. This occurs after the binding of antigens with their corresponding antibodies in solution and involves the formation of larger insoluble aggregates which may be visible as a cloudy precipitate.

The relative proportions of antigen and antibody are important in the precipitin reaction. In conditions of

antibody excess all antigen will be found in the precipitate; although with certain antisera the complexes formed in extreme excess may be soluble, due to the solubility of the reacting antibodies (Perlmann and Hammarström, 1983). At optimal proportions of antigen and antibody, all of both will precipitate out. When there is an excess of antigen the amount of precipitate decreases with increasing antigen concentrations. In conditions of extreme antigen excess precipitation may be completely inhibited; although antigen and antibody still react together only small complexes form, instead of large insoluble lattices.

Immunological methods using precipitation are relatively insensitive, detecting between 1-10μg/ml of antigen or antibody, while radio- or enzyme immunological techniques may detect 1-10ng/ml or less (Perlmann and Hammarström, 1983). Immunodiffusion methods do, however, have good qualitative discriminating power. In Ochterlony double diffusion (the version of agar gel immunodiffusion used in this study) the antigen solution and antiserem are placed in holes, punched out of agarose gel, at suitable distances from each other. As antigen and antibody diffuse against each other, precipitin zones (lines) develop where they meet in optimal proportions for precipitation. The position of lines is dependent on the diffusion properties and relative concentrations of reactants. Hence if several non-cross-reacting antigen-antibody systems are present in the same mixture, each should precipitate out in a different position. Although precipitin zones are visible as cloudy lines in the agar gel, staining of the preparation can assist visualisation and prevent further diffusion.

The preparation of slides for AGID is described in section 2.5.3. Each slide had two rosettes of wells cut out of the gel; one of each (the left-hand one) was always used to test rabbit A's serum, whether absorbed or unabsorbed, while the other was always used to test the corresponding sample of rabbit B's serum. A separate slide was used for

each of the canine liver preparations against which the serum samples were to be tested. The antibody solution (rabbit serum) was always placed in the central well of each rosette; the series of dilutions of the antigen solution were placed in the surrounding ring of six wells.

A few of the precipitin lines produced in this study were very close together or rather fuzzy, making estimation of their exact number rather difficult. In a few cases there was a suggestion of additional, exceptionally faint, precipitin lines. However, in the analysis of the results only precipitin lines which could be identified with certainty were counted. Some of the systems tested may therefore have contained slightly more antigen-antibody pairs than were attributed to them.

## 9.3 AGID EXPERIMENT ONE

### 9.3.1 Experiment Design

The aim of this experiment was to discover whether the rabbits had been successfully stimulated to produce antibodies and if so, whether any of them appeared to be related to CACH infection.

The two rabbit sera were tested against material from six dogs: four experimentally-infected CACH cases (dogs D, H, I and J) and two "normal" animals (dogs K and L). The preparation of their liver samples is described in section 2.5.3.

The six liver preparations were also tested against two pairs of absorbed sera from the same rabbits. These were the results of the absorption of serum samples from both rabbits A and B against each of two different canine liver powders (1 and 2), using the procedure described in section 2.5.2. Both liver powders had been prepared 3

years previously and they were used routinely for laboratory work requiring powdered normal canine liver.

Liver from dog D, an 18-week-old, male crossbreed, had been used to immunise the two rabbits originally (see sections 2.5.1 and 9.1.1). Dog D had itself been inoculated with serum and liver from a CACH field case (FC2 / dog F) (Jarrett and O'Neil, 1985). It was also used as the source case for the fifth rat experiment (section 5.7) and the first hamster experiment (section 7.3). Its history is given in section 5.7.2).

Dogs H and I [dogs 13 and 15 in Jarrett and O'Neil (1985)], both male crossbreeds, had been euthanased 13 weeks and 17 months respectively after inoculation with both serum and a liver preparation from experimentally-infected dog D. After infection dog H had exhibited episodes of raised transaminase levels and at postmortem had been found to have marked diffuse CACH.

Dog J [dog 18 in Jarrett and O'Neil (1985)] was also a male crossbreed which had been killed 17 months after inoculation with a liver preparation from experimentally-infected dog E [dog 9 in Jarrett and O'Neil (1985)]. The latter animal was also used as the source case for dog experiment 2 and its history is given in section 4.4.2. Both dogs I and J had exhibited episodic peaks in alanine aminotransferase levels and episodes of pyrexia (Jarrett et al., 1987). At postmortem they were both found to have liver lesions consisting of fibrosis, piecemeal necrosis and acidophil cells round portal tracts.

Dogs K and L were considered to be normal, uninfected animals. Although frozen liver from these two dogs was available, their identities and origins were not known.

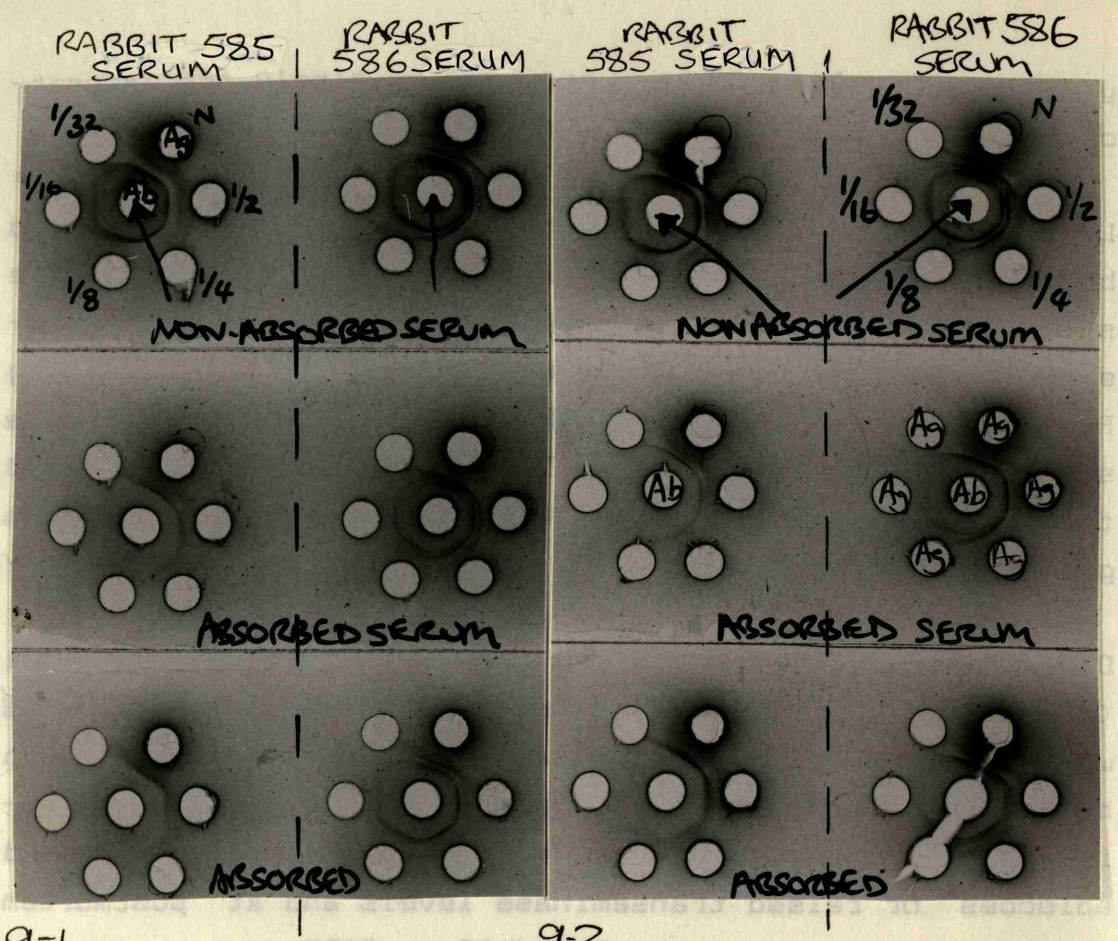



Fig 9-1 AGID expt 1 plates with dog J's liver as Ag in outer ring (dog 18 of Jarrett + O'Neil '85)

Fig 9-2 AGID expt 1 plates with dog D's liver as Ag in outer ring (dog 8 of Jarrett + O'Neil '85)

### 9.3.2 Results

Precipitin lines were produced when the two rabbits' sera were tested against both the experimentally-infected CACH cases and the normal, uninfected dogs. After absorption with either of the dried dog liver preparations, both rabbit sera were still able to form precipitin lines with most of the antigen preparations tested. Rabbit A's serum produced 3 precipitin lines against most of the dog livers tested.

Although absorption of the sera with liver powder 1 did reduce the number of antigen-antibody systems in all but one of rabbit A's and all but two of rabbit B's tests, some precipitin lines were still produced in eleven of the twelve systems tested.

After absorption with liver powder 2, rabbit A's serum produced less precipitin lines than the unabsorbed serum. In contrast, there appeared to be no reduction in the number of precipitin lines formed when rabbit B's serum was tested after absorption with liver powder 2.

Figures 9-1 and 9-2 show the reaction with dogs J and D respectively. Unfortunately the slides were not photographed at the time of the experiment; by the time they were photographed the gel had dried out and often split or distorted.

### 9.3.3 Conclusion

The results of this experiment suggested that both rabbits A and B appeared to have produced immune responses to several components of canine liver, but they did not clarify whether any of the antibodies produced were specific for antigens related to CACH. Although absorption with the normal liver powders failed to remove some of the antibodies from the antiserum, liver from the two normal,

uninfected dogs also reacted with both the absorbed and unabsorbed sera.

The basis of the classification of dogs K and L as "normal" was not known and the lack of information about them meant that the possibility of their having been exposed to CACH at some time could not be completely ruled out. Similarly, since the identity of the dogs from which the "normal" liver powders were obtained was unknown, the possibility of them having been exposed to CACH could not be totally discounted.

Another unknown variable in this experiment was the efficacy of liver powders 1 and 2, when used for the absorption of antibodies to normal canine liver components. If they were not sufficiently "active" (e.g. due to possible production faults or the ill-effects of storage) to remove antibodies to normal liver components, results like those seen in the tests of absorbed sera versus liver preparations from dogs K and L might be seen. However, such a lack of efficacy would also mean that antigen/antibody systems seen in tests of absorbed sera versus CACH cases could not be presumed to be abnormal i.e. related to infection.

A second AGID experiment was designed to attempt to eliminate some of the unknown variables present in this experiment.

## 9.4 AGID EXPERIMENT TWO

### 9.4.1 Experiment Design

In this experiment two of the experimentally-infected CACH cases (dogs D and I) checked in the previous experiment were retested, as was one of the normal dogs (dog K). Liver samples from three other dogs were also tested: a

field case of CACH (dog A/FC1), a field case of liver disease thought not to be CACH (dog M) and an uninfected control from dog experiment 1 (dog C).

Two canine liver powders were freshly prepared, because of doubts about the efficacy of those used to absorb the rabbit sera in the previous experiment (powders 1 and 2). The new freeze-dried preparations were made, from the liver of normal dog K (liver powder 3) and experimental control dog C (liver powder 4), using the protocol given in section 2.5.2. They were both used to absorb serum samples from rabbits A and B, as described in section 2.5.2. The two new pairs of absorbed sera were tested against the same six liver preparations as the two unabsorbed sera.

Dog A, a field case (FC1) of CACH from the West of Scotland, had been used as the source case for the first rat, mouse and guinea pig transmission experiments (sections 5.3, 6.2 and 6.3 respectively) and for the second hamster experiment (section 7.4). This male German shepherd dog had been found to have chronic active hepatitis with fibrosis at 8 months of age. Further details of its history and pathology are given in section 5.3.2).

Dog M was a male German shepherd dog from the East of Scotland which had been euthanased, at 19 months of age, after two episodes of ascites within a month (see section 1..5.5). It had been presented with ascites and pyrexia, although still bright. After an initial improvement on medication, the ascites had recurred within a fortnight, accompanied by an elevated ALT level of 80 IU/l, an alkaline phosphatase level of 120 IU/l and a urea of 3.4 mmol/l. The ascitic fluid was found to be a noninflammatory transudate. At postmortem the dog was found to have severe macronodular cirrhosis and splenic fibrosis. Rubeanic acid staining of liver sections failed to reveal any accumulation of copper. The owners' previous dog had

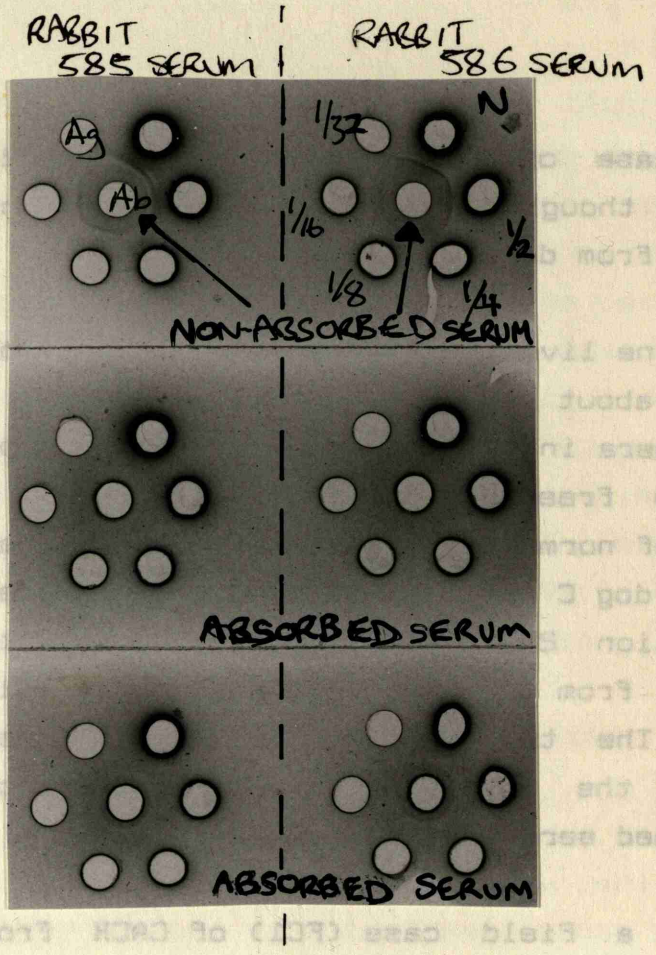



Fig 9-3 AGID plates of expt 2 with dog D's liver as Ag in outer ring (dog 8 of Jarrett + O'Neil, 85)

been destroyed, because of cirrhosis, 18 months earlier. It had also been a male German shepherd dog, but was only 8 months of age at the time of its death. Although the two animals had never been in contact with each other, they did originate from the same kennels. It was one of a number of cases of apparently similar hepatic fibrosis in GSDs discussed in section 1.5.5.

Dog C, a 4-month-old, female border collie, was a control animal (35) from the first dog transmission experiment (section 4.3). Liver from this animal had been used to inoculate rats in the fourth rat experiment (section 5.6), to show that normal dog liver did not produce CACH-like lesions in rats.

### 9.4.2 Results

Precipitin lines occurred in all the tests of both rabbits' unabsorbed serum. Rabbit A's unabsorbed serum produced two precipitin lines with five of the liver preparations tested and three lines when tested against normal dog K's liver. Three precipitin lines were found in all six of the tests involving rabbit B's unabsorbed serum.

However, no visible precipitation was seen when the same six liver preparations were tested against rabbit sera which had been absorbed with either of liver powders 3 and 4. Figure 9-3 shows dog D's reaction, for comparison to the previous year.

### 9.4.3 Conclusion

Both rabbits' unabsorbed serum produced one less precipitin line than in the previous experiment, when tested against liver preparations from dogs D (figure 9.3) and I. This may have been due to the 1 in 2 dilution of all the sera for this experiment. In the first AGID experiment the

serum samples were tested undiluted and one of the precipitin lines in each of three of the four tests in question was very short, only showing up against the neat antigen (liver) preparation.

The repeated tests of unabsorbed serum from both rabbits against "normal" dog K's liver produced three precipitin lines each. This was the same result as experiment 1 for rabbit A and one less line than was seen in experiment 1 for rabbit B. One of the precipitin lines produced by the latter's undiluted serum in experiment 1 had been short, only showing up against the neat liver preparation. That antigen/antibody system may not have shown up with the diluted serum used in experiment 2.

The absence of any precipitin lines in the tests involving absorbed rabbit serum suggested that the antigen/antibody systems seen in the tests of unabsorbed serum were probably related to normal canine liver constituents. If there were any antibodies related to CACH in the rabbit serum they were not present in appropriate concentrations to produce visible precipitation.

## 9.5 PAP IMMUNOCYTOCHEMISTRY THEORY AND METHOD

Immunohistochemistry employs the specificity of antigen-antibody reactions and a labelling system (either fluorescent, radioactive or enzymic) for the detection of antigens in tissue. The antibody itself is labelled for detection in direct techniques; but the label is attached to an anti-immunoglobulin antibody in indirect techniques (Morgan-Capner and Pattison, 1985).

In enzyme immunocytochemistry the label used is an enzyme (e.g. peroxidase or alkaline phosphatase) which can produce a coloured deposit with an appropriate substrate. Its location, and therefore that of the antigen, in the

tissues can then be demonstrated with the light microscope (Fleming, 1985). The peroxidase-antiperoxidase (PAP) technique used in this study was an indirect type of reaction. The protocol followed is detailed in section 2.5.4.

The liver sections to be tested were subjected to a series of preparatory treatments before the application of the primary antiserum (rabbit B's serum). Slides were then washed to remove the antiserum, leaving only specific antibodies attached to antigens in the section. A bridging antibody was then applied: swine antibody directed against rabbit immunoglobulin G. Any which did not bind to the rabbit IgG attached to the section, was washed off before the application of a rabbit antibody specific for, and already bound to, peroxidase. The anchored swine anti-rabbit IgG bridging antibody could also bind to some of the second rabbit antibody with its attached peroxidase. The site of the original antigen was therefore labelled and demonstrable by the development of a brown colour when the enzyme was supplied with a substrate.

As with other immunological techniques there is an optimum range of relative concentrations of antigen and antibody, within which the best results are obtained.

## 9.6 PAP IMMUNOCYTOCHEMISTRY EXPERIMENTS

The aim of the PAP experiments was to attempt to help identify the antigens which the rabbits had been sensitised to and, if a positive reaction could only be obtained with CACH-infected cases, to develop the technique as a test for CACH infection.

Rabbit B's serum was used as the antiserum for the PAP experiments because it had produced more precipitin lines than rabbit A's serum in most of the AGID experiments.

This suggested that rabbit B had been stimulated to produce either a greater variety of antibodies, to constituents of dog D's liver, than rabbit A; or at least greater amounts, enabling the detection of more antigen/antibody systems.

### 9.6.1 PAP Experiment One

The first PAP experiment was designed to check that the rabbit antiserum could produce a positive reaction against liver from the dog used to immunise the rabbit. This dog's liver could then be used as a positive control for further investigations, once the optimum dilution of antiserum had been determined.

Five sections of liver from dog D were tested against dilutions, of rabbit B's unabsorbed serum in PBS, of 1/10, 1/20, 1/100, 1/200 and 1/400. Two controls were also employed. Normal rabbit serum, at a dilution of 1 in 10 in PBS, was used in place of rabbits B's serum on one section of dog D's liver. In the second control the primary antibody was omitted and PBS used instead, to test for endogenous peroxidase activity and nonspecific binding of the secondary antibody. Both controls and the three most concentrated solutions of antiserum produced negative results, with no cells staining brown. Positive staining of hepatocytes was only seen in sections treated with serum dilutions of 1/200 and 1/400.

### 9.6.2 PAP Experiment Two

In this experiment further dilutions of the antiserum were tested. Starting with the highest one used in the previous experiment (1/400) five two-fold dilutions in PBS were made (1/800, 1/1600, 1/3200, 1/6400 and 1/12800). Two controls used normal rabbit serum, at dilutions of 1/400 and 1/3200, as the primary serum with negative results. Only small numbers of very palely stained cells were

Fig 9-4 PAP CACH expt. dog D's liver with NORMAL rabbit serum 1:400 = NEGATIVE CONTROL (×87.5)

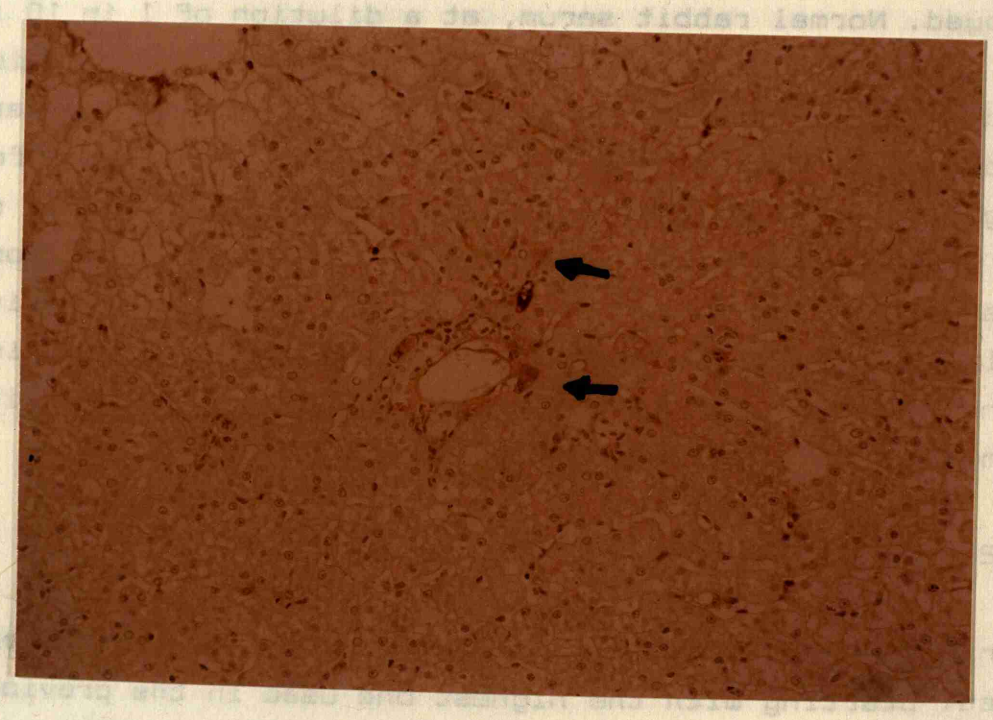


Fig 9-5 PAP Expt 3. CACH expt dog D's liver with absorbed rabbit serum 1:500 (×175)

produced by the most dilute and the most concentrated of the unabsorbed rabbit antiserum dilutions. Pale positive reactions were seen in the sections treated with the dilutions of 1/800 and 1/6400. Good positive staining of hepatocytes was obtained with the 1/1600 and 1/3200.

Sections of dog D's liver were also tested against six dilutions in PBS of absorbed antiserum (rabbit B's serum absorbed with dog liver powder 1): 1/10, 1/20, 1/100, 1/200, 1/400 and 1/800. The first two dilutions did not appear to produce any staining. Although some suspicious cells were seen with the 1/100 dilution, it could not be described as a positive reaction. The best positive reaction was obtained with the 1/200 dilution of absorbed antiserum, as staining became paler with the two further dilutions. Only small numbers of cells were stained in the three positive reactions.

### 9.6.3 PAP Experiment Three

In this experiment five further dilutions of the unabsorbed serum (1/5000, 1/10000, 1/50000, 1/100 000 and 1/500 000) and four further dilutions of the absorbed serum (1/500, 1/1000, 1/5 000 and 1/10 000) were tested against dog D's liver sections. A control of normal rabbit serum at a dilution of 1/10 000 was also included (see figure 9-4). Positive reactions were obtained with the first two dilutions of both unabsorbed and absorbed serum. The other tests were all negative. Figure 9-5 shows dog D's liver tested against absorbed serum at a dilution of 1/500.

### 9.6.4 PAP Experiment Four

Since positive reactions had been obtained with rabbit B's serum absorbed with dog liver powder 1 this was used, at dilutions of 1/500 and 1/1 000, to test liver sections from four dogs thought to be field cases of CACH (dogs A, F, N and O). A section of dog D's liver was included as a

Fig 9-6 PAP. CACH exptl. dog D's liver with absorbed rabbit serum 1:500 = POSITIVE CONTROL (X 175)

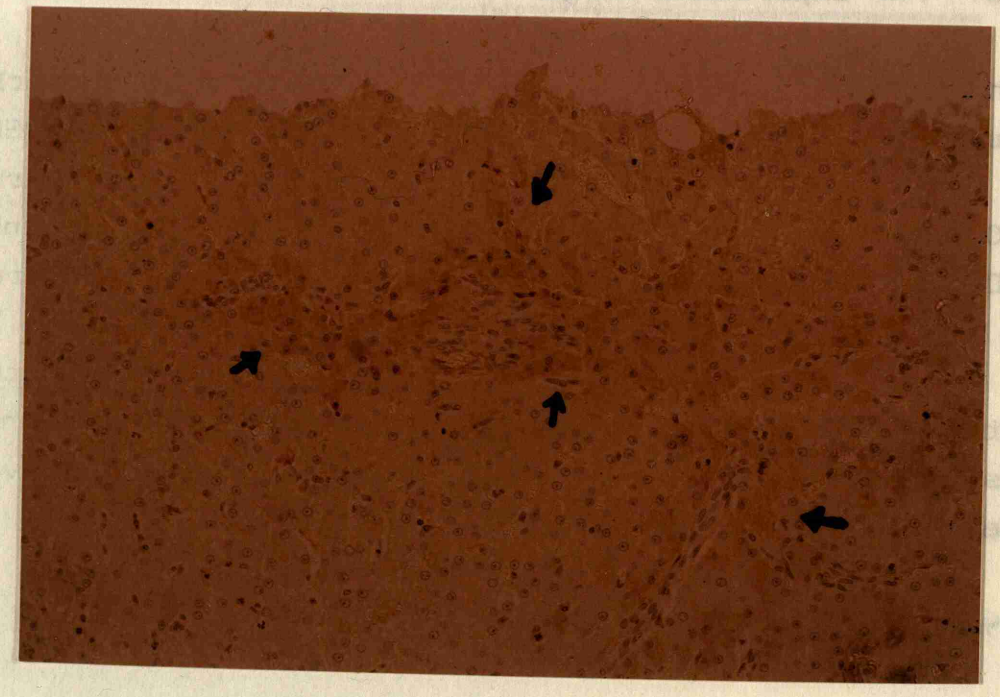


Fig 9-7 PAP. CACH field case dog A's liver with absorbed rabbit serum 1:500 (x175)

positive control; this gave a good positive reaction, which can be seen in figure 9-6. A negative control employed PBS, instead of the primary antibody, on a section of dog A's liver and gave the expected negative result. All four of the CACH field cases produced positive results (brown-stained cells) when treated with the more concentrated antiserum (1/500); three of them are shown in figures 9.7 to 9.9). No obvious positive reactions were detected in the sections treated with the higher dilution of absorbed antiserum.

Liver from dog A, the source case for the first rat, mouse and guinea pig transmission experiments and the second hamster experiment, had also been tested in the second AGID experiment. Details of this 8-month-old German shepherd dog are given in sections 9.4.1 and 5.3.2.

Dog N's liver had exhibited marked acidophil cell hepatitis with obvious piecemeal necrosis.

Details of dog F, a 13-month-old cocker spaniel are given in section 5.7.2. This was the source case used to inoculate dog D (Jarrett and O'Neil, 1985) which was used to immunise rabbits A and B.

Dog O was a 4-year-old, male cocker spaniel with a history of vomiting, anorexia, weight loss and ascites. It had a BSP retention of 50% before euthanasia. At postmortem the liver was found to be reduced in size, mottled yellow, firm and nodular, with an accessory portal circulation. Histology revealed chronic hepatitis progressing to cirrhosis, with bridging fatty vacuolation, obvious piecemeal necrosis and fibrosis extending out from portal tracts. Acidophil cells were noticeable around portal tracts.

fp336

Fig 9-8 PAP. CACH field case dog N's liver with absorbed rabbit serum 1:500 (x140)

Fig 9-9 PAP. CACH field case dog O's liver with absorbed rabbit serum 1:500 (x87.5)

### 9.6.5 PAP Experiment Five

Rabbit B's serum absorbed with dog liver powder 1 was used again at a dilution of 1/500 on liver sections from: five dogs with liver diseases other than CACH (dogs Q, R, S, T and U), two uninfected control dogs (P and C) from the first canine transmission experiment (section 4.3) and two of the CACH field cases tested in the previous experiment (dogs A and F). A liver section from dog D was used successfully as a positive control. One negative control employed PBS, instead of the primary antiserum, on a section of dog U's liver. In eight other negative controls dilutions of 1/500 of normal rabbit serum were used instead of rabbit B's serum on sections from dogs Q, R, S, T, P, C, A and F.

Dog Q was a 7-year-old, male Bedlington terrier with hepatitis and hepatic fibrosis due to copper accumulation. The liver used in the PAP experiment was from a biopsy sample.

Dog R, a neutered female collie cross, had died at 6 years of age after only one week's illness. At postmortem it was found to have massive hepatic necrosis, apparently due to leptospirosis.

Dogs S, T and U were all cases of infectious canine hepatitis (ICH). Dog S came from England, its liver histology can be seen in figure 9-10. The other two were both 9-week-old Cavalier King Charles spaniels from the West of Scotland, which had died within 2 days of purchase. Dog T was a male and dog U was a female.

Liver from dog C had been used in rat experiment 4 (section 5.6) and investigated in AGID experiment 2 (section 9.4). Dogs C and P were female border collie littermates who's details are given in section 4.3. Dog P was 13½ weeks of age when killed and dog C (35) was 16

Fig 9-10 Liver of ICH field case dog S (Mass-OG x175) with classical ICH inclusion bodies (↗)

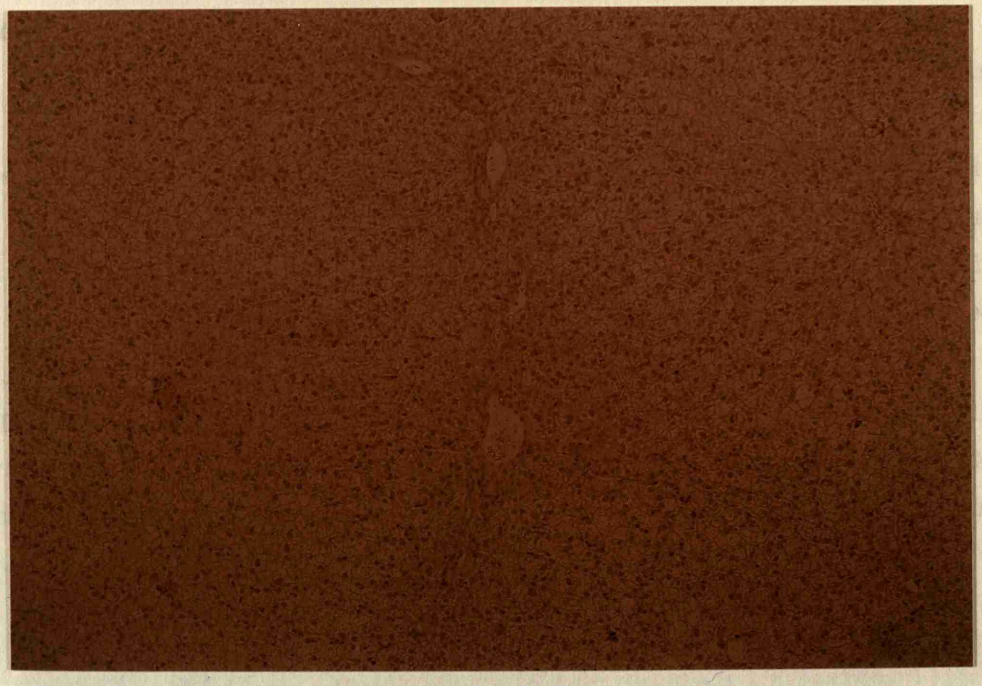


Fig 9-11 PAP. Normal control dog C liver with absorbed rabbit serum 1:500 (X87.5) no staining

weeks of age.

The nine negative controls all produced the expected negative results. The two CACH field cases gave some, rather indistinct, positive staining with the absorbed antiserum. No staining was seen with the two uninfected control dogs; part of dog C's section is shown in figure 9-11. The five cases of other liver diseases appeared to give negative results with the absorbed antiserum, although occasional suspicious staining was seen in a few on them (see figure 9-12 for dog S). The rather indistinct appearance of the two positive reactions and some of the negative reactions meant that the results of this experiment could not be considered conclusive.

### 9.6.6 Conclusion

In the first three PAP experiments rabbit B's unabsorbed and absorbed antiserum appeared to react with antigens in the liver of the CACH case (dog D) used to immunise rabbits A and B.

In the fourth experiment the absorbed antiserum also reacted to antigens in the liver of four CACH field cases, including the dog used to infect dog D (dog F) and another which had been used as the source case in several of the laboratory animal transmission experiments (dog A).

When liver sections from dogs A and F were tested again in the fifth experiment they still reacted with the absorbed rabbit antiserum, although to a lesser degree. There was no reaction between the antiserum and liver from two uninfected control dogs. Although the antiserum also appeared not to react with five cases of liver disease other than CACH, some of the results were not as obviously negative as their negative controls.

The results of the PAP immunohistochemistry experiments

fp 338

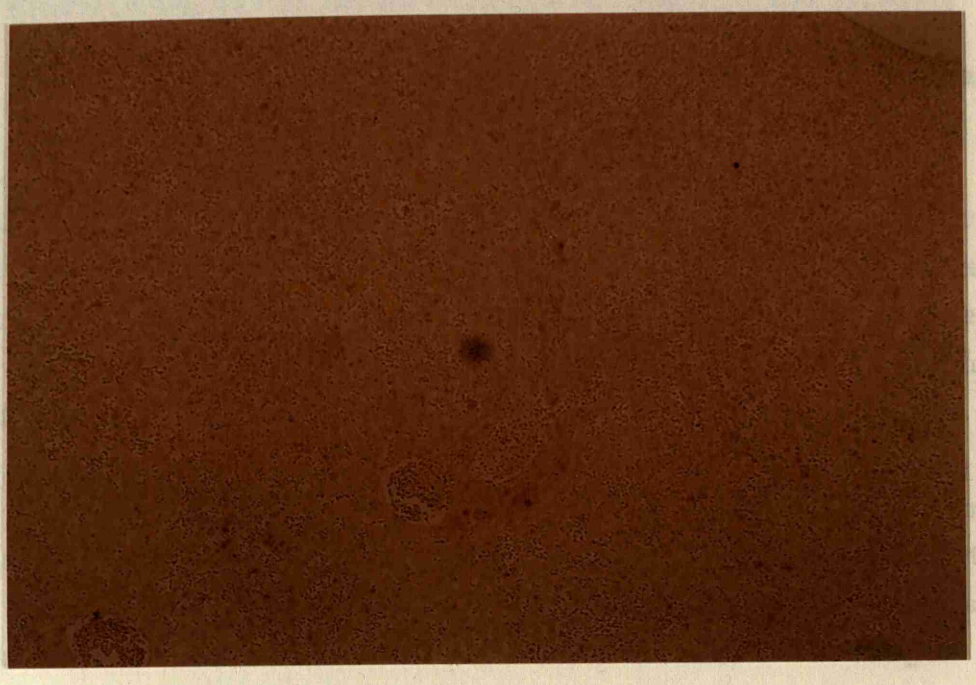


Fig 9-12 PAP. ICH "Control" liver of dogs with absorbed rabbit serum 1:500 (x87.5)

show that the rabbit antiserum, even after absorption with normal dog liver, contained antibodies to one or more antigens in the CACH-infected livers. However they do not prove unequivocally that the antibodies are specifically directed against antigens related to CACH infection. In the light of the results from absorbed sera in AGID experiment 2, further PAP investigations using different sera absorbed with different liver powders might help clarify the situation.

## 9.7 IMMUNE ELECTRON MICROSCOPY

The use of specific antibody to aggregate virions for electron microscopy, can help their visualisation in specimens containing only small amounts of them. As with other antigen-antibody reactions, their relatilve ratio is an important factor in the optimum formation of complexes.

The incubation and processing protocol used in this study is given in chapter 2 (section 2.5.5). After incubation, any complexes formed were collected by pelleting in a microcentrifuge and negative stains were made of the resuspended pellets. The supernatants were respun, for longer, to pellet any smaller complexes or particles and another set of negative stains were made of the new pellets.

### 9.7.1 Samples investigated by immune electron microscopy

The antiserum used for this study was rabbit B's serum which had been absorbed with normal liver powder 4 (from control dog C). Two different dilutions of antiserum (1 in 2 and 1 in 10, as described in section 2.5.5) were used with some of the antigen samples to increase the chances of achieving the optimum ratio for complex formation. The antigen solutions investigated were fractions (4 and A) from two different isopycnic centrifugal separat-

fp 339

Fig 9-13 Expt. dog 38's lymph node prep. fract 4 with 1:2 immune serum (x20 000)

ions of lymph node preparations, from dog 38, described in chapter 8 (section 8.3.3).

### 9.7.2 Results

In the first investigation numerous small, loose groups of particles were found in the respun version of fraction 4 treated with the 1 in 2 dilution of serum. Although similar in appearance, they were not identical sizes, as can be seen in figure 9-13. Figures 9-14 and 9-15 show examples of the most similar particles.

In conditions of antibody excess, heavy coating of particles can obscure detail and reduce the degree of clump formation. Whether such a mechanism was involved in this case is not known. Figure 9-16 shows a particle purified from the adjacent fraction.

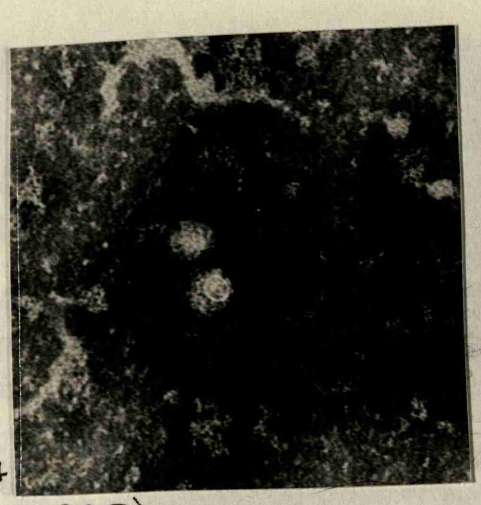
In the repeat investigation using the respun version of the comparable fraction A, from the second sample of dog 38's lymph node, not many of the particles were seen (figure 9-17). However some larger, more pleomorphic particles were found in the sample incubated with the 1 in 10 dilution of serum (see figures 9-18 to 9-19).

## 9.8 SEROLOGICAL TESTING FOR EVIDENCE OF HEPATITIS B INFECTIONS

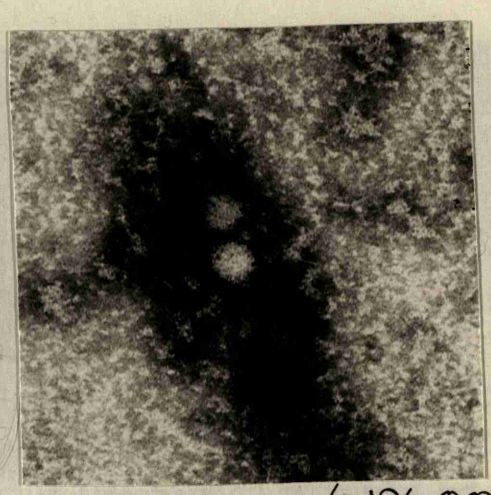
Dr Follett of the Glasgow Hepatitis Reference Laboratory was kind enough to blind test twenty canine sera samples for evidence of both HBsAG and HBC.

The samples tested included preinoculation ones from experiment 1 dogs 31 and 35 and experiment 2 dogs 38 to 41, samples collected 5, 10 and 30 weeks postinoculation from dogs 38 and 40. Serum from seven field cases was also tested, including FC10 (source dog B) and dog M, one of

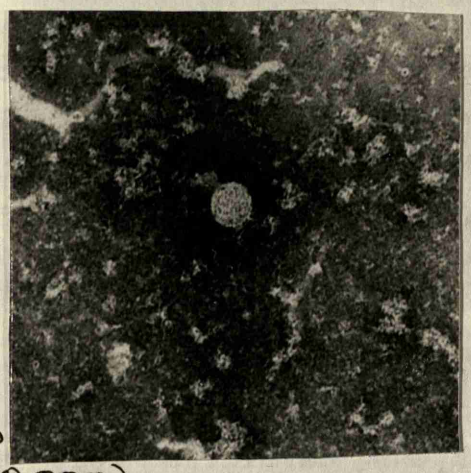
fp340



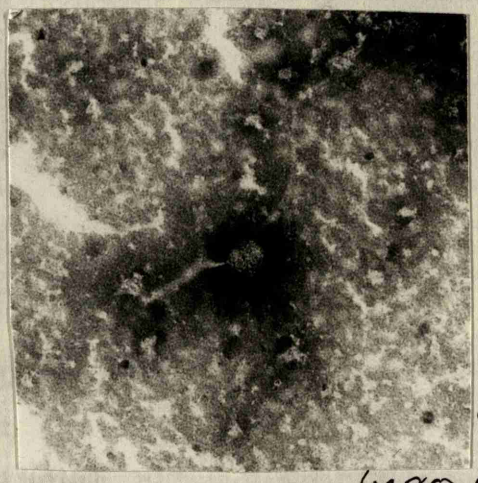
9-14
(x120 000)



9-15
(x126000)

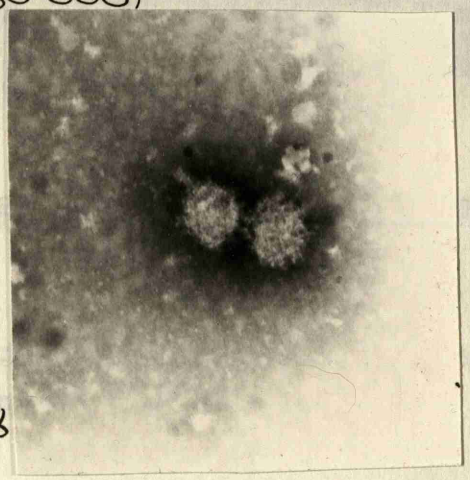


9-16
(x80 000)

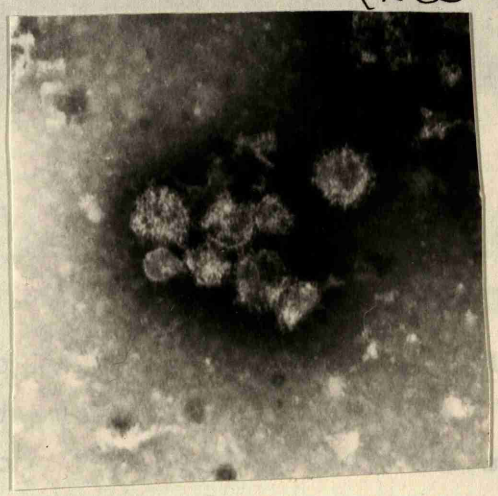


9-17
(x80 000)

(x80 000)



9-18



9-19

Fig 9-14 Exptl. dog 38's lymph node prep. fract 4 +1:2 immune serum
Fig 9-15 Exptl dog 38's lymph node prep. fract 4 + 1:2 serum
Fig 9-16 Dog 38's lymph node prep. fract. 3 +1:2 immune serum
Fig 9-17 Dog 38's 2nd lymph node prep. fract A + 1:10 immune serum
Fig 9-18 Dog 38's 2nd lymph node prep. fract A +1:10 serum
Fig 9-19 Dog 38's 2nd lymph node prep. fract A + 1:10 serum

the GSDs with hepatic fibrosis discussed in chapter 1 (section 1.5.5).

All tha samples were negative for both antigens.

# CHAPTER 10 CONCLUSION

Canine acidophil cell hepatitis would appear to occur throughout much of the British Isles since field cases were received from Scotland, England, Ireland and Wales. Clinical signs exhibited by field cases were those common to many liver diseases; no pathognomonic signs were identified for CACH. Multiple cases were not a feature of this condition, although several apparently unrelated cases were received from at least one veterinary practice. The lack of multiple cases may possibly be due to a low infectivity, or an unusual natural transmission of CACH.

The transmission experiments included first and second passages of CACH infection in dogs (dog experiments 1 & 2) and a third experimental passage back to dogs using experimentally-infected rat material (dog experiment 3). The second dog experiment showed that neither clarification of the inoculum nor subsequent filtration, through a 0.65μm filter, prevented transmission of the aetiological agent to dogs.

Rats developed liver lesions, like those of CACH in dogs, after inoculation with material from field and experimental dogs (first and second passages of infection). It is possible that CACH may be transmissible by inoculation to mice and/or guinea pigs although the very small numbers of positive reactions at the end of small experiments could not be considered definitive proof of this.

Inoculation of rats with liver from a normal dog failed to produce CACH lesions, like those resulting from inoculation with affected case material. This result lends support to the theory that the lesions produced in experimental dogs were also not due to any reaction to normal liver components.

The experimentally-infected dogs appeared outwardly healthy throughout the experiment, despite periodically elevated transaminase levels. If the same can occur in

field cases then CACH may be more prevelent than indicated by overt cases. If the trend of increasing degrees of fibrosis with time in subclinical cases, (see dog expt 2), also occurs in the field then CACH may be responsible for a proportion of cases of cirrhosis of unidentified aetiology.

# APPENDIX 1 HISTOLOGICAL PROCEDURES

24 Hour Processing Cycle

30 minutes in no.1, then 1 hour in each of the rest until 2 hours in last two waxes.

1) 70% spirit
2) 2% phenol meths
3) 2% phenol meths
4) 2% phenol meths
5) 2% phenol meths
6) Absolute alcohol
7) Absolute alcohol
8) Absolute alcohol
9) Absolute alcohol/xylene (50:50)
10) Xylene
11) Wax
12) Wax
13) Wax
14) Wax

Mayer's haematoxylin

| | |
|---|---|
| Haematoxylin | 1g |
| Distilled water | 1l |
| Potassium or ammonium alum | 50g |
| Citric acid | 1g |
| Chloral hydrate | 50g |
| Sodium iodatae | 0.2g |

Dissolve the haematoxylin, potassium alum and sodium iodate in the water, add citric acid or choral hydrate.

Haematoxylin and Eosin

Dewax sections in xylene, hydrate through graded alcohols to water.

1) Sections to water via iodine and hypo
2) Mayer's haematoxylin
3) Differentiate in 1% acid alcohol
4) Wash in water
5) Blue in STWS (Scott's tap water substitute)
6) Wash in water

7) Putts eosin (1:6)
8) Wash in water
9) Dehydrate through graded alcohols, clear in xylene and mount in DPX

Results: Nuclei = blue
Cytoplasmic components = varying shades of pink

Masson's Trichrome

1) Sections to water via iodine and hypo
2) Celestine blue 5 minutes
3) Rinse in water
4) Mayer's haematoxylin 5 minutes
5) Water rinse
6) Differentiate nuclei in 1% acid alcohol
7) Water rinse
8) Blue in STWS
9) Water rinse
10) Stain in Masson stain * = 2 parts 1% ponceau 2R in 1% acetic acid, 1 part 1% acid fuchsin in 1% acetic acid for 10 minutes
11) Water rinse
12) Differentiate in 1% phosphotungstic acid with collagen to decolourise muscle, fibrin and rbcs remain red
13) Water rinse
14) Counterstain in 1% lightr green
15) Water rinse
16) Dehydrate, clear in Xylene and mount

Results: nuclei = blue/black
muscle, fibrin, rbcs = red
collagen, reticulin, amyloid, mucin = green

Masson-orange G

* Rinse in meth spirit, stain with Picro - Mallory orange-G
(80% picric acid, 0.2% orange-G) for 30 secs. Rinse in water then follow Masson's trichrome from step 10.

# APPENDIX 2 ACIDOPHIL CELL SCORING SYSTEM

In all experimental animals a minimum of three liver sections, from different lobes, were examined systematically with the light microscope. The number of acidophil cells, around each affected portal tract in the section, was counted. For ease of assessment, affected portal tracts were assigned to one of the numeric groups below. Unaffected portal tracts, with no acidophil cells around them, were not counted.

portal tracts with 1-2 acidophil cells around them
3-4 acidophil cells
5-9 acidophil cells
10-19 acidophil cells
20-39 acidophil cells
⩾ 40 acidophil cells
too many acidophil cells to easily count

The numbers of portal tracts falling into each of the above groups were added up, to give an indication of the severity of acidophil cell reaction in that liver section. The results from all of an animal's liver sections were considered when assessing its acidophil cell score, since lesion distribution was not always uniform.

Animals were scored using a combination of the numbers of acidophil cells around individual portal tracts, the number of affected portal tracts and an impression of the proportion of portal tracts affected. The last was most significant in the longer laboratory animal experiments involving a wide age, and hence size, range of animals. Since each of their liver sections consisted of a slice of a whole lobe, those of early culls were much smaller than those of later culls. In contrast, the canine liver sections tended to be larger blocks of more uniform size, each just a piece out of a lobe.

A scoring scale of - to ++++, +++++ or ++++++, depending on the species, was used. A score of - indicated either no

acidophil cells or only 1 or 2 at a tiny number of portal tracts. The score increased from +, with increasing numbers of acidophil cells. An animal with small numbers of acidophil cells at many portal tracts could have the same acidophil cell score as one with more acidophil cells around fewer portal tracts. Similarly the acidophil cell score first suggested on assessment of a liver section with many acidophil cells, would be reduced if the animal's other liver sections were markedly less affected.

The scoring scales and the severity of lesions required for classification as a definate positive reaction are not comparable between species, due to interspecies variation in normal liver appearance. Normal mice for example were found to have either no acidophillic hepatocytes or only a tiny number, whereas other species may have small numbers scattered through the parenchyma of normal liver. The two most affected experimental mice (39 and 40), with distinctly more liver lesions than the others, would not have been considered significant if they had been rats (section 6.2.3).

Since small numbers of acidophil cells were sometimes found in controls of certain species, it was important to ensure that a diagnosis of a definate acidophil cell reaction was only made in those animals with a marked, easily noticeable, quantitative difference between them and controls of their species. A positive acidophil cell reaction was considered to be present with scores of ++ and higher, or +++ and higher, depending on the species. This was an arbitrary cut off point, deliberately chosen to err on the side of caution. Animals scoring below this level were classified as not having a definate acidophil cell reaction. Many of the latter had more acidophil cells than their controls, but there was not a large enough distinction, between the histological appearance of their livers and that of controls, to be taken as significant.

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