VARIOUS STUDIES ON LIVER DISEASE

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INTRODUCTION

VARIOUS STUDIES ON LIVER DISEASE

This thesis consists of one clinical and four experimental studies of various aspects of liver disease. The first of these was carried out between 1947 and 1950 when I was a resident medical officer in Belvidere Infectious Diseases Hospital, Glasgow, and comprises an investigation into the hepatic lesions found in association with infectious diarrhoea and vomiting of infants. At that time, compared with recent years, this was a relatively lethal disease and I had the opportunity to carry out numerous autopsies on fatal cases. One was impressed by the severity of fatty change in the livers of many of these babies. The possible importance of this was frequently emphasized by the absence of other gross morbid changes. This led me to undertake a clinical study of the liver in infantile gastro-enteritis, using various liver function tests and liver biopsy. Attempts are made to relate the results to various other clinical features and, in particular, to the nutritional state of the patient. The effect of various lipotropic factors, the value of which at the time of the investigation was mostly controversial, is also studied. Note is made of the infrequent occurrence of liver necrosis as a complication of this infection, and various minor hepatic changes are described.

My interest in liver disease has continued during the past ten years during which I have held appointments in the pathology department of Glasgow Royal Infirmary. My first experimental projects were

investigations into the effect of infection and stress on the livers of rodents, including the influence of adrenal medullary and cortical hormones. No notable results having been obtained, it was decided to investigate the effect of cortisone and corticotrophic hormone, which had recently become available for experimental work, on hepatic cirrhosis induced by carbon tetrachloride. Neither hormone seemed to influence the development of the lesion and this was thought at first to lend support to the idea of passive condensation of hepatic stroma in the pathogenesis of cirrhosis. Two further experiments were then undertaken to study the nature of the hepatic fibrous tissue directly. Firstly, the cirrhotic liver has been studied by autoradiography following administration of S^{35}-labelled sodium sulphate to detect newly-formed sulphated mucopolysaccharide. Secondly, the Coons' fluorescent antibody technique has been used to determine the antigenic nature of the connective tissue in cirrhosis, using antibodies to glomerular basement membrane and granulation tissue from quartz granulomata. These experiments and their results are set out and discussed in Part II.

Preliminary work with cortisone and ACTH suggested that these hormones were deleterious to the acute hepatic lesion induced by a single dose of carbon tetrachloride. Experiments leading to confirmation of this are described in Part III. Included also in this part, are studies on the influence of the same hormones on the damage induced by several other hepato-cellular poisons.

Tritium-labelled thymidine has become available recently, and its

use in investigating liver regeneration is described in Part IV. Thymidine is utilized exclusively in the synthesis of deoxyribonucleic acid and autoradiographs of regenerating liver from animals so treated show positive images over newly-formed cell nuclei. This work is still in progress, but the results so far obtained are thought to have sufficient interest for inclusion in this thesis.

Part V is also an autoradiographic study of liver, in this instance following the administration of S^{35}-labelled methionine and cystine. The uptake of these labelled amino-acids was studied in experimental liver injury, particularly that induced by two methionine antagonists, ethionine and bromobenzene.

Since these studies on liver disease are not all closely related, the results of each part are discussed and summarized separately. It was considered that the figures and tables would be followed more readily by placing them in a separate volume.

PART I

HEPATIC LESIONS IN INFANTILE GASTRO-ENTERITIS

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INTRODUCTION

Post-mortem examination of infants who have died of infectious diarrhoea and vomiting, so-called "gastro-enteritis", frequently reveals severe fatty change of liver which may be all the more conspicuous for the absence of other obvious morbid anatomical lesions. Consequently, it has been noted by many authors in their descriptions of this disease, such as Nabarro (1923); Cooper (1937); Lowdon (1954). Some have regarded fatty liver as a serious complication responsible for relapse or even death (Paterson & Nabarro, 1922; Alexander & Eiser, 1944), but did not support such a presumption with clinical evidence of hepatic dysfunction. Attempts to evaluate the importance of hepatic steatosis in infantile gastro-enteritis have been carried out by a few workers, e.g., Marie *et al.* (1947); Lévesque *et al.* (1947), but conclusions were based on small numbers of autopsy results, little or no attempt having been made to study liver structure or function during life. Modrewska-Winowska (1954) noted fatty change, siderosis or round-cell infiltration of liver in 109 cases of infantile cachexia; in common with many other authors, she attributed steatosis to deficiency of protein or specific lipotropic factors, but suggested that minor degrees of fatty change could be caused by infection *per se* or by a diet rich in fat or carbohydrate. More thorough studies using specific liver function tests and liver biopsy were under-

taken by Meneghello et al. (1949) and by Albeggiani (1954), but their patients appear to have had a multiple deficiency syndrome analagous to kwashiorkor rather than infantile diarrhoea and vomiting as known in this country. Stowens (1960) studied various liver diseases in 1,000 young children using needle biopsy. Thirty-one of these patients had malnutrition, and brief reference was made to fatty change associated with starvation; this change developed from the periportal zones of liver lobules and was extensive within three days of illness, but regression following the establishment of a normal diet was a much slower process.

The present investigation is based on a study of 167 infants, 131 with gastro-enteritis and the remainder with other conditions. Needle biopsy of the liver was carried out in every case, and in many this procedure was repeated on one or more occasions. In this way attempts are made to assess the importance of fatty change of liver in these infants and to correlate its severity with various clinical manifestations, liver function tests, prognosis and treatment including lipotropic therapy.

In contrast to fatty change, liver necrosis has been described as a complication of infantile diarrhoea and vomiting (Schlesinger et al., 1949). The present series includes three possible cases, a low incidence compared with the numbers quoted by Bonham-Carter (1947) and Wainwright (1950). The condition has been mentioned frequently in review articles on gastro-enteritis usually, as in the instance of fatty liver, in a rather superficial way; indeed, some authors have made no clear distinction between liver necrosis and fatty liver, e.g., Gunn (1945); Giles and

Sangster (1948); Giles et al. (1949); Lawson (1951); Mann et al. (1952). The three cases in this study have varied histo-pathological features and their relationship to gastro-enteritis is uncertain.

METHODS

The cases on which this investigation is based were admitted to the gastro-enteritis unit of Belvidere Infectious Diseases Hospital, Glasgow, during a two-year period. Since all biochemical and histological procedures were carried out without technical assistance, the 167 cases were not consecutive admissions, but were divided into six groups, each of 25 consecutive cases, and a final group of 17. Study of a new group was not undertaken until the investigations required for the previous group were almost complete. In addition, 10 patients were investigated again when re-admitted after periods of several months from the times of their original illnesses. None of these could be regarded as a relapse of gastro-intestinal infection. Consequently this study is based on 177 cases of illness in 167 patients.

One hundred and thirty-one of these 177 cases had gastro-enteritis; 32 had infections other than gastro-enteritis; 14 had no detectable infection. Sixty-seven of 131 gastro-enteritis cases were complicated by a parenteral infection. Diagnostic details are given in Table I. The isolation of pathogenic strains of Bact. coli was not available as a routine bacteriological procedure during the greater part of the investigation, but positive results were obtained latterly from all cases diag-

nosed as gastro-enteritis on clinical grounds. It is not difficult to recognize moderately or severely ill cases who suffer from watery diarrhoea with green or orange-coloured stools, vomiting of variable severity, dehydration and toxaemia. Mild cases all had diarrhoea with green stools but little or no toxaemia or vomiting and no dehydration. The following were not classified as gastro-enteritis:

(1) Very mild cases of diarrhoea which co-existed with obvious parenteral infection and which cleared up with successful treatment of that infection by sulphonamides or antibiotics.

(2) Very mild cases of diarrhoea, in infants who were well otherwise, which cleared up when the diet normal for the patient's age and expected weight was supplied.

The treatment of gastro-enteritis in this series followed generally accepted principles. In particular, the methods recommended by Alexander (1948) were carried out, especially with regard to initial starvation and gradual return to normal feeds; relief of dehydration by intravenous infusions, or, in the case of very small infants, intramedullary infusions; treatment of parenteral infection with sulphonamides and antibiotics; prevention of cross-infection as far as was practicable with the limited nursing staff available and the disadvantage of an open ward. The fluids for parenteral infusion were either Hartmann's solution diluted with an equal volume of 5% glucose or reconstituted human plasma similarly diluted. Pint doses of these fluids were given alternately. Whole blood in quarter and half pint amounts was transfused to a few babies with anaemia

which became evident after the relief of dehydration. The administration of fluid subcutaneously with hyaluronidase to aid dispersion was abandoned as unsatisfactory after a few attempts.

Certain cases received streptomycin, 1-2 g. daily by mouth for 6 to 14 days, sometimes with marked clinical improvement. Others received oral aerosporon in 100 to 200 mg. doses for similar periods, but without effect.

Carob flour was used in several severe cases including four members of the present series. This treatment was soon abandoned since no case showed any improvement apart from altered consistency of the stools.

Patients were discharged home only when they were able to retain a full diet for 2 to 3 days without recurrence of diarrhoea or vomiting.

In addition to routine therapy, certain babies, as already indicated, received one of the following substances which might be expected to exert some lipotropic action:

(1) Dl-Methionine (22 cases). 0.25 g. of the white powder was dissolved in two of the daily feeds and administered daily for 6 to 8 days.

(2) Choline chloride (33 cases). A 5% aqueous solution was prepared and added to 4 daily feeds. In a few cases with severe vomiting it was mixed with the glucose-Hartmann infusion fluid until the patients could retain a substantial amount of fluid orally. The dose by either method did not exceed 100 mg./Kg. body weight/day. Thus, a 15 lb. child received 16 ml. 5% choline in one pint of infusion fluid or 4 ml. in each

of four feeds daily. This treatment was continued for 7 or 14 days.

(3) Casein hydrolysate (14 cases). Three patients received oral casein hydrolysate for 2 days, 2 days and 7 days respectively. The dose was 5 g. in each feed, 7 feeds being given daily. All three (as well as some others not included in this series) tolerated these feeds very badly; consequently, the method was discontinued.

Eleven others received the hydrolysate by intravenous drip. "Casydrol" diluted with an equal volume of 5% glucose was substituted for diluted plasma in the scheme already outlined. The longest duration of this therapy was 6 days. In most cases it was discontinued after a few days because of local venous thrombosis.

(4) Crude liver extract - "Heparglandol B" (Roche) (9 cases). This extract is claimed to be rich in Vitamin B complex including aneurine, riboflavine, nicotinamide, pyridoxine and pantothenic acid. 0.5 ml. was given by intramuscular injection daily for 7 to 14 days.

(5) Vitamin B compound - "Oral Hepatex" (Evans Medical Supplies) (4 cases). This includes Vitamin B_{12}, 6 µg. per ml. One half teaspoonful was given thrice daily for 14 days.

(6) Inositol (3 cases). Each case received 100 ml. by mouth twice daily for 14-15 days.

(7) Pantothenic acid (6 cases). 25 mg. was given by mouth twice daily for 7-9 days.

(8) Pyridoxine (3 cases). This was administered orally in 10 mg. doses twice daily for 14 days.

(9) Riboflavine (4 cases). Each case received 1 mg. thrice daily by mouth for 14 days.

Clinical examination on admission and daily thereafter during the acute illness included a careful search for parenteral infection, especially in lungs and middle ears. To avoid excessive catheterization of female patients, evidence of urinary infection was sought only in specimens removed for investigation of ketonuria, etc. (see below), and exceptionally in cases of unexplained pyrexia. Clinical assessment of the severity of toxaemia and dehydration was recorded each day, as well as frequency of vomiting, numbers and character of the stools, and liver size as estimated by palpation and percussion. Records were kept of fluid intake and the calorie value of retained food and parenteral fluid.

Rectal swabs were examined bacteriologically for specific shigella and salmonella infections, and the appropriate treatment undertaken in positive cases. Some stools were examined for Giardia Lamblia but no case of this infestation was found. Capillary blood from the heel was taken for haemoglobin and blood cell investigations, both on admission and following the relief of dehydration. The tuberculin jelly skin test was done in each case. In many, the gelatin film digestion test for trypsin in faeces was performed and was always positive; the value of this is now discredited, but no case of fibro-cystic disease of pancreas was discovered at autopsy. Other special investigations such as urine culture, X-ray examination of chest, examination of cerebro-spinal fluid and serological tests for syphilis were undertaken if indicated.

Liver biopsy was performed in the early afternoon. At 10.00 a.m. on the same day, 10-20 ml. whole blood was withdrawn from the superior sagittal sinus and subjected to various liver function tests and other biochemical procedures. These were as follows:

(1) Prothrombin time. The one-stage technique devised by Quick et al. (1935), but using Russel viper venom as a source of thromboplastin, was employed. Normal clotting time was taken as 25 seconds ± 5 seconds, confirmed by observations on normal sera.

(2) Serum bilirubin. "Direct" and "Indirect" Van den Bergh reactions were performed and total bilirubin estimated roughly by means of a Lovibond comparitor.

(3) Plasma protein. Only total protein estimations by the copper sulphate method were obtained.

(4) Thymol turbidity (MacLagan, 1944a).

(5) Serum colloidal gold reaction (MacLagan, 1944b).

(6) Takata-Ara reaction (Ucko, 1942).

(7) Blood sugar (Folin and Wu).

(8) Total serum cholesterol. The colorimetric method of Sackett (1925) was employed, using a "one-cell" photoelectric colorimeter.

(9) Serum cholesterol esters. Free cholesterol was precipitated by 1% alcoholic solution of digitonin, the esters extracted with hot petroleum ether and quantitated as for total cholesterol.

(10) Serum phospholipid (Youngburg & Youngburg, 1930: normal plasma levels by this method are 300-350 mg. in each 100 ml.).

(1) to (4) were undertaken at the time of initial liver biopsy in all cases and the remainder in some cases only.

Urinary examination on the days of liver biopsy included qualitative tests for the presence of reducing substances, bilirubin, urobilinogen and ketone bodies. Catheter specimens were obtained from female infants.

A few minims of syrup of chloral were given one hour before liver biopsy. No attempt was made to undertake the operation on any baby who was gravely ill or in whom the prothrombin time exceeded 40 seconds.

Liver was obtained by a trochar and cannula introduced through the ninth inter-costal space in the right mid-axillary line. Local anaesthesia was obtained by 1% planocaine injected into skin and subcutaneous tissues and then down to the pleural surfaces of chest wall and diaphragm and the liver capsule. Judging by experience in adults and by the lack of disturbance in many of these small patients, anaesthesia appeared to be quite adequate.

No special apparatus such as the Vim-Silverman needle was used. The simple cannula had an internal diameter of 2 mm. and was 20 cm. long. Along with the trochar it was introduced by a sharp thrust through the anaesthetized area and into the liver for a distance of ¾" to 1". The trochar was then withdrawn and a clean dry record syringe of 50 ml. capacity attached. Suction was applied as the cannula was advanced medially and slightly upwards for a further inch, and maintained during its rapid withdrawal. In the majority of cases a substantial column of

tissue appeared within the barrel of the syringe with the inrush of air which followed the withdrawal of the cannula.

The operation is easy to perform and causes little or no generalized upset. Possibly it is easier to obtain liver in this way from infants compared with adults, since a suitable quantity was obtained in 95% cases. (The percentage failure in adults by the same method is usually much higher than 5%). One child developed pneumothorax as a result of the procedure, but recovered. No death could be attributed directly to the operation; and the death rate for the experimental group was no higher than that for other patients during the same two-year period. There was no sign of internal haemorrhage in any case which came to autopsy at a later date. Histological examination of the liver at the site of puncture was carried out in such cases. As shown in Figures 1 and 2, the needle track is filled with fibrin and surrounded by a thin zone of necrotic liver parenchyma. In a few cases, bleeding occurred from the cannula on withdrawing the trochar; in this event the cannula was withdrawn quickly, no attempt being made to obtain hepatic tissue, and in no case did any ill effect ensue. The cause of failure to obtain adequate tissue in other cases was not apparent.

It is concluded that the operation is simple and reasonably safe if carried out by an experienced operator. I had experience of adult liver biopsy and performed the operation on the cadavers of infants on many occasions before undertaking the present investigation.

The column of liver tissue was divided by a sharp scalpel blade into

three portions and fixed in (1) 10% neutral formol-saline, (2) Flemming's fluid and (3) Bouin's fluid. If insufficient material was available only the first or the first and second of these were used. After adequate fixation, all were dehydrated, passed to paraffin and 7 μ sections cut.

(1) Sections from tissue fixed in formol-saline were stained by Ehrlich's haematoxylin and eosin; haematoxylin and van Gieson's stain; Heidenhain's azan modification of Mallory's method, or, in some cases, Masson's trichrome method, for fibrous connective tissue; Gordon and Sweet's method for reticulin; Heidenhain's iron haematoxylin to show mitochondria; Giemsa's stain applied for 24 hours to show cytoplasmic basophilia associated with ribonucleic acid; Perls's method for iron-containing pigment.

(2) Sections from tissue fixed in Flemming's fluid already showed fat stained black by perosmic acid and some were counterstained with safranin.

(3) Sections from tissue fixed in Bouin's fluid were stained for glycogen by Best's method, and, in some cases, by the periodic acid-Schiff technique.

In about a third of the cases, there was sufficient material left over to fix in formalin and embed in gelatin. The frozen sections cut on a modified Cambridge rocking microtome were stained for lipid with Sudan IV, Sudan black and Nile blue sulphate, the results being compared with those obtained with perosmic acid.

In a few cases a little liver tissue was placed in nutrient broth and incubated aerobically for periods up to one week at 37° C. No growth of organisms was obtained from any of these.

In all, 354 successful biopsies were carried out in 167 patients. In addition, material was obtained from post-mortem examinations in 26 of the 47 fatalities in the experimental group. Autopsy was performed as soon as possible, and in many cases within a few hours of death. Each autopsy comprised an examination of all organs, including middle ears and paranasal sinuses. In every case histological examination was undertaken on liver, gall-bladder, pancreas, kidneys, stomach, jejunum, ileum, colon, heart, lungs, cerebrum and cerebellum, pituitary, thyroid, thymus, adrenals, gonads, spleen and mesenteric lymph nodes. Table II gives the numbers of liver examinations, both biopsy and autopsy on these patients. Generally, there was an interval of two weeks between any two biopsies in one patient. Cases receiving lipotropic substances were examined immediately before and soon after such treatment.

RESULTS

(A) Fatty Change in Liver

Variable degrees of this condition were present in the large majority of specimens. Chemical estimations of lipid were not undertaken and the degree of fatty change was estimated arbitrarily from the microscopic appearance of tissue sections. The following grades were recognised:

(1) ++++ The entire liver parenchyma consists of cells partly or almost completely filled with fat globules.

(2) +++ A few cells contain no fat; alternatively the majority of fat droplets are small compared with the first group.

(3) ++ About 50% of each lobule consists of fat-laden cells; alternatively many small droplets have a wider distribution in the liver.

(4) + Obvious fatty change is confined to 25% of each lobe or a fine dusting of fat is seen throughout the lobule.

(5) ± A few droplets of fat are seen especially with fat stains, although often inconspicuous in H. & E. sections.

(6) 0 No fat seen in H. & E. sections and not more than a very fine deposit detectable by specific fat stains.

Figures 3 to 21 illustrate examples of these grades.

Generally the parenchymal cells most heavily involved are those closest to the portal canals. Occasionally the centrilobular cells are most severely affected (Figs. 22 & 23). Severity was assessed from the preparations showing most fat, generally the osmium-stained sections. H. & E. sections are almost as informative in this respect but would be misleading occasionally (Figs. 24 to 27). In some autopsy material it was noted that the right lobe is slightly more fatty than the left (Figs. 28 & 29); sections of the former were used in the estimation of fatty change, as biopsy material was obtained from the right side.

In most frozen sections treated with Nile Blue sulphate the fat stains pink (Fig. 30), but in some autopsy specimens it appears dark

purple or blue (Fig. 31), even if previous biopsy material from the same case had pink-staining fat. According to some authorities, e.g., Lorrain Smith (1908); Cain (1947), neutral fat stains pink and fatty acids blue by this method. Possibly some chemical change may occur in liver fat soon after death with production of fatty acids from triglycerides.

The degree of fatty change assessed in the manner described above was correlated with various clinical features:

(1) Age of patient. Table III correlates severity of hepatic steatosis with age, regardless of other clinical findings. Extreme degrees of fatty change were not found in any infant less than 15 weeks' old, although such cases were often of comparable clinical severity to the older age groups. Frequently, the livers of infants who died of gastro-enteritis within the first four weeks of life showed little if any stainable fat.

(2) Sex. The series comprised 98 males and 79 females. Fatty liver appeared to be rather more severe in females compared with male patients in the younger age groups (Table III), but because of the small numbers involved these findings are probably without significance.

(3) Clinical diagnosis. The relationship between primary disease and fatty liver is shown in Table IV. It will be seen that all cases of the most advanced degree of steatosis were found in patients with gastro-enteritis with or without parenteral infection; however, moderately severe steatosis may be found in association with infections other than gastro-enteritis. Fatty change was usually minimal or absent in non-

infective conditions which consisted of wrong feeding (9 cases), primary megacolon (1 case) and babies with no detectable abnormality (4 cases). Even among the last of these one had + liver fat and another ± liver fat; consequently it is probable that such minor degrees have little significance.

Reference to Table I will show that the series included examples of a wide variety of parenteral infections. There was no correlation between any one type and the severity of fatty liver.

In a few instances the clinical diagnosis altered between liver biopsies, while autopsy occasionally revealed a parenteral infection unsuspected during life. Table V shows the results obtained for all liver examinations and from these results conclusions similar to those made from the data on Table IV may be drawn. (Of the 177 cases, liver fat was maximal at the first examination in 142, at the second examination in 27 and at the third in 8).

(4) Severity of symptoms. Gross fatty liver could be expected in patients over 6 weeks of age with severe symptoms of gastro-enteritis. In particular, cases with frequent vomiting were liable to fatty liver, but a few exceptions with much fat and little or no vomiting were encountered. The association with severity of diarrhoea was not so marked, and no relationship could be established with pyrexia or dehydration.

In those cases subjected to repeated liver biopsy, clinical improvement was generally associated with diminution in liver fat but in some a

moderate amount of fat remained after clinical cure.

(5) Duration of illness and clinical relapse. One example of ++++ fatty change (Fig. 6) was found in a baby within 24 hours of the onset of a first attack of gastro-enteritis. It is claimed that, in adults, upper abdominal pain may be caused by stretching of the liver capsule from rapid enlargement of the liver due to fatty change (Himsworth, 1947). It seems possible that in infants too, fatty change may develop comparatively rapidly, but in the present investigation no estimation could be made of rate of development since most cases had been ill for several days before admission to hospital. (The average interval between onset of symptoms and first biopsy was 11 days, and between subsequent biopsies, 12 days).

In several cases with initially severe fatty change, reversion to normal as shown by subsequent biopsies did not occur in less than five weeks, although the patients were clinically almost well for part of these periods. While the development of fatty liver may be rapid, regression is probably a prolonged process (Figs. 32 to 36).

Table VI correlates severity of steatosis with duration of illness. If fatal cases are excluded, it can be seen that those with the most severe fatty liver initially had the longest clinical course of illness.

Fifty-one patients suffered one or more relapses of gastro-enteritis. As shown in Table VII, there is no obvious relationship between the degree of fatty liver and the tendency to relapse. Relapse can occur in very young babies in the absence of fatty liver.

(6) Body weight. As Table VIII shows, there is no obvious relationship between fatty liver and either actual or expected body weight at the time of biopsy. Loss of weight between biopsies was associated with an increase in liver fat only in a minority of cases (Table IX). The most severe degrees of fatty liver were found generally in babies with much subcutaneous fat; if such cases had lost weight, this might be explained in part by dehydration.

(7) Calorie intake. Careful records were made of the food intake of all patients in the series during their stay in hospital. From these the daily intake of protein, carbohydrate and fat has been calculated in grams/Kg./day, and also daily calorie deficiencies. Allowance has been made for loss through vomiting, but this could only be estimated roughly; moreover it is by no means certain how much retained food is utilized by these infants. The nutritional value of transfused fluids is included in the calculations.

The large majority of cases received National Dried Milk since this was the food to which they were accustomed prior to admission. Each ounce of reconstituted full-cream milk was estimated to contain 7.4 g. protein, 11.5 g. carbohydrate and 8.1 g. fat with a calorie value of 144. The corresponding figures for half-cream National Dried Milk were taken as: protein 6.2; carbohydrate 13.0; fat 4.6; calories 112. When special proprietory brands of milk and other foods were used, these values were calculated from the information supplied by the manufacturers. Each teaspoonful of sugar was reckoned as 4 g. carbohydrate; each ounce

of infused plasma, 1.4 g. protein; each ounce of infused 5% glucose, 1.4 g. carbohydrate; each ounce of casein hydrolysate, 1.4 g. protein, 1.4 g. carbohydrate and 11.5 calories. Tables X, XI, XII and XIII correlate these food values with severity of fatty liver at various intervals prior to biopsy or autopsy, irrespective of other factors such as diagnosis. It would appear that deficiency of calories and of all three energy-producing components of the diet is related to the severity of fatty change. It should be stressed, however, that these are mean values for the whole series and that the standard deviations for all figures are relatively high, except in the fat ++++ group (Figs. 37 & 38). Correlation is weakest at the other end of the scale and this may be explained by (a) inclusion in the fat negative group of several tiny infants with severe illness and low food intake; (b) inclusion in the fat + and fat $\pm$ groups of babies whose clinical recovery with improvement in food intake preceded reduction in liver fat, which appears to be a gradual process; (c) the possibility that a mild degree of hepatic steatosis may be produced as a physiological phenomenon in healthy infants through the ingestion of full-cream milk.

(8) Liver function tests and liver size. The results of liver function tests are summarized in Table XIV. All cases with elevation of serum bilirubin in the absence of detectable liver necrosis ("hepatitis") suffered from parenteral infection with anaemia and it is probable that excessive haemolysis was present. If there was impaired ability of the liver to conjugate and excrete bilirubin it could not be correlated with

fatty change. Of the flocculation tests, thymol turbidity was equivocal in a few patients without hepatitis, but in these same patients the Takata-Ara and colloidal gold reactions were always negative. Hyperlipaemia following milk feeds may cause difficulty in estimating thymol turbidity although, whenever possible, blood was withdrawn before feeding times.

Total plasma protein was somewhat depressed in the majority of infants in this investigation, even during dehydration. The level was notably low in those with an extreme degree of fatty liver; otherwise there was no correlation with the severity of hepatic steatosis. Likewise, some cases of gastro-enteritis had low plasma values for cholesterol and phospholipid which could not be correlated with the degree of fatty liver.

Contrary to expectations, no relationship at all could be made out between the degree of fatty change and clinical estimations of liver size based on palpation and percussion. In a few cases the liver fat decreased although the liver was thought to increase in size.

(9) Carbohydrate metabolism.

(a) Blood sugar. This was estimated in about a third of the cases at the time of initial liver biopsy. These fasting blood sugar levels varied considerably and profound hypoglycaemia was not uncommon in severe gastro-enteritis, especially in those cases with gross fatty liver. The correlation between less severe hypoglycaemia and fatty liver is not so marked (Table XV).

(b) Urinary ketones. Almost invariably, a high initial liver fat

was associated with ketonuria. Less frequently ketonuria was found in cases with little or no stainable liver fat, especially if the liver appeared to be depleted of glycogen and the blood sugar level was depressed (Table XV).

(c) Liver glycogen. A rough quantitative assessment was undertaken based on histochemical findings, in a manner similar to that for quantitation of liver fat. Examples of various liver glycogen accumulations are illustrated in Figures 39 to 41. From Tables XVI and XVII it is obvious that, with a few exceptions, the two are inversely proportional quantitatively; as one diminishes the other tends to increase.

From these findings it would appear that severe fatty liver in gastro-enteritis is associated with an upset in carbohydrate metabolism which involves lowering of the blood sugar, depletion of liver glycogen and a tendency to ketosis.

(10) Treatment. The results of this part of the investigation are shown in Tables XVIII and XIX, in which are listed those substances with an alleged lipotropic action used in the experiment together with other forms of therapy which might have had some effect on the degree of steatosis. Inositol, pyridoxine, pantothenic acid and riboflavine were used in too few cases for any conclusion to be drawn from the results, but in none of these was there any very striking improvement. Neither did methionine, choline, Vitamin B complex or intravenous casein have any notable effect on liver fat in the average case. All three cases receiving casein hydrolysate orally showed perceptible decrease in liver

fat, but this was not investigated further, because of the severe vomiting apparently aggravated by this form of therapy. None of the other factors noted, including intravenous plasma and glucose, had any appreciable effect on liver fat in the majority of cases.

(B) Liver Necrosis

Liver necrosis has been reported as a serious complication of infantile diarrhoea and vomiting by several authors and it was expected that some cases including minor degrees of the condition would be revealed by an investigation of this failure. In fact, only three babies had clinical evidence of parenchymal liver damage and one of these may have had a primary viral infection of liver. Moreover, I had experience of no other example of this condition outwith the present series of cases during a three-year period.

The following clinical details refer to the three cases in question.

Case 1 (No. 152 of series). Female baby, aged 15 weeks, admitted on account of vomiting and diarrhoea with green stools of 24 hours' duration. The patient was mildly toxic and dehydrated and showed no evidence of parenteral infection or jaundice.

3rd day - Clinical condition improved and dehydration relieved without necessity for parenteral fluid therapy. Blood examination revealed normal flocculation tests and a negative van den Bergh reaction. Total plasma protein measured 5.25 g.%. Liver biopsy shows slight fatty change but no sign of necrosis, fibrosis or mesenchymal cellular proliferation or

infiltration (Figs. 42 & 43).

14th day - Loose green stools and occasional episodes of vomiting had persisted. In spite of this, liver biopsy shows almost no fatty change and no other abnormality (Figs. 44 & 45). Liver function tests remained negative.

16th day - Vomiting subsided since reversion to reconstituted half-cream dried milk. The patient became febrile and examination now revealed bilateral basal bronchopneumonia.

21st day - The respiratory infection had responded well to penicillin but on this day there was a severe relapse of vomiting and diarrhoea leading to dehydration. Oral feeding was discontinued and intravenous fluid therapy commenced. The fluids administered were Hartmann's solution and casein hydrolysate according to the schedule already described.

24th day - In spite of the relief of dehydration the child remained toxic and jaundice was noticed for the first time. Bilirubin was present in the urine and blood examination revealed a direct positive van den Bergh reaction with serum bilirubin measuring 5.5 mg.%. All three flocculation tests were now notably positive and the plasma protein level had fallen to 4.25 g.%. Since the prothrombin time had not risen above 35 seconds, a third liver biopsy was carried out. Compared with the previous biopsy specimen there is a considerable increase in periportal fat and condensation of stroma in the portal areas (Figs. 46 & 47). Necrosis is not obvious, but neutrophil polymorphs are present in the portal tracts (Fig. 48), and there appears to be some cholangiolar proliferation. This

is suggestive of mild acute cholangio-hepatitis, but there is no bile duct dilatation and no bile thrombi.

25th day - The child was ill and febrile but there was no evidence of venous thrombosis or recurrence of pulmonary infection. In view of the possibility of a reaction to casein hydrolysate this was replaced by diluted plasma in the intravenous drip.

31st day - Fever subsided following withdrawal of casein hydrolysate but the patient remained ill with deepening jaundice, diarrhoea and occasional episodes of vomiting. There being no evidence of a haemorrhagic tendency or untoward elevation of prothrombin time, a fourth biopsy was undertaken. The histopathological features noted in the third biopsy persisted in this specimen and are rather more severe (Figs. 49 & 50). Perilobular cholangiolar proliferation with loss of adjacent liver parenchyma are illustrated (Fig. 51), but otherwise there is no evidence of frank liver necrosis.

36th day - The patient's condition continued to deteriorate and she died on this day. A further course of penicillin together with sulphamezathine had been given, but without effect. Because of persistent vomiting and diarrhoea, intravenous infusion was continued.

Post-mortem examination confirmed the presence of liver necrosis. The liver was of unusually soft consistency and presented a mottled appearance due to points of haemorrhage in an otherwise pale fatty organ. Histological examination now reveals extensive loss of liver parenchyma,

although this may be exaggerated by post-mortem autolysis (autopsy performed 18 hours after death). Necrosis is extensive throughout each lobule and there is haemorrhage in some centrilobular zones (Fig. 52). Fatty change and portal fibrosis persist (Figs. 53 & 54). There was no evidence of infection of the extra-hepatic bile-ducts or gall-bladder and inflammation of the alimentary canal was entirely absent, the bowel being empty apart from some green mucus in the colon. There was no sign of parenteral infection with the exception of a little muco-purulent material in both middle ears; the broncho-pneumonic condition had subsided. In spite of prolonged infusion of intravenous casein hydrolysate, venous thrombosis was not evident. Macroscopic and microscopic examination of other organs revealed no additional information of note.

It is claimed that liver necrosis complicating infantile gastro-enteritis is often periportal in distribution (Wainwright, 1950). This case conforms in as far as there is destruction of limiting cell plates adjacent to inflamed portal tracts, but frank necrosis is evident only in the autopsy specimen. The case might be regarded as an example of cholangiolitic hepatitis; however, it is unlike any examples which I have seen in liver biopsies from adult patients; in these, hydropic swelling of liver parenchymal cells and numerous lymphocytes are present, but are not seen in this case. It is interesting to note that the liver complication occurred during administration of casein hydrolysate, which incidentally appeared to aggravate the symptoms of the initial illness. Moderately severe fatty change also developed in the liver during this period, but

may have been one manifestation of a toxic factor peculiar to the case, and not comparable to the rest of the series. The presence of periportal fibrosis is also of interest; this was rarely found to such a degree in uncomplicated fatty liver and appears to develop fairly quickly with the onset of portal inflammation.

Case 2 (No. 72). Male child, aged 19 weeks, admitted with diarrhoea and vomiting of three days' duration. The baby was markedly toxic and dehydrated necessitating immediate intravenous fluid therapy, using Hartmann's fluid and diluted reconstituted plasma according to the method already described. There was no jaundice and no evidence of parenteral infection.

3rd day - Intravenous fluids were discontinued because of rapid improvement in the clinical condition and success with oral feeding.

22nd day - The child was kept in hospital because of slight intermittent attacks of diarrhoea with green stools and occasional vomiting. On this day slight icterus was noted in the conjunctivae and bile pigment was found in the urine. Blood examination revealed a direct positive van den Bergh reaction with a serum bilirubin level of 2.5 mg.%. All flocculation tests were distinctly positive and the total plasma protein measured 4.67 g.%. Liver biopsy reveals small foci of necrosis having a haphazard distribution throughout the liver lobules (Fig. 55). Periportal cells show loss of cytoplasmic basophilia but there is no cholangitis (Fig. 56). As in the previous case there is evidence of stromal condensation (Fig. 57).

34th day - The child was sent home on this day since he had been feeding normally and had normal stools for four days. The jaundice had practically disappeared although liver function tests were still abnormal. A second liver biopsy was attempted two days before dismissal, but only a very small amount of tissue showing no abnormality was obtained.

This child was seen as an out-patient two weeks later, when he appeared quite well. There had been no relapse of diarrhoea and vomiting and jaundice was no longer evident.

This would appear to be another example, possibly a different type, of liver necrosis complicating infantile diarrhoea and vomiting. It was notably milder than the first case and resulted in recovery. Unfortunately, there was no biopsy before the onset of jaundice as it had not been intended originally to include this case in the present series. There was never any evidence of parenteral infection and no form of lipotropic therapy had been employed.

Case 3 (No. 93). Female child, aged one year, admitted with vomiting and diarrhoea of three days' duration. As is usual in children of this age, the symptoms were not very severe and did not call for any special treatment. There was evidence of bronchitis.

8th day - Mild diarrhoea persisted for a few days although the patient was able to take a normal mixed diet fairly well. Bacteriological examinations for dysentery and other specific infections were negative. Jaundice was noted for the first time on this day. Bile was present in the urine and the serum bilirubin measured 2.8 mg.%. Flocculation tests

were positive and the plasma protein level was 5.67 g.%. Liver biopsy shows some periportal fatty change. There is no obvious necrosis but a striking infiltration of the portal areas and adjacent sinusoids with inflammatory cells, mostly lymphocytes together with a few neutrophils (Figs. 58 & 59). Periportal fibrosis is slight (Fig. 60).

21st day - The jaundice still persisted although the patient had now been free from symptoms for the past week and taking a normal diet readily. A second blood examination revealed abnormalities of liver function almost identical to the first examination. All the features of the first liver biopsy are now more marked in a second biopsy (Fig. 62), while portal fibrosis is established (Fig. 61). There is still no conclusive evidence of liver cell necrosis but small collections of inflammatory cells are seen which may obscure pin-point lesions of this nature.

35th day - The patient having remained well and the jaundice having now subsided, she was dismissed home. Three days earlier a third liver biopsy was undertaken and shows persistence of the features already found in preceding biopsies but are slightly less marked (Figs. 63 & 64). Liver function tests also continued to be abnormal but showed some improvement.

The case has not been designated "gastro-enteritis" since the initial vomiting and diarrhoea were mild and possibly the prodromal stage of a primary hepatic infection. That this may have been viral in type is suggested by the clinical course of the illness and the predominance of lymphocytes in the liver tissue. Hydropic degeneration of liver cells and pin-point focal necrosis, which are typical of virus A (IH) or virus

B (SH) infections, are not found, however. Milder types of infection such as that associated with glandular fever were considered, but at the time of the third biopsy the Paul-Bunnell test was done with negative results and the characteristic cells of the disease were not seen in stained blood films. Cytomegalic inclusion disease was not excluded but seems unlikely in a child of this age. The co-existence of bronchitis suggests the presence of an influenzal or adeno-virus infection, although hepatitis is not described as a complication of such conditions. Plasma cells are inconspicuous and this would seem to exclude the type of hepatitis recently described by Page and Good (1960).

(C) Other Structural Changes in the Liver

(1) Changes in mitochondria. Liver cell mitochondria are demonstrated readily by staining biopsy material with iron haematoxylin (Fig. 65). Owing to distortion wrought by chemical fixation, no assessment of early degenerative changes in these cytoplasmic structures was possible in this investigation, but considerable variation in quantity and distribution was apparent between different cases. In a few instances mitochondria could not be differentiated adequately from cytoplasmic basophilic material.

In many biopsy specimens mitochondria could be seen in all liver cells although the concentration is usually greatest and sometimes confined to the peripheral part of each lobule. Contrary to expectation this was not related to fatty change and mitochondria can be seen in the marginal

cytoplasm of liver cells laden with fat (Fig. 66). In normal liver cells the distribution is usually diffuse throughout the cytoplasm, but it may be marginal (Fig. 67).

Individual variations could not be correlated with severity, duration or type of illness (apart from loss of stainable mitochondria in cases of "hepatitis"). In several biopsies from a single patient the amount and distribution is not infrequently constant in spite of variation in the amount of fat and severity of illness. Mitochondria cannot be demonstrated in autopsy material, even if examined within a few hours of death (Fig. 68).

(2) Changes in cytoplasmic basophil bodies. Liver cell cytoplasm is normally rich in basophilic material, which, in this series of cases, was demonstrated in both autopsy and biopsy material by Giemsa's stain applied for 24 hours (Figs. 69 & 70). Staining is generally most intense in the periportal zones and is occasionally confined to the cells of these regions (Fig. 71). Within individual liver cells basophilic staining may be diffuse or granular, and occasionally may appear to be concentrated adjacent to the walls of bile canaliculi (Fig. 72). Basophil bodies are usually taken to represent ribonucleic acid within the liver cells; they disappear following treatment with ribonuclease (Rich & Berthrong, 1949).

As with mitochondria, basophil bodies are often found in fat-laden liver cells (Fig. 73), but there are some exceptions (Fig. 74), and no correlation could be made out between the apparent amount of this substance and the severity or variability of hepatic steatosis or the

clinical state of the patient. In liver necrosis, however, there is complete lack of ribonucleic acid staining (Fig. 56).

In view of its relationship to protein synthesis, the degree of basophilia was assessed roughly and related to the quantity of circulating plasma protein (Table XX). The degree of correlation is slight and possibly without significance, although a more informative result might have been obtained with values for plasma albumen, rather than total protein.

In nutritional liver disease such as alcoholic cirrhosis, the liver cell cytoplasm may contain dense hyaline bodies ("alcoholic hyalin" or "Mallory bodies"). I am familiar with this entity but have found no convincing evidence of its presence in this series of cases.

(3) Mesenchymal cellular proliferation or infiltration, etc. Sinusoidal cells appear unusually prominent in five cases, e.g., Figure 75, but the significance of this was not evident. Apart from cases of liver necrosis, minor degrees of lymphocytic or polymorphonuclear leucocytic infiltration were detected in 28 specimens from 22 patients, but could not be correlated in any way with clinical diagnosis or severity of illness. Three of these 22 had no infective condition clinically. These cells may be most conspicuous in or around portal tracts (Fig. 76) or scattered diffusely throughout the sinusoids (Fig. 77). There was no correlation with age or anaemia and extra-medullary haematopoiesis appears to be an improbable explanation. Figure 76 incidentally shows the phenomenon of liver cell nuclear vacuolation; this was found also in two

other biopsies but its significance was not apparent; there was no clinical evidence of liver necrosis or diabetes mellitus in these cases. Bacilli are seen in one biopsy specimen stained by Giemsa's method (Fig. 78); this was a case of severe gastro-enteritis without parenteral infection who recovered eventually.

(4) Increase in connective tissue stroma of liver. Apart from liver necrosis, minor degrees of condensation of periportal reticulin (Figs. 79 & 80) and portal fibrosis (Fig. 81) were noted in 10 specimens from 8 patients. This need not be related to severe fatty change; conversely the reticulin pattern can be quite normal in association with fatty liver. In no case is there any indication of disturbances of the normal lobular architecture or nodular regeneration, indicative of cirrhosis. It is important in autopsy material to disregard the liver structure immediately under the capsule (Fig. 82: The remainder of this liver was not fibrotic).

(5) Haemosiderin in liver. This was demonstrated by the Prussian Blue reaction in all specimens fixed in formalin and passed to paraffin (Fig. 83). Arbitrary assessment of the amount of pigment, presumably haemosiderin, was made as for fat and other liver cell constituents. Some examples of variable degrees of positive staining are illustrated (Figs. 84 to 87). Haemosiderin is in the form of intra-cytoplasmic granules, usually confined to liver cells in the periphery of each lobule. These granules may be seen in cells distended with fat (Fig. 87). Occasionally the iron-containing pigment is largely or completely

contained within sinusoidal cells (Fig. 88).

Staining is intense in material from very young infants, but traces persist in the liver cells of older babies, often to the fortieth week of life. The obvious relationship between liver haemosiderin and age is shown in Table XXI, which is based on examination of all liver specimens with the exception of a few recovered from patients who had received previously a blood transfusion or oral iron therapy. Table XXII shows the relationship to the results of certain haematological investigations carried out at the times of biopsy. Intensive iron staining is generally associated with excessive numbers of circulating reticulocytes, above average corpuscular haemoglobin concentration and indirect positive van den Bergh reaction.

No relationship was evident between the extent of iron staining and clinical diagnosis, severity or duration of illness or degree of fatty liver, and consequently there was no relationship also to the nutritional state of the patient. Intense siderosis seems to be associated with hypoproteinaemia (Table XXII), but this may be explained by the frequency of both in the youngest age groups.

(D) Other Features noted at Post-mortem Examination

Of the 26 autopsies performed, extensively severe enteritis with superficial erosions of bowel mucosa and phlegmonous exudate was noted in 4. Eight others showed mild inflammation especially in the ileum. In the remainder the alimentary canal was macroscopically normal, although

histological examination sometimes revealed very mild inflammatory congestion and cellular infiltration (Figs. 89 & 90). A mild non-specific inflammatory reaction commonly involved the mesenteric lymph nodes, but no splenic abnormality was noted.

Cholecystitis and extra-hepatic cholangitis were never observed. Reference has already been made to the various types of parenteral infection found in this series of cases.

Histological examination of the pancreas from each case showed none of the degenerative changes which have been described in association with nutritional liver disease, or which, in a more severe form, would have been suggestive of fibrocystic disease.

Hydropic and fatty degeneration were commonly found in renal tubular epithelium but no other changes of note. No abnormal deposits of glycogen indicative of von Gierke's disease were detected at this site or in any liver specimens.

In 6 fatal cases there was dilatation of the right side of heart, together with pulmonary oedema and haemorrhage, suggestive of excessive administration of fluid by intravenous infusion. Cerebral oedema was also noted in such cases but no specific form of encephalopathy or intracranial haemorrhage.

No abnormality of note was detected in the endocrine glands from any case. The zona fasciculata of the adrenal cortex was usually depleted of stainable lipid.

DISCUSSION

Infectious diarrhoea and vomiting in infants is one of several communicable diseases which have undergone a notable reduction in virulence, particularly in the last decade (Derham & Rogerson, 1957). This change has been noted in various parts of the world, e.g., in Australia by Croll (1946). However, at the time of this investigation it was still a common and serious condition responsible for many deaths during the first year of life, particularly among children of the lower social groups living in overcrowded conditions in cities such as Glasgow (Bloch, 1941). During the period in question (January 1947 to December 1949), I had clinical responsibility for 608 cases, including those which are the subject of this investigation. Even during these few years there was a notable drop in mortality, from 38 per cent in 1947 to 16 per cent in 1949. It is unlikely that this was due entirely to improvements in therapy, important as these were, such as early and effective relief of dehydration and the use of antibiotics, together with increasing experience of senior nursing staff.

It seemed at that time that any investigation into this important disease might be of value and would justify the use of liver biopsy as a means of investigation. In addition, it seemed that such a project would be of value in assessing the importance of fatty liver in general and not necessarily that occurring in infantile gastro-enteritis. Fatty liver is found not infrequently in association with a variety of human

illnesses and its importance may be a matter of conjecture in spite of the large amount of experimental work which has been done on this subject. Nutritional deficiency in general and lack of lipotropic factors in particular, have been regarded as causes, and such a hypothesis could be studied fairly readily in young bottle-fed infants, the nutritional value of whose diet could be calculated and supplementary factors easily added.

This being in part a clinical investigation, certain data are of necessity based on subjective impressions. Certain other data, such as the amounts of liver fat and glycogen, although measurable, are similarly estimated and would have been of more value if accurate quantitation had been carried out. These omissions are due to a lack of sufficiency in time and certain laboratory facilities. What is presented in this investigation was carried out by the author unaided by any technical assistance and included the setting up and use of all apparatus for histology and for certain biochemical methods not previously in use at Belvidere Hospital. However, it is claimed that the conclusions have some value being based on notable qualitative changes in the liver.

Investigation and Treatment of Infantile Diarrhoea and Vomiting

The diagnosis of this disease was one matter which depended on clinical judgment and could not be subjected to laboratory analysis. However, an illness of bottle-fed infants causing watery diarrhoea with green or orange stools, vomiting of variable intensity, dehydration and irritability, is quite typical of the condition. There are many

descriptions of the disease in the medical literature which confirm this, e.g., Paterson and Nabarro (1922); Young (1933); Cooper (1937); Smellie (1939); Campbell and Cunningham (1941); Alexander and Eiser (1944); Gunn (1945); Gairdner (1945); Mann et al. (1952); Friedman (1953); Lowdon (1954). Mild cases were recognized as such if diarrhoea and vomiting persisted after correction of other possible causes of these symptoms, such as faulty feeding or parenteral infection, and in the absence of bacteriological evidence of dysentery or food-poisoning. During the last year of the investigation, facilities were available for typing pathogenic strains of Bact. coli, described originally as Bact. coli neapolitanum by Bray (1945), and subsequently by Giles and Sangster (1948) and Giles et al. (1949). Numerous more recent investigations have been reviewed by Payne (1960). A close correlation was found between the recovery of these organisms and the clinical diagnosis of gastro-enteritis. Not infrequently positive results were obtained from relatively mild cases and from symptomless contacts in other wards of the hospital.

It is unlikely that any persistent case of epidemic diarrhoea of neonates is included in this series, since this infection generally arises in the nursery units of maternity hospitals whence none of my cases came. It would appear to be a viral infection and cell-free filtrates of the faeces can cause diarrhoea in calves (Light & Hodes, 1949). Other viral infections associated with enteritis are recorded in older children and in adults (e.g., Brown et al., 1945); no case in this series occurred as part of an outbreak of diarrhoea and vomiting among

older persons. Joncas and Pavilanis (1960) undertook viral cultures of rectal swabs from 74 infants and children with diarrhoea. Fourteen viruses were isolated, including adeno-viruses which may have been pathogenic. Echo virus has also been recovered from similar cases (Sommerville, 1958).

In some accounts of epidemic neonatal diarrhoea, the liver has been reported as normal, e.g., Ormiston (1941); Scott et al. (1952). Rice et al. (1937) mentioned parenchymatous degeneration of liver but did not specify fatty change. Sakula (1943) found fatty liver in a few of his cases, but the significance of this was not investigated.

Allergic diarrhoea has also been borne in mind, but in the absence of urticaria and eosinophilia there was no suggestion of this condition in any patient. According to Rothman (1953), certain cases of infantile diarrhoea and vomiting may be explained on this basis, since it is possible for proteins of cow's milk to enter the circulation undigested and act as antigens, especially in very young subjects or in those with some other intestinal disease. Schloss and Worthen (1916) found foreign protein in the urine of children with nutritional disorders, possibly derived from ingested food.

Certain primary metabolic diseases causing illness in infancy are associated with fatty liver, and conceivably such cases could be admitted to a gastro-enteritis unit because of vomiting or diarrhoea. No patient in this series had reducing substances in the urine so that both galactosaemia and diabetes mellitus may be excluded. In the hepato-renal form

of glycogen-storage disease the liver parenchymal cells are swollen with abnormal quantities of fat and glycogen; the cytoplasm is vacuolated and the nuclei small and centrally placed. These features were not noted in any specimen of liver in this series. Fibrocystic disease of pancreas may be associated with fatty liver (Craig et al., 1957) but no evidence of this disease was found in the autopsy material. Likewise, early cases of hepato-lenticular degeneration may have fatty liver (Anderson & Popper, 1960), but in addition there are degenerative changes in liver cell nuclei and in sinusoidal cells which were not noted in my cases. These are all uncommon diseases, and it is clear that the majority of my patients suffered from an endemic enteral infection now accredited to pathogenic strains of Bact. coli.

While it is unsatisfactory to have no laboratory confirmation of the clinical diagnosis, this is not regarded as a serious disadvantage in the present investigation; fatty liver is likely to be part of a metabolic upset common to a variety of enteral infections. Marriott et al. (1933) noted its occurrence in their series of cases of infantile diarrhoea and vomiting, a third of which proved to be dysentery. In the present investigation it has been noted that fatty liver, at least in its less extreme degrees, is by no means confined to patients with the clinical diagnosis of "gastro-enteritis". Consequently, while correlations have been sought with diagnosis and the incidence of complications, relapse and co-existing parenteral infection, more attention has been given to the general aspects of nutrition and fat metabolism.

All patients were seen twice daily so that any alteration in the clinical condition could be detected early. Apart from the initial examination on admission, evidence of parenteral infection was always looked for in those cases failing to respond to treatment, and in the event of relapse. Catheterization of female patients was restricted, however, except when urine was required for investigation of ketonuria, since urinary infection is not very common in this young age group and may be induced by the procedure (Smellie, 1939). Otitis media was recorded only from distinct redness of the ear-drum, with or without bulging, and these signs may only be apparent after the relief of dehydration (Marshall, 1933; McConkey & Cooper, 1938; Leathart, 1943). Conversely, during dehydration, the drum can have an opaque appearance which is probably not significant (Karelitz & Schick, 1931; Cohen *et al.*, 1933). There appears to be some difference of opinion regarding the importance of otitis media in relation to the aetiology and course of infantile gastro-enteritis. Gunn (1945) recorded an 80 per cent incidence among his fatal cases and some authors go so far as to regard the ear infection as the underlying cause of the disease. Other observers, e.g., Maizels and Smith (1934), have held the contrary opinion, with which I agree. Like Johnston *et al.* (1933), I have seen many cases of infantile diarrhoea and vomiting with no detectable evidence of otitis media and some in whom the ear infection appeared to develop after the intestinal infection. Most cases of otitis in my series responded readily to penicillin, and paracentesis was required very rarely; but penicillin had no influence on

the course of the gastro-intestinal condition. Again, I have noted evidence of otitis media in infants dying of conditions other than the one in question, and this also has been noted by others, e.g., Patterson and Smith (1944); Wright and Wright (1946). Microscopic examination of the mucoid exudate in the middle ears of infants at autopsy usually failed to reveal acute inflammatory cells, while the presence of bile staining on occasion was indicative of regurgitation of alimentary canal contents into the middle ears via the Eustachian tubes (Wishart, 1930). This may have been mistaken for an inflammatory exudate by some authors.

Apart from lipotropic therapy and the very infrequent use of myringotomy, treatment followed the methods generally accepted at the time of the investigations, especially those based on the recommendations of Powers (1926) and described in detail by Field et al. (1943); Alexander and Eiser (1944) and Alexander (1948). Particular attention was paid to the early and efficient relief of dehydration. Since severe illness usually lasted for many days on end, fluid was administered by continuous intravenous drip as first recommended by Karelitz and Schick (1931). It was found best to administer fluid via a cannula tied into the internal saphenous vein at the ankle and anchored by a stitch through the adjacent skin. The limb and lower part of infusion tubing were fixed to one splint which was tied to the cot but with minimum interference to the general management of the patient. With experience and maintenance of a ready supply of infusion equipment, sterile instruments and splints, this operation could be done with little delay, and temporary emergency

infusions into peritoneum were unnecessary. Scalp vein infusions were easier to assemble but less easy to maintain for prolonged periods. In a few very small infants, fluid was given by bone marrow drip using the lower end of femur or upper end of tibia as described by Drinker et al. (1922); Gimson (1944); Massey (1950). This procedure was rarely satisfactory for more than 36 hours. Owing to the tendency to oedema in these small patients (Young, 1942), great care was taken to check fluid intake and output at frequent intervals, but the latter can only be assessed roughly. Overloading of the circulation did occur in a few cases in which it may have been the main cause of death. For this important matter it was necessary to rely on the vigilance of nursing staff, and this was not always satisfactory, especially at night. Considerable improvement was apparent as senior nurses and sisters gained experience. Improvement in prevention of cross-infection was noted likewise, although this was an almost unsurmountable problem incurred by the admission of infectious cases to an open ward.

Certain methods of treatment which are occasionally recommended were tried and discarded as useless or even harmful. Carob flour was found to be quite ineffective, and it is difficult to understand the claims for this form of therapy made by certain Continental paediatricians, e.g., Fortier et al. (1956). Walker (1952) found that carob flour may even be dangerous as it can aggravate dehydration. Other methods which were not employed were initial purgation (Field et al., 1943; Gunn, 1945), initial gastric lavage (Lawson, 1951; Lowdon, 1954), and administration of fluid

by intra-gastric drip (Giles & Sangster, 1948; Berkeley, 1947).

Owing to lack of laboratory facilities, a proper control of blood electrolytes was not undertaken in these cases. Much reliance was placed on the oral or intravenous administration of Hartmann's solution (Hartmann & Elman, 1929; Hartmann & Senn, 1932) for the supply of electrolytes in a manner likely to prevent severe acidosis and alkalosis. Hartmann's solution was always diluted with 5% glucose or plasma. No extra potassium salt was added, although this has been recommended by Darrow (1946) and others in view of the low levels of exchangeable potassium associated with persistent protein insufficiency in infants (Smith & Waterlow, 1960). Without measurement of blood electrolyte levels, it is impossible to estimate the importance of conditions such as hypernatraemia (Finberg & Harrison, 1955; Skinner & Mole, 1956; de Young & Diamond, 1960) and hypocalcaemia (Rapaport et al., 1947) as causes of morbidity and death in my cases. A few fatal cases had signs of excessive sodium and water retention. None had intracranial bleeding suggestive of gross hypernatraemia or any evidence of hypokalaemia as described by Perkins et al. (1950).

Effective antibiotics such as neomycin were not available at the time of this study. Some cases were treated with streptomycin or aerosporon. The former had a remarkable curative effect in many cases, but symptoms were liable to recur after the antibiotic was discontinued. This has been the experience of others, e.g., Lowdon (1954).

Investigation of Liver Structure and Function

There are few reports on the use of liver biopsy in paediatric practice and only that of Stowens (1960) is based on any substantial number of cases. Reference has been made already in the introduction to some investigations of this sort. In addition, Bruton et al. (1955) gave their experience of the operation in 50 children whose ages ranged from 19 days to 10 years. In a few of these cases the operation was repeated. These authors used the infra-costal approach since all their cases had hepatomegaly (mostly not steatosis). As a result of their experience, they believe that liver biopsy is a safe and useful adjunct to clinical investigation. They did not give their patients the benefit of any sedation prior to biopsy, which seems unreasonable. Pomponio (1956) reported his experience of liver biopsy in 100 children, mostly over the age of one year, and stated that the operation is easier in children than in adults and can be repeated safely after a few days; my experience is in agreement with this.

Vernois (1844: quoted by Rossett, 1956) is credited with the first successful liver biopsy. However, its use as an established procedure in clinical work is generally attributed to Iversen and Roholm (1939), whose method I have used in this investigation with only minor modifications, viz., the use of a larger (50 ml.) syringe for aspiration, and omission of a preliminary skin incision before the insertion of trochar and cannula. My 5 per cent failure rate compares favourably with most reports; Iversen and Roholm had a failure rate of 22.5 per cent. By the

use of more modern techniques such figures can be reduced substantially; Nelson (1954) failed to obtain liver tissue in only 2 per cent of his series of young adult patients using a modified Vim-Silvermann needle.

Most authors are agreed that liver biopsy is a reasonably safe procedure, at least in the hands of those with experience of the technique. Terry (1952) has reviewed reports totalling 10,000 cases and finds a recorded death rate of 0.12 per cent and important non-fatal complications in 0.33 per cent. While there is a wide range of complications (Rossett, 1956), only one example, viz., right-sided pneumothorax, was found in my series and fortunately this resolved within a few days. Although many babies who had been subjected to liver biopsy eventually died, there was no indication that death was due in any instance to the operation. Thus, no examples of haemothorax, haemoperitoneum, biliary peritonitis or hepatic rupture were noted in those cases subjected to autopsy. The death rate of this series of cases was slightly lower than that of other cases of gastro-enteritis in my charge during the same period.

It may be assumed that needle biopsy can afford a reasonable evaluation of a diffuse liver lesion like steatosis. On the contrary one would not expect this to apply to a lesion of irregular distribution such as focal necrosis, coarse cirrhosis, or those cases of fatty change due to some local vascular disturbance with liver parenchymal ischaemia. I can confirm the former assumption from examination of blocks of tissue taken from various parts of the livers of fatal gastro-enteritis cases. Occasionally, fatty change is slightly more severe throughout the right

lobe compared with the left. Of course, all biopsy material was taken from the right side and so is comparable, while records of autopsy material are assessed on the appearance of the right lobe. The fatal case of liver necrosis had a fairly uniform distribution of liver damage which was recorded accurately in the biopsy specimens.

A few authors of articles on this subject do not agree with these opinions. Coppo et al. (1956) took biopsies from right and left lobes of liver during laparotomy in patients with hepatitis and found considerably more damage on the right side. Bogoch and Casselman (1955) performed multiple biopsies at short time intervals (3 hours, 25 minutes and 5 minutes) in three patients, in an attempt to show that the liver structure may vary with alteration in functional activity from time to time and in different parts of the organ. In fact their third patient did show variable fatty change during these short time intervals, but as the diagnosis was biliary cirrhosis, it is possible that the distribution of fat represented variable degrees of ischaemia in different parts of the liver.

Only standard histological techniques were employed and these require little comment. The efficacy of methods for the demonstration of glycogen was tested by staining control sections treated with diastase. Fixation in a picric acid-dioxane mixture had no advantage over Bouin's fluid. Even the coating of slides with celloidin prior to the application of Best's stain was found to be unnecessary. The osmic acid sections were examined soon after preparation, since fading can occur

within a few weeks. No enzyme studies or special cytochemical methods of investigation were undertaken although additional information would no doubt be gained by such a project. Studies of this sort have been reported by Waterlow (1950) in cases of kwashiorkor.

Reports on the use of liver function tests in young children are, like those on liver biopsy, relatively few. Harris (1952) investigated three well-known causes of jaundice in infants, viz., hepatitis, erythroblastosis foetalis and bile duct atresia, and found such tests of less value than straight-forward clinical examination, supplemented if necessary by liver biopsy. The association of an obstructive type of jaundice with hepatitis and with erythroblastosis is possible and would be expected to cause difficulty in the interpretation of liver function tests in certain cases. Kaye et al. (1959) also found biopsy superior to chemical tests of liver function in differentiating jaundice of bile duct atresia from that of viral hepatitis. Weller (1951) is of the opinion that tests of liver function may be misleading when applied to infants; he found negative flocculation tests in hepatitis and low levels of alkaline phosphatase in obstructive jaundice, which is the reverse of the generally accepted findings. Weller investigated 53 infants suffering from a variety of hepatic diseases and 12 normal controls. Four were cases of gastro-enteritis with hepatic failure, but unfortunately the exact nature of the lesion in the liver is not clear from the author's description.

In the present series it is remarkable how sensitive the flocculation tests were in detecting liver necrosis confirmed by biopsy. All remain-

ing cases had negative tests irrespective of the degree of fatty change and presumably there was no associated elevation of plasma globulin (Kunkel & Hoagland, 1947). Serum for these tests was collected before feeds to avoid the turbidity sometimes noted after ingestion of milk. Wilson and Hanner (1934) observed that cream or cod-liver oil caused in children a marked rise in serum lipids, especially neutral fat. Apart from undesirable turbidity following milk feeds, elevation of blood fat might invalidate the results of thymol turbidity tests which are dependent on the blood lipid level in addition to the level of globulin.

The finding of negative flocculation tests in the presence of fatty liver is in agreement with most observations made in adult patients with a similar condition, e.g., Franklin et al. (1948), although misleading negative results may sometimes occur in the presence of focal necrosis and viral hepatitis (Popper et al., 1949a & b). Ulevitch et al. (1951) noted positive results in patients with fatty liver, but some of these may have been alcoholic; in alcoholism, variable degrees of liver cell damage in addition to fatty change are almost always present (Popper & Schaffner, 1952; Popper et al., 1955).

Plasma protein levels are slightly lower in normal infants compared with older persons (Darrow & Carey, 1933; McMurray et al., 1948), but are not reduced to the degree found in many cases in this series. The total plasma protein in two of the three cases with liver necrosis and the group with extreme fatty change was particularly low. It is possible that production of protein by the liver, especially albumen and fibrinogen, is

impaired by an extreme degree of fatty change, but the persistence of stainable ribonucleic acid suggests that this function is not completely inhibited. Unfortunately, figures for the individual plasma proteins are not available. Diminution of protein intake and dilution of plasma with intravenous fluid would no doubt contribute to hypoproteinaemia and consequently its significance as an index of liver dysfunction is difficult to estimate.

Apart from the three cases of liver necrosis, elevation of serum bilirubin was generally associated with anaemia, increase in circulating reticulocytes and prominent staining of haemosiderin in hepatic tissue. It is assumed, therefore, that in such cases there was excessive haemolysis rather than damage to liver parenchyma. It may be postulated that a fatty liver has a diminished capacity to conjugate and excrete bilirubin especially if the bilirubin is being produced in excess. Bilirubin excretion tests were not undertaken, but it would appear that in many cases an extremely fatty liver is still capable of excreting normal amounts of endogenous bilirubin.

No case of marked elevation of prothrombin time was found in association with severe hepatic steatosis. The prothrombin response to parenteral vitamin K injections gives a more sensitive indication of this particular hepatic function (Ungar et al., 1948) but was not undertaken in this investigation.

The mean values for serum cholesterol and phospholipid seem rather low but are probably within the normal range for this age group. Low values

are recorded in nutritional deficiency including choline deficiency (Lucas, 1958). Higher levels would have been expected if the fatty liver under investigation were associated with increased mobilization of fat from fat depots under the stimulus of starvation. It is felt, however, that to be significant, this part of the investigation should have included more cases, blcod levels of total lipid and figures from normal controls of the same age group. Owing to the technical difficulties involved and lack of adequate facilities, this was impracticable. As expected the serum cholesterol esters were reduced in two cases with liver necrosis.

Other accredited liver function tests, e.g., blood levels of alkaline phosphatase and clearance of hippuric acid, were not undertaken. It was hoped to carry out bromsulphthalein excretion tests, but this dye was unobtainable at the time of the investigation. Impaired excretion of bromsulphthalein has been reported in cases of uncomplicated fatty liver, e.g., by Popper (1954), and apparently this is not due to compression of sinusoids by fat-laden cells (Kessler et al., 1954).

The notable effect of established liver necrosis on the liver function tests which were performed is an indication of the efficiency of these. Consequently it is reasonable to assume that no severe degree of liver dysfunction was present in the remaining cases, and that the severity of the illness in infantile gastro-enteritis cannot be attributed to this cause. By contrast, the third case of "hepatitis" was never severely ill in spite of the presence of established liver dysfunction.

Pathogenesis of Fatty Liver

It is now commonly accepted that the literal interpretation of the terms "fatty infiltration" and "fatty degeneration", introduced by Virchow, can be confusing when applied to the liver. On the other hand, the unqualified use of the term "fatty change in liver" may mislead by suggesting a single pathological entity. The terms "physiological fatty liver" and "pathological fatty liver" have been used (Peters & van Slyk, 1946) and seem more appropriate to a proper understanding of the condition.

(1) Physiological fatty liver. In cases of this sort, plasma lipid levels are raised but their pattern is not distorted, and the liver receives abnormal amounts of fat through normal fat transport mechanisms. It is assumed that the intra-hepatic metabolism of fat is not deranged but that fat accumulates within liver cells as a result of increased intake. The condition may be found:

(a) following ingestion and absorption of a fatty meal;

(b) following accelerated mobilization of fat from fat depots in the body secondary to some deficiency in carbohydrate utilization, as in starvation and untreated diabetes mellitus with ketosis.

(2) Pathological fatty liver. This term implies a primary defect in fat metabolism involving transportation of fat by phosphorylated compounds or cholesterol esters in the blood stream, or its metabolism within liver cells. Examples of this type are:

(a) Fatty liver associated with toxic hepatitis, especially that due

to ingestion of chemical hepatotoxins. Liver necrosis is usually evident, but fatty change can be more conspicuous, as in phosphorus poisoning. In these cases there is good clinical evidence of liver dysfunction.

(b) Fatty liver associated with anoxia, other than the histoxic type which would be included under 2(a). Liver parenchymal cells, like other specialized cells, are relatively sensitive to deprivation of oxygen so that fatty change may be a manifestation of minor degrees of cell damage in severe anaemia and systemic venous congestion; in cirrhosis fatty change may be found within regenerating nodules rendered relatively ischaemic by the development of intra-hepatic vascular shunts.

(c) Fatty liver induced through deficiency of lipotropic factors not produced endogenously. The normal transportation and metabolism of fat are dependent on these factors which include certain amino-acids, fatty acids and components of the vitamin B complex. This type of fatty liver has been studied extensively with experimental animals.

In the cases under investigation, liver necrosis was a feature of only about 2 per cent and it seems reasonable to dismiss toxic factors as causes of fatty liver in the remainder. There was no correlation between fatty liver and any form of medicinal therapy which might have had a minor deleterious effect on hepatic cells. Fatty liver has been attributed to aureomycin (Yesner et al., 1951; Sutherland et al., 1951; Di Luzio & Zilversmit, 1956) but this antibiotic, being difficult to obtain at the

time of this study, was given to one patient only in the series (a case of staphylococcal pneumonia with septicaemia).

Anoxia, whether of the anaemic, stagnant or anoxic types, may likewise be dismissed from a consideration of the pathogenesis of fatty liver in this series of cases. Many patients were shown to be moderately anaemic following relief of dehydration, but there was no correlation between the roughly estimated haemoglobin value and the severity of fatty liver. Anaemia was never present to the profound degree generally associated with fatty liver in adults.

It has already been observed that a marked degree of lipaemia may be found in infants following a full-cream milk feed. This physiological fluctuation in blood fat level has been noted frequently, e.g., Hejda (1930); Man and Gildea (1932). It is a variable phenomenon (McClure & Huntsinger, 1928), being exaggerated if the diet is deficient in protein and carbohydrate (Bang, 1918). Dogs starved for 7 to 14 days become relatively intolerant of ingested fat and abnormally high blood levels follow a fatty meal in these circumstances (Rony & Ching, 1930). However, Albrink and Neuwirth (1960) obtained the opposite effect in a few human adult subjects starved for periods of up to one week. The association of fatty liver with hyperlipaemia is also well-known. Both are produced in fowls by over-feeding and this is utilized for the production of pâté from goose liver. Hyperlipaemia and fatty liver also occur in geese during the egg-laying season (Flock et al., 1937). Meyer and Hartroft (1960a) induced polyphagia in rats by hypothalamic damage,

and this resulted in a choline-resistant fatty liver. The rate of development of physiological fatty liver can be rapid, possibly taking an hour or less (Dury & Treadwell, 1953).

The fatty liver under investigation cannot be explained so readily. Many liver biopsies showing advanced steatosis were performed when the infants were taking little or no milk. It is possible, however, that the persistence of minor degrees of fatty change during convalescence, and in some cases with no detectable clinical abnormality, may have had this simple physiological explanation.

Mobilization of endogenous fat may be associated likewise with fatty liver. In starvation, the iodine number of liver fat becomes the same as that in subcutaneous tissues and other fat depots. Dible (1932) showed that fatty liver will persist in fasting rats so long as these animals retain a store of endogenous fat, and this was confirmed later in rabbits (Dible & Libman, 1934). The phenomenon is most pronounced in mice, whose liver fat may rise by 300 per cent after one day's starvation (Hodge et al., 1941). These animals are very extravagant in their use of this fat as a source of energy and death ensues in a few days if starvation is not relieved. By contrast the fatty liver induced by high fat diet may be reduced by a spell of starvation, although studies with C^{14} labelled fat reveals impaired fat metabolism in these cases (Whitney & Roberts, 1955). Fine and Williams (1960) studied the effect of starvation, epinephrine, glucose and insulin on the lipid content of

portal and hepatic venous blood and concluded that the liver plays a homeostatic rôle in fat metabolism; in particular, the blood levels of non-esterified fatty acid is controlled by the amount withdrawn or released by the liver.

The fatty liver of infants with gastro-enteritis would appear to have a good deal in common with some of these experimental results. Depletion of food intake amounting to starvation was certainly present in many babies at the time of their admission to hospital when fatty liver might be extreme. The relief of this state of undernutrition was often a matter of great difficulty as long as symptoms of the infection persisted and accordingly steatosis would remain for days or weeks and resolve only gradually with clinical recovery. This investigation has shown a relationship between the degree of fatty change and reduction in the intake of all energy-producing food components, including carbohydrate, and a relationship also with hypoglycaemia, reduction in liver glycogen and ketosis. The absence of severe hepato-steatosis among the very young infants in this series could be explained in the light of this theory by the relative sparseness of subcutaneous fat in the very young. Conversely, it was noted that obese babies frequently had very fatty livers.

The incidence of ketonuria was high in this series compared with other reports. No attempt was made to study ketosis by any quantitative method. Blood electrolyte studies would have contributed little to this, since acidosis in infantile gastro-enteritis may exist for reasons

other than ketosis (Steinitz, 1903; Howland & Marriott, 1916; Schloss & Stetson, 1917; Marriott, 1920), the most usual cause being renal functional impairment associated with dehydration (Schloss, 1918; Maizels & McArthur, 1929; Karelitz & Schick, 1931; Schoenthal et al., 1933; Marples et al., 1934). Indeed, minor degrees of renal tubular damage are frequent (Black, 1959). Other factors contributing to acidosis include the accumulation of metabolites, such as lactic acid, and the transfer of sodium ions into cells (Darrow, 1946).

A great deal of experimental work has been done on the production of fatty livers by means of certain dietary deficiencies, especially in small rodents, which has led to the discovery of lipotropic factors. These have been reviewed recently by Cornatzer (1960). It would be reasonable to suspect a deficiency of these factors in a disease with nutritional disturbance, such as gastro-enteritis, and so their therapeutic effect became part of the investigation.

These experimental diets are generally rich in fat and poor in protein and their effect on the liver can be relieved by the addition of protein, such as casein which is rich in the amino-acid methionine (Best & Huntsman, 1939). Methionine can be transmethylated in vivo to form choline (du Vigneaud et al., 1940; 1941) which is required for the transport of fat to the liver and its metabolism therein (Perlmann & Chaikoff, 1939a & b), particularly with regard to lecithin formation. Consequently, the addition of choline to the experimental diet has the same effect in reducing liver fat as casein or methionine and may even be superior to

methionine, which cannot replace choline completely (Young et al., 1956). There are various other types of experimental fatty liver which are unaffected by choline, but all fail to respond to the appropriate therapy if choline is completely lacking in the diet (Lucas, 1958).

Fatty liver can also be induced in animals receiving normal amounts of methionine or choline if substances are added which antagonise their lipotropic action. Thus, cystine may aggravate a tendency to fatty liver by diverting methionine to the accelerated anabolism of protein, rather than the synthesis of choline (Griffith, 1941a; Mulford & Griffith, 1942). Ethionine has the same effect, especially on female rats, apparently by acting as a methionine antagonist. Nicotinamide may interact with methionine to form N-methyl-nicotinamide, thereby inhibiting its lipotropic action (Handler & Dubin, 1946). Transmethylation of methionine to choline is dependent on the methyl transporting agents, vitamin B_{12} and folic acid; consequently a relative choline deficiency may arise from deficiency of these factors.

In this series of cases the administration of choline or methionine in substantial doses had no notable effect on the fat content of the liver. There may have been impaired intestinal absorption of these substances given orally, but parenteral administration was equally ineffective. Popper et al. (1951) found no inhibition of choline absorption in patients with acute symptoms of severe liver disease, while in cirrhosis the absorption rate is actually accelerated.

Experimental diets deficient in choline or methionine are all highly

artificial and it is doubtful if there is ever a deficienty of this sort in human diets or indeed in any mammalian diet under natural circumstances. A normal adult diet contains about 300 to 600 mg. choline daily, but much of this may be broken down to trimethylamine by intestinal bacteria (de la Huerga & Popper, 1951). Man may share with guinea-pigs and rabbits a natural resistance to choline deficiency (Blumberg et al., 1942; Spellberg et al., 1942; Handler, 1949). Endogenous production of choline from ethanolamine and methyl groups derived from sulph-hydryl containing amino-acids has been shown to occur in man (Simmonds et al., 1943).

The fatty liver of gastro-enteritis has some features which differ from the hepatic lesion of experimental choline deficiency. In most of my cases fat was limited to the periportal hepatic cells, or was maximal in the periportal zone of each lobule, while, in choline deficiency, liver fat has a centrilobular distribution. Best et al. (1955) and Meyer and Hartroft (1960b) found that choline had no effect on a periportal-type fatty liver; in fact, it may aggravate it. However, it is possible that the distribution of liver fat depends on species as well as aetiological factors; monkeys fed a choline-deficient diet for a year or more have the periportal type of lesion which does not progress to cirrhosis (Mann et al., 1953; Wilgram et al., 1958).

By contrast with gastro-enteritis, animals with choline-deficiency fatty liver have hyperglycaemia due to abnormal glucose tolerance (Waldstein et al., 1957). Choline has no effect on the fatty liver induced by starvation (Goldschmidt et al., 1939). McKay et al. (1939)

found otherwise, although ketosis remained unchanged.

Several other substances possess a lipotropic effect on experimental fatty liver and a few of these have been investigated in this work. The numbers of patients so treated are small since initial results were not encouraging. Pyridoxine, which was given to six babies, is said to augment the lipotropic action of choline (Engel, 1942). Moreover, deficiency of this vitamin alone will cause fatty liver in rats (Halliday, 1938) and aggravate the effect of choline deficiency (Griffith, 1941b). Rinehart and Greenberg (1956) report similar results in monkeys; these animals develop fatty liver if maintained on a pyridoxine-deficient diet for three to eight months. Monkeys, however, may be relatively susceptible to this type of nutritional deficiency as their daily maintenance requirement of pyridoxine (3 to 5 mg.) exceeds the average daily human intake.

Like choline, inositol is essential for normal fat metabolism, and its absence from synthetic diets causes fatty liver in rats, even if such diets are poor in fat as well as protein (Engel, 1942).

Lack of pantothenic acid causes fatty liver in dogs (Severi & Fonnesu, 1956). Rats usually die of adrenal haemorrhage before the liver change is evident. Activated pantothenic acid (Co-enzyme A) is one component of liver cells damaged by the ingestion of chemicals such as carbon tetrachloride (Christie & Judah, 1954), and fatty change as well as necrosis is a feature of the liver lesion in such cases of toxic hepatitis.

An increase in liver fat has been noted in riboflavin-deficient dogs

(Potter et al., 1942) and swine (Waisman, 1944), but is not described in reviews of this deficiency in man (Parsons, 1944; Copping, 1945).

Opinions vary on the value of crude liver extract as a lipotropic agent. Positive results are described in experimental animals which could not be explained by the choline content of the extract (Hall & Drill, 1948) or the presence of inositol and folic acid (McCormick & Drill, 1950). Possibly the trans-methylating effect of the vitamin B_{12} in the compound was responsible (Shils & Stewart, 1954). A negative result with liver extract was reported by György and Goldblatt (1942), while McHenry and Gavin (1940) obtained a lipogenic effect which might have been due to excessive thiamine in their extract, thiamine being an inositol antagonist (Griffith, 1941b). Two different liver preparations were employed in the present investigation, one for oral and the other for parenteral administration. Neither had any notable effect on liver fat.

Lipotropic agents not investigated in this series of cases include vitamin C, effective in guinea-pigs (Spellberg & Keaton, 1939; 1940); heparin, effective in dogs (Di Luzio & Zilversmit, 1956); and betaine, which like methionine, is a source of methyl groups for choline synthesis (Best et al., 1950; Young et al., 1956); manganese; certain amino-acids, e.g., lysine, threonine and arginine (Scott, 1960). Poly-unsaturated fatty acids are also lipotropic (Engel, 1942; Decker et al., 1950), while a high percentage of C^{14} to C^{18} fatty acids in the diet has the opposite effect (Channon & Wilkinson, 1936). Consequently, a synthetic diet with butter and lard in place of vegetable oil is more effective in the

experimental production of fatty liver (Benton et al., 1956, 1957; Spellberg et al., 1942). The efficiency of choline as a lipotropic agent may be impaired in the absence of certain fatty acids from the diet (Engel, 1942). Such experimental results can have no bearing on the present investigations, since the type of fat ingested was the same for all cases.

In the experimental production of dietary fatty liver, reduction in protein, irrespective of casein, methionine or betaine, may be just as important as excessive administration of saturated fatty acids (Deo & Ramalingaswani, 1960). The level of chicken liver fat is usually inversely proportional to dietary protein within the range of 14 to 30 per cent (Donovan & Balloun, 1955). The same is true of rats (Harper et al., 1954; Lucas & Ridout, 1955), when the liver fat so induced has a periportal distribution (Lucas, 1958).

Kwashiorkor, which is a multiple nutritional deficiency syndrome including fatty liver, responds to administration of protein or a mixture of essential amino-acids. This is discussed in more detail below. In this series casein hydrolysate did appear to reduce liver fat in some cases but unfortunately had a tendency to exaggerate the symptoms of gastro-enteritis, and one case developed liver necrosis during this treatment. For these reasons its use in a larger number of cases was considered to be undesirable. The administration of dilute reconstituted human plasma as a source of protein had no notable effect on liver fat.

From a consideration of the complex pathogenesis of fatty liver and

the results of this investigation it would appear that this hepatic manifestation of gastro-enteritis is probably related to depletion of food in general, rather than to the lack of any particular vitamin or other lipotropic factor. Depletion of food intake in these infants naturally includes carbohydrate, protein and fat in appreciable amounts without any alteration in the relative quantities of each. Consequently, there is no disproportion of any one food component as is obtainable in experimental work. Carbohydrate depletion could most readily explain the development of fatty liver in these babies, but it is quite possible that the associated depletion of protein was a contributing factor.

The Influence of Hormones on the Development of Fatty Liver

Deoxycorticosterone acetate has been recommended in the treatment of refractory dehydration and malnutrition in infancy (Bigler & Traisman, 1951). Zeizel and Ströder (1956) found evidence of adrenal cortical dysfunction in such cases, based on the study of corticoid excretion, electrolyte imbalance and eosinopenia, but only in the presence of infection. Changes in endocrine function associated with stress are no doubt brought into play in severe gastro-enteritis. In infants, as in adults, there is a rise in the excretion of corticoids under such circumstances (King & Mason, 1950) but starvation may have an inhibiting influence (Landau et al., 1948). The present investigation was undertaken before most of the recent work on stress and no attempt was planned to study adrenal function. A review of stained blood films of the first

two groups of twenty-five patients taken at or soon after admission shows no remarkable change in eosinophil counts. (Within a week of birth, the eosinophil count of full-term infants should be equivalent to or slightly higher than that of adults (Bongiovanni, 1950; Read et al., 1950; Burrell, 1953)). No notable lesions were found in the pituitary or adrenal glands in any fatal case; sudanophilic material was usually depleted in the zona fasciculata but this observation is unlikely to give much information on the functional state of the adrenal cortex during life.

Increased fat mobilization is a feature of stress and may lead to ketosis and fatty liver (Leblond et al., 1939). Selye (1951) regards fatty liver, and also focal necrosis of this organ, as manifestations of stress. Intact pituitary and adrenal function is necessary for the development of starvation ketonuria in experimental animals (Fry, 1937), but possibly not in man (Kinsell et al., 1951). Stressors such as cold and endotoxin are similar to epinephrine in stimulating fat mobilization, and adrenergic blocking agents are inhibitory (Wertheimer et al., 1960).

Cortisone has a marked effect on plasma lipids, causing elevation of cholesterol and phospho-lipid and fall in neutral fat, irrespective of diet (Adlersberg, 1950). Adrenalectomy has the opposite effect (di Luzio et al., 1954). It is often reported that cortisone will increase liver fat as well as glycogen, at least in certain laboratory animals. In fowls this effect is obtained with hydrocortisone and corticosterone, but not cortisone (Stamler et al., 1954; Dulin, 1956).

An intact adrenal cortex is apparently necessary for the development of hepatic steatosis, but it is not clear which pituitary hormone influences this adrenal function. Weil and Ross (1949) implicate growth hormone, but this hormone will suppress fatty acid synthesis (Allen et al., 1956). Morrison (1952) found that both ACTH and STH increase liver fat, the latter in the absence of the adrenals. A special pituitary fat-mobilizing factor has been postulated by Payne (1949) which may act independently of the adrenals (Levin & Farber, 1950). Zarafonetis et al. (1959) believe that this is derived from the posterior pituitary and that its output is increased in conditions of stress following a rise in circulating corticosteroids. It acts mainly on omental and mesenteric fat.

It follows that adrenalectomy should inhibit the development of fatty liver and this is generally accepted, e.g., Barnes et al. (1941); Ingle (1943). It prevents the fatty change normally induced by starvation (MacKay, 1937). Tracht et al. (1956) found that the operation did not inhibit mobilization of fat, especially epididymal fat, although it prevented starvation fatty liver; on the other hand, hepatectomy was inhibitory. It may be incorrect to assume that the liver is only affected passively by increased mobilization of fat; on the contrary, this aspect of fat metabolism may be under active control by the liver.

In view of these findings and the fact that severe fatty liver was found frequently in association with severe illness, it cannot be denied that adrenal cortical hyperfunction may have contributed to its pathogenesis. This would appear to be a suitable project for further study

now that methods are available for quantitation of circulatory corticosteroids. Of course, it is obvious that the hepatic steatosis cannot be explained by this mechanism alone because of the associated lack of liver glycogen, hypoglycaemia and the normal eosinophil counts.

Other Types of Human Fatty Liver

Fatty liver is not described in acute alimentary infections of comparable severity in adults, such as cholera, acute dysentery and bacterial food poisoning. It is frequently associated, however, with chronic digestive disturbances, such as chronic pancreatitis and ulcerative colitis, and may be complicated by liver fibrosis and cirrhosis. Cirrhosis associated with fatal ulcerative colitis can be of the coarse post-hepatitic type rather than the finer fatty type, and Hoffbauer et al. (1953) suggest that this is due, not to a nutritional deficiency, but to virus B hepatitis derived from parenteral infusions.

Dible (1951) has reviewed the numerous conditions in man associated with fatty liver and lists anaemia, toxaemia (both chemical and bacterial) and starvation as the important causes. None of these need progress to liver fibrosis or cirrhosis. In this country fatty cirrhosis is commonest in obese females in association with alcoholism or an infection; however, the incidence of alcoholic cirrhosis is much lower here compared with many other countries, notably U.S.A. and France. Unlike the findings in this series of gastro-enteritis cases, liver function tests in nutritional and alcoholic liver disease in adults are often abnormal,

but the degree of dysfunction is not necessarily related to the degree of fatty change (Leevy et al., 1953). Moreover, obesity alone in adults can be associated with the same hepatic structural and functional defects; Zelman (1952) found this to be so in nineteen out of twenty males who were 50 to 100 per cent overweight.

It is well known that experimental animals with fatty liver are unduly susceptible to hepatoxins and possibly the same applies to human cases. This could be a factor in the pathogenesis of hepatic necrosis in infantile gastro-enteritis, although in the present series the incidence of this complication was low in spite of the co-existence of severe steatosis and parenteral infection with toxaemia. Popper et al. (1955) suggest that liver cell destruction in the fatty livers of alcoholics leading to cirrhosis may be due to the toxaemia of parenteral infection, usually in the respiratory tract. Broad-spectrum antibiotics prevent cirrhosis in choline-deficient rats (Rutenburg et al., 1957). There are, of course, other explanations for liver fibrosis and cirrhosis in fatty liver; thus, fluctuation in the amount of liver fat could lead to disorganization of cell plates with altered lines of stress in which connective tissue septa would appear. The work of Hartroft and Sellers (1952) on choline deficiency in rats has revealed the importance of fat cysts in the liver. These cysts, formed by coalescence of fat-laden cells may rupture causing both fat embolization in distant organs and condensation of liver stroma. This has been described also in man by Hartroft (1953). Fat cysts were seen in some cases during the present

work and stainable fat could be detected within hepatic vessels, but not renal glomeruli. In spite of this there was seldom more than minimal condensation of reticulin or collagen fibre formation at sites of fatty change. Fat cysts are probably of much less importance in human cirrhosis compared with that induced in rodents by dietary means.

These cases of nutritional and alcoholic liver disease in man have been the subject of many investigations into the therapeutic value of lipotropic agents, and the results are conflicting. At one time, a diet rich in protein and supplemented with choline, methionine or cystine was regarded as beneficial (Franklin et al., 1948), even if alcohol was not withdrawn (Patek et al., 1948). Apart from associated malnutrition, the body's requirements of choline may be raised in alcoholism, as increased amounts of acetyl co-enzyme A, a metabolic product of choline, are required for the oxidation of alcohol (Klatskin, 1959). Recently, in the treatment of alcoholism, more value has been attributed to hospitalization and withdrawal of alcohol than to the composition of the diet (Popper et al., 1950; Leevy et al., 1953). In fact, there may be considerable clinical improvement in the presence of a persistently negative nitrogen balance and severe fatty liver (Eckhardt et al., 1950). Phillips and Davidson (1954) report improvement in a few cases given only glucose-saline and choline, although a proper nutritious diet was better. Others recommend a high protein diet but regard supplementary methionine, choline or cystine as without value (Buck, 1948; Morey et al., 1952). It would appear that a wide variation in diet is compatible with clinical, if not

pathological, improvement. Even a high fat diet can be beneficial; Mindrum and Schiff (1955) record improvement in both liver structure and function in alcoholics treated in this way. The presence of fatty liver does not imply that the body is incapable of utilizing fat.

The fatty liver of infantile gastro-enteritis is similar to that associated with these more chronic nutritional deficiencies in adults in so far as it appears to regress with improved nutrition but is unaffected by supplementary lipotropic factors. It is different in that there is rarely any associated liver necrosis, fibrosis and impaired hepatic function. In the adult type under discussion, there is a tendency to "pin-point" focal necrosis, infiltration with inflammatory cells including neutrophil polymorphs, and fibrosis, even at an early stage and before cirrhosis is established (Popper et al., 1955). Death may occur from liver failure rather than the effects of cirrhosis (Klatskin, 1959). There may be chemical peculiarities in the type of fat within the liver cells (Thannhauser & Reinstein, 1942). Masses of dense hyaline material ("alcoholic hyalin" or "Mallory bodies") are described in the liver cell cytoplasm of these adult cases (Norkin et al., 1960). They were not observed in my material although the optimal conditions for demonstration, viz., alcohol fixation followed by phosphotungstic acid haematoxylin staining, were not carried out.

No discussion on fatty liver in children would be complete without reference to kwashiorkor ("infantile pellagra"). This condition arises in young children after weaning and is due to a diet rich in carbohydrate

but grossly deficient in protein; or there may be deficient absorption and synthesis of protein from an unsatisfactory diet (Craviato, 1958). The illness varies somewhat in intensity in different parts of the world, especially in many tropical and sub-tropical countries, but it is not found in Britain. Among the various lesions which comprise this syndrome, fatty liver is a constant and usually very striking feature and could have a pathogenesis similar to that of the fatty liver in gastro-enteritis, although the two conditions are dissimilar in other respects. Diarrhoea is not uncommon in kwashiorkor but usually mild in comparison to that occurring in gastro-enteritis (Dean, 1960). Sometimes, however, the onset of kwashiorkor may date from an acute gastro-intestinal infection (Gerbasi, 1960), and may benefit from treatment with antibiotics (Gerbasi & Burgio, 1955).

The diet of these patients is poor in fat as well as protein so that carbohydrate may be the source of liver fat. As in adults with nutritional fatty liver, this fat is unusual in composition, being rich in saturated fatty acids and poor in non-saponifiable fat and phospho-lipid (Macdonald, 1960). Unfortunately, there is no information for comparison from gastro-enteritis material.

As in gastro-enteritis, the clinical severity and prognosis of kwashiorkor have been correlated with and attributed to fatty liver without supporting evidence of impaired liver function. Gillman and Gillman (1945d; 1946) at one time regarded severe hepatic steatosis as a cause of death in these cases and assessed prognosis on the amount of stainable

fat present in biopsy specimens. It was suggested also that those children who survived would develop cirrhosis (Gillman & Gillman, 1945c), although the presence of excess iron-containing pigment in these livers ("cyto-siderosis") might induce fibrosis (Gillman & Gillman, 1944; 1945b).

The results of more recent investigations contradict these ideas (Trowell, 1954; Gillman & Gilbert, 1954). Campbell (1956) found focal liver necrosis in a few cases of kwashiorkor but no evidence of cirrhosis. Higginson et al. (1957) have made an extensive study of liver disease in the Bantu. It is well known that cirrhosis and primary cancer of liver are common among this race whose children frequently suffer from kwashiorkor. These authors could not demonstrate any sequence of structural change from fatty liver to cirrhosis and cancer. Cirrhotic livers of adult Bantu natives are not fatty in type and need not be associated with any nutritional deficiency or alcoholism. Cirrhosis may be the result of infection such as viral hepatitis and malaria (Walters & Waterlow, 1954) or of ingestion of certain hepato-toxins such as senecio (Sherlock, 1955). The high iron content of the Bantu diet may also be significant in this respect (Higginson & Keeley, 1960).

Serial liver biopsies have been carried out in kwashiorkor to study the therapeutic value of lipotropic factors. Gillman et al. (1944) tested a number of substances, including crude liver extract, brewer's yeast, nicotinic acid and thiamine, but found all these to be ineffective or even dangerous. Extract of hog's stomach did have a beneficial effect both on the clinical condition and in reducing liver fat (Gillman &

Gillman, 1945a). This was confirmed by Meneghello et al. (1949). A similar benefit was obtained in rats which developed fatty liver when fed diets similar to those consumed by these African children (Gillman & Gillman, 1945a). Black-coated mice are also susceptible (Hodson, 1960); the addition of single food factors, such as lysine or casein, seems to exaggerate the already unbalanced diet and aggravates the illness in these mice. Substances other than stomach extract found to be of value in treatment are methionine (Channon et al., 1940; Gillman & Gillman, 1945c) and various protein hydrolysate preparations (Daft et al., 1938; Madden et al., 1945; Gillman & Gillman, 1946; Cartwright et al., 1945). Although it may not reduce minor abnormalities in established liver function tests, methionine restores the abnormal amino-acid pattern of the plasma in kwashiorkor and improves the utilization of ethanolamine for choline synthesis (Edozien et al., 1960). Waterlow and Wills (1960) describe the striking uptake of S^{35}-labelled methionine by the tissues in a single case of the disease.

Treatment of kwashiorkor has been simplified recently by the discovery of Hansen et al. (1956) that the condition can be cured completely by a diet containing eleven essential amino-acids irrespective of the presence of any vitamins or other supplements. Kwashiorkor would appear to be the result of a multiple amino-acid deficiency provided there is no serious secondary infection to impair recovery (Trowell & Muwazi, 1945). Higginson et al. (1957) found that Bantu breast milk is adequate in protein but that the weaning diet is grossly deficient in amino-acids,

except, curiously enough, methionine.

It is concluded that multiple amino-acid deficiency is a possible cause of fatty liver in children although in kwashiorkor co-existent infection and anaemia probably play a part in its development. However, there is no good reason for the assumption that fatty liver by itself influences clinical severity, prognosis or response to treatment (Waterlow & Weisz, 1956). Kinnear and Pretorius (1956) found little evidence of liver dysfunction by routine liver function tests apart from some impairment of bromsulphthalein excretion. Plasma protein levels were very low (average 3.74 g.% in 107 cases), but this could be due to causes other than impaired liver function. It is a feature of any condition in which there is failure to thrive (Coles, 1960). In kwashiorkor there is no evidence that the liver has lost its capacity to produce albumen since the low blood levels of this protein are quickly restored to normal following effective treatment (Edozien, 1960). Baron (1960) found elevation of plasma transaminase in kwashiorkor but this could be derived from sources other than a damaged liver. Waterlow and Weisz noted a marked depletion of protein and ribonucleic acid and an excess of fat, glycogen and water within liver cells in kwashiorkor. Thus, the cytochemistry of the liver cell in this disease may differ somewhat from that in infantile gastro-enteritis. Some similarities are apparent however, viz., relatively normal liver function; lack of response to many lipotropic factors; no obvious tendency towards the development of cirrhosis.

Pancreatic degeneration has been noted in fatal cases of kwashiorkor (Howard & Meriweather, 1952; Campbell, 1956), and is reminiscent of a similar lesion associated with chronic malnutrition in adults. Bras et al. (1957) found this lesion in every fatal case which they examined in the West Indies, while Badr el Din and Aboul Wafa (1957) report clinical confirmation of pancreatic insufficiency based on a study of the enzyme content of duodenal juice. It seems probable that both hepatic and pancreatic lesions are the common result of malnutrition, and that one is not a sequel of the other. In rabbits fed diets taken by children with kwashiorkor, both fatty liver and pancreatic acinar atrophy develop concurrently (Volk & Lazarus, 1960). Bras et al. describe the lesion as atrophy with or without dilatation of acini, loss of zymogen granules and proliferation of small ducts; no such lesions were seen in any fatal case of gastro-enteritis in the present investigation, although histological examination of the pancreas was always undertaken.

Infant Nutrition in General

All cases of gastro-enteritis in this series were bottle-fed at the time of onset of the initial illness. This is in keeping with the generally accepted observation that the infection is much commoner in bottle-fed compared with breast-fed babies, e.g., McNeil (1940). Doubtless this is due to contamination of prepared feeds by pathogenic micro-organiams. The low standard of hygiene among the poorer classes of the community rather than their breast-feeding habits would explain the high

incidence of the disease in this social group (Deeny & Murdock, 1944).

As breast-fed infants are not included in this series of cases, it cannot be stated whether they are more or less susceptible to fatty liver. There is no reason to suppose that cow's milk would predispose to the condition. According to Lucas (1958), 5.2 per cent of bovine lact-albumen is methionine. Lawrence et al. (1945) have studied the vitamin B content of both human and bovine milk. The latter contains an average of 14.7 µg. choline in each 100 ml., which is unaffected by drying, but no figures are available for choline in human milk. Human milk is relatively rich in inositol, and poor in pyridoxine, pantothenic acid and riboflavine. Pasteurization and drying do not affect these substances in cow's milk. Storage of dried milk results in loss of pyridoxine as this slowly combines with and is inactivated by sulphur-containing compounds (Bernhart et al., 1960). Coursin (1954; 1955) has found a wide variation in the pyridoxine content of different samples of milk and milk preparations, and attributes a syndrome of gastro-intestinal disturbance, irritability and convulsions to deficiency of this vitamin. A similar report is published by Rabe and Plonko (1956). There is no mention of fatty liver in either of these communications, although this can be a result of experimental pyridoxine deficiency and has been discussed above.

Apart from the risk of enteral infection, there is no evidence that children fed cow's milk are at a disadvantage compared with breast-fed children or that they suffer from under-nutrition to such an extent that

fatty liver might be expected to develop. In a study of 98 per cent of all infants born in Rochester, Minnesota, during a 3½ year period, Harris (1951) found a slightly greater gain in weight with bottle-feeding compared with breast-feeding, and that this occurred irrespective of the addition of sugar to the prepared feeds. Vignec et al. (1948), from a study of height, weight, head and chest circumference, haemoglobin, blood cell counts and plasma protein levels, concluded that cow's milk was adequate for infant nutrition without the necessity for any supplement.

It is assumed that malnutrition in young bottle-fed infants is due, not to the quality of milk, but to its administration in wrong amounts, or to disease which interferes with nutrition. A few examples of wrong feeding are included in this series and some had minor degrees of hepatic steatosis. Prolonged malnutrition leading to marasmus (athrepsia) has been described frequently, e.g., by Marriott (1920) and apparently may occur in the absence of infection. The present series of cases included many examples of marasmus, but always in association with severe gastro-enteritis.

Not infrequently the symptoms of gastro-enteritis persist for several weeks, sometimes with one or more acute relapses. The resulting reduction in food intake is obviously a serious matter to a young infant quite apart from any possible deleterious effect on the liver. Even in the absence of persistent infection, prolonged protein deficiency sets up a vicious circle by reducing appetite (Clements, 1946), and so recovery is delayed. In addition, there will be an increasing tendency to oedema,

lowered resistance to concurrent or new infections, a reduced capacity of the plasma to bind iron, copper and calcium, and a lowering of the metabolic rate which becomes so marked in marasmus and which is so refractory to treatment. The very low levels of plasma protein found in many cases of severe gastro-enteritis are evidence of protein depletion; these were frequently much less than the rather low figures (5.8 g.% or less) given for normal infants under one year of age (Poyner-Wall & Finch, 1950).

It is obviously desirable in these circumstances to supply as much protein as can be utilized by the patient. Plasma protein administered intravenously would be beneficial (Daft et al., 1938), although its action is said to be slow in comparison with hydrolysates of protein (Eckhardt et al., 1948). At the time of this investigation the risk of virus B hepatitis associated with plasma infusions was not fully appreciated.

Shohl et al. (1939) and Mueller et al. (1940) reported that infants can be nourished successfully with hydrolysed casein as the only source of protein in the diet. The same given intravenously will reverse a negative nitrogen balance in dogs provided the amino-acids present include tryptophane and methionine (Elman, 1939). Nitrogen retention is accomplished more readily if glucose is included in the infusion fluid (McNair, 1960). This nitrogen sparing effect of glucose had been noted previously by Cuthbertson and Munro (1937). Intravenous administration of casein hydrolysate to infants was described by Shohl and Blackfan (1940) and by Glück and Wilson (1946). According to these authors the infusions are

well tolerated, but a febrile reaction may occur if the dose exceeds a certain level. This reaction does not appear to be due to any impurity since it may follow administration of a pure amino-acid mixture. Possibly, the fever may be part of a general rise in metabolic rate which is stimulated by this form of therapy. In theory it should not be an allergic response since hydrolysis of protein will destroy antigens (Hopps & Campbell, 1953). These authors suggest that undesirable side-effects are due to a histamine-like substance in the digest, the action of which can be demonstrated experimentally by its effect on guinea-pig uterus. Another undesirable side-effect from casein hydrolysate administration is a tendency to acidosis due to excess chloride in the digest, calcium chloride being used to precipitate casein (Hoffman et al., 1948).

Albanese et al. (1948) found no difference in nitrogen retention between two groups of infants fed dried milk and casein hydrolysate respectively, but these were normal subjects and probably had no need of a high protein intake. Shohl (1943) had considerable success with casein hydrolysate in the treatment of infantile gastro-enteritis; he used the preparation "Amigen" both orally (20 per cent of diet) and intravenously (2.5 to 4 per cent in glucose-saline). It is possible that different preparations vary in their side-effects. The only one available to me, viz., "Casydrol", tended to aggravate vomiting if given orally and always produced a febrile response, often with severe toxaemia and vomiting if given intravenously. This was unfortunate, both with regard to its theoretical advantages and its apparent lipotropic effect in some

cases. It is of interest that liver necrosis developed in one case during this form of treatment. Hopps and Campbell (1953) report one example of hepatic failure in a series of 17 autopsies on patients who had toxic symptoms following casein digest therapy.

The Significance of the Liver Lesions in Gastro-Enteritis

Attempts to assess the importance of hepatic steatosis in infants have been made by certain French paediatricians. Lévesque et al. (1947) noted a clear association between severity of toxaemia and severity of fatty change in a cholera-like illness of infants which they regarded as a special type of gastro-enteritis, due to a virus. Those authors found no urobilin in the urine but performed no other tests of liver function; nevertheless, they attributed death to liver failure. Parenchymatous hepatitis and hydropic fat-laden cells, reminiscent of phosphorus poisoning were described but not illustrated. Marie et al. (1947) also found a correlation between clinical severity and hepatic steatosis in 29 cases. In these, increase in prothrombin time and diminution in blood cholinesterase were suggestive of liver dysfunction. These authors put forward the curious idea, not convincingly established, that fatty liver is the result of damage to α-cells of the pancreatic islets and diminution in output of lipocaic. My results are confirmed to some extent by the work of Chaptal et al. (1955) based on liver biopsy studies. These authors note that the fatty change can develop quickly but recedes slowly, that it is associated with loss of liver glycogen and aggravation of ketosis,

that there is no other liver parenchymal damage and that liver function tests are not abnormal (but only the results of galactose tolerance tests are detailed). Contrary to my findings, they believe that choline is of value therapeutically and that the condition may progress occasionally to cirrhosis. In support of the hypothesis that the fatty liver of gastro-enteritis may be physiological in type, Chaptal et al. found, in addition to evidence of impaired carbohydrate utilization, an increase in blood lipid and in α2- and β-lipoprotein. They believe, however, that there is also an upset in the metabolism of liver cells leading to failure in the utilization of fat. Increase in liver cell phosphatases is described but is obviously artefact over cell nuclei. Results very similar to those of Chaptal et al. are described by Sarrouy and Clausse (1956) from a study of 35 cases which included repeated liver biopsy. In this group of infants, blood lipid levels were low, but it is possible that some may have been examples of kwashiorkor, since the report comes from Algeria and includes children older than one year.

It is not possible from the results of the present work to reach definite conclusions on all aspects of fatty liver in infantile diarrhoea and vomiting. From a consideration of these results, however, together with a consideration of the infrequent reports of others on the same subject and of the pathogenesis of fatty liver in general, some hypotheses can be suggested. Thus, the condition may well be "physiological" in type, the effect of excessive fat mobilization induced by severe nutritional impairment. It is probably co-existent with and not

contributory to the severity of the illness, and there is no evidence that any serious liver dysfunction exists. Probably its main importance is an undue susceptibility to noxious influences such as bacterial toxaemia and gross abnormalities in protein intake when hepatic necrosis and fibrosis may supervene. Liver dysfunction is then evident clinically and, in contrast to uncomplicated steatosis, the condition becomes a serious complication to the primary disease. Fortunately, this is rare. There is no evidence that special lipotropic factors have any value in treatment, which should be directed to the re-establishment of a normal food intake by the elimination of the alimentary infection.

Further investigations, such as blood lipid estimation, the degree of ketonaemia and other aspects of lipid metabolism, would certainly be of value, while an estimation of adrenal cortical function would also be of interest in view of its rôle in fat metabolism. With the recent fall in severity and importance of the disease, however, it may be argued that there is no longer sufficient justification for a project which requires liver biopsy as part of its investigational procedure. Consequently, no attempt has been made to pursue this matter further in recent years.

In adults with malnutrition, examination of the liver frequently reveals foci of necrosis, infiltration with inflammatory cells and a tendency to fibrosis, in addition to fatty change. Another common feature is the presence of stainable iron-containing pigment; this may be quite prominent although not usually comparable in severity to established idiopathic haemochromatosis or transfusion siderosis (Dubin, 1955;

Finch & Finch, 1955). Gillman and Gillman (1944) describe hepatic siderosis in children aged nine years or older suffering from malnutrition, but not in younger patients with kwashiorkor. The haemosiderosis in my material was regarded as normal when related to the patients' age. This corresponds with the findings of Langley (1951) who investigated the matter in detail in normal and premature infants from birth until the seventh month of life. This author regards stainable iron in the liver as the manifestation of a disproportion between supply and utilization, rather than an indication of excessive haemolysis. McCance and Widdowson (1951) found that, in mammals including man, there is an unusually high level of body iron during suckling, this iron being derived from milk. The decrease in liver during infancy may only be apparent but not real because of the rapid growth of the organ during this period.

In spite of what is held regarding the susceptibility of fatty liver to toxic damage, liver necrosis was uncommon in this series of cases. At the time of the investigation it was recognized in other gastro-enteritis units and was the subject of two communications from this country. The first of these, by Schlesinger et al. (1949), described six cases, all of whom had rapidly developing jaundice, oedema, or a haemorrhagic tendency associated with increased prothrombin time and potassium deficiency. Plasma protein levels were very low, reaching 3.2 g.% in one instance, but thymol turbidity (not estimated in every case) was normal. Since one only of these patients died and came to autopsy, and since no liver biopsy studies were carried out, this paper

gives limited information regarding the pathology of the disease. The liver in the single fatal case showed severe fatty change, dilatation of sinusoids, "pin-point" focal necrosis, round-cell infiltration and a suggestion of early fibrosis. Casein hydrolysate, both oral and intravenous, vitamin B complex and choline chloride were used in the treatment of all cases. Wainwright (1950) gave a fuller account of the pathology based on sixteen fatal cases with jaundice. In some of these the liver was enlarged and congested, fatty or bile-stained. Necrosis was zonal in type and often had a periportal distribution. Inflammatory cell infiltration, bile duct proliferation and early fibrosis were additional features, but there was no cholangio-hepatitis. This author noted also that fatty liver alone was not associated with any tendency to fibrosis, and that jaundice could occur with no demonstrable liver necrosis.

It will be evident that my fatal case of liver necrosis had features similar to many of Wainwright's cases. Frank necrosis was seen only in the autopsy material, but in the biopsies there was some indication of liver cell damage adjacent to inflamed portal canals.

In experimental periportal zonal necrosis it has been postulated that the greatest damage is suffered by those liver cells first exposed to the circulating toxin (Himsworth, 1947). In gastro-enteritis it may be that a blood-borne bacterial toxin derived from the alimentary canal has a similar action. The histo-pathological features of toxic periportal necrosis, e.g., that induced by allyl formate (v. Part III of this thesis), are quite unlike those of the fatal case under discussion.

The biopsy findings of this case were more like those found in adults suffering from "cholangiolitic" hepatitis, frequently regarded as a form of viral hepatitis but with clinical features of obstructive jaundice (Gall & Braunstein, 1955). Bacterial infection of the upper biliary tract could explain the change noted in my case, but its true pathogenesis must remain uncertain.

It is well-known that liver necrosis as distinct from fatty change can be produced experimentally by dietary means. Such diets are deficient in sulph-hydryl containing amino-acids (György, 1944; György & Goldblatt, 1949), or lack vitamin E and a factor ("factor 3") in certain types of yeast used as a component of the diets (György & Goldblatt, 1951). Unlike the zonal necrosis of liver hepatotoxins, this dietary necrosis is irregular and massive in type and appears after a latent period of about six weeks, although structural change may be preceded by a functional metabolic disorder (Chernick et al., 1955). It is possible also to produce periportal zonal necrosis by dietary means, if excess cystine is added to the diet (Curtis & Newburgh, 1927), and this lesion can progress to cirrhosis (Earle & Victor, 1941; 1942). Cysteic acid has the same effect (Earle et al., 1942). This cannot be the explanation for a possible association between liver necrosis and the administration of casein hydrolysate, since the alkali used in the preparation of this material tends to destroy cystine (Jones & Gersdorff, 1934). It may be, however, that any gross disproportion of amino-acids is necrogenic. McLean and Beveridge (1952) found that protein-deficient yeast

diets are productive of liver necrosis only if the casein content lies between 2 and 4 per cent of the diet, while no necrosis occurs if the yeast content is less than 5 per cent or greater than 30 per cent. These results are obtained irrespective of the amount of vitamin E, carbohydrate or fat in the diet. It seems possible that the administration of casein hydrolysate to babies in a state of acute starvation may set up an unnatural dietary situation conducive to liver damage, especially if fatty change is already present.

The other cases with clinical evidence of liver cell damage presented different pathological features from the fatal case. One had focal necrosis in a very fatty liver which might have been expected more frequently in patients suffering from severe toxaemia and stress. The other had an illness not typical of infantile gastro-enteritis, and was probably a primary viral infection involving liver.

SUMMARY

(1) Three hundred and fifty-four liver biopsies were performed on 167 babies, the majority of whom suffered from infectious diarrhoea and vomiting of infancy. These biopsies, together with the results of 26 autopsies from the 47 fatalities in the same series of patients, form the basis of the investigation.

(2) Gross fatty liver is found in many but by no means all cases of severe gastro-enteritis, and especially in those with frequent vomiting.

Moderately severe fatty liver may co-exist with infections other than gastro-enteritis, but is rare in the absence of an infective condition.

(3) Very small infants, usually in the first month of life, and with little subcutaneous fat, rarely have severe fatty liver in spite of severe illness. There is no correlation between severity of hepatic steatosis and actual or expected body weight.

(4) Severe fatty liver can develop quickly but subsides slowly, lagging behind clinical improvement. There is no correlation between the severity of the condition and the duration or relapse rate of gastro-enteritis.

(5) There is an inverse relationship between severity of fatty liver and calorie intake, irrespective of clinical diagnosis.

(6) There is also a relationship between fatty liver, especially if severe, and depletion of liver glycogen, ketonuria and hypoglycaemia. From this it is deduced that the hepatic condition may follow excessive mobilization of fat from fat depots in the body secondary to depletion of carbohydrate.

(7) No notable effect on fatty liver was obtained from the administration of various lipotropic factors including methionine and choline.

(8) Severe fatty liver is compatible with normal liver function. By contrast, three cases in the series with additional liver cell injury presented positive results with the common liver function tests.

(9) These three cases had differing liver pathology which is described and discussed.

(10) The differences between the liver changes in infantile gastro-enteritis and those known to be associated with illnesses induced by protein deficiency, including kwashiorkor, are discussed. The exact rôle of protein deficiency in the pathogenesis of gastro-enteritis-fatty liver could not be determined from the present investigation.

(11) The amount of iron-containing pigment in the livers of these babies was inversely proportional to their ages. This is regarded as a natural phenomenon and not as a manifestation of malnutrition.

(12) Minor structural changes in the liver could not be correlated with clinical features of disease. These included changes in mitochondria and cytoplasmic basophilic bodies, mesenchymal cellular infiltration or proliferation and minor degrees of portal fibrosis. There is no evidence that the fatty liver of infantile gastro-enteritis may be complicated by cirrhosis.

PART II

THE NATURE OF THE FIBROUS CONNECTIVE TISSUE

OF HEPATIC FIBROSIS AND CIRRHOSIS

THE NATURE OF THE FIBROUS CONNECTIVE TISSUE OF HEPATIC FIBROSIS AND CIRRHOSIS

The origin of collagen fibres which develop within the liver in a variety of circumstances, sometimes culminating in hepatic cirrhosis, is still a matter of doubt. In certain instances, fibrogenesis would appear to be identical with that described in wound healing and in chronic inflammation. In suppurative cholango-hepatitis, for instance, fibroblasts are prominent in the vicinity of inflamed bile ducts, and the portal fibrosis which results is due, no doubt, to the activity of these cells.

By contrast, a number of hepatic diseases are known which may terminate in cirrhosis, but in which fibroblastic activity is not a conspicuous feature. This is so in human viral hepatitis, although cells of mesenchymal origin other than typical fibroblasts may be numerous within the affected liver. The same has been noted frequently in experimental animals during the development of carbon tetrachloride-induced cirrhosis. Moreover, collagen fibres may appear along lines of stress in the hyperplastic nodules of a cirrhotic liver with little or no apparent activity of connective tissue cells. It has been suggested that, in such examples of chronic liver disease, fibrosis is the result of passive condensation of the reticulin framework of the liver following destruction of the

parenchymal cells. It is claimed that the basement membranes of glandular organs are not precursors of collagen (Robb-Smith, 1957); consequently, proof of the "condensation" hypothesis would imply that hepatic reticulin is different from these basement membranes, and recent work with the electron microscope suggests that this is so (Popper et al., 1960).

Certain aspects of the problem have been investigated in the following experiments, in all of which two distinct types of liver fibrosis were induced for comparison, viz.,

(i) diffuse hepatic fibrosis progressing to cirrhosis, the result of repeated administration of carbon tetrachloride;

(ii) a focal zone of fibrosis induced by the implantation of catgut into the liver.

In the first group of experiments, the effects of cortisone and ACTH were studied on the development of these two lesions. It is, of course, well-known that these hormones have an inhibitory influence on the repair of wounds.

Secondly, the uptake of radio-active sulphur, administered as S^{35}-sodium sulphate, was studied in the lesions by autoradiography. Again, positive results using this technique have been obtained in healing wounds, because of incorporation of sulphur into acid mucopolysaccharides of inter-cellular ground substance and into the cytoplasm of fibroblasts.

Thirdly, the antigenic nature of the two types of connective tissue was studied by the Coons fluorescent antibody technique, using antibodies

to glomerular basement membrane and to reticular fibres from granulation tissue. In previous reports of similar investigations, little attention has been given to hepatic reticulin, possibly on account of its comparative thinness in the normal liver so that it remains inconspicuous by this technique.

A. THE INFLUENCE OF CORTISONE AND ACTH ON EXPERIMENTALLY-INDUCED HEPATIC FIBROSIS AND CIRRHOSIS

The methods and results of four experiments are described, followed by a general comment.

I. The Influence of the Hormones on the Development of Cirrhosis from previously Healthy Livers by Carbon Tetrachloride Induction

Methods. Young Swiss albino mice of both sexes, weighing 15 to 20 g. at the commencement of the experiment, received carbon tetrachloride twice weekly by oesophageal tube. Four parts of carbon tetrachloride were diluted with six parts of liquid paraffin and each dose consisted of 0.1 ml. of this mixture. Two control groups were given similar doses of pure liquid paraffin.

Certain mice received, in addition, either cortisone or ACTH, commencing two days before the first dose of carbon tetrachloride and continuing on six days of each week, until the animals died. Cortisone (Cortisone Acetate, Roussel Laboratories Ltd., Batch No. 883) was given

as a single daily intra-peritoneal injection, each dose being 0.25 mg. For ease of measurement, this was diluted with four times its volume of isotonic saline just before use. ACTH ("Acthar" Gel, Armour Laboratories, Lot No. M.22007) was given by a similar route twice daily. Each dose was 2 Armour units and was undiluted. Isotonic saline was injected in lieu of hormone in one control group.

The effectiveness of hormone therapy was evident from the profound eosinopenia and thymic atrophy induced in these animals. Adrenal atrophy in the cortisone-treated mice and hyperplasia in those receiving ACTH were also evident without exception. The same batches of hormones were used concurrently in certain experiments described in Part III of this thesis, in which details regarding their effect on circulating eosinophils and thymus gland weights are tabulated (Table XXX).

Animals surviving more than a few weeks were subjected to liver biopsy and this was repeated at approximately monthly intervals. The technique of biopsy was as follows: the mouse was anaesthetized with ether, the abdominal wall depilated, and an incision made in it just to the right of the mid-line and extending posteriorly from the inferior costal margin for a distance of 1.0 to 1.5 cm. The liver was exposed readily and its gross morphology noted. A thin wedge of tissue was snipped off with scissors, in the first instance from the lower right portion of the middle lobe, including a small part of the inferior margin. In cases of established cirrhosis, the biopsy site was altered if necessary in order to obtain a representative portion away from large

regenerating nodules. Since no hormone-treated animal lived into such a late stage of the disease, this matter was not a problem in the comparison between the various experimental groups. Sites of previous biopsies were also avoided. Following removal of liver tissue the abdominal wall was closed in two layers with interrupted catgut sutures and sealed with a celloidin dressing. No attempt was made to control haemorrhage, since this was never troublesome. The over-all death rate was 8 per cent and became somewhat less when, with practice, the procedure could be completed within a few minutes. Carbon tetrachloride was discontinued temporarily for two or three days before and after the operation.

Animals were killed when moribund and their livers examined macroscopically and microscopically. Gross evidence of cirrhosis was evident usually by the fourth month of treatment with carbon tetrachloride and well-established by the sixth (Figs. 91 to 93). Animals dead for more than a few hours were discarded because of rapid autolysis and shrinkage of hepatic tissue rendering it useless for comparison with fresh tissue. Laparotomy wounds were also examined histologically.

All biopsy specimens (e.g., Fig. 94) and at least three representative portions of the liver after death were fixed in Rossman's picric acid-alcohol-formalin mixture. 7 μ sections from paraffin blocks were stained by haematoxylin and eosin, by van Gieson's method, by Gordon and Sweet's method for reticulin and by the periodic acid-Schiff technique.

The degree of hepatic fibrosis was assessed arbitrarily from examination of these sections. Sections stained for reticulin (without

counter-stain) were obviously the most suitable for comparison with each other, as this shows most clearly both the normal thin reticular fibres and the thicker young collagenous fibres which also stain red by van Gieson's method (Figs. 94 & 95). Good examples of the varying severity of the lesion were selected following a preliminary survey, and photographed. Four of these are illustrated in Figures 97 to 100. Uniform sections of all specimens were then stained with the same freshly prepared ammoniacal silver solution. These sections were examined histologically in a haphazard order, each assessment being made with reference to the photographs before the slide number was noted. Examination of all slides was repeated with results almost identical to the first assessments.

Van Gieson sections corresponding to two of the five grades of fibrosis are illustrated (Figs. 101 & 102). In livers with nodular hyperplasia (Figs. 103 & 104), assessment of fibrosis was made as far as possible from the hepatic tissue lying between nodules. This usually appeared to be well-advanced although accurate assessment was often impossible because of compression. Fortunately, comparison between the various experimental groups at this stage of the disease was rarely required (v. infra).

The animals used in these experiments were divided into five groups as follows:

Group 1 - received cortisone and carbon tetrachloride

Group 2 - received ACTH and carbon tetrachloride

Group 3 - received carbon tetrachloride alone

Group 4 - received cortisone alone

Group 5 - received ACTH alone

Table XXIII gives the numbers of animals and numbers of liver examinations in each group.

Results. Hepatic fibrosis induced in the manner described develops very gradually with the appearance of thin fuchsinophil fibres radiating out from centrilobular zones, the sites of maximal liver cell damage. In animals dying at this stage soon after a dose of carbon tetrachloride both necrosis and fibrosis may be located together giving the appearance of "reversed lobulation" (Fig. 105). Fibroblasts may be inconspicuous especially in areas removed from the macrophage activity around necrotic hepatic cells (Fig. 106). In more advanced cases the thicker fibrous bands contain mesenchymal cells which are likely to include some fibroblasts (Fig. 107), although it may be impossible to determine whether elongated cells adjacent to collagen fibres are of this nature or whether they may not be sinusoidal cells persisting after the collapse of liver parenchyma (Fig. 108). Rarely in these mouse livers one finds small spheroidal or elongated foci of what appears to be osteoid tissue (Fig. 109) and which is presumably the product of recent mesenchymal cellular activity. Heterotropic ossification has been noted in mouse livers

damaged by radio-active colloidal gold (Upton et al., 1956).

Figure 110 shows severity of liver fibrosis plotted against duration of carbon tetrachloride poisoning. Animals from Groups 4 and 5 are not represented, since these were, without exception, free from any trace of hepatic fibrosis. Apart from a little round cell infiltration of the portal tracts in seven specimens from Group 4 and two from Group 5 there was no suspicion of any liver damage. Fatty and glycogenous infiltration were common, sometimes very marked, and attributable, no doubt, to hormone therapy.

Perusal of Figure 110 reveals the following:

(i) The duration of life of mice receiving repeated doses of carbon tetrachloride is shortened by cortisone and ACTH (animals in Groups 4 and 5 also had shorter survival periods than Group 3). Further comparison between Groups 1, 2 and 3 is limited, therefore, to those mice receiving up to forty doses of carbon tetrachloride.

(ii) Within this limitation, there is no notable difference in severity of hepatic fibrosis between the three groups. If anything, hepatic fibrosis is slightly more severe in hormone-treated animals compared with those of Group 3.

(iii) In Group 3, hepatic fibrosis appears to develop rather more rapidly in male compared with female mice and the latter tend to survive longer. Sex differences cannot be studied in Groups 1 and 2 because their numbers are too small.

(iv) There is a fairly wide variation in the response to carbon

tetrachloride at all stages of poisoning. Comparison is possible only between groups of animals and not individuals.

(v) No member of Group 1 and only one of Group 2 lived long enough to develop true cirrhosis with nodular hyperplasia. It is not misleading, therefore, to show in Figure 110 this feature as a further development of the liver disease although it does not necessarily imply any further increase in fibrous tissue.

Changes in the severity of hepatic fibrosis in individual animals subjected to multiple liver examinations are summarized in Table XXIV. While the numbers in Groups 1 and 2 are not high, it is obvious that neither cortisone nor ACTH have any appreciable effect in preventing the development of hepatic fibrosis in the manner described.

By contrast, examination of the laparotomy wounds of three Group 1 mice dying within a week of biopsy showed notable inhibition of the reparative response compared with comparable material from the control group (Figs. 111 to 114).

II. The Influence of Cortisone on the Progress of Hepatic Fibrosis already Induced and Maintained by Carbon Tetrachloride

Methods. Young Swiss albino mice of both sexes were treated with diluted carbon tetrachloride in the manner described in the previous experiment. About three months later each animal was subjected to wedge biopsy of liver. On the following day, cortisone therapy, also identical to that previously described, was commenced (Group 6), control animals

(Group 7) receiving isotonic saline. ACTH was not used in this experiment. Cortisone was continued until the animals died or were killed.

Liver biopsies were repeated at approximately monthly intervals as long as the mice survived. The preparation and assessment of liver sections were carried out as in the previous experiment.

Table XXV shows the numbers of animals and examinations of liver tissue in this experiment.

Results. The relationship between severity of hepatic fibrosis and duration of carbon tetrachloride administration is shown in Figure 115 and summarized in Table XXVI. From these it would appear that:

(i) Cortisone tends to shorten duration of life, even if hepatic damage is not well established. No animal of Group 6 survived for longer than 35 weeks, while several members of Group 7 survived for periods of up to one year.

(ii) Females appear somewhat more resistant than males to carbon tetrachloride and may survive for a year despite continued administration of the liver toxin and the presence of severe cirrhosis.

(iii) Cortisone appeared to inhibit temporarily the development of fibrosis especially during the few weeks between first and second liver examinations. Eleven of the 28 mice in Group 6 actually showed a perceptible reduction in the severity of fibrosis and in two of these the change was very noticeable.

(iv) Evidence of early nodular hyperplasia was found in only one

cortisone-treated animal; consequently this feature is included in Figure 115.

The livers of cortisone treated mice usually showed less mesenchymal reaction (Figs. 116 & 117) and thicker fibrous bands, but this was by no means constant, especially after several weeks' treatment when there may have been an acquired resistance to the hormone.

In both groups the hepatic fibrous tissue stained faintly by the periodic acid-Schiff technique, rather less intensely than the scar tissue developing in the abdominal wall wounds. Impairment of early healing of these laparotomy wounds was quite evident in animals of Group 6 compared with Group 7, and was confirmed by microscopic examination of the wound edges. Completion of healing was often delayed into the second week after operation in many Group 6 mice.

III. The Influence of Cortisone on Hepatic Fibrosis Induced by Carbon Tetrachloride, which was Withdrawn before the Commencement of Hormone Therapy

Methods. Similar to experiment A.II with the following exceptions:

(i) Carbon tetrachloride was discontinued permanently at the time of first biopsy when cortisone therapy commenced (Group 8). (A control Group 9 received no hormone).

(ii) There were no repeat biopsies, the animals being killed at variable intervals as shown in Figure 118.

The preparation of sections and assessment of hepatic fibrosis were

identical to that of the previous experiments. ACTH was not used.

Results. These are shown in Figure 118 and are summarized in Table XXVII. It would appear that the disease had not reached an irreversible stage since reduction in fibrous tissue was noted in many control animals. Cortisone failed to accelerate this process; indeed the opposite impression is gained from this small series of animals.

No sex differences were noted in this experiment.

IV. The Influence of Cortisone and ACTH on Intra-hepatic Scarring Induced by Catgut Implantation

Methods. Young Swiss albino mice of male sex only were used. Initial weights were 20 to 25 g. Implantation of catgut was carried out as follows: The animal was anaesthetized with ether and the abdominal wall depilated. The abdominal wall was opened by a right paramedian incision extending for 1.0 to 1.5 cm. posteriorly from the infracostal margin. The anterior surface of liver was depressed gently with forceps and the end of a small piece of dry 0000 non-chromisized catgut, held vertically in pressure forceps, was pressed through the middle lobe, about 5 mm. above the inferior margin, from anterior to posterior surface. A small piece of catgut was left projecting from both surfaces to facilitate its localization at a later date. The abdominal wall was then closed in two layers with interrupted catgut sutures and sealed with celloidin.

The operation can be performed quickly and there is no troublesome haemorrhage. The death rate of 6 per cent was due apparently to excessive

administration of ether rather than surgical trauma.

Animals so treated were killed at intervals of 1,2, 3, 4, 6, 10, 14, 21 or 28 days after the operation. Liver taken for histological examination included the site of implanted catgut. Fixation, paraffin embedding and staining were carried out as in the previous experiments. The presence of catgut in the tissue did not interfere to any extent with the cutting of sections. The abdominal wall laparotomy wounds were also examined histologically.

Certain animals received injections of cortisone or ACTH. The dosage and frequency of administration of these hormones were identical to that of the previous experiments. The treatment commenced two days before catgut implantation and continued until the animals were killed.

The numbers of mice used were as follows:

Group 10	- implantation of catgut + cortisone	16 animals
Group 11	- implantation of catgut + ACTH	4 animals
Group 12	- implantation of catgut + saline injection (control)	25 animals

Further details are given in Table XXVIII.

Results. Animals of Group 12 showed a marked local reaction to the presence of catgut within the liver which varied with time.

1 day after operation (Figs. 119 & 120) - The lesion consists of -

(a) Catgut, surrounded by

(b) a thin zone of neutrophil polymorphs and P.A.S. positive granules, surrounded by

(c) a zone of necrotic liver tissue, the thickness of which is variable and may depend on the amount of trauma inflicted during the operation. This is surrounded by

(d) a thin zone of glycogen-depleted but viable liver cells.

2nd day (Fig. 121) - Scanty mesenchymal cells, possibly of sinusoidal-cell origin are seen within layers (c) and (d). There is no increase in reticulin, but possibly a little condensation due to displacement by the catgut.

3rd day (Figs. 122 & 123) - Early absorption of catgut is now evident through the action of acute inflammatory cells. Fibroblasts and fine faintly P.A.S.-positive fibrils are now recognizable in the outer zones. (The crushed appearance of catgut in Figure 122 is artefact).

4th day (Figs. 124 & 125) - Similar to the 3rd day, with the exception of a very thin zone of faintly-staining fuchsinophil fibres at the margin of the lesion.

7th day (Figs. 126 & 127) - A distinct zone of granulation tissue is now evident, surrounding the partially absorbed catgut and zone of necrotic liver tissue. Young collagen fibres, stained by van Gieson's stain are evident. The glycogen-free zone of liver cells has disappeared and there is P.A.S.-positive intercellular ground substance.

10th day (Figs. 128 to 130) - Absorption of catgut is now almost complete and the surrounding young connective tissue has become thicker. Reticulin and collagen fibres appear more condensed.

14th day (Fig. 131) - Further condensation is apparent in the developing

scar tissue.

21st day (Figs. 132 & 133) - The lesion is now represented by a small relatively acellular scar, in which small remnants of foreign material may persist.

28th day - The scar has apparently been absorbed since no trace of any lesion can be detected.

Certain modifications are evident in the reaction in the livers of the cortisone-treated animals (Group 10).

1st day (Fig. 134) - The polymorph infiltration in zone (b) is inconspicuous.

2nd day (Fig. 135) - The mesenchymal cell reaction in the outer zones is notably less than in the control group.

3rd and 4th days (Figs. 136 to 139) - A very thin zone of granulation tissue with early collagen formation is seen surrounding a wide zone of necrotic liver tissue and unabsorbed catgut.

7th day (Fig. 140) - There is no evidence of catgut absorption and some necrotic liver tissue may persist. Although the mesenchymal response is still inhibited, a thin zone of collagen has formed surrounding the catgut and necrotic liver.

14th and 21st days (Figs. 141 to 143) - Much of the catgut is still unabsorbed and is surrounded by a thin relatively acellular band of collagen.

28th day (Fig. 144) - The catgut appears to lie within a columnar space having a very thin lining of mature collagen.

Inhibition of catgut absorption was evident also in the two mice treated with ACTH for two weeks, but the findings at the 21st and 28th days were similar to those of the control group at these periods.

The influence of these hormones in delaying the absorption of the catgut was quite notable as was the reduction in mesenchymal response. Nevertheless, collagen formation was not inhibited completely despite the continued administration of the hormones. The laparotomy wounds of these hormone-treated mice show inhibition of the cellular reaction associated with normal healing, as in the previous experiments; nevertheless a thin relatively acellular scar has formed by the end of the second week (Figs. 145 & 146).

COMMENT

The experimental production of liver cirrhosis by agents which cause death with autolysis of liver parenchymal cells is well-established. Carbon tetrachloride is probably the hepatotoxin most commonly employed for this purpose, judging by the numerous reports of its use in the medical literature. Much helpful information was obtained from the article by Cameron and Karunaratne (1936), although these authors used rats and not mice.

Just as the degree of susceptibility to a single sublethal dose of carbon tetrachloride varies slightly among mice, identical with regard to strain, age, weight and sex, so the rate of development of cirrhosis varies between individuals. For this reason it is desirable to study

the progress of the lesion in individual animals by repeated liver biopsy. The influence of some factor such as cortisone can be judged only in this way or by the use of a large control group. Nevertheless, in the work which has been described the various experimental groups were as similar as possible with regard to the animals employed, as well as their environment and diet. Body weights did not vary by more than 5 g. at the commencement of each experiment. The sex variant has been shown in the results since it is known that male rodents are slightly more susceptible than females to the acute lesion of carbon tetrachloride poisoning; it would appear that the same applies to the chronic lesion.

The investigation by Daniel et al. (1952) on the portal circulation of the carbon tetrachloride-induced cirrhotic liver in the rat included repeated liver biopsy. Considerable individual variation in the severity of liver damage was noted among individual animals, particularly after the second month of treatment. Steinberg and Martin (1946), using a radiological technique following administration of thorium dioxide to assess severity of liver disease, also noted considerable variation between rats suffering from pre-malignant cirrhosis induced with butter-yellow. Morrione (1947) undertook a quantitative chemical study of collagen in the cirrhotic livers of experimental animals and found a good correlation between his results and the histological appearance of the liver, judged especially from sections stained to show reticulin. The same is true of human material, at least in the early stages of cirrhosis (Warren & Wahi, 1947).

Ungar and Feldman (1953) described the sequence of events within rat liver following implantation of 000 surgical catgut. Those are similar to the results recorded above, although the process was rather more protracted; absorption of the catgut in the rat was incomplete at the third week after implantation, while scarring persisted for two months. The inflammatory response would appear to be a reaction to foreign material and not to focal liver damage, since it is much less marked round implanted homologous tendon (Ungar & Neuman, 1953).

The inhibitory influence of cortisone on wound healing and on the cellular response to various forms of injury is well-known. Most authors agree that the action of ACTH on wound healing is mediated through the adrenal cortex (Asboe-Hansen, 1954). The subject has been reviewed recently by Pintar (1960). Glucocorticoids seem to act, at least in part, at the cellular level, and in the case of healing wounds, inhibit the activity and proliferation of mesenchymal cells and the production of both connective tissue fibrils and intercellular ground substance (Baker & Abrams, 1955). Thus, there is inhibition of fibroblastic and endothelial cell proliferation and a reduction in metachromatic ground substance around turpentine abscesses in cortisone-treated rats (Taubenhaus *et al.*, 1953). Ground substance never disappears completely however, and its positive periodic acid-Schiff staining is retained. Scanty, but nevertheless mature, collagen does appear at the margin of the lesion. These effects were noted in the present work, both in the reaction to the implanted catgut and in the laparotomy wounds. A variable response in

some of the latter may have been due to secondary infection which inhibits the antiphlogistic effect of cortisone (Lattes et al., 1954). No indication of bacterial infection was evident within the liver or peritoneal cavity of any animal and, without exception, the influence of both cortisone and ACTH on the intrahepatic lesion was striking.

Ungar et al. (1956) have studied the influence of cortisone on the reaction to catgut implanted in rat liver. They also have observed a marked inhibition of granulation tissue production, provided the hormone is given prior to implantation. Unlike its effect on mice in the present work, cortisone did not inhibit the initial polymorphonuclear leucocyte response, but seemed to stimulate, in spite of mesenchymal cell inhibition, rather abundant reticulin fibres and markedly hyaline scar tissue. Lattes et al. (1953) noted impaired giant-cell formation and impaired absorption of foreign material during administration of corticosteroids.

The failure of the hormones to influence the development of carbon tetrachloride-induced cirrhosis is in marked contrast to the effect obtained in the catgut-implantation experiment. The progressive nature of the early lesion appears to be quite unimpeded by the administration of cortisone or ACTH; in fact, in some cases it may have been slightly aggravated by them.

Diengott and Ungar (1954) gave cortisone to rats after their tenth dose of carbon tetrachloride (administered three times weekly) and killed the animals eight days later following continuation of both treatments. Compared with their control group, the hormone-treated animals had more

numerous and broader fibrous septa as well as prominent bile ducts and fibroblasts. Liver parenchymal cells also were affected, numerous pleomorphic and giant cell forms being noted. Many of these features were not apparent in my material in which the dosage of cortisone was much smaller.

Regniers et al. (1955) undertook a similar experiment in rabbits and noted an acceleration in the development of cirrhosis, provided the cortisone is given in large amounts and commences one week before the first dose of carbon tetrachloride. An opposite beneficial effect was obtained with small doses of cortisone administered to established cases of cirrhosis. This tends to confirm my findings in certain animals of the second experiment. Many of these results in rabbits appear to be unreliable, however, being based on the comparison of small groups of animals with no attempt to assess progress in individuals. Rabbits are unsuitable animals for this type of experimental work because of the high incidence of naturally-occurring liver infection among them.

Wahi et al. (1956a, b) studied the influence of cortisone on experimental cirrhosis in rats, using reticulin-stained sections to assess severity of the hepatic lesion. In rats compared with mice, cirrhosis seems to develop more rapidly, being well-established after treatment with carbon tetrachloride for eight weeks. These authors then gave large doses of cortisone, but this had no effect on the rate of regression on the lesion following withdrawal of the hepatotoxin, apart from some thinning of mature fibrous bands. Eger and Stratakis (1958) studied the

influence of prednisone on cirrhosis produced in rats by allyl alcohol. The development of the lesion was unaffected and parenchymal cell regeneration was impaired.

In human cirrhosis, these hormones may have some value in cases complicating viral hepatitis (Havens et al., 1952; Winkler & Tygstrup, 1957). Clinical improvement may be due to diminution in jaundice following reversal of bile stasis, and not to any beneficial effect on the basic pathological condition. This is discussed more fully in Part III.

Animals receiving repeated doses of hormones for periods exceeding a few weeks may become refractory to their effects. Antibodies to ACTH have been demonstrated in mice after treatment for 25 days (Chase, 1949), and the same is true of the rat (Gordon, 1949), even when the adrenals remain enlarged. Persistence of adrenal enlargement could be due, of course, to contaminating growth hormone in the ACTH preparation. Refractoriness of rats to cortisone is also known (Winter et al., 1950).

The administration of carbon tetrachloride would be expected to produce a non-specific stress effect on the adrenals and this would appear to be the interpretation of the findings of Wahi et al. (1956a). Such an effect is probably much less pronounced after the repeated administration of small doses. It had been hoped to repeat the experiment in adrenalectomized animals, but no means were found to overcome the rapidly fatal effects of single small doses of the toxin in both rats and mice treated in this way.

Cirrhosis also may interfere with the physiological effects of the

hormones, because the diseased liver causes incomplete or abnormal steroid metabolism (Miller & Axelrod, 1954).

While the normal influence of cortisone and ACTH may have been inhibited or modified in the experiments described, either by a refractory state or by cirrhosis, it is unlikely that this would be so during the early development of diffuse hepatic fibrosis when no effect could be produced on its progress by either hormone.

B. THE UPTAKE OF S^{35}-SULPHATE BY DEVELOPING SCAR TISSUE WITHIN THE LIVER

Methods

Sodium sulphate labelled with the radio-active isotope of sulphur (S^{35}) was dissolved in isotonic saline and administered to mice by intraperitoneal injection. All animals were killed 24 hours after a single dose which consisted of 100 μc. S^{35} Na_2SO_4 in 1 ml. saline. (Obtained from the U.K. Atomic Energy Authority, Amersham, Bucks).

The animals used in this investigation included a number of those from several groups in Experiment A, viz.,

Group 3 - Induction of cirrhosis by repeated administration of carbon tetrachloride but without hormone therapy. Nine animals from this group were used, all of which had been on treatment with carbon tetrachloride for five months.

Group 10 - Implantation of catgut into the liver and daily

cortisone injections - 9 animals.

Group 11 - Catgut implantation and daily ACTH injections - 4 animals.

Group 12 - Catgut implantation without hormone therapy - 9 animals.

In addition to these, a new group of 25 mice (Group 13) was studied, these animals having the combined lesions of carbon tetrachloride cirrhosis and catgut implantation. Cirrhosis was produced as described previously. After the continued administration of the liver toxins for five, six or seven months, a piece of 0000 surgical catgut was implanted within the liver, avoiding hyperplastic nodules whenever possible. Owing to the firm consistency of the cirrhotic liver, it was necessary in many cases to employ suture material attached to a small "non-traumatic" round-bodied needle which was passed through the gland. Carbon tetrachloride was discontinued for three or four days prior to the operation and recommenced only in those animals kept alive for one week or longer.

Animals from Groups 10, 11, 12 and 13 were killed at intervals after catgut implantation and always 24 hours after administration of S^{35}-sodium sulphate. Details of the numbers employed are shown in Table XXVIII.

Tissues for autoradiography were taken from liver and healing abdominal wall wounds. Every care was taken to have implanted catgut near the centre of the block of liver tissue. All specimens were fixed in formol-alcohol, passed to paraffin wax and sections cut in the usual way. Autoradiographs were prepared in a dark room lit by a Wratten

No. 1A red Safelight. Sections from which wax had been removed and passed to water were covered with Kodak stripping film AR 50 floated on a tank of water, as described by Doniach and Pelc (1950). Both coarse-grain and fine-grain film were used. After drying the slides were stored within sealed containers in a refrigerator at 5° C. for 60 to 90 days, the longer period being used for fine-grain preparations. The slides were then developed with Kodak developer DX 80 for four minutes, followed by fixation in "Amfix" for twice the period required to clear the preparations, usually four minutes. Staining with safranin and light green (Curran & Kennedy, 1955) was carried out before mounting.

Adjacent sections from the same blocks of liver were stained by conventional methods and by certain histochemical procedures for mucopolysaccharides. Autoradiographs could thus be compared with preparations stained as follows: haematoxylin and eosin; van Gieson's method for collagen; Gordon and Sweet's method for reticulin fibres; periodic acid-Schiff technique (method of McManus as described by Pearse, 1960); 0.5% aqueous toluidine blue for metachromasia; Hale's dialyzed iron method for mucopolysaccharides; Alcian Blue for mucopolysaccharides (method of Steedman as described by Pearse, 1960). For Hale's method, several batches of colloidal iron reagent (Rinehart & Abu'l Haj, 1951) were prepared and gave uniform results; their efficacy was shown by positive staining of glomerular capillary basement membrane. Standard methods of staining with toluidine blue originally gave negative results with the cirrhotic fibrous tissue; consequently, this was repeated on formalin-fixed

frozen sections, and ethanol dehydration avoided, as recommended by Curran (1960).

Results

No difficulty was experienced in cutting sections of cirrhotic liver with implanted catgut and several examples are illustrated (Figs. 147 to 149). It will be evident that the two types of fibrous tissue under investigation can be included in a single section.

An intense uptake of radio-active sulphate is evident from the autoradiographs of the laparotomy wounds at all stages of healing within the time limits of the experiments (Figs. 150 & 151). As would be expected, this occurs at the wound edges where there is profusion of metachromatic ground substances and proliferation of fibroblasts. By contrast, there is little or no evidence of radio-activity over collections of pus cells seen in some sections around embedded catgut (Fig. 152). In wounds of several weeks' duration with well-developed scar tissue, there is still a notable concentration of radio-active sulphur.

In the absence of wound sepsis, cortisone has the effect of minimizing the early cellular response to injury and there is a reduction in metachromatic ground substance. Reduction in the extent of S^{35} uptake corresponds with the reduction in these histological features, but the intensity of uptake where present is not reduced (Figs. 153 & 154).

The concentration of radio-active sulphate within the liver around the implanted catgut is of comparable intensity and is seen at all stages

of absorption and repair. During the early stages, the reaction is obtained over the zone of proliferating mesenchymal cells outwith the zone of necrotic liver tissue (Figs. 155 & 156). It becomes more extensive by the seventh or tenth days when the zone of young connective tissue is widest and persists during healing (Figs. 157 & 158). Later, when the catgut is completely absorbed, and the site of liver damage reduced to a small scar, there is still some evidence of sulphur uptake in this region (Fig. 159). As in the abdominal wall wounds, hormone therapy reduces the extent but not the concentration of radio-activity at the margin of the lesion (Figs. 160 & 161); in material from the small ACTH-treated group the reaction is notably intense (Fig. 162).

These results were obtained in cases of catgut implantation without exception. Autoradiographs of the cirrhotic lesions, however, give a more variable picture. Nevertheless, it is apparent that in most cases of cirrhosis there is some uptake of sulphur in the fibrous tissue within the liver. A completely negative result was obtained in two cases only. In the remainder intensity of radio-activity is not closely related to severity of cirrhosis or its duration within the five to seven month period. While rarely of comparable intensity to that of the catgut lesions, it is usually sufficient to show the pattern of cirrhosis quite distinctly on the autoradiographs (Figs. 163 to 166). Fine-grain preparations show that sulphation is confined within the narrow bands of fibrous tissue and associated mesenchymal cells. A certain amount of background radio-activity of low density is seen over all normal liver

tissue with a slight increase over the walls of blood vessels (Fig. 167). This persists in spite of washing the sections in water during the preparation of the autoradiographs and avoiding the use of celloidin as a protective coating.

Periodic acid-Schiff positive ground substance and fibres are found in the new connective tissue around embedded catgut. At a later stage the scar shows faint but distinct staining by this method even in hormone-treated animals. The same is apparently true of cirrhotic fibrous tissue; P.A.S. staining is frequently very faint but never completely absent, and occasionally is distinctly positive (Fig. 168).

Metachromasia of scar tissue around catgut, following toluidine blue staining, is pale but distinct, being of the pink or γ-type (Fig. 170). Very faint metachromasia can sometimes be recognized in the cirrhotic lesion (Fig. 169), provided alcohol is not used in the dehydration of sections. Sections of the catgut lesion prepared by the standard method are also unsatisfactory although they do reveal cells with purple (β-metachromatic) granules, presumably mast cells (Fig. 171). No mast cells are seen in the cirrhotic lesions.

The results obtained with the dialyzed iron technique are similar to these other histochemical methods, i.e., distinct positive staining of connective tissue formed around the implanted catgut (Fig. 173) but indistinct or negative staining of the cirrhotic lesion (Fig. 172), although the reagents used stain glomerular basement membrane readily (Fig. 174). The Alcian Blue technique gives results almost identical to

those obtained by Hale's method.

COMMENT

The demonstration of sulphated mucopolysaccharides through labelling with radio-active sulphur is now a well-established procedure. In mice, healing cutaneous wounds have been studied in this way by Glücksman et al. (1956). These authors described uptake over periodic acid-Schiff positive ground substance as well as over fibroblasts and new connective tissue fibres. Similar results were reported by Loewi and Kent (1957) from investigations on healing tendon wounds in guinea-pigs.

Autoradiographic evidence for the concentration of radio-active sulphur within connective tissues is indicative that this element has been incorporated into sulphated mucopolysaccharides (Boström, 1952). It resists washing with water which would presumably remove unbound sulphate. This was confirmed by Oppenheimer et al. (1960) in their study of connective tissue pockets round embedded plastics. Such compounds exist in metachromatic intercellular ground substance and possibly on the surface of reticulin fibres; mature collagen fibres, however, contain little or no sulphated material (Gross, 1950a). Its presence within fibroblasts has been reported (Curran & Kennedy, 1955) and it is probable that these cells are the source of certain sulphated mucopolysaccharides, particularly chondroitin sulphate B. Endothelial cells may be capable of producing a different sulphated compound, viz., heparitin sulphate

(Meyer et al., 1959). This might explain the slight uptake of S^{35} over vessel walls within the liver. A possible incorporation of sulphate into the sulph-hydryl groups of certain amino-acids may also explain the faint uptake over liver parenchyma generally.

In the present work, the uptake of S^{35} in the vicinity of early healing wounds is no more than would be expected. Its presence in scars which stain red by van Gieson's method, is indicative that young, if not mature, collagen is closely associated with a sulphated compound. It is alleged that cortisone inhibits the synthesis of sulphated mucopolysaccharides (Layton, 1951; Nordlie & Fromm, 1958; Kowalewski, 1959), but the experiments just described have shown that, while cortisone and ACTH reduce mesenchymal cellular proliferation, they do not nullify completely the production of sulphated compounds or the formation of scar tissue. The uptake of radio-active sulphur by the fibrous tissue in cirrhosis must indicate mesenchymal cellular activity at these sites in the liver with synthesis of sulphated mucopolysaccharides. Since this synthesis seems to be an essential step in scar tissue formation it would appear that the cirrhotic lesion cannot result entirely from passive condensation of pre-formed fibres but must involve the activity of connective tissue cells.

The presence of sulphate as anionic groups in polysaccharide molecules should render these substances metachromatic with toluidine blue (Pearse, 1960). It is obvious from the present work that autoradiography following the administration of radio-active sulphur is a

much more sensitive method for the detection of newly-formed sulphated compounds. However, it would appear from this and from the results of the other histochemical methods that the cirrhotic lesion is not rich in acidic mucopolysaccharides.

C. THE ANTIGENIC NATURE OF INTRA-HEPATIC FIBROUS TISSUE, STUDIED BY THE FLUORESCENT-ANTIBODY TECHNIQUE

Antibodies were prepared to mouse glomerular basement membrane and to quartz granulomatous tissue, conjugated with fluorescein by the Coons technique and applied to sections of mouse liver containing the fibrous lesions already described. The object of this investigation was to compare the antigenic properties of the argyrophilic fibres present in the cirrhotic lesion and in the new connective tissue formed around implanted catgut.

Methods

1. Production of antibody to glomerular basement membrane. Fifty pairs of mouse kidneys were obtained from healthy young Swiss albino mice. Each animal was killed quickly and the thorax and abdomen opened. The inferior vena cava was cut and the body perfused with 50 to 100 ml. of sterile isotonic saline introduced through a fine needle into the left ventricle. The bloodless kidneys were then removed, stripped of their

fibrous capsules and the cortices dissected from the medullae.

The pooled renal cortices were homogenized briefly in a Waring Blender with ice-cold isotonic saline and pressed through 120-mesh stainless steel gauze with a spatula. The gauze retains coarse fragments of fibrous tissue and blood vessels. The material remaining was centrifuged at 1,000 r.p.m. and the supernatant fluid, which contains blood cells and isolated tubular epithelial cells, decanted off. The deposit was re-suspended in ice-cold saline and allowed to settle by gravity. After a few minutes, the upper 4/5, which contains many tubular fragments but few isolated glomeruli, was aspirated. This process of aspiration and re-suspension was repeated several times, when the final deposit consists mainly of glomeruli. The method is almost similar to that applied to dog kidneys by Krakower and Greenspon (1951). The final preparation was homogenized with 25 ml. isotonic saline and merthiolate added to a concentration of 1:10,000. It was stored at -20° C.

Two young adult male albino rabbits each received 1 ml. of the thawed suspension by intraperitoneal injection twice weekly for four weeks. Adjuvants were not used. Ten days after the last injection, each animal was anaesthetized and 25 to 30 ml. blood removed by cardiac puncture. The blood was collected in heparinized tubes and centrifuged to obtain plasma. One week later each animal received a further course of injections and was again bled.

Globulin was obtained from the rabbit plasma by precipitation with 40% ammonium sulphate. This was redissolved in distilled water and

dialyzed to remove sulphate. Merthiolate was added to a concentration of 1:10,000 and the solution stored at -20° C.

2. Production of antibody to quartz granulomatous tissue. 4 g. precipitated silicon dioxide was added to 100 ml. isotonic saline and the mixture sterilized by boiling. 0.5 ml. of a uniform suspension was then administered to each of 25 young adult Swiss albino mice by injection into the loose subcutaneous tissue of the dorsal region. Each animal was killed ten days later, when a white disc-like lesion was present at the site of injection. These lesions consist of quartz particles bound together with gelatinous extra-cellular material and with a margin of organizing connective tissue including many young argentophil fibres (Curran, 1953). They can be dissected out very readily from the surrounding tissues. Each granuloma was carefully homogenized with a little isotonic saline and the homogenates pooled. By allowing the ground-up tissue to settle by gravity some of the liberated silica could be decanted off in the supernatent fluid. This was repeated several times. The final product was made up to 25 ml. with isotonic saline and merthiolate added to a concentration of 1:10,000. It was stored at -20° C.

Two rabbits each received intraperitoneal injections of the thawed-out suspension of ground-up granulomata through a wide-bored needle. Each dose was approximately 1 ml., and adjuvants were not used. The number of injections, subsequent bleeding and preparation of globulin

were similar to those described for the production of antibody to glomerular basement membrane.

3. Preparation of fluorescein isocyanate. The method described in detail by Coons and Kaplan (1950) was employed. In summary, this involves the suction of a stream of phosgene gas through a solution of fluorescein-amine in dry acetone. The resultant fluorescein isocyanate is precipitated by evaporation and redissolved in a dry acetone-dioxane mixture, so that 25 mg. of original amine compound produces 3 ml. of the final solution.

4. Conjugation of antibody. A 100 ml. conical flask containing 9 ml. isotonic saline and 3 ml. sodium carbonate:bicarbonate buffer at pH 9.0 was set inside a large beaker and the whole placed on a magnetic stirrer. Chopped ice was placed between flask and beaker and the stirring mechanism set in motion. When the contents of the flask were chilled, 4 ml. of thawed rabbit serum fraction were added dropwise to it over a period of ten minutes. Stirring was allowed to continue for a further ten minutes.

With stirring still in progress 1 ml. of freshly prepared fluorescein isocyanate solution was added dropwise and very slowly. This was most conveniently done from a tuberculin syringe.

On completion of the procedure the flask, ice-bath and magnetic stirrer were transferred to a refrigerator containing an electric power point for the stirring motor. Stirring of the mixture was carried out in this way for 24 hours at 0° to 2° C.

At the end of this period the apparatus was removed from the

refrigerator and the content of the flask transferred to dialysis tubing. It was dialyzed in the refrigerator against M/15 phosphate buffer in isotonic saline. The buffered saline was changed twice daily and dialysis continued until all unconjugated fluorescein was removed; this usually takes seven or eight days. The conjugate was then returned to a flask, shaken up with dry guinea-pig liver powder (100 mg./ml. serum in the original mixture), filtered and stored in the refrigerator. No preservative is necessary as the dye is bacteriostatic.

5. Material for investigation. A further series of twelve Group 13 mice were prepared as already described. All had received carbon tetrachloride for five months and were cirrhotic. Intra-hepatic catgut lesions varied in duration from three days to two weeks.

Small blocks of liver tissue were placed deeply within clean dry test-tubes which were subsequently stoppered and plunged into a slush of carbon dioxide snow and alcohol at approximately -70° C. After freezing of the tissue, the tubes were transferred to a cryostat at -20° C. Care was taken that one block of tissue from each liver contained implanted catgut. Material was fixed also in Rossman's picric acid-alcohol-formalin for routine histological examination.

6. Staining of sections with fluorescein-antibody conjugate. Frozen sections of hepatic tissue were cut within the cryostat at 6 μ. These were flattened on to clean glass slides with heat from the finger tip. Slides on removal from the cryostat were immediately dried with warm air

from a hair dryer. Sections from each block of tissue were fixed in 10% formalin for twenty minutes and stained by van Gieson's method and by Gordon and Sweet's method for reticulin. Other unfixed sections were covered with a few drops of conjugated antibody for two hours at room temperature. Slides were placed on damp filter paper within Petri dishes to prevent drying of conjugate. At the end of this period they were well washed with M/15 phosphate buffer (pH 7.0) to remove excess dye and the sections mounted in a glycerol-phosphate buffer mixture. The use of special quartz slides or cover-glasses is unnecessary.

7. Microscopic examination and photography. The preparations were examined with a Leitz BX 20 fluorescence microscope, using a 150-watt compact source mercury vapour lamp (Phillips ME/D 103803) as the source of light. The eyepiece contained a Euphos barrier filter.

Photographic records were obtained from this microscope on high-speed daylight-type Ektachrome film. Suitable exposure times were 20 to 25 seconds for low power fields (10:1) and 30 to 40 seconds for high power fields (25:1). Slightly longer exposures are preferable for photography of negative results. Satisfactory black and white photographs have been obtained using Kodak P 300 panchromatic plates, 2 minutes for low power and 4 minutes for high power fields. Exposure times much in excess of these are to be avoided as they cause fading of the fluorescence.

8. Control methods. When fluorescence of connective tissue fibres was

observed, the following controls were set up:

(a) Sections were treated with fluorescein conjugated serum from healthy rabbits with no antibody relevant to these experiments.

(b) Sections were treated for one hour with normal rabbit serum followed after washing by treatment with antibody conjugate as described.

(c) Sections were treated for one hour with unlabelled antibody-containing serum followed by the application of the same antibody conjugate.

(d) Sections were treated for one hour with unlabelled antibody to glomeruli followed by the application of the granuloma antibody conjugate, and vice versa.

(e) Samples of both conjugates were absorbed for two hours with one or other antigen homogenate, filtered, and used on liver sections as already described.

9. Staining of sections by the anti-antibody method. Further sections from this material were re-examined at a later date by the method whereby the appropriate antibody, unconjugated, is "sandwiched" between tissue antigen and a fluorescein-labelled antibody to rabbit globulin (Mellors et al., 1955). It is claimed that sensitivity is increased by this procedure.

Frozen unfixed sections were treated with unconjugated antibody for

two hours at room temperature. Following a period of washing in phosphate buffer, they were then stained with goat serum containing an antibody to rabbit globulin, this antibody being conjugated with fluorescein. The goat anti-rabbit preparation was obtained commercially from "Microbiologicals, Inc.", Baltimore, Md., U.S.A.

Results

Distinct apple-green fluorescence of connective tissue fibres comprising the cirrhotic lesion was obtained following treatment with either antibody. Usually, only one or two of these fibres could be distinguished along any one septum, and in many cases may not have accounted for its entire thickness. Each fluorescing fibre had the dimensions of a deeply staining argyrophilic fibre and seemed to correspond to those staining by van Gieson's method. Fluorescent staining was indistinct or absent in some thinner septa and was frequently obscured by ceroid pigment which has a natural white fluorescence. This pigment was copious in many specimens, especially in the neighbourhood of portal tracts and along the fibrous septa of the cirrhotic liver. It may be demonstrated readily in paraffin sections with fat stains (Fig. 175) or by the periodic acid-Schiff technique (Fig. 176).

Figures 177 to 181 show examples of the positive results in cirrhosis which were obtained by both antibodies. Fluorescence was less intense if either serum was diluted with more than the same amount of phosphate buffer, whether used in the direct or in the "sandwich" method.

No attempt has yet been made on an exact quantitative comparison.

The results suggest that either (a) two antigens have been identified in the hepatic connective tissue fibres or (b) the two antibodies are similar in so far as they have an affinity for a single antigen in this connective tissue. The results of the various controls which were undertaken support this second suggestion, as pre-treatment with one unconjugated antibody quenched subsequent fluorescence normally obtained by the other. In addition, both could be absorbed by either tissue antigen. Details of these results are as follows:

(a) Normal rabbit serum conjugate - negative fluorescence (Fig. 182).

(b) Normal rabbit serum → either conjugated antibody - positive fluorescence (Fig. 183).

(c) Unconjugated antibody → same antibody conjugated - negative fluorescence.

(d) (i) Unconjugated anti-glomerular serum → anti-granuloma serum conjugate - negative fluorescence (Fig. 184).

(ii) Unconjugated anti-granuloma serum → anti-glomerular serum conjugate - negative fluorescence.

(e) (i) Anti-glomerular serum conjugate absorbed with homogenates of glomeruli or granulomata - negative fluorescence.

(ii) Anti-granuloma serum conjugate absorbed with homogenates of glomeruli or granulomata - negative fluorescence.

No fluorescence of comparable intensity was observed with either serum in the young scar tissue around implanted catgut. Faint indistinct fluorescence is present in the outer margin which is the active zone of argentophil fibre formation, but fibres could not be distinguished on high power examination (Figs. 185 & 186). Occasionally tiny tubular structures could be made out with thin fluorescing walls, possibly capillary blood vessels.

In the remainder of the sections fluorescence with both sera marked the basement membrane of most interlobular bile-ducts. No normal hepatic reticular fibres in the walls of sinusoids could be identified.

The abdominal wall wounds of some animals were subjected to similar examination and showed positive staining of sarcolemma, basement membrane of skin appendages and a few tubular structures, probably capillary blood vessels in granulation tissue.

Sections from the kidneys of these animals showed distinct fluorescence of glomerular and tubular basement membranes with both sera.

The opportunity was obtained recently of investigating a case of human cirrhosis in a similar fashion. Fresh frozen sections were prepared from a wedge biopsy of liver taken from a case of fatty cirrhosis associated with chronic alcoholism. These were treated with a rabbit

anti-human glomerular serum conjugated with rhodamine. Fluorescence of the finer connective tissue fibres was obtained (Figs. 187 & 188).

COMMENT

Since the first report on its usefulness in demonstrating bacteria within tissues (Coons et al., 1942), this technique, which allows the visual representation of sites of antigen-antibody reaction, has become an established procedure in histology. Its application to tissue components was reported nine years later as a means of demonstrating ACTH in pituitary cells (Marshall, 1951). Hill and Cruickshank (1953) prepared antibodies to homogenates of renal tissue and to isolated glomeruli and showed positive staining of basement membranes by fluorescein conjugates of these, not only on the glomerular walls, but in a variety of tissues (Cruickshank & Hill, 1953). Other fibres including the reticular network of spleen and lymph nodes, capillary walls and sarcolemma were stained also, but hepatic reticulin was not convincingly demonstrated.

Interest in the nephrotoxic property of glomerular basement membrane antigen has stimulated many investigators along similar lines and in the use of other immunological techniques such as the labelling of antibody with radio-active iodine (Pressman & Sherman, 1951) and tests of its ability to produce nephritis in experimental animals (Baxter & Goodman, 1956). It seems reasonably certain that glomerular basement membrane has antigenic properties similar to those of basement membranes of other

organs such as lung and placenta (Baxter & Goodman). These authors claim that many tissues can be used to produce nephrotoxic antibodies most of which, on injection, will be absorbed readily by renal glomeruli because of their large capillary bed and blood flow. Others believe that glomerular basement membrane contains at least two antigens, only one of which is nephrotoxic (Goodman et al., 1955; Markowitz, 1960). Malazzo (1957), who holds the former view, has shown that glomerular antigen is a periodic acid-Schiff staining material resistant to trypsin and hyaluronidase, probably a glycoprotein. Such compounds are generally thought to confer specificity to antigens and haptens.

In spite of negative or unconvincing results with normal liver reticulin by the fluorescent antibody technique, some evidence exists that the organ contains antigen with similar properties to glomerular basement membrane antigen. Thus Pressman and Sherman showed by their I^{131} labelling technique that some glomerular antibody could be absorbed by the liver. Smadel (1936) had noted previously the removal of nephrotoxic antibody by liver. Baxter and Goodman separated liver stroma from parenchymal cells and showed that the antigenic property was present in the former.

Possibly negative staining of liver reticulin by glomerular basement membrane antibody conjugate is due only to the sparseness of these thin hepatic fibres. However, Mellors et al. (1955) have reported that there is no staining of the relatively more profuse splenic reticulin, although spleen has the capacity of absorbing the antibody (Hill & Cruickshank). Scott (1957) produced an antibody to connective tissue reticulin as

distinct from epithelial basement membrane by the use of normal synovial tissue as antigen. His basement membrane antibody stained bile duct basement membrane but no other reference was made to the liver. Basement membrane may have something in common with fibrous connective tissue since 70 per cent of its antibody can be absorbed by collagen (Markowitz, 1960).

From the results of the present work it is apparent that some fibres comprising the membranous septa which develop within the liver in cirrhosis contain material antigenically similar to that found in glomerular basement membrane and probably other basement membranes in the body. If it is assumed that normal liver reticular fibres also are basement membranes this would be in favour of the supposition that these are condensed in the cirrhotic lesion. This supposition might be further strengthened by the absence of comparable fluorescence in the developing scar tissue around implanted catgut (apart from a few capillary walls), although it must be remembered that many argyrophilic fibres in these lesions, like normal hepatic reticulin, are very thin.

This hypothesis is made less certain by the positive results obtained with quartz granuloma antibody. This was prepared originally with the expectation that its conjugate would demonstrate the reticular fibres of the catgut lesion but not basement membranes or the cirrhotic lesions. It has been shown that capillary walls have an affinity for basement membrane antigen, and it may be that the granulomata had a sufficiency of these small vessels to produce a similar antibody. Contamination with sarcolemma is unlikely as the granulmatous lesions

were separated carefully from underlying muscle during dissection. It may be that young connective tissue in quartz granulomata contains a glycoprotein chemically similar to that responsible for specific antigenicity of basement membranes; this is discussed more fully below. In this event rather more prominent fluorescence of the catgut lesion would have been expected. The fact that faint fluorescence could be detected at the margin of this lesion might imply that such an antigen is in fact present in fine reticular fibres or inter-cellular material, but in a relatively low concentration. However, Taylor et al. (1961) have failed to demonstrate reticulin fibres in granulation tissue with glomerular and pulmonary basement membrane antibody conjugates.

GENERAL DISCUSSION

Hepatic cirrhosis is a disease common to man and produced readily in animals by experimental procedures. Although the subject of numerous investigations, there is still much regarding its pathology which remains obscure. Even the term "cirrhosis" causes confusion, there being no generally accepted definition of this. From the standpoint of clinical medicine, the mere presence of excess fibrous tissue in the liver is unimportant; it is the abnormal intrahepatic vascular shunts and distortion which matter, as these cause ischaemia of surviving nodular liver parenchyma and the establishment of portal hypertension. Some pathologists, e.g., Popper and Schaffner (1957) suggest that these functional

abnormalities should be implied by the term "cirrhosis", otherwise "hepatic fibrosis" should be used. On this basis many cases of so-called "biliary cirrhosis" and "cardiac cirrhosis" could not be regarded as true cirrhosis.

Further confusion is caused by the adoption of different classifications of hepatic cirrhosis or fibrosis by different authors. Thus, to many American pathologists the terms "Laennec's cirrhosis" or "portal cirrhosis" are synonymous with the fine fatty cirrhosis commonly associated with alcoholism. In this country, where alcoholic cirrhosis is relatively uncommon, the same terms have a wider meaning.

It is difficult to find a counterpart to experimental carbon tetrachloride cirrhosis in human pathology. Prolonged or repeated exposure to a hepatoxin of this type would be unusual. Post-toxic cirrhosis in man is generally of the coarse "post-necrotic scarring" type of lesion, similar to that which may complicate an unusually severe bout of viral hepatitis, and which is taken to represent the result of extensive destruction of liver parenchyma by a single episode of poisoning. The experimental toxic lesion develops gradually and is reminiscent of the "creeping" type of cirrhosis described by Gall (1960) in his account of alcoholic liver disease and cryptogenic portal cirrhosis in man. It has been suggested that antigens from damaged liver cells set up an auto-immune reaction causing a continuation of tissue cell destruction, even in cases of experimental nutritional cirrhosis (Wilgram, 1959). One would not expect this in carbon tetrachloride cirrhosis, the development

of which depends on the continued administration of the poison. During the early stage of this development the fibrous tissue extends from centri-lobular veins and recalls the "reversed lobulation" pattern of human cardiac cirrhosis, but there is no venous or sinusoidal congestion. It is only the advanced case with gross nodular hyperplasia which resembles coarse well-established cirrhosis in man.

In the present investigation, these questions of definition and relevance to the human disease are probably unimportant. I have been concerned with the origin of fibrous connective tissue within the liver and, doubtless, this involves processes common to many cases of the disease regardless of aetiology. Several hypotheses require consideration.

(a) Hepatic fibrosis is the result of collagen formation by fibroblasts. It is evident from the catgut implantation experiment that proliferation of fibroblasts with formation of connective tissue fibrils is possible within mouse liver. In the rat, intra-hepatic injections of carrageenin induce foci of fibrosis which also undergo fibrolysis after a few weeks (Zak et al., 1958).

Necrotic liver undergoing autolysis can be a stimulus to fibroblastic activity and proliferation. Scar tissue develops around autologous liver transplants, unless these be boiled before transplanting in order to destroy the enzymes liberated by autolysis (Cameron & Karunaratne, 1936). Prominent fibroblasts are described, especially in

the portal tracts, in biopsy material from patients with viral hepatitis, particularly those cases likely to develop cirrhosis at a later date (Weinbren, 1952). Leevy et al. (1955) obtained fibroblasts readily by tissue culture of liver biopsy material from various types of cirrhosis, but it seems likely that a similar result could be obtained from any specimen of fresh liver. These authors noted also the disappearance of fibroblasts and the shrinkage of fibrous tissue in serial biopsies of chronic cases. Gall (1960) noted prominent fibroblasts even in "post-collapse" cirrhosis but mitotic activity of these cells was inconspicuous. Electron microscopic studies of human cirrhotic liver by Cachera and Darnis (1955) showed collagen fibrils extending from within mesenchymal cells and the same has been reported in the portal canals of normal human and mouse liver (Cossel, 1959). Many histologists would disagree that these fibrils are formed within cells, although they would regard mesenchymal cells in their proximity as probable fibroblasts.

Conversely, others have been impressed by the relative lack of fibroblastic activity in cirrhosis. Popper and Elias (1955), in their three-dimensional study of human cirrhotic livers, described the development of collagenous membranes and portal fibrosis in the absence of prominent fibroblasts. Smetana (1956) made a similar observation in human post-necrotic cirrhosis. In the periductular type of hepatic fibrosis described by Schaffner and Popper (1961), electron microscopy reveals few if any recognizable fibroblasts in relation to new fibre formation.

In experimental carbon tetrachloride cirrhosis, the condition develops insiduously without prominent fibroblastic activity. Mesenchymal cells are numerous around necrotic liver but appear to be macrophages. Some at least are derived from liver sinusoidal cells (v. Part IV of this thesis). There is nothing comparable to the prominent fibroblastic reaction around implanted catgut. Scanty spindle-shaped cells are seen in association with collagen fibres, but these might be sinusoidal cells entrapped by condensed reticulin.

(b) Connective tissue fibres are deposited from tissue fluids without prominent fibroblastic activity. Popper and Elias (1955) suggested an origin from plasma protein.

The exact rôle played by fibroblasts in collagen formation is still uncertain. Electron microscopic studies of these cells in tissue culture show condensation of material at cell margins and the development of filamentous projections which precede the appearance of cross-striated collagen fibrils in their vicinity (Yardley et al., 1960). It is uncertain whether the fibroblasts are necessary for the continous formation of collagen or whether they only initiate the process. It is well-known that collagen fibrils can be precipitated from collagen solution in vitro in the absence of cells. Collagen solvents are generally weak organic acids or neutral saline, while solubility depends to some extent on the age of the collagen (Jackson & Bentley, 1960). (In cirrhosis a high percentage of liver collagen is of the immature

alkali-soluble type, according to Hutterer et al., 1960). Precipitation of collagen is readily obtained by varying pH or increasing the concentration of salt (Gross, 1950b). Addition of mucopolysaccharides, especially chondroitin sulphates A and C, will also accelerate the effect (Highberger et al., 1951; Wood, 1960). It may be that fibrils can form in vivo at a distance from the cells producing their constituents.

It is claimed that collagen fibrils can be produced in vitro by the action of detergents on plasma proteins and that this may be connected with the development of cirrhosis in mice following the application of detergents to the skin (Holsti, 1956). This author suggested a similar mechanism to explain cirrhosis in rabbits caused by feeding bile salts.

(c) Connective tissue fibrils are formed by the condensation of the reticulin framework of the liver following absorption of necrotic parenchymal cells. Many hepatotoxic agents selectively destroy parenchyma and leave intact the other structures including sinusoidal cells and reticulin. Following a single incident of this sort, the intact stroma acts as a scaffolding for regenerating liver. In cases of more extensive liver necrosis, involving numbers of complete lobules adjacent to each other or in cases of repeated exposure to toxin with inhibition of regeneration, the surviving stroma collapses. The reticular nature of the condensed structure persists and can be seen by silver impregnation (Steiner, 1960). Popper and Elias (1955) believe that collagen appears in the collapsed stroma which is condensed but not augmented. Popper

(1954) described condensation of reticulin and the appearance of collagen along lines of stress passing radially into regenerating nodules of hyperplastic liver tissue.

This theory may afford an explanation for the increased incidence of cirrhosis in leukaemic children since the use of modern chemotherapeutic measures for this disease. Leukaemic cells replace liver cells and keep the stroma open, but when they in turn are destroyed the stroma collapses and cirrhosis develops (Hutter et al., 1960).

Epithelial basement membrane differs structurally from reticulin and has not been regarded as a collagen precursor. Consequently if it be shown that collagen fibres appear in the liver as a result of stromal condensation, this stroma should not have the structural characteristics of basement membrane. Recent results obtained by electron microscopic examination of the liver suggest that this is so. Ruettner et al. (1956) failed to recognize a continuous membrane separating sinusoidal from hepatic parenchymal cells in the rat. According to Popper et al. (1960) there is no basement membrane but scattered reticulin fibres having the same axial periodicity as collagen. As they are usually cut transversely, they may be seen as isolated argyrophil points in ultra-thin sections studied by the light microscope, but appear as continuous membranes in thicker sections (Popper et al., 1958). These fine reticular fibres contain mucopolysaccharide and are periodic acid-Schiff positive. It seems reasonable that aggregates could form collagen fibres.

(d) <u>There is condensation of hepatic stroma following rupture and collapse of fat cysts</u>. Hartroft and Sellers (1952) described these cysts and their rupture in the livers of choline-deficient rats and it was later suggested that a similar phenomenon is a factor in human cirrhosis (Hartroft, 1953). Hepatic fibrosis is the result of stromal condensation; extra-cellular fat is apparently inactive in this respect (Gall, 1960). While this may be important in the pathogenesis of fatty cirrhosis it is inapplicable to carbon tetrachloride cirrhosis, in which fatty change does not proceed to cyst formation.

(e) <u>There is aggregation of ductular basement membrane leading to periductular fibrosis</u>. Electron microscopic studies have shown that the small intra-hepatic biliary ductules or cholangioles, unlike hepatic sinusoids, have a continuous lining of basement membrane (Popper <u>et al</u>., 1960). These authors have found that ductular proliferation and an associated periductular fibrosis can be induced readily in rats by feeding ethionine over a period of weeks. In addition they have found the same phenomenon in various examples of human cirrhosis including post-necrotic cases and those due to haemochromatosis and cholangio-hepatitis. In these conditions the proliferating ductular cells are small and oval and may be mistaken for mesenchymal cells. However, they can be distinguished readily by electron microscopy and there are no transitional forms between them and hepatic parenchymal cells (Schaffner & Popper, 1961). They form tiny channels each with a distinct basement membrane. This

spreads for a short distance over the surface of liver parenchymal cells where biliary canaliculi pass into the ductules. In ductular proliferation the basement membrane also increases in amount and young collagen fibres are seen in association with it. Whether these do in fact arise out of this membranous proliferation and condensation is still uncertain. Mesenchymal cells are numerous in areas of periductular fibrosis but do not include recognizable fibroblasts.

In addition to these possible causes of fibrosis, factors may exist of which nothing is known at present. There is much in the pathogenesis of human cirrhosis which is unexplained, e.g., the factors which determine its development in a minority of clinically mild cases of viral hepatitis; the numerous cryptogenic cases (Weissbein & Scott, 1960); the absence of such a complication in yellow fever and leptospirosis; the minimal scarring of healed amoebic abscess of liver (Paul, 1960), while other focal lesions such as gumma cause gross scarring. The mechanism by which connective tissue can disappear from a cirrhotic liver is also obscure although this undoubtedly occurs, especially in nutritional cirrhosis both in man and experimental animals (Patek et al., 1960).

A brief review of the structure and chemical composition of the components concerned in the pathogenesis of cirrhosis is given before discussion of my experimental results in relation to these theories.

(a) Adult or mature collagen, as obtained from tendo Achilles, consists of non-branching fibres with little or no argyrophilia,

metachromasia or positive P.A.S. staining. The bound carbohydrate recoverable from the tissue is qualitatively similar to that of normal basement membrane, but relatively scanty (Glegg et al., 1953). Pure collagen obtained by precipitation from collagen solutions may contain no carbohydrates at all (Moss, 1955); nevertheless, the addition of small amounts of mucopolysaccharide to such solutions is one way of obtaining precipitates (Gross et al., 1952). Scanty mucopolysaccharide in adult collagen is probably present in the matrix between fibrils and is not an integral part of the collagen molecule. Its sparseness would account for the negative staining properties described above and for the apparent absence of antigenicity.

(b) Reticular fibres are found characteristically in embryonic skin and in granulation tissue. They have many of the properties of collagen, consisting of fibrils with the same 640 Å axial periodicity, but are considerably thinner (Gross, 1950a, b). Mature collagen is likely to be formed by aggregations of these but, unlike mature collagen, they lie in and are coated with an amorphous glycoprotein matrix which imparts the positive argyrophil property and P.A.S. staining to the fibres. Much, but not all, of this matrix can be removed by washing with water (Gross, 1950b). It is apparent that young collagen also contains some glycoprotein which gradually disappears with aging.

(c) Intercellular ground substance. This is rich in sulphated acid mucopolysaccharides, particularly certain chondroitin sulphates, and in hyaluronic acid. The latter may be scanty or absent from granulation

tissue in which metachromasia is unaffected by streptococcal hyaluronidase (Bunting & White, 1950). Recent work by Jackson et al. (1960) has thrown some doubt on the importance of sulphated compounds in the production of young connective tissue fibres; chemical studies of granulation tissue have shown that these compounds do not appear until the ninth day after wounding when new collagen is well-established. Intercellular polysaccharide present during the earlier stages of healing is thought to be derived from serum mucoprotein and not from the activity of local mesenchymal cells. In the present work autoradiography showed an earlier uptake of sulphur in the catgut lesions, and in the laparotomy wounds.

(d) Basement membranes are similar to reticular fibres in so far as they are demonstrable by silver impregnation techniques and are P.A.S.-positive (Little & Kramer, 1952). However, they consist of branching fibres with no axial periodicity. The poor affinity of the material for perosmic acid renders it fairly homogeneous on electron microscopic examination. The central axis is somewhat denser than the margins and tiny transverse pores are sometimes described. According to Robb-Smith (1957), it is not a collagen precursor. While reticular fibres and their precursors are probably products of fibroblasts, the origin of basement membrane is uncertain. Gersch et al. (1950) thought that it is merely a condensation of intercellular ground substance, but the constant relationship to epithelium or endothelium at certain sites suggests that it is a more intimate product of cell activity.

Two groups of connective tissue mucopolysaccharides are described, "acid" and "neutral." The former include sulphated compounds found in a variety of normal tissues and in the intercellular ground substance of granulation tissue. A number of these compounds are known and have been tested by Meyer and Rapport (1951) and more recently by Dorfman (1958). They include chondroitin sulphate A, B and C, kerato-sulphate, heparin and heparin monosulphate. (The acid mucopolysaccharides, hyaluronic acid and chondroitin, are not sulphated). Those found in intercellular ground substance and most likely to be present in the hepatic lesions are chondroitin sulphates A and C. Scanty foci of intense sulphate uptake in the young connective tissue round the implanted catgut probably represented heparin in mast cells; as already noted, mast cells were not seen in the cirrhotic lesions.

These acid mucopolysaccharides consist of repeating disaccharide units, each with an acetylated hexosamine and a uronic acid. The presence of a free carboxyl or sulphate group on each repeating unit confers a highly negative change to the material which may account for its positive metachromatic staining, and for positive results obtained by the Hale and Alcian blue techniques. While all acid mucopolysaccharides can be shown in tissue section by these methods, the metachromatic reaction is probably best for the demonstration of the sulphated compounds (Curran, 1960). The exact significance of positive staining is still uncertain, however, and is discussed by Pearse (1960). Conversely, negative results by any method do not necessarily imply that small amounts of acid

mucopolysaccharides are not present.

Mature collagen contains little or no sulphated material (Gross, 1950a). Neither does normal basement membrane so far as it is possible to detect within the limits of autoradiography, and the absence of metachromasia would tend to confirm this. No sulphated material was recovered from renal basement membrane by Windrum et al. (1955). Nevertheless, the presence of some form of acidic mucopolysaccharide in glomerular walls is likely as they stain well by Hale's colloidal iron technique. Curran (1957) has reported uptake of S^{35}-sulphate by endothelial cells lining vascular channels, but I have never been convinced of this in liver sinusoidal cells or in adjacent reticulin.

Windrum (1958) described in granulation tissue a second protein-bound polysaccharide which is not sulphated, but which can be rendered metachromatic by an applied sulphation technique. Conversely, methylation will reverse this metachromasia by inducing de-sulphation (Spicer & Meyer, 1960). The substance in question is noted particularly on the surface of reticular fibres and is P.A.S.-positive; it is apparently a so-called "neutral" polysaccharide or glycoprotein. The sulphation metachromasia technique of Windrum shows that it too may be elaborated by fibroblasts and that it is scanty or absent in mature collagen.

The periodic acid-Schiff technique gives positive staining of tissues which contain "neutral" mucopolysaccharides. The reaction as described by McManus (1948) depends on the formation of aldehyde from 1-2 glycol groups by periodic acid (Leblond, 1950). Accordingly, carbohydrate-

containing compounds such as mucopolysaccharides will stain by this method; chondroitin sulphate and hyaluronic acid, however, do not do so, being resistant, apparently, to the action of periodic acid.

Windrum et al. (1955) undertook an extensive investigation into the chemistry of "reticulin"; this was obtained from the mid-cortical tissue of normal young adult kidneys and would consist mostly of basement membrane from glomeruli and tubules, but some collagen, particularly from small vessel walls, might have been present. This composite material was claimed to be a lipo-protein-carbohydrate complex consisting of fatty acids (mainly myristic), 17 amino- and imino-acids, glactose, mannose, fucose and a trace of hexosamine, but no uronic acid or sulphated material. I have obtained the same simple sugars with the addition of glucose from the cation-exchange resin hydrolysis of isolated human glomeruli (Patrick et al., 1961). Glegg et al. (1953) also recovered similar sugars from "reticulin" of lymph nodes, perirenal fat, testes and lung; ribose was also isolated, but may have been derived from incompletely digested nucleic acid.

Amino-acid differences between collagen and renal "reticulin" are described, e.g., by Eastoe (1955). These may be due to the presence of sclero-protein in addition to glycoprotein in one but not the other. The carbohydrate portion is constant but can vary from species to species throughout the animal kingdom (Gross et al., 1958). This could explain species antigenic specificity, although a few closely related species do share a common basement membrane antigen, e.g., mouse and rat.

According to Bairati (1958), lymph node reticulin is similar to pre-collagenous reticular fibres while the stroma of red splenic pulp is basement membrane, these observations being based on electron microscopic observations and on amino-acid content. Tomlin (1953), on the other hand, regards splenic reticulin as pre-collagenous from his electron microscopic observations. Jackson and Williams (1956) describe solubility differences between basement membrane and reticulin. The latter, obtained from granulation tissue around carrageenin foci, behaved like collagen, and in citrate solution contained little or no carbohydrate. Clerici and Pernis (1958) studied similar tissue around silicotic foci; compared with mature collagen, the fibres contained more hexose and hexosamine.

Basement membrane has obvious structural and functional differences compared with the argentophil fibres of granulation tissue, embryonic connective tissue and, possibly, splenic and hepatic reticulin. It may be, however, that they share something in common, viz., a protein-bound carbohydrate or glycoprotein, the "neutral" polysaccharide already referred to. This may contribute largely to the structure of basement membrane. In pre-collagenous reticular fibres it must serve some purpose, such as a template for sclero-protein molecules, but it may not be an integral part of the final product.

Cytochemical and exhaustive histochemical studies were not undertaken in the present investigation. In so far as developing connective tissue

stained red by van Gieson's method and in a positive fashion with acid aniline dyes, it was regarded as collagen. Nevertheless, it retained its affinity for silver impregnation and was usually P.A.S.-positive, even in cases of well-established carbon tetrachloride cirrhosis with nodular hyperplasia. Slight metachromasia was detected in some cases. Both Hale's colloidal iron technique and the Alcian blue techniques for acid mucopolysaccharides were only faintly positive. All these findings are similar to those reported by Balasubrahmanyan (1953) in his study of the same lesion in rats. He noted that P.A.S. staining became negative with aging of the collagen or after treatment of sections with hyaluronidase.

It is obvious that the carbon tetrachloride induced cirrhotic lesions contain less acid mucopolysaccharides than the new connective tissue around implanted catgut and this could be attributed to a passive process of condensation of basement membrane or liver reticulin, rather than to the recent activity of fibroblasts. However, from the autoradiographic studies following administration of S^{35}-labelled sodium sulphate, there is no doubt that a recently synthesized sulphated compound is present at the sites of fibrogenesis in cirrhosis. It is now generally accepted that this must be the product of the activity of mesenchymal cells, presumably fibroblasts, and sulphate uptake has been noted within these cells (Curran & Kennedy, 1954).

It is apparent from these results that the collagen in the cirrhotic livers is immature. Possibly no animal survived long enough for maturity to be established. The continued administration of carbon tetrachloride

up to or within a few days of obtaining hepatic tissue probably maintained sufficient stimulus for mesenchymal cell activity with production of sulphated material.

Antigenic specificity of certain bacteria, such as the various types of pneumococci, depends on the quality of their capsular glycoprotein and the same may be true of connective tissues. The demonstration by the Coons technique of antigen to basement membrane antibody in the cirrhotic lesion does not necessarily imply that this consists of thickened or condensed basement membrane since, as already suggested, the same glycoprotein may be a component of reticular fibres and immature collagen. In my experience this technique is not highly sensitive and it is difficult to interpret negative results; the argyrophil fibres round the implanted catgut were finer than the fibres of the cirrhotic lesion and, like normal liver reticulin, may have been beyond the limits of sensitivity of this method. The positive results with quartz granuloma antibody strengthen the view that basement membrane and reticulin share a common glycoprotein antigen. My results confirm that excessive amounts of this material are present in the cirrhotic lesions but throw no light on its origin; it could be a recent product of mesenchymal cell activity but could equally well be a normal component of condensed hepatic reticulin or ductular basement membrane.

Humoral factors affecting wound healing seem to act on mesenchymal cells, particularly fibroblasts. These cells can be stimulated by so-called "trephones" or "wound hormones", which appear to be enzymes

liberated by leucocytes (Davidson, 1943). Cortisone seems to have the opposite effect on these cells although there may also be a direct effect on mature collagen as shown by the prolonged application of the hormone directly to the skin (Castor & Baker, 1950). This direct effect may have been responsible for the temporary reduction in severity of established hepatic fibrosis obtained with cortisone, but little is known at present on the process of fibolysis which can occur naturally in cirrhosis.

The failure to demonstrate any important influence of cortisone or ACTH on the development of cirrhosis might be taken to indicate the absence of fibrogenesis dependent on fibroblastic activity. These hormones clearly inhibit the mesenchymal response to a single large dose of carbon tetrachloride with delay in absorption of necrotic tissue, but healing will occur eventually despite continued administration of either hormone (Patrick, 1955). In developing carbon tetrachloride cirrhosis smaller doses of the toxin are employed and a refractory state may develop with the continued administration of hormone.

It is notable that while similar doses of these hormones inhibit the early cellular response to injury round implanted catgut and in laparotomy wounds, some scar tissue does develop with little delay compared to non-hormone treated animals. This delaying but impermanent effect on wound healing is reminiscent of that produced by a protein-deficient diet (Clark, 1919), particularly diets deficient in sulphur-containing amino-acids (Williamson et al., 1951). Ungar (1953) noted delayed

absorption of catgut implanted in rat liver when the animals were fed a low-protein, high-fat diet; this was not due, apparently, to the presence of fatty liver or choline deficiency. These findings lend support to the view that cortisone may act through accelerated protein catabolism.

Pearse et al. (1960) describe two stages in wound healing, viz., an early cellular response and a later stage of collagen fibre production. Testosterone stimulates the first and inhibits the second, whereas cortisone inhibits the first but does not influence the second. The tensile strength of healing wounds (an index of the second stage) is actually enhanced slightly by giving cortisone to wounded rats with protein depletion.

Gross (1958) has pointed out that substantial amounts of granulation tissue are unnecessary for wound healing; experimental wounds heal satisfactorily if their granulation tissue is removed repeatedly, provided their edges are undamaged. Cirrhosis appears to be a condition in which scarring can develop insiduously without any marked activity of connective tissue cells; there is no reason to suspect that cortisone would have much influence on such a process.

Of the three investigations described, it is considered that most information on the development of cirrhosis was derived from the study on the uptake of labelled sulphur, since the result was taken to indicate cellular activity for the production of a substance known to be concerned in healing. The results of the other experiments would, on first consideration, support the hypothesis of the more passive process of

reticulin or basement membrane condensation, but arguments have been advanced to show that they may not in fact be incompatible with the other theory. The problem of the exact relationship between sulphate-utilizing cells and production of new collagen fibres in cirrhosis remains and will probably require electron microscopy for its elucidation. It is my intention to make use of recently acquired equipment for a study of the lesion induced by carbon tetrachloride. The application of methods I have employed for the analysis of the carbohydrate component of normal and diabetic glomerular basement membrane glycoprotein may also yield information of interest when applied to the newly-formed hepatic fibrous tissue.

SUMMARY

1. Cirrhosis of liver was induced in mice by feeding carbon tetrachloride twice weekly for periods of up to one year. The progress of the disease in individual animals was followed by repeated liver biopsy.

2. A second focal type of scarring was induced in some of these livers, and also in the livers of otherwise healthy mice, by the implantation of fine catgut. This, together with the laparotomy wounds, provided a means whereby certain properties of the fibrous tissue in cirrhosis could be compared conveniently with those of the new fibrous connective tissue normally associated with wound healing.

3. Cortisone and ACTH given daily in doses sufficient to depress circulating eosinophils and induce thymic atrophy, failed to inhibit the development of cirrhosis. The life-span of these mice was shortened by hormone therapy.

4. When given after the establishment of cirrhosis and during the continued administration of carbon tetrachloride, cortisone appeared to inhibit temporarily the further development of the lesion; in several cases there appeared to be a temporary reduction in the amount of fibrous tissue in the liver. The cause of this, taken with the other results, is not apparent.

5. Cortisone given to mice with established cirrhosis failed to accelerate the regression of the lesion following withdrawal of the liver poison; in fact, the opposite effect was obtained in a few cases.

6. The same doses of cortisone and ACTH had a notable inhibitory effect on the mesenchymal response around intra-hepatic catgut and delayed its absorption. Nevertheless, hormone therapy failed to inhibit the development of fine scar tissue around the catgut, just as it failed to inhibit scar tissue formation with healing of the laparotomy wounds.

7. The synthesis of sulphated acid mucopolysaccharide in the various lesions was studied by autoradiography following administration of S^{35}-labelled sodium sulphate. There was intense uptake of the isotope at the laparotomy wound edges and around the implanted catgut, which was not prevented by hormone therapy. A less intense but quite distinct

uptake was obtained also over the fibrous tissue in cirrhosis.

8. Conventional histochemical techniques for the demonstration of acid mucopolysaccharides revealed very little of this material in the cirrhotic lesion compared with the margins of the catgut lesion.

9. By the Coons fluorescent antibody technique it was found that the cirrhotic fibrous tissue contained antigen which reacted with antibodies to glomerular capillary basement membrane and to quartz granulomatous tissue. Staining of reticular fibres around the catgut lesions was faint and inconclusive with both conjugated antibodies. Results of antibody absorption experiments indicated that the two antibodies were probably one and the same.

10. A comparison of the two types of connective tissue has therefore shown:

(a) that both are similar in that they contain collagen fibres, the development of which is resistant to the administration of cortisone and ACTH

(b) that the cirrhotic lesion is relatively rich in material with antigenic properties, presumably a neutral polysaccharide. Similar material is present normally in glomerular basement membrane and probably in other basement membranes and reticulin fibres throughout the body.

(c) that the cirrhotic lesion is relatively poor in acidic mucopolysaccharides but is not completely lacking in a sulphated compound of this nature; this is synthesized presumably from mesenchymal cells in the lesion.

11. An attempt is made to relate these results to the pathogenesis of cirrhosis. While passive condensation of hepatic stroma may contribute to the development of the lesion, it is apparent that there must be some mesenchymal cell activity which is usually concerned with fibrogenesis. The exact relationship of these cells to the new collagen fibres in the lesion has not been determined.

PART III

THE INFLUENCE OF CORTISONE AND ACTH

ON EXPERIMENTAL ZONAL NECROSIS OF LIVER

THE INFLUENCE OF CORTISONE AND ACTH ON EXPERIMENTAL ZONAL NECROSIS OF LIVER

Experiments designed to study the influence of cortisone and ACTH on carbon tetrachloride cirrhosis of liver are described in Part II of this thesis. During this work it was noted, especially at the commencement of each experimenta, that mice which received both liver poison and hormone appeared more ill and suffered a higher mortality than controls which received no hormone. Moreover cortisone and ACTH frequently prolonged the acute illness of carbon tetrachloride intoxication, and it seemed that some inhibition in the process of recovery from acute liver damage had been induced.

These original impressions are confimred by the results of the experiments to be described. In addition to carbon tetrachloride certain other hepatotoxins were used, viz., tannic acid, allyl formate and phosphorus. When many of these experiments were carried out, supplies of both hormones were restricted and it was advantageous to work with small animals. The mouse was, therefore, the animal of choice. Later, rats and guinea-pigs were also employed, the latter being the most suitable for studies with allyl formate.

In each experiment the extent of liver damage and manner of recovery have been studied in animals receiving one or other hormone and in animals

receiving no hormone. The majority of experiments have been concerned with the carbon tetrachloride lesion in mice, and include determinations of the minimum effective dosage of both liver poison and cortisone and an investigation into the influence of various diets on the lesion.

Most of published reports on the relationship of hormones to liver disease are concerned with the effects of the latter on the former; e.g., Furlong et al. (1949) have shown that carbon tetrachloride cirrhosis in guinea-pigs is associated with an increased output of oestrogen. There is evidence that cortisone is quickly inactivated by normal liver if given orally or by intrasplenic injection (Robbins et al., 1957). Normal liver is apparently able to reduce steroid hormones to inactive substances and to aid their excretion through conjugation with glucuronic acid (Cameron, 1957a). These functions are disturbed in liver disease but the significance of this in clinical medicine is uncertain; thus, in nutritional cirrhosis, clinical features commonly attributed to hyperoestronism may really be due to an imbalance between oestrogens and androgens, the latter being reduced, not by liver dysfunction, but by associated malnutrition with gonadal atrophy (Cameron, 1957b; Martini, 1960). Martini gives an up-to-date review on all aspects of the subject.

There have been fewer reports on investigations into the influence of hormones on liver diseases. Aterman (1950) and Cavallero et al. (1951) claimed that cortisone reduced the severity of carbon tetrachloride cirrhosis in rats and noted both a reduction in inflammatory cells within the liver and some inhibition of mitotic activity in liver cells.

Aterman and Ahmad (1953) found in these animals an exaggeration of the hydropic degeneration of liver cells associated with carbon tetrachloride poisoning and also a worsening of liver function studied by the bromsulphthalein excretion test. Schwarz (1951) found that cortisone had a beneficial effect on dietary liver necrosis of young rats, but this lesion is in many ways dissimilar to that of chemically-induced zonal necrosis.

Simultaneously with the publication of the results of some of this work (Patrick, 1955), Hoffman et al. (1955a & b) reported similar findings in rats treated with cortisone and carbon tetrachloride, but failed to note much effect of the hormone on liver cell regeneration. They suggested that the removal of necrotic liver tissue in the absence of macrophages is due to the action of proteolytic enzymes from the blood and from viable liver cells. They attributed regeneration to amitotic division of liver cells in spite of inhibition in the synthesis of deoxyribonucleic acid.

Cortisone may be ineffective in preventing normal regeneration after partial hepatectomy (Canzanelli et al., 1949) but the process is hastened in adrenalectomized animals (Drabkin, 1950) and by somatotropic hormone (Cater et al., 1957). With regard to primary liver cancer, however, cortisone may accelerate growth and adrenalectomy inhibit it (Chany & Boy, 1960).

MATERIALS AND METHODS

Most of the animals were young Swiss albino mice of both sexes weighing 15 to 25 g. The remainder were male Wistar albino rats, 80 to 150 g. in weight, and male guinea-pigs, 230 to 450 g. in weight. The animals in each experiment were divided thus:

Group A — Animals given a single sub-lethal dose of liver poison plus hormone therapy.

Group B — Animals given liver poison alone.

Group C — Animals given cortisone or ACTH alone.

The groups comprising any one experiment were closely similar with regard to age, weight, sex and living conditions. Unless otherwise stated rats and mice were fed on "Animal Diet Rowett Institute No. 86 (Rat Cake)". Guinea-pigs received "Animal Diet Rowett Institute No. 18".

Carbon tetrachloride was administered to Groups A and B by oesophageal tube. Rats received a single dose of pure liquid, 0.2 ml./100 g. body weight; mice were given 0.1 ml./20 g. body weight of a mixture of carbon tetrachloride 4 parts, liquid paraffin 6 parts. Control (Group C) animals received similar quantities of pure liquid paraffin. Details of alternative liver poisons are given below.

Cortisone acetate (Roussel Laboratories Ltd., Batch No. 883) was given daily by intramuscular injection in a dose of 2 mg./100 g. body weight/day. For mice, the accuracy of small doses was assured by dilution of the hormone suspension with nine times its volume of isotonic

saline immediately before use. ACTH ("Acthar Gel", Armour, Lot No. M 22007) was given to mice only, 0.25 mg. twice daily by intramuscular injection.

For all animals of Group A, hormone therapy, whether cortisone or ACTH, commenced on the day prior to the administration of hepatotoxin, and was repeated daily until the animals were killed. Group C animals received identical doses while those of the control Group B were given daily injections of isotonic saline.

Animals from each group were killed by stunning at intervals ranging from one hour to one week after poisoning. Until hormone dosage was established, the thymus glands were weighed and the circulating eosinophils enumerated by Randolph's method from blood obtained by cutting the throat.

Thin slices of fresh liver were placed in Rossman's picric acid-alcohol-formalin fixative, embedded in paraffin, and sections stained by haematoxylin and eosin, by the periodic acid-Schiff technique, and with Best's stain for glycogen. Cytoplasmic basophil bodies, regarded as representative of ribonucleic acid, were demonstrated by staining with 0.5% aqueous toluidine blue or with diluted Giemsa's stain applied for 24 hours. Other liver slices were fixed in 10% formalin containing calcium chloride 10 g./litre and embedded in gelatine. Frozen sections from these blocks were stained for fat with Sudan IV or Sudan Black.

EXPERIMENT 1

To observe the effects of constant doses of cortisone and ACTH on animals at variable intervals after a constant dose of carbon tetrachloride

In all 420 animals were used in this experiment. Members of each group were killed at intervals of 1 hour, 3 hours, 5 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days and 7 days after the administration of carbon tetrachloride (Table XXIX). Eight Group A mice and two Group B mice died prematurely and were discarded.

Results

Group B animals, with carbon tetrachloride alone, showed general evidence of poisoning within 24 hours, when they became listless, sat huddled together and had ruffled coats. After a further 24 hours, many appeared to recover and by the third day the large majority were well again. Control Group C animals given cortisone or ACTH remained well, but the combination of hormone with carbon tetrachloride resulted in prolongation of the illness produced by the latter, often to the fifth, sixth or even seventh day after poisoning (Fig. 189).

On naked-eye examination, the livers with established zonal necrosis were somewhat larger and more friable than normal, with exaggeration of the normal lobular pattern which produced a characteristic diffuse mottling (Fig. 190). These liver changes showed gradual improvement though they persisted to some extent for a day or two after the apparent clinical recovery of the animals. In keeping with the prolonged illness

of many Group A animals, there was corresponding delay in the resolution of this liver lesion. The typical appearance of mouse livers in this experiment four days after poisoning is shown in Figure 191. The small thymus glands of the animals after daily cortisone injections is indicative of the efficacy of such hormone therapy.

An early histological feature indicative of liver cell damage is loss of cytoplasmic basophilia. This is noted especially in swollen mid-zonal cells within an hour of poisoning, when the centrilobular cells lose the normal granular appearance of their cytoplasm (Fig. 192). This apparent loss in ribonucleic acid becomes more obvious later (Figs. 193 & 194), but necrosis is not seen until 24 hours after poisoning. The lesion is centrilobular in distribution and fairly uniform throughout the liver. Surviving periportal cells are strongly basophilic (Fig. 195). Many specimens at this stage show notable hydropic degeneration of mid-zonal cells at the margins of necrosis (Fig. 196). These features were observed in specimens from both Group A and Group B mice.

Histological evidence of regeneration was generally found in Group B by the second day. Around the eosinophilic necrotic tissue of the centrilobular zones are numbers of mesenchymal cells, presumably derived from circulating leucocytes and from undamaged sinusoidal cells (Figs. 197 & 198). Phagocytosis of the necrotic liver parenchyma is well-advanced by the third day. Simultaneously, viable liver parenchymal cells show signs of regeneration; many are swollen and multinucleated, and mitotic figures are frequently seen (Fig. 199). On the fourth day

after poisoning, most or all of the necrotic liver has been replaced by regenerated parenchyma (Fig. 200).

By contrast, such indications of repair were greatly minimised and often absent in animals of Group A. Microscopic examination commonly reveals relatively acellular areas of eosinophilic necrosis around the central veins, while the apparently undamaged liver parenchyma shows no proliferative activity (Figs. 201 & 202). Excessive cytoplasmic lipid and glycogen are noted in the periportal cells which thus present a vacuolated appearance in H. and E. sections. This is a known effect of cortisone and ACTH alone and was commonly found in Group C animals also (Fig. 203).

The features of delayed recovery just described were not uncommon in the livers of Group A animals killed as late as the fourth and fifth days after poisoning (Figs. 204 & 205). Thereafter the microscopic evidence of recovery in this group, though similar in pattern to that of Group B, never obtained comparable intensity. Mitotic figures were less numerous, phagocytic activity was less prominent and the whole process appeared depressed. Nevertheless, by the seventh day, despite the continuation of hormone therapy, necrotic tissue had disappeared from the centrilobular zone and recovery was well established.

For the purpose of recording results, an arbitrary estimate was made of the amount of eosinophilic necrotic tissue within the average lobule. Sections from at least two, and in many cases three, blocks of each liver were examined and the extent of necrosis recorded as a percentage of the

total lobule, to the nearest 10 per cent. Various examples are illustrated (Figs. 206 to 209).

The results of Experiment 1 are shown on Figure 210, in which each symbol represents the findings obtained from a single animal, plotted according to the degree of necrosis and to the time after carbon tetrachloride administration. Clearly there is an association between hormone therapy and the duration of liver damage. Accentuation of this damage is also apparent in many members of Group A, compared with Group B. Group C animals are excluded since none showed evidence of liver necrosis.

EXPERIMENT 2

To observe the influence of variable doses of cortisone on mice suffering from carbon tetrachloride poisoning

In this experiment, the amount of cortisone given to the animals of Groups A and C varied; the doses received daily for four days by the twelve mice of each group were 0.05, 0.10, 0.20, 0.30, 0.40, 0.60, 0.80, 1.00, 1.50, 2.00, 3.00 and 5.00 mg. The twelve animals of Group A plus twelve of Group B received 0.1 ml. carbon tetrachloride 40% in liquid paraffin on the second day of the experiment. All 36 mice were killed on the fourth day, i.e., two days after the administration of liver poison.

Results

In Experiment 2 only those animals which received less than 0.2 mg.

cortisone daily failed to show the adverse effect on liver damage which has been described above. On the other hand, the deleterious influence of cortisone did not increase with increased dosage. Likewise, thymic atrophy and eosinopenia, two well-known effects of cortisone, were notable in all but one animal which received at least 0.2 mg. cortisone daily.

Details of thymus weights and eosinophil counts are shown in Table XXX.

EXPERIMENT 3

To observe the influence of constant doses of cortisone on mice given variable doses of carbon tetrachloride

As in Experiment 2, each group consisted of twelve mice. Those forming Groups A and C each received 0.4 mg. cortisone acetate daily for five days. The amount of carbon tetrachloride given as a single dose varied between individual members of Group A as follows: 0.01, 0.02, 0.03, 0.04, 0.05, 0.75, 0.10, 0.20, 0.30, 0.50, 0.75, 1.00 ml. pure liquid. The smaller amounts were suitably diluted with liquid paraffin. Group B animals were treated in a similar way. The larger doses of liver poison proved quickly fatal. The remaining mice along with those comprising Group C were killed on the fourth day of the experiment, i.e., two days after the administration of carbon tetrachloride.

Results

Animals of Group B which received 0.03 ml. carbon tetrachloride or more, developed obvious liver necrosis. In the case of Group A animals,

however, all suffered a substantial degree of liver damage; even 0.01 ml. produced 70 per cent necrosis. Doses of carbon tetrachloride as high as 0.2 ml. or more were generally fatal to mice of both Group A and Group B in a relatively short time.

Carbon tetrachloride is known to increase liver weight due to oedema. Accumulation of water, glycogen and fat within the liver may follow the administration of cortisone. Group A animals (CCl_4 + cortisone) as would be expected, had a liver weight somewhat greater than that of the animals which received either substance alone. The livers were weighed from all animals of Experiments 2 and 3 and from certain mice of Experiment 1. The average wet liver weight in 56 animals of Group A was 7.6 per cent total body weight. The corresponding figure for Group B (57 animals) was 6.0 per cent and for Group C (46 animals) 6.8 per cent.

Details of this experiment are included in Table XXXI.

EXPERIMENT 4

To observe the influence of cortisone and various diets on carbon tetrachloride poisoning in mice

In other experiments, food intake was not measured, but it was not notably impaired by hormone therapy. Because the deleterious effect of cortisone may have been related to its action on protein metabolism, it was decided to repeat the work with this hormone and carbon tetrachloride on mice receiving either (i) high protein diet or (ii) low protein-low fat diet or (iii) water only. The composition of diets (i) and (ii) was as follows:

	High Protein Diet	Low Protein Diet
Casein (fat and vitamin free). (B.D.H.)	300 g.	50 g.
Lard	50 g.	50 g.
Sucrose	597 g.	847 g.
l-Cystine	5 g.	5 g.
*Salt Mixture	40 g.	40 g.
**Vitamin Powder	8 g.	8 g.
***Fat-soluble Vitamins	0.4 ml.	0.4 ml.

*Salt Mixture:	Sodium chloride	4.35%
	Magnesium sulphate	13.70%
	di-Sodium hydrogen orthophosphate	8.72%
	Potassium phosphate-tribasic	23.98%
	Calcium phosphate-mono acid	13.58%
	Ferric citrate	2.97%
	Calcium lactate	32.70%
**Vitamin Powder:	Aneurine hydrochloride	0.3125 g.
	Riboflavine	0.5000 g.
	Pyridoxin hydrochloride	0.3125 g.
	Calcium pantothenate	1.2500 g.
	Nicotinic acid	1.2500 g.
	Menaphthone	0.3125 g.
	Sucrose	996.0625 g.
***Fat-soluble Vitamins:	Vitamin A palmitate	500,000 I.U.
	Calciferol (Vitamin D_2)	100,000 I.U.
	dl-α-Tocopherol acetate (0.95 g./ml.)	4 ml.
	Arachis oil	3 ml.

The dry components were mixed carefully with a large mortar and pestle. The lard was melted by heat and the fat-soluble vitamins added. The powder and melted fat were then thoroughly mixed in a Waring blender. 500 gram amounts of each diet were prepared at one time and stored in a

refrigerator before use.

Mice were fed such diets exclusively with water ad lib for 10 to 14 days before the commencement of the experiment which was otherwise similar to Experiment 1. Food was withdrawn from the "water only" group (iii) 18 hours before the administration of carbon tetrachloride; these animals were starved until the end of the experiment.

The numbers of animals employed are shown in Table XXXII.

Results

These also are set out in Table XXXII. It is considered that:

(a) the deleterious influence of cortisone is unaffected by both high and low protein diets;

(b) starvation by itself has a deleterious influence, all animals in this group being dead within four days of poisoning with extensive liver necrosis and no evidence of repair. The death rate of the cortisone-treated sub-group was notably high during the early stages of this experiment.

No Group C (i), (ii) and (iii) animal had evidence of liver necrosis. Nevertheless, the four Group C (iii) animals died by the end of the fourth day of starvation.

EXPERIMENT 5

To observe the influence of cortisone on animals suffering from tannic acid poisoning

The plan was similar to that of Experiment 1 with tannic acid

substituted for carbon tetrachloride. Aqueous solutions of tannic acid (B.D.H.) were given by intramuscular injection in doses of approximately 100 mg./kilogram body weight. Mice received a single injection of 0.2 ml. 1% tannic acid solution, and rats 0.1 ml. 10% tannic acid solution. Group C animals received intramuscular injections of isotonic saline. ACTH was not used in this investigation. Cortisone was given to Group A and Group C animals as in Experiment 1. Animals from each group were killed 1 day, 2 days, 3 days, 4 days, 5 days and 7 days after the administration of tannic acid. The numbers employed are shown in Table XXXIII.

Results

Figure 211 gives the results of this experiment. Four mice of Group A and one of Group B died prematurely on the second day after poisoning. There were no fatalities among the rats.

Owing to the rather more variable, and often more prolonged, effect of tannic acid on the liver and because of the smaller number of animals used, these results are not so conclusive as those of Experiment 1. Nevertheless, they suggest a similar delaying effect of cortisone on the resolution of the liver lesion. Examples of this lesion on the third day after poisoning are illustrated (Figs. 212 & 213).

Figure 214 summarizes in graph form the results of Experiments 1 and 5 in both of which the liver lesion was a centrilobular zonal necrosis.

EXPERIMENT 6

To observe the influence of cortisone on allyl formate poisoning in guinea-pigs

Allyl formate (B.D.H.) was diluted twenty times with isotonic saline and administered in a single dose by intramuscular injection.

This substance is highly toxic to adult rats, even in a dosage of 0.1 ml. of the diluted material. Most animals died within 24 hours of its administration with little evidence of liver damage apart from depletion of glycogen and basophilic cytoplasmic granules. One rat, also receiving cortisone, had recognizable periportal necrosis of liver (Fig. 215).

The guinea-pig proved to be a more suitable animal, being more resistant to the general toxic effect of allyl formate, but showing more liver damage. As in previous experiments, cortisone was given daily throughout the experiment, commencing on the day before administration of liver poison. Group A and Group B each consisted of eighteen animals; seven Group C animals showed no ill effects from cortisone alone, and are not considered further.

Results

Group B animals became ill within a few hours of administration of allyl formate. The response was variable, some being only mildly upset while others died within 24 hours. Of those less severely affected, recovery was evident after the second day and complete by the fourth day.

Glycogen disappears from the periportal portion of each liver lobule within three hours of poisoning (Figs. 216 & 217) and soon after these cells become swollen. Periportal zonal necrosis is evident in 24 hours; peripheral liver cell plates are disorganized and individual liver cells have a strikingly eosinophilic cytoplasm and pyknotic nuclei. Küpffer cells are prominent and there may be some neutrophil polymorph infiltration (Figs. 218 to 220). Such changes were associated with an exaggeration of the normal lobular pattern of the liver as seen on naked-eye examination.

Although essentially a zonal type of necrosis, more massively necrotic areas may be found where several adjacent lobules are completely destroyed (Fig. 221). In some cases periportal oedema and haemorrhage are additional features, being reminiscent of the human liver in fatal eclampsia (Fig. 222).

By the second day macrophages and polymorphs are now prominent and there is evidence of repair (Figs. 223 & 224). Glycogen may return first to the rim of viable cells which are marginal with the necrotic zones (Fig. 225). Repair proceeds rapidly and each lobule is usually regenerated completely by the third day. There may be some persistent portal oedema or lymphatic dilatation (Fig. 226).

Considerable variation was found in the extent of liver damage between individual animals receiving constant doses of poison. Apparently the severity of illness does not depend only on the extent of liver damage and some guinea-pigs dying within 24 hours had minimal hepatic

lesions. For this reason, allyl formate is unsatisfactory, compared with carbon tetrachloride, as an agent by which the effect of cortisone on liver necrosis may be studied, and it would be unjustifiable to draw conclusions from this work. Cortisone did appear to minimize the toxic effects of allyl formate but may have accelerated the onset of liver damage. There is also a suggestion of a slight delay in healing under the influence of the hormone.

The results of this experiment are set out in Table XXXIV.

In view of the inconclusive nature of the results, it was decided not to continue the experiment with larger numbers of animals or to investigate further the toxic action of allyl formate on organs other than the liver.

EXPERIMENT 7

To observe the influence of cortisone on phosphorus poisoning in mice

Like allyl formate, phosphorus is claimed as an agent which will produce periportal zonal necrosis of liver. As with allyl formate also, it is difficult with phosphorus to establish experimental conditions whereby the liver can be damaged without killing the animals in a short period of time. Indeed, it was impossible for this reason to use either rats or guinea-pigs and in neither could any hepatic necrosis be induced; loss of liver glycogen did occur, but often spared the periportal regions of the organ.

Structural changes in the liver were more apparent in mice which were

chosen for the experiment. Forty animals were used, half receiving daily injections of cortisone as in the previous experiments. Animals surviving the original toxic illness were killed at intervals (Table XXXV).

For administration to these mice, yellow phosphorus was added to a weighed quantity of arachis oil and the mixture reweighed. The amount of oil was then adjusted without delay so that the phosphorus was approximately one per cent of the mixture. The phosphorus dissolves slowly in the oil. A suitable single dose for young 20 g. Swiss albino mice was found to be 0.05 ml. given subcutaneously.

Results

Mice so treated appeared unwell within a few hours and some died within a day of poisoning. Others recovered gradually, being well again by the fifth or sixth day. The rate of recovery was slower compared with the illness of allyl formate poisoning.

The livers of these animals were swollen and extremely pale due to fatty change. Fat forms fine droplets scattered throughout the cytoplasm of the liver cells which have a foamy appearance; the nuclei are not displaced. There is no stainable glycogen. This degenerative change is always more marked at the periphery of each liver lobule (Figs. 227 to 229) but may extend to involve most liver cells. In no case, however, was there evidence of frank necrosis of periportal cells. On the contrary, a few animals show, at 48 hours after poisoning, a narrow but distinct zone of coagulative necrosis around each congested centrilobular vein (Figs. 230 to 232).

Macrophages and polymorphonuclear leucocytes are evident during the second and third days after poisoning. These are scattered throughout each lobule, but are sometimes more profuse in the centrilobular zones where small eosinophilic bodies, presumably derived from necrotic liver cells, may be numerous (Figs. 233 & 234). (They are reminiscent of the inclusion bodies described by Doniach and Weinbren (1952) following partial hepatectomy, and by Anderson et al. (1961) in liver cells adjacent to areas of physically induced hepatic injury. They are regarded by these authors as swollen intracellular lysosomes or lipofuscin, or, possibly, imbibed plasma protein). The inflammatory process gradually subsides with return to normal within a week.

Owing to the high mortality rate in the early stages of illness and the variable effect of phosphorus on the livers of survivors, it was considered that this was not a suitable hepatic lesion for the study of cortisone effects. Consequently, only a small number of animals is reported. The difference in the liver lesions between Groups A and B is not striking but the most obvious example of centrilobular necrosis was in a cortisone-treated animal. The mesenchymal response described during the period of recovery was minimal or absent in the cortisone-treated group, but this did not appear to delay complete recovery to any extent.

DISCUSSION

There are numerous substances which may damage the liver functionally and structurally and these have been reviewed recently by Stoner and Magee (1957). As these authors suggest, probably all act by disturbing the internal metabolic processes occurring within the liver cell. The exact mechanism of such action and the factors determining the maximum extent of liver damage within the lobule in any specific case are still matters of debate. Only a small selection of these hepatic poisons have been employed in this work, as the main purpose was to study liver regeneration following injury by any convenient method. However, the adverse influence of cortisone and ACTH on the severity of the carbon tetrachloride lesion made it desirable to repeat the work with other chemically distinct liver poisons. Those which were chosen proved to be rather less suitable for this experimental project on account of the variable severity of the resulting liver lesion in apparently identical animals. Nevertheless, the results obtained do suggest that the hormones exert the same deleterious effects on the extent of liver damage and healing, regardless of the type of toxic agent employed.

Liver damage from halogenated hydrocarbons, especially chloroform and carbon tetrachloride, has been the subject of innumerable investigations. They are convenient to use and generally produce notable liver damage well within the minimal lethal dose. Carbon tetrachloride is, perhaps, preferable to chloroform, as it may produce less renal damage, at least in

small male rodents (Culliford & Hewitt, 1957). The development of centrilobular zonal necrosis and the rapid repair of the damaged liver as described in my Group B animals are quite in keeping with various published reports, particularly those by Cameron and Karunaratne (1936) who described the lesion in rats, and by Stowell and Lee (1950) who made an extensive study of the condition in mice. In mouse liver, mitotic activity is greatest on the second or third day after injury, so that regeneration of each damaged lobule is well advanced by the fourth day and complete by the sixth (Tsuboi et al., 1951).

In order to study the effect of extrinsic factors on such a lesion, it is desirable to use a large number of animals as the severity of liver damage by a constant dose of carbon tetrachloride varies among apparently identical animals. This discrepancy can be reduced to some extent by administration of the poison orally (McCloskey & McGehee, 1950), or by inhalation. I have invariably given carbon tetrachloride by oesophageal tube and not by parenteral injection. With a little practice the tube can be passed easily and quickly without much disturbance to the animals. In spite of this, the results were not quite uniform among similar animals so treated.

Several workers, e.g., György et al. (1946) have noted a sex difference in the response to carbon tetrachloride, females showing less liver damage than males. I have noted some evidence in support of this in my work on carbon tetrachloride-induced cirrhosis (Part II). In Experiment 1, ten young female mice are included in each of the three

Groups A, B and C, with results similar to those obtained with male animals. In all other experiments males only were used.

The age of the experimental animal is an additional factor which can affect the character of liver damage. Post et al. (1960) noted that recovery from the carbon tetrachloride lesion in rats is delayed with advancing age irrespective of dosage. All rats and mice in my experiments were young animals, mostly eight to ten weeks old.

The development of centrilobular zonal necrosis of liver as a complication of burns treated with tannic acid suggested that this substance might be a liver poison. Experimental proof was forthcoming, e.g., by Cameron et al. (1943) and Barnes and Rossiter (1943). I have used smaller doses of tannic acid than these authors, but still find a high degree of susceptibility to its toxic effects among some of my experimental animals. Like carbon tetrachloride, tannic acid seems to damage liver mitochondria, particularly in the centrilobular zones, soon after administration (Cameron et al.)

It has been suggested that peripcrtal zonal necrosis results from the initial severe damage to those liver cells first exposed to the circulating toxin (Himsworth, 1947). This is possibly so in the allyl formate lesion, although the peripcrtal oedema and haemorrhage in some cases were suggestive of primary vascular damage. Heinemann (1937) had most success with rabbits, using 0.2 ml. to 0.5 ml. for 2 kilogram animals. She noted that rats usually develop fatal cerebral symptoms but no liver damage. In order to conserve available supplies of

hormone I preferred to use guinea-pigs, in many of which obvious liver damage could be produced without killing the animals.

Marshall and Rowntree (1915) described periportal zonal necrosis in the livers of dogs given two daily injections of phosphorus (10 mg. and 7.5 mg.) dissolved in oil. Most experimental work with this poison has been carried out in dogs and it may be that rodents are unsuitable for the purpose. These animals clearly develop liver cell damage but this does not progress to frank periportal necrosis. On the other hand, centrilobular zonal necrosis was noted in some instances, which might be an ischaemic phenomenon from sinusoidal constriction secondary to swelling of more peripherally placed cells. If this is so the lesion could be essentially similar to carbon tetrachloride necrosis for which the same mechanism has been proposed to explain the distribution of liver cell damage.

This ischaemic theory of centrilobular zonal necrosis was advanced in 1948 by Glynn and Himsworth who showed sinusoidal constriction by means of India ink injected into the spleens of animals which also received carbon tetrachloride. Conversely, the liver lesion is reduced by the administration of oxygen (Andrews, 1957). Autologous grafts of liver in omentum are relatively resistant, possibly because of a rich blood supply which reduces the tendency to ischaemia (Cameron & Karunaratne, 1936; Myren & Vinje, 1952).

The results of some other investigations throw doubt on this theory. Daniel et al. (1952), using an angiographic technique to study hepatic

circulation could not demonstrate sinusoidal constriction after carbon tetrachloride. Stoner (1956) has devised a method of internal calorimetry of the liver; with this apparatus he found evidence of increased blood flow after intoxication. It has been suggested that in Glynn and Himsworth's experiment the general anaesthesia preceding intrasplenic injection of India ink, and not carbon tetrachloride, caused sinusoidal constriction.

Myren (1956) believes that the initial damage in carbon tetrachloride poisoning involves mid-zonal hepatic cells. I have noted slight hydropic change and loss of basophilic cytoplasmic material in this zone within an hour of poisoning. Myren described swelling of these cells within minutes of subcutaneous injection and within seconds of intrasplenic injection. It seems possible that there is an early and perhaps transient sinusoidal constriction which might be sufficient to aggravate the action of the poison on the centrilobular zone and so determine the maximum site of damage. If this is so, the mechanism might be further aggravated by cortisone which increases the bulk of liver cells through increasing their content of water, glycogen and fat. On the other hand, Myers and Taylor (1952) found that cortisone increased liver blood flow in eight human subjects with normal liver function.

Lacquet (1932) noted mitochondrial damage in the livers of rats three hours after carbon tetrachloride administration. Cameron and Karunaratne suggested that this change may occur within one hour. Loss of basophilic cytoplasmic granules thought to be ribonucleic acid is also an early

feature of liver damage (Rosin & Doljanski, 1946), but chemically the amount of ribonucleic acid is not reduced (Farber et al., 1951; Hoffman et al., 1955b). Carbon tetrachloride probably damages endoplasmic reticulum of liver cells with dispersion of ribonucleic acid throughout the hydropic cytoplasm (Oberling & Rouiller, 1956; Leduc & Wilson, 1958).

Damaged mitochondria undergo increased permeability and disorganization but their enzymes concerned with the tricarboxylic cycle may not be destroyed (Christie & Judah, 1954). Oxidative phosphorylation is disturbed nevertheless because of loss of co-factors and pyridine nucleotides. Feeding rats with pyridine nucleotide precursors such as nicotinic acid and DL-tryptophane can protect against carbon tetrachloride injury (Gallagher, 1960). The remarkable accumulation of calcium in these damaged mitochondria at the expense of potassium cations will also interfere with their function (Thiers et al., 1960). Histochemical evidence of recovered enzyme activity follows the reappearance of normal mitochondria about the fifth day after poisoning (Tsuboi & Stowell, 1951).

It has been shown recently, especially by Recknagel and his co-workers, that such enzymatic dysfunction does not explain the early liver cell changes which appear within a few hours of poisoning (Recknagel & Anthony, 1957; Recknagel et al., 1958). Calvert and Brody (1960) believe that these early changes are the result of abnormal sympathetic impulses initiated by the hepatotoxin and mediated by the adrenal medulla. It is claimed that these impulses cause both liver sinusoidal constriction and fatty liver following mobilization of depot fat. This theory is

supported by reduction of liver damage by adrenergic blocking agents, reserpine, adrenalectomy and especially by cervical cord transection. The adrenal medulla is apparently more important than the cortex in this mechanism. Schotz and Recknagel (1960) criticized these results in so far as they could demonstrate no elevation of plasma lipid following administration of carbon tetrachloride. Recknagel and Litteria (1960) found that the liver may contain measurable amounts of carbon tetrachloride for up to nine hours before its fat content begins to rise and before there is any loss of enzyme function normally associated with mitochondria. During this time detoxicating enzyme systems associated with microsomes may be deranged (Neubert & Maibauer, 1959).

Lowe & Williams (1953) found that cortisone had no effect on the mitochondrial enzymes of rat liver cells, and Myren (1960) noted that ACTH had no influence on liver dehydrogenases. However, according to Kerppola and Pitkänen (1960) there is inhibition of respiratory function at the cytochrome oxidase level. By the use of isocitrate or α-ketoglutarate as substrates, depression of oxidative phosphorylation and ATP synthesis can be demonstrated, especially in old female rats (Kerppola, 1960). This effect can be reversed with oestradiol. It seems possible that cortisone, by acting in this way, could aggravate the deleterious influence of carbon tetrachloride on mitochondrial function.

Other liver enzymes are influenced by corticosteroids. The effect on glucose-6-phosphatase and fructose diphosphatase leads to hyperglycaemia (Weber et al., 1956; Kvam & Parks, 1960), while the liver

content of glycogen is raised through the effect on phosphorolase (Hess et al., 1960). It seems unlikely that liver injury would be aggravated by these results unless indirectly through swelling of cells from excessive glycogen storage. It has been noted, however, that glycogen disappears from the liver within a few hours of poisoning, whether cortisone is given or not.

In addition to glycogen, liver cell water and probably fat are increased by cortisone and ACTH. Before the isolation of these hormones it was recognized that anterior pituitary extract would produce ketosis and fatty liver (Fry, 1937; Ingle, 1943), while adrenalectomy prevented fatty liver (Long et al., 1936). With regard to cortisone, Williams et al. (1953) found rabbit liver fat unchanged after its administration, while Harris et al. (1953) found a decrease in guinea-pig liver fat. The majority of reports, however, describe the opposite effect, e.g., Rich et al. (1951); Dury (1955); Kief and Schürmeyer, 1956. ACTH also induces fatty liver according to Baker et al. (1948) and Morrison (1952). It is generally assumed that cortisone acts as a stimulant to the mobilization of fat from fat depots. In addition Altman et al. (1951) noted its action in stimulating lipogenesis in perfused rat liver. Adrenalectomy depresses this hepatic function while stimulating fat catabolism (Perry & Bowen, 1956).

In the present investigation, quantitative estimations of fat and glycogen were not undertaken, but histochemical tests showed excess of both substances in the livers of control rats and mice which received

cortisone or ACTH alone, and also in the viable portions of the livers of many Group A animals after the acute illness had subsided. These effects were not pronounced in guinea-pig liver.

Liver necrosis is aggravated by steatosis, at least by that associated with low-protein high-fat diet (Moise & Smith, 1924; Goldschmidt et al., 1939; Forbes et al., 1941). Goldschmidt and his co-workers noted that the glycogen content of the liver which could be varied by dietary measures did not influence the extent of the lesion. It seems possible that the fatty change induced by the hormones under investigation may have had a similar deleterious effect.

Cortisone may also aggravate liver necrosis indirectly through its effect on protein metabolism, viz., inhibition of anabolism and acceleration of catabolism (Cannon et al., 1956). Liver damage is accelerated and recovery is delayed in conditions of protein deficiency induced by low-protein diet (Smith & Moise, 1924). However, it appears that cortisone stimulates protein synthesis in the liver as distinct from the rest of the body (Clark, 1953), even in animals on a protein-free diet (Pesch & Clark, 1956). This might explain the lack of benefit obtained by a high-protein diet (Experiment 4). It is well-known that starvation has a profound deleterious influence on liver necrosis, e.g., Graham (1915), and this seems to be beyond the influence of cortisone.

Some observations on the influence of cortisone and ACTH on wound healing have been given in Part II; it is not surprising that a similar inhibitory effect was noted on the repair of necrotic liver. It is

possible that this also may be independent of any effect on protein metabolism (Baker & Whitaker, 1948; 1950), but may be a local action by the hormones at the site of injury. Cortisone applied locally to mouse skin inhibits mitosis (Green & Ghadially, 1951). So may ACTH even in adrenalectomized animals (Green, 1950). ACTH may also inhibit the emigration of leucocytes into an inflamed area in adrenalectomized animals (Menkin, 1953b). This author described a different local action for cortisone, viz., the suppression of leucotaxine and reduction of capillary permeability (Menkin, 1953a).

The "antiphlogistic" action of these hormones was among the first of their properties to be investigated. Selye (1949) noted that both reduced the inflammatory response to injected formalin. Some reports describe the inhibition of all mesenchymal cells involved in inflammation and wound healing, e.g., Michael and Whorton (1951), while others stress the relative susceptibility of fibroblasts and endothelium (Lattes et al., 1950). Ragan et al. (1951) found relatively less inhibition of blood capillary growth in granulation tissue of rabbit ear wounds.

To be effective in the retardation of healing, either hormone must be administered from the start of wounding (Spain et al., 1950). These authors describe inhibition of phagocytosis of India ink particles injected intraperitoneally in mice receiving cortisone. Others believe that there is no inhibition of phagocytosis but only in the mobilization of cells and their emigration to the site of injury (Germuth et al., 1952; Gell & Hinde, 1953; Magee & Palmer, 1953). Marcus et al. (1953) actually found

stimulation of phagocytosis in animals receiving cortisone.

In the present work, the effect of cortisone and ACTH in inhibiting the mesenchymal response within the liver during the early stages of repair was most noticeable. Some of these mesenchymal cells were probably derived from the blood but many seem to come from local sinusoidal cells. Studies with tritiated thymidine described in Part IV show evidence of new DNA formation in these cells during recovery from centrilobular zonal necrosis. Co-existent with this depression of phagocytosis was a transient inhibition of liver parenchymal regeneration. In spite of the continued administration of either hormone there was, eventually, disappearance of necrotic liver tissue and its replacement by viable parenchyma. There is an analogy with this and cutaneous wound healing; repair is delayed but is followed to completion in spite of continuous cortisone administration, as described in Part II of this thesis. Reference has already been made to the views of Hoffman et al. (1955) on the possibility of enzymatic autolysis of necrotic material and amitotic division of liver cells during cortisone administration. Others have also noted absence of mitosis in regenerating liver of animals receiving cortisone, e.g, Roberts et al. (1952); Einhorn et al.(1954). It appears, however, that a little corticosteroid is necessary for liver growth as regeneration is also inhibited by adrenalectomy (Friedgood et al., 1950).

The sensitivity of animals to exogenous cortisone seems to vary among different species. Thus, rats, mice, rabbits and ferrets are all very sensitive to its influence on weight loss and inhibition of antibody

production, whereas men, monkeys and guinea-pigs are relatively resistant (Shewell & Long, 1956). Upton and Coon (1951) noted the relative resistance of guinea-pigs to the wound-healing influence of both hormones in spite of the ready production of eosinopenia. This seems to be borne out to some extent by the results of my experiments on allyl formate damage in guinea-pigs. Cortisone had no very marked effect on the lesion although it was given in a dose sufficient to cause thymic atrophy, reduction in circulating eosinophils and even exopthalmos, an effect noted by Williams (1953).

The dosage of both hormones in all these experiments was adequate to induce the well-known effects of eosinopenia and thymic atrophy. It was considered undesirable to use the very large doses employed by some investigators owing to the possibility of liver damage by this means alone. Focal necrosis of mouse liver was attributed by Antopol (1950) to high cortisone dosage. It is possible that cortisone may activate some viral infection of liver to which mice and other laboratory animals are susceptible and so produce or aggravate liver necrosis. Mice and hamsters are rendered more susceptible to poliomyelitis virus in this way (Shwartzman, 1950), and to coxsackic virus (Kilbourne & Horsfall, 1951). Melcher et al. (1952) claimed ability to induce susceptibility in rats to human viral hepatitis by treatment with cortisone, but this has never been confirmed.

Gledhill and Andrewes (1951) described a mouse hepatitis virus causing extensive liver damage. The histological characteristics of

of this disease include cytoplasmic inclusions, an irregular or massive type of necrosis and giant cell formation during recovery, none of which were features of the carbon tetrachloride and tannic acid lesions under investigation. A different variety of murine hepatitis is described by Jordan and Mirick (1951), but may be a type of protozoan infection (Morris et al., 1956). This has as one of its features a diffuse infiltration of liver with mesenchymal cells reminiscent of leukaemia, but not seen in any of the animals used in the present investigation. Lackey et al. (1953) described a condition similar to and possibly identical with that noted by Jordan and Mirick which was aggravated by large doses of cortisone or ACTH. Jordan and Mirick (1955) confirmed this. Dick et al. (1956) described a third type of murine hepatitis, probably viral, which appeared during attempts to induce human viral hepatitis in mice by the aid of cortisone injections. This disease spreads inwards from the peritoneal surface of the liver and is associated with severe ascites; again these features are in no way typical of the lesions under investigation.

The possibility of stimulation of such infection by cortisone or ACTH cannot be excluded with certainty, but there is no positive evidence that this did happen. Liver necrosis was never seen in any Group C animals and the lesions found in Groups A and B were typical of chemically-induced liver damage.

Carbon tetrachloride and other toxins used in these experiments would be expected to act as stressing agents. Following their administration

there was depletion of lipid from the adrenal cortex but no gross reduction in circulating eosinophils. The effect of cortisone or ACTH would be to exaggerate grossly this physiological process. The adrenal glands of the experimental animals were examined in some cases only, but never showed any evidence of toxic effects such as those described by Phelps and Hu (1924) and Gardner et al. (1925), and attributed to carbon tetrachloride.

The liver may suffer some damage from conditions of abnormal stress not necessarily involving exposure to any hepatoxic agent. Selye (1951) described both fatty liver and focal necrosis of liver as manifestation of stress. Abnormal liver function tests are found in human patients with extensive burns and some fatal cases may show severe hepatic congestion and centrilobular zonal necrosis (James et al., 1951). In experimental shock there appears to be portal and sinusoidal constriction (Daniel & Pritchard, 1951), together with hepatic venous congestion which follows spasm of muscular tissue in the walls of hepatic veins. This throttling mechanism is particularly well developed in the dog (Elias & Popper, 1955) and in the racoon (Andrews, 1957). It would appear, however, that these mechanisms for the production of hepatic ischaemia and congestion are due to the local action of adrenaline or noradrenaline or to autonomic nerve impulses, and not to any direct specific action of corticosteroids (Andrews, 1957).

It is naturally of interest, in view of the results of these experiments, to note the reports on the use of cortisone and ACTH on

on cases of acute liver disease in man. Most of these reports refer to viral hepatitis, and it would appear that the euphoria and improved appetite which follow hormone therapy are beneficial in such cases. In many there is a fall in serum bilirubin with clinical improvement in the severity of jaundice and its concomitant itching (Thorn et al., 1950; Colbert et al., 1951; Rifkin et al., 1952; Eichman et al., 1953; Sborov et al., 1954; Siede & Klampe, 1960). What is perhaps of more importance is the claim that cortisone may help in recovery from hepatic coma (Ducci & Katz, 1952; Shane et al., 1955). These reports are difficult to assess because of the concomitant use of other forms of therapy, especially aureomycin which in itself can be beneficial in hepatic failure (Phear et al., 1956). In a later paper, however, Ducci and Katz (1955) claim some success with cortisone therapy in the absence of tetracycline administration.

There are, however, many reports in which this treatment is regarded with scepticism. The most important contra-indication is increased susceptibility to relapse especially on withdrawal of the hormone, and such a relapse can be more severe than the original illness. Evans et al. (1953) found this to be true of both ACTH and cortisone. They also had no success with these hormones in severe cases complicated by hepatic failure and coma. Other untoward side effects are a tendency to portal thrombosis (Bongiovanni & Eisenmenger, 1951) especially in cirrhotic patients, and aggravation of ascites and generalized oedema.

In a review article on the place of hormone therapy in liver disease,

Spellberg (1956) recommended that cortisone be tried in cases of hepatic failure and obviously there would be little to lose by doing so. Its use is also recommended in certain chronic diseases of liver expecially in those with an obstructive type of jaundice in the absence of extra-hepatic blockage, e.g., primary biliary cirrhosis and so-called cholangiolitic hepatitis complicating viral infections of liver. It would appear that cortisone acts in some way on the permeability of biliary canaliculi with increase in bilirubin secretion.

It would be unwise to look too closely for an analogy between the experimental results of the present work and these reports on naturally-occurring human liver disease. The latter do not include any condition quite similar to that of chemically-induced zonal necrosis in experimental animals, while there is probably a species difference in response to the administration of cortisone and ACTH. All that can be stated is that there is no experimental proof to justify this form of therapy in acute liver disease in man.

SUMMARY

1. The extent of centrilobular zonal necrosis of liver, induced in young adult mice and rats by carbon tetrachloride, was accentuated by the daily administration of cortisone.

2. Recovery was also delayed by cortisone but was gained eventually in spite of its continued administration.

3. Histological examination of the livers of these hormone-treated animals showed depression of mesenchymal cell proliferation with inhibition in the phagocytosis of necrotic tissue. Complete repair was attained in spite of this and in spite of the apparent inhibition of mitotic activity in surviving liver cells.

4. The minimum dose of carbon tetrachloride for the induction of hepatic necrosis in mice was reduced by cortisone.

5. The minimum dose of cortisone for the production of these effects in mice was 1 mg./100 g. body weight. Effectiveness did not increase with dosage.

6. Similar results were obtained in mice with long-acting ACTH in a dose of 0.25 mg. twice daily.

7. A high-casein diet did not reverse the deleterious influence of cortisone on liver necrosis in mice.

8. Less conclusive but essentially similar results were obtained in mice and rats suffering from centrilobular zonal necrosis of liver induced by tannic acid.

9. Periportal zonal necrosis of liver was induced in guinea-pigs by allyl formate. The variable severity of liver damage among individual

animals made it impossible to assess the effect of cortisone on this lesion.

10. Liver damage was produced in mice with phosphorus. Damage consisted of fatty and hydropic degeneration of liver cells and, in some cases, narrow zones of centrilobular necrosis. Some inhibition of mesenchymal cell activity during repair seemed to be effected by cortisone; otherwise its influence on the lesion could not be determined, because of variation in the response of individual mice to phosphorus.

11. Reasons for the deleterious influence of cortisone and ACTH on liver necrosis are discussed. Possible factors include sinusoidal constriction from excessive swelling of viable cells, induction of fatty change and inhibition of certain mitochondrial enzymes. No evidence was obtained in favour of a direct necrogenic effect of either hormone on the liver; neither was there evidence for the induction of murine hepatitis or for protein depletion as possible explanations of the results obtained.

PART IV

STUDIES ON LIVER REGENERATION

WITH TRITIATED THYMIDINE

STUDIES ON LIVER REGENERATION

WITH TRITIATED THYMIDINE

An important property of liver tissue in the adult animal is its remarkable ability to revert to a labile state and undergo rapid proliferation when a portion of its substance is destroyed. Liver regeneration has been the subject of much research and has included the investigation of a wide variety of topics, e.g., the importance of blood supply and nutrition; the role of bile duct epithelium as a source of liver parenchyma; the susceptibility or resistance of newly-formed tissue to further damage. Reviews on the subject of liver regeneration have appeared from time to time and I have referred to those by Podwyssoski (1886), v. Meister (1894), Muir (1908), Fishback (1929), Cameron (1952), Harkness (1957) and Leevy (1960). Two projects relating to this subject are included in this thesis. The inhibitory action of cortisone and ACTH on liver regeneration has been described in Part III. This part gives an account of some autoradiographic studies of mouse liver following the administration of thymidine labelled with tritium.

Thymidine is a pyrimidine forming part of the complex deoxyribonucleic acid molecule, but is not present in ribonucleic acid or in any other tissue component. Thymidine is quickly incorporated into DNA which is undergoing synthesis at the time of its administration. Consequently, the nuclear chromatin of newly-formed cells will contain the radio-active

label of H^3-thymidine and will be demonstrated on autoradiographs.

DNA synthesis is followed by mitosis within a few hours and it might be expected that this and subsequent episodes of cell division would soon lead to substantial dilution of the radio-activity of labelled cells. It has been found, however, that some labelled liver cells can retain a considerable intensity of radio-activity for many weeks after regeneration of the organ. An assessment of early regenerative activity can be obtained from mitotic count, but this method is probably much less accurate than the enumeration of labelled cells seen on autoradiographs. Schultze and Oehlert (1960) found that counts of labelled cells in a variety of growing rat tissues were ten times the mitotic counts, and in the present work I have found an even greater difference.

By this technique it has been planned to study the pattern of liver regeneration following chemically-induced zonal necrosis and partial hepatectomy, the persistence of labelled cells in the completely regenerated liver, and the activity of bile duct epithelium and sinusoidal cells during regeneration. Tritiated thymidine of satisfactory radio-activity has only recently become available commercially in this country, and these projects are at present incomplete. Nevertheless, the results obtained so far are regarded as having sufficient interest to justify their inclusion in this thesis.

EXPERIMENT 1

To ascertain the time of greatest uptake of H^3-thymidine by mouse liver following carbon tetrachloride injury

Methods. Twelve adult Swiss albino mice weighing 25 to 30 g. each received by oesophageal tube a single dose of 40% carbon tetrachloride in liquid paraffin, the amount of the mixture being 0.10 ml./10 g. body weight as in previous experiments. Six of these twelve animals received the liver poison at 9 p.m. and the rest at 9 a.m. on the following day. One animal from each of these two groups was killed at the following time intervals after poisoning: 12, 24, 36, 48, 60 and 72 hours.

Each mouse received one injection of tritiated thymidine 12 hours before killing, i.e., at 0, 12, 24, 36, 48 or 60 hours after carbon tetrachloride. Therefore, six animals received thymidine at 9 a.m. and the remainder at 9 p.m. This procedure was adopted to ensure that half the animals would receive thymidine shortly before a period of DNA synthesis, which is known to undergo diurnal fluctuation.

2 mc. H^3-thymidine was obtained from the Radiochemical Centre, U.K. Atomic Energy Authority, Amersham, about one week before use. In the interval it was stored at -20° C. It was dissolved in 20 ml. cold isotonic saline for intraperitoneal injection and each animal received 1 μc./g. body weight. When not in use the remainder of the solution was stored at -20° C.

Small blocks of liver were taken immediately after killing the

animals and fixed in 10% formalin in 70% ethanol. Samples of other tissues were also retained, including spleen, kidneys, adrenals and intestine. Autoradiographs were prepared from paraffin sections using the stripping film technique and also the immersion technique as recommended by Messier and Leblond (1957). The former was carried out as described in Part II for the demonstration of S^{35}-labelled compounds in liver sections. Fine grain film was used which had an optimum exposure time of 130 days, although faint images could be obtained after much shorter periods. For the immersion technique, Ilford G 5 nuclear emulsion was employed. This is supplied as a gel and was heated in a water bath at 50° C. before use. Following dipping, slides were placed on end to dry and then stored within sealed boxes in a refrigerator at 5° C. for periods of 100 to 130 days. Development and fixation were carried out as for stripping film preparation, using Kodak "DX 80" for four minutes followed by "Amfix" for a similar period.

From a comparison of the two methods, the images appeared slightly more intense by the immersion method. In theory the degree of resolution should be better as the emulsion layer is thinner compared to the thickness of the stripping film. Counterstaining is much simpler, requiring only a few minutes with 1% aqueous neutral red.

For comparison with the autoradiographs, other sections from the same blocks of tissue were stained by haematoxylin and eosin and by the Feulgen technique for chromatin, using light green as a counterstain.

Results. The first six animals of the series to be killed, i.e., at 12, 24 and 36 hours after carbon tetrachloride showed no images at all on the autoradiographs of liver. By contrast a positive result was obtained from all but one of the remainder, the majority of surviving liver parenchymal cells being labelled in cases given thymidine at 9 a.m. No images of cell nuclei were present in the 72 hour specimen which received thymidine at 9 p.m. As DNA synthesis is thought to be at a maximum during the night it was expected that all mice given thymidine at 9 a.m. would present relatively poor or negative results, but this was not so; the most intense uptake was obtained in the animal which received thymidine at 9 a.m. 36 hours after poisoning.

As this was merely a pilot experiment designed to find the optimum time for administration of labelled thymidine, no quantitative estimation of its uptake was undertaken. Epithelial cells lining the base of intestinal crypts were labelled in all animals including those which gave negative results with autoradiography of liver sections.

EXPERIMENT 2

To study the origin and fate of liver cells labelled with tritiated thymidine during regeneration following carbon tetrachloride necrosis

Methods. From the result of Experiment 1 it was decided to administer the same amount of thymidine at 9 a.m. and 36 hours after carbon tetrachloride. Twenty-two adult male Swiss albino mice weighing 25 to 30 g. were so treated and killed at intervals varying from ½ hour to 8 weeks

after thymidine. Details are given in Table XXXVI.

Autoradiographs of liver and certain other tissues were prepared as in Experiment 1. Adjacent sections from the same blocks of liver, stained by the Feulgen technique, were used to enumerate mitotic figures. These were counted in each of two sections from the same specimen and related to paper weights of the camera lucida drawings of the whole sections.

Enumeration of nuclear images in the autoradiographs was found to be difficult because of their large numbers in comparison to the mitotic counts and because some areas could be obscured by artefact. Consequently it was thought preferable to estimate total radio-activity of individual liver sections. Each 6 μ section was mounted separately on a $\frac{3}{4}$" circular glass coverslip and placed in a Tracerlab SC 16 windowless gas flow counter. The average of three separate counts was taken, each count being obtained from exposure to a flow of helium for 1,000 seconds and enumerated on a Panax automatic scaler.

Results. The uptake of labelled thymidine showed some variation throughout the experimental group and even between individuals killed at the same time after carbon tetrachloride intoxication. However, no specimen was completely devoid of images.

In the early stages of regeneration both hepatic parenchymal cells and sinusoidal cells may be labelled, not necessarily in a uniform manner. Figure 235 shows labelling confined mainly to sinusoidal cells when much necrotic tissue is still present. Examples of autoradiographs from two

mice 41 hours after carbon tetrachloride and 4 hours after H^3-thymidine are illustrated (Figs. 236 to 240); in these there is labelling of both types of cell. It is evident that the endothelial lining of centrilobular veins shows the same regenerative activity as adjacent sinusoidal cells (Fig. 239). In many portal tracts are small clusters of cells with positive images (Fig. 240). A few may represent new bile duct epithelium but the majority are presumably of mesenchymal cell origin. Considerable uptake of labelled thymidine was noted in the splenic white pulp of these animals (Fig. 241), and it seems possible that this tissue may be the source of some labelled macrophages within the liver.

These illustrations also show that new hepatic parenchymal cells are not derived from any narrow zone of the lobule, but have a fairly haphazard distribution. This is more evident when regeneration is well-advanced (Fig. 242). At this stage many cells which were originally labelled may have undergone further mitosis with dilution of their H^3-thymidine content (Fig. 243). Nevertheless, many cells with an intense uptake of radioactive material persist for longer periods and indeed for the duration of the experiment (Figs. 244 to 249). The uptake is particularly intense in the three-week specimen, almost every liver cell containing some radioactive material (Fig. 247). In this immersion specimen the specificity of the method is illustrated by the lack of uptake over the nucleoli of several cell nuclei, nucleoli being devoid of DNA. Figure 248 shows a scanty positive result while that of the eight-week specimen is fairly intense (Fig. 249); clearly a good many cells formed in the liver soon

after carbon tetrachloride intoxication can persist undamaged for at least this period of time.

Autoradiographs of adrenal cortex (Fig. 250) and renal cortex (Fig. 251) show an uptake of thymidine in excess of what might be expected in normal adult animals. It is possible that carbon tetrachloride causes some damage to the specialized cells of these organs which is followed by regenerative activity.

Details of mitotic counts and radio-activity of liver sections are given in Table XXXVI. It will be evident that there is no uniformity in the uptake of tritiated thymidine in relation to duration of recovery from liver necrosis, and that the mitotic counts give a very poor indication of the degree of DNA synthesis in the early stages of regeneration. The reasons for this lack of uniformity are not apparent. Possibly the normal rhythm of DNA synthesis is upset in the damaged liver and varies from animal to animal.

EXPERIMENT 3

To study the uptake of tritiated thymidine in mouse liver following partial hepatectomy

Methods. Partial hepatectomy was performed in nine adult male Swiss albino mice weighing 25 to 30 g. Under ether anaesthesia the abdominal wall was depilated and a transverse incision made in the skin of the anterior portion. The large left lobe of liver was delivered through a small incision in the muscle wall in the left hypochondrium. This

lobe was excised following the application of a catgut ligature to its base. The muscle wound was then extended across the epigastrium to expose the middle lobe, which was dealt with in the same way, but more carefully to avoid damage to stomach and upper intestine. In some cases it was found easier to remove the two main portions of the middle lobe separately. The abdominal wall was then closed in two layers with interrupted catgut sutures and sealed with celloidin. Glucose was added to the drinking water to make a 5% solution. The excised portions of liver from each animal were weighed and subjected to routine histological examination.

H^3-thymidine, 1 μc./g. body weight was given in solution by intraperitoneal injection 36 or 48 hours after the operation. Three of the nine mice were moribund on the following day and were killed. The remainder which appeared to be undergoing a normal recovery from the operation were killed at the following intervals after the administration of thymidine, viz., 1 day, 2 days, 4 days, 7 days, 14 days and 28 days.

Immediately after killing the entire remnant of liver was removed, weighed and portions prepared for autoradiography and for routine histological examination as in previous experiments. Radio-activity in individual sections was measured as in Experiment 3, but mitotic counts were not undertaken.

Results. Data relating to this experiment are given in Table XXXVII. Maximum uptake of the labelled thymidine seems to occur during the latter

half of the first week after the operation, but the numbers of animals employed are too small for this to be conclusive. In spite of this radio-activity and increasing weight, the autoradiograph of the liver six days after partial hepatectomy was negative, as were those from all animals killed at shorter intervals after the operation. Images were readily seen in the liver section of the remainder but were generally fainter than those obtained from many animals in Experiment 2. Possibly a greater quantity of hepatic tissue is renewed after partial hepatectomy compared to that which regenerates in a comparable period during recovery from zonal necrosis. If this is so there would be a greater dilution of the labelled thymidine in the regenerating liver in this experiment.

As in the previous experiment, the labelled parenchymal cells make no particular pattern in the regenerated tissue but have a haphazard distribution throughout the liver lobules (Fig. 252). Labelling of bile duct epithelium was more apparent in this experiment, even in the walls of well-formed interlobular ducts (Fig. 253).

DISCUSSION

It is known that certain changes take place in the composition of liver cells during regeneration. These include loss of glycogen and protein, various alterations in enzyme activity such as inhibition of glucose-6-phosphatase, and gain in lipid and nucleic acids. Nucleic

acid precursors are utilized readily at such a time. These may be administered with a radio-active isotope label which appears subsequently in the newly synthesized product. During the last few years tritium-labelled thymidine as a precursor of deoxyribonucleic acid has been used to study the growth of various plant and animal tissues in vivo and to study cells in culture. The high degree of resolution obtainable on autoradiographs of tissues containing tritium-labelled DNA together with the stability of this substance have made it possible to study various aspects of regeneration beyond the scope of routine histological methods.

Hughes et al. (1958) gave H^3-thymidine to normal young adult mice. The uptake was rapid, occurring within a few minutes of intravenous administration. Before it can enter tissue cells, thymidine is probably phosphorylated to thymolytic acid. This substance gradually deteriorates, the labelled material forming tritium hydroxide, unless it be used in the synthesis of DNA. Hughes et al. found that in the absence of DNA synthesis, degeneration was almost complete in an hour; it follows that only those cells in the process of DNA synthesis will be labelled when a single dose of tritiated thymidine is given. In normal subjects these will be the cells of labile tissues such as bone marrow and the lining of the alimentary canal. Only a very occasional liver cell shows evidence of DNA synthesis in the adult animal.

MacDonald and Mallory (1959) reported the result of a similar study in 40- to 60-day old rats. An adequate dose of tritiated thymidine for these animals was 0.7 μc./g. body weight. With proper storage the

material remained active for six months. In these young growing rats, 2 to 3 per cent of liver cells were labelled, including some sinusoidal cells; labelled cells had a haphazard distribution throughout the liver lobules. In adult rats which were also studied, hardly any liver cells were seen on autoradiographs. It seems that in recovery from liver injury, regeneration is merely an exaggerated form of the normal process in the growing animal. MacDonald and Mallory also noted the uptake of thymidine by a very few myocardial cells and neurones, so it is apparent that DNA can be replaced in cells which do not undergo mitotic activity. This may be possible also in other tissues including liver. Schultze and Oehlert (1960) gave H^3-thymidine to young rats and mice and compared their results with the results of counting mitotic figures. They found that a wave of DNA synthesis may persist for five hours and that it is followed by a spell of mitotic activity which lasts for only 20 to 30 minutes.

The specificity of the method is evident from the fact that only cell nuclei are labelled and that this can be removed completely by deoxyribonuclease (Amano et al., 1959). Taylor et al. (1957) in a study of growing bean roots have shown that one half of each labelled chromosome appears after mitosis in each daughter cell with an equal amount of new unlabelled chromatin. Labelled DNA may be dispersed throughout the subsequent cell progeny, but none is lost from the tissue as a whole. It seems unlikely that there is any turnover of DNA during cellular reproduction. Daoust et al. (1956) in a chemical study of P^{32}-labelled

DNA in regenerating rat liver also noted that the material was augmented, but none of that already synthesized was lost.

With H^3-thymidine, MacDonald et al. (1960) have demonstrated considerable regenerative activity in the livers of choline-deficient rats. Autoradiographs of liver biopsy material taken four hours after administration of thymidine were compared with those of autopsy material obtained ten days later. Counts of labelled nuclei fell by fifty per cent, but possibly due to dilution from further liver cell proliferation during this period.

The activity of enzymes involved in DNA synthesis has been studied by Schneider et al. (1960). They found that in rat liver after partial hepatectomy this activity was increased 27 times and that it can be abolished by ethionine. Bollum and Potter (1959) also noted that partial hepatectomy stimulated phosphorylation of thymidine and polymerization of deoxynucleotide, but the exact nature of the enzymes concerned is unknown.

Studies with other labelled nucleic acid precursors followed by quantitation of extracted DNA have confirmed that this is a substance which remains stable for a considerable period after synthesis. Hecht and Potter (1956) used C^{14}-orotic acid in this fashion; after 100 days most of the labelled RNA had disappeared, while almost all the labelled DNA remained. According to Swick et al. (1956) the life span of DNA in rat liver nuclei is at least 150 days. It is not surprising, therefore, that many labelled cells were found in mouse liver eight weeks

after DNA synthesis.

Wilson and Leduc (1948) found that the natural growth of hepatic tissue in young mice cannot be explained entirely on mitotic activity, especially in the later stage of development. Possibly there is a process of amitotic reproduction which may also become exaggerated during regeneration. This, together with the relative sensitivity of autoradiography and the relatively brief periods of mitotic activity, may account for the wide discrepancy between my autoradiographic results and mitotic counts.

The phenomenon of diurnal rhythm in DNA synthesis was studied by Barnum et al. (1958) using P^{32}-labelled precursors. In the young growing mouse liver cell DNA synthesis occurred in the early hours of the morning, followed by a wave of mitosis six to twelve hours later (conversely the synthesis of liver cell RNA and phospholipid occurred in the evening). From this it might be considered advantageous to give labelled thymidine shortly before the onset of synthesis. However, the very brief period during which intracellular thymolytic acid is available for DNA synthesis probably explains the frequent failure of my 9 p.m. injections. It has been noted that synthesis extends over several hours and was probably still in progress in my cases at 9 a.m. The optimum time for thymidine administration is probably somewhat earlier, about 6 a.m., and this is being used in subsequent work.

Higgins and Anderson (1931) in their detailed account of the technique and sequelae of partial hepatectomy in the rat, described the

first histological evidence of regeneration 24 hours after the operation. This became maximal at the third day and abated gradually over the next week or two, and occasionally during a longer period. Partial hepatectomy is usually carried out as described by these authors but I have found it easier in mice to remove the organ in parts through a transverse incision.

Regeneration after partial hepatectomy involves all tissue components of the liver (Fishback, 1929), but there may be a slight lag in formation of collagen in the walls of blood vessels (Harkness & Harkness, 1954). Blood flow increases during regeneration (Benacerraf et al., 1957), but the process may not depend on portal blood supply, contrary to what is generally supposed (Weinbren, 1955). Damaged liver may produce a humoral substance with the property of stimulating cell regeneration. Hurowitz and Studer (1960) found some evidence for this in parabiotic rats, one of which had a partial hepatectomy. Fisher and Fisher (1960) suggested a similar explanation for the enhanced growth of tumour implants in animals with liver damage. The various phenomena associated with liver regeneration, especially that induced by partial hepatectomy, have been reviewed recently by Harkness (1957) and Leevy (1960). Harkness stated that mitosis is most active in cells adjacent to portal tracts, but my autoradiographs have shown a less localized distribution of DNA synthesis.

Biochemical studies on regenerating liver after partial hepatectomy have shown rapid synthesis of RNA but very inconsistent formation of DNA during the four days immediately following the operation (Novikoff & Potter, 1948). Ficq (1959), using labelled amino-acids to study DNA

synthesis, found depressed metabolism in isolated liver cell nuclei obtained from rats after partial hepatectomy; this effect lasted for 48 hours. It may explain my observation of slight delay in uptake of H^3-thymidine compared with that in carbon tetrachloride treated mice. Once started the process of DNA synthesis after the operation probably proceeds as in the normal growing animal. Fresco and Bewdich (1960), using C^{14}-glycine and N^{13}-adenine as precursors of DNA found that it was stable for at least three months after partial hepatectomy.

Following injury by carbon tetrachloride, a proportion of regenerated liver cells are thought to possess abnormal nuclear characteristics, e.g., abnormal ploidy, relatively short life span and a tendency to neoplastic change (Hoffman et al., 1956). Livers with carbon tetrachloride-induced cirrhosis show more mitotic activity than normal after partial hepatectomy, according to Bartok et al. (1960). Grisham et al. (1960) noted an increase in volume of liver cell nuclei and nucleoli after regeneration. Recently regenerated cells also utilize excessive amounts of oxygen and are easier to grow under tissue culture conditions (Perkinson & Irving, 1956). The tritiated thymidine technique could be used to determine the life-span of these allegedly abnormal cells. At present I have undertaken to follow the intensity of thymidine uptake in individual mice by repeated liver biopsy. A comparison is being made between animals which received the labelled thymidine during normal development with those which were similarly treated during recovery from carbon tetrachloride necrosis.

Kelly et al. (1957) studied DNA synthesis in mouse liver after carbon tetrachloride, using P^{32}-di-sodium hydrogen phosphate as a label. A good uptake was obtained between 30 and 40 hours after poisoning. Leevy et al. (1959) have used H^3-thymidine for the same purpose but have also limited their observations to the optimum time of uptake. They too have found that this is maximal at 36 hours with a gradual falling off to zero at 120 hours after poisoning.

The results of the partial hepatectomy experiment are unsatisfactory because of the small number of animals used and because of an incorrect choice of time for the administration of thymidine. It is planned to repeat the experiment with larger doses of thymidine given at periods later than 36 hours after the operation.

SUMMARY

1. Tritiated thymidine in a single dose of 1 μc./g. body weight was given to 12 mice following carbon tetrachloride poisoning. Autoradiographs of liver obtained 12 hours later showed uptake of thymidine in the nuclei of newly-formed cells in those animals which received the tritiated compound not earlier than 36 hours after poisoning.

2. Some animals received H^3-thymidine at 9 a.m. and others at 9 p.m. More satisfactory results were obtained following administration in the morning.

3. Twenty-two mice received the same dose of H^3-thymidine in the morning, 36 hours after carbon tetrachloride, and were killed at intervals thereafter. Autoradiographs of liver showed that:

(a) labelling of liver parenchymal cells occurs in a haphazard manner throughout the lobules;

(b) many labelled cells persist in the liver for at least 8 weeks after regeneration;

(c) subsequent mitosis probably dilutes the labelled DNA in many liver cells;

(d) a high proportion of sinusoidal cells may be labelled at an early stage in recovery. This cellular activity extends to the endothelial lining of hepatic veins;

(e) the portal tracts contain many labelled cells, mesenchymal as well as bile duct epithelium.

4. Labelled cell nuclei were greatly in excess of, and showed a poor correlation with, the number of mitotic figures.

5. Radio-activity of individual sections was measured in a gas-flow counter. The results gave a good correlation with intensity of H^3-thymidine uptake as seen on autoradiographs, and a poor correlation with mitotic counts.

6. Partial hepatectomy was carried out on 9 mice, which received H^3-thymidine 36 hours later. Autoradiographs of remaining hepatic tissue showed:

(a) many negative results suggesting that regenerative activity is delayed beyond 36 hours in such cases;

(b) in positive cases, a less intense uptake compared with that observed in the liver during recovery from carbon tetrachloride zonal necrosis;

(c) as in the previous experients, a haphazard distribution of labelled cells throughout the liver lobules and evidence of bile duct regeneration.

PART V

STUDIES WITH S^{35}-LABELLED AMINO-ACIDS

IN EXPERIMENTAL LIVER DISEASE

STUDIES WITH S^{35}-LABELLED AMINO-ACIDS IN EXPERIMENTAL LIVER DISEASE

An account has been presented in Part II of this thesis of the uptake in mouse liver of radio-active sulphur given as S^{35}-sodium sulphate. Autoradiography showed that the element was incorporated into material in cirrhotic fibrous tissue and to a less extent in the walls of the larger blood vessels. Very little was detected in liver parenchyma although this includes various sulphur-containing organic compounds, e.g., methionine, cystine, glutathione and taurine.

A striking difference is apparent if the S^{35} isotope of sulphur is given incorporated in methionine or cystine. The normal liver parenchyma is then diffusely labelled in autoradiographs. This seemed to afford a method for studying the uptake or depletion of these amino-acids under various experimental conditions, and particularly in liver diseases induced by the administration of substances antagonistic to methionine.

Two methionine antagonists have been studied, bromobenzene and ethionine. The former eliminates sulph-hydryl groups from the tissues through the production of mercapturic acid, which is excreted by the kidneys. This contitioned depletion of methionine is given as the explanation for the centrilobular zonal necrosis of liver induced experimentally by bromobenzene (Koch-Weser et al., 1953). These authors noted that the hepatic damage could be aggravated by fasting, and reduced

in severity by feeding methionine or cystine. If this is so, it is a different mechanism from that attributed to carbon tetrachloride and many other liver poisons which produce the same morphological lesion. However, I have observed in this work certain structural differences between the lesion induced by bromobenzene and that induced by carbon tetrachloride. Cornatzer and Gallo (1956) have, in addition, observed absence of fatty change in the liver damaged by bromobenzene. In the present work a comparison is made between the effects of these two hepatotoxins on liver methionine and cystine as demonstrated by autoradiography.

The second methionine antagonist, ethionine, causes liver and pancreatic degeneration, and occasionally neoplastic change, if given in small amounts over a prolonged period. Larger doses given within 12 hours are followed, especially in female rats, by severe fatty liver. This regresses gradually after 48 hours unless the dose is sufficient to cause death within a few days (Farber et al., 1951). Like bromobenzene poisoning, the condition is alleviated by feeding methionine, but choline or other lipotropic factors are ineffective (Farber et al., 1950). Unlike bromobenzene, ethionine does not deplete the liver of methionine; in fact the level of this amino-acid may rise concurrently with the development of fatty change (Levy et al., 1955). There is probably no depletion of sulph-hydryl groups but inhibition in the utilization of methionine. Consequently, results different from those of the bromobenzene experiments were expected and obtained from autoradiographic

studies of the liver in ethionine poisoning.

MATERIALS AND METHODS

Bromobenzene is a dense viscid liquid miscible with oil but not with water. It was necessary to dilute it to twenty times its volume with liquid paraffin in order to obtain accurate measurement of the small non-fatal doses required for mice. The oily mixture was administered as a single dose by oesophageal tube from a tuberculin syringe.

Preliminary studies with amounts comparable to those used by Kooh-Weser et al. (1953) for the production of liver damage in rats had little or no effect on mice. Larger doses were rapidly fatal but caused only hepatic congestion. It was then discovered that a short preliminary period of starvation prior to poisoning had the desired effect of producing non-fatal liver necrosis. Consequently this has been practised in all experiments with bromobenzene, and also in control experiments and experiments with carbon tetrachloride; food, but not water, was withdrawn at 6 p.m. and replaced the next morning at 9 a.m. after the administration of liver poison. All animals were young male Swiss albino mice weighing 20 to 30 g. and fed on Rowett Institute Animal Diet No. 86.

Dl-ethionine is a white, slightly waxy powder with an odour similar to that of methionine or casein. It dissolves slowly in warm water containing a trace of weak alkali, such as sodium carbonate. A 1% solution was prepared in this way.

Young adult Wistar strain female albino rats were given four doses of this solution by intraperitoneal injection, equally spaced over a period of 9 hours. As with bromobenzene, this was preceded by a 15-hour period of starvation. Some animals killed early in the experiment received fewer than four injections.

Preliminary attempts were made to produce fatty liver in mice with ethionine, given both orally and by intraperitoneal injection. Unexpectedly, the most striking results were obtained in male animals, but being very variable it was decided to confine this investigation to female rats.

Although the S^{35} isotope has a half-life of 80 days it was planned to use the labelled amino-acids without delay after delivery, to gain the maximum effect and comparable results between different experimental groups. All the experiments with bromobenzene or carbon tetrachloride (3 to 12) were carried out at the same time, and the autoradiographs prepared with the least possible delay. The ethionine experiments (14 to 17) were performed at a different time with different samples of comparable radio-activity.

S^{35}-methionine and S^{35}-cystine were obtained from the Radiochemical Centre, U.K. Atomic Energy Authority, Amersham. The methionine was dissolved readily in a few drops of isotonic saline and transferred to a universal container, where its volume was made up to 10 ml. with saline. Cystine was treated in a similar way with 10 ml. N/10 hydrochloric acid, later neutralized with a few drops of N/2 sodium

hydroxide. Both solutions were stored at 5° C. Dosage was 100 µc. for rats and varied between 25 µc. and 50 µc. for mice.

The preparation of autoradiographs from liver sections was similar to that described in other experiments (Parts II and IV). 10% formalin in 70% ethanol afforded adequate fixation without apparent loss of radioactive material. In view of this possibility, a few additional specimens in Experiments 3 and 4 were prepared by freeze-drying. Small blocks of fresh liver were frozen by immersion in iso-pentane surrounded by liquid nitrogen. These were then transferred quickly to an Edwards tissue dryer and maintained *in vacuo* for 48 hours surrounded by a sludge of solid carbon dioxide and alcohol. The tissues were then embedded in paraffin *in vacuo* and sections cut in the usual way, pressed on to slides without floating on water and fixed with formalin vapour. Autoradiographs were prepared from these. As the result did not differ in any way from the preparations which had been fixed in formol-alcohol, the method was considered to be unnecessary. Both coarse and fine grain stripping films were used, the exposure times being 85 days. Coarse grain preparations without counterstain were generally preferable for this work.

Radioactivity was measured in material from all animals in Experiments 3 to 12 and 14 to 17. As described previously this was carried out in a gas-flow counter using 6 µ sections mounted on coverslips. Attempts were made to weigh these sections on coverslips, but this was beyond the limits of accuracy of the balances available to me. The counts were

related, therefore, to paper weights of camera lucida drawings of the sections and then to the weights of the livers from which the sections had been obtained. This method is probably inaccurate as it fails to account for possible variations in the thickness of sections counted.

Adjacent sections from the same blocks of liver were stained by conventional methods. In the ethionine experiments, frozen sections were obtained from liver fixed in formol-calcium and embedded in gelatine. These were stained with Sudan IV and haematoxylin. An additional block of liver from each animal was fixed in 1% trichloracetic acid in 80% alcohol and passed to paraffin wax. Sections from these blocks were treated by the method of Barrnett and Seligman (1952) for the demonstration of sulph-hydryl groups in tissues.

EXPERIMENT 1

To determine the minimum effective dose of bromobenzene in mice

Twelve mice were used in this experiment. Bromobenzene was given diluted in liquid paraffin as described and after preliminary starvation. The amounts of pure bromobenzene varied between 0.025 and 0.25 ml./ 100 g. body weight. Details are given in Table XXXVIII. Mice which survived the initial illness were killed one or two days after poisoning and their livers examined by conventional histological procedures.

Results

These are also given in Table XXXVIII. It is evident that a suitable dose following 15 hours' starvation is 0.05 ml./100 g. body weight. This was adhered to throughout subsequent experiments. A description of the liver lesion is given below (Experiment 2).

EXPERIMENT 2

To study the effects of a suitable non-lethal dose of bromobenzene on mouse liver

Twelve mice each received a single dose of bromobenzene, 0.05 ml./100 g. body weight, and were killed at intervals ranging from 1 hour to 4 days, as shown in Table XXXIX. Blocks of liver from each animal were fixed in Rossman's picric acid-formalin-alcohol and passed to paraffin. Sections were stained by haematoxylin and eosin, by the periodic acid-Schiff technique and by the long Giemsa method.

Results

Compared with carbon tetrachloride, the onset of illness in these mice is more rapid, being apparent within an hour or two. At this stage the liver is not necrotic but appears congested. Necrosis may be seen in 12-hour specimens, especially on the under surface of the liver. Necrotic areas are irregular in shape and may be pale or haemorrhagic. At 24 hours they are better defined but of variable extent (Figs. 254 & 255). The remainder of the liver presents a mottled appearance with exaggerated

lobular markings as in carbon tetrachloride poisoning.

Histologically there is little of note during the first few hours of the illness. An early change is seen in the basophilic material of the liver cell cytoplasm; this becomes diffuse and apparently augmented within the cells of the centrilobular zones (Fig. 256). At the same time glycogen is lost from these areas (Fig. 257).

Apart from massive necrosis which varied in extent from case to case, some degree of zonal necrosis is always present in the remainder of the liver. Usually this is centrilobular in distribution but occasionally it is mid-zonal. There may be very striking mid-zonal hydropic degeneration (Fig. 258) but unlike the carbon tetrachloride lesion it may proceed to mid-zonal necrosis with escape of the centrilobular cells (Fig. 259). In other cases, or in other parts of the same liver, the zonal lesions may be extensive (Fig. 260) or may merge with areas of massive necrosis (Figs. 261 to 263).

Histological evidence of repair is evident by the second or third day and may also have a mid-zonal distribution (Fig. 264). Massive necrosis may persist after healing of the zonal lesion (Fig. 265).

EXPERIMENT 3

To study the uptake of S^{35}-methionine in normal mice

Five mice were starved for 15 hours and then given 1.5 μc. S^{35}-methionine/g. body weight by intraperitoneal injection. Subsequently they were fed and killed at intervals, viz., 1, 5, 24, 48 and 96 hours

after the injection. Autoradiographs were prepared and radio-activity assessed as described.

Results

A heavy uptake of S^{35}-methionine was evident throughout the liver parenchyma in all specimens (Figs. 266 to 267). There was no detectable loss of radio-activity over the 4-day period. These values, together with those for Experiments 4, 5, 6 and 7 are shown in Table XL. The histochemical demonstration of sulph-hydryl groups in the liver sections by Barrnett and Seligman's method was unsatisfactory. Only faintly positive results were obtained compared with control sections of skin and panoreas. Although carried out in all experiments it was obviously very inferior to autoradiography and warrants no further reference.

EXPERIMENT 4

To study the influence of bromobenzene on S^{35}-methionine already incorporated in the liver

Ten mice received 1.5 μc. S^{35}-methionine/g. body weight at 9 a.m. Food was withdrawn at 6 p.m. and replaced the next morning at 9 a.m. when bromobenzene was given as in Experiment 1. Animals were killed in pairs at 1, 4, 8, 12 and 24 hours after poisoning and their livers examined as described above.

Results

What was expected was a depletion of methionine in the mid-zonal or

centrilobular areas where necrosis would subsequently develop if the animals had survived. At 1 hour there is no detectable loss of radioactivity. At 4 hours it is just possible to make out a slight accentuation in the centrilobular zone (Figs. 268 & 269) and this is quite definite at 8 hours and 12 hours (Fig. 270 to 273), being the opposite of the expected result. Labelled methionine persists even in the necrotic tissue whether of zonal or massive distribution (Figs. 274 & 275) although it may have been depleted in the surviving viable tissue.

EXPERIMENT 5

To observe the uptake of S^{35}-methionine by mouse liver already damaged by bromobenzene

The procedure of the last experiment was reversed, each animal receiving bromobenzene before S^{35}-methionine. The interval between the two administrations varied from 1 hour to 48 hours, while the animals were killed at times which varied from 7 hours to 48 hours after S^{35}-methionine. Details are shown in Table XL.

Results

As expected, the uptake of the labelled amino-acid was inhibited in the zones of liver damage, and so the autoradiographs show a reverse picture to that obtained from Experiment 4 (Figs. 276 to 285). When the administration of S^{35}-methionine is delayed for 48 hours after bromobenzene there is a diffuse uptake which is rather less dense than normal (Figs. 286 and 287).

EXPERIMENT 6

To study the influence of carbon tetrachloride on S^{35}-methionine already incorporated in the liver

The procedure was similar to that described in Experiment 4, with carbon tetrachloride substituted for bromobenzene. Each animal received 0.2 ml. 40% carbon tetrachloride in liquid paraffin by oesophageal tube.

Results

No loss of radio-activity is apparent 1 hour after poisoning (Figs. 288 & 289). Thereafter, as in the bromobenzene experiment, a faint decrease is detected in the periportal zones of all specimens examined at 4, 8 and 12 hours after poisoning; one of these is illustrated (Figs. 290 & 291). This feature is not apparent in the 24-hour specimens (Figs. 292 & 293).

EXPERIMENT 7

To observe the uptake of S^{35}-methionine by mouse liver already damaged by carbon tetrachloride

The plan of this experiment was similar to that of Experiment 5, with carbon tetrachloride substituted for bromobenzene.

Results

These are also similar to those of Experiment 5, the uptake of labelled amino-acid being inhibited in the areas of liver damage. This is illustrated in Figures 294 to 297.

EXPERIMENT 8

To study the uptake of S^{35}-cystine by normal mouse liver

This was similar to Experiment 3 with S^{35}-cystine substituted for S^{35}-methionine. The specific activity of this sample being slightly less than the methionine preparation, each animal received 1.25 μc./g. body weight. This dose was also used in subsequent experiments.

Results

A diffuse uptake is evident throughout the liver parenchyma. It is particularly intense in blood cells within vessels. Compared with S^{35}-methionine it seems less concentrated but is nevertheless well marked (Figs. 298 & 299). This was so in all specimens in the experiment.

The results of the measurement of radio-activity in these sections and in all others comprising Experiments 9, 10, 11 and 12 are shown in Table XLI.

EXPERIMENT 9

To study the influence of bromobenzene on S^{35}-cystine already incorporated in the liver

This experiment was carried out as for Experiment 4 with cystine substituted for methionine.

Results

These also are similar to Experiment 4, i.e., no change at one hour (Figs. 300 & 301), and thereafter a relatively less intense uptake in the

periportal zones (Figs. 302 to 309).

EXPERIMENT 10

To study the uptake of S^{35}-cystine by mouse liver already damaged by bromobenzene

The procedure was similar to that in Experiment 5 with cystine substituted for methionine.

Results

Results similar to those of Experiment 5 were to be expected, but the opposite was obtained in every case, i.e., there appears to be a concentration of cystine in the tissues surrounding the centrilobular veins, even when these have undergone necrosis (Figs. 308 to 311). In one 48-hour specimen this feature is not obvious, but it is at least apparent that radio-activity is no less marked in the necrotic compared with the viable zones (Figs. 312 & 313).

EXPERIMENT 11

To study the effect of carbon tetrachloride on S^{35}-cystine already incorporated in the liver

Similar to Experiment 6, with cystine substituted for methionine.

Results

These were again similar to the bromobenzene experiment, cystine being concentrated in the centrilobular zones, even when these have become necrotic (Figs. 314 to 323).

EXPERIMENT 12

To study the uptake of S^{35}-cystine by mouse liver already damaged by carbon tetrachloride

Similar to Experiment 7, with cystine substituted for methionine.

Results

These are dissimilar to Experiment 7 but similar to Experiment 10, i.e., the amino-acid seems to gain access to and have an affinity for the zones of liver damage (Figs. 324 to 327).

All results obtained from these various autoradiographic examinations are summarized in Table XLII.

EXPERIMENT 13

To study the effect of a suitable non-lethal dose of dl-ethionine on rat liver

Eight young female rats, 90 to 105 g. in weight, were chosen for this experiment. After a preliminary 15-hour spell of starvation, each received 0.05 g. dl-ethionine as already described. For those animals not killed early in the experiment, this dose was repeated three times during 9 hours. Rats were killed at intervals after the first injection ranging from 1 hour to 5 days. Details are given in Table XLIII.

Results

These are given also in Table XLIII. Slight fatty change was evident in the 12-hour specimens, and was well-marked on the following day. It appeared to reach a maximum on the second day (Fig. 328),

after which it gradually subsided.

Histological examination confirms the presence of fatty liver which seems to be most severe in the periportal zones (Fig. 329), although fat stains show a fairly diffuse distribution (Fig. 330). Fat forms small discrete droplets in liver cell cytoplasm and fat cysts are not seen. There seems to be a general reduction in cytoplasmic basophilic material. Necrosis is not detected.

EXPERIMENT 14

To study the uptake of S^{35}-methionine by normal rat liver

Five young female rats, each approximately 100 g. in weight, were starved for 15 hours and given 100 μc. S^{35}-methionine. They were killed at intervals, viz., 1, 5, 24, 48 and 96 hours later. Autoradiographs of liver were prepared as described and the intensity of radio-activity in liver sections counted.

Results

Specimens from all animals showed an intense uptake of methionine throughout the liver. The results of quantitation of radio-activity in this experiment and in Experiments 15, 16 and 17 are shown in Table XLIV.

EXPERIMENT 15

To study the influence of ethionine on S^{35}- methionine already incorporated in rat liver

Seven young female rats were used, weighing 95 to 120 g. Each

received 100 μc. S^{35}-methionine at 9 a.m. Food was withdrawn at 6 p.m. and restored at 9 a.m. the following day after the first dose of ethionine. The animals were killed at intervals varying from 2 hours to 72 hours after this. Details are given in Table XLIV.

Results

Each autoradiograph shows an intense uptake of S^{35}-methionine which is in no way affected by ethionine (Fig. 331).

EXPERIMENT 16

To study the uptake of S^{35}-cystine by normal rat liver

Similar to Experiment 14, with cystine substituted for methionine.

Results

A uniform uptake of cystine is apparent in all animals killed at intervals varying from 1 hour to 96 hours after its administration.

EXPERIMENT 17

To study the influence of ethionine on S^{35}-cystine already incorporated in rat liver

Similar to Experiment 15, with cystine substituted for methionine.

Results

All specimens show a good uptake of cystine throughout the liver. In a few there appears to be a slight reduction in the periportal zones, but probably due to attenuation of liver cytoplasm with fat (Fig. 332).

DISCUSSION

Methionine and cystine are unique in being the only important dietary sources of organic sulphur for protein synthesis. Studies with S^{35}-sulphate have shown that very little of the isotope becomes incorporated into tissue protein; in fowls, for example, there is a ready uptake into taurine and an unknown compound in feathers, but only 1.5 per cent reaches either cystine or methionine (Machlin & Pearson, 1956).

Autoradiography has shown a rapid uptake of S^{35}-methionine into tissue proteins. Compared with liver, it is more intense in intestinal mucosa, pancreas, spleen, kidneys and blood plasma, and less intense in testes, heart, brain and striped muscle (Friedberg et al., 1948). Feeding labelled methionine has given similar results (Tarver & Morse, 1948). These authors found that about 30 per cent of the isotope was excreted in the urine and faeces within two weeks.

The sulph-hydryl compound, methionine, is an essential amino-acid, the lack of which causes retardation of growth and a negative nitrogen balance which cannot be corrected by cystine (Womack et al., 1937). In the liver, methionine may undergo a process of demethylation and reduction to form homocysteine. Cysteine is derived from homocysteine through the action of serine. This in turn is a source of both cystine and glutathione, the latter being compounded from cysteine, glycine and glutamic acid. Glutathione is essential for the reduction and re-activation of various oxidizing enzymes such as co-enzyme A and lipoic

acid conjugates, and for maintaining tissue ascorbic acid in a reduced form. It also plays a part in DNA synthesis and has an important role in special circumstances, such as the elimination of heavy metals and the reduction of toxic effects induced by ionizing radiation (Barrow, 1953).

The importance of methionine as a source of choline has been discussed. This is brought about by transfer of methyl groups to ethanolamine, a product of serine. Methylation of other substances is also dependent on methionine, e.g., creatine and nicotinamide (Marquez et al., 1955). Vitamin B_{12} plays an essential rôle in these reactions. In its absence, there is defective choline synthesis with the development of fatty liver (Bennett et al., 1955). There is also failure in reduction and re-activation of co-enzyme A (Ling & Chow, 1951).

The disulphide compound cystine is not an essential amino-acid as it can be synthesized in vivo from methionine. Moreover, it cannot replace methionine completely in the diet. With cysteine it forms an important oxidation-reduction system. Like methionine, it is a source of taurine and taurocholic acid. Both amino-acids, but cystine in particular, seem to be important in wound healing (Williamson & Fromm, 1955). The muscle level of methionine falls during healing, while its hepatic level rises, the liver being the site of its conversion to cystine (Fromm & Nordlick, 1956). Methionine also stimulates growth of fibroblasts in vitro (Kieler, 1954) and, possibly, the regeneration of liver after partial hepatectomy (Hopsu & Härkönen, 1960).

The sulphur-containing intermediary compounds in the metabolism of

methionine and cystine, viz., homocysteine, cysteine and homocystine, have a very transient existence, and it seems unlikely that they would be represented to any extent in the liver autoradiographs. Glutathione also has a brief existence in the liver, although it can persist for prolonged periods in the brain (Douglas & Mortensen, 1956). The hepatic level is reduced by starvation or low protein diet (Barford & Eden, 1956), which was a condition of my experiments. Moreover, glutathione is protein-free and water-soluble and may not be retained in histological preparations.

Awapara (1957) gave S^{35}-cystine to rats in order to study the metabolism of taurine. Although used in bile salt synthesis, the amount of this substance in liver was found to be low. If S^{35}-taurine is injected into rats, much is taken up by tissues other than the liver.

From these considerations, it is assumed that autoradiographs of the liver after the administration of S^{35}-cystine show mostly cystine incorporated in the hepatic tissues. After S^{35}-methionine, a positive image may represent methionine or possibly cystine. The different behaviour of the two amino-acids administered after the induction of liver damage indicates that much of the methionine could not have changed to cystine, at least before its incorporation within the liver.

Albino rats are generally used in studies with ethionine. Both acute and chronic illnesses are described in these animals, depending on dosage and duration of administration. Only the acute effect of giving relatively large amounts within a short period has been investigated in

this work. Such doses if continued for a further day or two would probably be fatal, especially in female animals. Testosterone has a protective effect and castration increases the susceptibility of males (Farber et al., 1951). Liver necrosis is not evident but Koch-Weser et al. (1951) have demonstrated retention of bilirubin and bromsulphthalein.

The chronic illness induced by small repeated doses is associated with degenerative changes in salivary glands, gastric mucosa, liver and pancreas. Degeneration of renal tubular epithelium and testicular tissue may also occur (Kaufman et al., 1956). In pregnant rats there is a tendency to abortion and congenital defects in the foetus (Lee et al., 1956). An increase in iron absorption is recorded (Kinney et al., 1955). In the liver there is parenchymal degeneration, proliferation of bile ductular epithelium, fibrosis and round-cell infiltration. The presence of large numbers of small oval cells is a characteristic feature (Ungar & Goldberg, 1959); electron microscopy has shown these to be of cholangiolar origin (Schaffner & Popper, 1961). The relationship of this process to hepatic fibrosis has been discussed in Part II. Carcinoma of liver may supervene in these animals (Popper et al., 1953; Farber, 1956).

It has been suggested that these degenerative changes in the liver are secondary to the digestive disturbance caused by the pancreatic lesions (Loring & Hartley, 1955), but I have noticed no obvious structural change in that organ by routine histological examination during the acute illness. The therapeutic value of methionine indicates that ethionine may act by causing a disturbance in protein metabolism rather than by a

direct toxic action on liver cells (Stein et al., 1960). It seems probable that the acute effect involves the inhibition of the transmethylating property of methionine. In addition to this, ethionine decreases butyrate and pyruvate oxidation with inhibition of ketogenesis (Recant, 1956). There is, however, no evidence of any effect on Krebs cycle enzymes to explain diminished ketogenesis (Fischer & Recant, 1956).

Sidransky and Farber (1956) gave various labelled amino-acids, including S^{35}-methionine, to rats followed by dl-ethionine, but confined their observations to the pancreas. The methionine content of pancreas was increased by ethionine and there appeared to be no inhibition in protein synthesis, at least during the first 24 hours. However, one of these authors has found inhibition of liver protein synthesis within a five-hour period (Farber, 1955).

The results of the present work have demonstrated that ethionine does not appear to deplete the liver of sulph-hydryl or di-sulphide groups while effecting the development of fatty change. It confirmed that methionine need not be lost from the liver although its rôle in transmethylation is impaired.

The relationship of protein metabolism to liver necrosis as distinct from fatty change is one of considerable complexity. In spite of early reports by Opie and Alford (1914; 1915) which claimed a beneficial effect from high carbohydrate diet in liver necrosis and a deleterious effect from high fat and high protein diets, a high intake of protein has come to be regarded as therapeutically valuable, e.g., Messinger and Hawkins

(1940). Reduction in plasma protein by plasmaphoresis increases the susceptibility of dogs to chloroform poisoning (Miller & Whipple, 1940). With regard to individual amino-acids, a special claim has been made for methionine as a valuable agent in reducing the severity of liver damage, both in experimental animals and in man with naturally-occurring disease, e.g., Beattie et al. (1944); Beattie and Marshall (1944); Eddy (1945). Subsequently, some doubt has been cast on its therapeutic value, especially in viral hepatitis (Popper et al. 1948); indeed, excessive administration can be dangerous by precipitating hepatic coma (Phear et al., 1956).

Excessive doses of methionine might be of value in the elimination of some specific toxic substance such as bromobenzene or molybdenum (van Reen & Williams, 1956) but these are exceptional cases. Brunschwig et al. (1945) believed that sulph-hydryl groups which protected against the severity of chloroform liver damage in dogs need not be administered as methionine, since sodium thioglycollate was equally effective. Eger (1951; 1956) found methionine prophylactic in preventing liver damage from allyl alcohol but of no value if given after the development of liver necrosis; cystine however was beneficial before or after the development of the lesion.

Conversely, it is claimed that liver necrosis may be produced by diets deficient in protein, and especially sulphur-containing amino-acids. A diet completely free from protein causes haemorrhagic zonal necrosis of the liver in young rats, preventable by the addition of casein in

excess of 2 per cent but aggravated by cystine (Wachstein & Schwarz, 1960). Weichselbaum (1935) produced liver necrosis in rats with a cystine-deficient diet. On the other hand a diet with a gross disproportion of amino-acids, and particularly with an excess of cystine, is also necrogenic. Earle and Kendall (1942) produced a periportal type of zonal necrosis by feeding a diet, 5 to 10 per cent of which was cystine, or with 15 per cent as cysteic acid. Extra methionine, however, appears to be harmless and can protect against the damaging effect of excessive cystine. The complete absence of choline may also be beneficial in these circumstances (Handler & Follis, 1950).

The explanation of all these phenomena is uncertain. György et al. (1946) suggested that the beneficial effect of methionine lay in its lipotropic property, as fatty livers are unduly susceptible to various noxious factors. However, in many cases of dietary necrosis, fatty change is not a feature, and it is not apparent in bromobenzene poisoning. Dioguardi and Secchi (1960) have found a decrease in rat liver cell mitochondria and in cytochrome oxidase activity in protein deficiency which are reversed by the return to a normal diet.

Recent work on dietary liver necrosis has thrown considerable doubt on the importance of amino-acid deficiency in this condition. The condition, as first described by Weichselbaum (1935) may be produced in young weanling rats fed on a low protein, high fat diet, with yeast as the source of protein. Liver necrosis appears after a latent period of about six weeks; it is massive and often haemorrhagic in type, affecting

the left side of liver more than the right. It may lead to fatal hypoglycaemia or be followed by healing with gross scarring of the liver. The addition of methionine or cystine to the diet during the latent period can be prophylactic.

Himsworth and Glynn (1944) suggested that the uneven distribution of necrosis could be explained by a dual blood supply in the portal vein, the right side of liver receiving superior mesenteric venous blood and the left receiving splenic and colonic blood with a greater deficiency of amino-acids. György (1951) suggested that the left side might receive a bacterial toxin from the colon, as sterilization of the bowel with aureomycin helped to reduce the severity of the lesion. However, Daniel and Prichard (1951) have studied portal venous blood flow within the liver by serial angiography, using thorotrast, and have found no evidence for a dual blood supply. Cole et al. (1956) injected radio-active rose bengal into the various sources of portal blood in dogs and found that its localization in the liver did not depend on the site of inoculation.

It was noted that success in the production of liver necrosis depended on the type of yeast employed in the diet of experimental animals. György and Rose (1950) had positive results with Distillers Company yeast obtained from this country, but had no success with various American yeasts. Some brands of yeast appear to contain a factor ("factor 3") which protects the liver against the development of necrosis. The second of the three factors with a similar property (the first being sulphur-containing amino-acids) is vitamin E. This has been investigated

during the six-week latent period between the start of the experiment and the development of necrosis. During this time the liver is grossly normal but there is impairment of certain functions such as bromsulphthalein excretion (Linder et al., 1953). Fine granules with a tendency to calcify appear in the liver cell cytoplasm (Fite, 1954) and are probably of mitochondrial origin (Piccardo & Schwarz, 1958). Chernick et al. (1955a) demonstrated inhibition in oxygen consumption by liver slices taken during this period, a phenomenon which seems to be peculiar to this type of liver lesion. In addition, the inhibition of ketogenesis, lipogenesis and oxidation of acetate can be demonstrated. The metabolic upset can be reversed if vitamin E is added to the diet before liver slices are obtained (Rosecan et al., 1955), but there is also a depression of co-enzyme A activity which remains (Chernick et al., 1955b). As vitamin E prevents this type of liver necrosis, it seems that the pathogenesis of the lesion may be independent of the levels of co-enzyme A in the liver.

Vitamin E may have some protective influence on liver necrosis in general (Schwarz, 1944) and it is claimed to reduce the intensity of carbon tetrachloride necrosis (Hove & Hardin, 1951). In the instance of latent dietary liver necrosis it has no action on liver slices *in vitro* (Mertz & Schwarz, 1959), but is very effective *in vivo*, especially if given intravenously and in the form of α-tocopherol. Apparently, this is converted to some active metabolite after injection (Rodnan et al., 1956; Green et al., 1960).

Factor 3 has been isolated from certain brands of yeast and also from casein digests and pork kidney (Schwarz, 1960). It is an organic water-soluble compound smelling of garlic as it contains selenium; its activity, in part at least, depends on the presence of this element. It appears to behave as a vitamin and only trace amounts of the pure material, viz., 0.1 µg., can protect rats against fatal liver necrosis. It is possible that grossly excessive amounts would be toxic as are large doses of inorganic selenium compounds (Moxon & Rhian, 1943; Sellers et al., 1950; Halverson & Monty, 1960), but this is a feature common to many vitamins and other dietary constituents. Schwarz has found that traces of factor 3 can be recovered from samples of methionine and cystine. Once freed from this contamination they lose their ability to protect rats against the development of liver necrosis.

Deficiency offactor 3 seems to have different effects on different species of animals, and liver necrosis may be peculiar to small rodents. Even in mice as compared to rats, the liver lesion is often less severe than foci of damage elsewhere, especially in the myocardium (Schwarz, 1958). The same deficiency may inhibit growth in chicks, and may be the cause of "stiff-lamb disease" and "white muscle disease" in calves (Muth et al., 1958; Proctor et al., 1958 - quoted by Schwarz, 1960). Rabbits fed similar diets develop cirrhosis of liver without gross liver necrosis (Rich & Hamilton, 1940).

Factor 3 seems to act separately from vitamin E as one cannot replace the other; however, for the successful production of dietary liver

necrosis in rats, both should be absent from the diet. The presence of methionine or cystine is immaterial provided they are not contaminated with factor 3. It is suggested (Schwarz, 1958) that both activated vitamin E and factor 3 are catalysts in alternative pathways for the transfer of electrons and, when both are absent, there is a breakdown of oxidation functions, especially oxidative phosphorylation. This develops during the latent phase of the disease. What precipitates the acute necrosis is unknown, but the vascular reaction suggests blockage of the efferent blood flow in large areas of the liver.

During this latent period in dietary liver necrosis, individual members of the Krebs cycle, used as substrates, may increase the oxygen consumption of liver slices but fail to halt the development of respiratory decline (Chernick et al., 1955a). The metabolic lesion is probably a defect in the regeneration of co-enzymes such as pyridine nucleotides, flavin nucleotides or adenosine triphosphate. The addition of ATP or dipyridine-nucleotide halts the respiratory decline of these liver slices. While co-enzyme A may be depleted it is not related to this respiratory failure and is unaffected by vitamin E or factor 3. The addition of methionine to raise the co-enzyme A level in the liver could not be expected, therefore, to influence the basic lesion.

While these results relate to what may really be a peculiar disease of small rodents, they throw some doubt on the value of sulphur-containing amino-acids in liver disease generally. Methionine-deficient fatty liver seems to be an established entity and so the effects of substances

such as ethionine and methionine ethylester (Fehér et al., 1960) which block the transmethylation process can be understood readily. The same cannot be said for those toxins which are alleged to cause liver necrosis through the destruction of sulphur-containing amino-acids, such as bromobenzene and trinitrotoluene (Himsworth & Glynn, 1942). My experimental results show that the distribution of zonal necrosis is not determined directly by the loss of organic sulphur from the same zones; in fact, if there is any loss, it is from the areas of liver which survive the toxic effect of bromobenzene. It could be argued that bromobenzene may be like carbon tetrachloride, causing damage to periportal cells, with necrosis as a secondary phenomenon due to ischaemia. However, I have been unable to demonstrate loss of organically-bound sulphur before the onset of centrilobular degenerative lesions, such as loss of glycogen. Accordingly, it may be that bromobenzene has a direct toxic action on liver cells which is independent of any loss of methionine or cystine. If this is so, there is probably still some difference between its action and that of carbon tetrachloride, since there are morphological differences between the two types of lesion. Schwarz (1958) has suggested that in the massive necrosis of vitamin E and factor 3 deficiency there is a block in the efferent part of the hepatic vascular system which determines the extent of the lesion. Possibly, bromobenzene may be responsible for a similar vascular reaction.

Bromobenzene forms mercapturic acid by its action on cysteine (Koch-Weser et al., 1953). As already noted this is a metabolic product

of methionine, while cystine can be reversibly converted to cysteine by a reduction-oxidation procedure. It was to be expected, therefore, that autoradiography would show a depletion of both amino-acids by bromobenzene. The fact that carbon tetrachloride had the same effect on cystine and possibly also on methionine throws some doubt on the specificity of these results.

This work has shown also that while methionine is incorporated into viable liver parenchyma, cystine is taken up preferentially by degenerating and necrotic material. The explanation for this, from what is known of protein synthesis is not apparent.

A defect in this work has been the failure to obtain consistent results from quantitation of radio-active material in tissue sections. It is difficult to weigh de-paraffinized sections as these must be mounted on cover-slips which takes them beyond the range of micro-balances. It is my intention to repeat this work using larger quantities of desiccated liver which could be assessed in a scintillation counter. Alternatively, a chemical estimation could be made of sulphur-containing amino-acids from portions of liver and related to autoradiographs from other parts of the same organ. It would also be of interest to investigate further the apparent lack of specificity in the results by the use of additional liver poisons.

SUMMARY

1. Liver necrosis was produced in mice by the oral administration of bromobenzene following a short period of starvation. The lesion consisted of centrilobular or mid-zonal necrosis and a variable degree of massive necrosis.

2. Autoradiographs were prepared from liver sections following the intraperitoneal injection of S^{35}-methionine and S^{35}-cystine. The maximum sites of liver damage induced by bromobenzene were not depleted of either amino-acid, but there appeared to be some loss from periportal hepatic tissues which remained viable.

3. As a similar result was obtained with carbon tetrachloride, it may be that those changes detected by autoradiography are not specific for bromobenzene poisoning, although this substance is alleged to damage the liver by the removal of sulph-hydryl compounds.

4. S^{35}-methionine given to mice already suffering from bromobenzene or carbon tetrachloride poisoning was taken up mainly by viable periportal hepatic tissue. By contrast, S^{35}-cystine was taken up preferentially by damaged or necrotic tissue.

5. Fatty liver was produced in young female rats by the administration of dl-ethionine over a twelve-hour period, following a brief spell of starvation. Autoradiography showed no loss of S^{35}-methionine or S^{35}-cystine in such cases.

6. Quantitation of radio-activity in liver sections, using a gas-flow counter, failed to show a correlation with the autoradiographic findings. This was probably due to errors in the assessment of the masses of tissue so analysed.

7. These results are discussed in relation to current knowledge concerning the role of sulphur-containing amino-acids in liver disease. It would appear that:

(a) ethionine may interfere with the methyl-donating role of methionine and cause fatty liver without depleting the tissues of organic sulphur-containing compounds;

(b) liver necrosis in bromobenzene poisoning may depend on factors other than depletion of sulphur-containing amino-acids. Recent work on dietary liver necrosis has shown that the absence of these amino-acids may be unimportant in the production of liver damage.

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VARIOUS STUDIES ON LIVER DISEASE

THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF MEDICINE

OF THE UNIVERSITY OF GLASGOW

BY

ROBERT S. PATRICK, M.B.,Ch.B., D.P.H.

VOLUME II

SEPTEMBER 1961

VOLUME II

TABLE OF CONTENTS

P A R T I

HEPATIC LESIONS IN INFANTILE GASTRO-ENTERITIS

TABLES I to XXII

FIGURES 1 to 90

TABLE I

Clinical Diagnosis in 177 Cases investigated by Liver Biopsy

Diagnosis		Number
1. Gastro-enteritis alone		64
2. Gastro-enteritis and Parenteral Infection		
(a) Upper respiratory infection excluding otitis	24	
(b) Otitis media	3	
(c) Pneumonia	14	
(d) Measles	1	
(e) Pyuria	4	
(f) Skin sepsis	3	
(g) Conjunctivitis	1	
(a) + (b)	9	
(a) + (e)	1	
(a) + (f)	1	
(a) + (g)	1	
(a) + (f) + (g)	2	
(b) + (c)	1	
(b) + (f)	1	
(b) + (c) + (f) + (g)	1	67
3. Infections other than Gastro-enteritis		
(a) Upper respiratory infection excluding otitis	5	
(b) Otitis media	4	
(c) Pneumonia	5	
(d) Measles	1	
(e) Pyuria	1	
(f) Whooping-cough	1	
(g) Primary tuberculosis (Pulmonary)	3	
(h) Congenital syphilis	1	
(i) Dysentery	2	
(j) Meningitis	1	
(k) Staphylococcal septicaemia	2	
(l) Viral hepatitis	1	
(a) + (e)	1	
(a) + (i)	1	
(a) + (b) + (e)	1	
(b) + (i)	1	
(c) + (i)	1	32
4. Non-infective conditions		
(a) Wrong feeding	9	
(b) Hirschprung's disease	1	
(c) No abnormality detected	4	14

Total number of cases - 177.

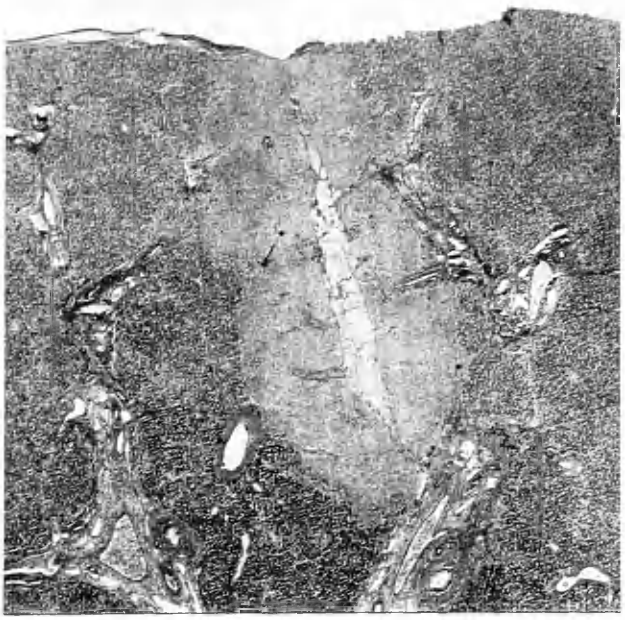


Fig. 1. Site in liver of biopsy performed 7 days before death.
H. & E. X 7½.

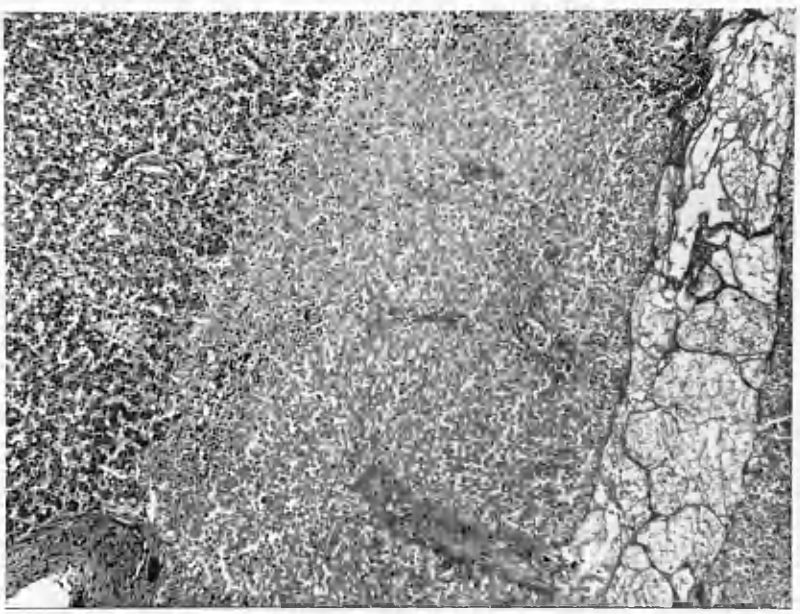


Fig. 2. Site of liver biopsy. The needle track is filled with fibrin and surrounded by necrotic liver tissue.
H. & E. X 60.

TABLE II

Numbers of Liver Examinations in 177 Infants

Biopsies in one patient	Autopsy	Number of patients	Total examinations
6	-	1	6 X 1 = 6
5	-	4	5 X 4 = 20
4	+	1	5 X 1 = 5
4	-	16	4 X 16 = 64
3	+	1	4 X 1 = 4
3	-	27	3 X 27 = 81
2	+	8	3 X 8 = 24
2	-	41	2 X 41 = 82
1	+	16	2 X 16 = 32
1	-	62	1 X 62 = 62
		177	380

Total liver biopsies 354

Total autopsies 26

Fig. 3. Liver biopsy specimen. Fat ++++
Osmic Acid X 32.

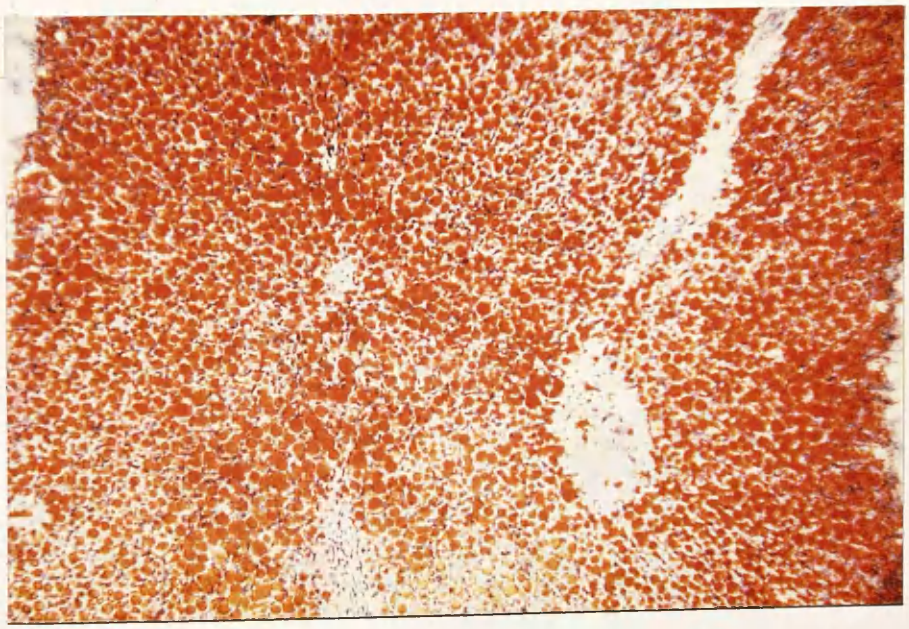


Fig. 4. Liver biopsy specimen. Fat ++++
Sudan IV & Haematoxylin X 90.

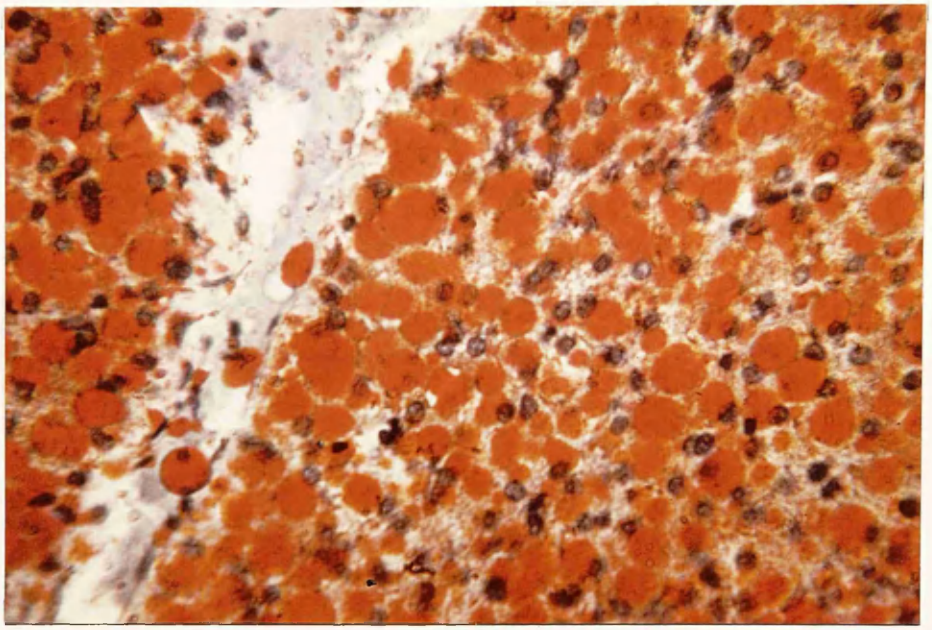


Fig. 5. Liver biopsy specimen. Fat ++++
Sudan IV & Haematoxylin X 310.

Fig. 6. Liver biopsy specimen.
Fat ++++. H. & E. X 124.

Fig. 7. Fat +++
Osmic Acid X 37.

Fig. 8. Fat +++
Osmic Acid X 41.

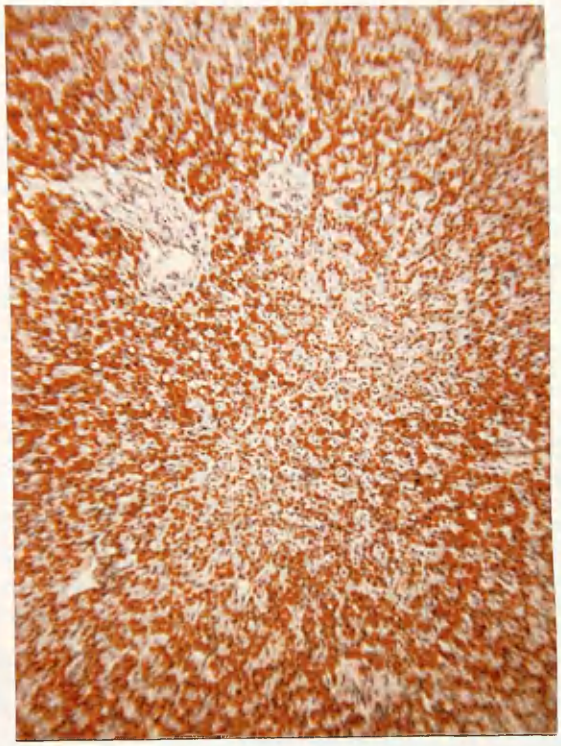


Fig. 9. Fat +++
Sudan IV & Haematoxylin X 90.

Fig. 10. Fat +++
H. & E. X 94.

Fig. 11. Fat ++
Osmic Acid & Safranin X 42.

Fig. 12. Fat ++
Osmic Acid X 70.

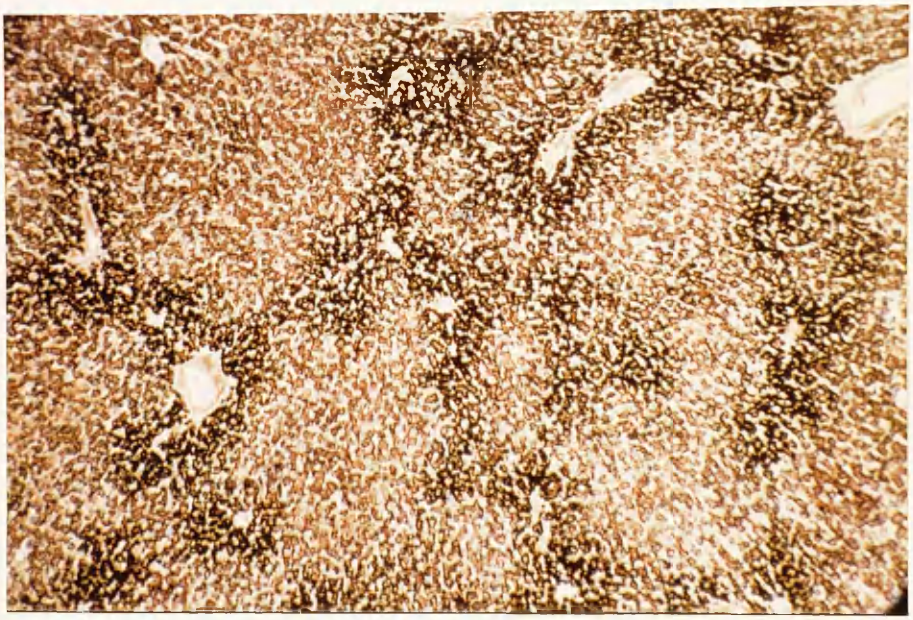


Fig. 13. Liver autopsy specimen. Fat ++
Osmic Acid & Safranin X 65.

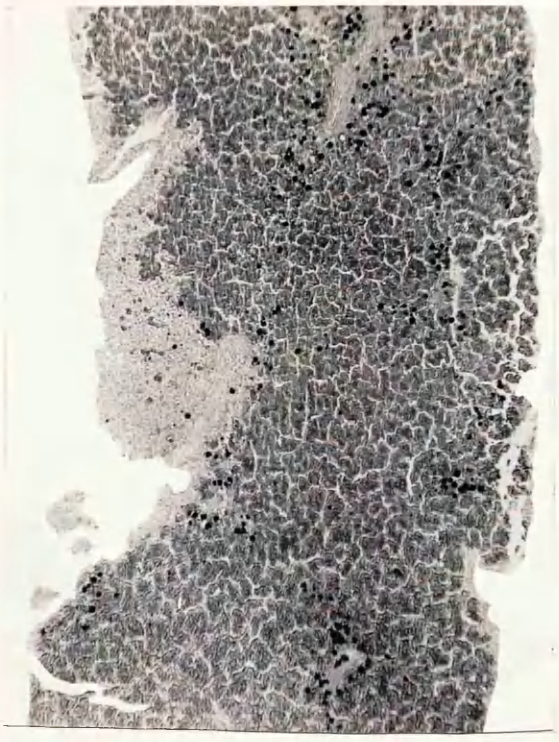


Fig. 14. Fat +
Osmic Acid & Safranin X 42.

Fig. 15. Fat +
Osmic Acid & Safranin X 64

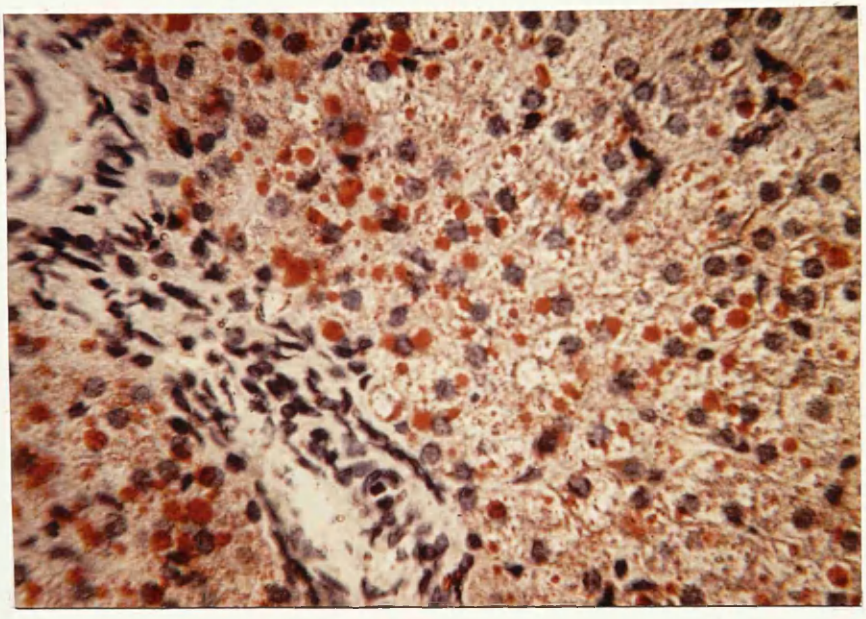


Fig. 16. Fat +
Sudan IV & Haematoxylin X 310.

Fig. 17. Fat +
H. & E. X 95.

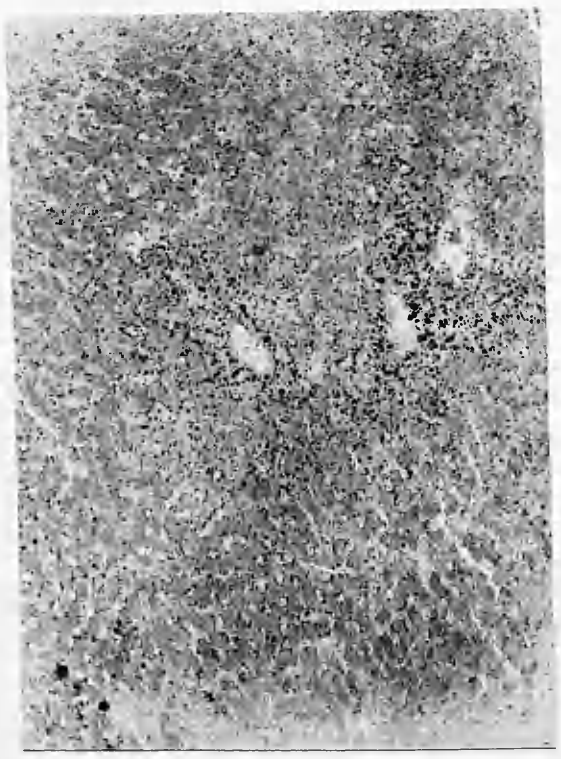


Fig. 18. Fat $\pm$
Osmic Acid & Safranin X 77.

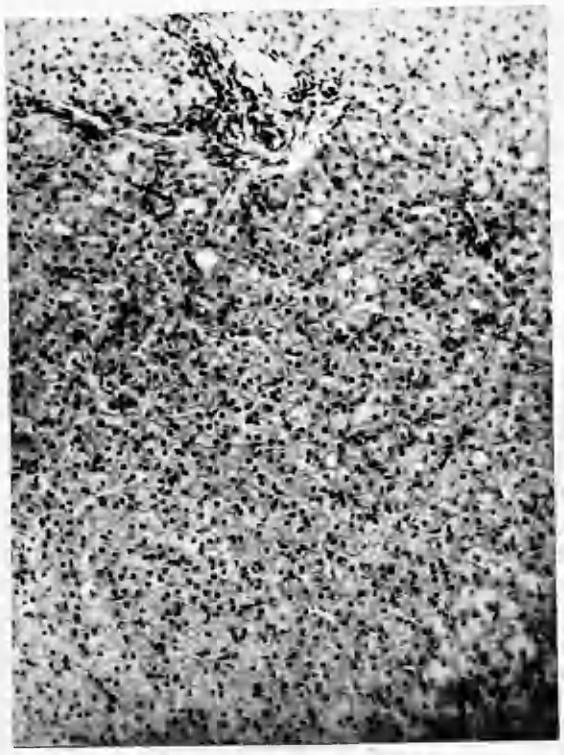


Fig. 19. Fat $\pm$
H. & E. X 144.

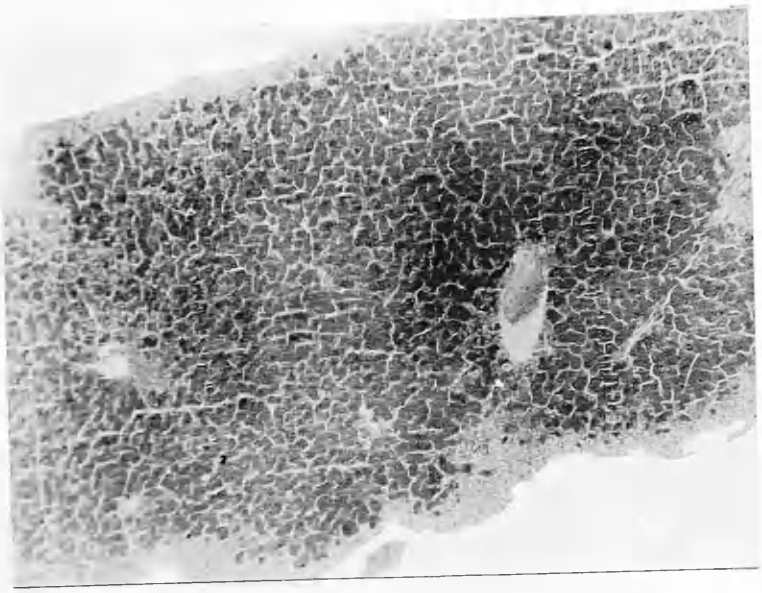


Fig. 20. Fat O
Osmic Acid & Safranin X 55.

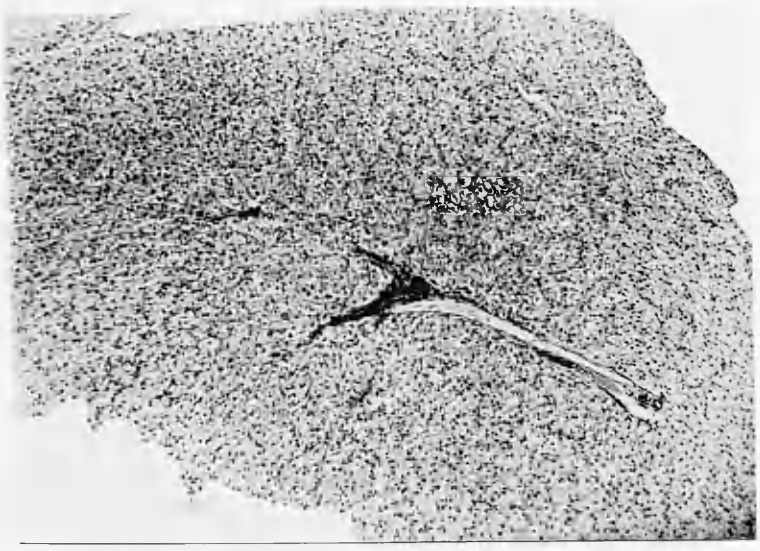


Fig. 21. Fat O
H. & E. X 85.

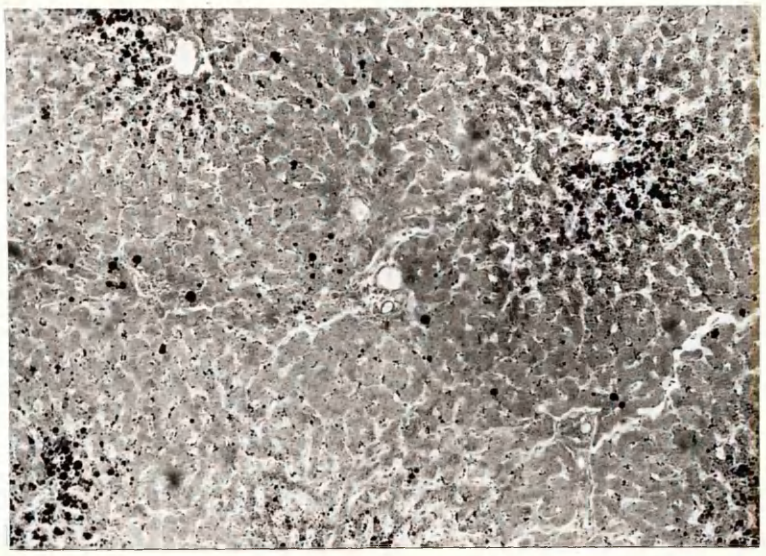


Fig. 22. Liver biopsy specimen. Fat mainly centrilobular in distribution.
Osmic Acid & Safranin X 67.

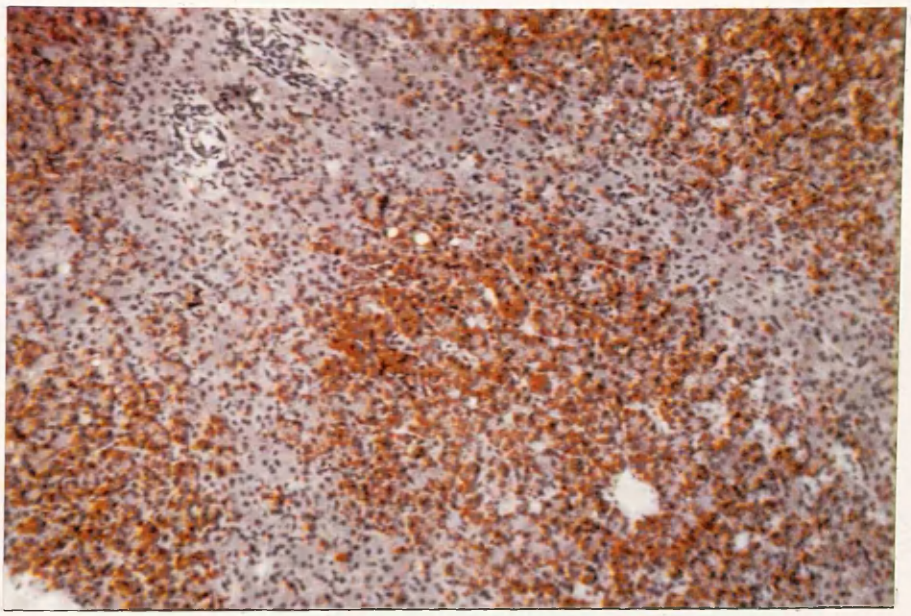


Fig. 23. Liver biopsy specimen. Centrilobular distribution of fat.
Sudan IV & Haematoxylin X 125.

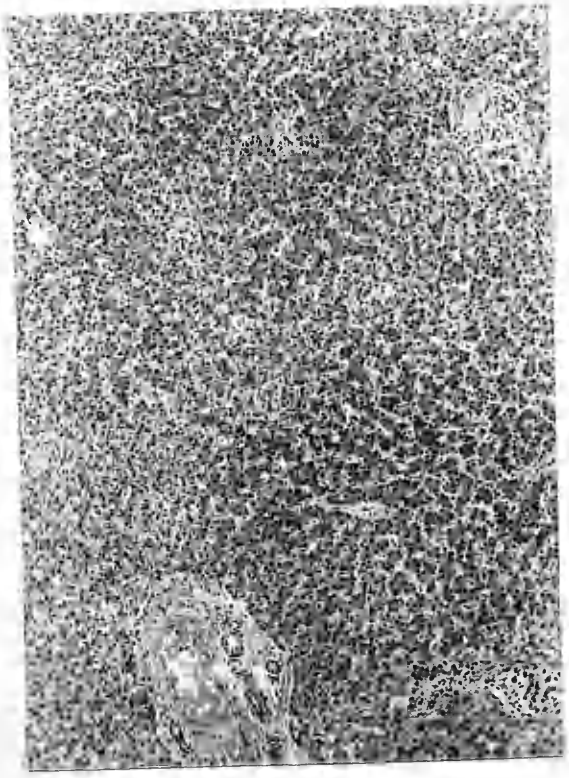


Fig. 24. Liver autopsy specimen. H. & E. X 77.

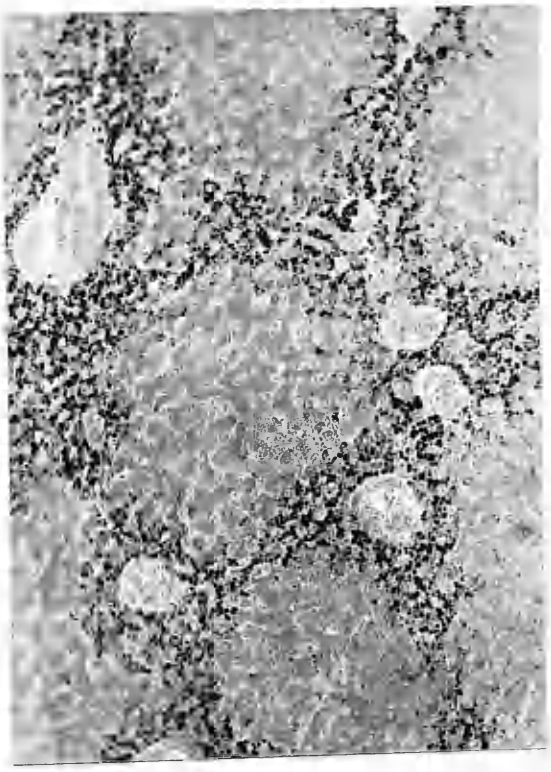


Fig. 25. The same specimen as shown in Figure 24 stained by osmic acid. X 77.

Fig. 26. Liver autopsy specimen. H. & E. X 102.

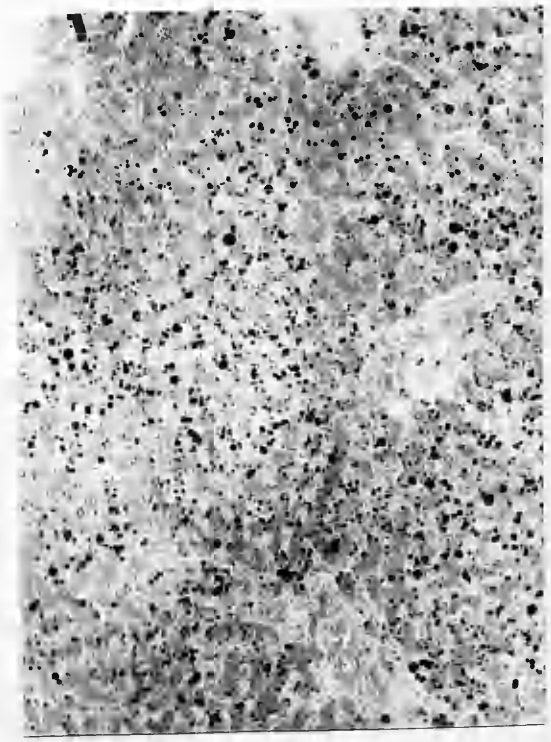


Fig. 27. The same specimen as shown in Figure 26 stained by osmic acid. X 102.

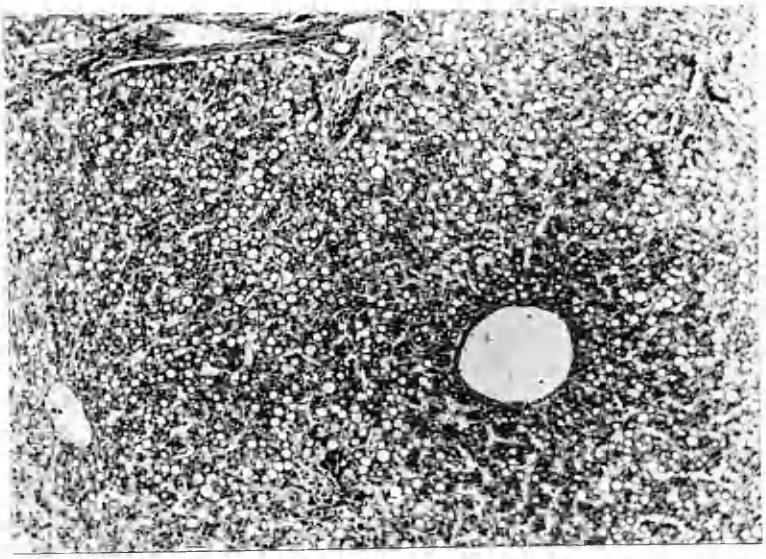


Fig. 28. Section from right lobe of liver.
H. & E. X 70.

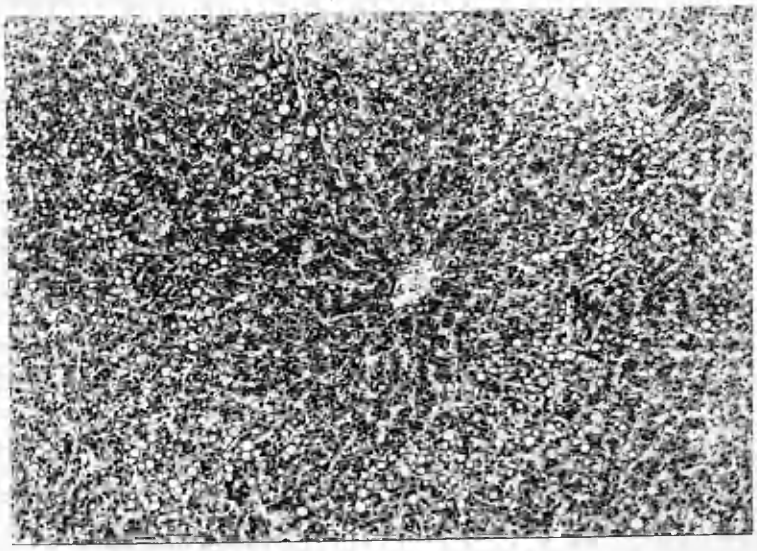


Fig. 29. Section from left lobe of the same liver.
H. & E. X 70.

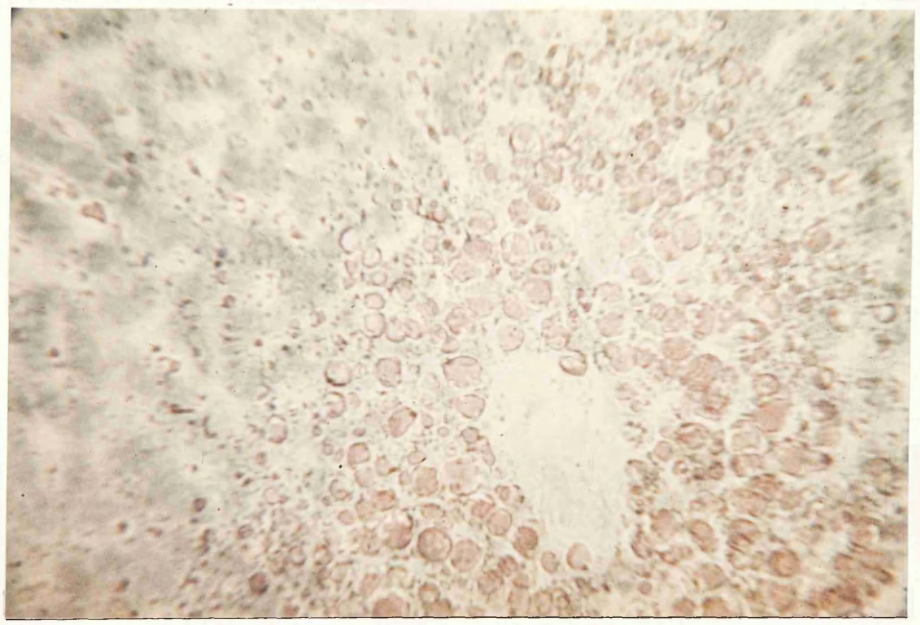


Fig. 30. Liver biopsy specimen.
Nile blue sulphate X 175.

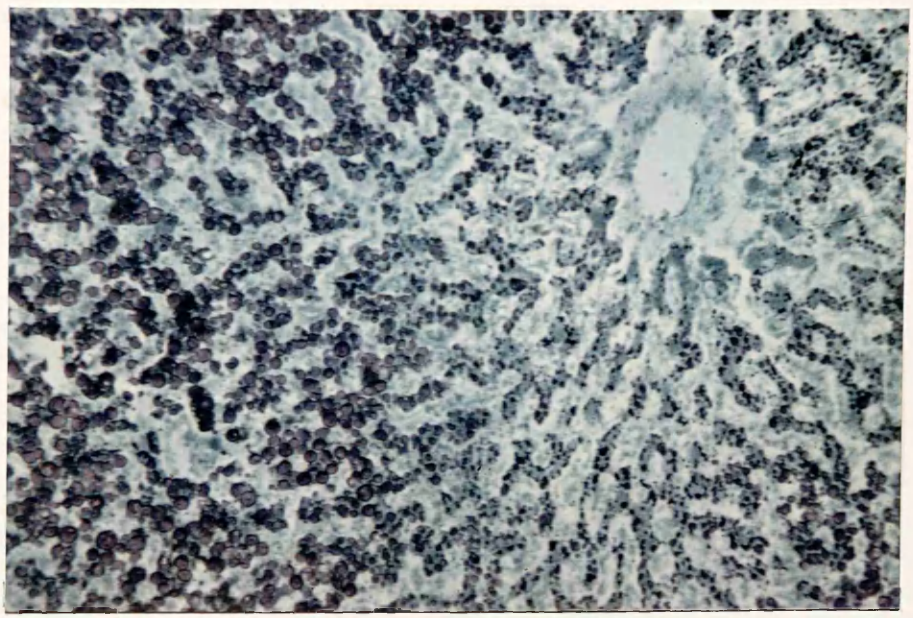


Fig. 31. Liver autopsy specimen.
Nile blue sulphate X 95.

TABLE III

Numbers of Cases classified to show the Relationship of Fatty Change in Liver to Age (at time of first Biopsy) and to Sex

Age (weeks)	Numbers of patients												All cases		
	Liver fat:	++++		+++		++		+		±		0			
	Sex:	M	F	M	F	M	F	M	F	M	F	M	F	M	F	Total
0-5						0	1	0	1	0	2	3	0	3	4	7
6-10						2	3	1	2	2	1	5	0	10	6	16
11-15				0	6	4	4	6	4	4	0	0	1	14	15	29
16-20		1	0	1	2	6	3	2	0	0	2	0	1	10	8	18
21-25		1	1	9	8	5	2	1	2	0	1	1	0	17	14	31
26-30		2	1	4	7	5	1	0	4	1	1	4	0	16	14	30
31-35				1	0	0	2	2	0			2	0	5	2	7
36-40		0	2	2	1	4	0			1	2	1	1	8	6	14
41-45				1	1			0	2			1	1	2	4	6
46-50						1	1					4	0	5	1	6
51-55				0	2			1	0	1	1			2	3	5
56-60												1	0	1	0	1
61-65						2	1	1	0					3	1	4
66-70												0	1	0	1	1
71+										2	0			2	0	2
Total		4	4	18	27	29	18	14	15	11	10	22	5	98	79	177

TABLE IV

Numbers of Cases classified to show the Relationship between Fatty Change in Liver and Clinical Diagnosis (at time of first Biopsy)

Diagnosis	Numbers of patients						Total patients
	Fat ++++	Fat +++	Fat ++	Fat +	Fat ±	Fat 0	
Gastro-enteritis alone	6	15	23	9	7	4	64
Gastro-enteritis with parenteral infection	2	25	14	12	10	4	67
Infections other than gastro-enteritis	0	5	8	4	9	6	32
Non-infective cases	0	0	0	4	2	8	14
Total	8	45	45	29	28	22	177

TABLE V

Fatty Change in Liver related to Clinical Diagnosis (at Time of all Examinations)

Diagnosis	Liver fat:	++++					+++					++					+					±					0						Total examinations
	Examination:	1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th	6th	
Gastro-enteritis alone		6	2	-	-	-	15	14	3	1	-	23	13	9	1	1	9	7	6	9	-	7	4	9	2	1	4	8	6	4	2	1	167
Gastro-enteritis with par-enteral infection		2	5	1	-	-	25	12	6	1	1	14	16	3	-	1	12	14	5	2	-	10	11	4	-	-	4	1	1	-	-	-	151
Infections other than gastro-enteritis		-	-	-	-	-	5	1	1	-	-	8	1	1	1	-	4	4	1	-	-	9	-	-	-	-	6	2	1	-	-	-	45
Non-infective cases		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	-	-	-	-	2	1	1	1	-	8	-	-	-	-	-	17
Total		8	7	1	-	-	45	27	10	2	1	45	30	13	2	2	29	25	12	11	-	28	16	14	3	1	22	11	8	4	2	1	380

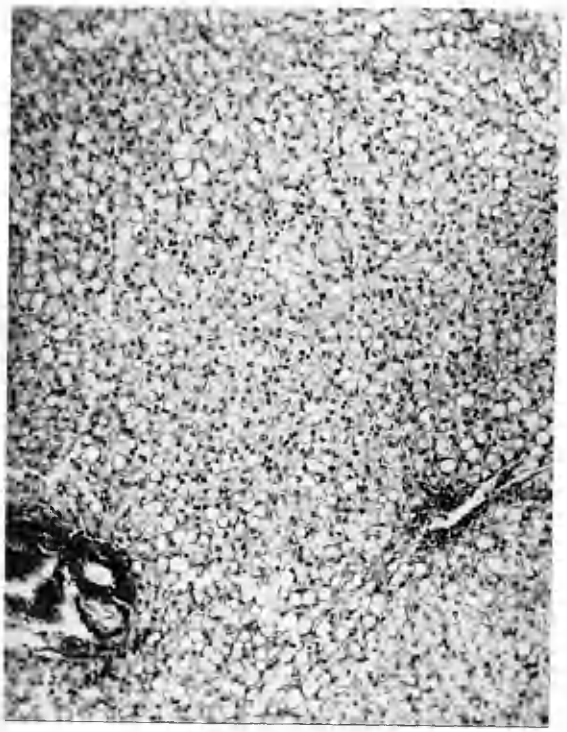


Fig. 32. Case No. 18. First liver biopsy. 6th day of illness. Fat +++
V.G. X 118.

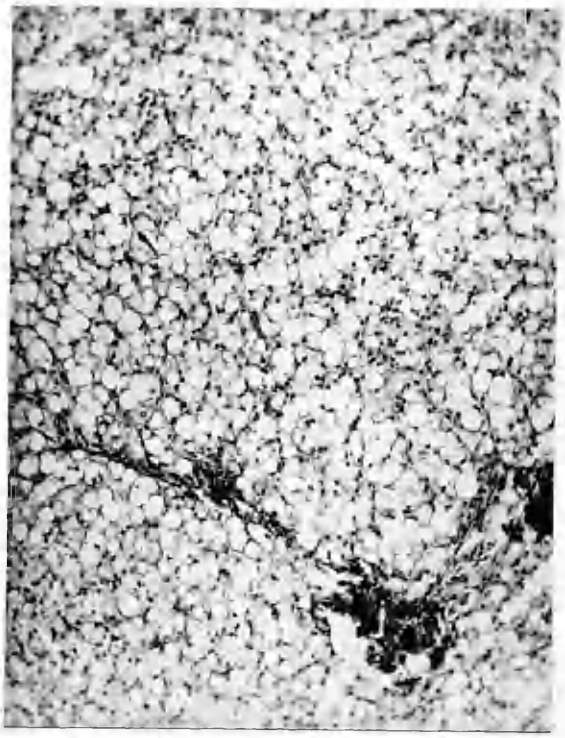


Fig. 33. Case No. 18. Second liver biopsy. 21st day of illness. Fat ++++
V.G. X 118.

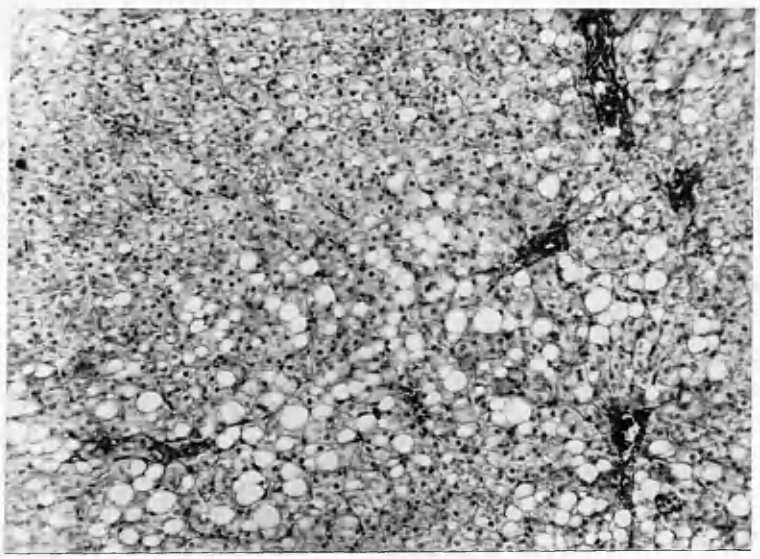


Fig. 34. Case No. 18. Third liver biopsy. 43rd day of illness. Fat +++
V.G. X 118.

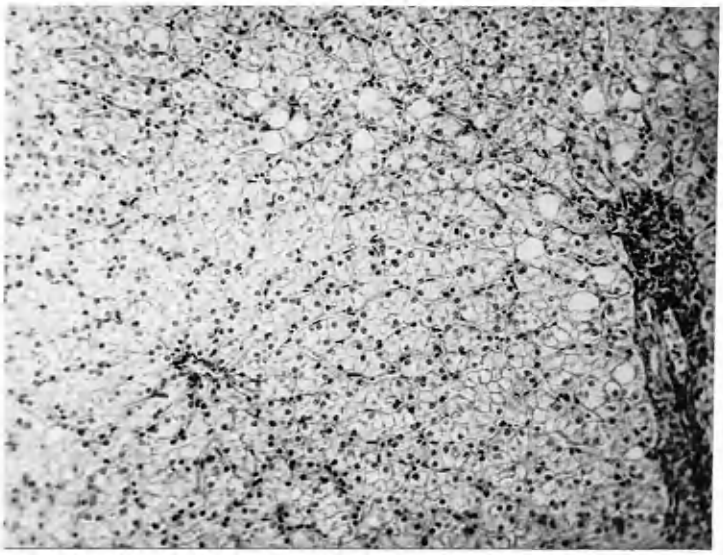


Fig. 35. Case No. 18. Fourth liver biopsy.
63rd day. Fat + V.G. X 118.

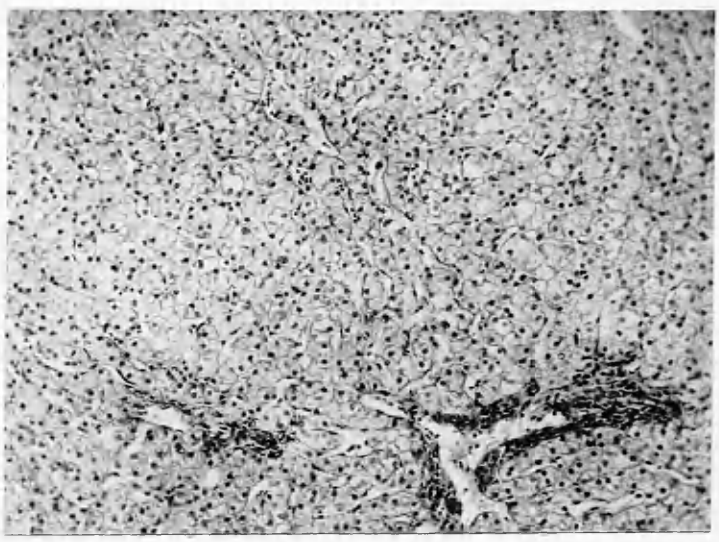


Fig. 36. Case No. 18. Fifth liver biopsy.
92nd day. Fat 0. V.G. X 118.

TABLE VI

Degree of Fatty Change in Liver related to Duration of Illness in Days

Diagnosis	Mean duration of illness (days)						Over-all mean duration (days)
	Fat ++++	Fat +++	Fat ++	Fat +	Fat ±	Fat 0	
Gastro-enteritis alone							
Survivors	34(5)	27(10)	34(11)	30(4)	22(5)	25(2)	31(7)
Fatal cases	24(1)	6(3)	31(7)	30(1)	23(1)	14(1)	23(14)
Gastro-enteritis with parenteral infection							
Survivors	71(4)	41(18)	47(13)	37(9)	39(4)	21(3)	43(51)
Fatal cases	20(4)	29(15)	21(5)	22(4)	19(1)	-	24(29)
Infections other than gastro-enteritis							
Survivors	-	32(5)	16(6)	13(4)	28(8)	11(6)	18(29)
Fatal cases	-	-	44(2)	33(1)	-	-	40(3)
Non-infective cases							
Survivors	-	-	-	6(3)	3(2)	4(8)	4(13)
Fatal cases	-	-	-	42(1)	-	-	42(1)
Total cases							
Survivors	53(8)	36(29)	35(31)	25(19)	27(15)	12(20)	30(130)
Fatal cases	17(6)	25(18)	29(14)	28(7)	21(2)	14(1)	25(47)

1. Figures in brackets indicate numbers of cases.

2. Maximum severity of fatty change is recorded for cases subjected to more than one investigation of liver.

TABLE VII

Degree of Fatty Change in Liver related to Number of Clinical Relapses of Gastro-enteritis

Diagnosis	Numbers of relapses						Total relapses
	Fat ++++	Fat +++	Fat ++	Fat +	Fat ±	Fat 0	
Gastro-enteritis alone	1	2	8	1	1	2	15
Gastro-enteritis with parenteral infection	3	14	11	6	2	0	36
Total	4	16	19	7	3	2	51

In each case subjected to multiple biopsies, liver fat is recorded from the investigation most nearly preceding the relapse.

TABLE VIII

Degree of Fatty Change in Liver related to Actual and Expected Body Weight at the Time of Liver Biopsy

Degree of fatty change	Actual weight (kilograms)		Expected weight (%)	
	Average	Range	Average	Range
++++	6.32	5.05- 8.72	89	80-103
+++	6.42	3.38-10.44	89	66-134
++	5.90	3.32-10.70	89	60-134
+	5.27	2.44-10.58	80	60-100
±	5.47	1.92-10.90	86	60-106
0	6.10	2.81- 9.39	83	53-110

TABLE IX

Variation in Degree of Fatty Change in Liver related to variation in Expected Body Weight

Alteration in expected weight during time interval between liver biopsies	Variation in degree of fatty change	Number of examinations
Gain in expected weight	Fat decreased	50
	Fat unchanged	27
	Fat increased	7
Expected weight unchanged	Fat decreased	3
	Fat unchanged	8
	Fat increased	5
Reduction in expected weight	Fat decreased	28
	Fat unchanged	22
	Fat increased	20

TABLE X

Degree of Fatty Change in Liver related to Daily Intake of Calories at Various Intervals prior to Examination of Liver

Degree of fatty change	Calories											
	Duration in days before liver examination											
	1	2	3	4	5	6	7	10	15	20	25	30
++++	27.0	29.5	28.3	27.5	26.9	27.1	26.7	26.5	28.8	29.0	-	-
+++	34.6	35.0	40.7	41.8	42.3	42.1	40.8	40.7	42.0	41.0	28.0	29.5
++	46.8	53.2	55.2	56.8	57.2	56.2	56.0	52.8	57.5	59.2	50.3	68.8
+	53.4	59.9	62.7	64.0	65.6	65.8	66.8	65.4	62.7	63.0	60.9	59.0
±	68.6	70.9	76.2	79.2	79.3	77.3	76.8	75.1	72.1	74.7	71.1	68.6
0	51.9	66.7	70.4	72.6	72.6	72.9	72.1	74.6	77.7	80.1	79.9	79.2

Figures give average daily intake of calories per kilogram body weight

TABLE XI

Degree of Fatty Change in Liver related to Daily Intake of Protein at Various Intervals prior to Examination of Liver

Degree of fatty change	Grams of protein											
	Duration in days before liver examination											
	1	2	3	4	5	6	7	10	15	20	25	30
++++	0.89	0.88	0.84	0.84	0.89	0.92	0.87	0.88	1.10	1.15	-	-
+++	1.48	1.46	1.68	1.66	1.66	1.66	1.62	1.72	1.67	1.62	1.53	1.30
++	1.93	2.18	2.27	2.33	2.35	2.30	2.27	2.16	2.36	2.61	2.59	2.96
+	2.40	2.66	2.78	2.86	2.90	2.92	3.00	2.99	2.88	2.87	2.78	2.80
±	2.92	3.10	3.28	3.46	3.47	3.45	3.42	3.39	3.39	3.28	3.21	3.17
0	2.06	2.85	3.00	2.96	2.94	2.96	2.96	3.14	3.08	3.26	3.11	3.05

Figures give average daily intake of protein in grams per kilogram body weight.

TABLE XII

Degree of Fatty Change in Liver related to Daily Intake of Fat at Various Intervals prior to Examination of Liver

Degree of fatty change	Grams of fat											
	Duration in days before liver examination											
	1	2	3	4	5	6	7	10	15	20	25	20
++++	1.71	1.94	2.00	1.94	1.98	1.90	1.88	1.85	1.84	1.78	-	-
+++	1.97	2.04	2.34	2.45	2.50	2.55	2.48	2.48	2.49	2.47	2.39	2.20
++	2.42	2.68	2.75	2.84	2.85	2.83	2.83	2.65	2.87	2.94	2.99	3.30
+	2.61	3.02	3.14	3.18	3.24	3.22	3.27	3.26	3.19	3.20	3.18	3.14
±	3.19	3.27	3.48	3.70	3.78	3.67	3.63	3.63	3.52	3.41	3.38	3.36
0	2.28	3.21	3.26	3.26	3.22	3.26	3.20	3.32	3.64	3.96	3.74	3.67

Figures give average daily intake of fat in grams per kilogram body weight.

TABLE XIII

Degree of Fatty Change in Liver related to Daily Intake of Carbohydrate at Various Intervals prior to Examination of Liver

Degree of fatty change	Grams of carbohydrate											
	Duration in days before liver examination											
	1	2	3	4	5	6	7	10	15	20	25	30
++++	3.11	3.11	3.24	3.15	3.03	3.01	3.00	3.03	3.30	3.43	-	-
+++	4.03	4.16	4.69	4.85	5.00	4.97	4.84	4.80	4.68	4.57	4.20	3.60
++	5.18	5.75	5.96	6.20	6.20	6.15	6.16	5.80	6.35	6.51	6.32	6.80
+	5.90	6.50	6.78	6.91	7.05	7.10	7.12	7.16	6.97	7.08	6.99	6.66
±	7.57	7.92	8.46	8.98	8.98	8.90	8.86	8.79	8.96	8.79	8.75	8.34
0	5.48	8.11	8.48	8.41	8.55	8.56	8.41	8.83	9.60	10.38	10.77	11.02

Figures give average daily intake of carbohydrate in grams per kilogram body weight.

FIG. 37 MEAN VALUES FOR DAILY INTAKE OF CALORIES OVER PERIODS OF 5, 10, 15 & 20 DAYS BEFORE LIVER EXAMINATION, RELATED TO SEVERITY OF FATTY LIVER

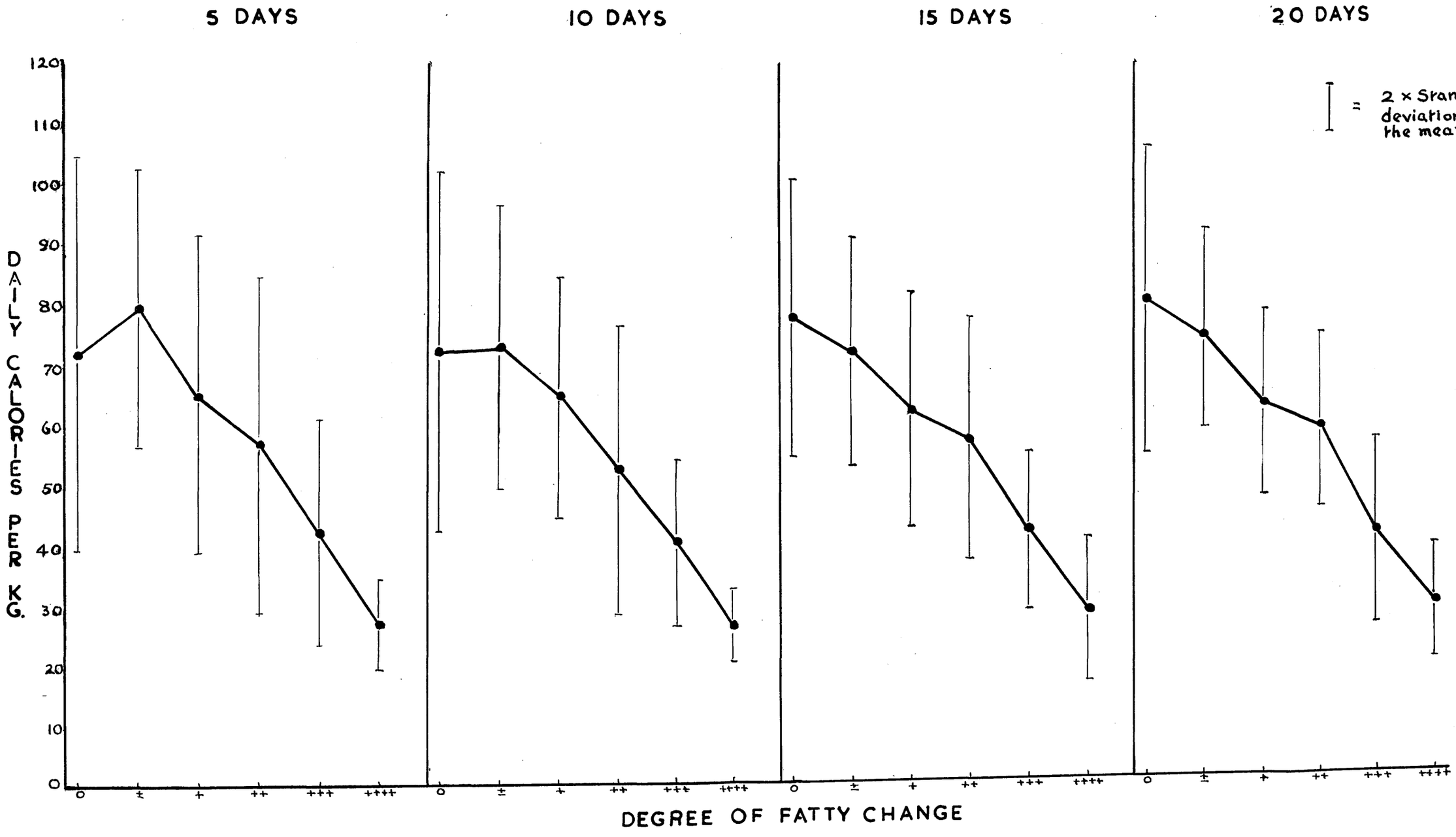


FIG. 38 MEAN VALUES FOR DAILY INTAKE OF CARBOHYDRATE, FAT AND PROTEIN OVER PERIODS OF 5, 10, 15 & 20 DAYS BEFORE LIVER EXAMINATION, RELATED TO SEVERITY OF FATTY LIVER

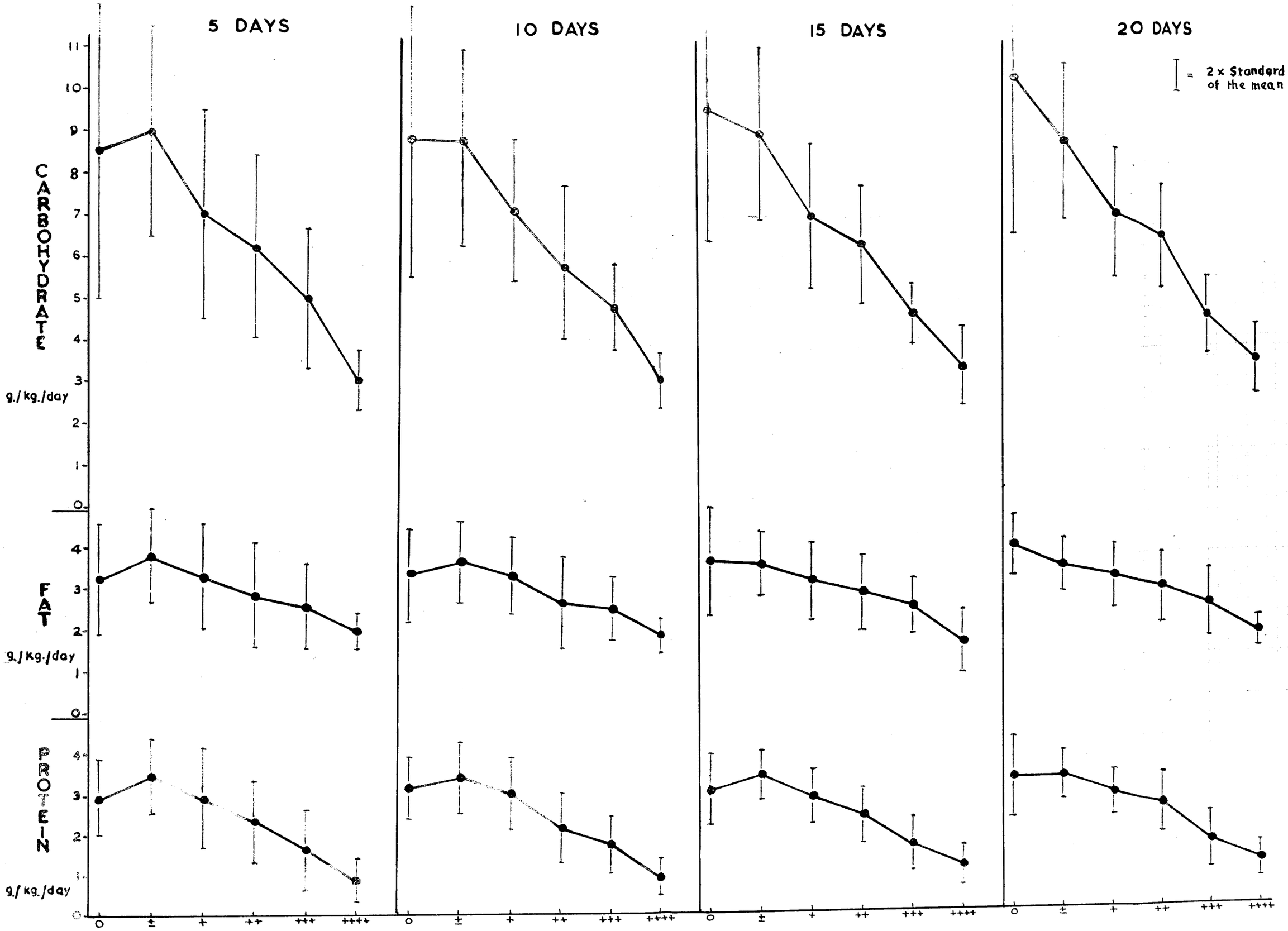


TABLE XIV

Results of Liver Function Tests related to Initial Liver Biopsy Results

Investigation	Numbers investigated		Degree of fatty change					
			++++	+++	++	+	±	0
Van den Berg Reaction (Indirect)	Without "Hepatitis"	174	1 +ve	1 +ve	6 +ve	2 +ve	1 +ve	1 +ve
" " " " (Direct)	With "	3	1 +ve	1 +ve	1 +ve	-	-	-
Urinary Urobilinogen	Without "	174	1 +ve	-	4 +ve	-	-	-
	With "	3	1 +ve	1 +ve	1 +ve	-	-	-
Total Serum Bilirubin - mg.%	Without "	174	0.8	0.5	0.8	0.4	0.5	0.4
	With "	3	2.5	5.5	2.8	-	-	-
Thymol Turbidity - Maclagan Units	Without "	174	3.3	2.0	2.5	2.5	2.5	3.0
	With "	3	6.0	13.0	17.0	-	-	-
Takata-Ara Reaction	Without "	70	← all negative →					
	With "	3	++	+	+++	-	-	-
Colloidal Gold Test	Without "	58	← all negative →					
	With "	3	++	+++	++++	-	-	-
Total Plasma Protein - g.%	Without "	164	4.50	5.64	5.77	5.56	5.55	5.62
	With "	3	4.67	4.25	5.67	-	-	-
Total Serum Cholesterol - mg.%	Without "	30	114	120	142	95	108	-
	With "	2	-	70	114	-	-	-
Serum Cholesterol Esters - mg.%	Without "	30	77	80	86	79	85	-
	With "	2	-	37	47	-	-	-
Serum Phospholipid - mg.%	Without "	25	230	259	324	268	295	-
	With "	0	-	-	-	-	-	-
Prothrombin Time - seconds	Without "	174	28	27	26	26	32	28
	With "	3	29	34	30	-	-	-

With the exception of Van den Berg and Urinary Urobilinogen findings, mean values are given for results obtained from "non-hepatitis" cases.

The results of one "hepatitis" case are shown in relation to the third liver biopsy (the first to show evidence of liver necrosis).

TABLE XV

Blood Sugar Levels and Ketonuria related to Amounts of Stainable Liver Fat and Glycogen

Liver fat	Liver glycogen	Numbers of biopsies	Tests for ketonuria related to these biopsies			Blood sugar - mg.%			
			Positive	Trace	Negative	Numbers examined	Highest	Lowest	Mean
++++	++++ - +++	0	-	-	-	-	-	-	-
	++ - +	0	-	-	-	-	-	-	-
	± - 0	13	13	-	-	6	44	26	36
+++	++++ - +++	0	-	-	-	-	-	-	-
	++ - +	19	11	5	3	4	72	28	56
	± - 0	44	30	10	4	14	78	29	53
++	++++ - +++	7	0	2	5	3	90	72	84
	++ - +	35	16	6	13	12	101	41	71
	± - 0	30	18	6	6	6	89	33	63
+	++++ - +++	15	0	1	14	3	104	71	96
	++ - +	19	0	4	15	5	111	49	71
	± - 0	24	8	8	8	3	102	58	77
±	++++ - +++	13	0	5	8	2	80	80	80
	++ - +	26	0	2	24	5	93	67	82
	± - 0	9	4	1	4	2	73	53	63
0	++++ - +++	13	0	1	12	6	128	93	107
	++ - +	19	2	3	14	3	121	60	85
	± - 0	6	4	1	1	1	58	58	58

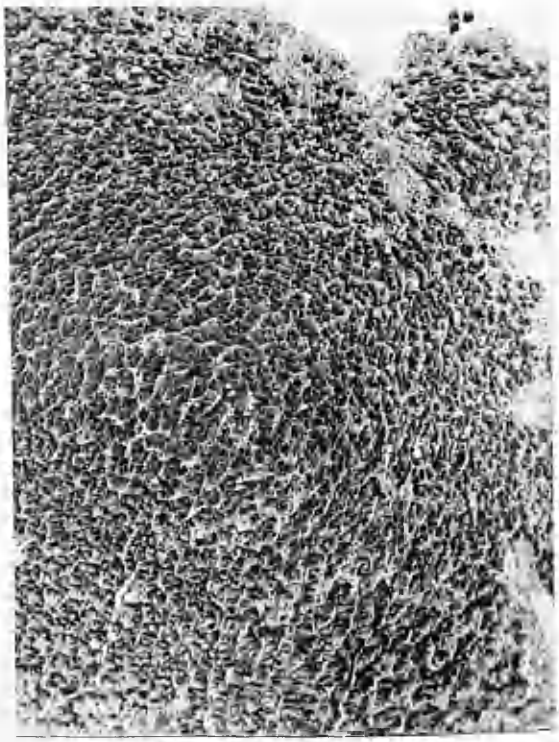


Fig. 39. Liver biopsy specimen.
Glycogen ++++ Fat o
Best's Stain X 65.

Fig. 40. Liver biopsy specimen.
Glycogen ++ Fat ++
Best's Stain X 74.

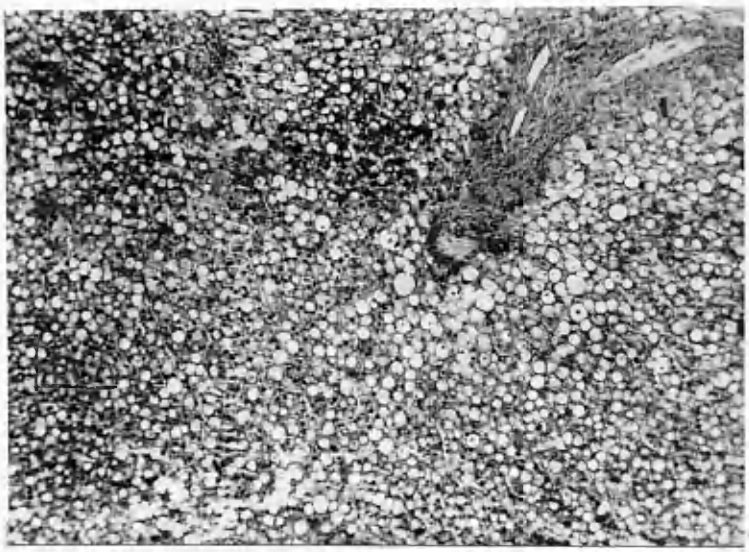


Fig. 41. Liver biopsy specimen. Glycogen $\pm$
Fat ++++
Best's Stain X 94.

TABLE XVI

The Amount of Stainable Fat related to the Amount of Stainable Glycogen in the Same Specimen of Liver (Biopsy Material only)

Degree of glycogen staining	Numbers of examinations					
	Fat ++++	Fat +++	Fat ++	Fat +	Fat ±	Fat 0
++++	0	0	2(0)	5(2)	8(2)	8(1)
+++	0	0	5(2)	10(6)	5(3)	5(3)
++	0	7(4)	27(11)	9(5)	16(3)	16(7)
+	0	12(5)	8(3)	10(4)	10(3)	3(2)
±	6(4)	22(12)	17(11)	17(8)	8(7)	3(3)
±	3(1)	13(9)	11(10)	5(2)	0	3(3)
0	4(2)	9(8)	2(2)	2(2)	1(1)	0
Average glycogen	±	±	+	+	++	++

Figures in brackets indicate numbers of first biopsy examinations.

TABLE XVII

Variation in Stainable Fat related to Variation in Stainable Glycogen in Livers of Patients subjected to Multiple Biopsies

Alteration in amount of stainable glycogen	Alteration in amount of stainable fat	Numbers of cases
Glycogen increased	Fat increased	3
	Fat unchanged	9
	Fat decreased	56
Glycogen unchanged	Fat increased	9
	Fat unchanged	14
	Fat decreased	10
Glycogen decreased	Fat increased	20
	Fat unchanged	3
	Fat decreased	5

TABLE XVIII

Numbers of Cases classified to show the Relationship between Fatty Change in Liver and Treatment (various forms including Lipotropic Therapy)

Treatment	Numbers of patients examined													
	++++ Fat		+++ Fat		++ Fat		+ Fat		± Fat		0 Fat		Total	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After*
Lipotropic Therapy														
Choline	3	2	12	13	11	9	4	6	2	2	0	1	32	33
Methionine	1	1	8	8	7	5	4	6	2	1	0	1	22	22
Casein (I.V.)	1.	0	3.	4	5	3	0	3	1	0	1	1	11	11
Casein (oral)	1	0	1	1	1	0	0	1	0	1	-		3	3
Vit. B Complex	1	0	9	9	5	3	2	4	2	2	0	1	19	19
Inositol	-		1	1	0	1	1	1	1	0	-		3	3
Pyridoxine	1	0	1	1	-		0	1	-		0	1	3	3
Pantothenic Acid	1	0	3	2	2	4	-		-		-		6	6
Riboflavine	-		-		2	1	2	1	0	1	0	1	4	4
Other Treatment														
Glucose (I.V.)	4	8	17	22	19	20	11	11	6	6	1	2	58	69
Plasma (I.V.)	3	9	17	18	15	21	10	7	5	7	2	4	52	66
Blood (I.V.)	0	1	-		1	3	2	2	1	0	-		4	6
Carob Flour	2	2	1	0	0	1	0	1	-		-		3	4
Streptomycin	0	1	5	3	6	6	4	8	1	5	-		16	23
Aerosporon	-		3	1	5	7	4	4	2	4	1	0	15	16
Penicillin	3	2	11	11	11	15	9	14	3	6	1	2	38	50
Sulphaguinidine	-		1	1	1	2	1	3	-		1	2	4	8
Sulphamezathine	3	2	9	9	11	14	10	14	3	7	2	8	38	54
Urinary Antiseptics	-		2	1	2	1	0	2	-		-		4	4

*In some cases the first liver biopsy was performed after treatment commenced.

TABLE XIX

Variation in Degree of Fatty Change in Liver related to Treatment (various forms including Lipotropic Therapy)

Treatment	Total	Number of cases studied before and after treatment		
		Liver fat decreased	Liver fat unchanged	Liver fat increased
Lipotropic Therapy				
Choline	32	10	18	4
Methionine	22	8	12	2
Casein (I.V.)	11	4	6	1
Casein (oral)	3	3	0	0
Vit. B Complex	19	7	8	4
Inositol	3	0	1	2
Pyridoxine	3	1	2	0
Pantothenic Acid	6	3	3	0
Riboflavine	4	3	0	1
Other Treatment				
Glucose (I.V.)	58	14	23	21
Plasma (I.V.)	52	11	22	19
Blood (I.V.)	4	1	0	3
Carob Flour	3	2	1	0
Streptomycin	16	9	5	2
Aerosporon	15	6	5	4
Penicillin	38	14	14	10
Sulphaguinidine	4	1	3	0
Sulphamezathine	38	16	14	8
Urinary Antiseptics	4	1	3	0

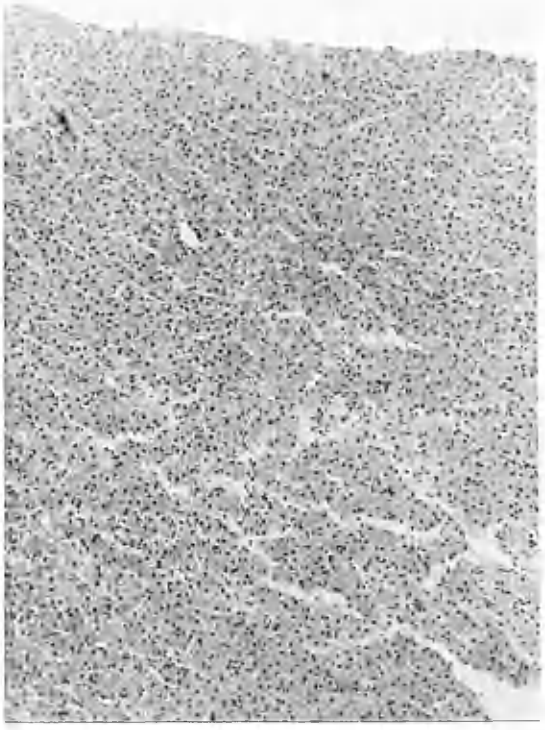


Fig. 42. Case No. 152. First biopsy. Fat +. No necrosis. H. & E. X 90.

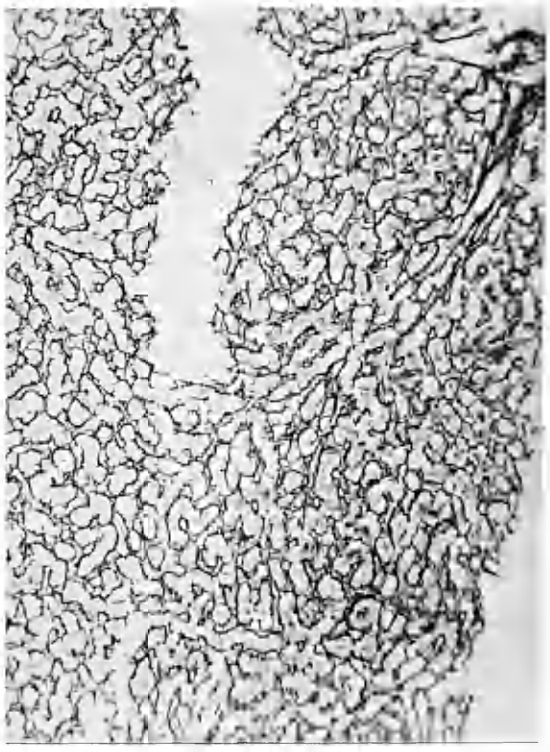


Fig. 43. Case No. 152. First biopsy. Reticulin Stain X 90.

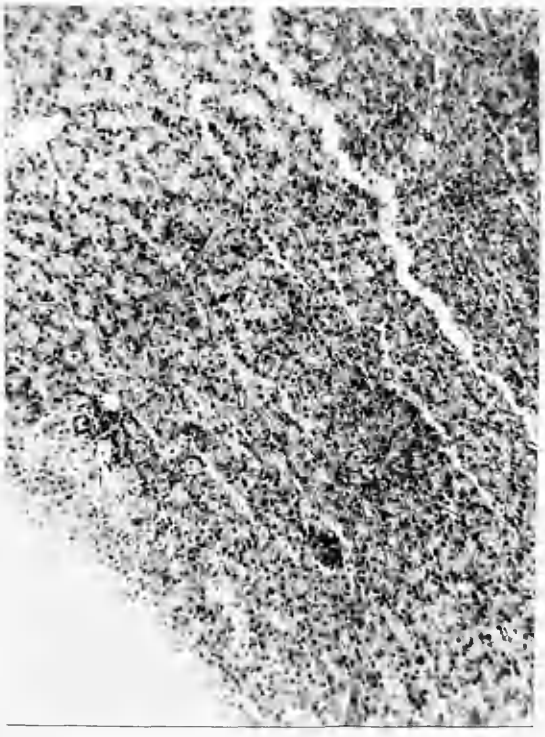


Fig. 44. Case No. 152. Second biopsy. Fat ±. No necrosis. Masson's Trichrome Stain X 90.

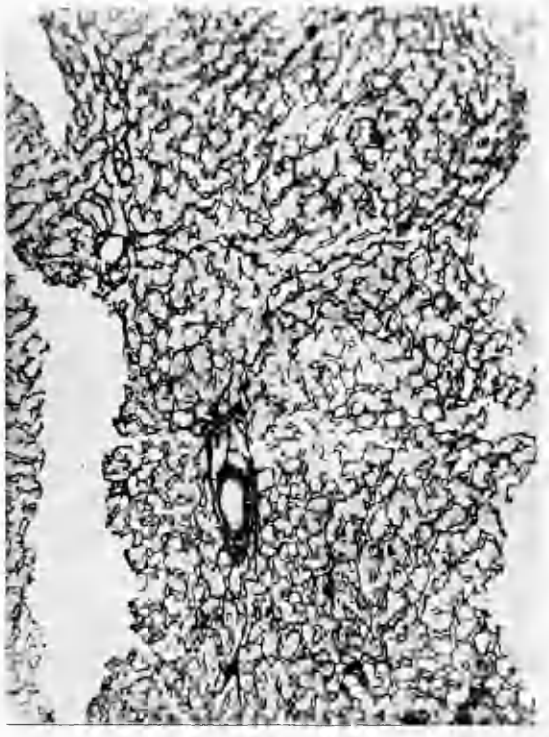


Fig. 45. Case No. 152. Second biopsy. Reticulin Stain X 90.

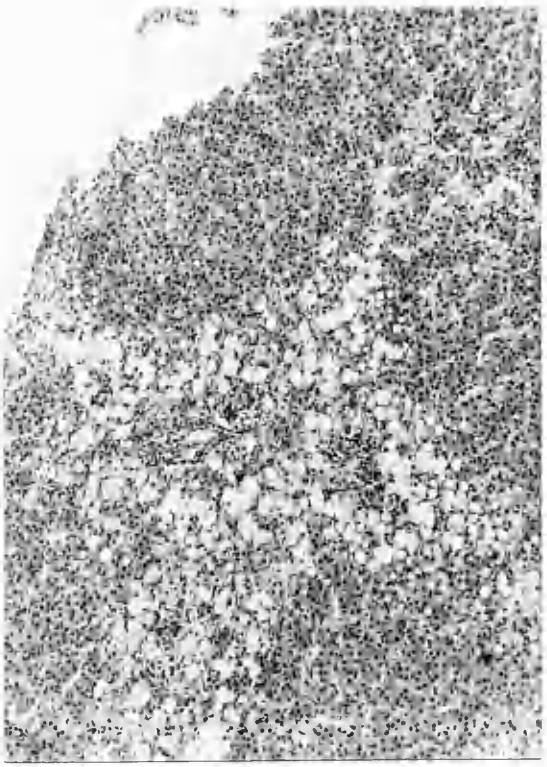


Fig. 46. Case No. 152. Third biopsy. Fat +++. H. & E. X 89.

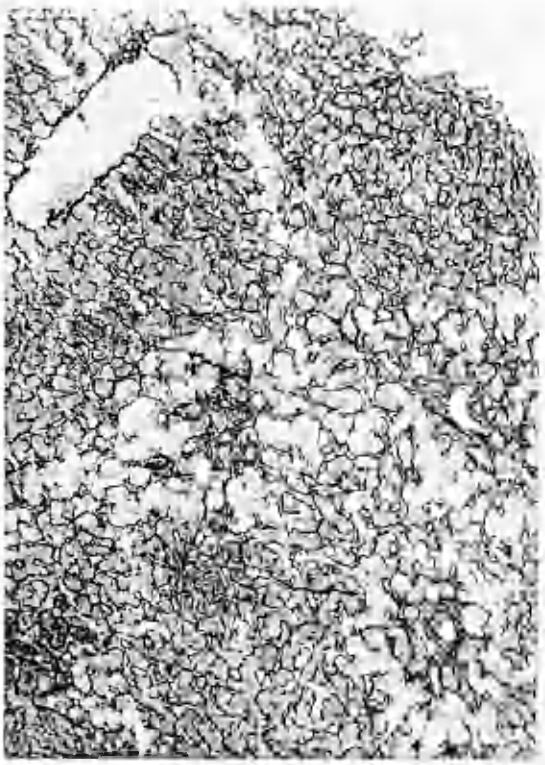


Fig. 47. Case No. 152. Third biopsy. Reticulin Stain X 89.

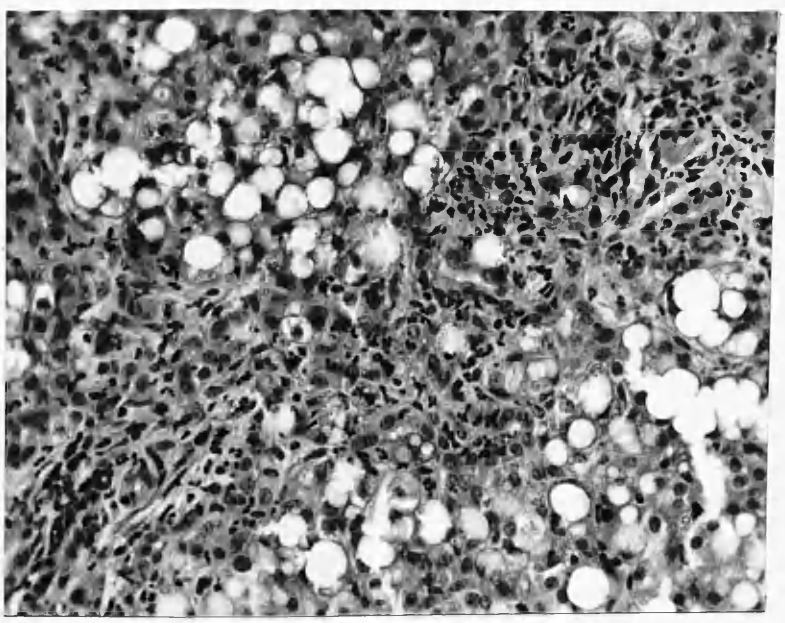


Fig. 48. Case No. 152. Third biopsy. Infiltration of portal areas by acute inflammatory cells. H. & E. X 240.

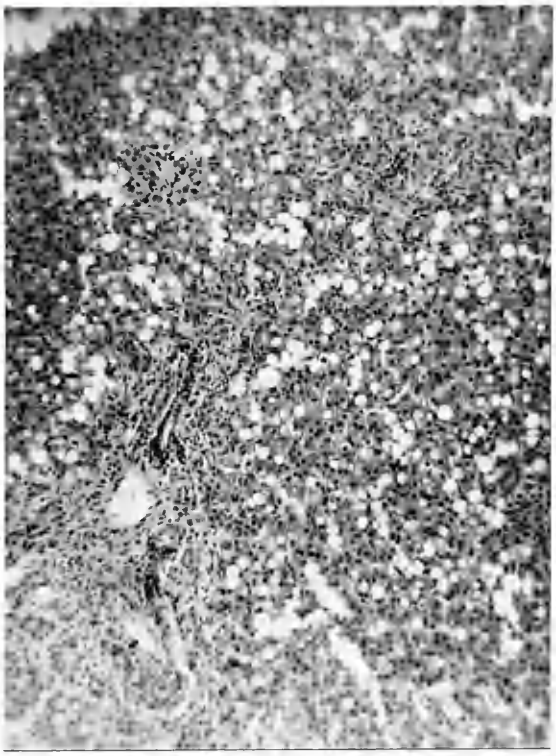


Fig. 49. Case No. 152. Fourth biopsy. H. & E. X 100.

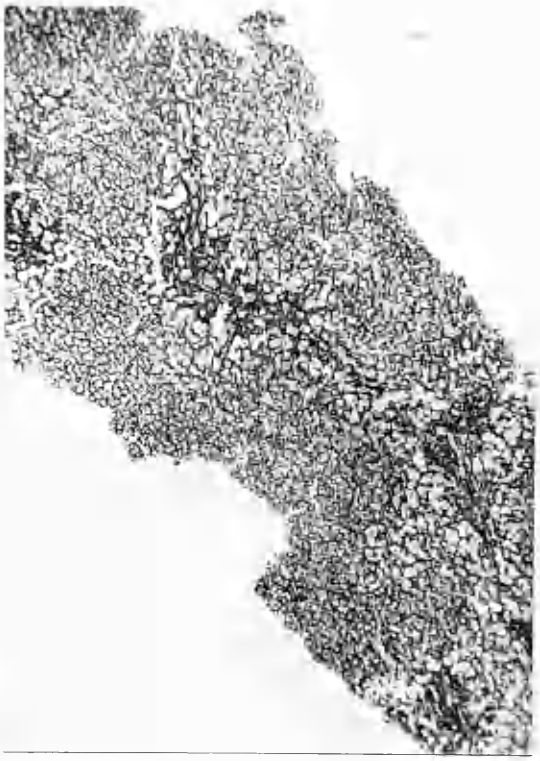


Fig. 50. Case No. 152. Fourth biopsy. Reticulin Stain X 50.

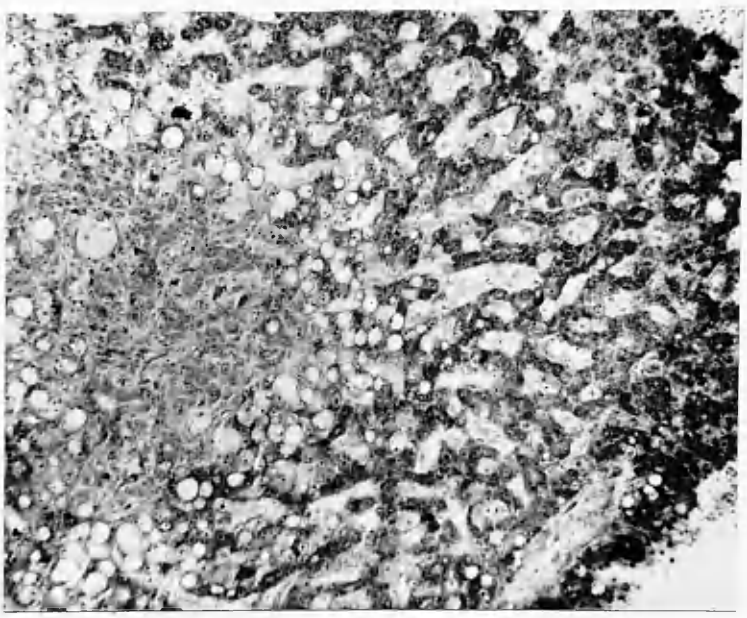


Fig. 51. Case No. 152. Fourth biopsy. Portal zone on left showing cholangiolar proliferation and damage to adjacent liver parenchyma, including loss of limiting plate, fatty change and reduction in R.N.A. Giemsa Stain X 150.

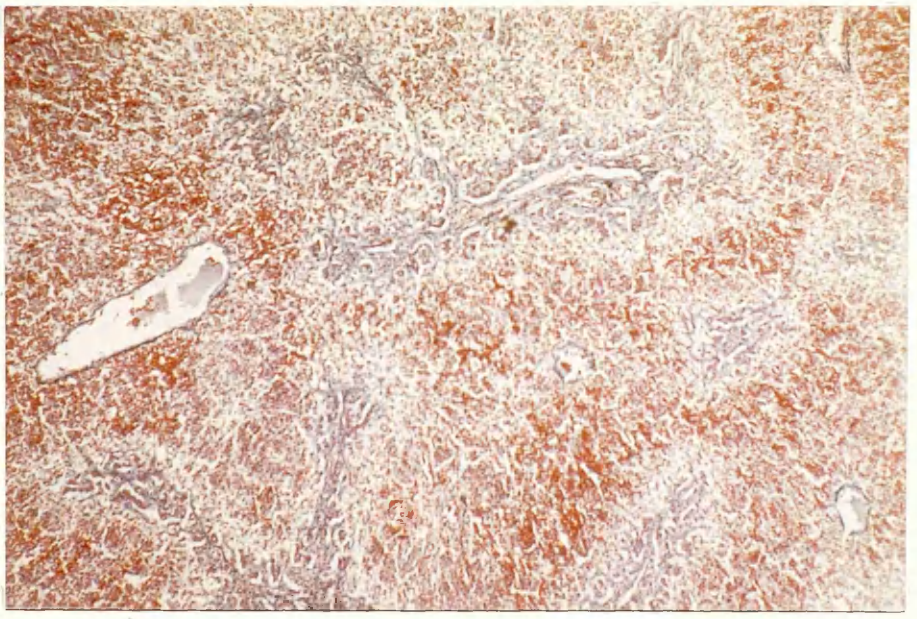


Fig. 52. Case No. 152. Autopsy specimen. Portal fibrosis and extensive haemorrhagic necrosis.
Heidenhain's Azan Method X 40.

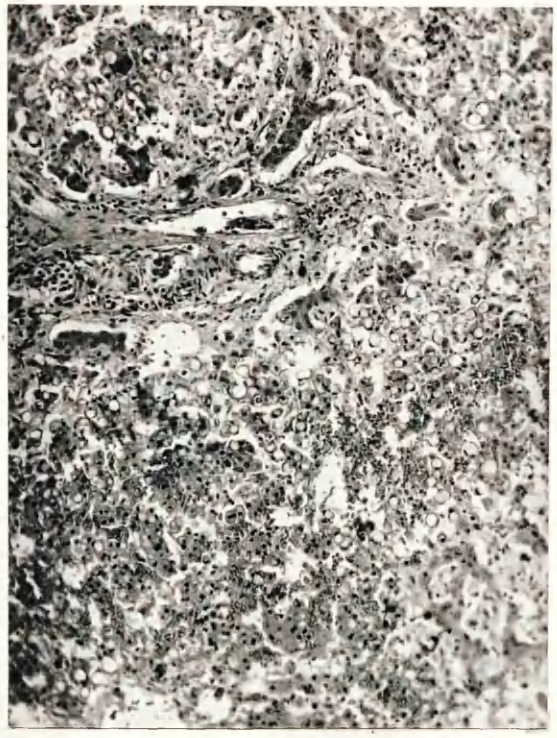


Fig. 53. Case No. 152. Autopsy specimen.
H. & E. X 118.

Fig. 54. Case No. 152. Autopsy specimen.
Reticulin Stain X 38.

Fig. 55. Case No. 72. Liver biopsy. Extensive fatty change and focus of necrosis.
H. & E. X 100.

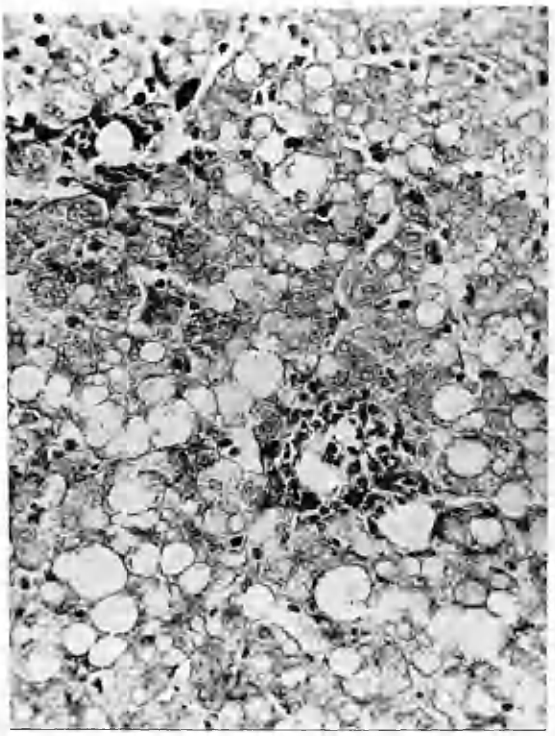


Fig. 56. Case No. 72. Liver biopsy. Specimen showing absence of R.N.A.
Giemsa Stain X 296.

Fig. 57. Case No. 72. Liver biopsy. Prominent periportal reticulin.
Reticulin Stain X 75.

Fig. 58. Case No. 93. First liver biopsy. Lymphocytic infiltration, predominantly portal. V.G. X 50.

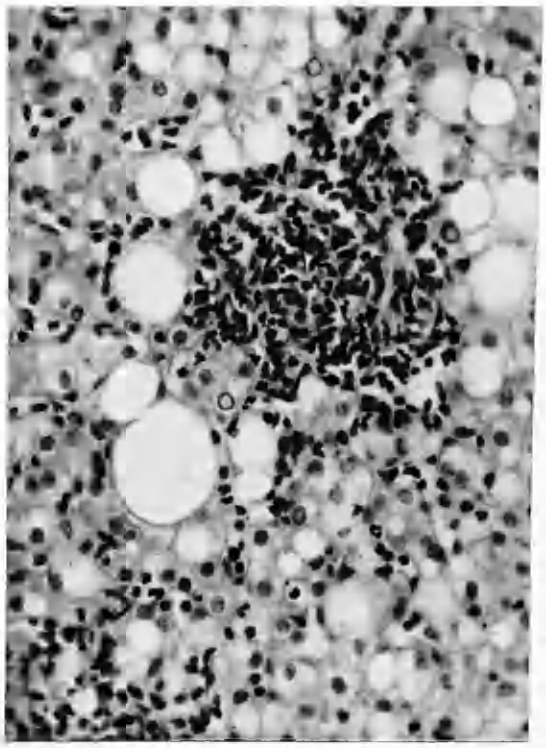


Fig. 59. Higher magnification. Inflammatory cell infiltration. Fatty change, including fat cysts. V.G. X 328.

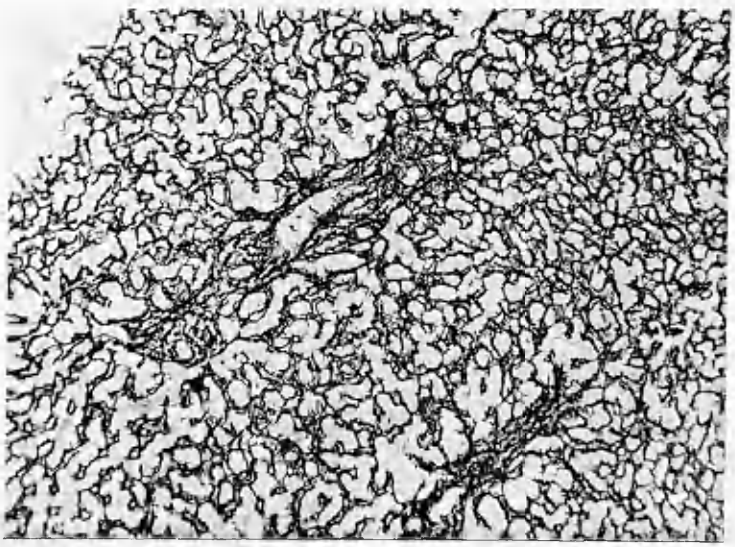


Fig. 60. Reticulin stain of same specimen. X 95.

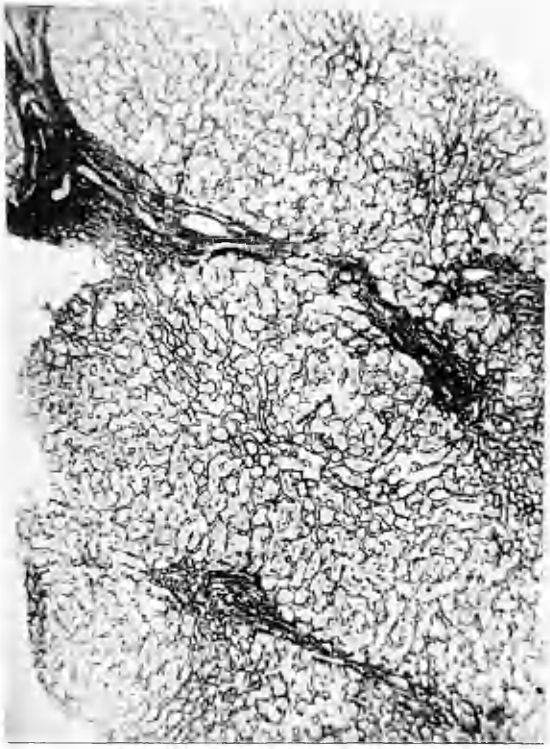


Fig. 61. Case No. 93. Second liver biopsy. Portal fibrosis. Reticulin X 60.

Fig. 62. The same specimen showing inflammatory cell infiltration. V.G. X 105.

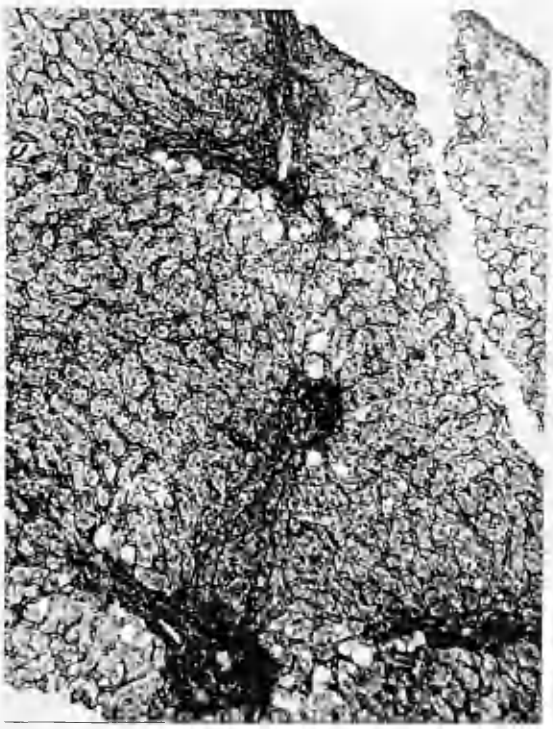


Fig. 63. Case No. 93. Third liver biopsy. Reticulin X 95.

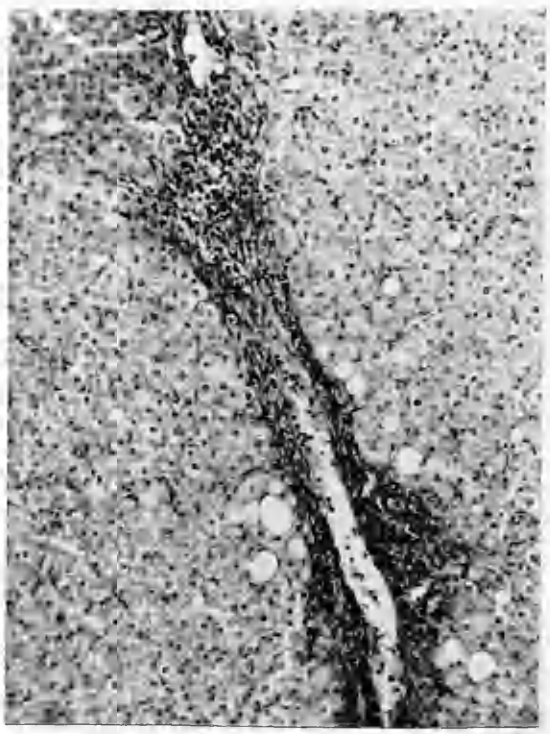


Fig. 64. The same stained by van Gieson's method. X 140.

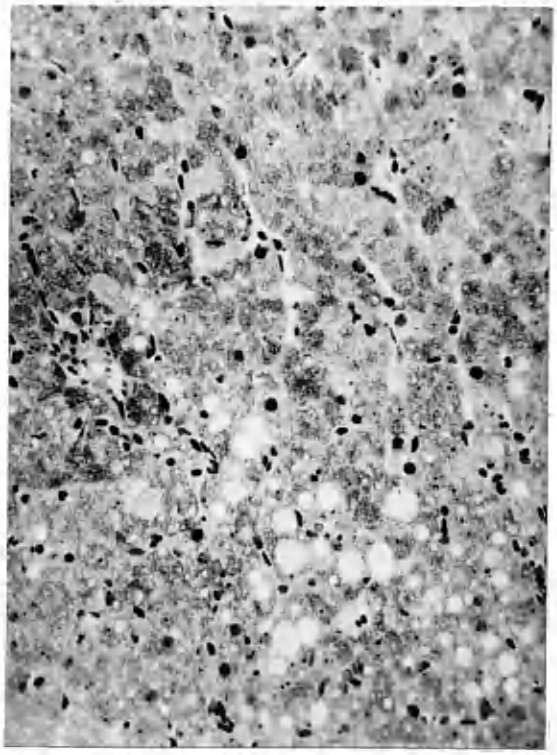


Fig. 65. Liver biopsy specimen. Patchy distribution of mitochondria. Iron haematoxylin X 312.

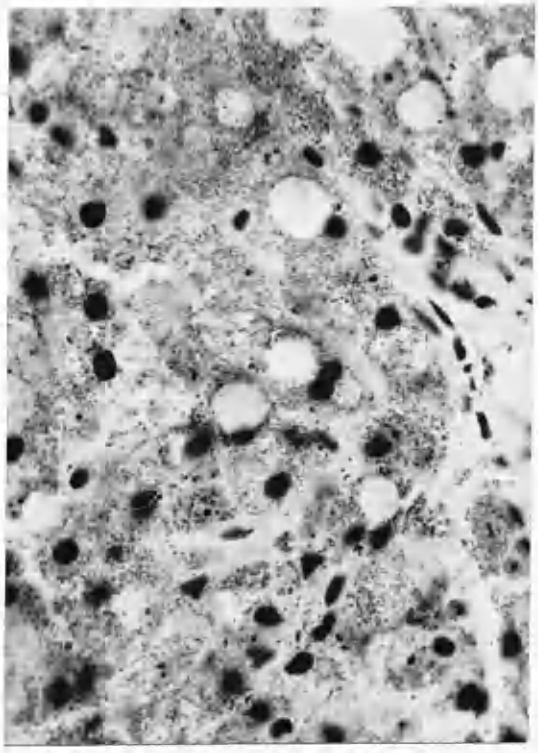


Fig. 66. Liver biopsy specimen. Mitochondria in fatty cells. Iron haematoxylin X 566.

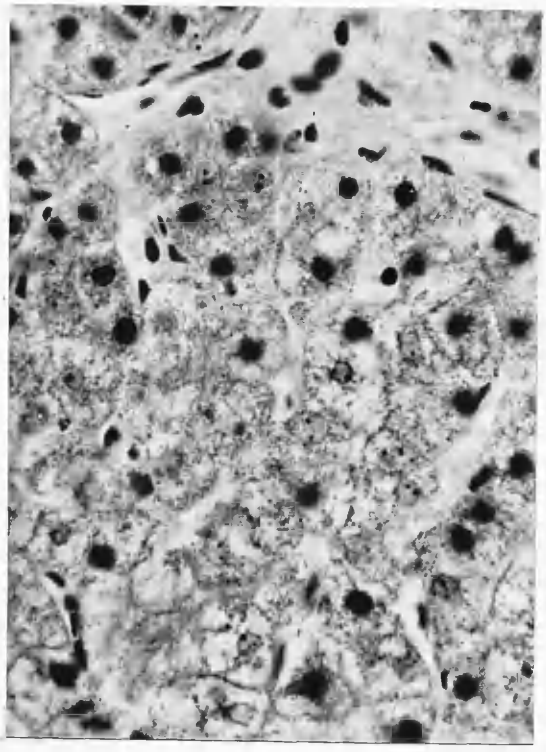


Fig. 67. Liver biopsy specimen. Marginal concentration of mitochondria. Iron haematoxylin X 566.

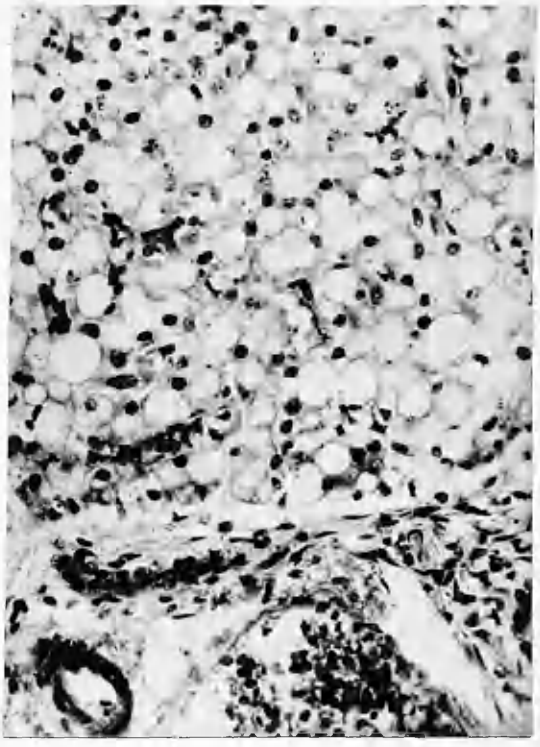


Fig. 68. Liver autopsy specimen. Mitochondria absent. Iron haematoxylin X 312.

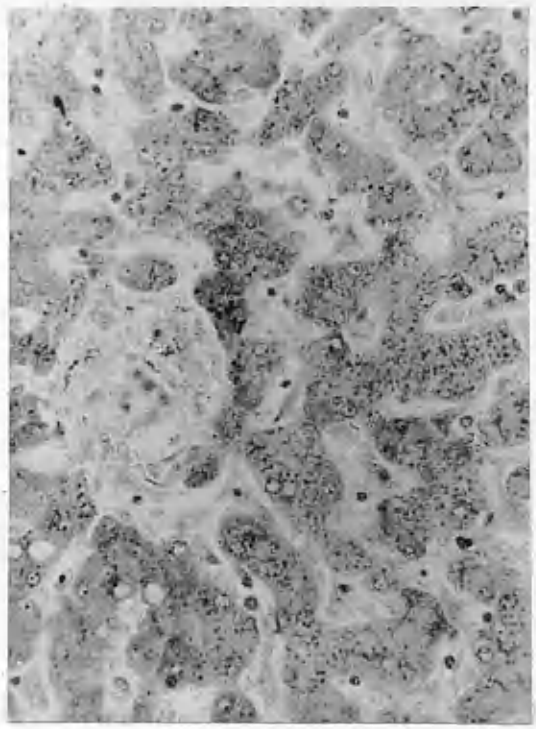


Fig. 69. Liver biopsy specimen stained to show R.N.A.
Giemsa stain X 328.

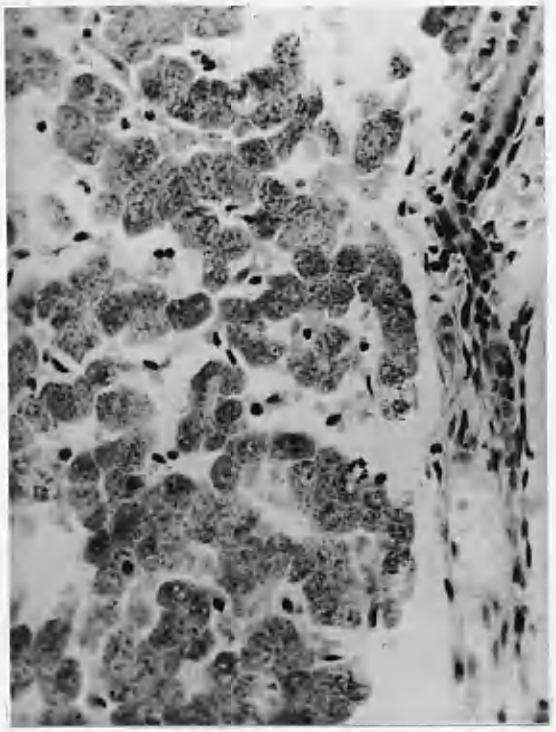


Fig. 70. Liver autopsy specimen stained to show R.N.A.
Giemsa stain X 312.

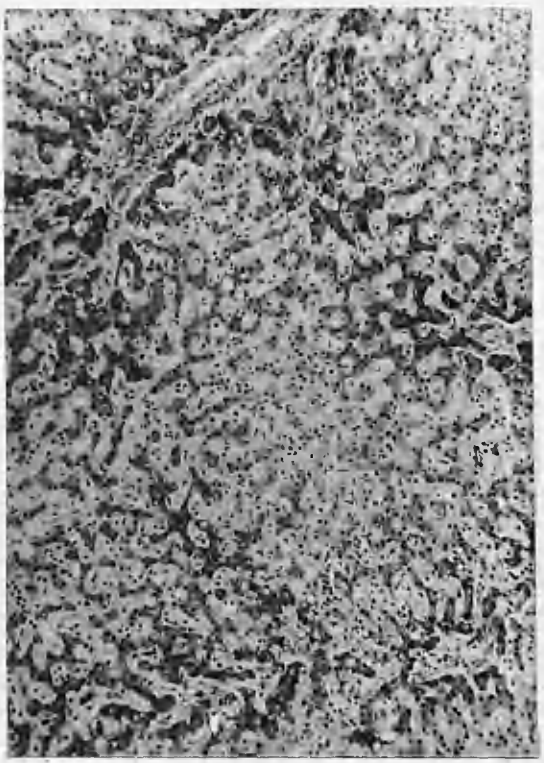


Fig. 71. Periportal concentration of R.N.A.
Giemsa stain X 90.

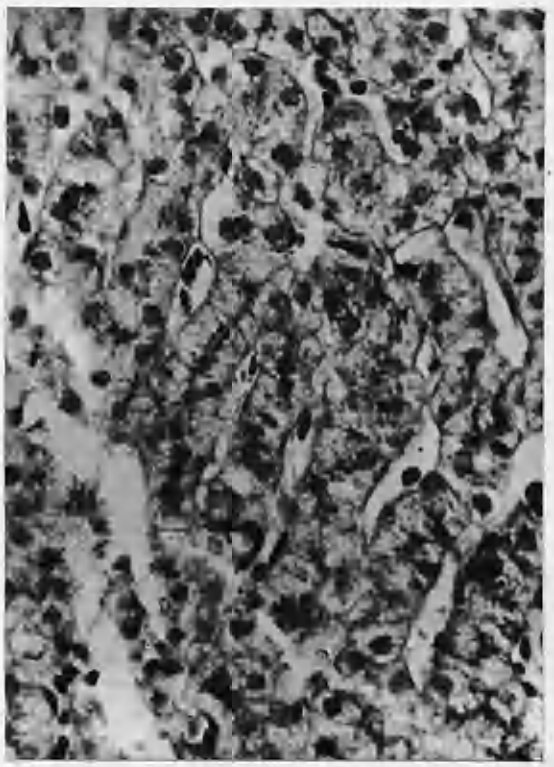


Fig. 72. Concentration of R.N.A. around bile canaliculi.
Giemsa stain X 430.

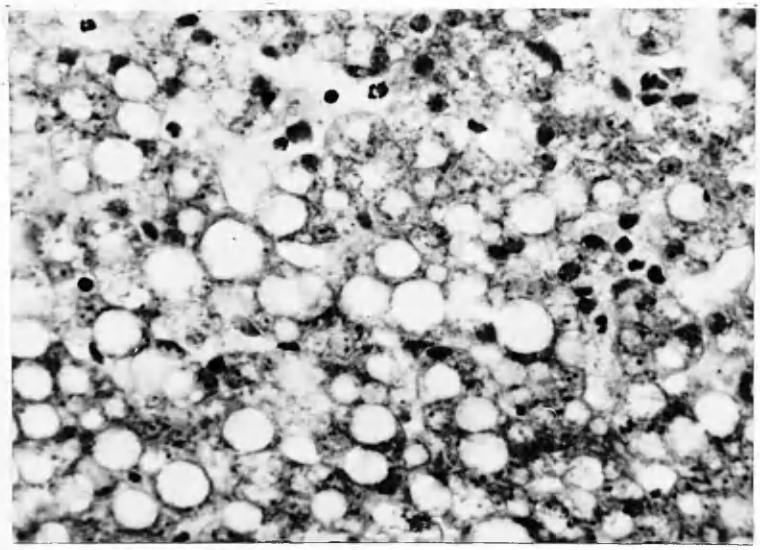


Fig. 73. Liver biopsy specimen. R.N.A.+ in fatty liver.

Giemsa stain X 430.

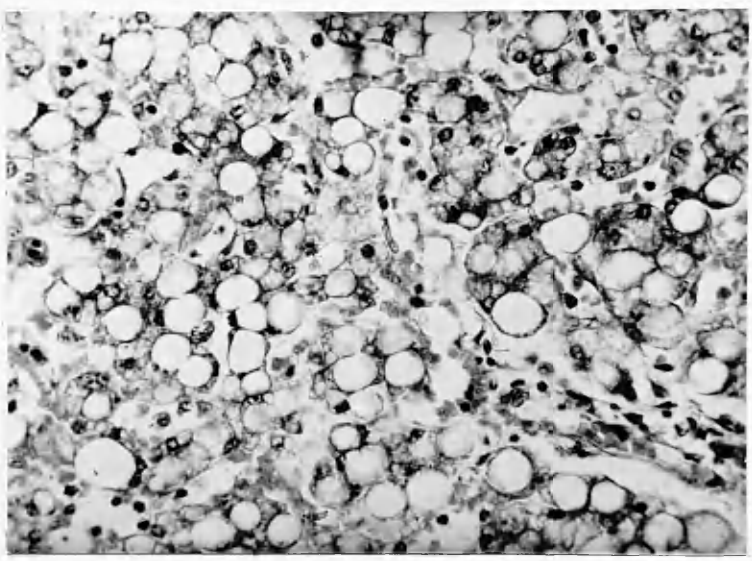


Fig. 74. Liver biopsy specimen. R.N.A.- in fatty liver.

Giemsa stain X 328.

TABLE XX

Liver Cell Cytoplasmic Basophilia related to Clinical Diagnosis and Plasma Protein Levels

Degree of basophilia	Numbers of biopsies so examined	Clinical diagnosis				Plasma protein - g.% (± S.D. of mean
		Gastro-enteritis	Gastro-enteritis + parenteral infection	Parenteral infection	Other	
++	27	15	12	0	0	6.18(± 0.92)
+	30	6	21	3	0	6.29(± 0.71)
±	90	30	39	9	12	5.28(± 0.72)
±	54	12	15	15	12	5.05(± 0.74)
0	12	9	3	0	0	5.27(± 0.66)

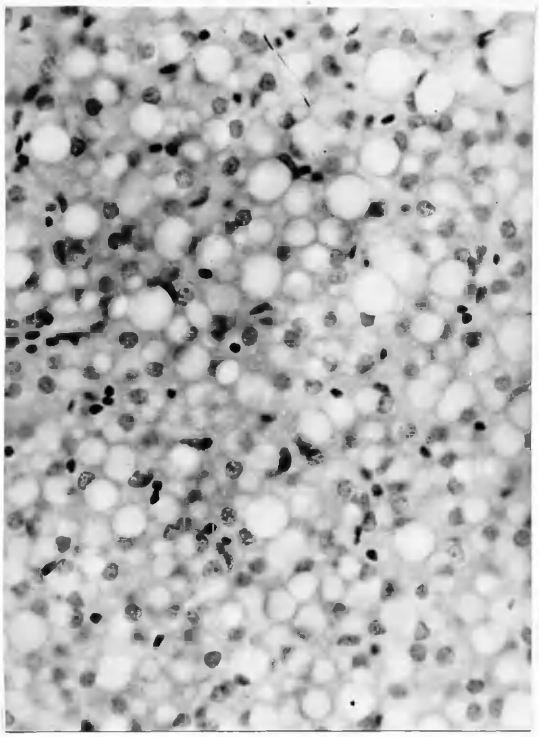


Fig. 75. Fatty liver and prominent sinusoidal cells. H. & E. X 362.

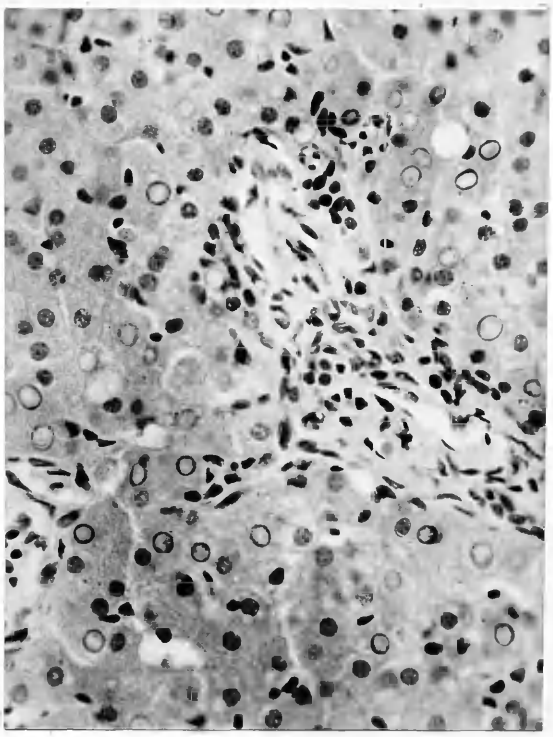


Fig. 76. Mild leucocytic infiltration. Vacuolation of liver cell nuclei. H. & E. X 328.

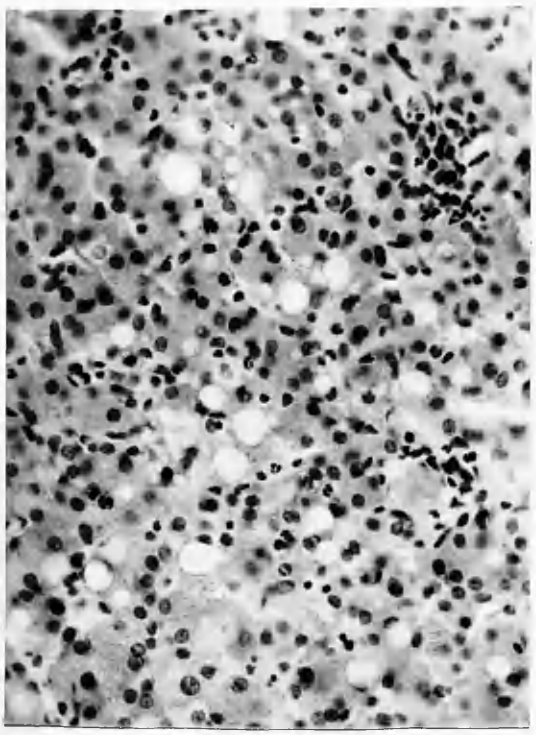


Fig. 77. Leucocytic infiltration of sinusoids. H. & E. X 328.

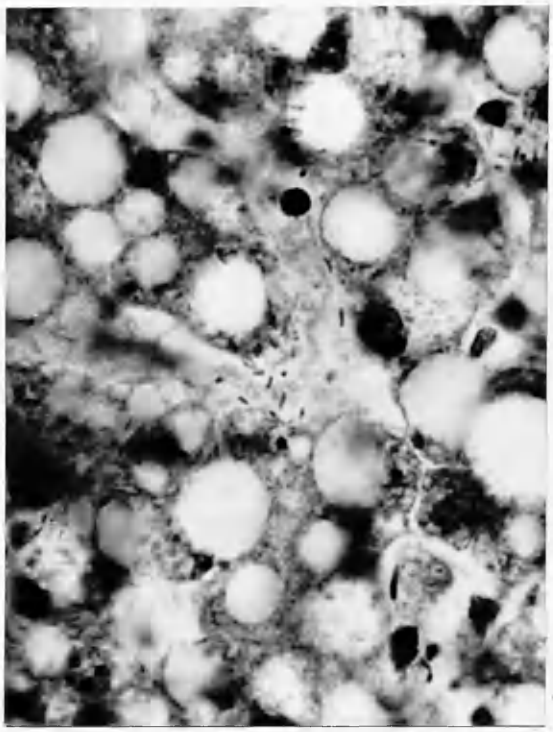


Fig. 78. Bacilli in liver biopsy specimen. Giemsa stain X 634.

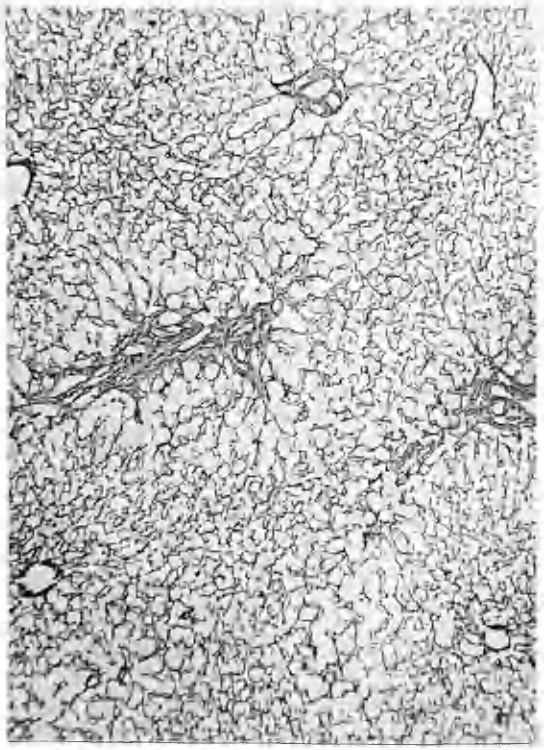


Fig. 79. Slight condensation of periportal reticulin.
Reticulin X 75.

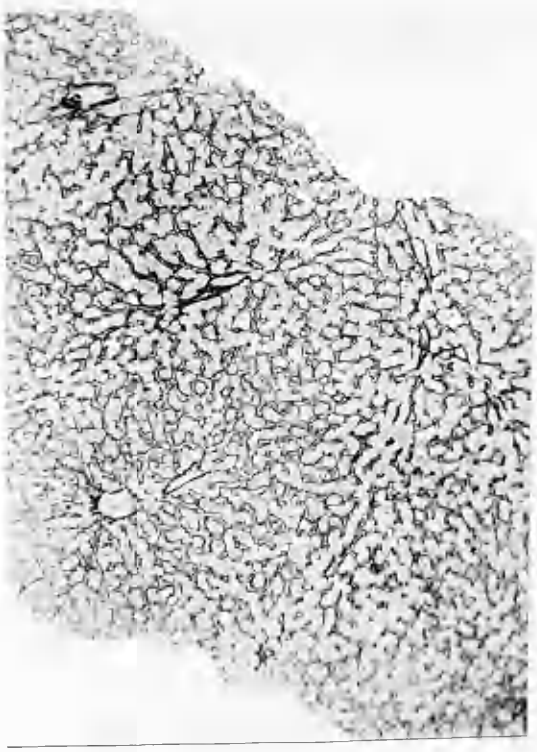


Fig. 80. Normal liver for comparison with Fig. 79.
Reticulin X 62.

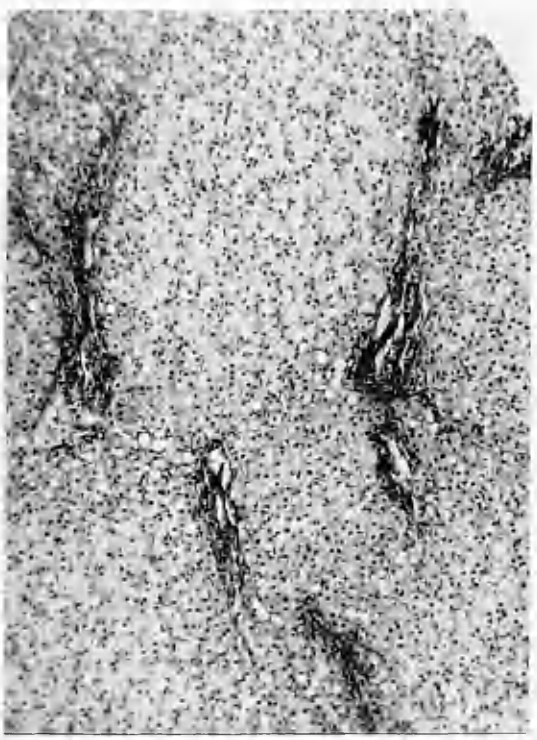


Fig. 81. Slight portal fibrosis.
V.G. X 95.

Fig. 82. Subcapsular fibrosis. Autopsy specimen.
Trichrome Stain X 38.

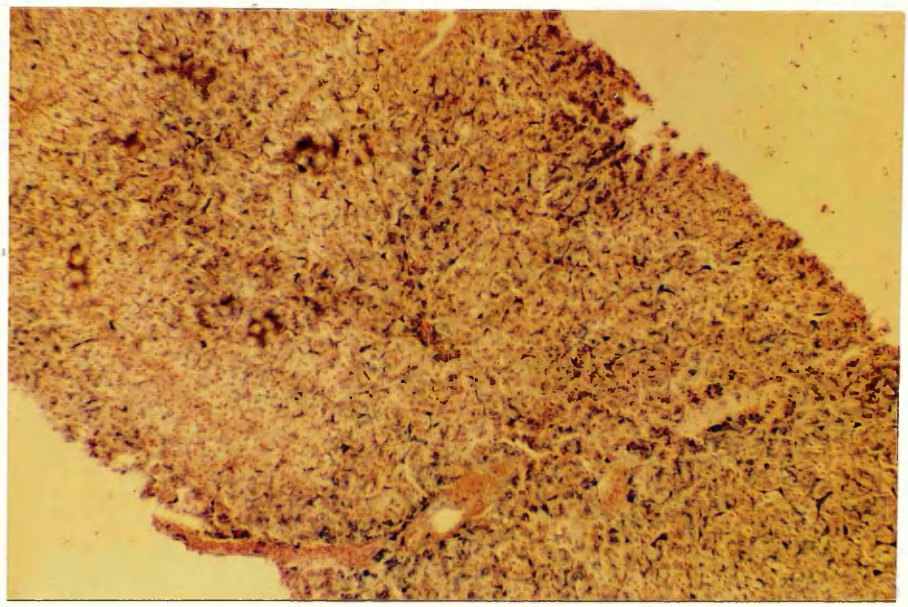


Fig. 83. Iron-containing pigment stained by the Prussian Blue technique.
Perls's Method & Carmalum X 70.

Fig. 84. Iron-containing pigment ++++.
Perls's Method X 95.

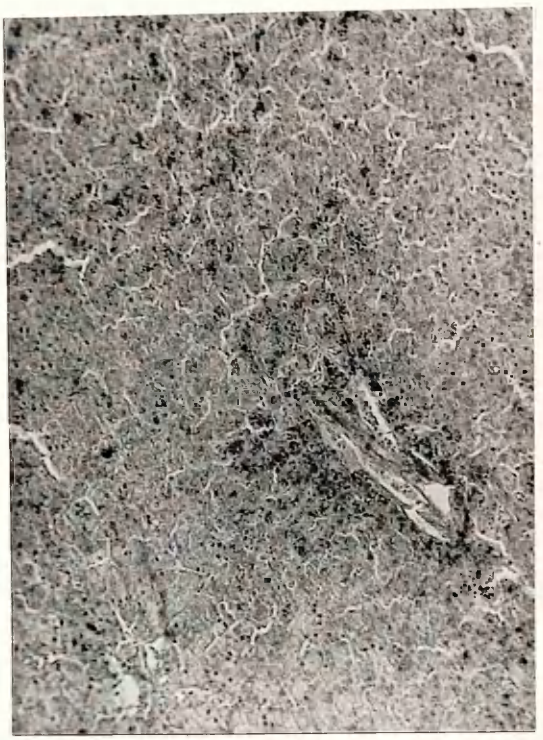


Fig. 85. Iron-containing pigment +++.
Perls's Method X 95.

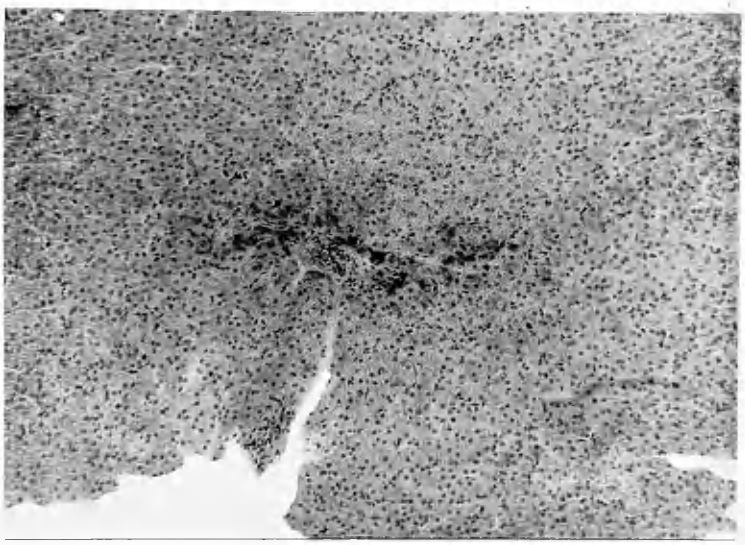


Fig. 86. Iron-containing pigment ++.
Perls's Method X 94.

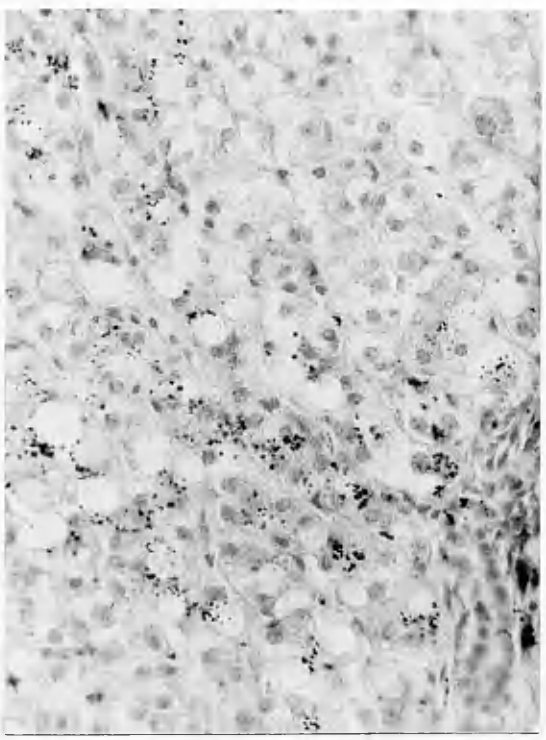


Fig. 87. Iron-containing pigment +.
Perls's Method X 294.

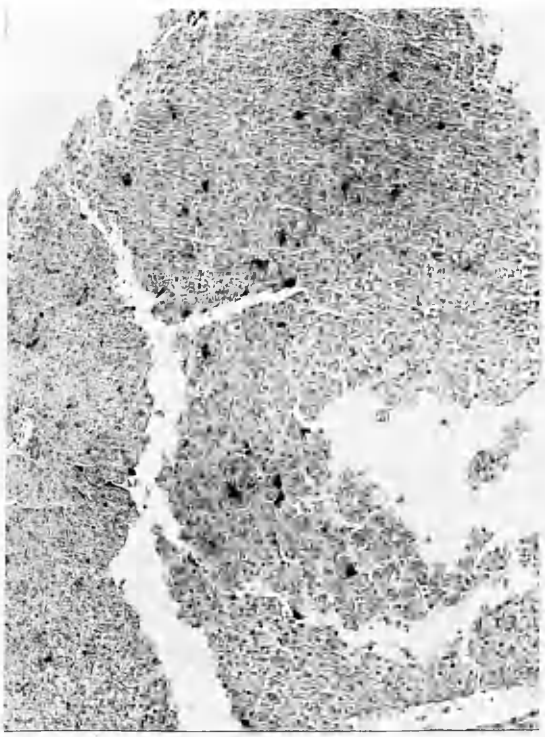


Fig. 88. Iron-containing pigment predominantly within sinusoidal cells.
Perls's method X 129.

TABLE XXI

Hepatic Siderosis related to Age, Gastro-enteritis and Parenteral Infection

Liver Iron	First biopsy						All biopsies	
	Number of cases	Age in weeks			Number with gastro-enteritis	Number with parenteral infection	Number of cases	Average age in weeks
		Youngest	Eldest	Mean				
++++	3	2	14	8.3	2	1	3	8.3
+++	17	2	21	9.8	15	5	32	10.9
++	54	4	39	19.1	39	28	101	19.1
+	37	11	44	26.3	30	20	96	27.3
±	23	17	66	30.5	16	16	51	30.2
0	36	15	87	44.5	21	15	80	40.7

TABLE XXII

Hepatic Siderosis related to the Mean Values obtained from Various Haematological Investigations

Liver iron	Haemoglobin g.%	R.B.C. x 10^6	P.C.V. ml.	M.C.V. μm^3	M.C.H.C. %	Reticulocytes % R.B.C.	Plasma protein g.%
++++	10.9	3.66	34.3	93.8	31.8	2.2	4.75
+++	10.3	3.20	34.1	112.7	30.3	2.8	4.86
++	10.7	3.72	38.6	95.6	28.2	1.7	5.43
+	10.3	4.91	35.6	81.5	28.2	0.8	5.56
$\pm$	10.3	5.26	37.3	77.0	27.6	0.6	5.42
0	8.9	4.86	35.1	77.2	26.1	0.8	5.77

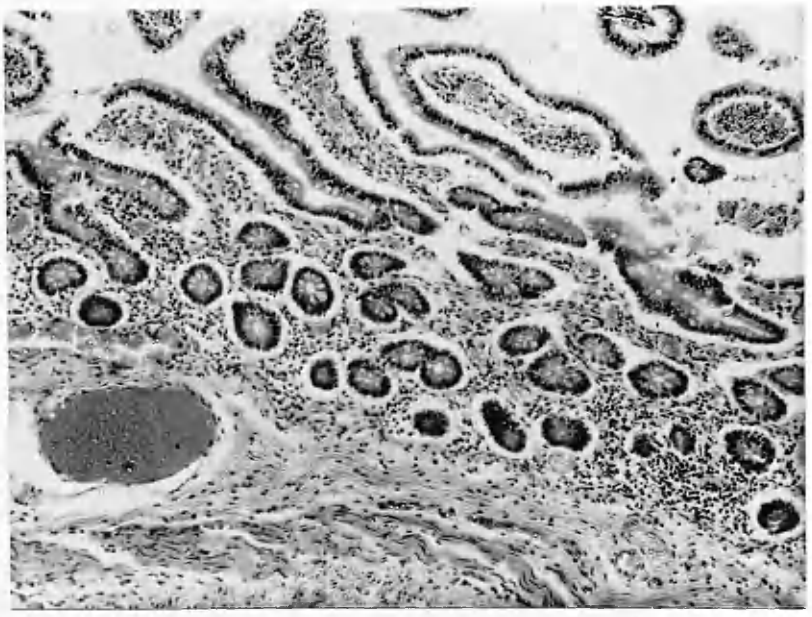


Fig. 89. Small intestine showing mild enteritis.
H. & E. X 90.

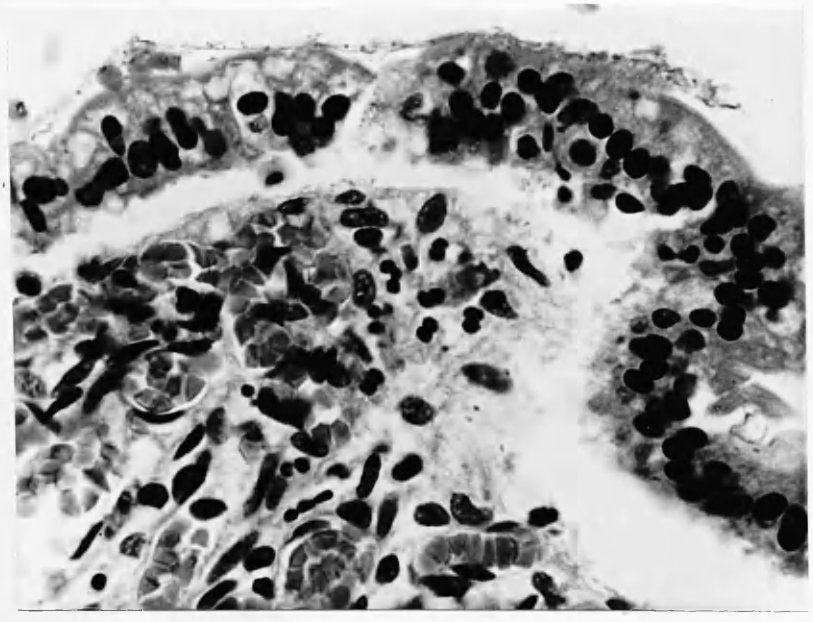


Fig. 90. Congestion and slight polymorphonuclear leucocytic infiltration of intestinal mucosa.
H. & E. X 630.

P A R T II

THE NATURE OF THE FIBROUS CONNECTIVE TISSUE

OF HEPATIC FIBROSIS AND CIRRHOSIS

TABLES XXIII to XXVIII

FIGURES 91 to 188

Fig. 91. Mouse liver with cirrhosis induced by 44 doses of CCl_4. Posterior aspect. X 2.

Fig. 92. Left: Liver from normal mouse.
Centre: Liver from mouse after 20 doses of CCl_4.
Right: Liver from mouse after 30 doses of CCl_4.
Anterior aspect and approximately normal size.

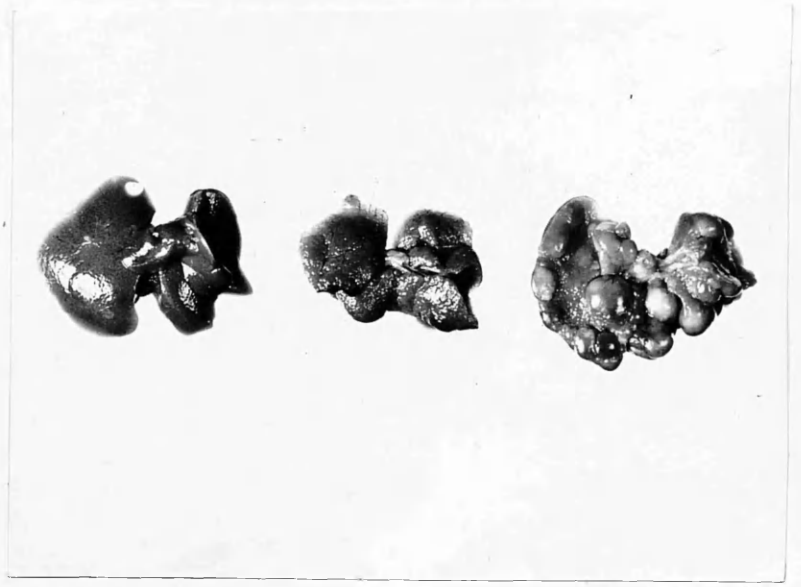


Fig. 93. As above. Posterior aspect of same livers.

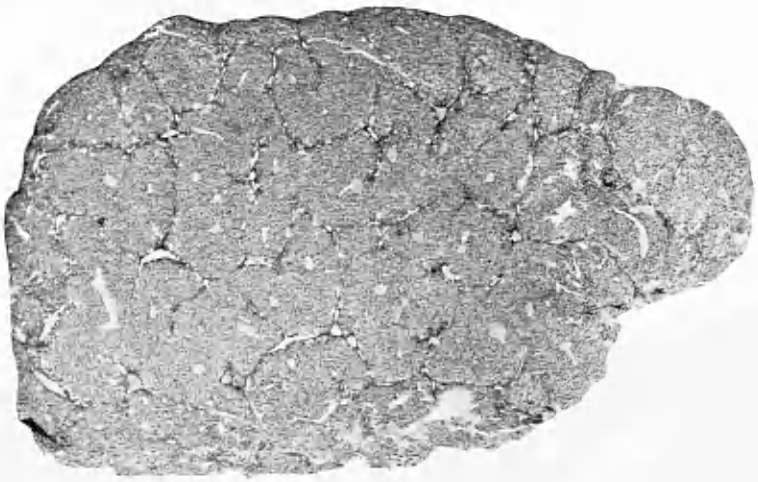


Fig. 94. Section from entire biopsy specimen of mouse liver with developing cirrhosis.
Van Gieson X 18.

Fig. 95. Established cirrhosis of mouse liver.
Reticulin Stain X 60.

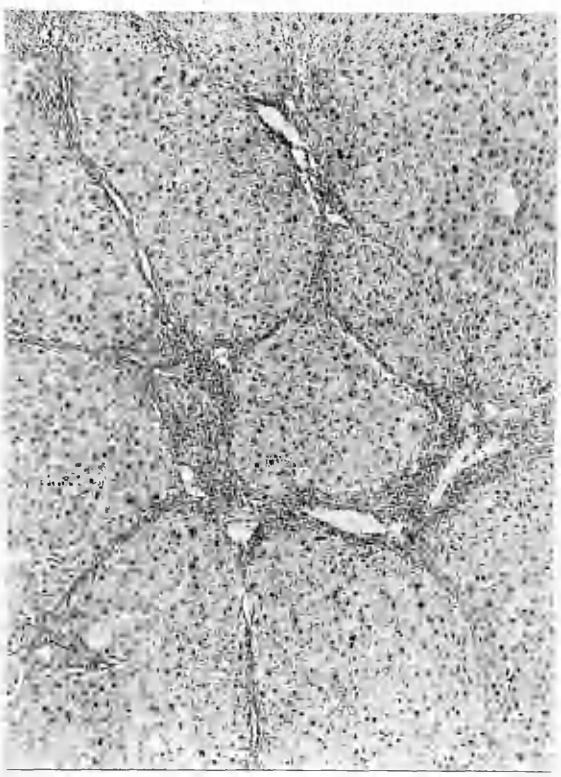


Fig. 96. The same stained by Van Gieson's method.
X 60.

Fig. 97. Fibrosis +.
Reticulin Stain X 70.

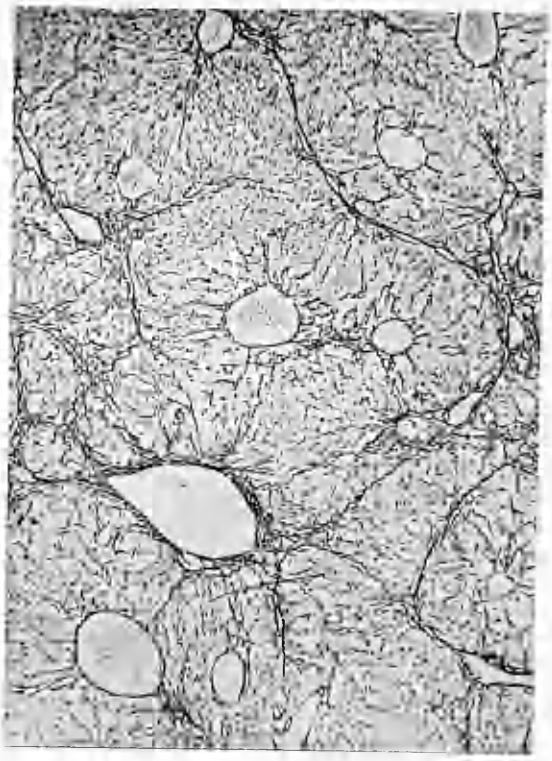


Fig. 98. Fibrosis ++.
Reticulin Stain X 70.

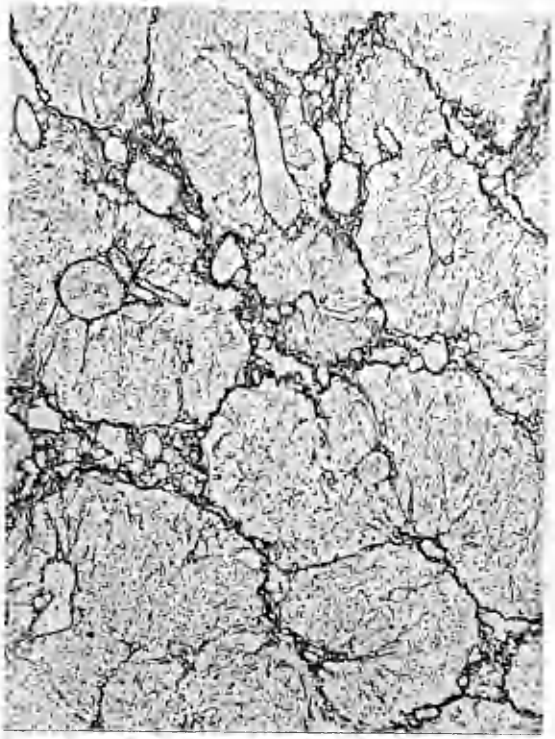


Fig. 99. Fibrosis +++.
Reticulin Stain X 70.

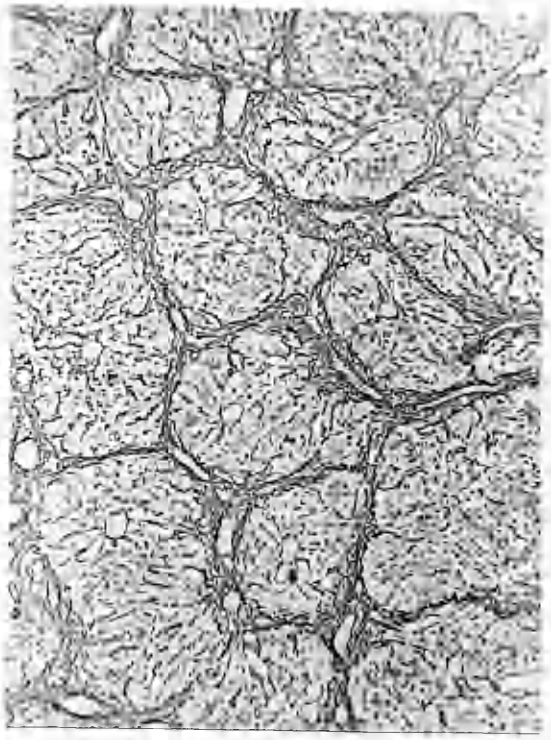


Fig. 100. Fibrosis ++++.
Reticulin Stain X 70.

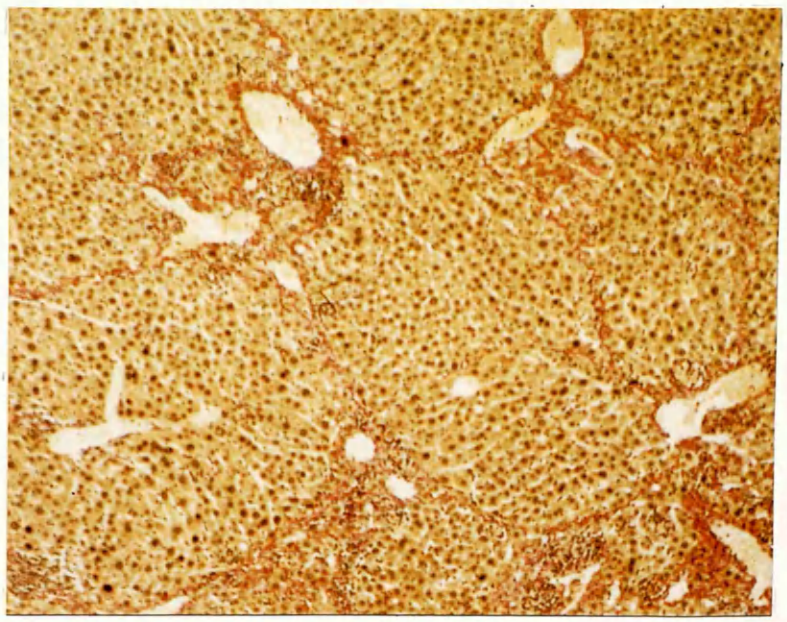


Fig. 101. Fibrosis ++.
Van Gieson X 65.

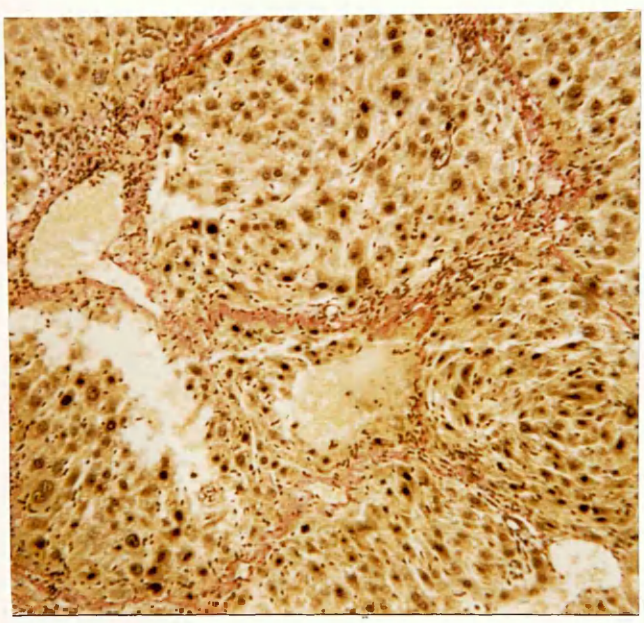


Fig. 102. Fibrosis +++.
Van Gieson X 150.

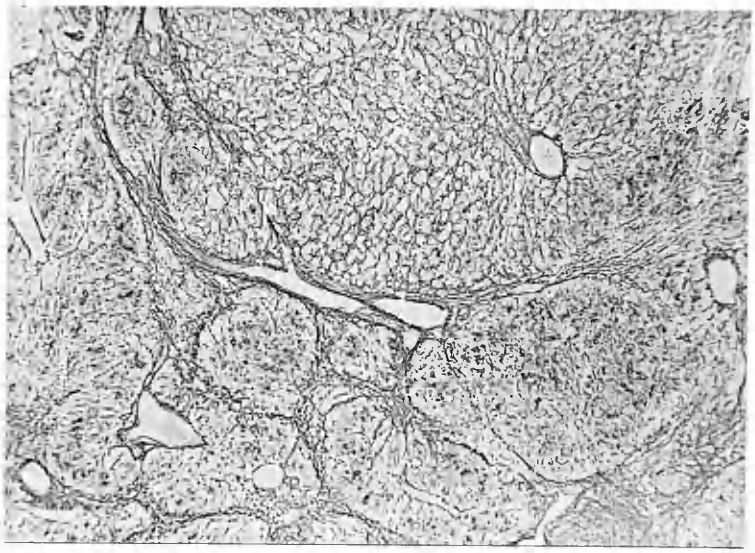


Fig. 103. Nodular hyperplasia +.
Reticulin Stain X 55.

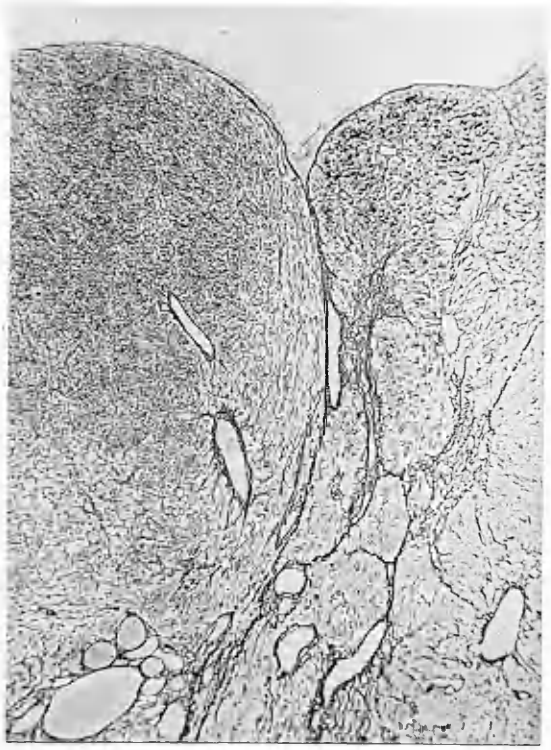


Fig. 104. Nodular hyperplasia ++.
Reticulin Stain X 40.

TABLE XXIII

Influence of Cortisone and ACTH on Development of CCl_4 Cirrhosis in Mice

Group	Numbers of animals	Numbers of examinations		
		Biopsy	Autopsy	Total
1. CCl_4 + Cortisone	42	15	42	57
2. CCl_4 + ACTH	26	12	26	38
3. CCl_4	91	80	91	171
4. Cortisone	11	18	11	29
5. ACTH	11	14	11	25

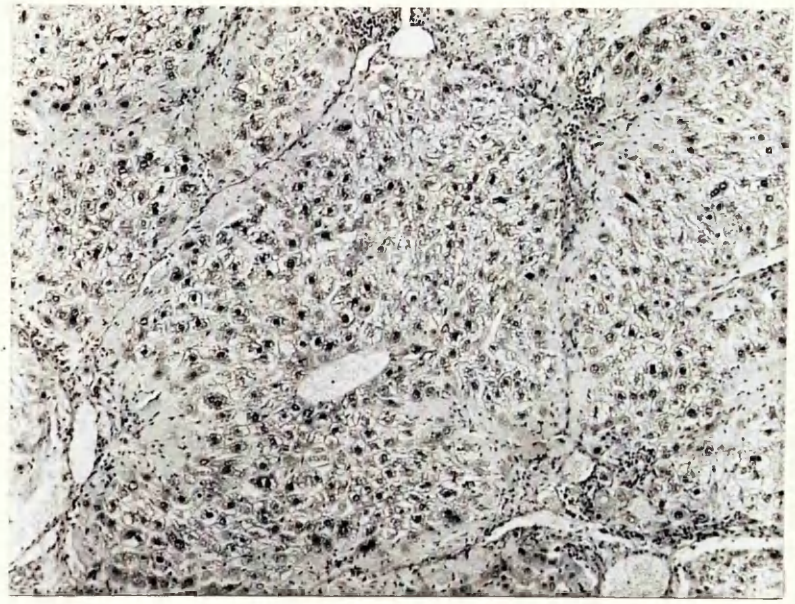


Fig. 105. CCl_4 necrosis and fibrosis, both affecting similar zones of liver.
Van Gieson X 90.

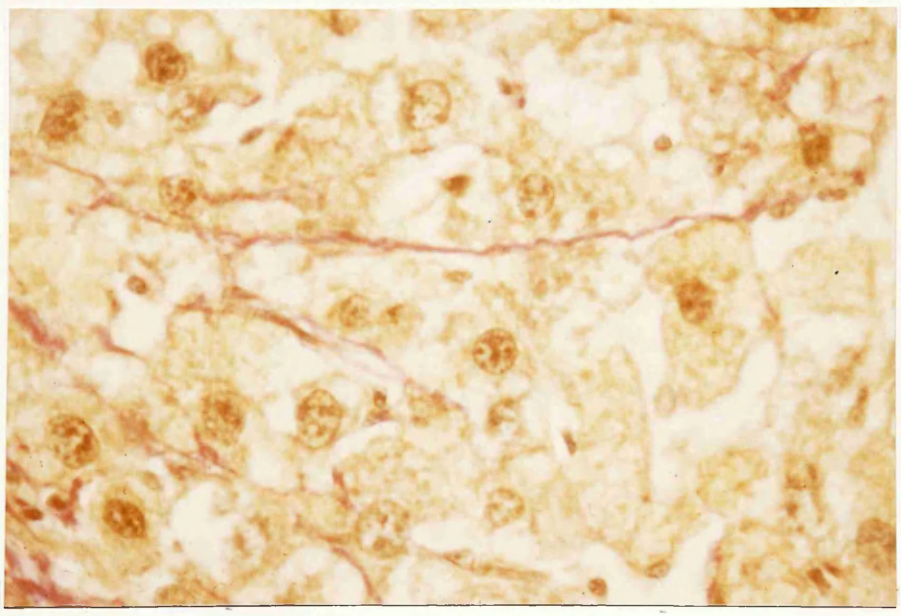


Fig. 106. Early hepatic fibrosis. Fibroblasts inconspicuous.
Van Gieson X 630.

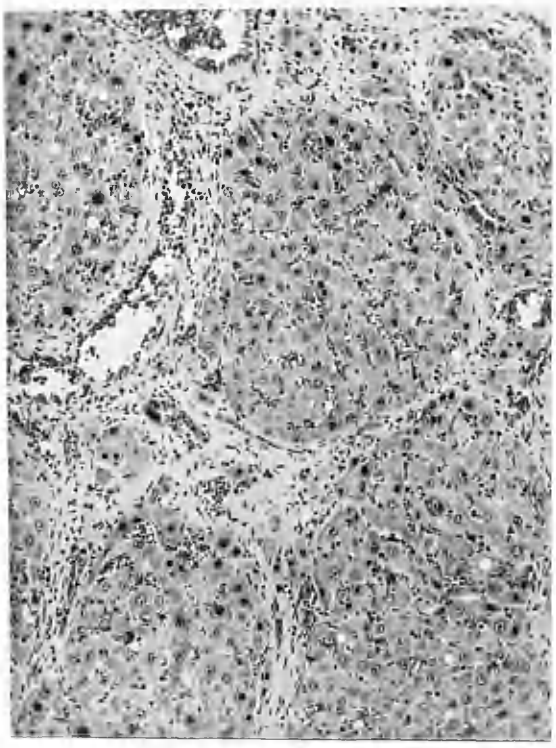


Fig. 107. Established CCl_4 cirrhosis. Mesenchymal cells in fibrotic areas presumably include fibroblasts.
H. & E X 100.

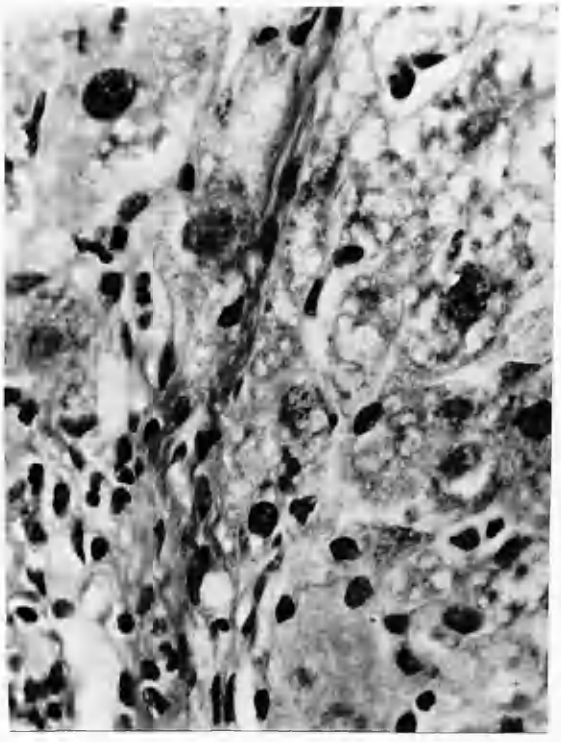


Fig. 108. Spindle-shaped cells associated with developing connective tissue fibres in cirrhotic liver. Van Gieson X 630.

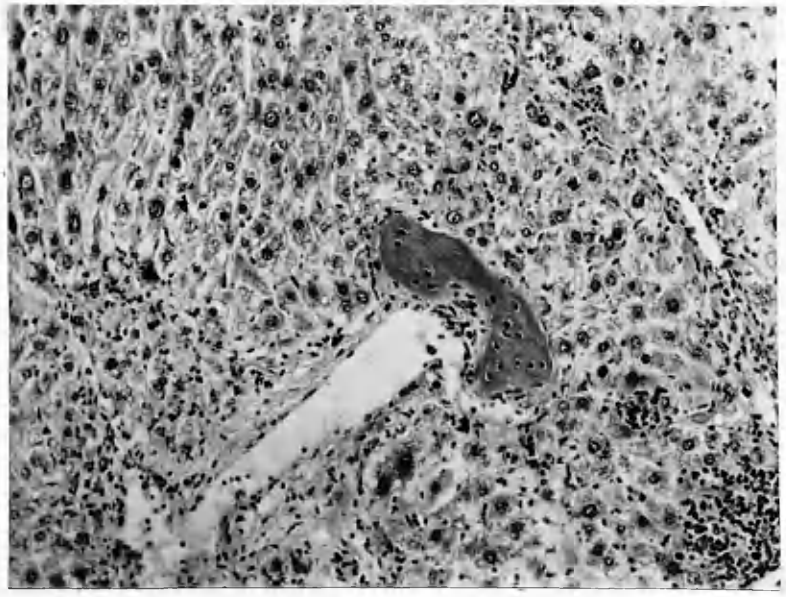


Fig. 109. Focus of osteoid metaplasia in cirrhotic mouse liver.
Van Gieson X 150.

FIG. 110 INFLUENCE OF CORTISONE AND ACTH ON THE DEVELOPMENT OF CCl_4 CIRRHOSIS

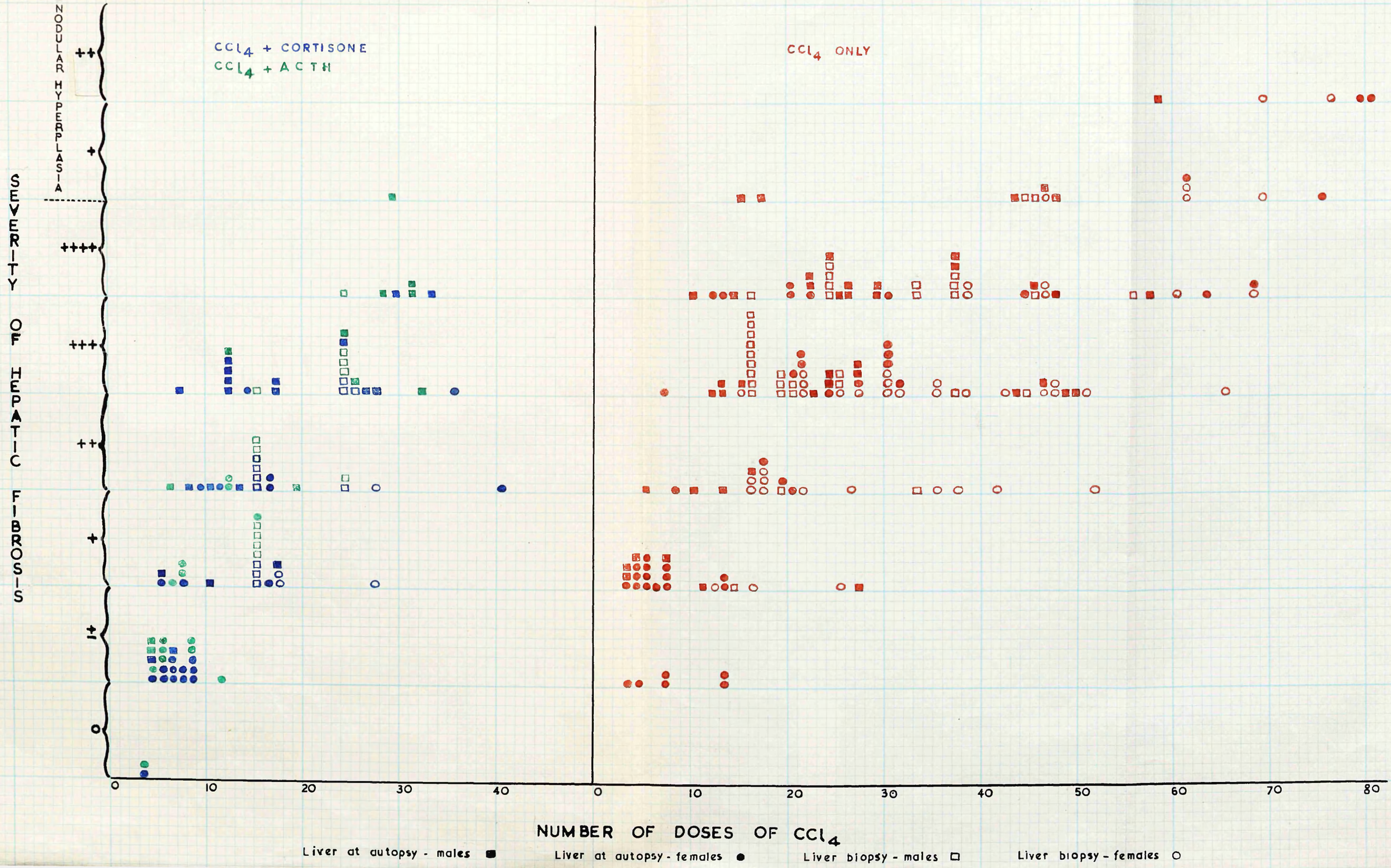


TABLE XXIV

Influence of Cortisone and ACTH on Development of CCl_4 Cirrhosis in Mice

Group	Numbers of repeat examinations	Comparison of 2nd with 1st examination			Comparison of all subsequent examinations with previous biopsies		
		Fibrosis Less	Fibrosis I.S.Q.	Fibrosis More	Fibrosis Less	Fibrosis I.S.Q.	Fibrosis More
1	15	0	1	9	3	3	9
2	12	0	0	6	0	2	10
3	80	3	2	23	12	23	45

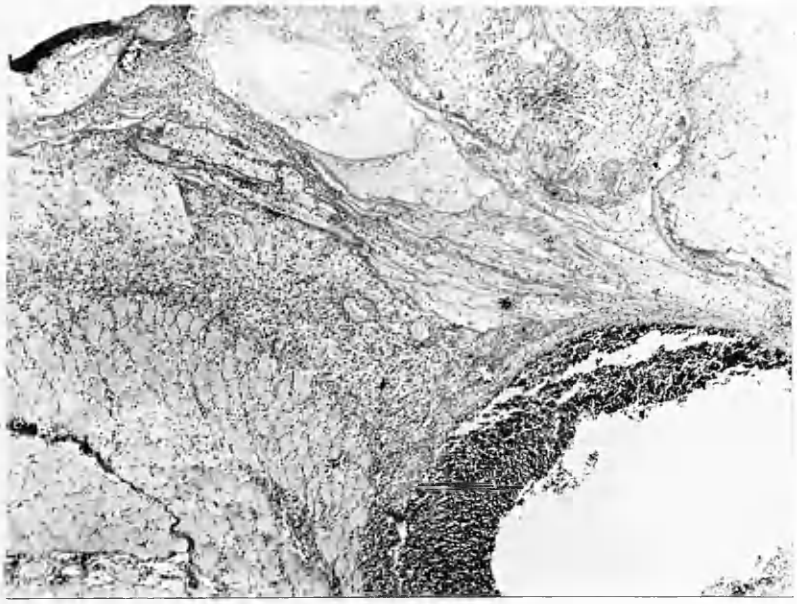


Fig. 111. 4-day laparotomy wound. Skin surface at top left-hand corner. Small stitch abscess at bottom right-hand corner.
Van Gieson X 60.

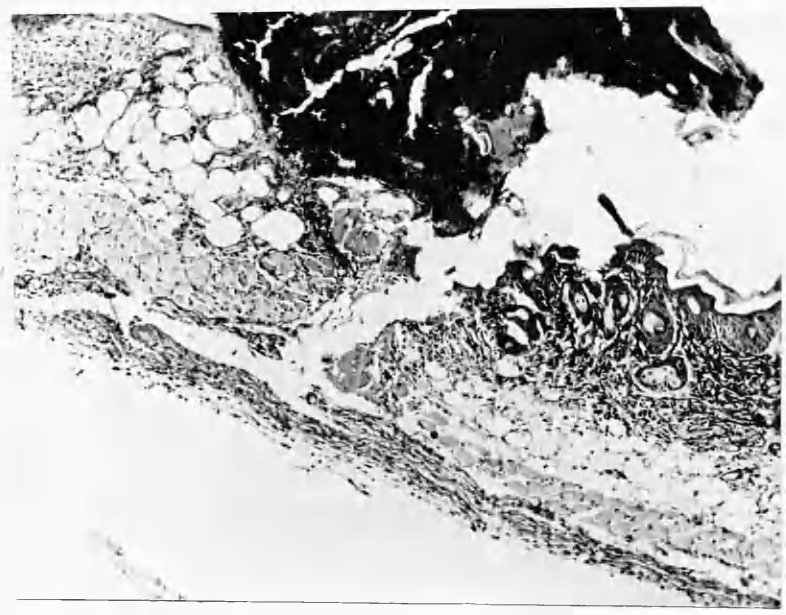


Fig. 112. 4-day laparotomy wound from cortisone-treated mouse. Skin surface with encrusted exudate at top right-hand corner.
Van Gieson X 90.

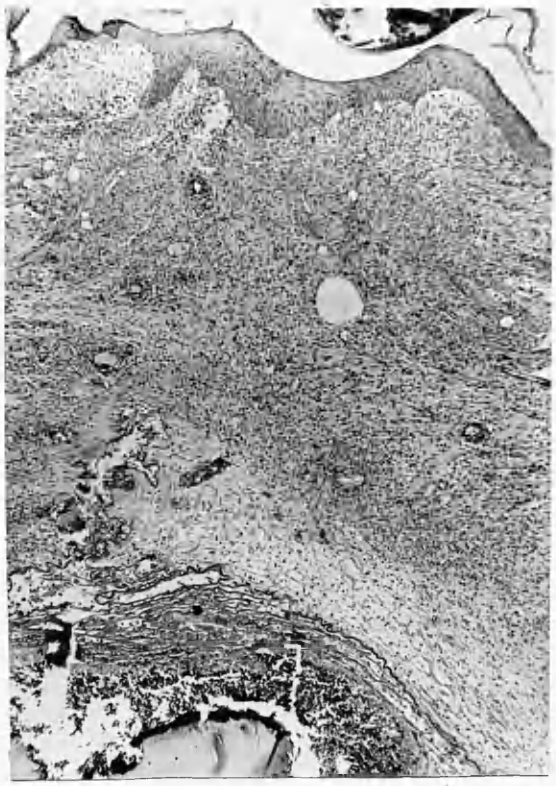


Fig. 113. 7 day laparotomy wound. Catgut at bottom of field.
Van Gieson X 60.

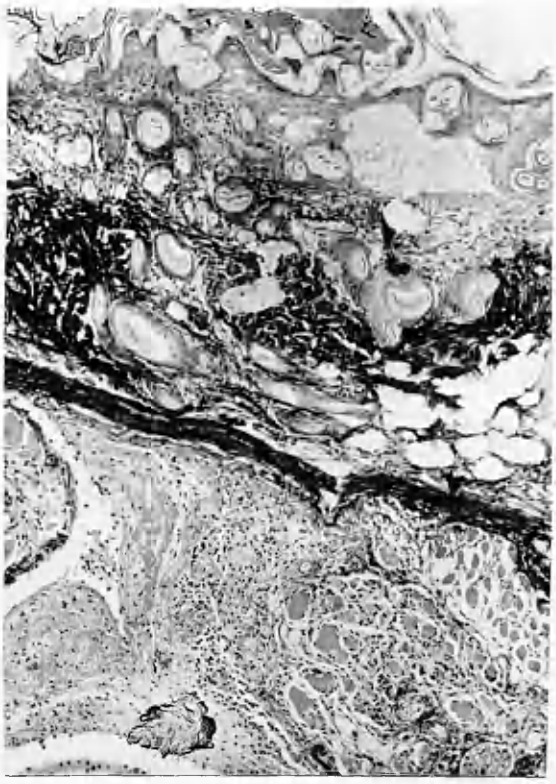


Fig. 114. 7 day laparotomy wound from cortisone-treated mouse.
Van Gieson X 60.

FIG. 115 INFLUENCE OF CORTISONE ON ESTABLISHED CCl_4 CIRRHOSIS

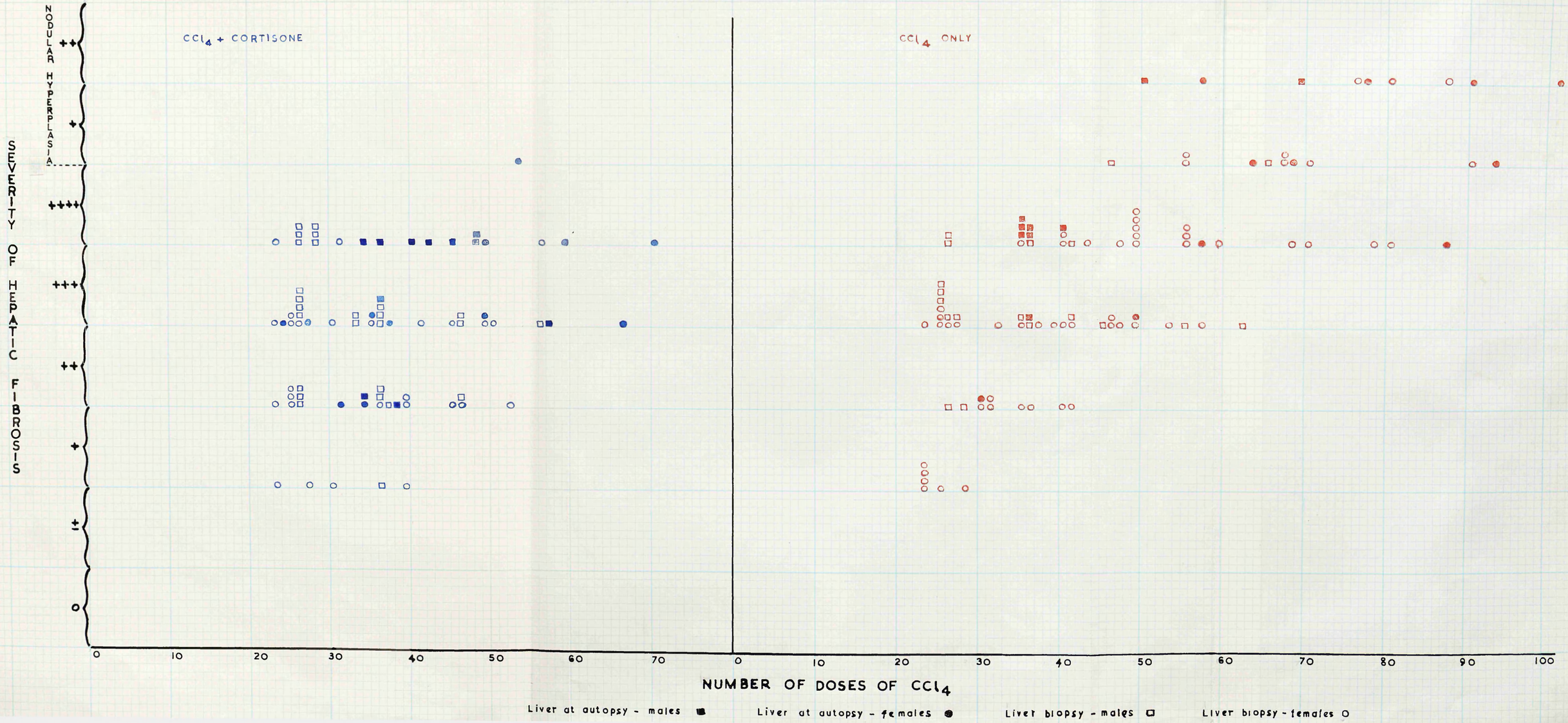


TABLE XXVI

Influence of Cortisone on Established CCl_4 Cirrhosis

Group	Comparison of 2nd with 1st examination			Comparison of all subsequent examinations with previous biopsies		
	Fibrosis Less	Fibrosis I.S.Q.	Fibrosis More	Fibrosis Less	Fibrosis I.S.Q.	Fibrosis More
6	11	11	6	12	13	20
7	1	7	19	1	29	41

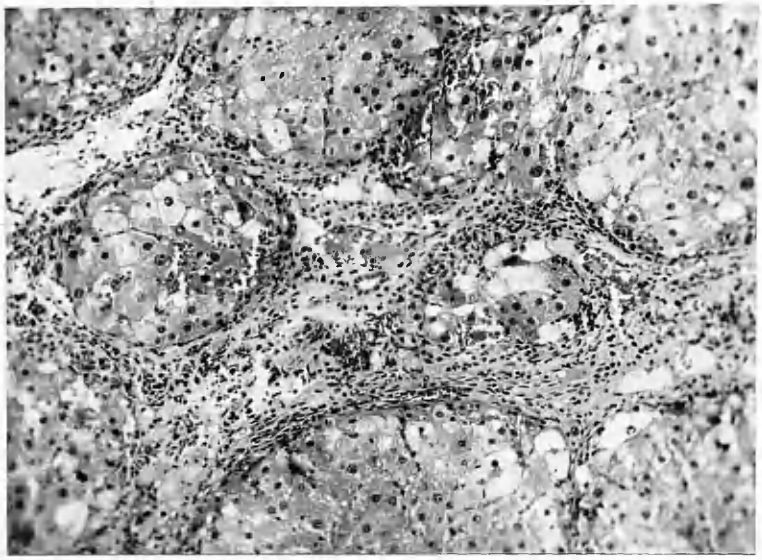


Fig. 116. Established CCl_4 cirrhosis. No cortisone or A.C.T.H.

H. & E. X 100.

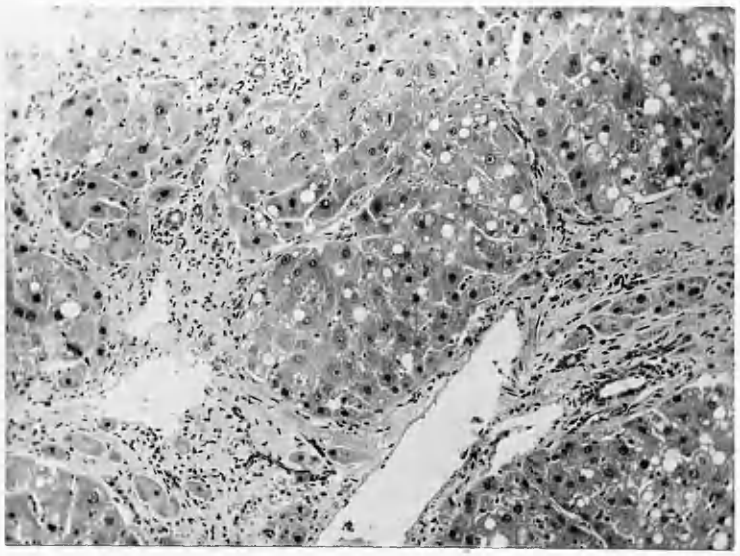


Fig. 117. Established CCl_4 cirrhosis. Daily cortisone injections.

H. & E. X 100.

FIG. 118 INFLUENCE OF CORTISONE ON THE REGRESSION OF CCl_4 CIRRHOSIS

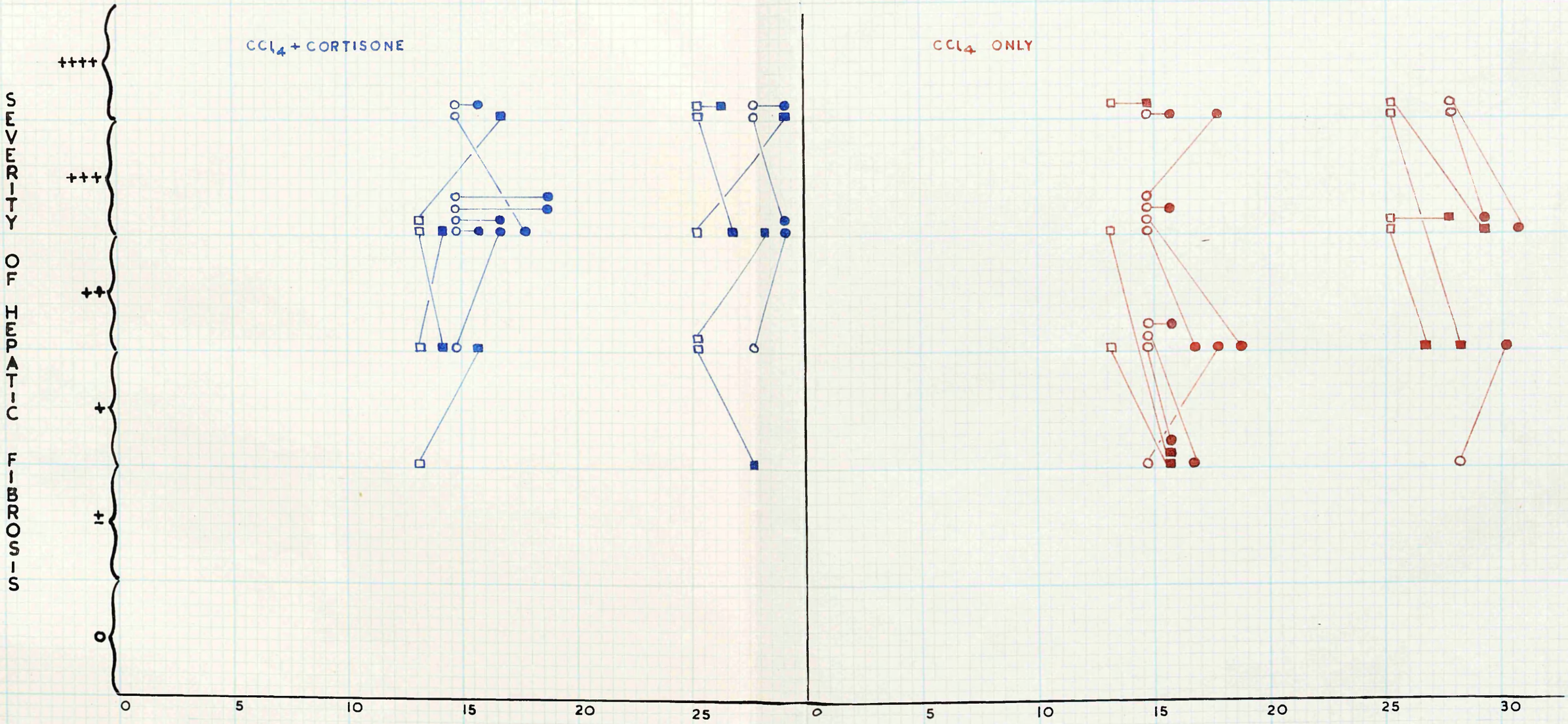


TABLE XXVII

Influence of Cortisone on Regression of CCl_4 Cirrhosis in Mice

Group	Numbers of animals	Comparison of autopsy with previous biopsy specimen (interval of 2 weeks)		
		Fibrosis Less	Fibrosis I.S.Q.	Fibrosis More
8. $CCl_4 \rightarrow$ Cortisone	19	5	7	7
9. $CCl_4 \rightarrow$ No hormone	19	11	5	3

TABLE XXVIII

Numbers of Mice with Cat-gut Implanted in Liver ± CCl_4 Cirrhosis

Group	CCl_4	Hormone	Cat-gut implantation - Duration in days										Total
			2	3	4	5	7	10	14	21	28	No. C-G	
10	Nil	Cortisone	1	0	1	0	1	0	2	2	2	0	9
11	Nil	ACTH	1	0	0	0	1	0	2	0	0	0	4
12	Nil	-	1	0	1	0	1	0	3	2	1	0	9
3 & 13	5 months	-	0	3	0	0	4	4	2	0	0	9	22
13	6 months	-	1	0	1	0	1	0	0	0	0	0	3
13	7 months	-	0	0	0	2	2	3	2	0	0	0	9
		Total:	4	3	3	2	10	7	11	4	3	9	56

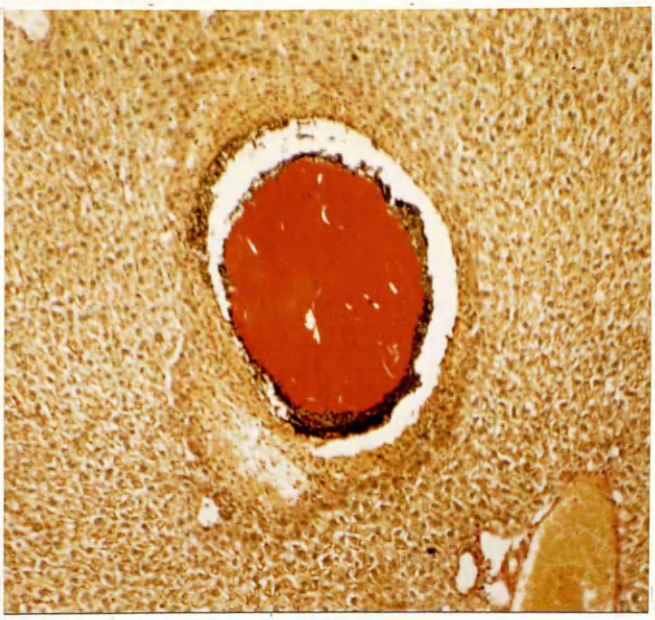


Fig. 119. Catgut implanted in liver for 1 day.
Van Gieson X 70.

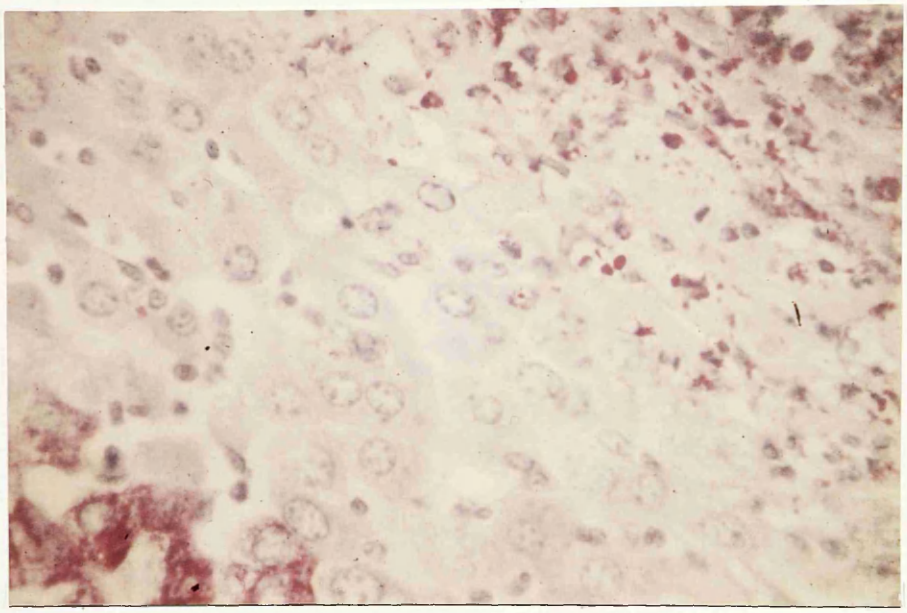


Fig. 120. Catgut implanted 1 day. Top right corner towards centre of lesion. Normal glycogen-laden liver cells at bottom left corner.
Periodic acid-Schiff reagent X 510.

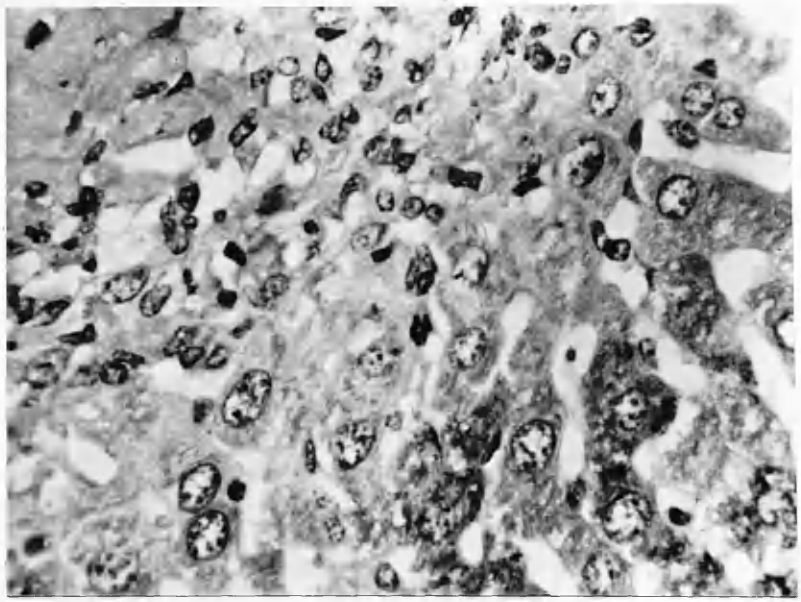


Fig. 121. Catgut implanted 2 days. Zone of proliferating connective tissue cells. Top left-hand corner nearest centre of lesion. P.A.S. X 630.

Fig. 122. Catgut implanted 3 days.

Van Gieson X 85.

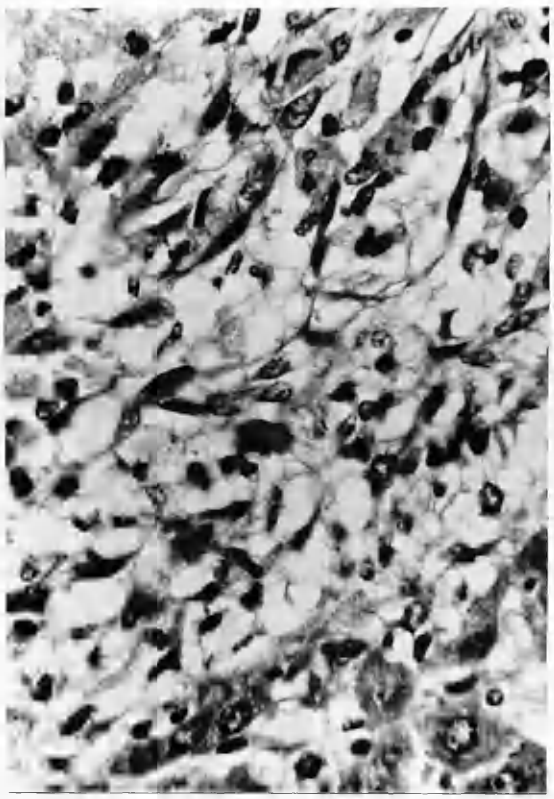


Fig. 123. Catgut implanted 3 days. Young fibroblasts in wall of lesion. P.A.S. X 630.

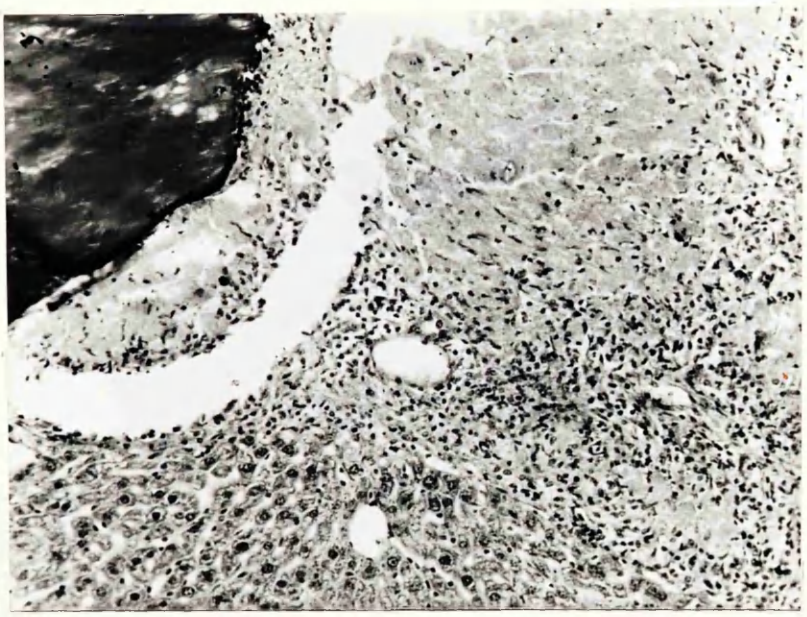


Fig. 124. Catgut implanted 4 days.
Van Gieson X 150.

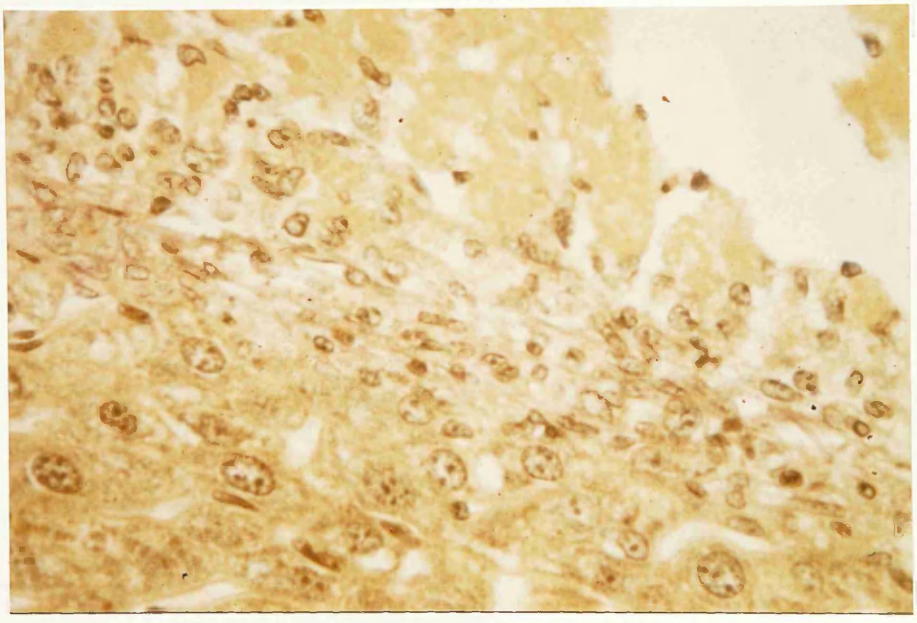


Fig. 125. Catgut implanted 4 days. Faint trace of fuchsinophil material between proliferating connective tissue cells.

Van Gieson X 525.

Fig. 126. Catgut implanted 7 days.
Van Gieson X 85.

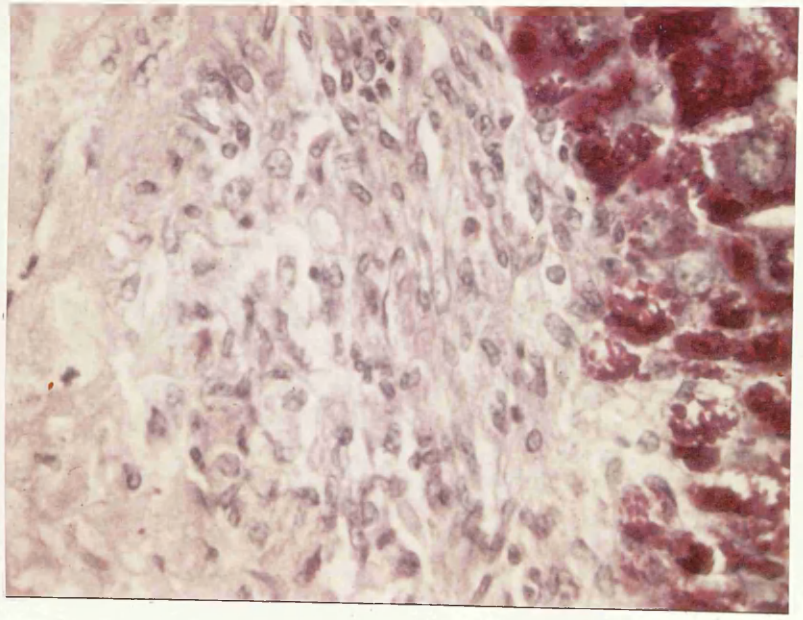


Fig. 127. Catgut implanted 7 days.
P.A.S. X 550.

Fig. 128. Catgut implanted 10 days.
Van Gieson X 85.

Fig. 129. Catgut implanted 10 days.
Reticulin Stain X 90.

Fig. 130. Catgut implanted 10 days.
P.A.S. X 110.

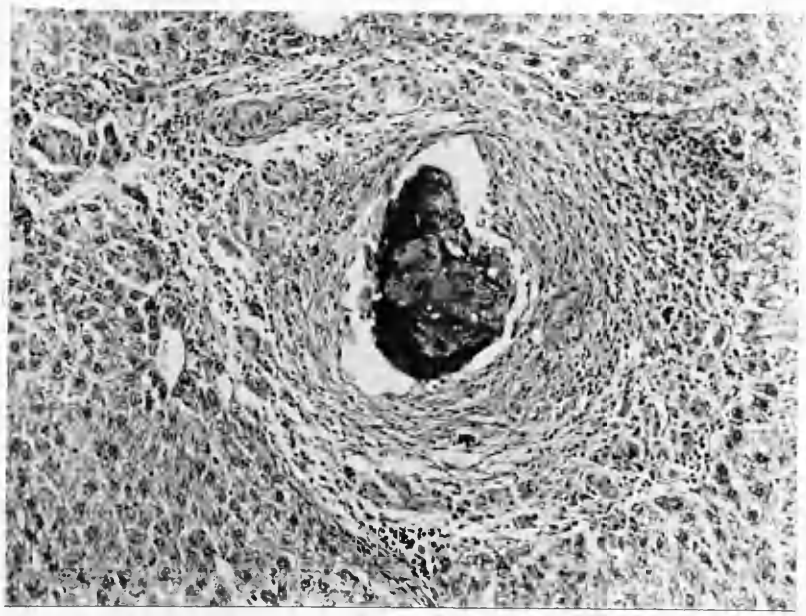


Fig. 131. Catgut implanted 14 days.
Van Gieson X 150.

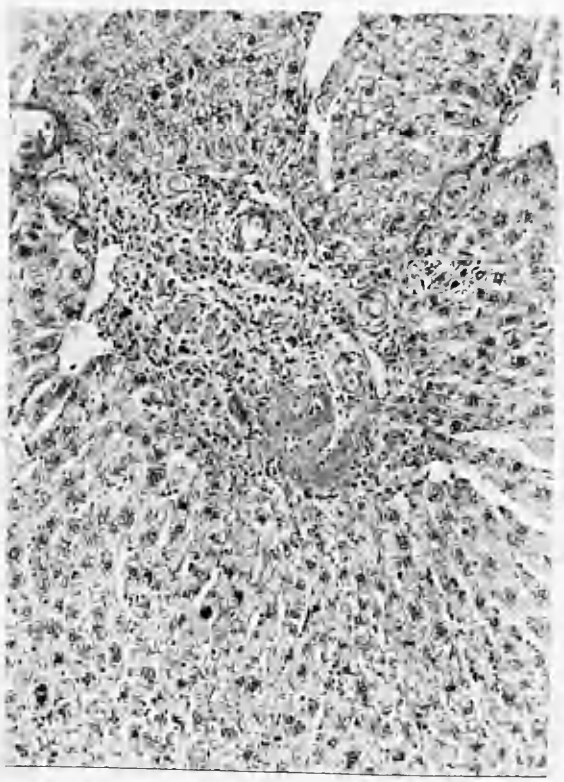


Fig. 132. Catgut implanted 21 days.
Van Gieson X 150.

Fig. 133. Catgut implanted 21 days.
Giant cell reaction around fragments of foreign material.
H. & E. X 150.

Fig. 134. Catgut implanted 1 day. Daily cortisone injection.
Van Gieson X 85.

Fig. 135. Catgut implanted 2 days. Daily cortisone injection.
Van Gieson X 120.

Fig. 136. Catgut implanted 3 days. Daily cortisone injections.
Van Gieson X 85.

Fig. 137. Catgut implanted 3 days. Daily cortisone injections.
Reticulin Stain X 85.

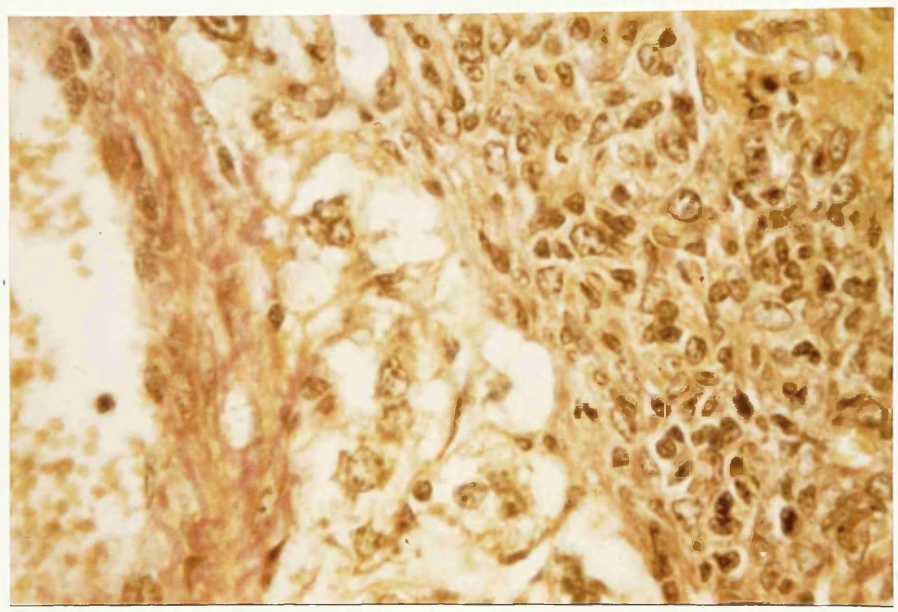


Fig. 138. Catgut implanted 3 days. Daily cortisone injections. Vessel wall on left. Faint trace of fuchsinophil fibres at margin of lesion on right.
Van Gieson X 525.

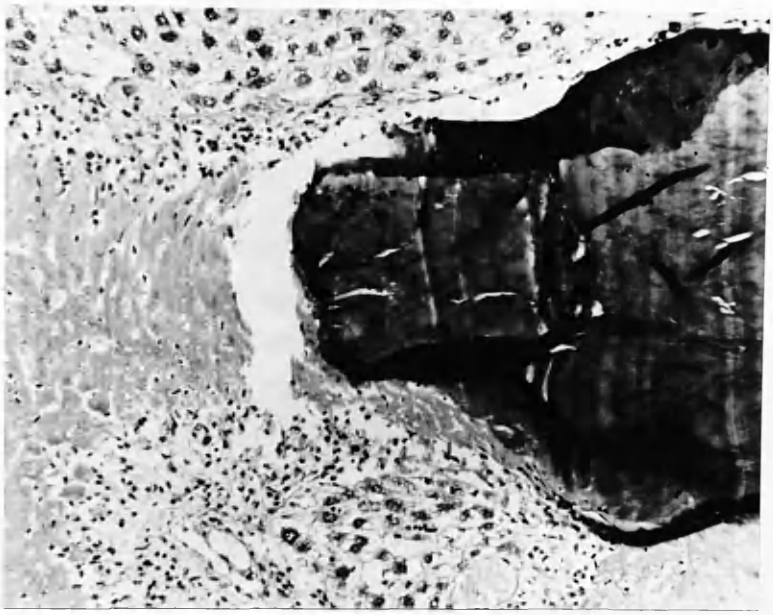


Fig. 139. Catgut implanted 4 days. Daily cortisone injections. Van Gieson X 150.

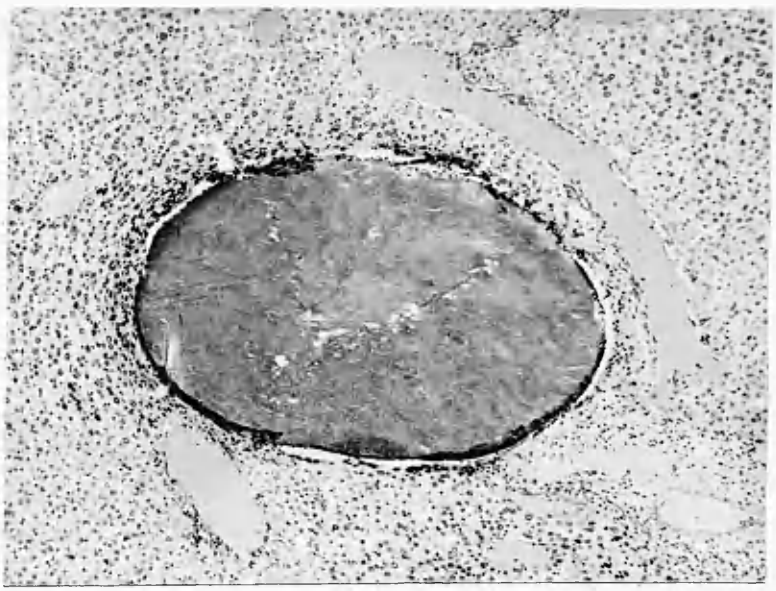


Fig. 140. Catgut implanted 7 days. Daily cortisone injections. Van Gieson X 85.

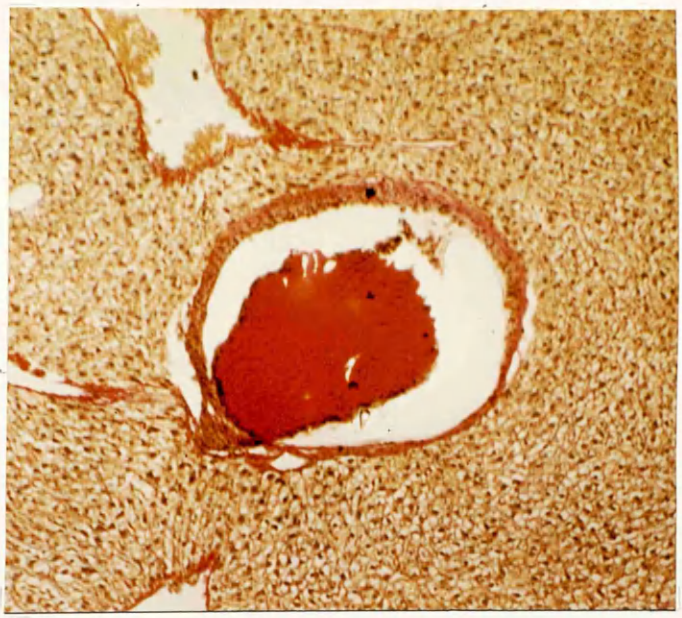


Fig. 141. Catgut implanted 14 days. Daily cortisone injections. Van Gieson X 70.

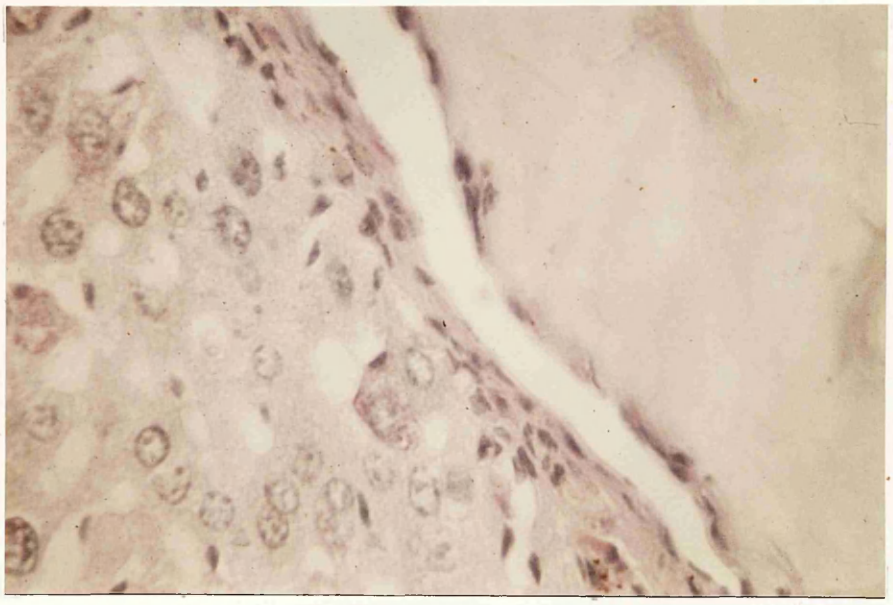


Fig. 142. Catgut implanted 14 days. Daily cortisone injections. Unabsorbed catgut on right.
P.A.S. X 510.

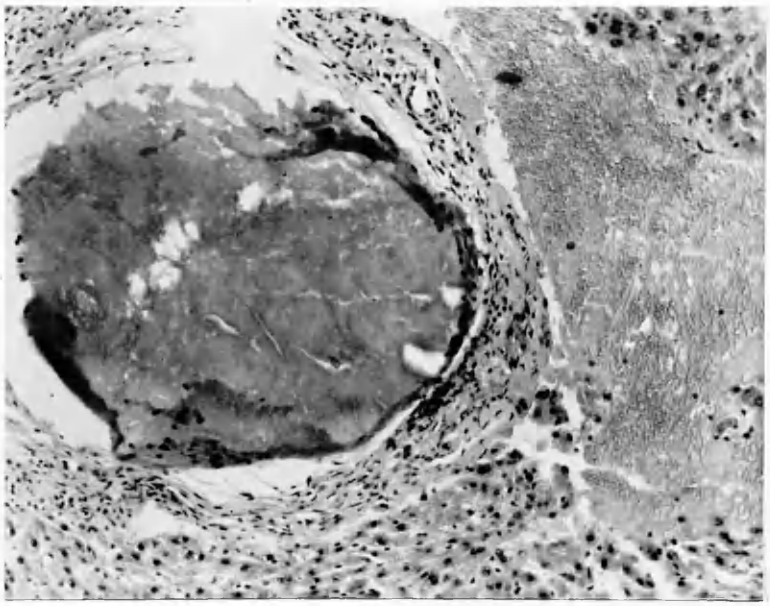


Fig. 143. Catgut implanted 21 days. Daily cortisone injections. H. & E. X 150.

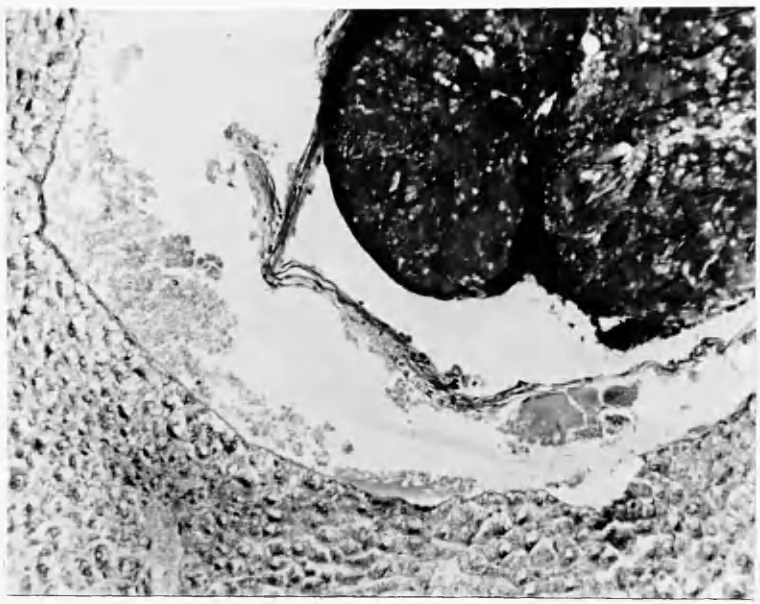


Fig. 144. Catgut implanted 28 days. Daily cortisone injections. Van Gieson X 150.

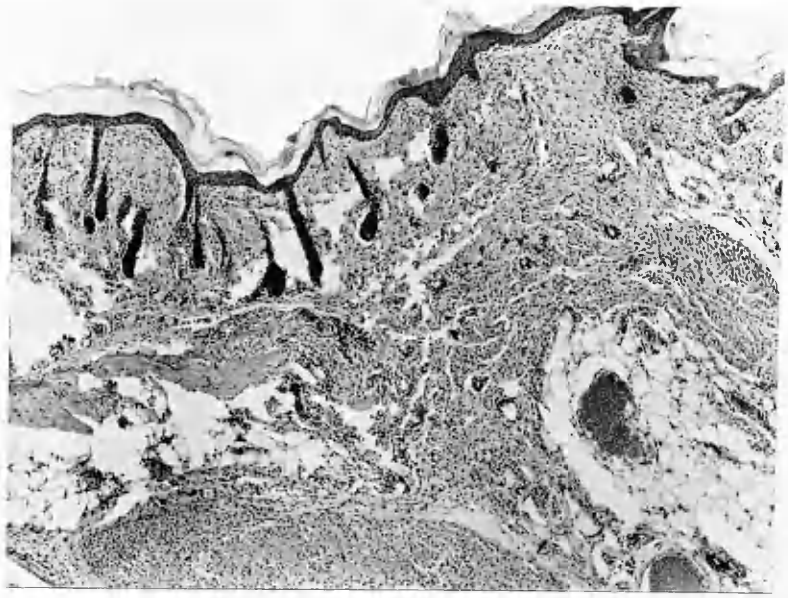


Fig. 145. Laparotomy wound 14 days after operation. Daily cortisone injections. H. & E. X 60.

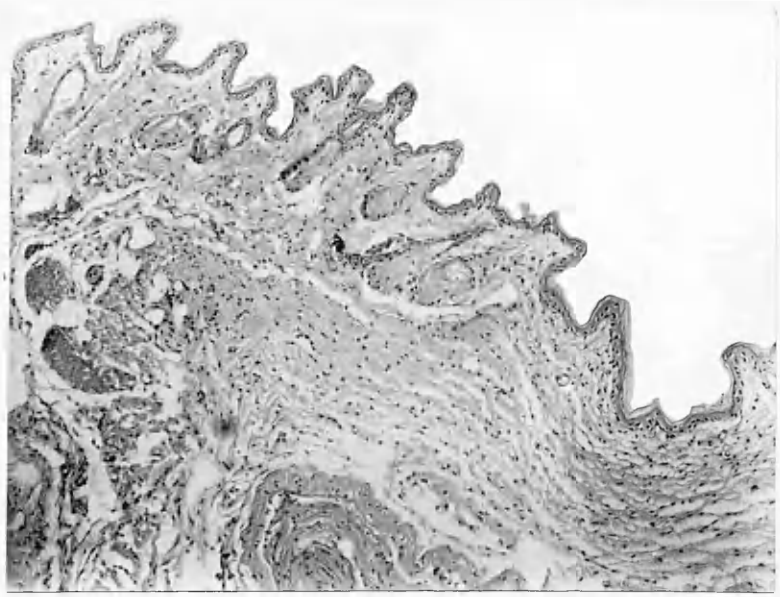


Fig. 146. Laparotomy wound 28 days after operation. Daily cortisone injections. H. & E. X 90.

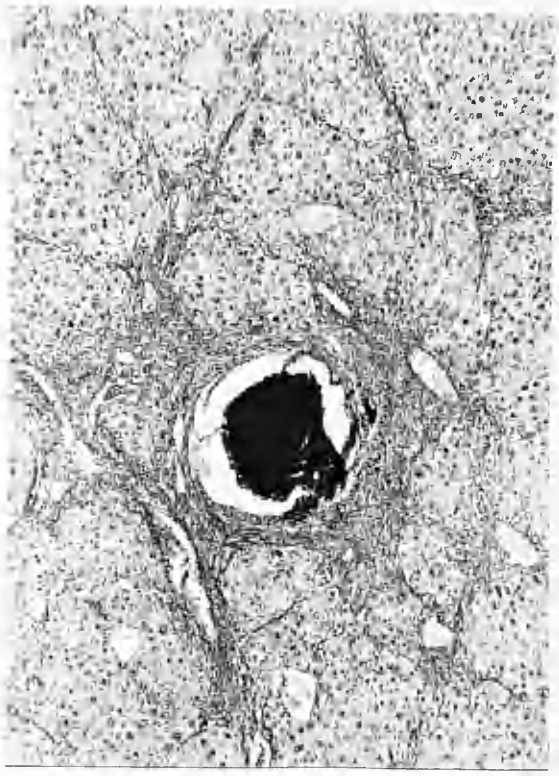


Fig. 147. Catgut implanted 7 days. CCl_4 cirrhosis. Van Gieson X 60.

Fig. 148. The same as shown in figure 147 stained to show reticulin. X 60.

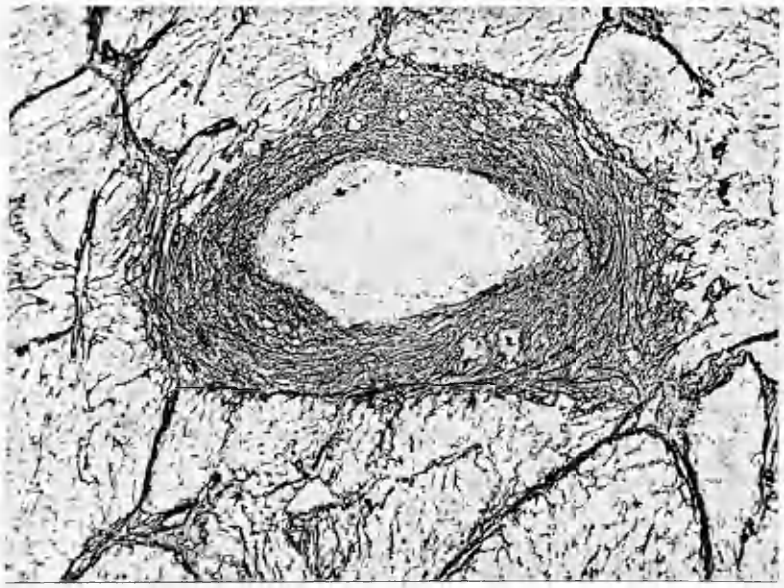


Fig. 149. Catgut implanted 10 days. CCl_4 cirrhosis. Reticulin Stain X 48.

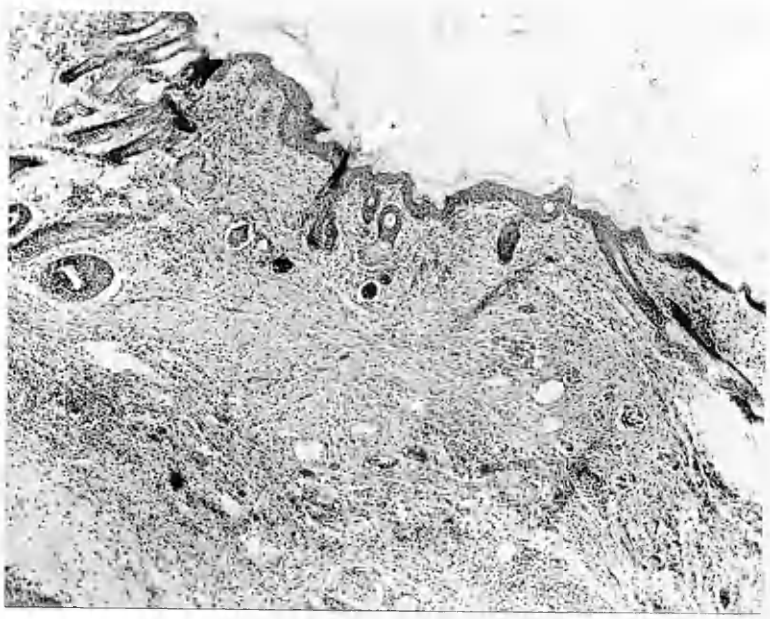


Fig. 150. Laparotomy wound 14 days after operation.
H. & E. X 60.

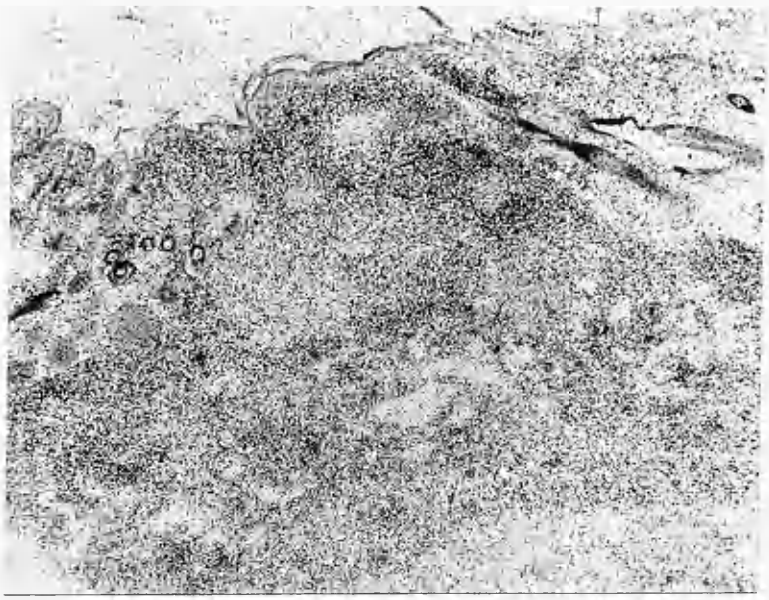


Fig. 151. Coarse-grain autoradiograph of same lesion.
X 60.

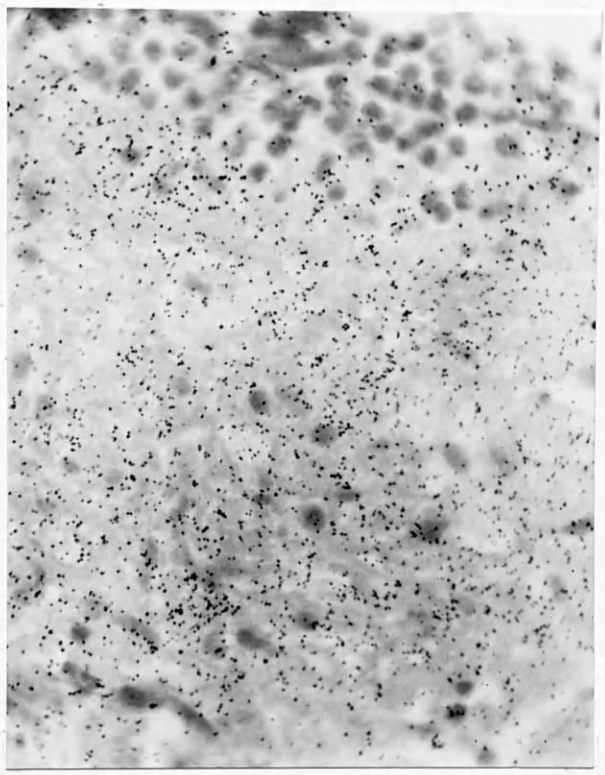


Fig. 152. Fine-grain autoradiograph of healing laparotomy wound. Acute inflammatory cells at top of photograph show very little sulphate uptake.
Fine-grain autoradiograph X 630.

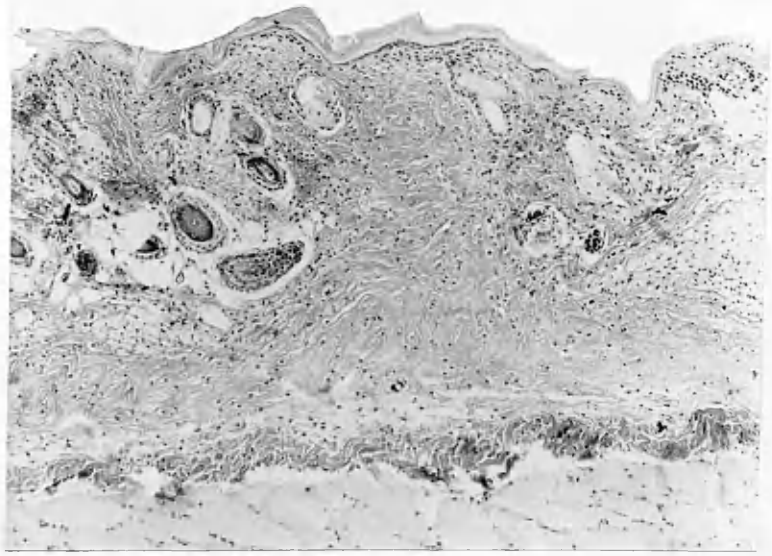


Fig. 153. Laparotomy wound 21 days after operation. Daily cortisone injections. H. & E. X 90.

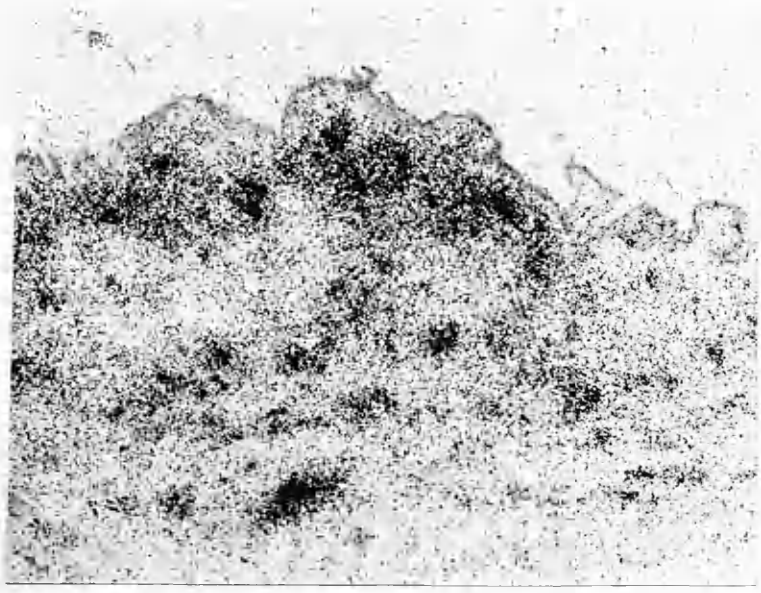


Fig. 154. Coarse-grain autoradiograph of same lesion. X 90.

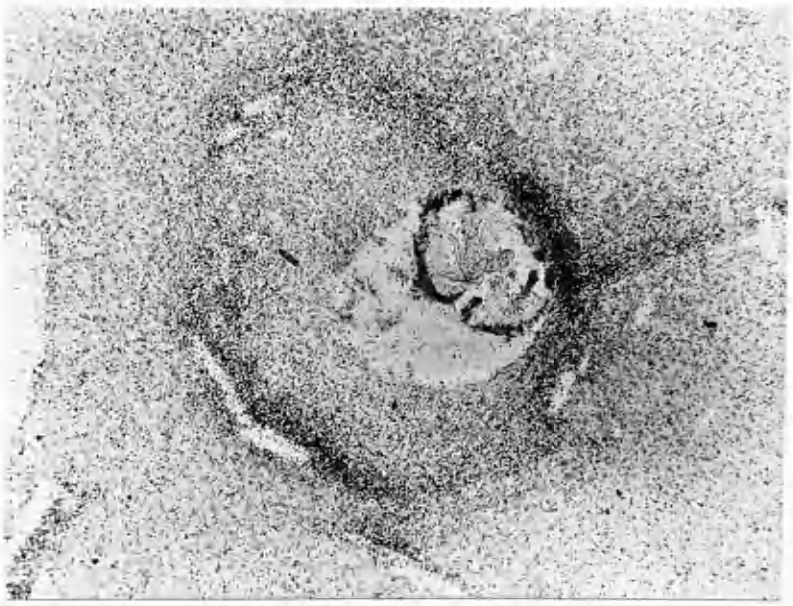


Fig. 155. Catgut implanted 2 days.
Coarse-grain autoradiograph X 60.

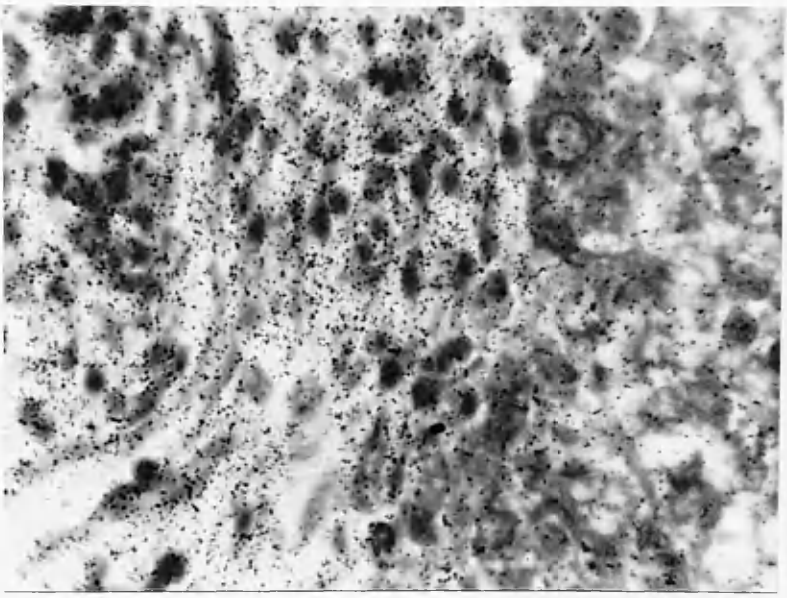


Fig. 156. Catgut implanted 2 days. Normal liver on right.
Fine-grain autoradiograph X 630.

Fig. 157. Catgut implanted 7 days.
Coarse-grain autoradiograph X 60.

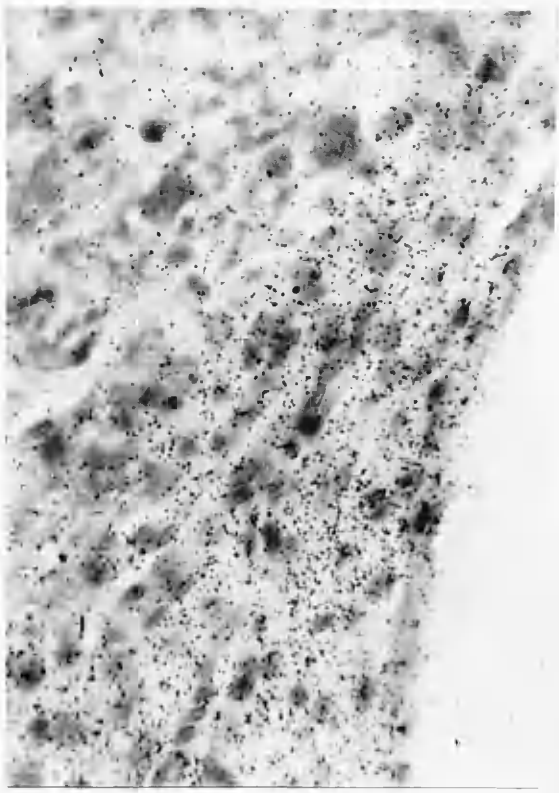


Fig. 158. Catgut implanted 7 days.
Margin of lesion on right.
Fine-grain autoradiograph X 630.

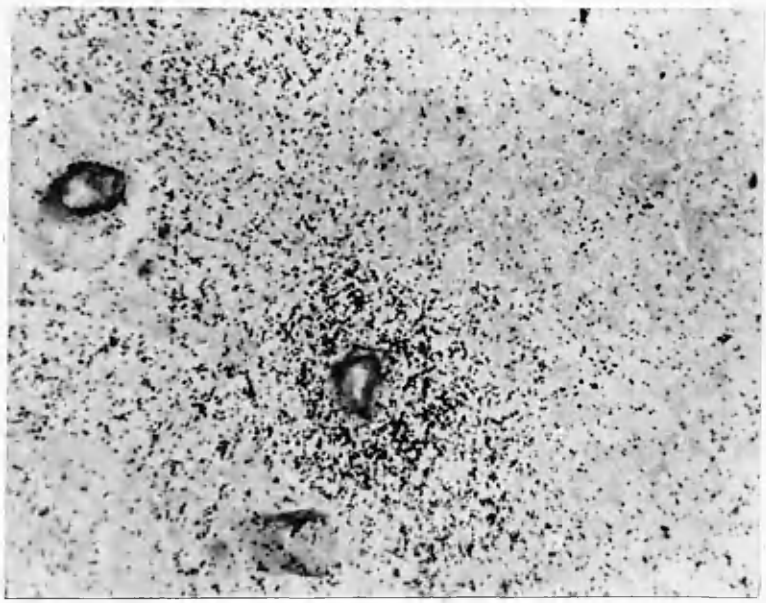


Fig. 159. Catgut implanted 21 days.
Coarse-grain autoradiograph X 630.

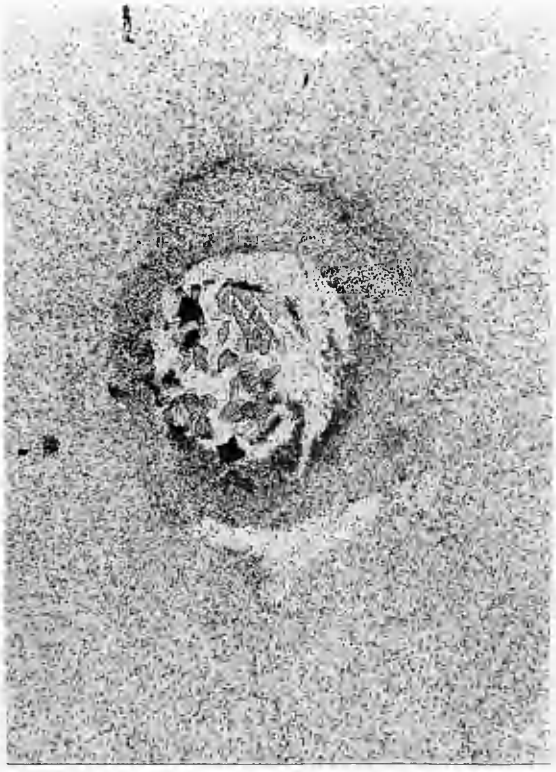


Fig. 160. Catgut implanted 2 days. Daily cortisone injections.
Coarse-grain autoradiograph X 60.

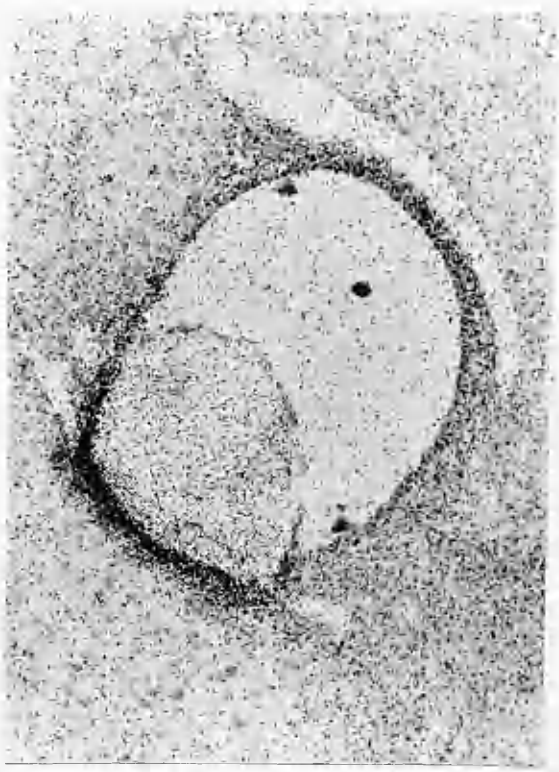


Fig. 161. Catgut implanted 7 days. Daily cortisone injections.
Coarse-grain autoradiograph X 90.

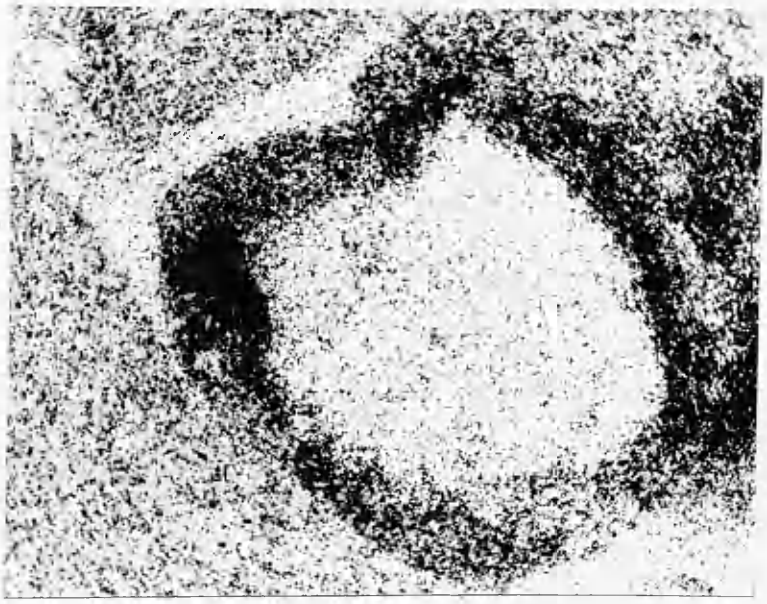


Fig. 162. Catgut implanted 14 days. Daily A.C.T.H. injections.
Coarse-grain autoradiograph X 150.

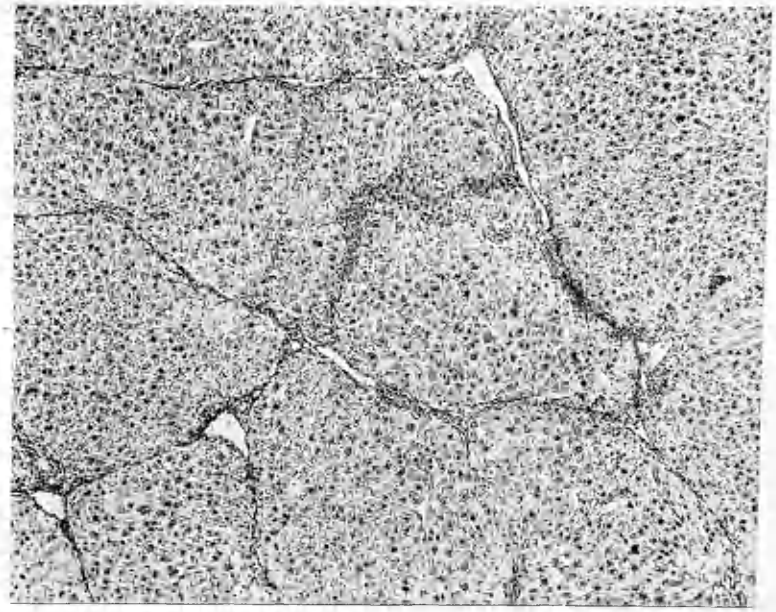


Fig. 163. CCl_4 cirrhosis induced over period of 20 weeks.
Van Gieson X 60.

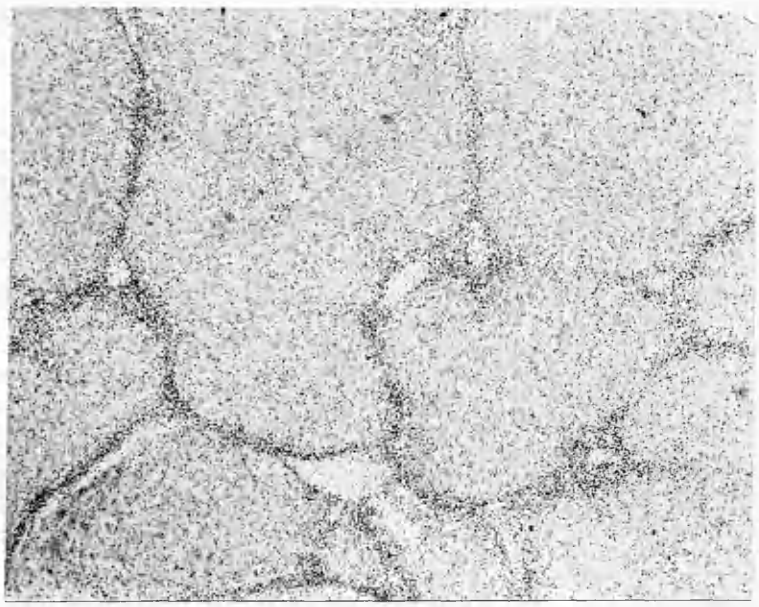


Fig. 164. Coarse-grain autoradiograph from same liver (not adjacent sections). X 60.

Fig. 165. CCl_4 cirrhosis. Coarse-grain autoradiograph X 120.

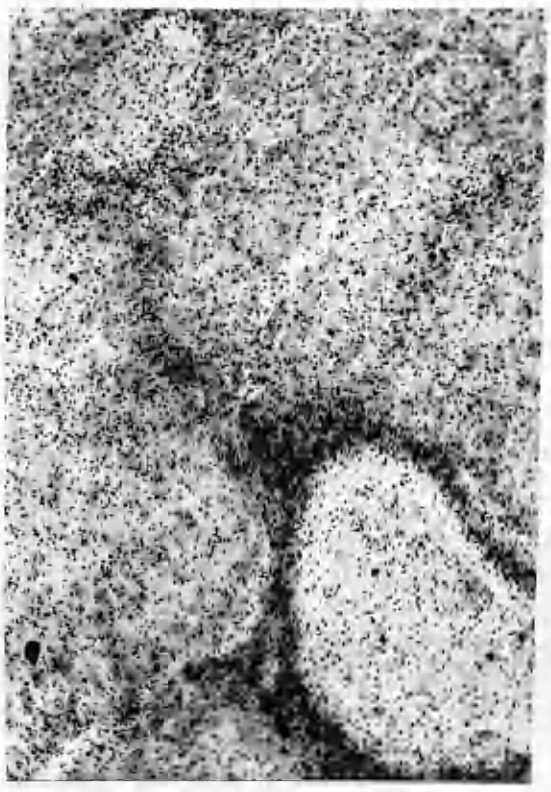


Fig. 166. CCl_4 cirrhosis. Catgut implanted in liver for 5 days. Coarse-grain autoradiograph X 120.

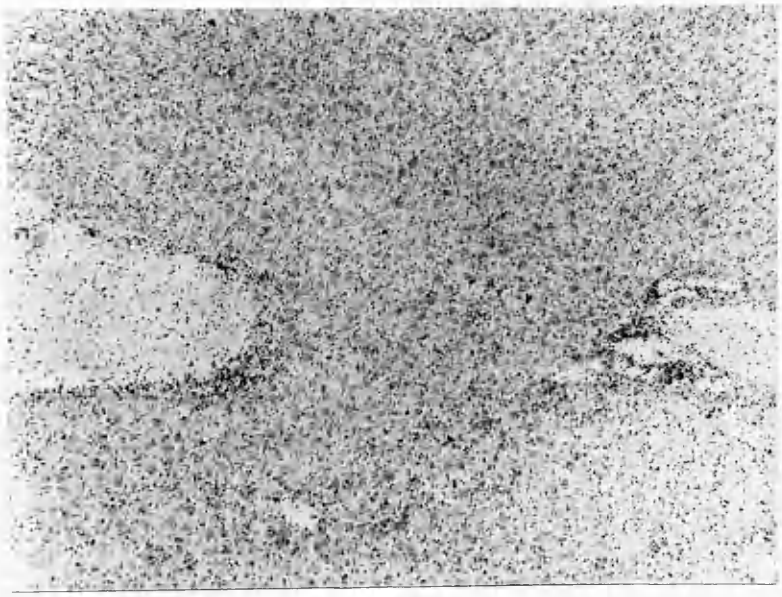


Fig. 167. Normal mouse liver. Coarse-grain autoradiograph following S^{35}- sulphate. Part of large hepatic vein on left. X 90.

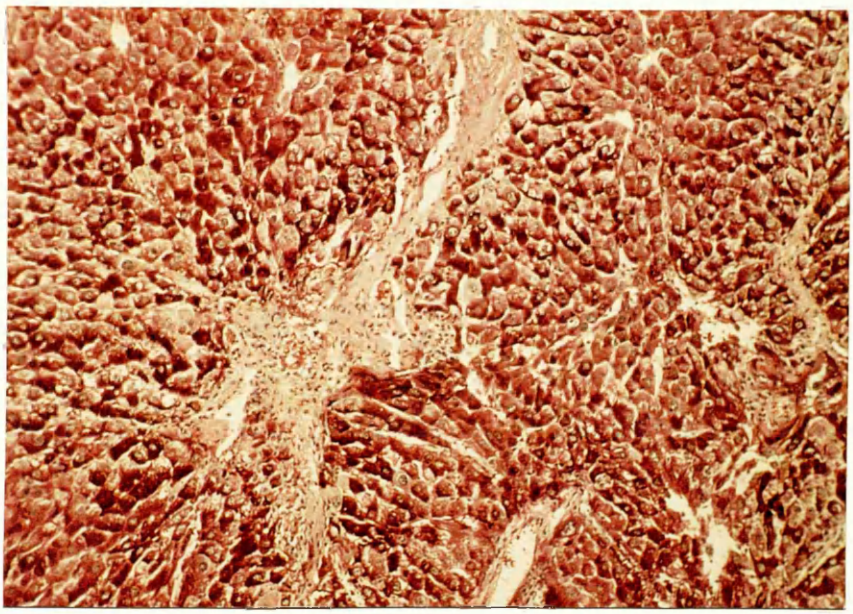


Fig. 168. CCl_4 cirrhosis. Mouse liver.
P.A.S. X 125.

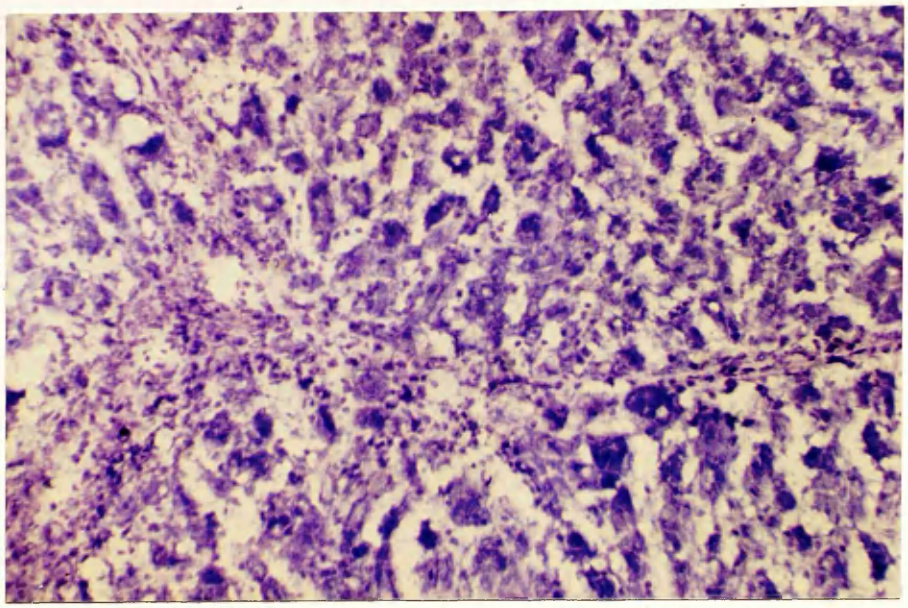


Fig. 169. CCl_4 cirrhosis of mouse liver. Fresh frozen section.
Toluidine Blue X 313.

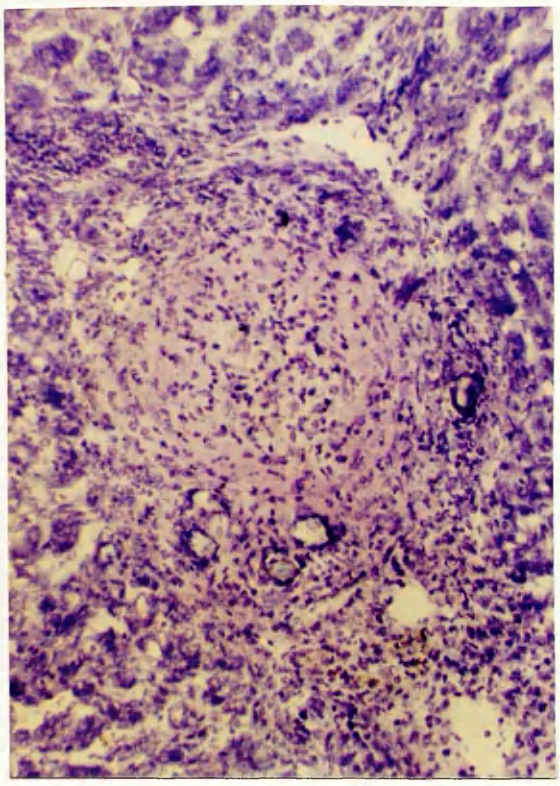


Fig. 170. Mouse liver with catgut implanted for 21 days. Fresh frozen section.
Toluidine Blue X 313.

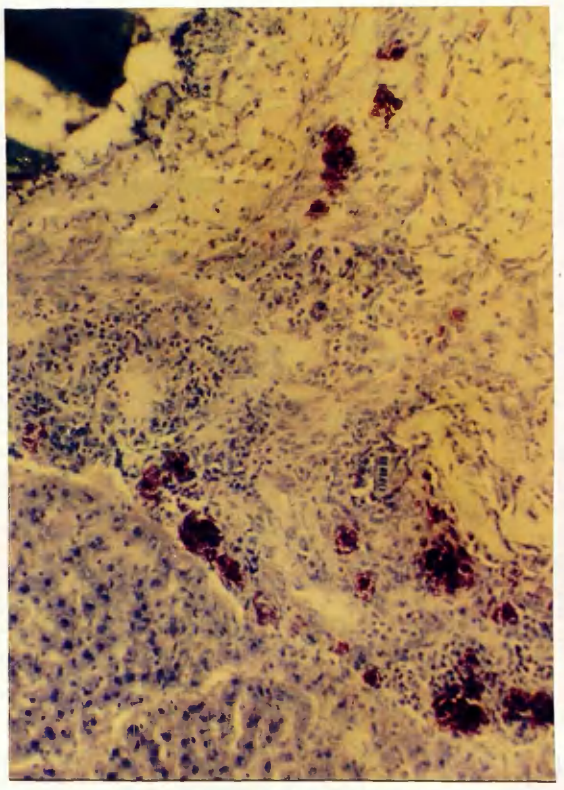


Fig. 171. Paraffin section of mouse liver. Prominent mast cells in wide scar around implanted catgut.
Toluidine Blue X 184.

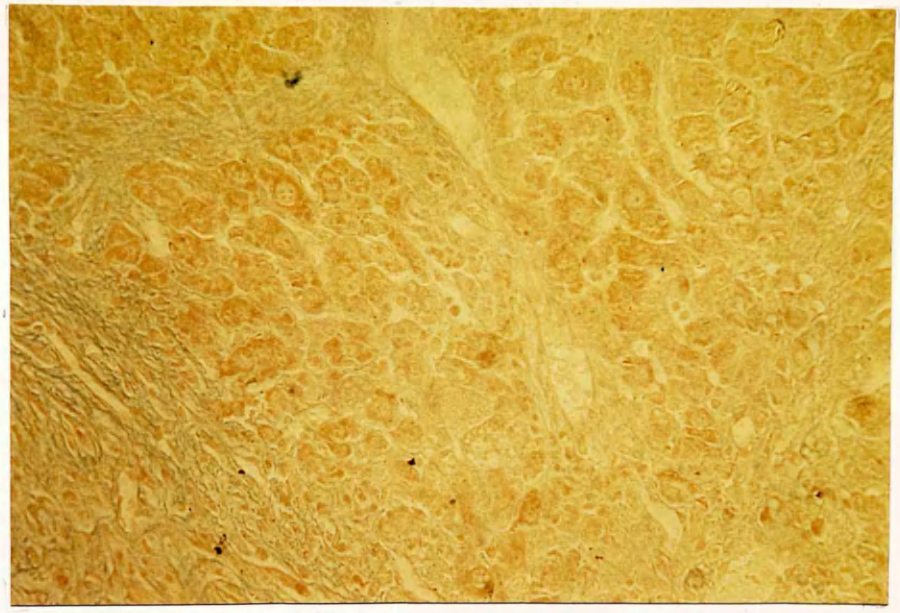


Fig. 172. CCl_4 cirrhosis. Mouse liver. Portion of scar around implanted catgut at bottom-left.
Hale's method for acid mucopolysaccharides X 313.

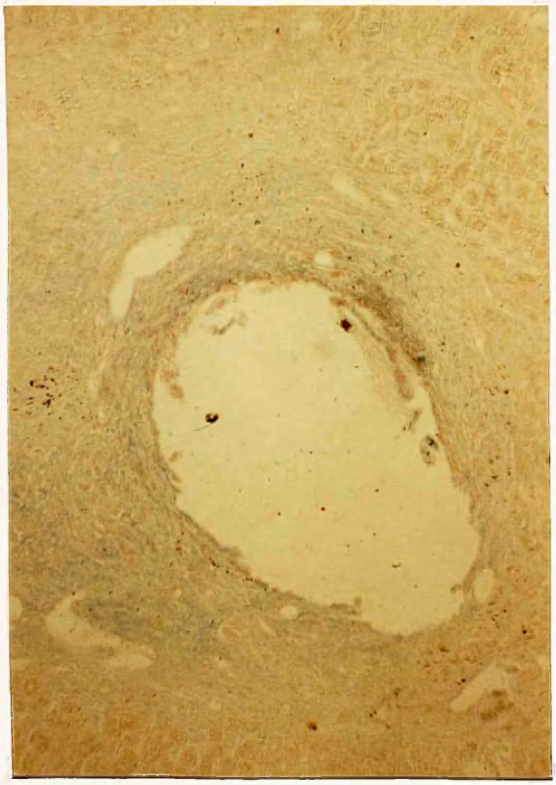


Fig. 173. Catgut implanted in mouse liver for 7 days.
Hale's method for acid mucopolysaccharides X 125.

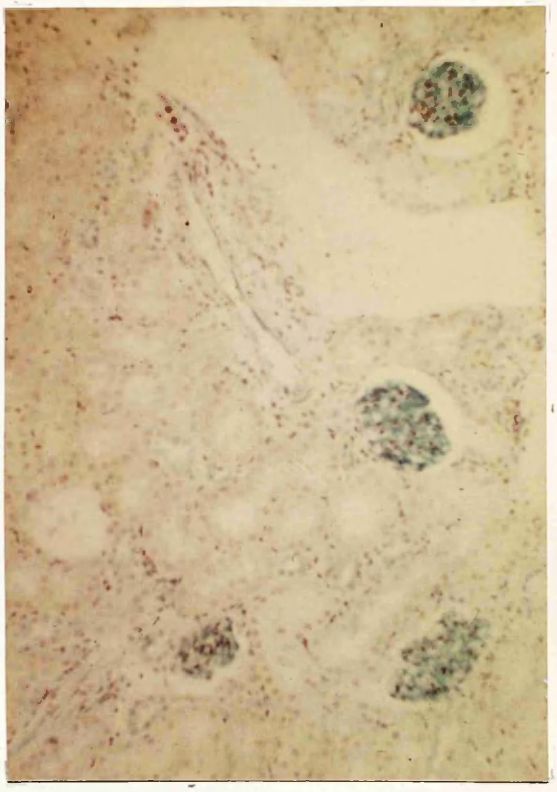


Fig. 174. Mouse kidney. Control section for Hale's method.
X 80.

Fig. 175. CCl_4 cirrhosis of mouse liver. Profuse ceroid pigment in paraffin section.
Sudan Black X 150.

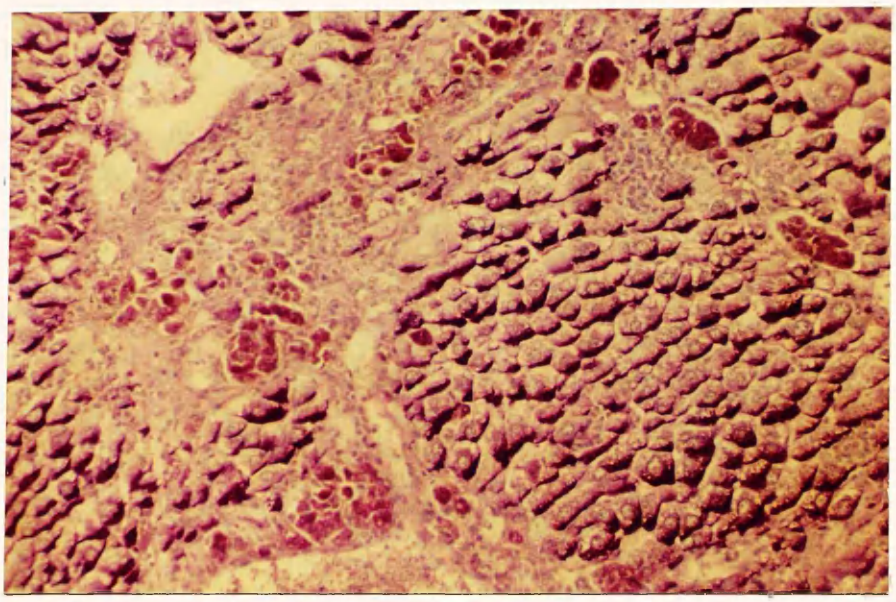


Fig. 176. CCl_4 cirrhosis of mouse liver. Profuse ceroid pigment in paraffin section. Glycogen also stained. P.A.S. X 200.

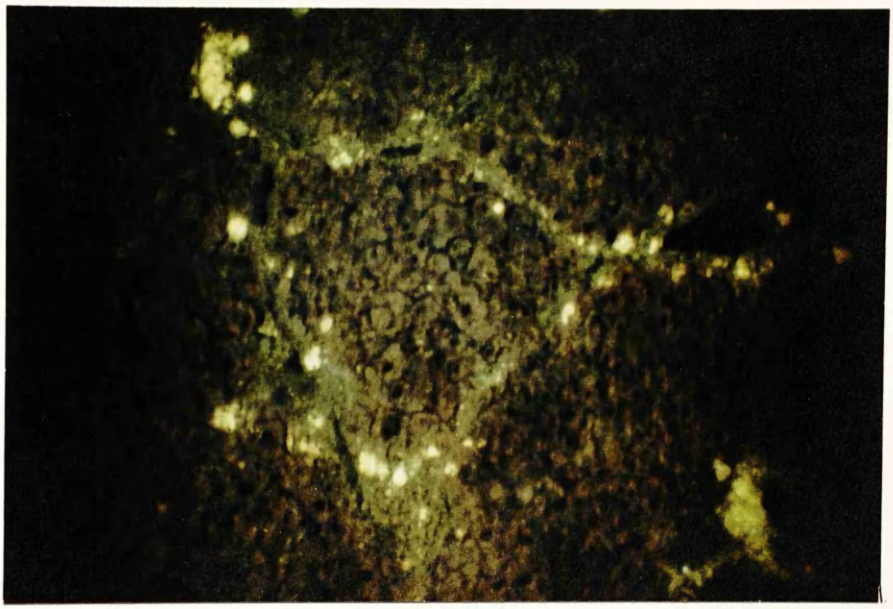


Fig. 177. Cirrhotic mouse liver. Rabbit anti-mouse glomerular serum → goat anti-rabbit-fluorescein conjugate. X 125.

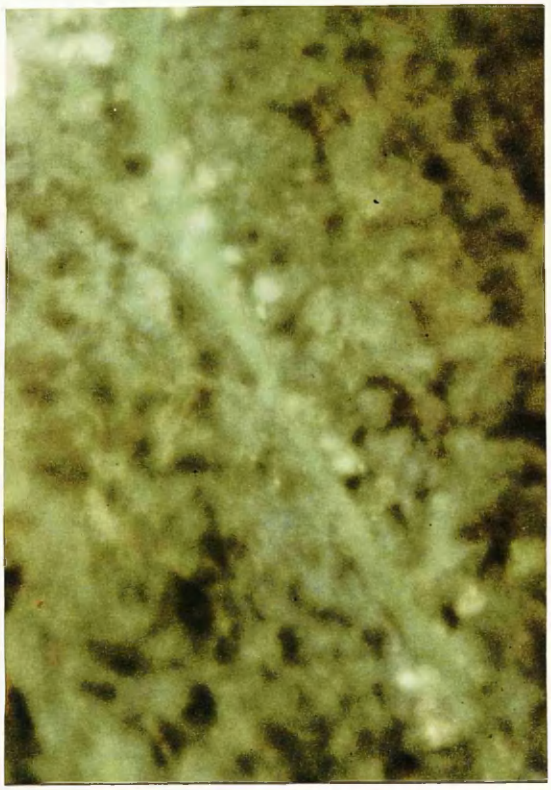


Fig. 178. Cirrhotic mouse liver. Rabbit anti-mouse glomerular serum → goat anti-rabbit-fluorescein X 313.

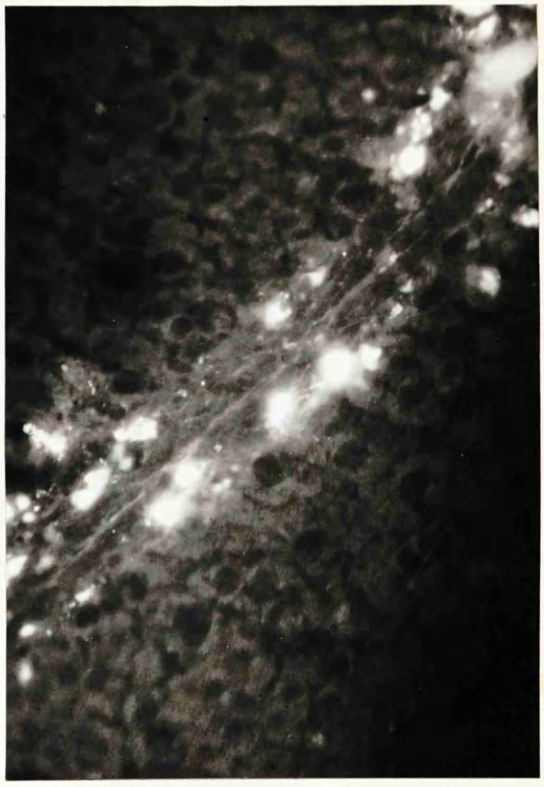


Fig. 179. Cirrhotic mouse liver. Rabbit anti-mouse glomerular serum conjugated with fluorescein. X 313.

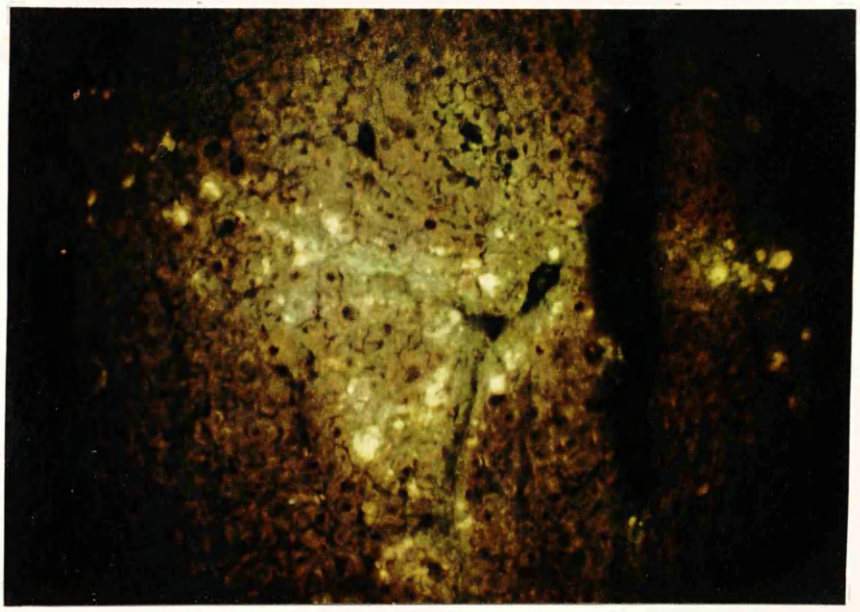


Fig. 180. Cirrhotic mouse liver. Rabbit anti-mouse granulation tissue serum → goat anti-rabbit-fluorescein conjugate. X 125.

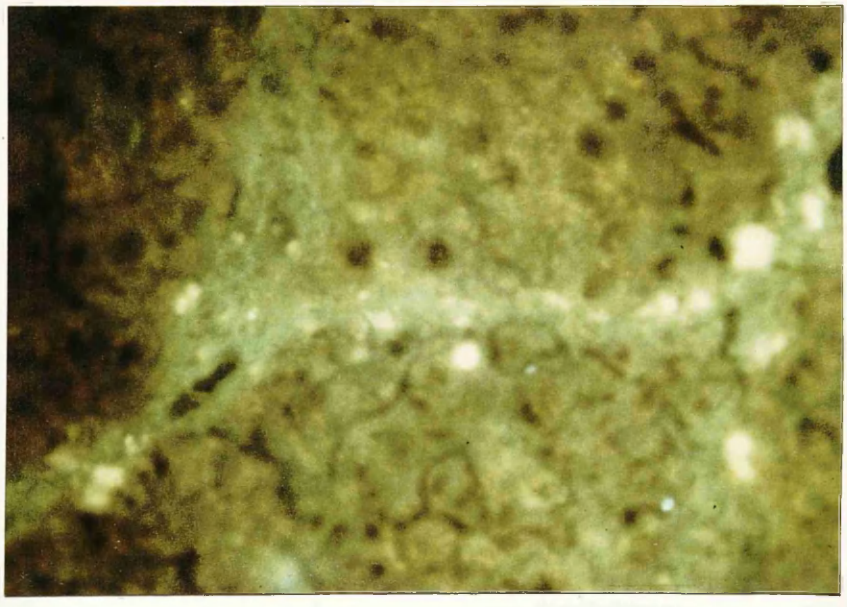


Fig. 181. Cirrhotic mouse liver. Rabbit anti-mouse granulation tissue serum → goat anti-rabbit-fluorescein conjugate. X 313.

Fig. 182. Cirrhotic mouse liver. Normal rabbit serum conjugated with fluorescein. X 125.

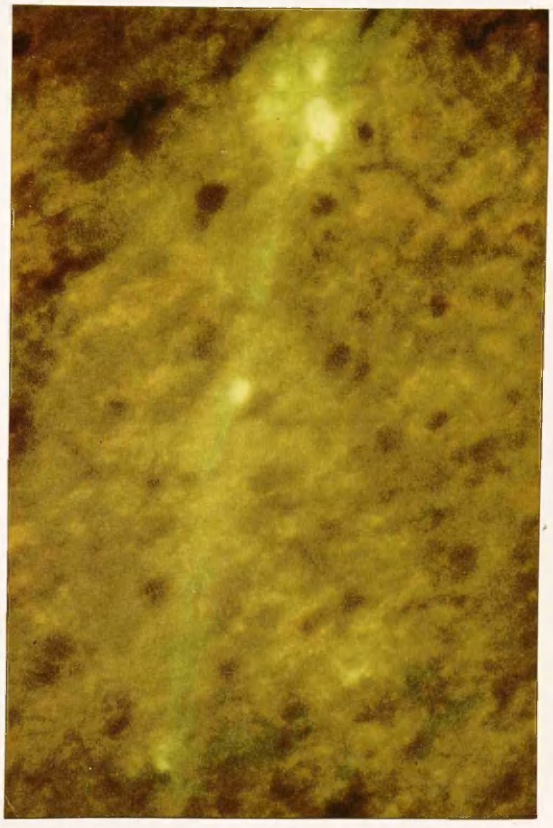


Fig. 183. Cirrhotic mouse liver. Unconjugated normal rabbit serum → conjugated anti-glomerular serum. X 313.

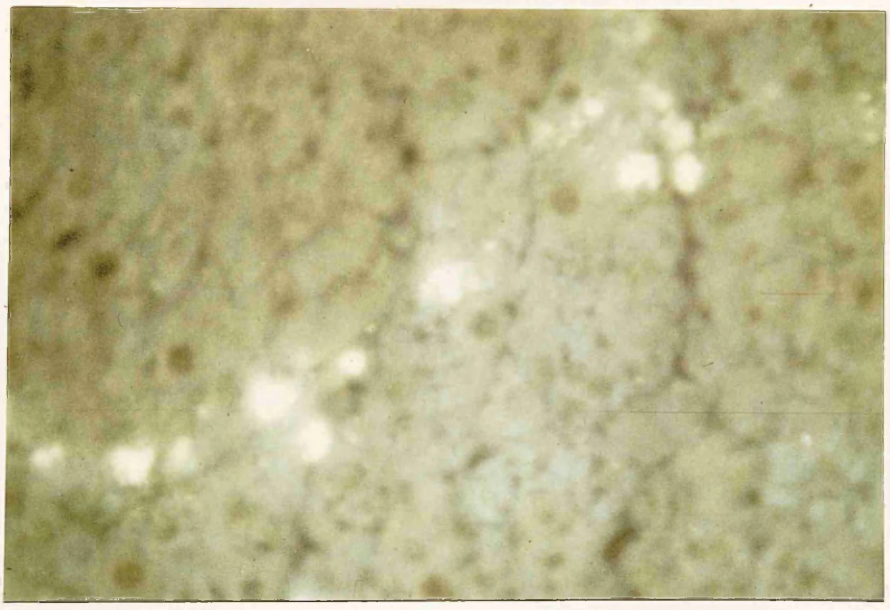


Fig. 184. Cirrhotic mouse liver. Unconjugated anti-glomerular serum → conjugated anti-granuloma serum. X 313.

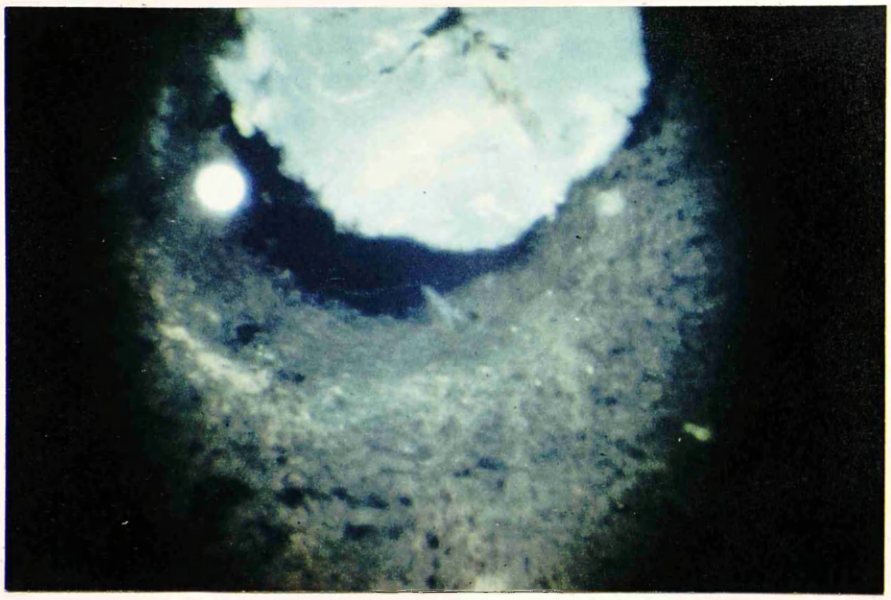


Fig. 185. Mouse liver with catgut implanted for 7 days. Anti-glomerular serum conjugated with fluorescein. Indistinct staining of outer margin of lesion. X 125.

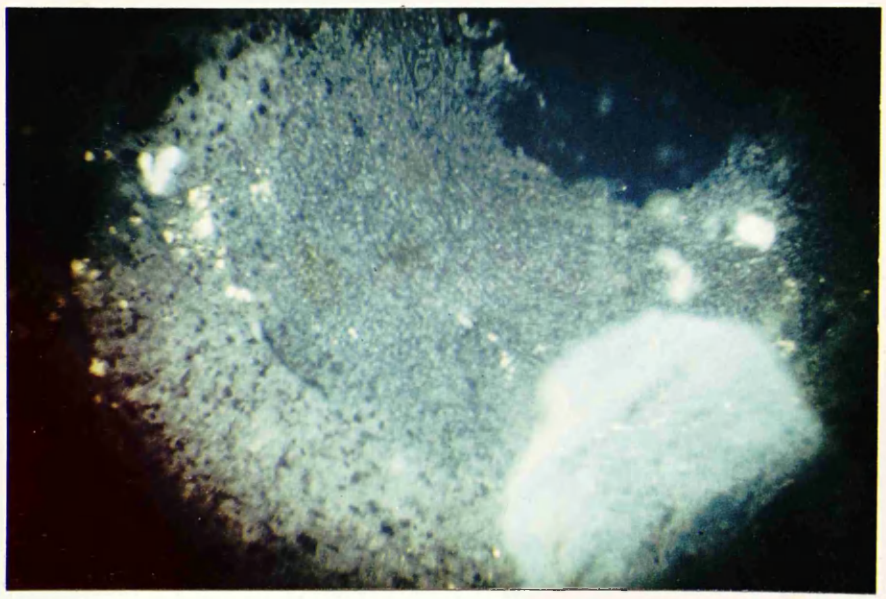


Fig. 186. Mouse liver with catgut implanted for 7 days. Anti-granuloma serum conjugated with fluorescein. Negative result. X 125.

Fig. 187. Cirrhotic human liver. Rabbit anti-human glomerular serum conjugated with rhodamine. X 125.

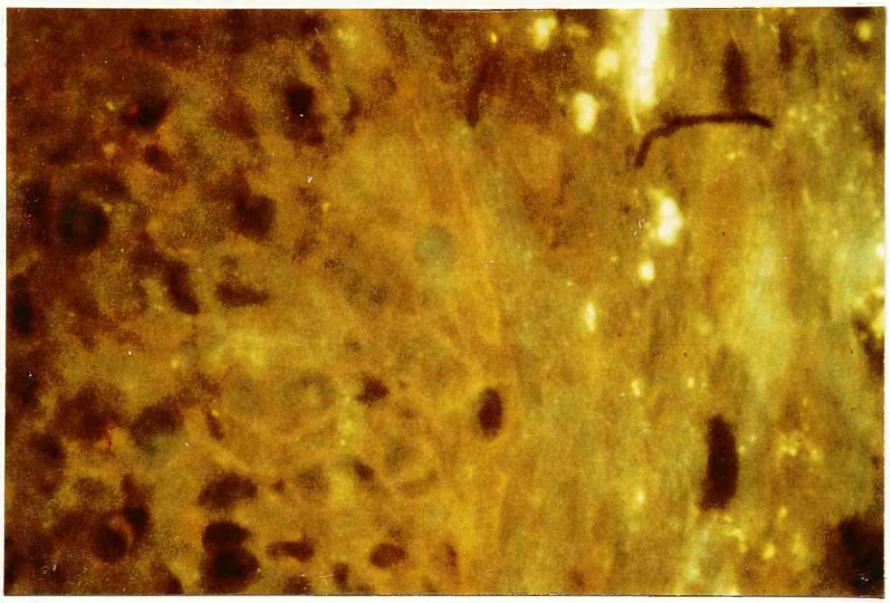


Fig. 188. Cirrhotic human liver. Rabbit anti-human glomerular serum conjugated with rhodamine. X 313.

P A R T III

THE INFLUENCE OF CORTISONE AND ACTH

ON EXPERIMENTAL ZONAL NECROSIS OF LIVER

TABLES XXIX to XXXV

FIGURES 189 to 234

TABLE XXIX

Experiment 1 - Numbers of Animals Employed and Duration of Poisoning

Group	Duration of poisoning										Total
	Hours			Days							
	1	3	5	1	2	3	4	5	6	7	
A. Animals receiving carbon tetrachloride and hormone											188
Mice - CCl_4 + Cortisone	2	2	2	10	32	28	34	11	7	6	134
Mice - CCl_4 + ACTH	-	-	-	2	6	9	9	2	2	-	30
Rats - CCl_4 + Cortisone	-	2	2	2	6	4	6	2	-	-	24
B. Animals receiving carbon tetrachloride alone											133
Mice	2	2	2	9	29	18	31	5	5	6	109
Rats	-	2	2	2	6	4	6	2	-	-	24
C. Animals receiving hormone alone											89
Mice - Cortisone	1	1	1	3	26	10	13	3	2	2	62
Mice - ACTH	-	-	-	1	6	2	2	1	1	-	13
Rats - Cortisone	-	1	1	1	6	1	2	2	-	-	14

Group C animals which received no carbon tetrachloride were killed at intervals to correspond with Groups A and B.

Fig. 189. 3 mice used in experiment 1 and treated daily for 4 days. From above downwards treatment consisted of: CCl_4; CCl_4 and cortisone; cortisone.

Fig. 190. Mouse liver showing CCl_4 zonal necrosis. X 3.

Fig. 191. Livers and thymus glands from 3 mice. From left to right these animals have received: CCl_4; CCl_4 + cortisone; cortisone. Approximately normal size.

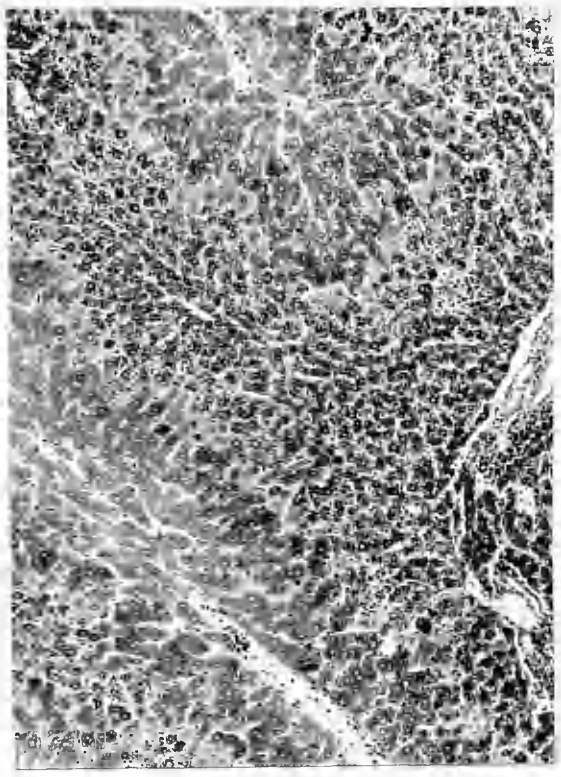


Fig. 192. Mouse liver 1 hour after CCl_4. Giemsa Stain X 90.

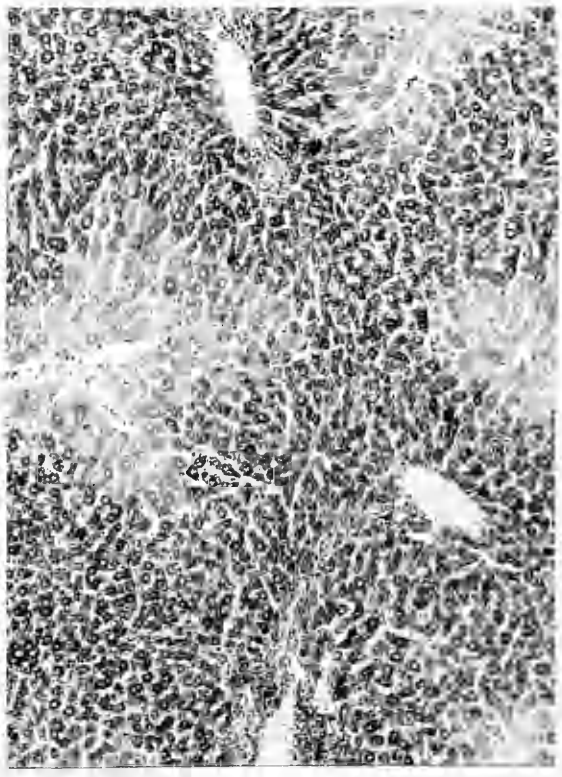


Fig. 193. Mouse liver 3 hours after CCl_4. Giemsa Stain X 90.

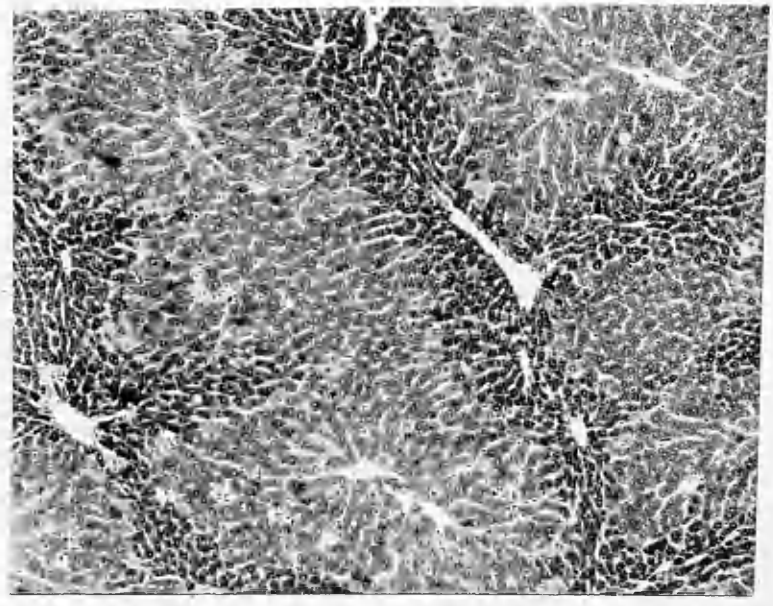


Fig. 194. Mouse liver 5 hours after CCl_4. Giemsa Stain X 90.

Fig. 195. Mouse liver 24 hours after CCl_4.
Giemsa stain X 90.

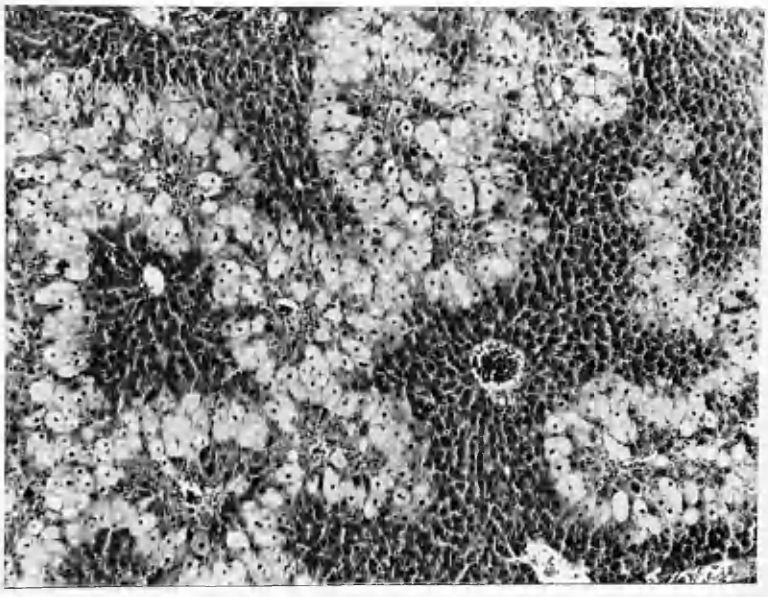


Fig. 196. Mouse liver 24 hours after CCl_4.
Prominent mid-zonal hydropic degeneration
together with centrilobular necrosis.
H. & E. X 90.

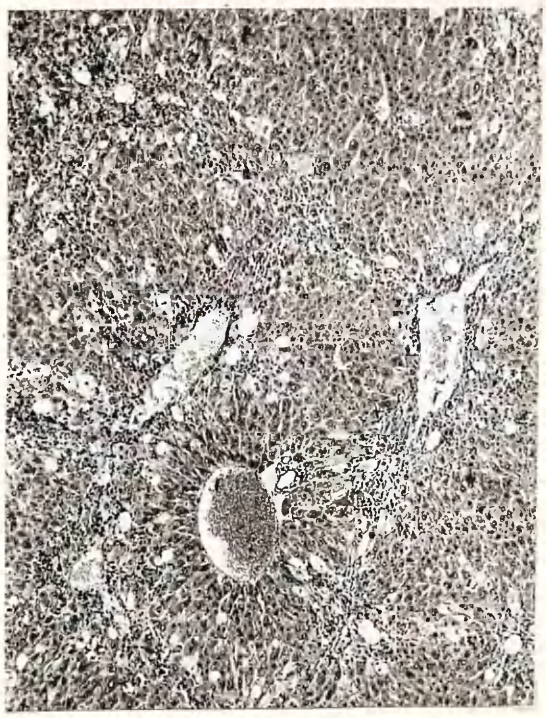


Fig. 197. Mouse liver 2 days after CCl_4. H. & E. X 60.

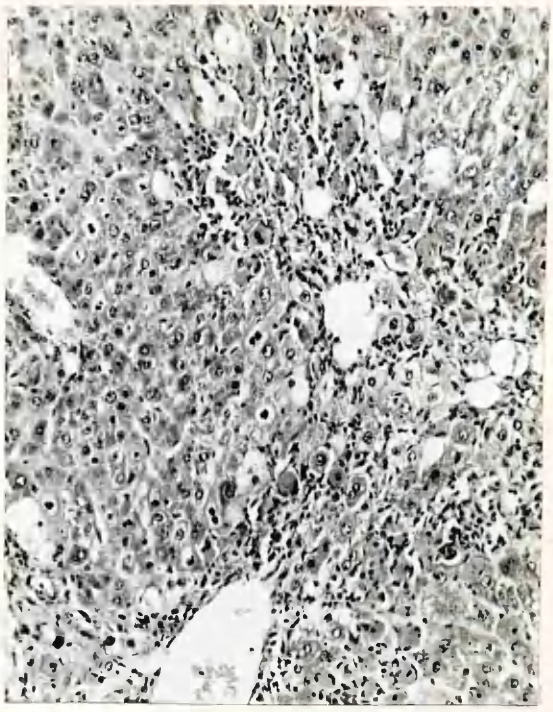


Fig. 198. Mouse liver 2 days after CCl_4, showing phagocytosis of centrilobular necrotic tissue. H. & E X 144.

Fig. 199. Mouse liver 2 days after CCl_4, showing regeneration of surviving parenchyma. H. & E. X 144.

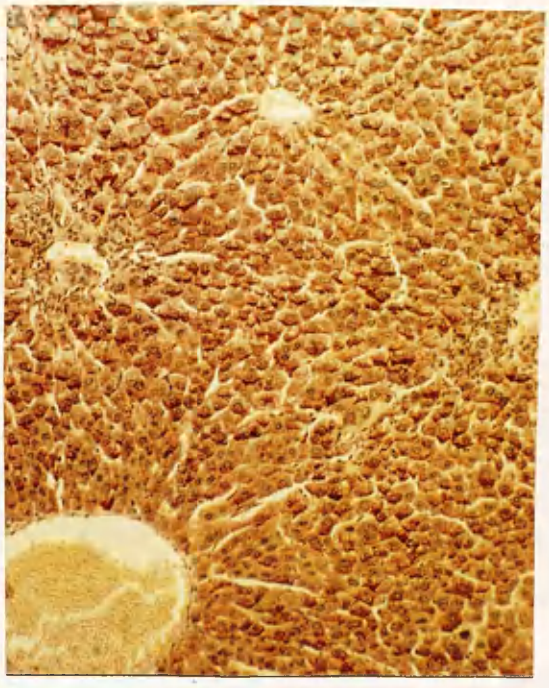


Fig. 200. Mouse liver 4 days after CCl_4. Repair well advanced and liver rich in glycogen. P.A.S. X 125.

Fig. 201. Mouse liver 2 days after CCl_4. Daily cortisone injections. Toluidine blue X 90.

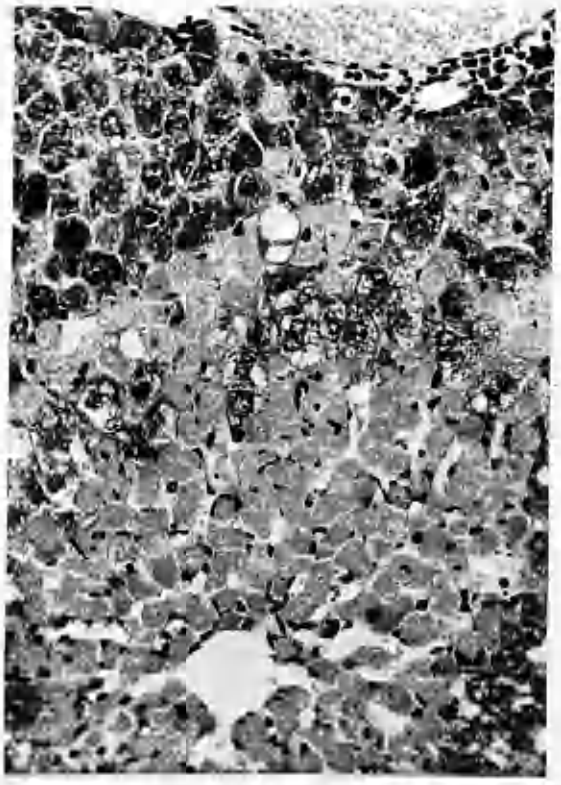


Fig. 202. Mouse liver 2 days after CCl_4. Daily cortisone injections. Toluidine blue X 240.

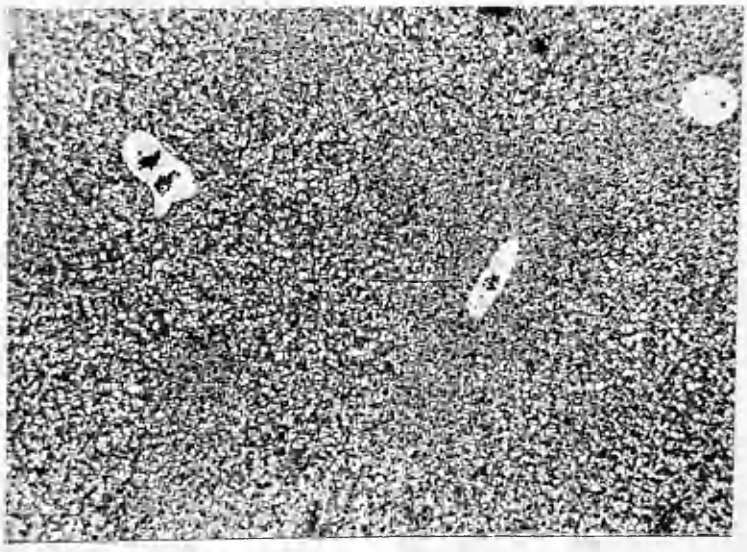


Fig. 203. Mouse Liver. Cortisone only for 3 days. No liver poison. H. & E. X 60.

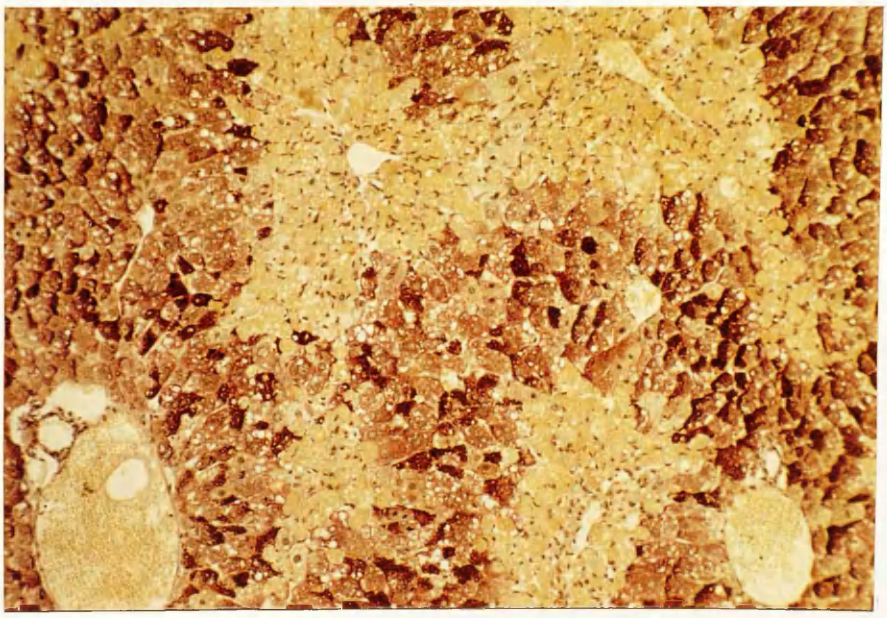


Fig. 204. Mouse liver 4 days after CCl_4. Daily cortisone injections (cf., Fig. 200).
P.A.S., Orange G X 120.

Fig. 205. Mouse liver 5 days after CCl_4. Daily cortisone injections.
P.A.S. X 60.

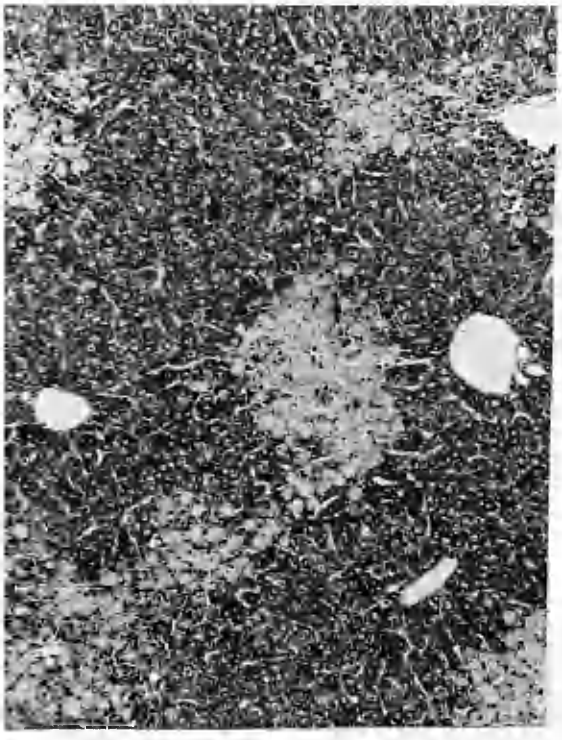


Fig. 206. 30% necrosis.
Giemsa's stain X 90.

Fig. 207. 50% necrosis.
Giemsa's stain X 90.

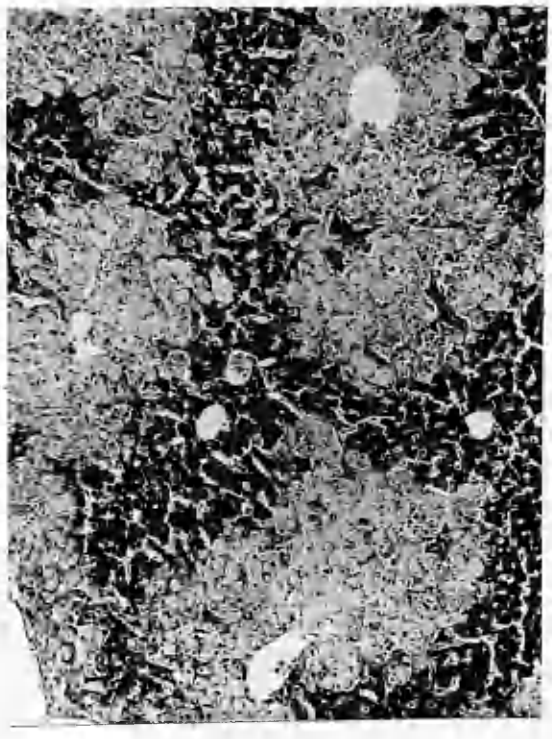


Fig. 208. 70% necrosis.
Giemsa's stain X 90.

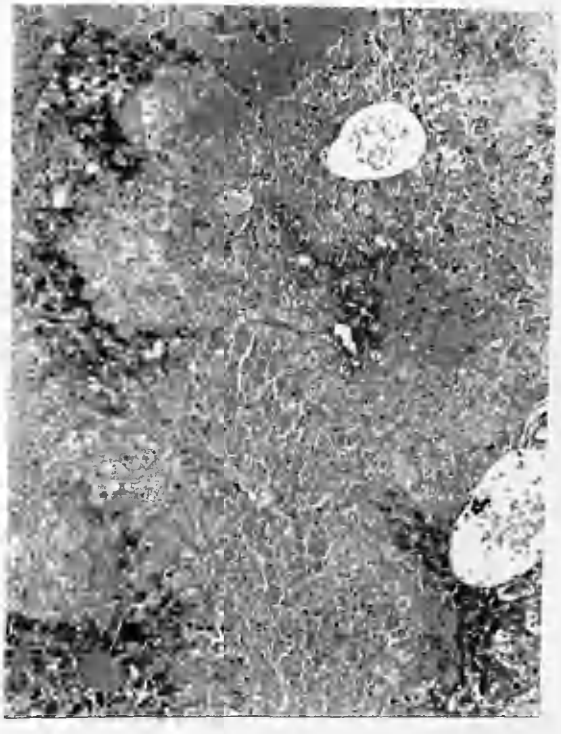


Fig. 209. 90% necrosis.
Giemsa's stain X 90.

FIG. 210 INFLUENCE OF CORTISONE AND ACTH ON THE DURATION AND SEVERITY OF LIVER NECROSIS INDUCED BY CCl_4

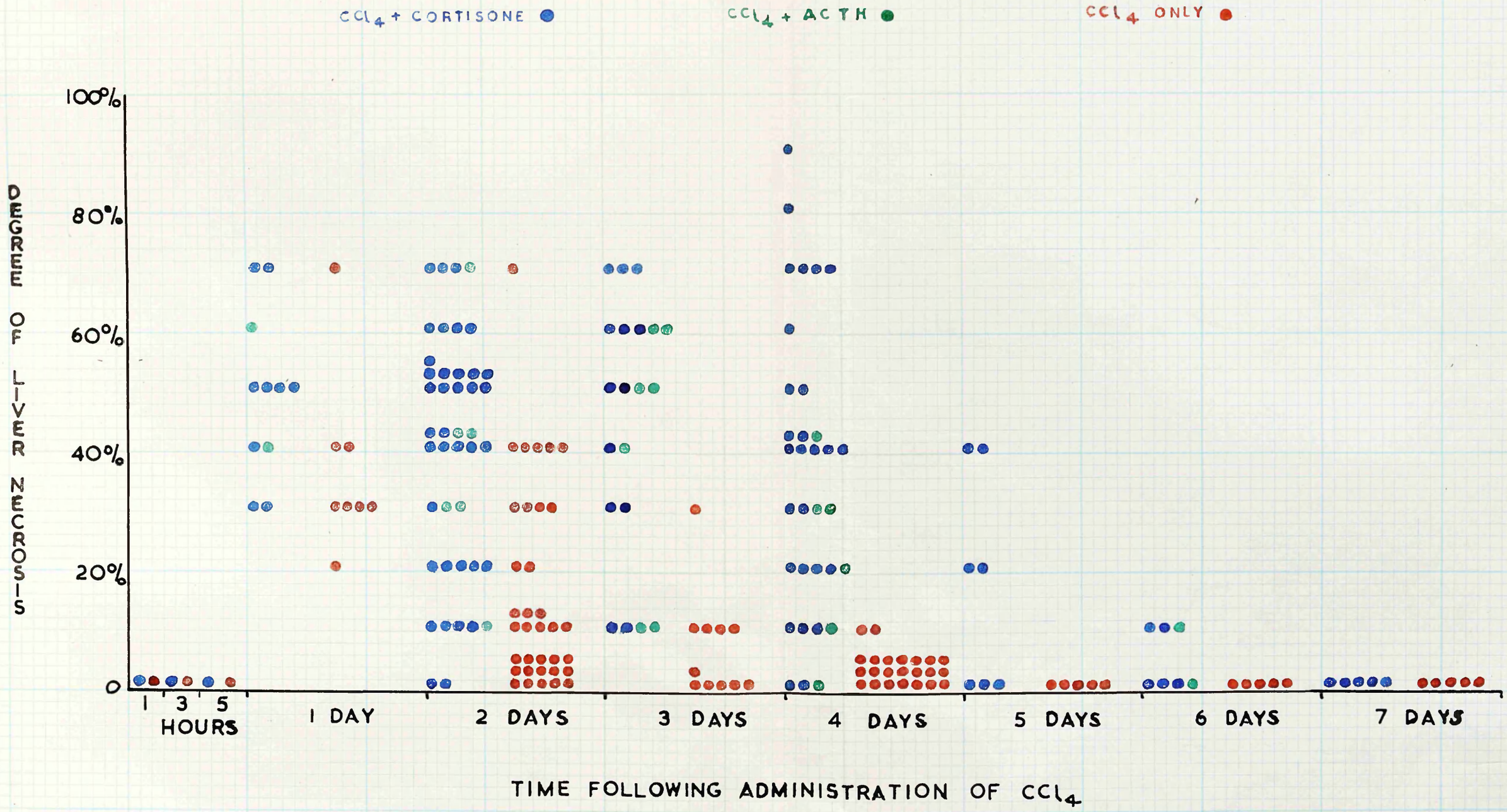


TABLE XXX

Thymus Weights and Eosinophil Counts Related to Dosage of Cortisone and Duration of Carbon Tetrachloride Poisoning

Group	Cortisone dosage A and C	Total quantity of cortisone A and C	Duration of CCl_4 poisoning (0.04 ml.) A and B	Number of Cases A	B	C	Average wet thymus weights as % total body weight A	B	C	Average eosinophil counts (cells/cub.mm. blood) A	B	C
	0.05 mg./day	0.2 mg. in 4 days	2 days	1	1	1	0.33	0.25	0.24	72	41	122
	0.10 "	0.4 " " 4 "	2 "	1	1	1	0.22	0.44	0.26	83	70	44
	0.20 "	0.8 " " 4 "	2 "	1	1	1	0.22	0.26	0.12	8	28	6
	0.30 "	1.2 " " 4 "	2 "	1	1	1	0.16	0.32	0.18	0	144	0
	0.60 "	2.4 " " 4 "	2 "	1	1	1	0.27	0.30	0.29	0	1117	761
	0.80 "	3.2 " " 4 "	2 "	1	1	1	0.21	0.39	0.22	13	19	44
	1.00 "	4.0 " " 4 "	2 "	1	1	1	0.12	0.36	0.13	0	3	0
	1.50 "	6.0 " " 4 "	2 "	1	1	1	0.29	0.36	0.15	0	240	2
	2.00 "	8.0 " " 4 "	2 "	1	1	1	0.13	0.30	0.25	6	63	2
	3.00 "	12.0 " " 4 "	2 "	1	1	1	0.13	0.20	0.19	0	78	0
	5.00 "	20.0 " " 4 "	2 "	1	1	1	0.07	0.23	0.16	6	28	0
	0.40 "	0.80 " " 2 "	1 hour	1	1	1	0.19	0.31	0.31	0	0	0
	0.40 "	0.80 " " 2 "	3 "	1	1	1	0.31	0.47	0.35	2	14	0
	0.40 "	0.80 " " 2 "	5 "	1	1	1	0.37	0.59	0.27	0	8	3
	0.40 "	1.20 " " 3 "	1 day	2	2	2	0.04	0.38	0.17	0	380	0
	0.40 "	1.60 " " 4 "	2 "	14	14	14	0.12	0.31	0.19	4	240	9
	0.40 "	2.00 " " 5 "	3 "	12	12	6	0.17	0.30	0.13	1	47	4
	0.40 "	2.40 " " 6 "	4 "	12	12	6	0.08	0.35	0.11	2	250	17
	0.40 "	2.80 " " 7 "	5 "	2	2	2	0.17	0.22	0.13	5	344	0
	0.40 "	3.60 " " 9 "	7 "	2	2	1	0.14	0.32	0.15	0	175	0

In this table are included all animals of Experiments 2 and 3 and some mice of Experiment 1.

All mice weighed 20-25 grams at the start of the experiments. Thymus weights are given as percentages of body weights at death.

TABLE XXXI

Fatalities and Liver Weights of Mice related to Dosage of Carbon Tetrachloride and Duration of Poisoning

CCl_4 dosage	Duration of CCl_4 poisoning	Number of cases			Premature deaths			Average wet liver * weights as % total body weight		
Group A & B	A & B	A	B	C	A	B	C	A	B	C
0.01 ml.	2 days	1	1	1	0	0	0	7.0	6.3	6.1
0.02 "	2 "	1	1	1	0	0	0	7.0	6.2	6.7
0.03 "	2 "	1	1	1	0	0	0	7.1	6.8	6.9
0.05 "	2 "	1	1	1	1	0	0	-	6.3	6.9
0.075 "	2 "	1	1	1	0	0	0	9.0	6.4	6.3
0.10 "	2 "	1	1	1	0	0	0	8.2	6.5	7.1
0.20 "	2 "	1	1	1	1	1	0	-	-	6.4
0.30 "	2 "	1	1	1	1	1	0	-	-	7.0
0.50 "	2 "	1	1	1	1	1	0	-	-	6.2
0.75 "	2 "	1	1	1	1	1	0	-	-	6.9
1.00 "	2 "	1	1	1	1	1	0	-	-	6.4
0.04 "	1 hour	2	2	1				8.6(2)	5.8(2)	8.4
0.04 "	3 "	2	2	1				8.9(2)	5.4(2)	8.7
0.04 "	5 "	2	2	1				7.6(2)	5.6(2)	6.0
0.04 "	1 day	12	9	4				6.2(2)	4.6(2)	6.0(2)
0.04 "	2 "	40	31	34	8	2	0	7.5(14)	6.2(14)	7.5(14)
0.04 "	3 "	37	18	12				7.5(12)	6.4(12)	6.5(6)
0.04 "	4 "	43	31	15				8.1(12)	5.5(12)	5.5(6)
0.04 "	5 "	13	5	4				7.0(2)	5.9(2)	7.0(2)
0.04 "	6 "	9	5	3				6.2(1)	5.9(1)	7.0(1)
0.04 "	7 "	6	6	2				6.6(2)	5.8(2)	6.2(1)

* Figures in brackets indicate numbers of mice from which liver weights were estimated.

Group B = All mice of this group in Experiments 1, 2 and 3.

Groups A and C = All mice of these groups in Experiments 1, 2 and 3 which received cortisone 0.4 mg./day or ACTH 0.25 mg. twice daily.

Group C mice received no liver poison but were killed at intervals corresponding to those of the other groups.

TABLE XXXII

Experiment 4 - Numbers of Animals Employed, and Severity of Liver Necrosis related to Cortisone Therapy and Diet

Duration of poisoning in days	Number of mice in each group*									Percentage degree of liver necrosis suffered by each animal (Mean values in brackets)								
	Group: Ai	Aii	Aiii	Bi	Bii	Biii	Ci	Cii	Ciii	Ai	Aii	Aiii	Bi	Bii	Biii	Ci	Cii	Ciii
1	1	2	1	1	1	1	1	1	1	10	40,30	40	60	60	40	0	0	0
2	7	7	9	4	4	5	2	2	1	50,40,30, 30,20,10, 10 (27)	50,50,30, 30,20,20, 10 (30)	80,70,60, 60,40,40, 40,40,30 (51)	40,20,20, 0 (27)	70,40,20, 10 (35)	80,80,50, 40,30 (56)	00	00	0
3	6	7	3	4	4	5	2	2	1	70,60,60, 40,20,20 (45)	80,60,60, 50,30,10, 0 (41)	70,70,70, (70)	10,10,0, 0 (5)	60,30,30, 10 (33)	70,50,50, 40,40 (50)	00	00	0
4	6	4	0	5	5	2	2	2	1	40,30,30, 20,10,0 (22)	30,30,20, 20 (25)	-	10,0,0, 0,0 (2)	30,10,10, 0,0 (10)	50,50 (50)	00	00	0
5	3	3	0	2	2	0	1	1	0	30,10,0 (13)	40,20,10 (23)	-	0,0	0,0	-	0	0	-

* All animals of Groups Aiii, Biii and Ciii, after the first day, were found dead at approximately the times indicated. The remainder were killed.

TABLE XXXIII

Numbers of Animals Employed in Experiment 5

Group	Duration of poisoning										Total
	Hours			Days							
	1	3	5	1	2	3	4	5	6	7	
A. Animals receiving tannic acid and cortisone											29
Mice	-	-	1	1	5	5	3	3	-	1	19
Rats	-	-	-	2	3	2	3	-	-	-	10
B. Animals receiving tannic acid alone											27
Mice	-	-	1	1	4	4	4	3	-	1	18
Rats	-	-	-	2	3	2	2	-	-	-	9
C. Animals receiving cortisone alone											11
Mice	-	-	1	1	1	1	1	1	-	1	7
Rats	-	-	-	1	1	1	1	-	-	-	4

Group C animals which received no tannic acid were killed at intervals to correspond with Groups A and B.

FIG. 211 INFLUENCE OF CORTISONE ON THE DURATION AND SEVERITY OF LIVER NECROSIS INDUCED BY TANNIC ACID

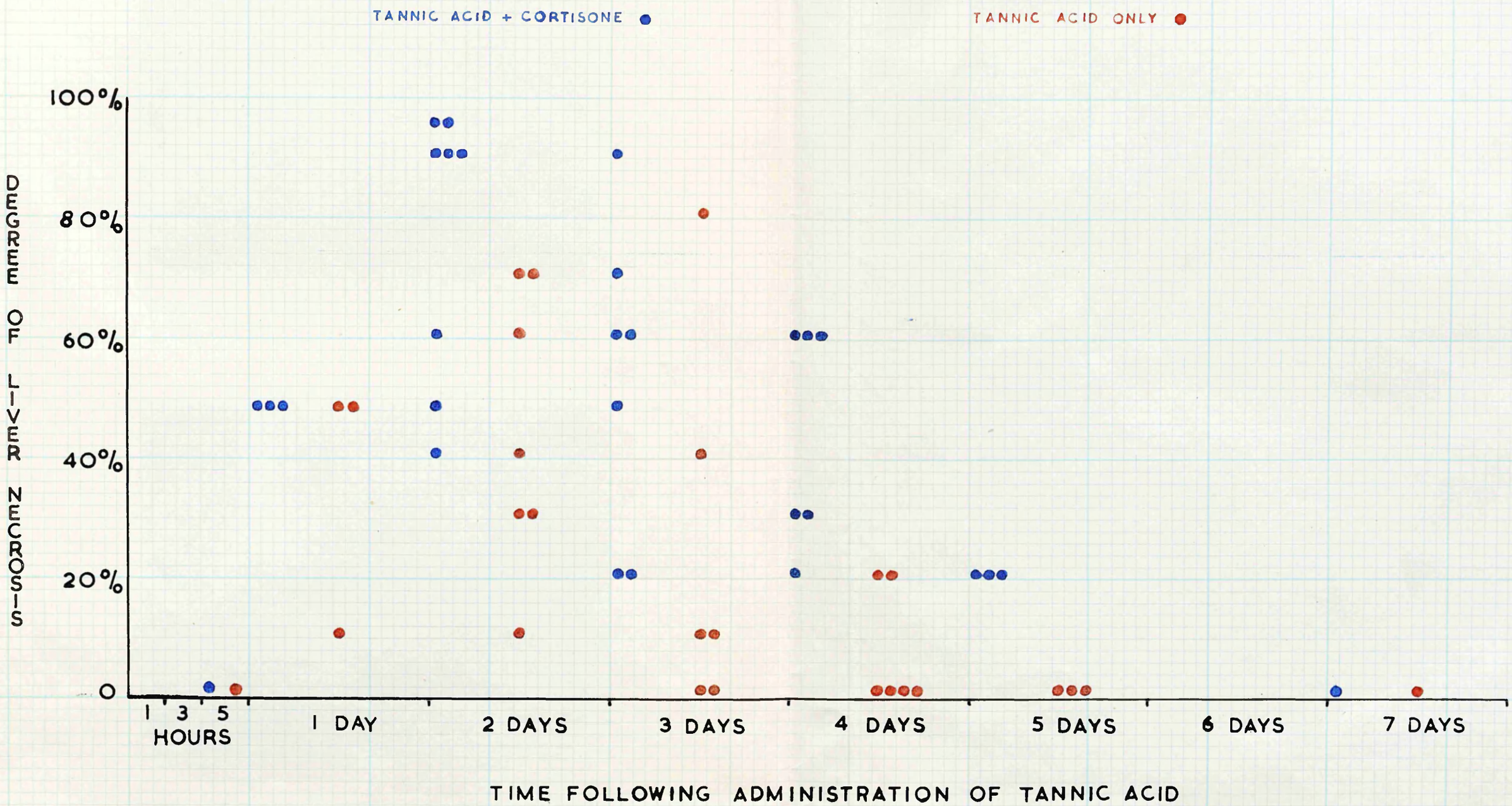
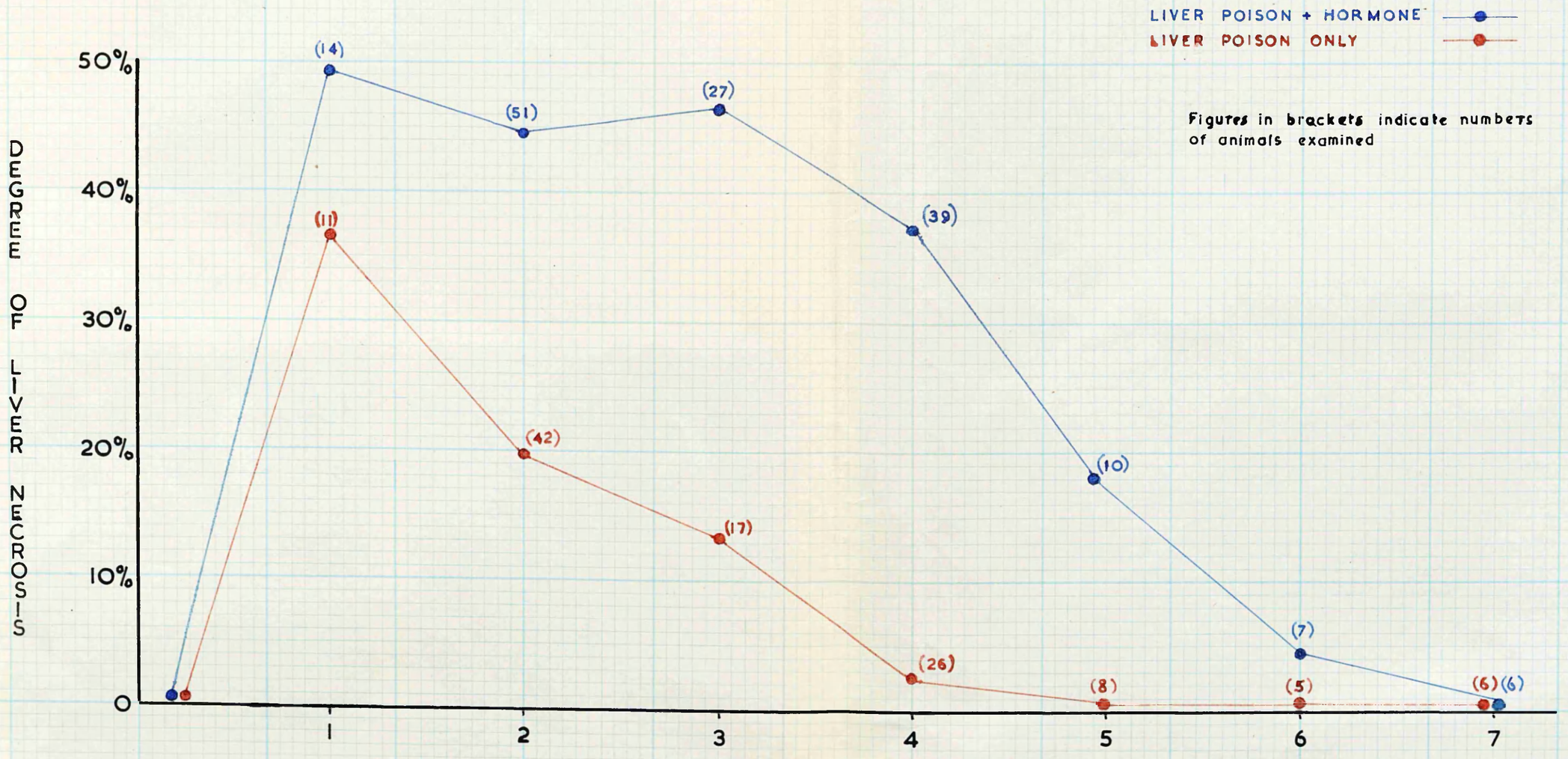


Fig. 212. Mouse liver 3 days after tannic acid. Repair well advanced. H. & E. X 80.

Fig. 213. Mouse liver 3 days after tannic acid. Daily cortisone injections. Extensive liver necrosis. H. & E. X 90.

FIG. 214 INFLUENCE OF CORTISONE AND ACTH ON LIVER NECROSIS. MEAN VALUES FOR SEVERITY OF NECROSIS RELATED TO DURATION OF POISONING



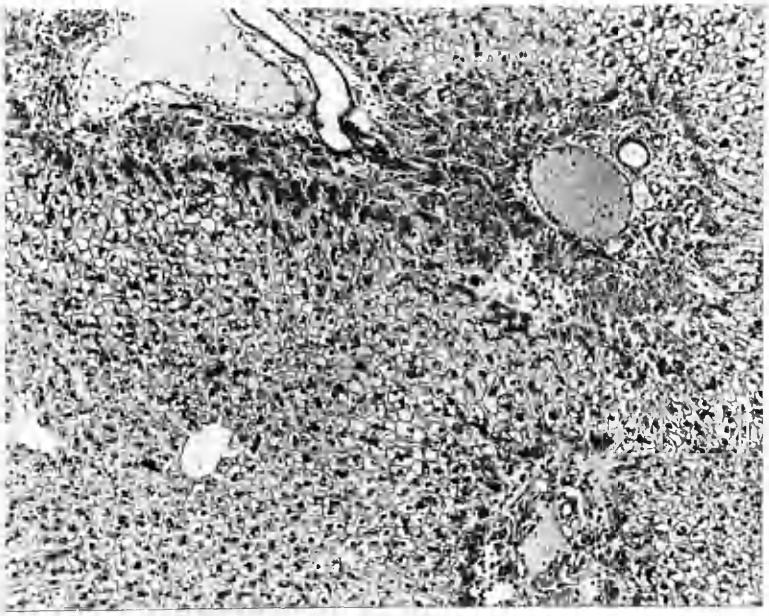


Fig. 215. Rat liver 2 days after allyl formate. Daily cortisone injections. Periportal zonal necrosis. H. & E X 125.

Fig. 216. Guinea-pig liver 3 hours after allyl formate. Loss of glycogen from periportal zones. P.A.S. X 90.

Fig. 217. Guinea-pig liver 4 hours after allyl formate. P.A.S. X 90.

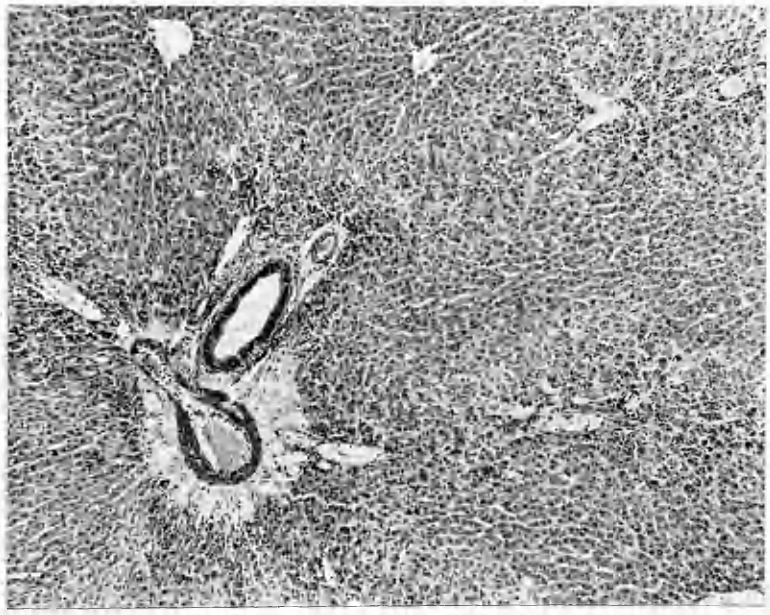


Fig. 218. Guinea-pig liver 24 hours after allyl formate. H. & E. X 60.

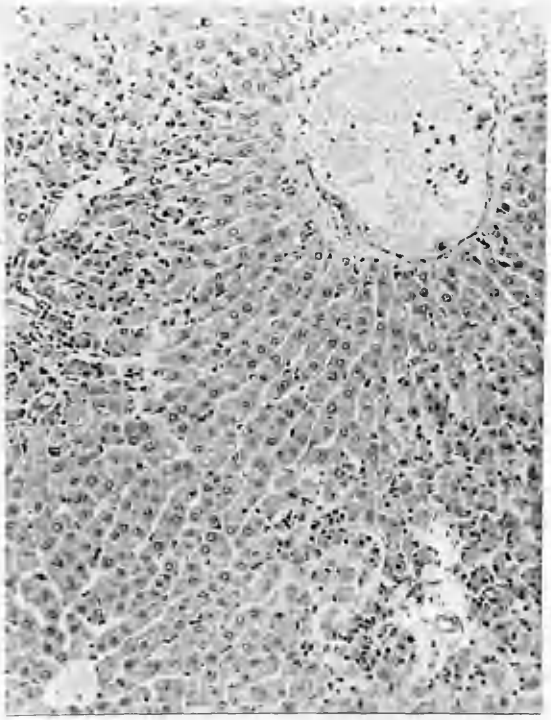


Fig. 219. Guinea-pig liver 24 hours after allyl formate. Centrilobular vein at top, right. H. & E. X 125.

Fig. 220. Guinea-pig liver 24 hours after allyl formate. Dense eosinophilic cells adjacent to portal tracts. H. & E. X 90.

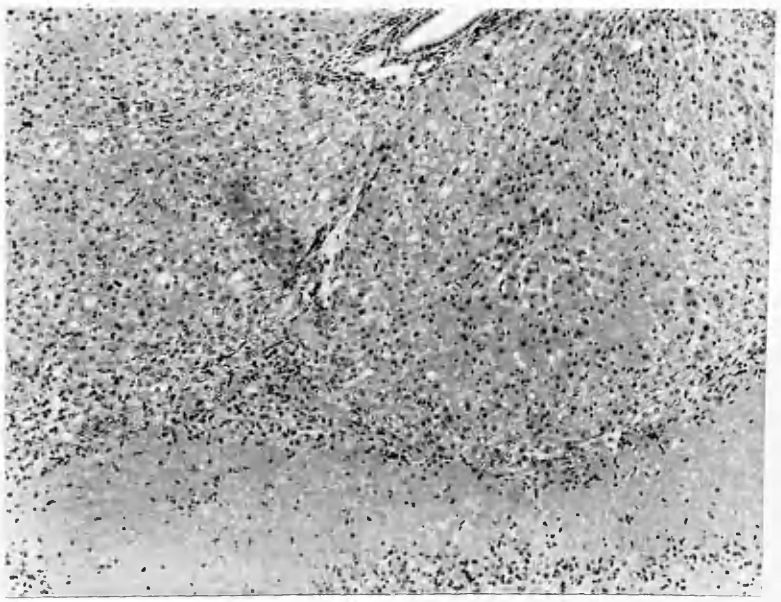


Fig. 221. Guinea-pig liver 24 hours after allyl formate. Band of massive necrosis near bottom of photograph. H. & E. X 90.

Fig. 222. Guinea-pig liver 24 hours after allyl formate. Perivascular haemorrhage.
H. & E. X 90.

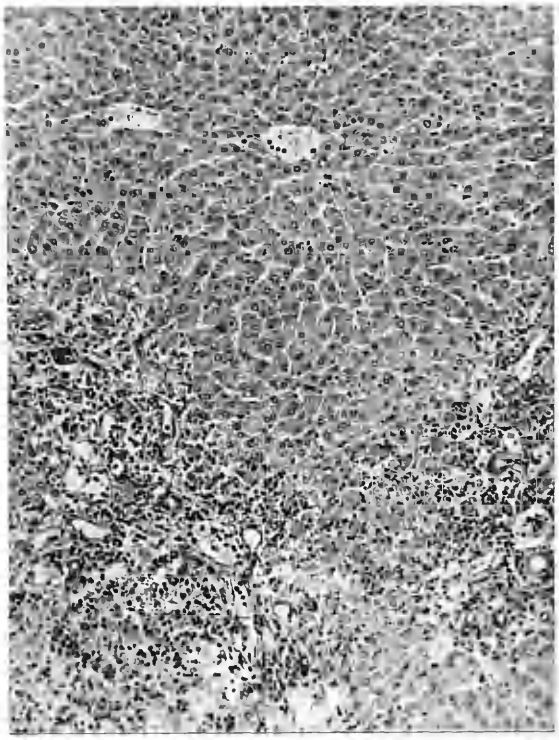


Fig. 223. Guinea-pig liver 2 days after allyl formate.
H. & E. X 100.

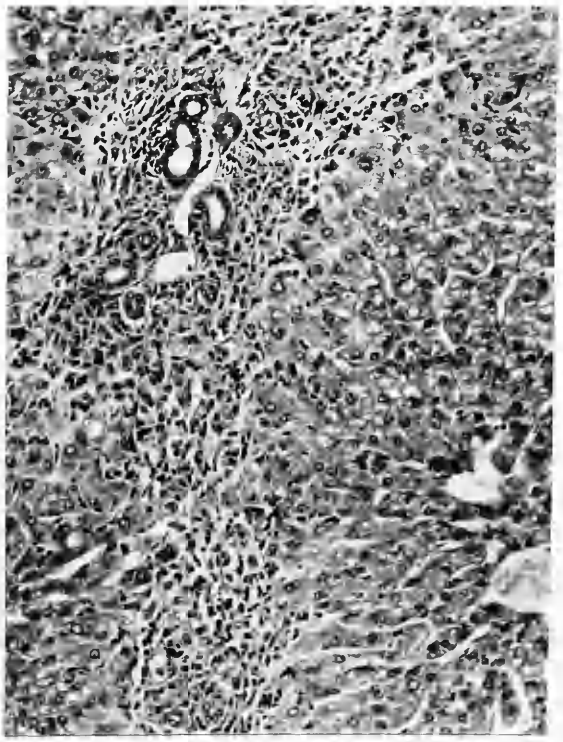


Fig. 224. Guinea-pig liver 2 days after allyl formate. Phagocytosis of necrotic tissue. H. & E. X 150.

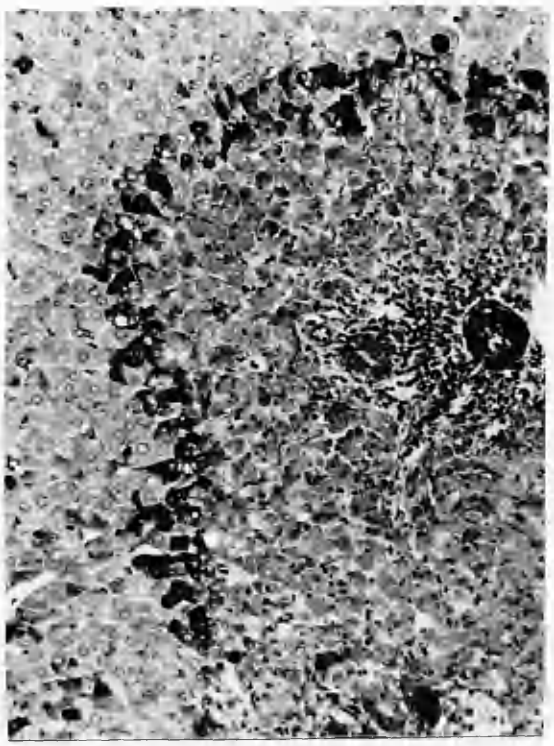


Fig. 225. Early regeneration of liver following allyl formate. Glycogen-laden cells at edge of regenerating parenchyma. P.A.S. X 150.

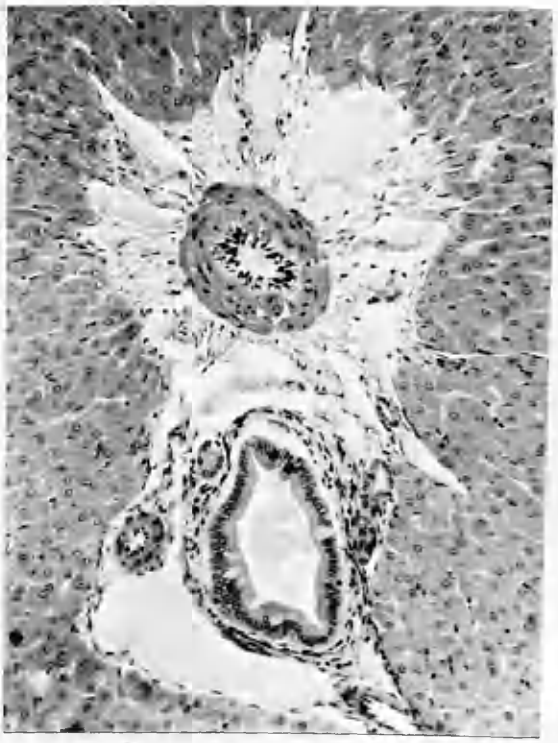


Fig. 226. Guinea-pig liver 4 days after allyl formate. There is no trace of necrosis but lymphatics are distended. H. & E. X 150.

TABLE XXXIV

Experiment 6 - Numbers of Animals Employed, and Severity of Liver Necrosis induced by Allyl Formate

Duration of poisoning	Number of guinea-pigs in each group			Percentage degree of zonal necrosis suffered by each animal		
	Allyl formate + Cortisone	Allyl formate	Cortisone	Allyl formate + Cortisone	Allyl formate	Cortisone
1 hour	1	1	0	0	0	-
3 hours	1	1	0	0	0	-
5 "	1	1	1	0	0	0
1 day	5	5	1	90,80,30,0,0	50,30,30,30, 20	0
2 days	4	4	2	90,70,0,0	50,50,0,0	0,0
3 "	4	4	1	0,0,0,0	20,10,0,0	0
4 "	1	1	1	0	0	0
5 "	1	1	1	0	0	0
Total	18	18	7			

Animals of the control (cortisone only) group were killed at intervals to correspond with the other groups.

TABLE XXXV

Experiment 7 - Numbers of Mice employed and Duration of Phosphorus Poisoning

Group	Duration of poisoning										Total
	Hours			Days							
	1	3	5	1	2	3	4	5	7	8	
A. Phosphorus + Cortisone											
Killed	1	1	1	2	2	3	2	1	1	1	15
Found Dead	0	0	0	4	1	0	0	0	0	0	5
B. Phosphorus alone											
Killed	1	1	1	3	3	3	2	1	1	1	17
Found Dead	0	0	0	3	0	0	0	0	0	0	3

Fig. 227. Mouse liver 1 day after phosphorus. Hydropic and fatty degeneration of periportal zones. H. & E. X 90.

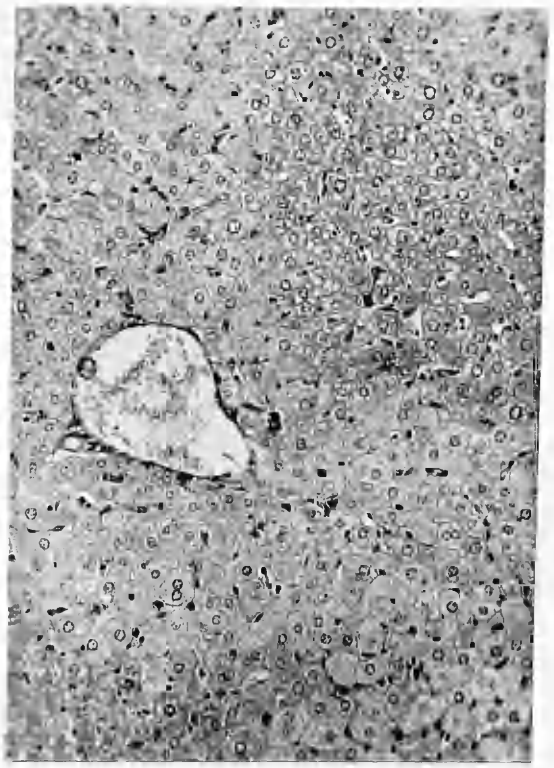


Fig. 228. Mouse liver 1 day after phosphorus. Portal tract on left. H. & E. X 164.

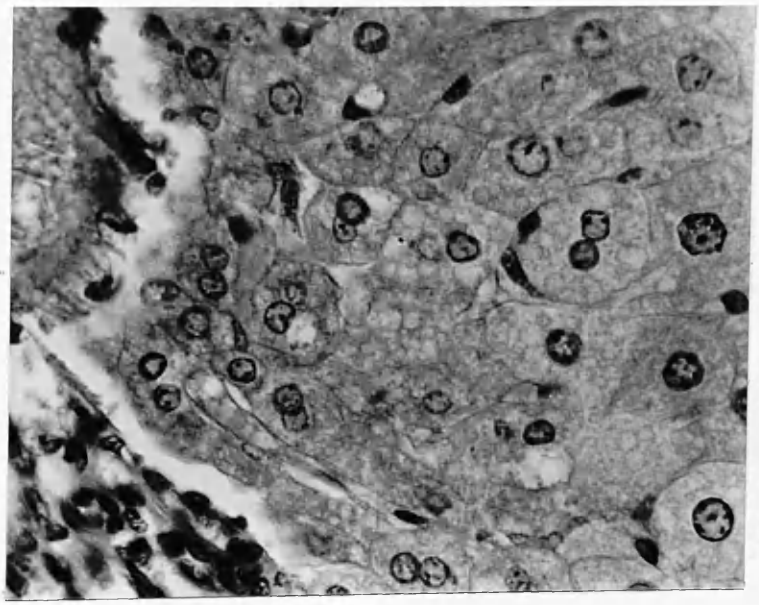


Fig. 229. Mouse liver 1 day after phosphorus. H. & E. X 630.

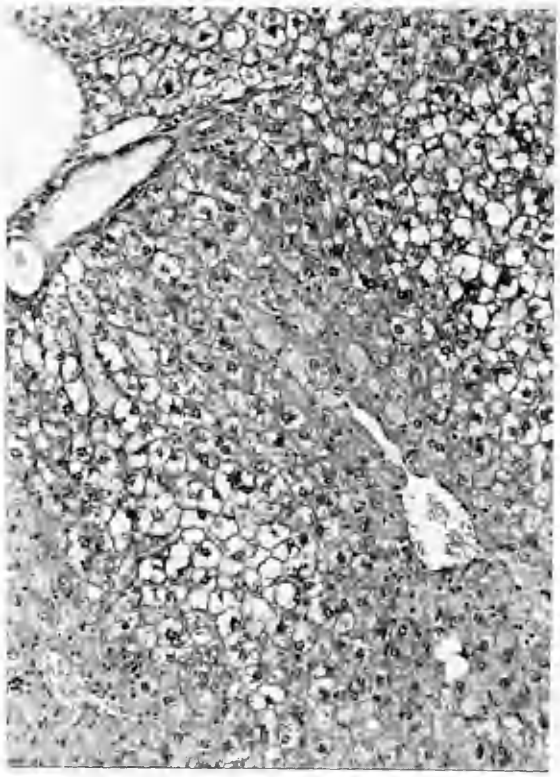


Fig. 230. Mouse liver 2 days after phosphorus. Centrilobular vein near bottom right. H. & E. X 150.

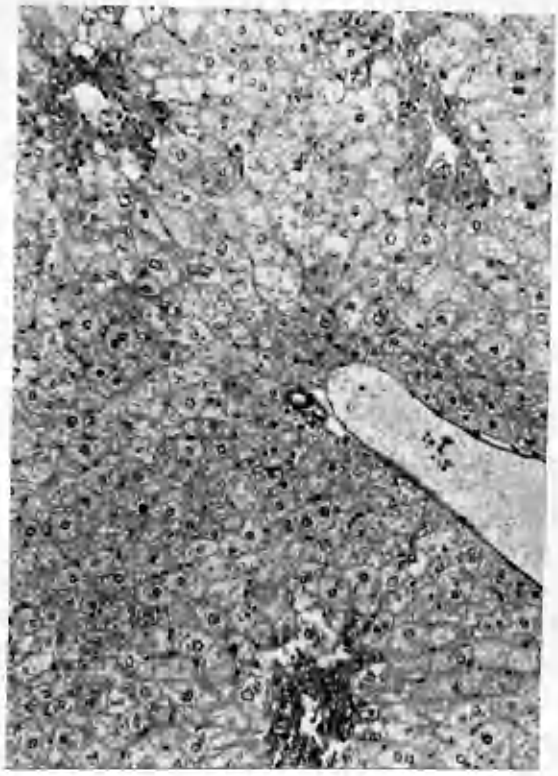


Fig. 231. Mouse liver 2 days after phosphorus. Narrow zones of centrilobular necrosis. H. & E. X 150.

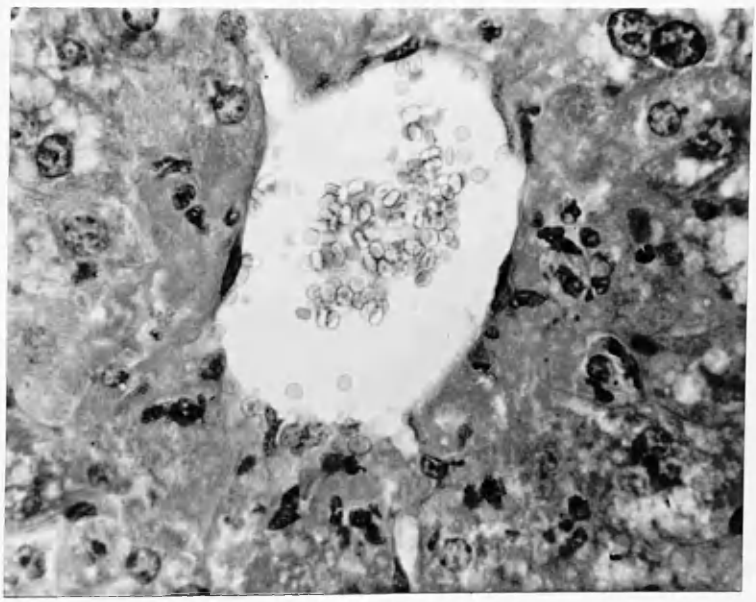


Fig. 232. Mouse liver 2 days after phosphorus. Centrilobular vein and adjacent tissue. H. & E. X 630.

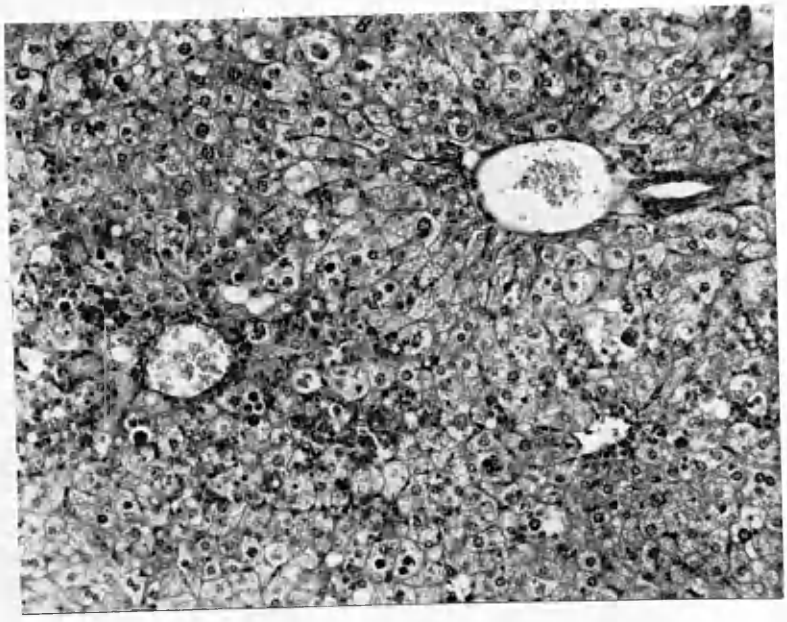


Fig. 233. Mouse liver 3 days after phosphorus. H. & E. X 150.

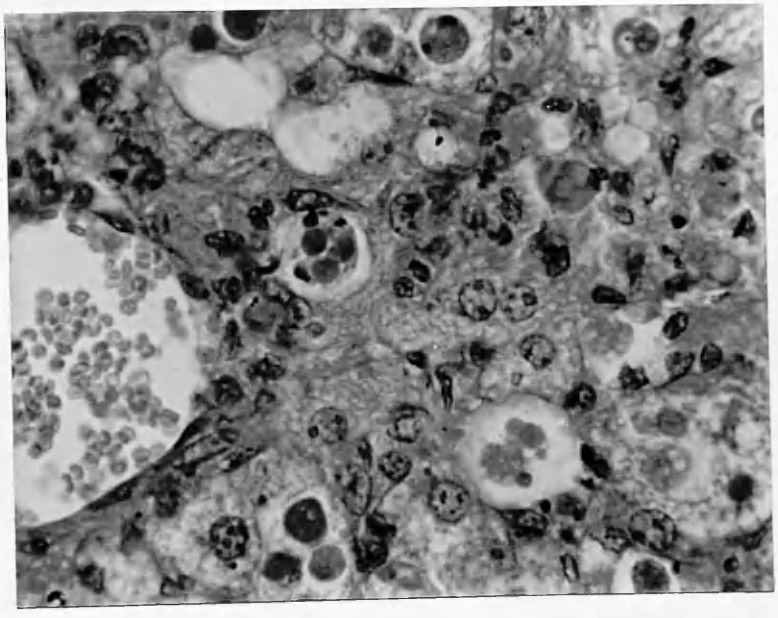


Fig. 234. Mouse liver 3 days after phorphorus. Centrilobular vein on left. H. & E. X 630.

P A R T IV

STUDIES ON LIVER REGENERATION WITH TRITIATED THYMIDINE

TABLES XXXVI to XXXVII

FIGURES 235 to 253

TABLE XXXVI

Radio-activity of Liver Sections and Mitotic Counts at Intervals after H^3-Thymidine and Carbon Tetrachloride

Mouse number	Time at which killed after H^3-Thymidine	Time at which killed after CCl_4	** % Liver necrosis	*** Gas-flow counts/min.	**** Mitotic counts
1	30 minutes	36½ hours	30	5.60	5
2	1 hour	37 "	50	46.44	27
3 *	2 hours	38 "	50	40.83	9
4 *	5 "	41 "	50	105.41	81
5 *	5 "	41 "	50	123.72	258
6	11 "	47 "	30	14.77	14
7	11 "	47 "	50	9.73	3
8	24 "	60 "	50	17.08	22
9	24 "	60 "	40	43.55	466
10	2 days	3½ days	20	25.38	700
11	2 "	3½ "	20	19.42	219
12	3 "	4½ "	0	35.35	404
13	3 "	4½ "	10	5.90	435
14 *	5 "	6½ "	0	39.30	0
15	6 "	7½ "	0	33.55	3
16 *	7 "	8½ "	0	49.20	0
17 *	10 "	11½ "	0	11.12	1
18 *	14 "	15½ "	0	40.41	0
19 *	21 "	22½ "	0	116.80	0
20	28 "	29½ "	0	18.25	0
21	42 "	43½ "	0	79.99	0
22 *	56 "	57½ "	0	49.54	0

* Illustrated (Figs. 235 to 249).

** See Figures 206 to 209.

*** Corrected for back-ground radiation and standardized for mean section paper weight and unit liver weight.

**** Standardized for mean section paper weight and unit liver weight.

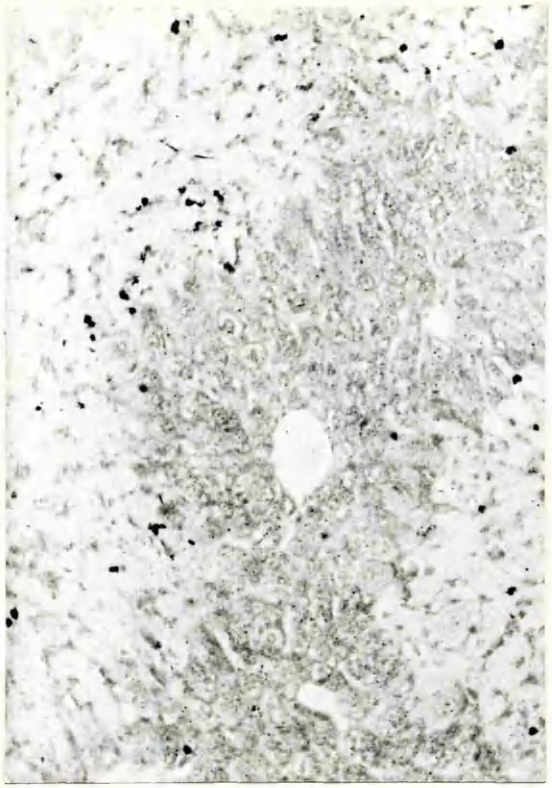


Fig. 235. Mouse liver 38 hours after CCl_4 and 2 hours after H^3-thymidine. Sinusoidal cells labelled.
Autoradiograph; neutral red X 150.

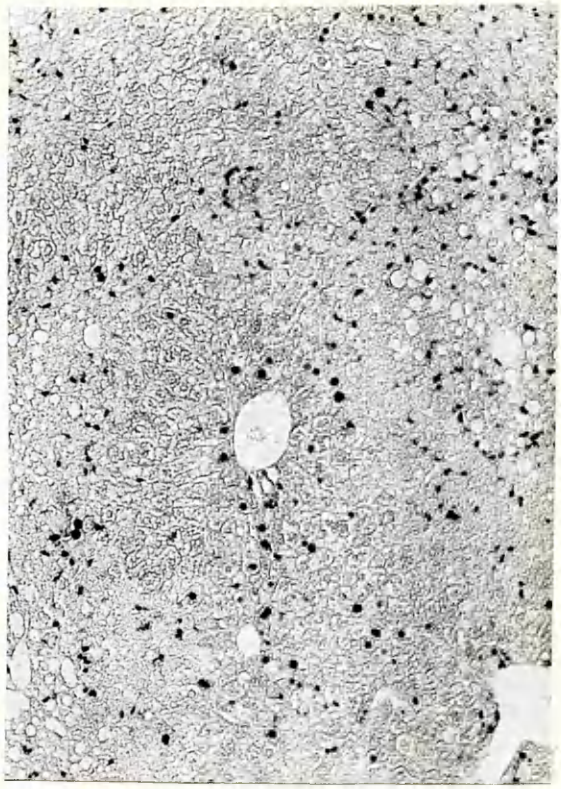


Fig. 236. Mouse liver 41 hours after CCl_4 and 5 hours after H^3-thymidine. Parenchymal and sinusoidal cells labelled.
Autoradiograph; neutral red X 90.

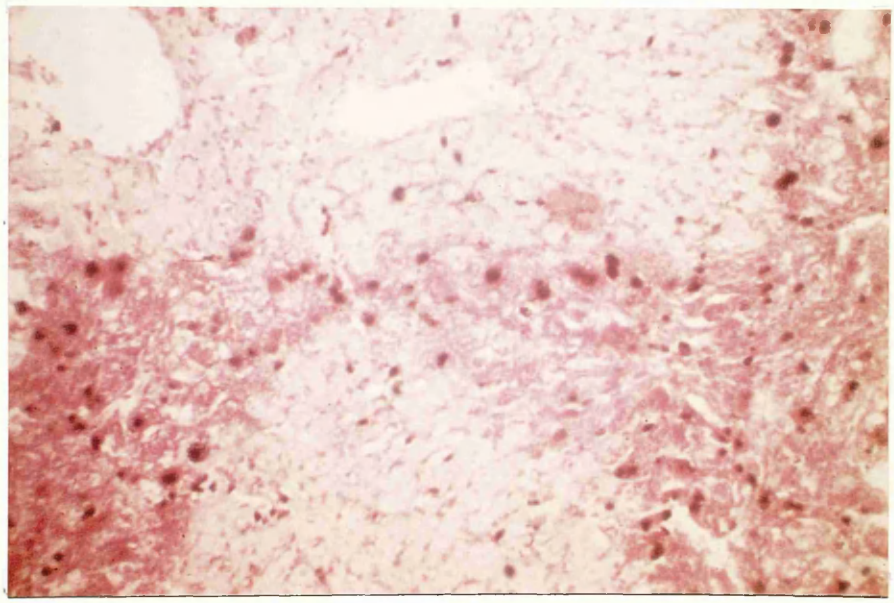


Fig. 237. Mouse liver 41 hours after CCl_4 and 5 hours after H^3-thymidine. Many parenchymal cells labelled.
Autoradiograph; neutral red X 125.

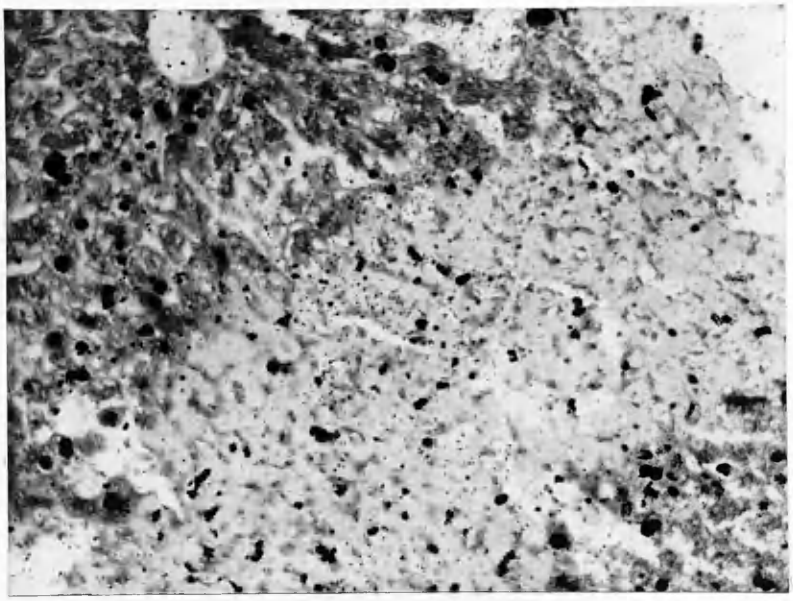


Fig. 238. Mouse liver 41 hours after CCl_4. Autoradiograph; neutral red X 150.

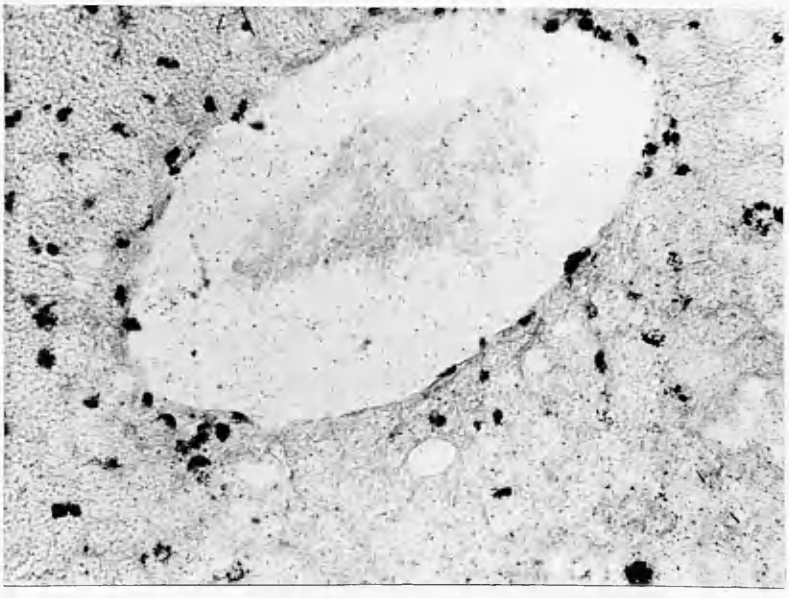


Fig. 239. Mouse liver 41 hours after CCl_4. Centrilobular vein and adjacent tissue. Sinusoidal cells and venous endothelium labelled. Autoradiograph; neutral red X 240.

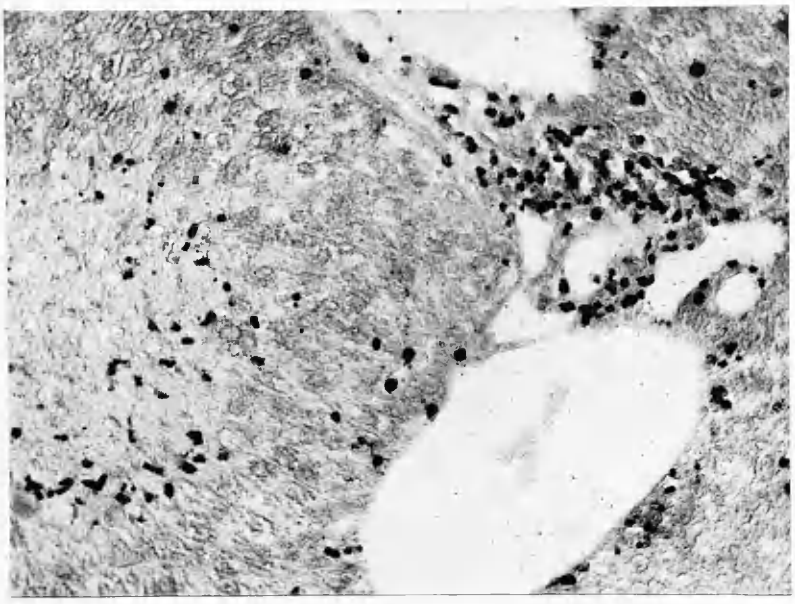


Fig. 240. Mouse liver 41 hours after CCl_4. Inflammatory cells in portal canal labelled. Autoradiograph; neutral red X 150.

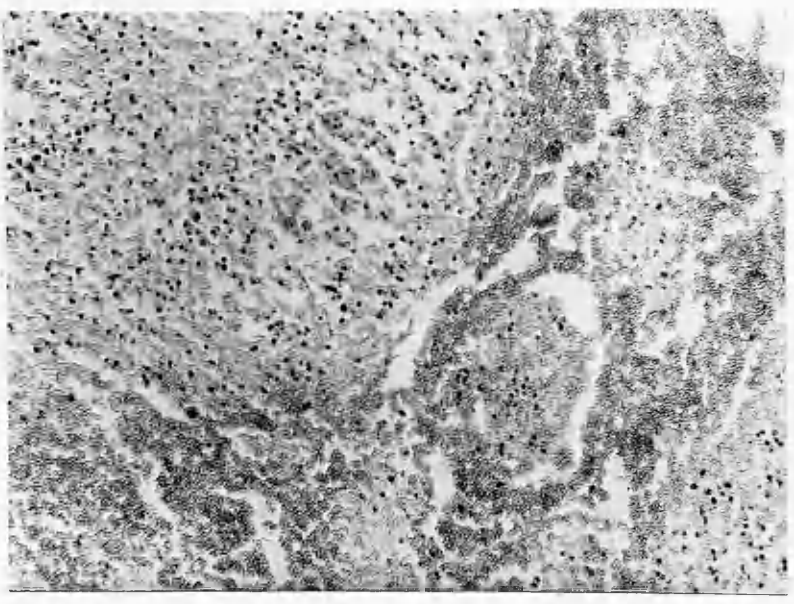


Fig. 241. Spleen of same animal. Autoradiograph; neutral red X 90.

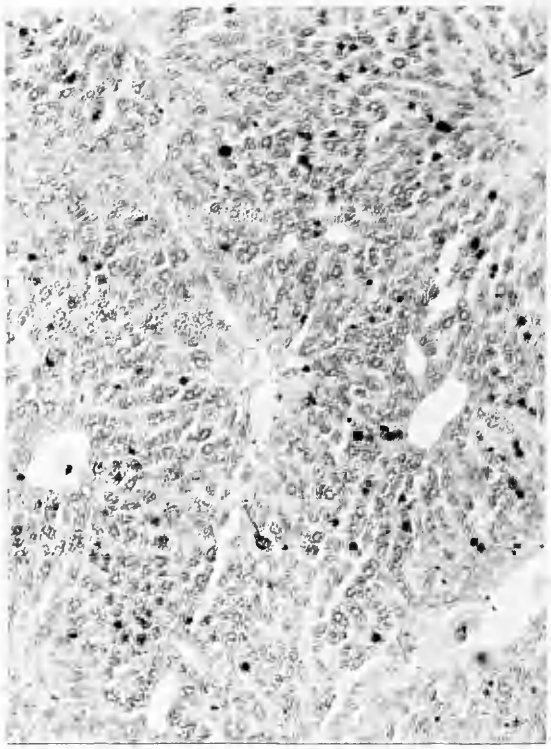


Fig. 242. Mouse liver 6 days after CCl_4. Regeneration complete. Autoradiograph; neutral red X 150.

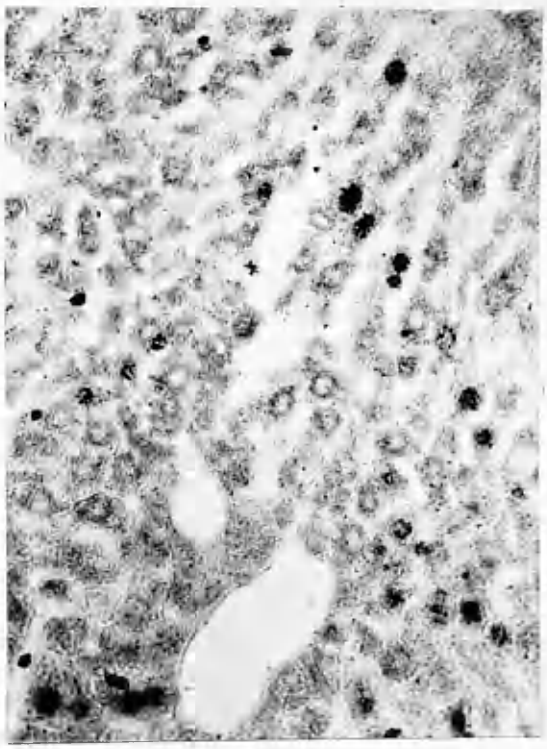


Fig. 243. Mouse liver 6 days after CCl_4. Variable intensity in labelling of liver cell nuclei. Autoradiograph; neutral red X 90.

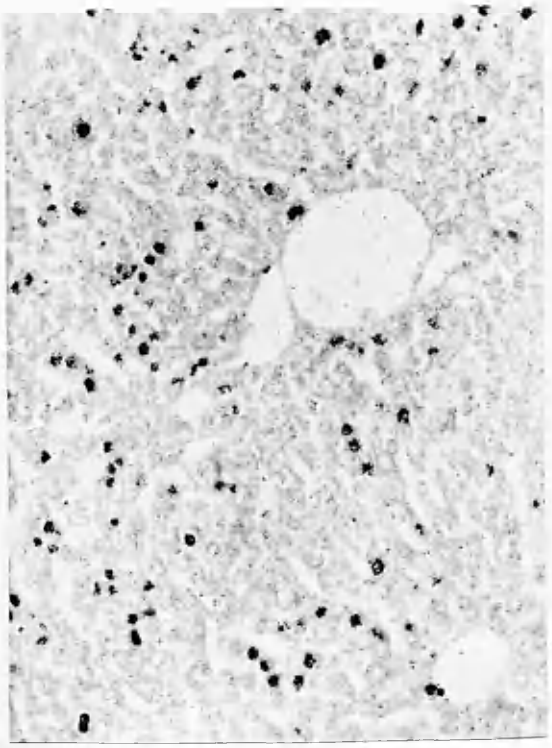


Fig. 244. Mouse liver 8 days after CCl_4. Autoradiograph; neutral red X 240.

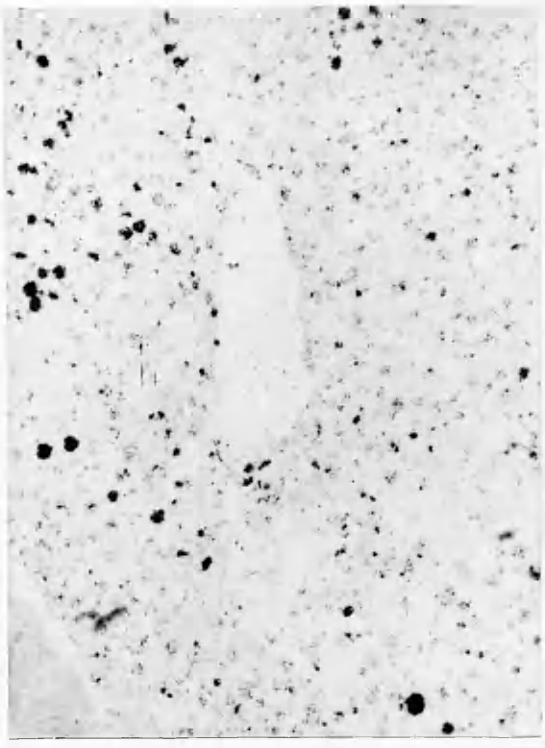


Fig. 245. Mouse liver 15 days after CCl_4. Autoradiograph; neutral red X 150.

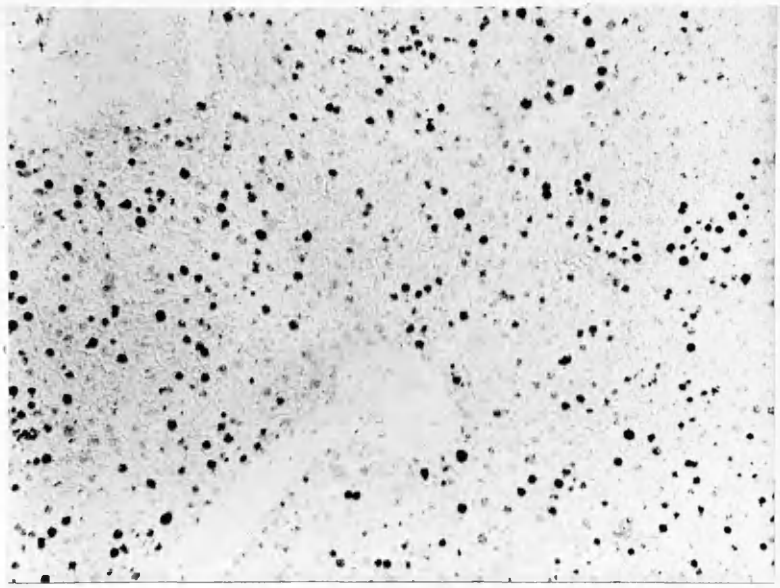


Fig. 246. Mouse liver 22 days after CCl_4.
Autoradiograph; neutral red X 90.

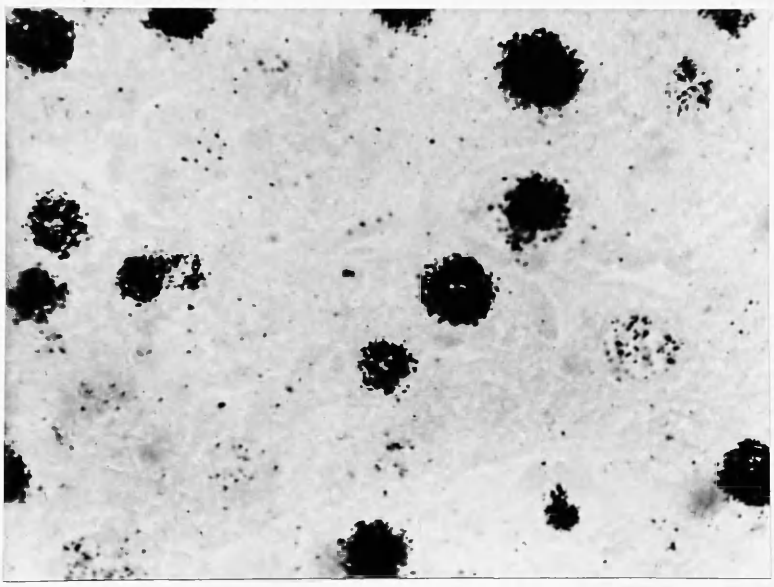


Fig. 247. Mouse liver 22 days after CCl_4.
Autoradiograph; neutral red X 630.

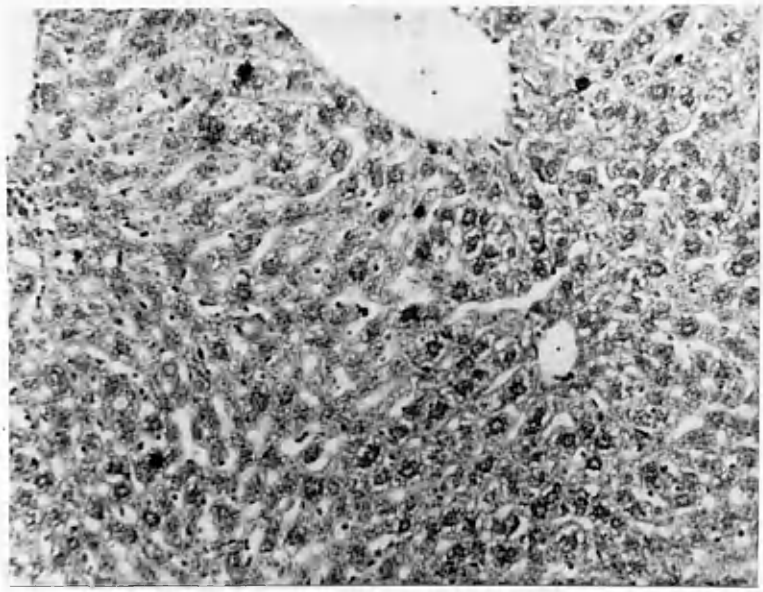


Fig. 248. Mouse liver 11 days after CCl_4. Four liver cell nuclei labelled in this field.
Autoradiograph; neutral red X 150.

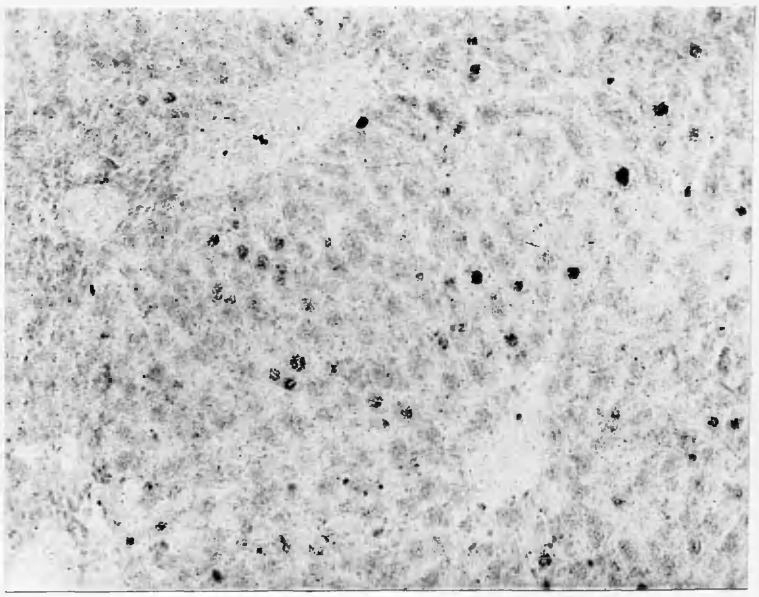


Fig. 249. Mouse liver 8 weeks after CCl_4. Numerous liver cell nuclei labelled.
Autoradiograph; neutral red X 150.

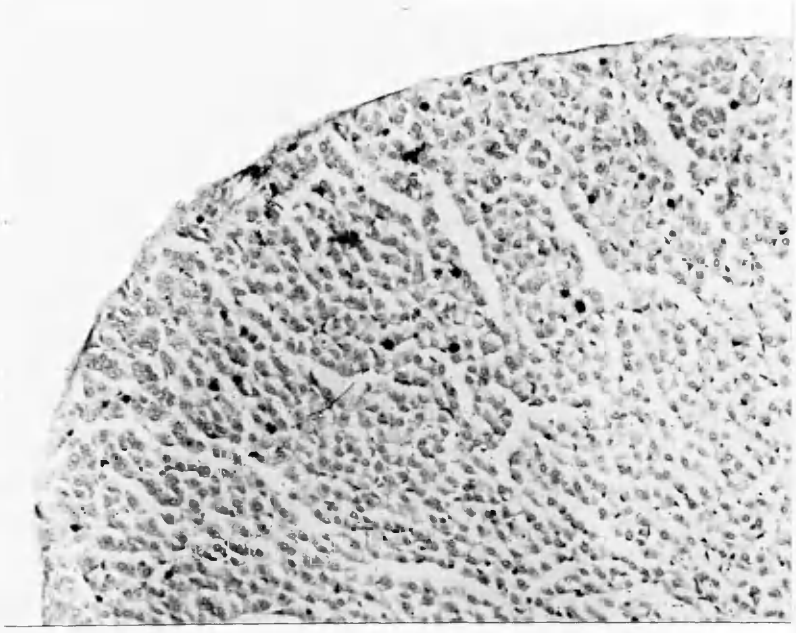


Fig. 250. Mouse adrenal gland 8 days after CCl_4.
Autoradiograph; neutral red X 150.

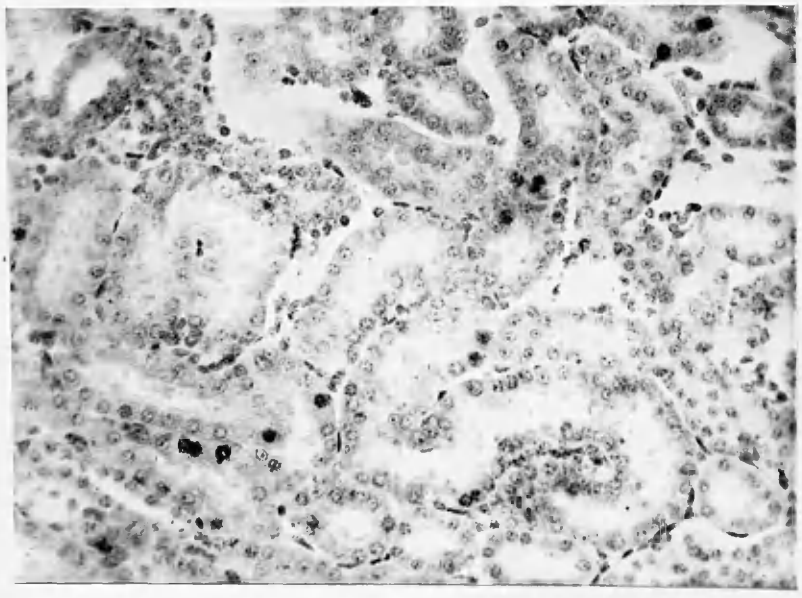


Fig. 251. Mouse renal cortex 15 days after CCl_4.
Autoradiograph; neutral red X 240.

TABLE XXXVII

Liver Weights and Radio-activity of Liver Sections after H^3-Thymidine and Partial Hepatectomy

Mouse liver	Weight of liver excised	Times at which killed after H^3-Thymidine	Times at which killed after Partial hepatectomy	Weight of liver at autopsy	* Counts/ minute
1	1.13 g.	1 day	2½ days	0.46 g.	1.89
2	1.12 g.	1 "	2½ "	0.41 g.	2.86
3	0.94 g.	2 days	3½ "	0.54 g.	4.27
4	1.20 g.	4 "	6 "	1.13 g.	18.95
5	1.01 g.	7 "	9 "	1.24 g.	25.22
6	1.14 g.	14 "	16 "	1.40 g.	18.25
7	1.25 g.	28 "	30 "	1.71 g.	19.20

* Corrected for back-ground radiation and standardized for mean section paper weight and unit weight of regenerating liver.

TABLE XXXVII

Liver Weights and Radio-activity of Liver Sections after H^3-Thymidine and Partial Hepatectomy

Mouse liver	Weight of liver excised	Times at which killed after H^3-Thymidine	Partial hepatectomy	Weight of liver at autopsy	* Counts/ minute
1	1.13 g.	1 day	2½ days	0.46 g.	1.89
2	1.12 g.	1 "	2½ "	0.41 g.	2.86
3	0.94 g.	2 days	3½ "	0.54 g.	4.27
4	1.20 g.	4 "	6 "	1.13 g.	18.95
5	1.01 g.	7 "	9 "	1.24 g.	25.22
6	1.14 g.	14 "	16 "	1.40 g.	18.25
7	1.25 g.	28 "	30 "	1.71 g.	19.20

* Corrected for back-ground radiation and standardized for mean section paper weight and unit weight of regenerating liver.

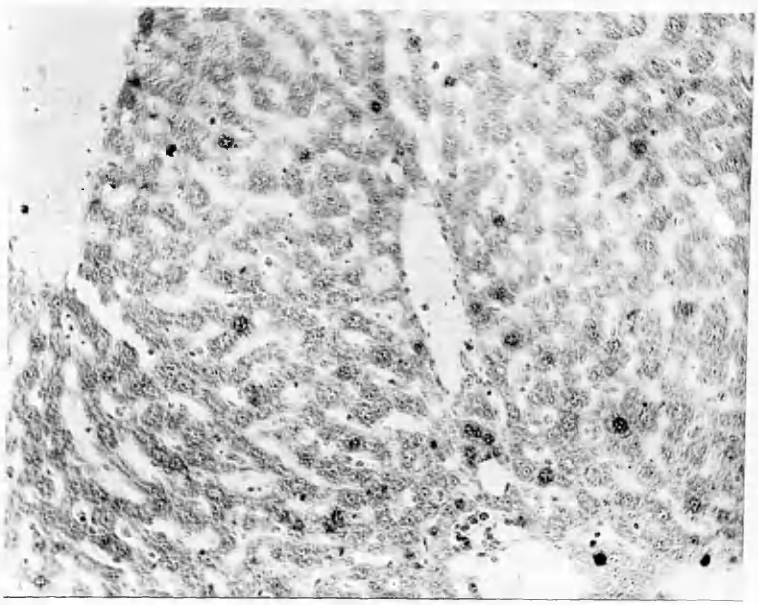


Fig. 252. Mouse liver 16 days after partial hepatectomy and 14 days after H^3-thymidine. Autoradiograph; neutral red X 150.

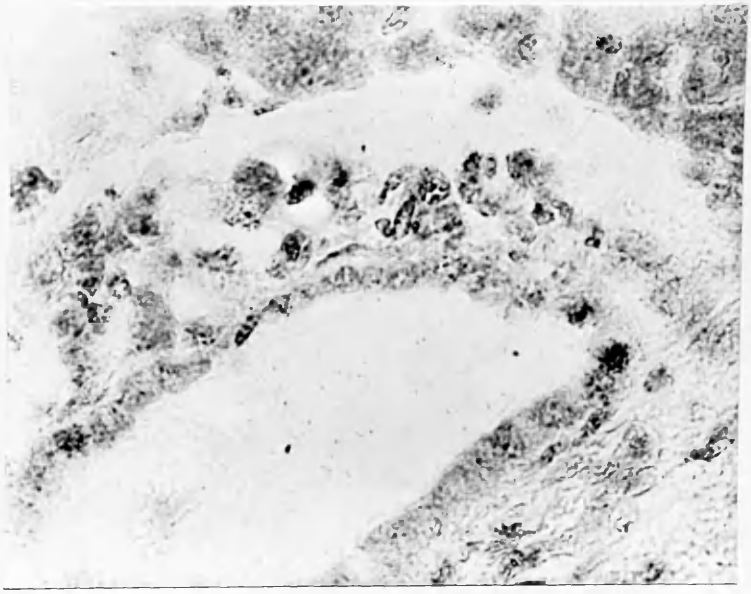


Fig. 253. Same specimen showing evidence of bile duct epithelial regeneration. Autoradiograph; neutral red X 630.

PART V

STUDIES WITH S^{35}-LABELLED AMINO-ACIDS

IN EXPERIMENTAL LIVER DISEASE

TABLES XXXVIII to XLIV

FIGURES 254 to 332

TABLE XXXVIII

Experiment 1 - The Effects of Variable Doses of Bromobenzene on Mice

Mouse number	Dose of bromobenzene ml./100 g. body weight	Died or killed	Interval after bromobenzene	Gross appearance of liver
1	0.025	Killed	24 hours	Slight zonal necrosis
2	0.025	Killed	48 hours	Zonal and massive necrosis
3	0.050	Killed	24 hours	Zonal and massive necrosis
4	0.050	Killed	48 hours	Zonal and massive necrosis
5	0.075	Killed	24 hours	Zonal and massive necrosis
6	0.075	Died	12 hours	Indistinct massive necrosis
7	0.100	Killed	24 hours	Zonal and massive necrosis
8	0.100	Died	?24 hours	Zonal and massive necrosis
9	0.150	Died	12 hours	Indistinct massive necrosis
10	0.150	Died	7 hours	Congested
11	0.200	Died	1 hour	Congested
12	0.250	Died	40 minutes	Congested

TABLE XXXIX

Experiment 2 - Changes in Mouse Liver at Intervals after Bromobenzene Intoxication

Mouse number	Dose of bromobenzene ml./100 g. body weight	Interval between bromobenzene administration and killing	Appearance of liver
1	0.050	1 hour	No abnormality detected
2	0.050	1 "	No abnormality detected
3	0.050	3 hours	Early degenerative changes
4	0.050	4 "	Early degenerative changes
5	0.050	7 "	Early degenerative changes
6	0.050	8 "	Early degenerative changes
7	0.050	11 "	Hydropic degeneration and early necrosis
8	0.050	12 "	Hydropic degeneration and early necrosis
9	0.050	24 "	Zonal and massive necrosis
10	0.050	2 days	Zonal and massive necrosis
11	0.050	3 "	Advanced regeneration
12	0.050	4 "	Zonal necrosis healed Massive necrosis persists

Fig. 254. Two livers from mice 24 hours after bromobenzene. Anterior aspect. X 2.

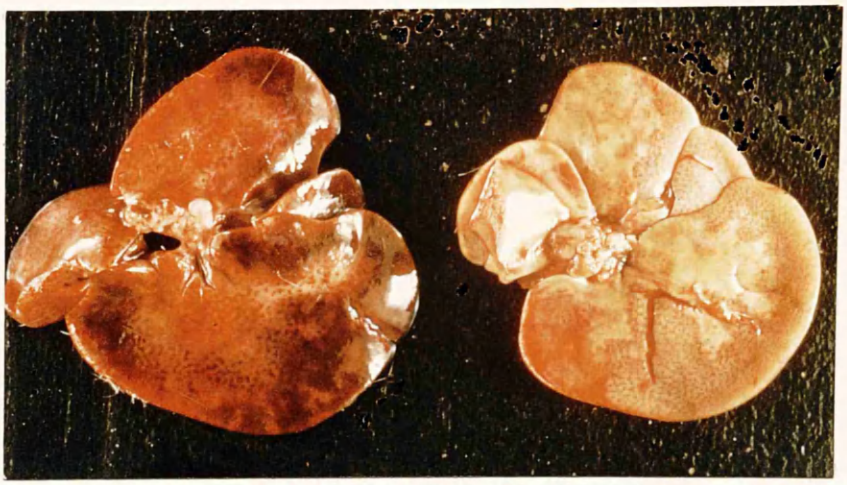


Fig. 255. Posterior aspect of same livers. X 2.

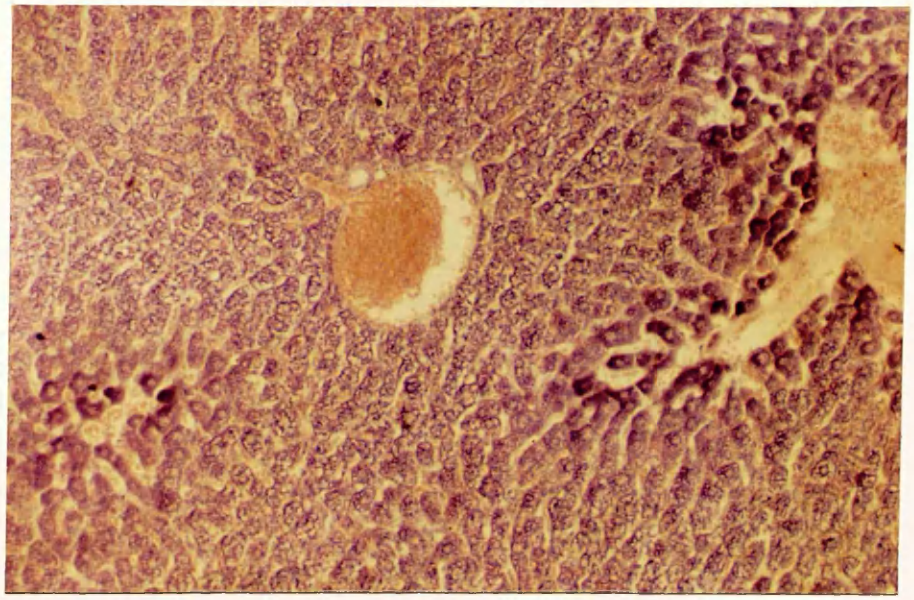


Fig. 256. Mouse liver 7 hours after bromobenzene. Diffuse basophilia of centrilobular cell cytoplasm. Giemsa's stain X 188.

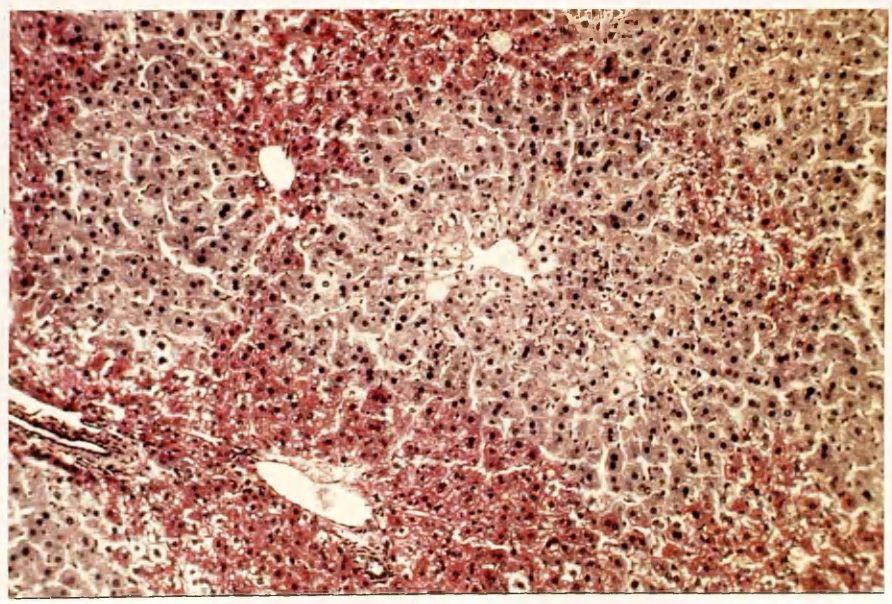


Fig. 257. Mouse liver 7 hours after bromobenzene. Loss of glycogen from centrilobular zones. P.A.S. X 125.

Fig. 258. Mouse liver 24 hours after bromobenzene. Mid-zonal hydropic degeneration. P.A.S. X 90.

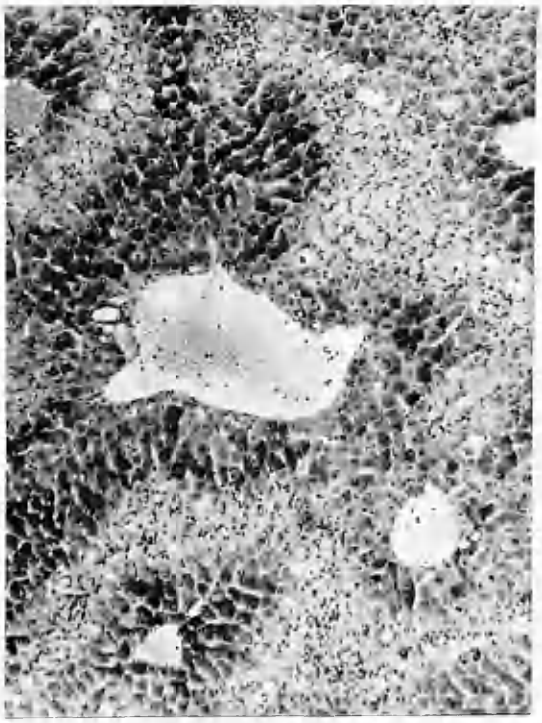


Fig. 259. Mouse liver 2 days after bromobenzene. Mid-zonal necrosis. P.A.S. X 90.

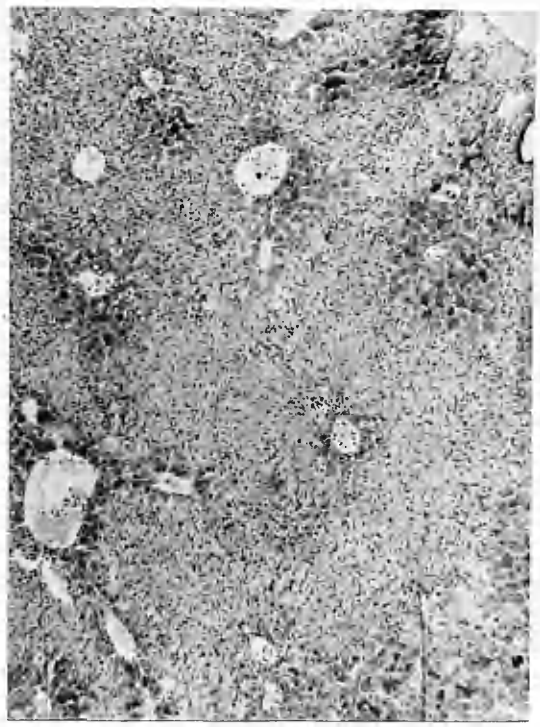


Fig. 260. Mouse liver 2 days after bromobenzene. Severe zonal necrosis. P.A.S. X 60.

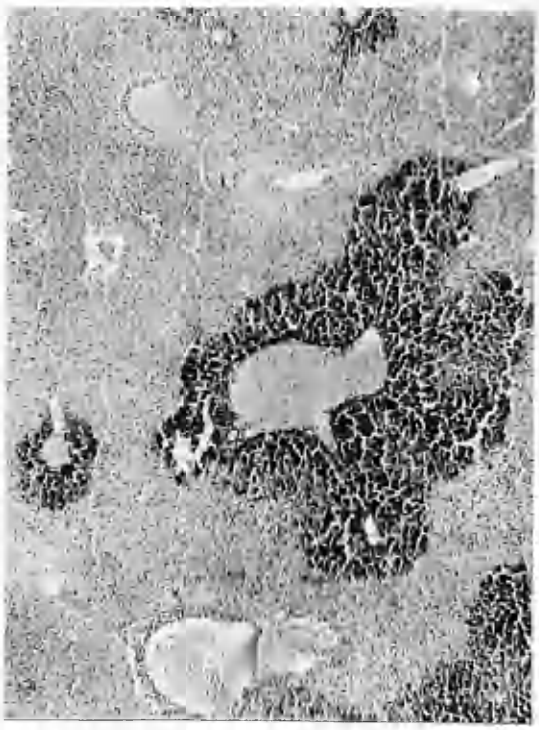


Fig. 261. Mouse liver 24 hours after bromobenzene. Massive necrosis. Giemsa's stain x 60.

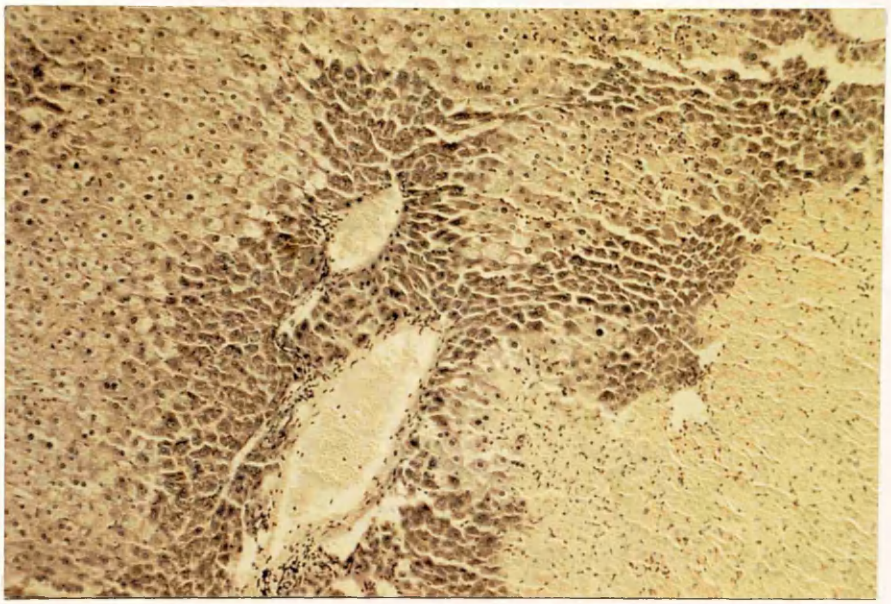


Fig. 262. Mouse liver 24 hours after bromobenzene. Centrilobular zonal degeneration and massive necrosis. Giemsa's stain X 75.

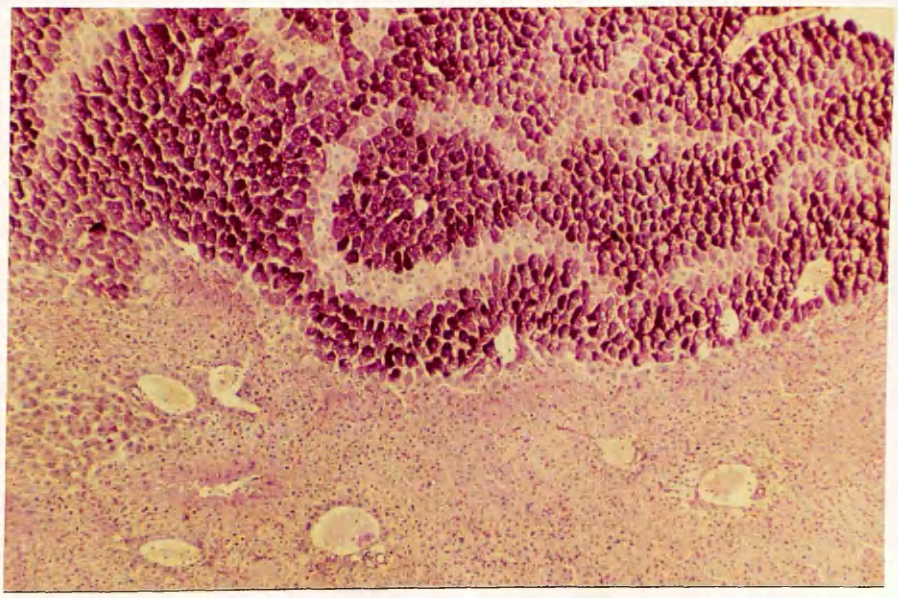


Fig. 263. Mouse liver 24 hours after bromobenzene. Mid-zonal degeneration and massive necrosis. P.A.S. X 75.

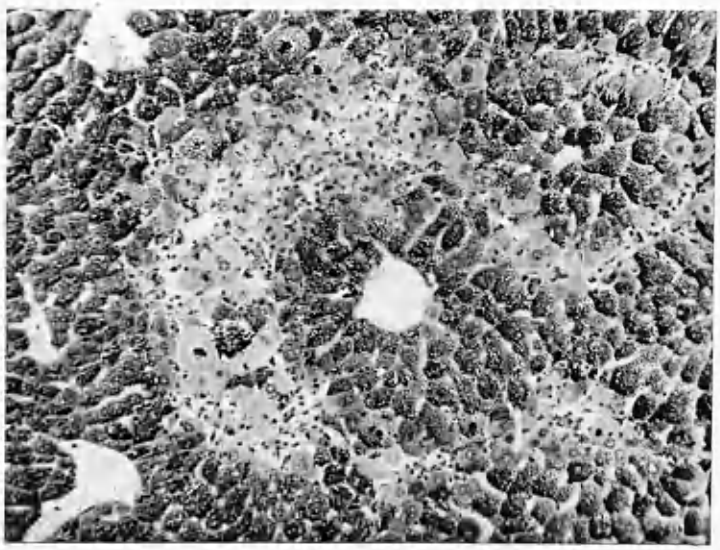


Fig. 264. Mouse liver 3 days after bromobenzene. Regeneration following mid-zonal necrosis.
P.A.S. X 180.

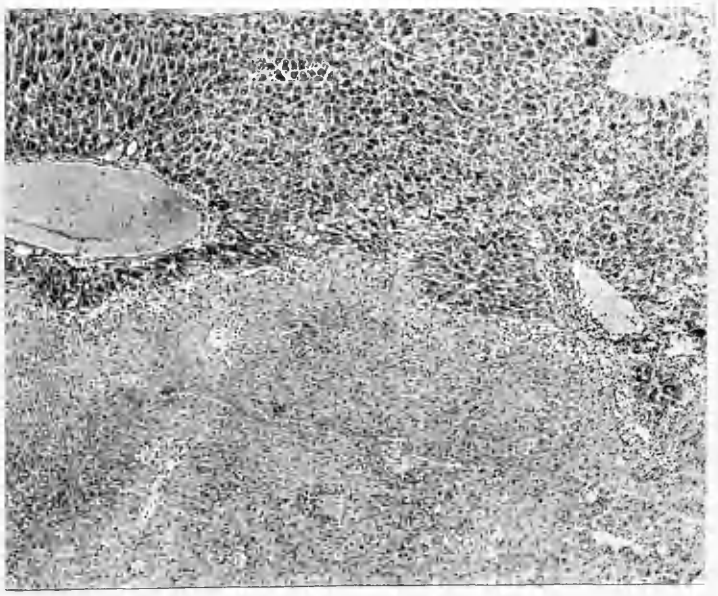


Fig. 265. Mouse liver 3 days after bromobenzene. Persistent massive necrosis. H. & E. X 60.

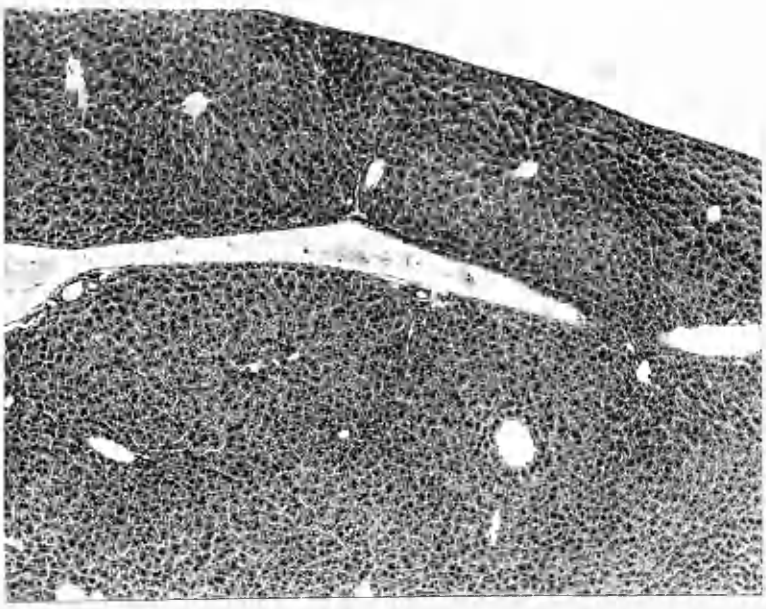


Fig. 266. Normal mouse liver. H. & E. X 60.

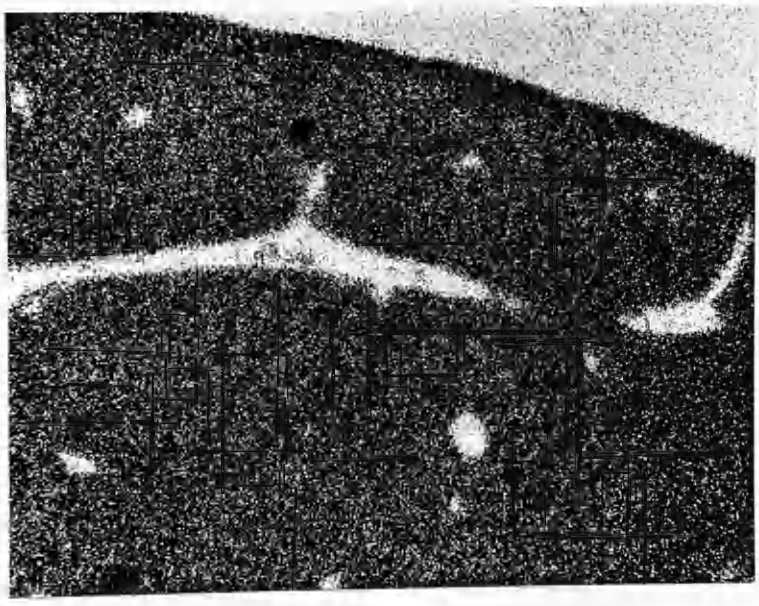


Fig. 267. Autoradiograph of same liver. Killed 24 hours after S^{35}-methionine. X 60.

TABLE XL

Experiments 3 to 7 - Numbers of Mice treated with S^{35}-methionine and Liver Poison, and Radio-activity of Liver Sections

Experiment number	Mouse number	Hours at which killed after S^{35}-methionine	Hours at which killed after Bromobenzene	** Counts/ minute
3	1	1	-	310.5
	2	5	-	334.4
	3 *	24	-	263.5
	4	48	-	340.0
	5	96	-	251.7
4	1	25	1	480.0
	2	25	1	193.8
	3 *	28	4	302.1
	4	28	4	421.5
	5 *	32	8	480.7
	6	32	8	125.8
	7 *	36	12	277.7
	8	36	12	368.9
	9 *	48	24	321.0
	10	48	24	446.2
5	1 *	7	8	685.1
	2 *	24	25	326.0
	3	24	25	332.7
	4 *	20	24	482.5
	5	20	24	154.8
	6 *	16	28	335.8
	7 *	24	48	154.1
	8 *	48	96	845.1
6	1 *	25	1	565.0
	2	25	1	178.0
	3	28	4	308.0
	4	28	4	282.4
	5 *	32	8	227.5
	6	32	8	78.0
	7	36	12	325.1
	8	36	12	315.1
	9 *	48	24	354.0
	10	48	24	257.8
7	1 *	6	7	402.1
	2	24	25	115.7
	3	20	24	306.7
	4	16	28	174.8
	5 *	24	48	226.6

Experiment number	Mouse number	Hours at which killed after S^{35}-methionine	Hours at which killed after CCl_4	** Counts/ minute

* Illustrated (Figs. 266 to 297).

** Corrected for back-ground radiation and standardized for mean section paper weight and unit liver weight.

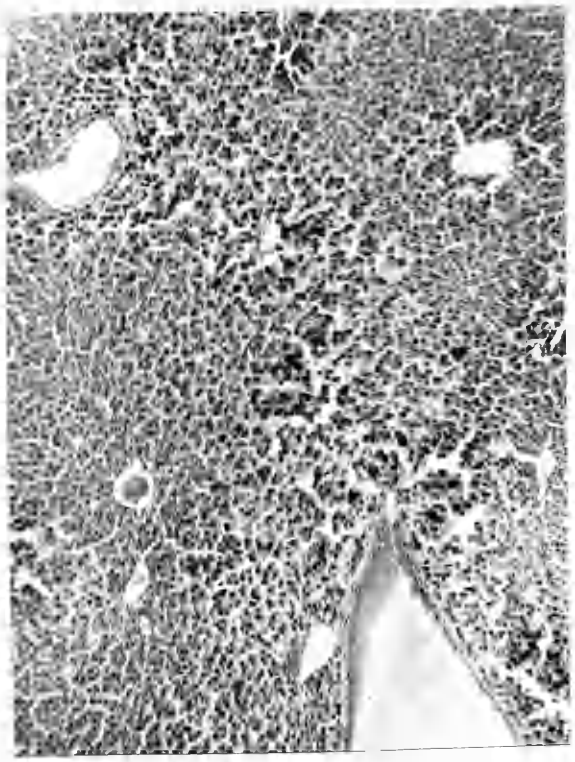


Fig. 268. Mouse liver 4 hours after bromobenzene. H. & E. X 60.

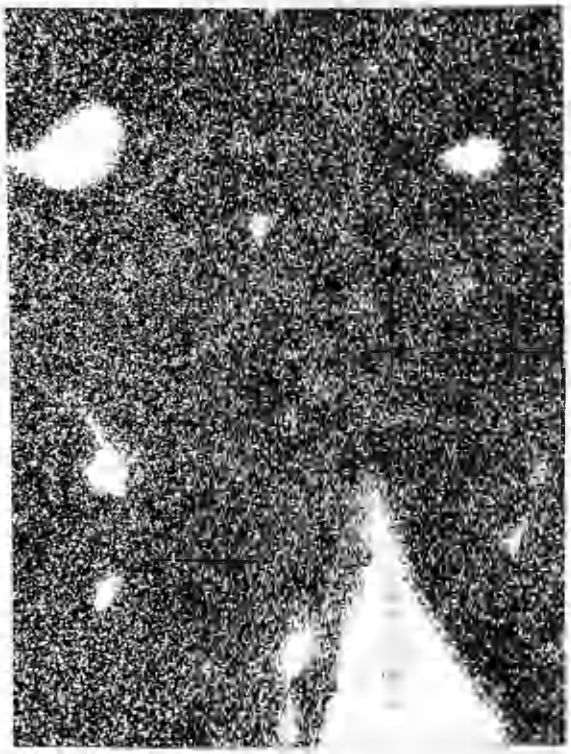


Fig. 269. Autoradiograph of same liver 28 hours after S^{35}-methionine. X 60.

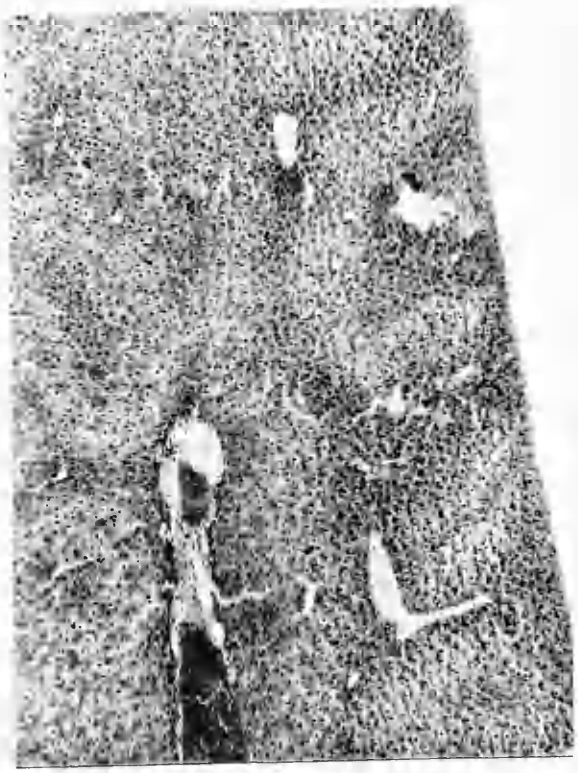


Fig. 270. Mouse liver 8 hours after bromobenzene. H. & E. X 60.

Fig. 271. Autoradiograph of same liver 32 hours after S^{35}-methionine. X 60.

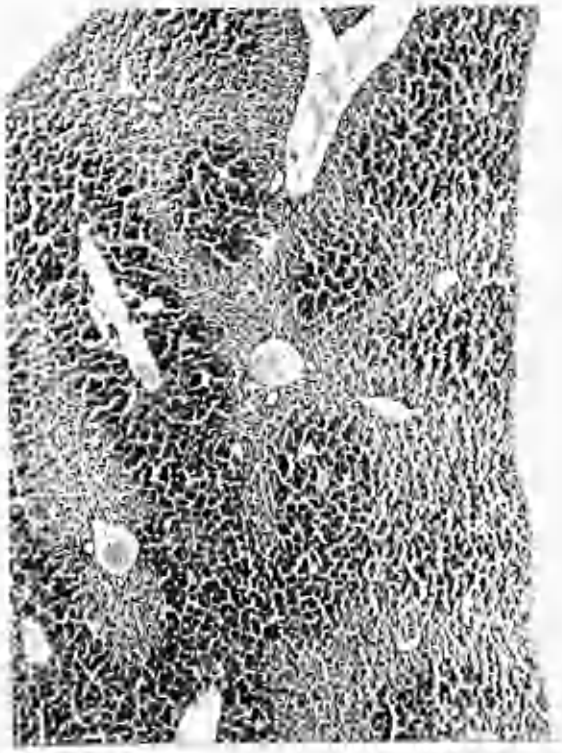


Fig. 272. Mouse liver 12 hours after bromobenzene. H. & E. X 60.

Fig. 273. Autoradiograph of same liver 36 hours after S^{35}-methionine. X 60.

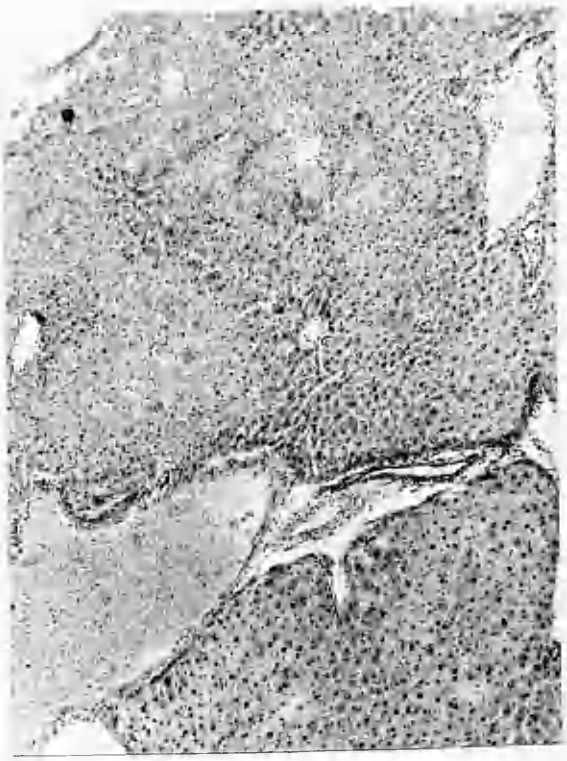


Fig. 274. Mouse liver 24 hours after bromobenzene. Upper portion necrotic. H. & E. X 90.

Fig. 275. Autoradiograph of same liver 48 hours after S^{35}-methionine. X 90.

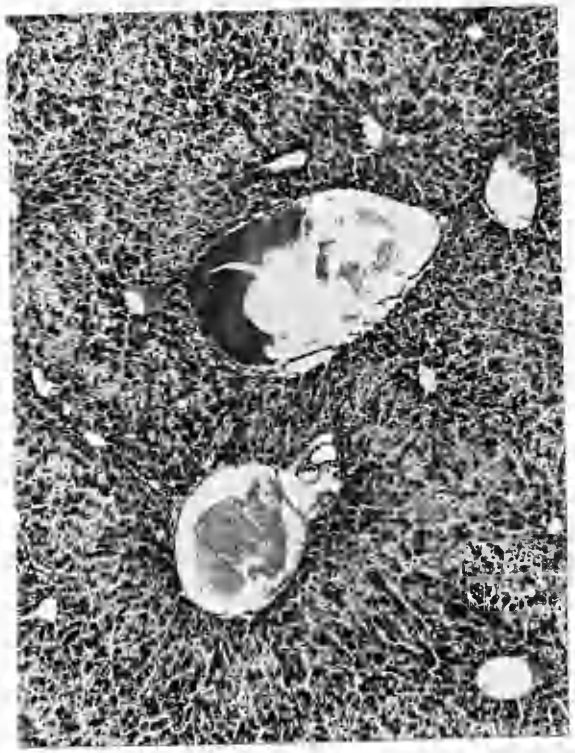


Fig. 276. Mouse liver 8 hours after bromobenzene.
H. & E. X 60.

Fig. 277. Autoradiograph of same liver 7 hours after S^{35}-methionine.
X 60.

Fig. 278. Mouse liver 25 hours after bromobenzene.
H. & E. X 60.

Fig. 279. Autoradiograph of same liver 24 hours after S^{35}-methionine.
X 60.

Fig. 280. Mouse liver 24 hours after bromobenzene.
H.& E. X 60.

Fig. 281. Autoradiograph of same liver 20 hours after S^{35}-methionine.
X 60.

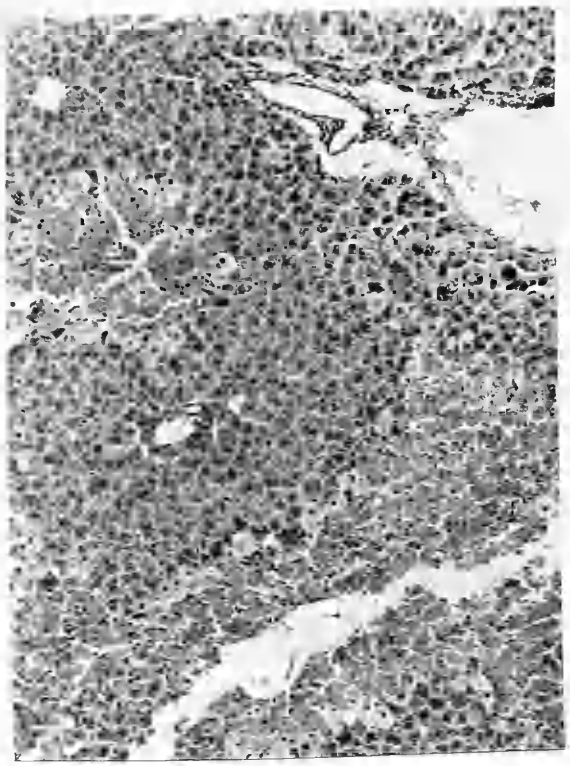


Fig. 282. Mouse liver 28 hours after bromobenzene.
H. & E. X 90.

Fig. 283. Autoradiograph of same liver 16 hours after S^{35}-methionine.
X 90.

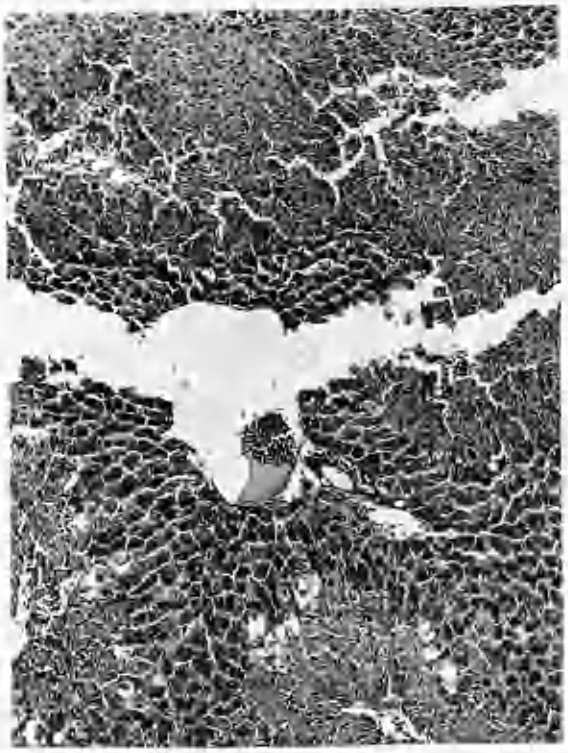


Fig. 284. Mouse liver 48 hours after bromobenzene.
H. & E. X 90.

Fig. 285. Autoradiograph of same liver 24 hours after S^{35}-methionine.
X 90.

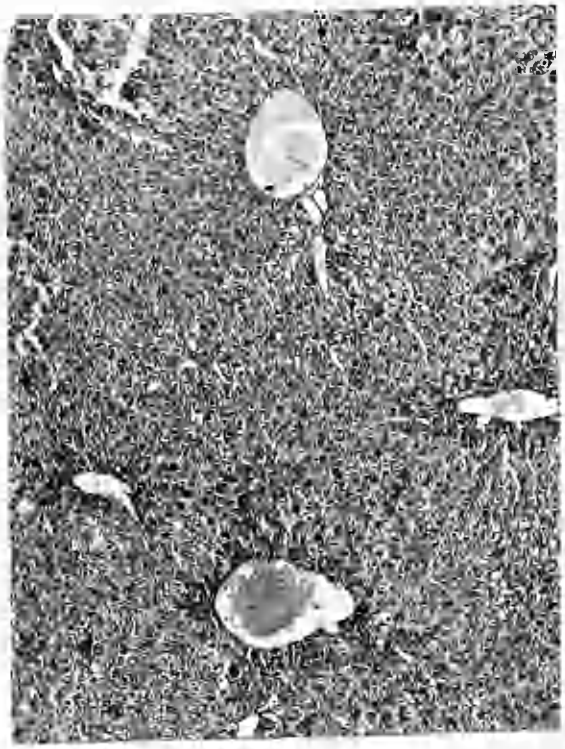


Fig. 286. Mouse liver 96 hours after bromobenzene. H. & E. X 60.

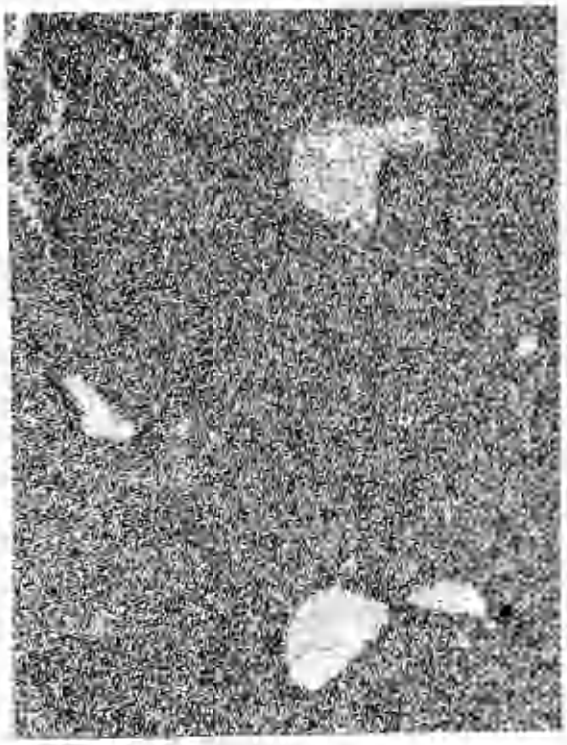


Fig. 287. Autoradiograph of same liver 48 hours after S^{35}-methionine.
X 60.

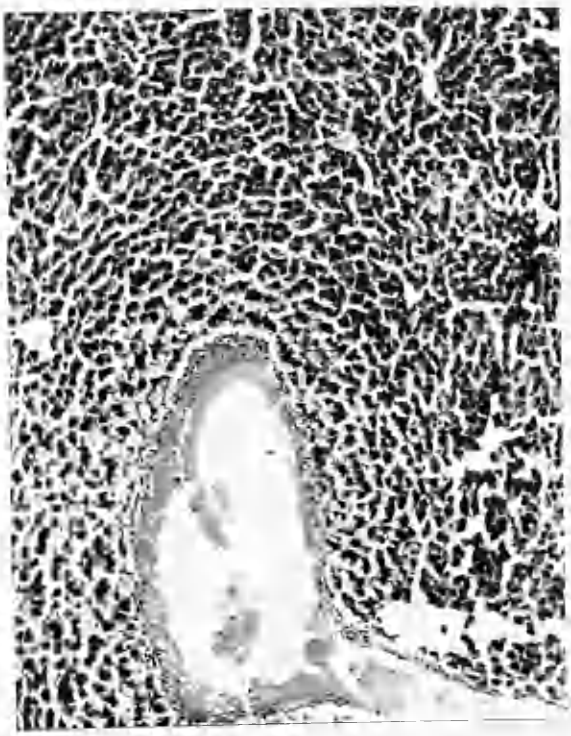


Fig. 288. Mouse liver 1 hour after CCl_4.
X 90.

Fig. 289. Autoradiograph of same liver 25 hours after S^{35}-methionine.
X 90.

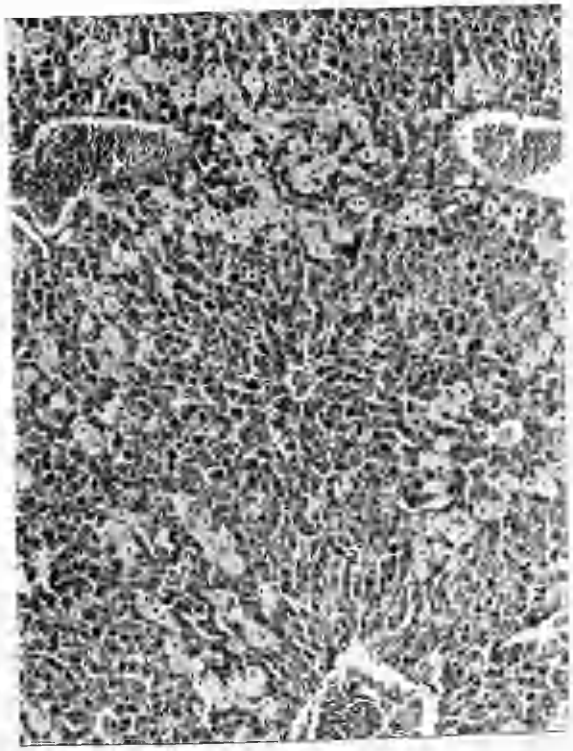


Fig. 290. Mouse liver 8 hours after CCl_4.
X 90.

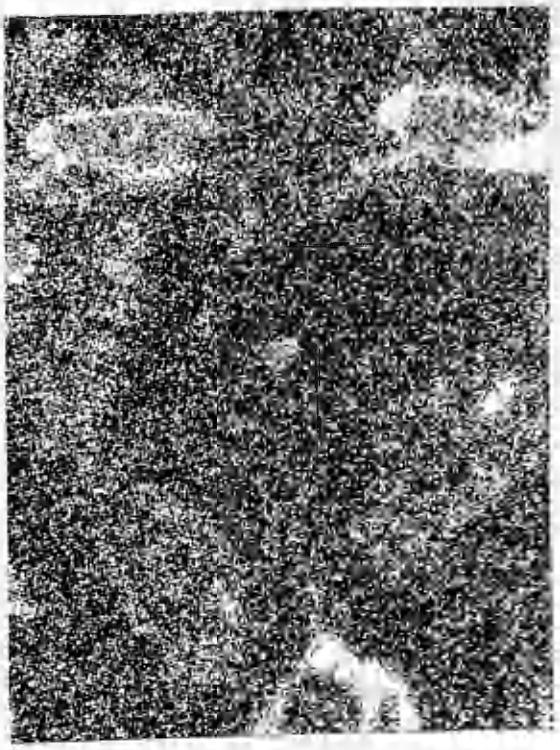


Fig. 291. Autoradiograph of same liver 32 hours after S^{35}-methionine.
X 90.

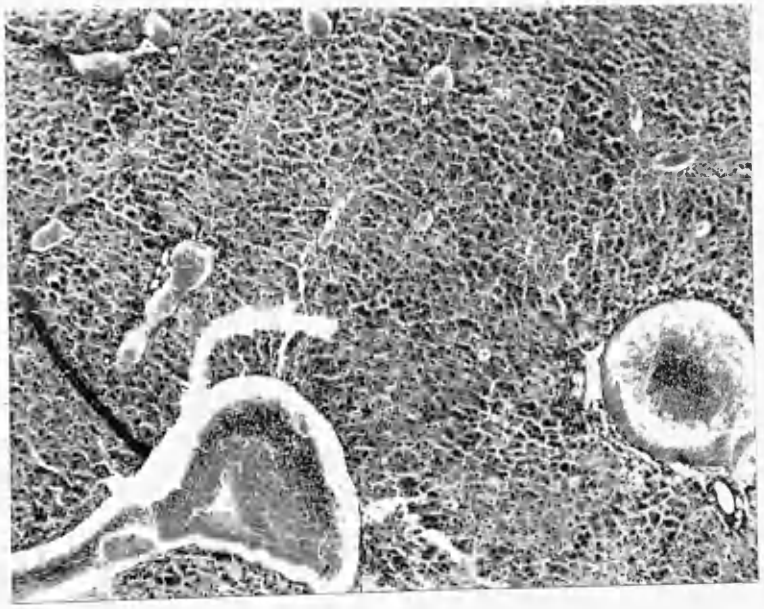


Fig. 292. Mouse liver 24 hours after CCl_4. H. & E. X 60.

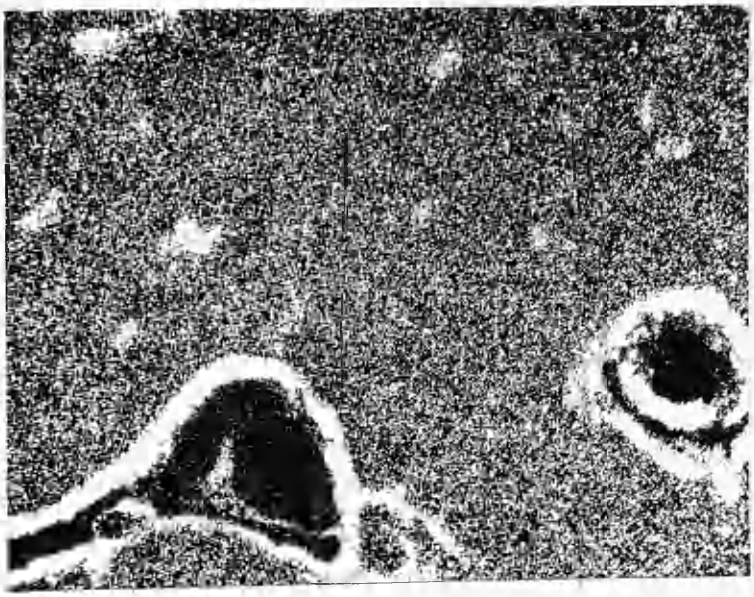


Fig. 293. Autoradiograph of same liver 48 hours after S^{35}-methionine. X 60.

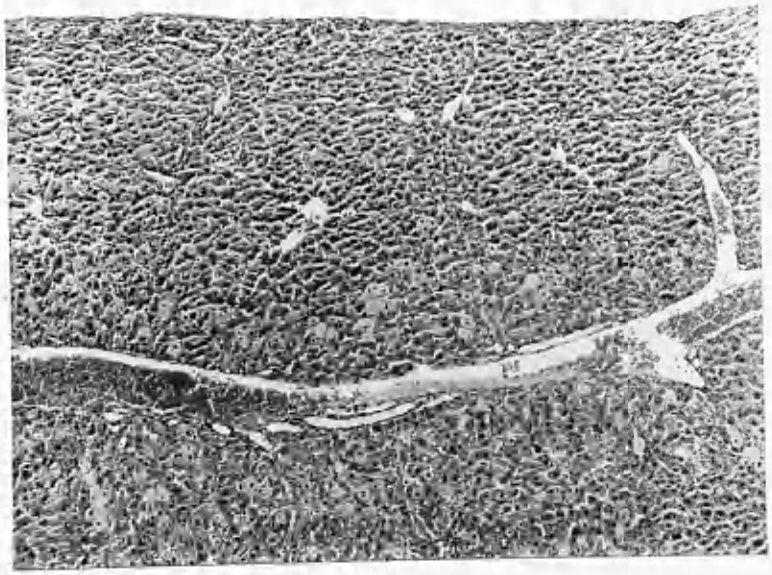


Fig. 294. Mouse liver 7 hours after CCl_4.
H. & E. X 60.

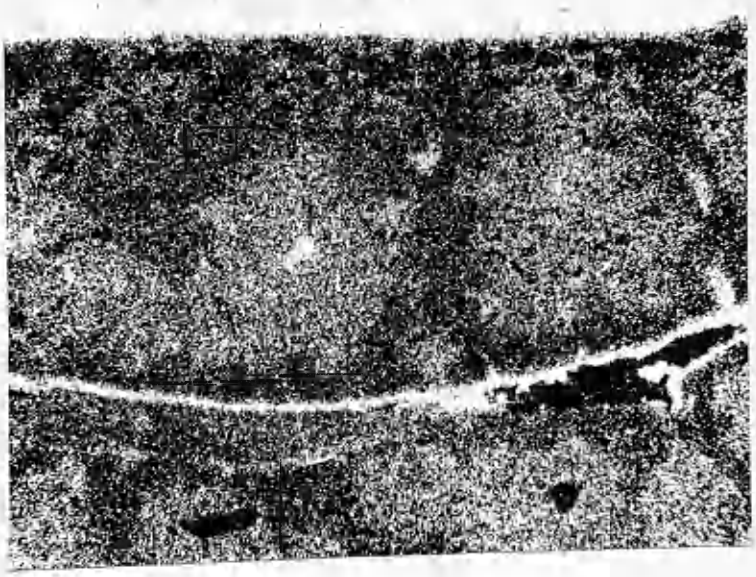


Fig. 295. Autoradiograph of same liver 6 hours after S^{35}-methionine. X 60.

Fig. 296. Mouse liver 48 hours after CCl_4.
H. & E.X 60.

Fig. 297. Autoradiograph of same liver 24 hours after S35-methionine.
X 60.

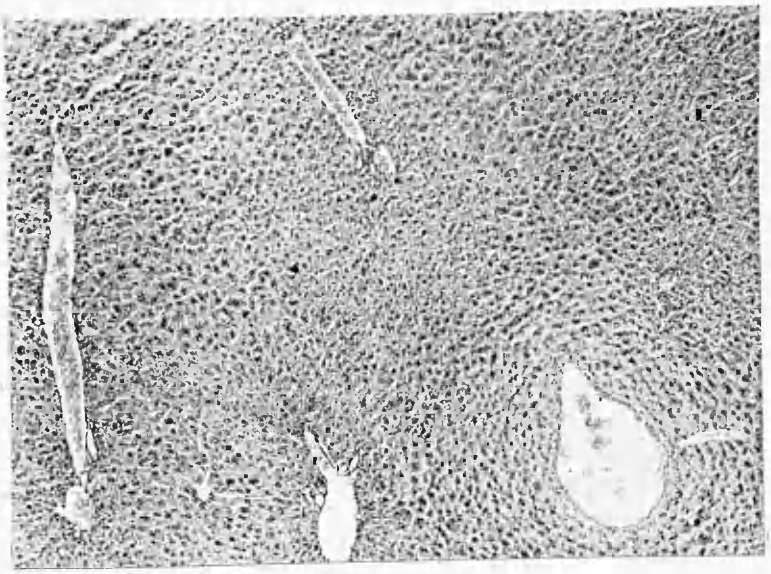


Fig. 298. Normal mouse liver. H. & E. X 60.

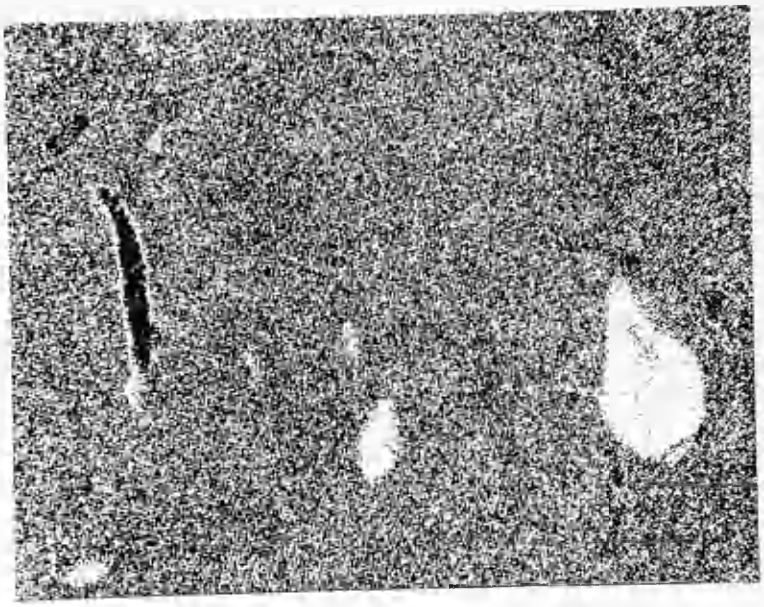


Fig. 299. Autoradiograph of same liver 24 hours after S^{35}-cystine. X 60.

TABLE XLI

Experiments 8 to 12 - Numbers of Mice treated with S^{35}-cystine and Liver Poison, and Radio-activity of Liver Sections

Experiment number	Mouse Number	Hours at which killed after S^{35}-cystine	Bromobenzene	** Counts/ minute
8	1	1	-	118.8
	2	5	-	133.0
	3 *	24	-	128.4
	4	48	-	98.4
	5	96	-	92.9
9	1 *	25	1	85.9
	2	25	1	75.9
	3	28	4	132.0
	4	28	4	58.5
	5 *	32	8	89.4
	6	32	8	126.7
	7 *	36	12	98.9
	8	36	12	82.8
	9 *	48	24	61.5
	10	48	24	73.5
10	1 *	11	12	38.9
	2	24	25	88.8
	3	24	25	88.1
	4 *	20	24	70.0
	5	20	24	40.9
	6	16	28	44.4
	7 *	24	48	40.8
	8	48	96	271.2

Experiment number	Mouse number	Hours at which killed after S^{35}-cystine	CCl_4	** Counts/ minute
11	1	25	1	98.4
	2	25	1	75.0
	3 *	28	4	114.0
	4	28	4	37.9
	5 *	32	8	76.3
	6	32	8	49.8
	7 *	36	12	67.3
	8	36	12	95.7
	9 *	48	24	-
	10 *	48	24	55.4
12	1	6	7	201.6
	2 *	24	25	55.5
	3	20	24	150.7
	4	16	28	31.8
	5 *	24	48	93.4

* Illustrated (Figs. 298 to 327).

** Corrected for back-ground radiation and standardized for mean section paper weight and unit liver weight.

Fig. 300. Mouse liver 1 hour after bromobenzene.
H. & E. X 60.

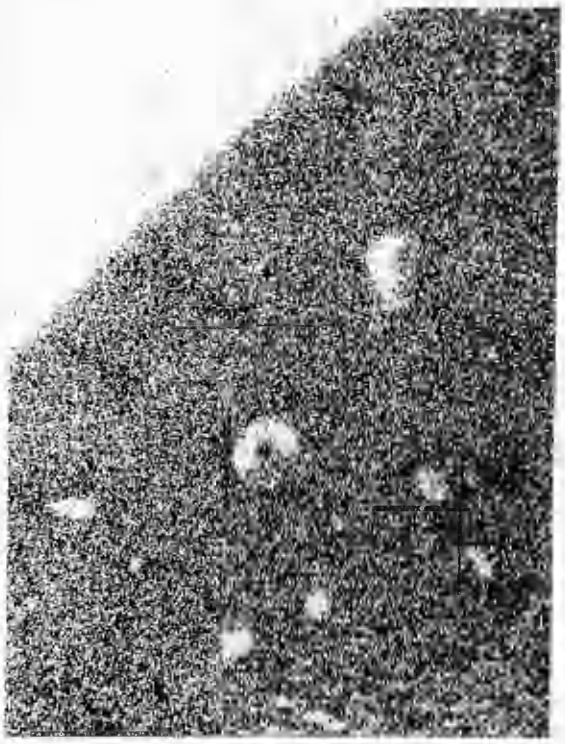


Fig. 301. Autoradiograph of same liver 25 hours after S^{35}-cystine.
X 60.

Fig. 302. Mouse liver 8 hours after bromobenzene.
H. & E. X 60.

Fig. 303. Autoradiograph of same liver 32 hours after S^{35}-cystine.
X 60.

Fig. 304. Mouse liver 12 hours after bromobenzene.
H. & E. X 60.

Fig. 305. Autradiograph of same liver 36 hours after S^{35}-cystine.
X 60.

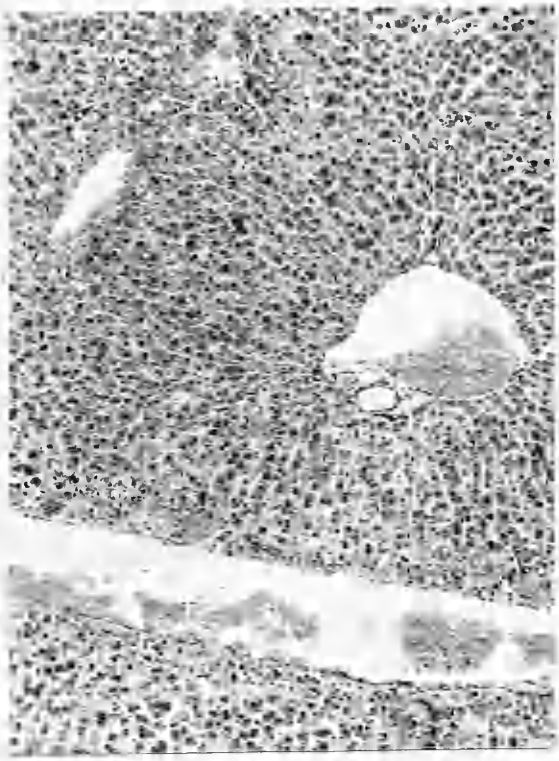


Fig. 306. Mouse liver 24 hours after bromobenzene.
H. & E. X 90.

Fig. 307. Autoradiograph of same liver 48 hours after S^{35}-cystine.
X 90.

Fig. 308. Mouse liver 12 hours after bromobenzene.
H. & E. X 90.

Fig. 309. Autoradiograph of same liver 11 hours after S35-cystine.
X 90.

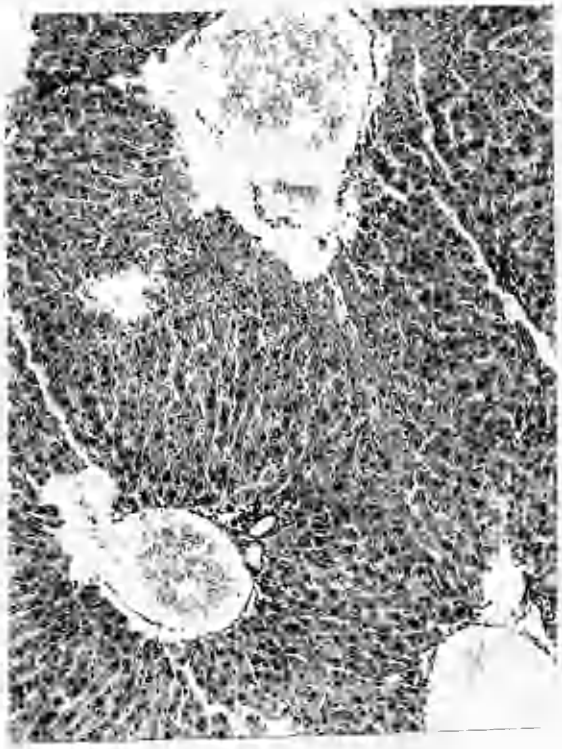


Fig. 310. Mouse liver 24 hours after bromobenzene.
H. & E. X 90.

Fig. 311. Autoradiograph of same liver 20 hours after S35-cystine.
X 90.

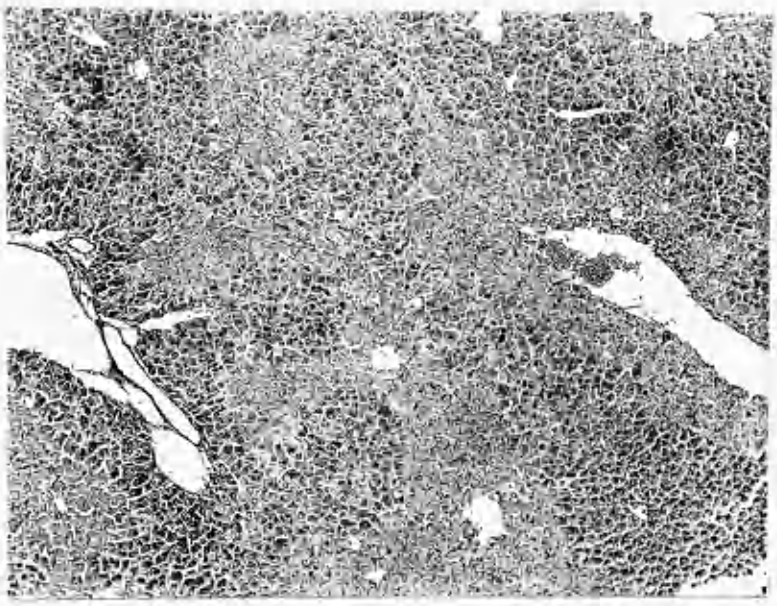


Fig. 312. Mouse liver 48 hours after bromobenzene.
H. & E. X 90.

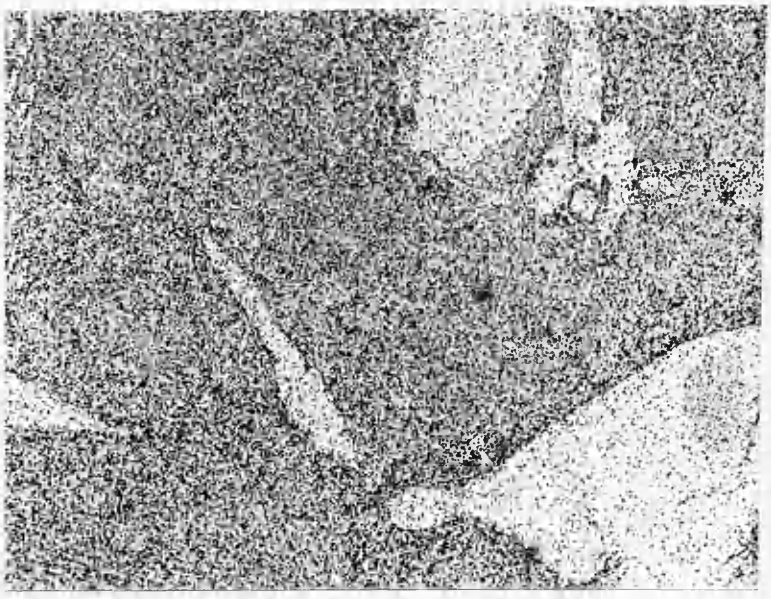


Fig. 313. Autoradiograph of same liver, but not comparable fields, 24 hours after S^{35}-cystine.
Neutral red counterstain X 90.

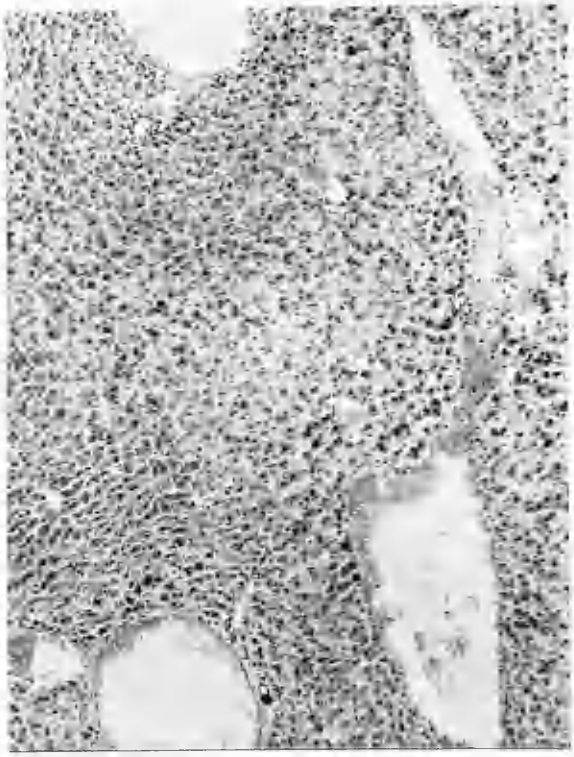


Fig. 314. Mouse Liver 4 hours after CCl_4.
H. & E. X 90.

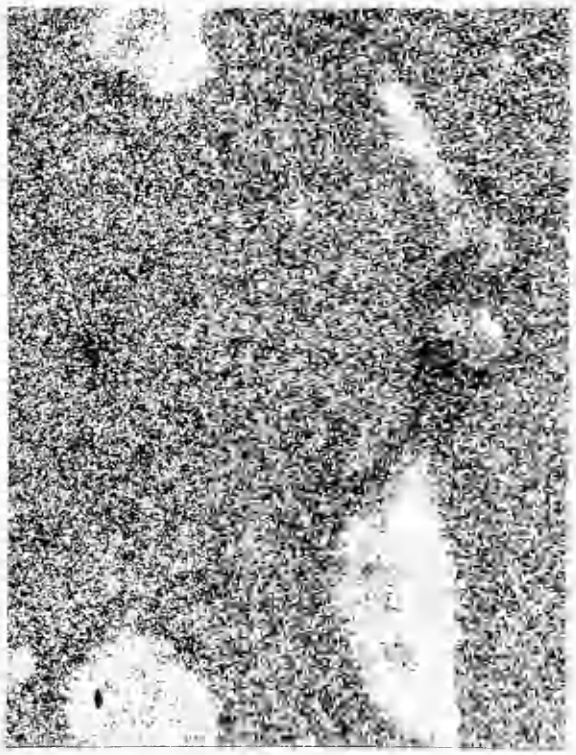


Fig. 315. Autoradiograph of same liver 28 hours after S^{35}-cystine.
X 90.

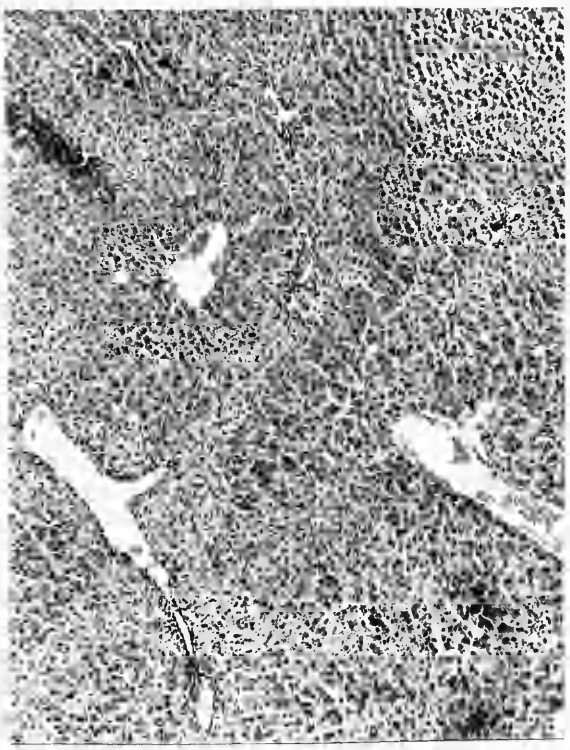


Fig. 316. Mouse liver 8 hours after CCl_4.
H. & E. X 60.

Fig. 317. Autoradiograph of same liver 32 hours after S^{35}-cystine.
X 60.

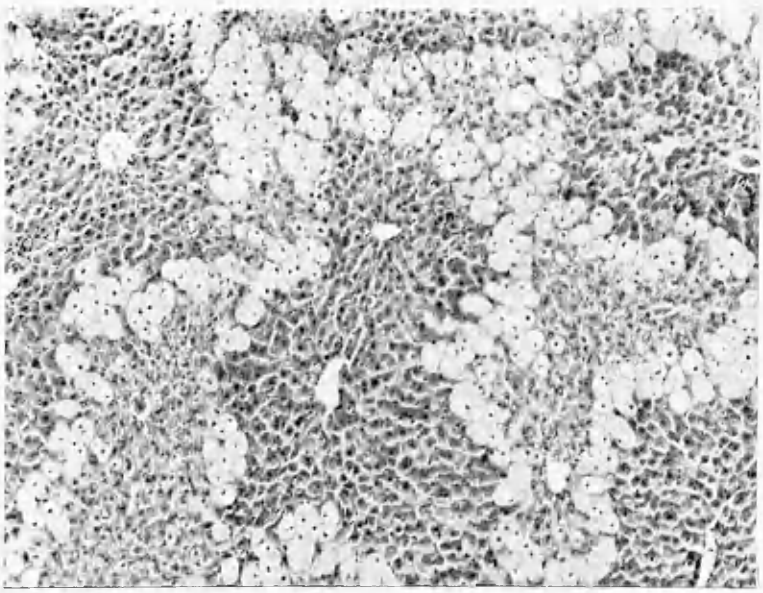


Fig. 318. Mouse liver 12 hours after CCl_4. H. & E. X 90.

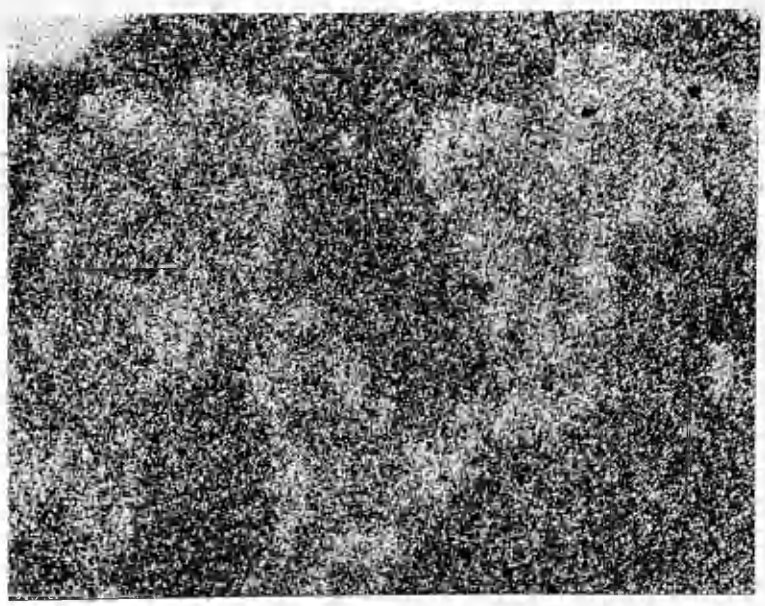


Fig. 319. Autoradiograph of same liver 36 hours after S^{35}-cystine. X 90.

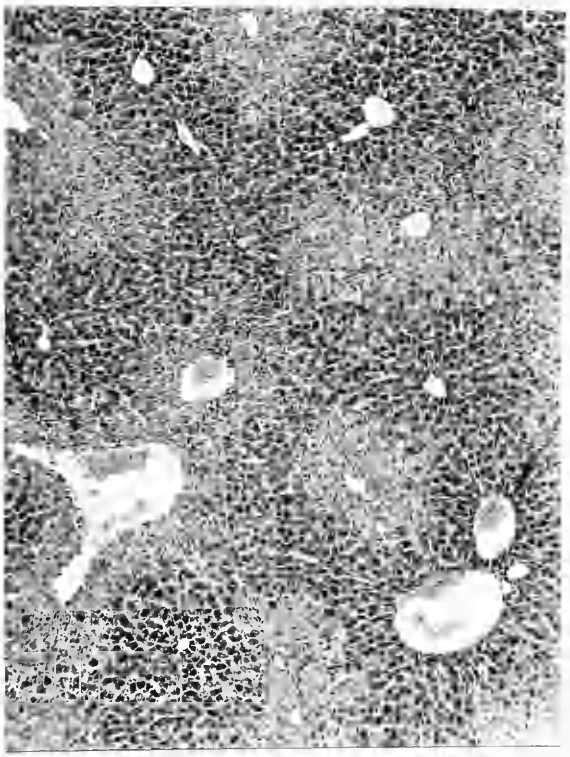


Fig. 320. Mouse liver 24 hours after CCl_4.
H. & E. X 60.

Fig. 321. Autoradiograph of same liver 48 hours after S^{35}-cystine.
X 60.

Fig. 322. Mouse liver 24 hours after CCl_4.
H. & E. X 90.

Fig. 323. Autoradiograph of same liver 48 hours after S^{35}-cystine.
X 90.

Fig. 324. Mouse liver 25 hours after CCl_4.
H. & E. X 90.

Fig. 325. Autoradiograph of same liver 24 hours after S^{35}-cystine.
X 90.

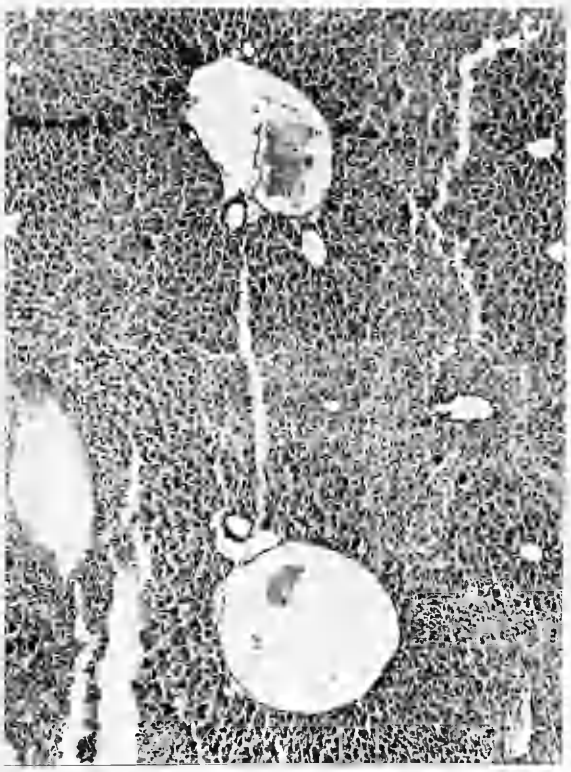


Fig. 326. Mouse liver 48 hours after CCl_4. H. & E. X 60.

Fig. 327. Autoradiograph of same liver 24 hours after S^{35}-cystine.
X 60.

TABLE XLII

Experiments 3 to 12 - Summary of Results

Experiment	S^{35} in liver
S^{35}-methionine - normal liver	Diffuse uptake
S^{35}-methionine followed by bromobenzene	Slight depletion in intact tissue
Bromobenzene followed by S^{35}-methionine	Depletion in damaged tissue
S^{35}-methionine followed by CCl_4	Slight depletion in intact tissue
CCl_4 followed by S^{35}-methionine	Depletion in damaged tissue
S^{35}-cystine - normal liver	Diffuse uptake
S^{35}-cystine followed by bromobenzene	Depletion in intact tissue
Bromobenzene followed by S^{35}-cystine	Slight depletion in intact tissue
S^{35}-cystine followed by CCl_4	Depletion in intact tissue
CCl_4 followed by S^{35}-cystine	Depletion in intact tissue

TABLE XLIII

Experiment 13 - Changes in Rat Liver at Intervals after Ethionine Intoxication

Rat number	Dose of ethionine	Interval between first dose of ethionine and killing	Appearance of liver
1	0.05 g.	1 hour	N.A.D.
2	0.10 g.	4 hours	N.A.D.
3	0.15 g.	8 hours	N.A.D.
4	0.20 g.	12 hours	Fat ±
5	0.20 g.	24 hours	Fat ++
6	0.20 g.	2 days	Fat +++
7	0.20 g.	3 days	Fat +
8	0.20 g.	5 days	Fat ±

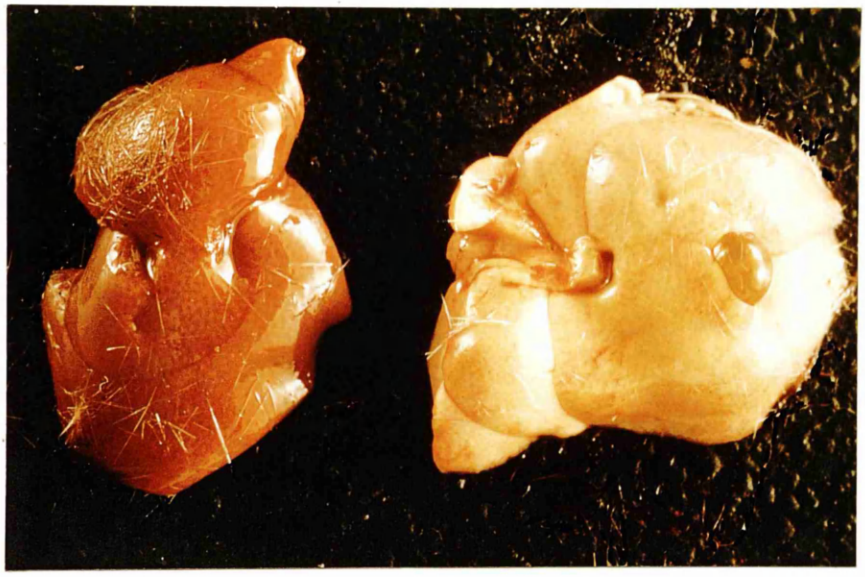


Fig. 328. Anterior aspect of two rat livers. Normal on left, ethionine-treated on right.

X 1.5.

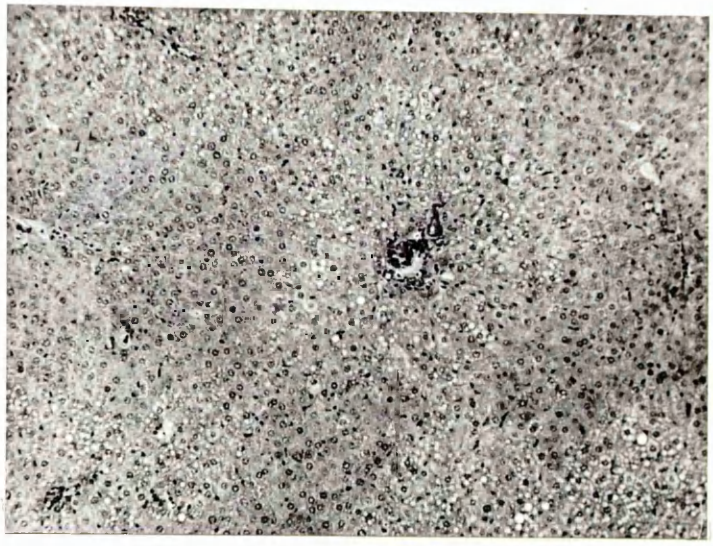


Fig. 329. Rat liver 24 hours after first dose of ethionine.

H. & E. X 75.

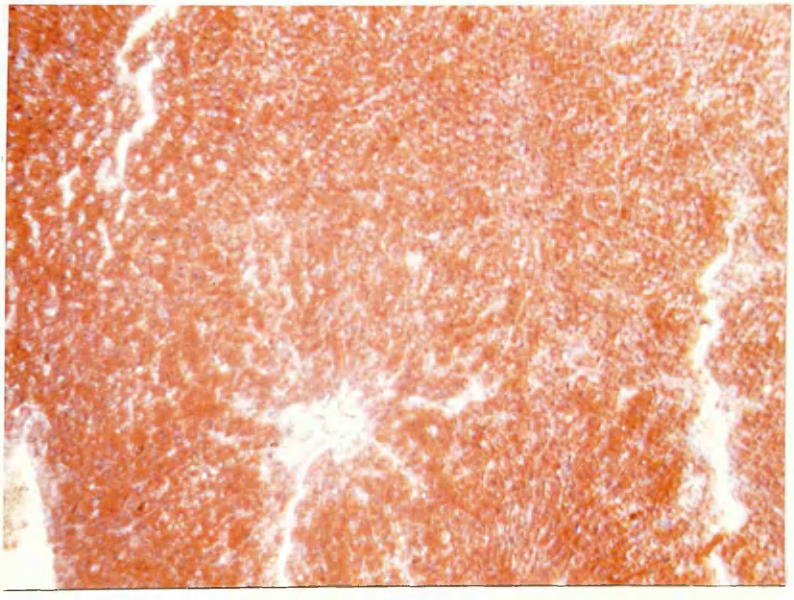


Fig. 330. Rat liver 24 hours after first dose of ethionine.

Sudan IV & Haematoxylin X 60.

TABLE XLIV

Experiments 14 to 17 - Numbers of Rats treated with S^{35}-Amino-acids and Ethionine, and Radio-activity of Liver Sections

Experiment number	Rat number	Hours at which killed after S^{35}-Methionine	Hours at which killed after S^{35}-Cystine	Hours at which killed after Ethionine	* Counts/minute
14	1	1	-	-	1160
	2	5	-	-	1049
	3	24	-	-	1128
	4	48	-	-	776
	5	96	-	-	944
15	1	26	-	2	1169
	2	28	-	4	1237
	3	31	-	7	1283
	4	36	-	12	856
	5	48	-	24	812
	6	72	-	48	613
	7	96	-	72	740
16	1	-	1	-	340
	2	-	5	-	377
	3	-	24	-	299
	4	-	48	-	372
17	1	-	26	2	416
	2	-	28	4	272
	3	-	31	7	409
	4	-	48	24	187
	5	-	72	48	292

* Corrected for back-ground radiation and standardized for mean section paper weight and unit liver weight.

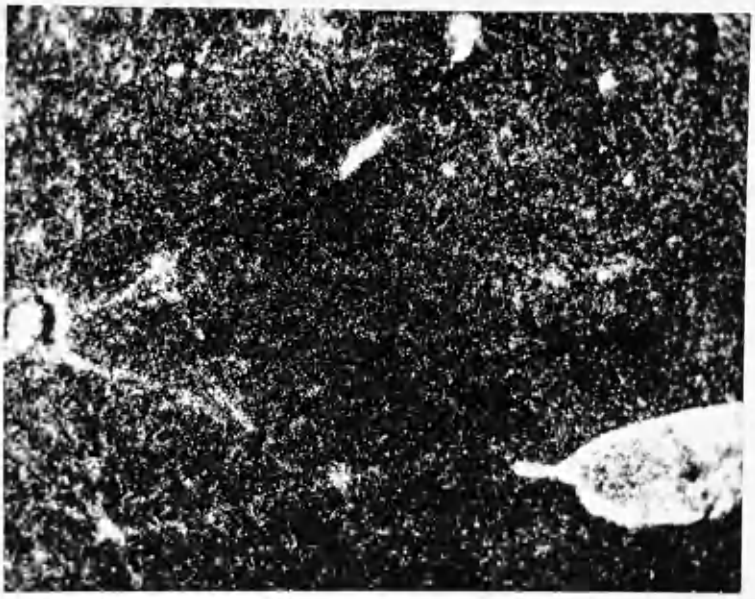


Fig. 331. Rat liver 24 hours after first dose of ethionine and 48 hours after S^{35}-methionine. Autoradiograph X 50.

Fig. 332. Rat liver 24 hours after first dose of ethionine and 48 hours after S^{35}-cystine. Autoradiograph X 50.