THE STRUCTURE OF FELINE CALICIVIRUS PARTICLES

by

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DECLARATION

The work recorded in this thesis was carried out in the Department of Veterinary Pathology, University of Glasgow Veterinary School between October 1975 and September 1978. All the results were obtained independently by the author apart from work described in Chapter 4 which was done in collaboration with James C. Neil, as indicated in the text.

SUMMARY

SUMMARY

Although the caliciviruses - San Miguel sealion virus, vesicular exanthema virus of swine and feline calicivirus - have been considered a provisional genus of the picornaviridae family (Melnick et al, 1974), as more information becomes available, especially in the area of molecular biology, it becomes increasingly clear that these are not true picornaviruses and hence the suggestion by various workers that these viruses be regarded as a separate virus family.

The aim of this study, therefore, was to extend our knowledge of feline caliciviruses: in particular, to examine the structure of the calicivirus particle and the way in which it is assembled. There were four main intentions: first, to determine whether the viral capsid was constructed from smaller subunits; secondly, to identify such subunit structures in FCV-infected cells and examine the roles they play in virion assembly; thirdly, to study and compare the antigenic properties of the whole virus particle relative to a major virus subunit; and fourthly, if both particles showed identical antigenic determinants, to exploit the potential of the subunit component as a source of material for vaccine production.

Chapter 3 describes the subunit structure of FCV. From virus harvests, two homogeneous populations of viral components called peak I and peak II were isolated from FCV-infected cell cultures. Peak I banded at a density of $1{\cdot}39 g.cm^{-3}$ in $CsCl_2$ gradients, had a sedimentation coefficient of 170S, contained RNA, was infectious and showed characteristic FCV particles by electron microscopy. This was the virus. The other component (peak II) had a buoyant density of $1{\cdot}26 g.cm^{-3}$, had a sedimentation coefficient of 15S, contained no RNA and was non-infectious. Electron microscopic examination showed that peak II contained a large mass of homogeneous materials, the detailed nature of which was unclear. However, polypeptide analysis by polyacrylamide gel electrophoresis revealed that both peaks I and II contained the major FCV capsid protein of 65,000 molecular weight. Also, as described in Chapter 5, the virus and the 15S material shared an antigenic determinant. These results showed that peak II probably contained virus-specified material, possibly viral subunits. A series of experiments showed that the 15S component was a stable product of FCV infection and was not generated from the virion by the conditions of viral growth and purification.

Subsequent studies showed that FCV is assembled from subunits in at least two stages. Purified virus was degraded by low pH in the presence of 0·7M $CsCl_2$ into a non-infectious 15S component which on exposure to reducing agents was converted to a 5S subunit. Both 15S and 5S subunits contained the single major capsid polypeptide of molecular weight 65,000 (p65). Similarly, "viral" and "natural" (peak II) 15S subunits cosedimented in sucrose gradients, and, as described in Chapter 5, are antigenically identical.

In Chapter 4, experiments are described which show that the FCV virion is assembled from these subunits. It was found that FCV infection rapidly inhibited cellular protein synthesis and that 120-150 minutes after the beginning of infection almost all newly synthesised protein was virus-specific. This was demonstrated by polyacrylamide gel electrophoresis of cell extracts examined at intervals after infection. The major polypeptide which appeared was the viral capsid protein, p65. Four other newly synthesised polypeptides of molecular weights 80,000 (2), 40,000 and 14,000 were apparent during the later stages of FCV infection. Only the component of 14,000 molecular weight could be shown to be related to the virus. The origin and purpose of the other three molecules was not determined; they did not appear, however, to be precursors or products of the p65.

Pulse-chase experiments showed that the virus was constructed from smaller particles. In FCV-infected cells pulsed for 5 seconds with ^{3}H-leucine at about 200 minutes after infection, newly synthesized virus polypeptide (p65) was extremely rapidly converted into a 5S component which, possibly through series of unstable intermediates, was incorporated within 30 seconds into a 15S subunit. Between 15 and 30 minutes after the initiation of protein synthesis, complete feline calicivirions were formed in infected cells.

In an analysis of the antigenic determinants of the virus (Chapter 5), purified, concentrated virus or 15S subunits were inoculated into rabbits to produce homologous antisera. These were then analysed by virus neutralization and immunodiffusion tests. The results showed that both antisera contained neutralizing antibodies to the virus. Analysis by immunodiffusion revealed that two antigenic determinants were present on the virus particle and one of these was also found on the 15S subunit. When the common antibody was absorbed from the antiviral serum with excess 15S antigen, the absorbed serum failed to neutralize virus

infectivity indicating that the neutralizing activities of the viral and 15S antisera were specific functions of antibody to the common antigen.

This result suggested the possible use of the 15S subunit as vaccine against calicivirus disease in cats and an experiment to this end is described in Chapter 6. A prototype vaccine was prepared by purifying the 15S subunit by rate zonal and equilibrium ultra-centrifugation followed by filtration of the preparation through membranes with a pore size of 25nm to exclude any infectious virus. The purified antigen was inoculated into cats with aluminium hydroxide adjuvant. The cats developed FCV neutralizing antibodies and resisted challenge with virulent FCV.

ABBREVIATIONS

BSA	Bovine serum albumin
CPE	Cytopathic effect
CPM	Counts per minute
DMSO	Dimethylsulphoxide
DOC	Deoxycholate
DTT	Dithiothreitol
EDTA	Ethylenediaminotetra acetate
EFC_2	Eagle's medium containing 2% FBS
EFC_{10}	Eagle's medium containing 10% FBS
EMC	Encephalomyocarditis virus
FBS	Foetal bovine serum
FCV	Feline calicivirus
FCV (EP)	Feline calicivirus (extra large plaque type)
FEA	Feline embryo cells of the FEA strain
FHV	Feline herpesvirus
FITC	Fluorescein isothiocyanate
FMDV	Foot-and-mouth disease virus
ME	Mouse-Elberfeld virus
2-ME	2-mercaptoethanol
MEM	Eagle's Minimal Essential Medium
mRNA	Messenger ribonucleic acid
NCVP	Non-capsid viral protein
p65	Polypeptide of molecular weight 65,000
PAGE	Polyacrylamide gel electrophoresis
PBS	Dulbecco's phosphate buffered saline 'A'
pfu.	Plaque-forming unit
POPOP	1,4-di-2(5-phenyloxazolyl-benzene
PPO	2,5, diphenyloxazole
PTA	Phosphotungstic acid
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N,N-tetramethylenediamine
TS	Tris saline buffer
VEV	Vesicular exanthema virus of swine

NOTE ON NOMENCLATURE

Throughout this thesis, the term "p65" is used to describe the virion polypeptide. This is because in early experiments its molecular weight was estimated to be 65,000 both by polyacrylamide gel electrophoresis in a continuous phosphate buffer system and by agarose chromatography in guanidine hydrochloride. In later experiments involving polyacrylamide gel electrophoresis in a discontinuous tris-buffered system an apparent molecular weight of 68,000 was found. For convenience the original term is used throughout.

CHAPTER 1

GENERAL INTRODUCTION

Feline caliciviruses (FCV) are major causes of respiratory disease in cats and may be isolated from cases of disease and from convalescent carriers (Gillespie & Scot, 1973). The virus can also be isolated from 8% of normal household cats, 24% of cats at shows and 41·5% of cats in colonies (Wardley et al, 1974).

Other caliciviruses have been isolated from pigs (Madin, 1970) and wild pinnipeds (Smith et al, 1973) and calicivirus-like particles have been observed in human infants (Madeley, 1976) and calves (Bridger & Woods, personal communication).

As discussed below, caliciviruses are small, simple, RNA-containing viruses which were provisionally classified as picornaviruses (Melnick et al, 1974) although they differ in structure (Zwillenberg & Burki, 1966) and certain biochemical features from other picornaviruses (Brown & Hull, 1973; Burroughs & Brown, 1974). Furthermore, they are unique among the picornaviruses in that the capsid consists of only one major polypeptide (Bachrach & Hess, 1973; Burroughs & Brown, 1974).

Discovery of feline caliciviruses

Soon after the original isolation of feline rhinotracheitis virus, now termed "feline herpesvirus", (Crandell & Maurer, 1958) it became apparent that respiratory disease in the cat was also associated with a distinctive group of viruses composed of several antigenic types (Crandell & Madin, 1960; Bittle et al, 1960; Percy & Prydie, 1963). These strains were later shown by Bürki (1965), Prydie (1966) and Crandell (1967) to exhibit the major properties of picornaviruses (Melnick et al, 1963) and as such were referred to as "feline picornaviruses".

The first isolation of a feline calicivirus was by Fastier (1957) in New Zealand. Subsequently, it has been recovered from cats in many parts of the world including Switzerland (Bürki, 1963), Austria (Zwillenberg & Bürki, 1966), Italy (Torlone, 1960), England (Prydie, 1966; Povey, 1969) Australia (Studdert et al, 1970), the United States of America (Bolin, 1957; Sinha, 1958; Bittle et al, 1960; Crandell & Madin, 1960; Kahn & Gillespie, 1970) and Scotland (Ormerod and Jarrett, 1978). The virus has been isolated from the oropharyngeal region of both infected (Crandell & York, 1966) and clinically healthy cats (Walton & Gillespie, 1970; Povey & Johnson, 1971) and also from the spleen, kidney and other organs (Fastier, 1957). Feline caliciviruses exert a rapid and cytopathic effect (CPE) in feline cell cultures and thus are easily distinguished from

feline herpesviruses which induce a slower CPE with syncytia formation and Cowdry A intranuclear inclusion bodies. The feline reoviruses cause a slow CPE characterised by ballooning and granular appearance of affected cells, and by the presence of large, irregularly shaped intracytoplasmic inclusion bodies. The whole process to its terminal stage of complete CPE requires 12-15 days for the reoviruses, compared to 1-3 days for the feline caliciviruses.

The signs of FCV disease vary markedly from inapparent infection involving only the upper respiratory tract to severe respiratory disease with pneumonia and terminal death (Kahn & Gillespie 1971). Cats that recover from the disease often become carriers and persistent shedding of the virus from tonsilar tissues for at least eleven months (Povey & Johnson, 1970) is perhaps the principal means by which the virus is maintained naturally.

Properties of feline calicivirus

The morphology of FCV is distinctive, the particles having a fringe-like and 'chunky' arrangement of the capsomeres creating an easily recognisable pattern after negative staining (Zwillenberg & Bürki, 1966; Almeida et al, 1968; Wawrzkiewicz, Smale & Brown, 1968). Although the capsomeres have been variously described as 'surface spikes' (Zee et al, 1968) or rod like (Strandberg, 1968), it was Wawrzkiewicz et al (1968) who first identified their true structures as hollow and cup-like, rather like the calyx of a flower from which the generic name "calici" is derived. Each virion is thought to consist of 32 capsomeres (Zwillenberg & Bürki, 1966; Almeida et al, 1968).

Estimation of the particle size has been made by filtration (Bürki, 1965; Crandell, 1967) and by electron microscopy (Zwillenberg & Bürki, 1966; Almeida et al, 1968; Strandberg, 1968) and it is generally agreed that the virus has a diameter of 35-40mm which makes the virus the largest of the picornavirus group.

Physico-chemical properties

The virion of FCV is non-enveloped and icosahedral in shape containing an infectious single-stranded ribonucleic acid (RNA) (Adldinger et al, 1969) with a chemical composition of 29·3% adenine, 19·9% cytidine, 21·0% guanine and 29·8% uridine (Newman et al, 1973).

The virions contain 18% RNA of molecular weight $2{\cdot}8 \times 10^6$ and 80% protein of molecular weight $12{\cdot}4 \times 10^6$ so that the total virion mass is $15{\cdot}2 \times 10^6$ daltons (Burroughs et al, 1978). The buoyant density of the virion in caesium chloride ($CsCl_2$) is 1·36 - 1·39g.cm^{-3} (Studdert et al,

1970, Newman et al, 1973). It is resistant to ether or chloroform treatment (Bürki, 1966; Bartholomew & Gillespie, 1968) and labile at pH 3 but not at pH 7 (Oglesby et al, 1971). The virion is not stabilized by 1M $MgCl_2$ (Crandell, 1967; Studdert et al, 1970) which instead, enhances thermal inactivation (Kahn & Gillespie, 1970). Furthermore, FCV can remain infective at -65°C for at least four years and is able to withstand the process of lyophylization (Gillespie & Scott, 1973).

Classification of small RNA-containing viruses

The International Enterovirus Group as part of a goal of classifying major groups of viruses on the basis of common biochemical and biophysical properties, defined a class of small RNA-containing viruses which they called "picornaviruses" ("pico" meaning very small and "RNA" referring to the type of nucleic acid in the virion) (Melnick et al, 1963) This group was defined to include those viruses which are small in size (15-30mm in diameter), bear no essential lipid envelope and contain single stranded RNA. On this basis, the picornavirus group ought to include the plant and bacterial small RNA viruses with which they have much in common. It did not; and the term 'picornaviruses' came to be applied exclusively to the small RNA-containing viruses that infect vertebrate hosts.

The International Committee on Viral Nomenclature recognised three genera in the family Picornaviridae: the Caliciviruses, Rhinoviruses and Enteroviruses (Wildy, 1971). Feline calicivirus together with vesicular exanthema virus (VEV) of swine were placed in the genus Calicivirus.

This classification soon ran into difficulties. By the Committee's definition, the caliciviruses by virtue of their large size (35-40mm in diameter) should not be included in the group, while certain physico-chemical properties suggest that foot-and-mouth disease virus and equine rhinovirus should not have been included in the rhinovirus genus. Similarly, the marked instability of the cardioviruses at pH 6 in the presence of chloride or bromide ions indicates that the members of this group are not typical enteroviruses (Speirs, 1962; Mark, O'Callaghan & Colter, 1970; Dunker & Ruekert, 1971). Hence, Newman et al (1973) suggested a division of the picornaviruses into six comprising the entero, cardio, calici, human rhino, equine rhino and foot-and-mouth disease virus subgroups.

At present, there is strong evidence that caliciviruses should be excised from the picornaviridae family to form a new virus group (Burroughs et al, 1974; Black & Brown, 1975/76). This proposal stemmed from the distinctive ultrastructure, certain physicochemical properties and mode of replication of caliciviruses which are quite different from the picornaviruses. It is interesting to note that this proposal already has the backing of the Study Group on Picornaviruses which is a subcommittee of the International Committee of the Taxonomy of Viruses (Melnick et al, 1974).

Relationship of FCV to other small RNA-containing viruses

Despite unique ultrastructural and certain physicochemical properties, FCV is similar in many respects to other small RNA-containing viruses found in other animal (vertebrate and invertebrate), plant and bacterial hosts. Tables 1.1, 1.2 and 1.3 contain details of comparative properties.

Viral RNA FCV, in common with other picornaviruses, has only one species of virion RNA (Brown, Newman & Stott, 1970). This is also true of all the small RNA-containing bacterial viruses and several of the plant viruses although members of the cowpea mosaic, NEPO and pea enation mosaic virus groups have two RNA species both of which are essential for infection (Van Kammen, 1967; 1968; Diener & Schneider, 1966; Harrison, Murant & Mayo, 1972). Some others like the alfalfa mosaic, Brome mosaic and Cucumber mosaic virus groups each have four or more RNA species (Kaper, 1968) and in most cases these split genomes exist as separate molecules of varying lengths and are bacilliform in shape (Hull, Hills & Markham, 1969).

Capsid components However, despite the great variation in their structural arrangements, all small RNA-containing viruses appear to possess icosahedral symmetry. The virions of most have diameters in the range of 22-32nm with the exception of satellite tobacco necrosis virus (17nm) and the caliciviruses (35-40nm). The large size and the overall shape of FCV is perhaps reflected in its high sedimentation value of 160-207s (Wawrzkiewicz et al, 1968; Oglesby et al, 1971) compared with 140-160s obtained for other picornaviruses (Rueckert, 1971; Newman et al, 1973). RNA bacteriophages sediment between 71-84s in $CsCl_2$ gradients (Watanabe et al, 1967; Kaper, 1968; Bendis & Shapiro, 1970; Hohn & Hohn, 1970).

Purification of small RNA viruses from infected cells by sucrose gradient or $CsCl_2$ equilibrium ultracentrifugation reveals a considerable degree of difference in the number of sedimenting components.

TABLE 1.1

COMPARATIVE PROPERTIES OF SMALL RNA-CONTAINING VIRUS PARTICLES

Group	No. of Sedimenting Particles	S-value	%RNA	Density ($g.cm^{-3}$)	Diameter(nm)
Vertebrate Viruses					
Entero	2	160, 74	30, 0	1·34, 1·30	28
Cardio	1	160	31	1·34	28
Calici(FCV)	2	170, 15*	18, 0*	1·39, 1·26*	35-40
Human rhino	2	155, 75	30, 0	1·40, 1·29	28
Equine rhino	1	150	NA	1·45	28
Foot-and-mouth disease (FMD)	2	146, 75	31, 0	1·43, 1·30	24
Invertebrate Viruses					
Acute paralysis of bees	NA	160	NA	1·34	29
Sacbrood	NA	160	NA	1·34	29
Gonometa	NA	160	NA	1·34	29
Nodamura	NA	140	NA	1·34	29
Plant Viruses					
Brome Mosaic Virus (BMV)	1	87	22	1·36	26
Cucumber Mosaic (CMV)	1	99	19	1·37	28
Turnip Yellow Mosaic (TYM)	2	116, 53	34, 0	1·42, 1·29	28
NEPO	3	126,91, 53	35,25 0	1·51, 1·42 NA	27·5
Alfalfa Mosaic (AMV)	4	99, 89, 75, 68	18,18, 18,18	1·39	Bacilliform structure
Bacterial Viruses					
R17	1	80	30	1·46	25
GA	1	76	NA	1·44	NA
$Q\beta$	1	84	30	1·47	25
θCb_5	1	71	NA	NA	23

Main source of information : Brown & Hull (1973)

NA = Not available

* = Information from present study

TABLE 1.2

COMPARATIVE PROPERTIES OF RIBONUCLEIC ACIDS

OF SMALL RNA-CONTAINING VIRUSES

Group	No. of Species	S-value	Molecular weight ($\times 10^{-6}$)
Vertebrate Viruses			
Entero	1	33-35	2·6
Cardio	1	35	2·6
Calici	1	37	3·0
Human rhino	1	32-33	2·2
Equine rhino	1	35	2·6
FMD	1	35	2·6
Invertebrate Viruses			
Acute panalysis	1	30	2·0
Sacbrood	1	35	2·6
Gonometa	1	NA	NA
Nodemura	2	22,15	1·0,0·5
Plant Viruses			
BMV	4	27,22,14	1·09,0·99,0·75,0·28
CMV	4	23,20,13	1·25,1·19,0·96,0·50
TYMV	1	30	2·0
NEPO	2	32,24	2·3,1·4
AMV	4	24,22,20,13	1·27,1·0,0·76,0·33
Bacterial Viruses			
R_{17}	1	26	1·1
GA	NA	NA	NA
$Q\beta$	1	32	1·1
θCb_5	1	30	NA

Main source of information : Brown & Hull (1973)

NA = Not available

Abbreviations as in Table 1.1

'Full' viral particles contain RNA and are infectious and sediment faster in rate zonal gradients than the 'empty' particles, which have a lower buoyant density, lack RNA and are non-infectious.

All the small RNA bacteriophages R_{17}, GA, Qβ, θ_cb5 (Watanabe et al, 1967; Kaper, 1968; Bendis & Shapiro, 1970; Hohn & Hohn, 1970), the cardio and the equine rhinovirus groups of picornaviruses have one homogenous population of sedimenting particles (Rueckert, 1971; Newman et al, 1973). However, the enteroviruses, human rhinoviruses and foot-and-mouth disease virus all have two sedimenting components: the 'empties' have an S-value of 74-75s and are procapsids. This differs significantly from the slower sedimenting components found in the present study of feline caliciviruses which are not procapsids but unassembled virus subunits sedimenting at about 15s.

Among the plant viruses, cucumber mosaic, tobacco necrosis and southern bean mosaic viruses have single sedimenting components whereas some others have two (turnip yellow mosaic and pea enation mosaic viruses); even more than four have been found in alfalfa mosaic virus (Kaper, 1968; Hull, 1969).

Capsid proteins

With the exception of the caliciviruses which contain one major polypeptide with a molecular weight of 65,000, the picornaviruses examined so far contain four polypeptides (Burroughs et al, 1974). However, like FCV, most plant small RNA viruses have single polypeptides; cowpea mosaic virus, broad bean wilt virus and tomato bushy stunt virus are among those having two capsid polypeptides but none is known to have four (Kaper, 1968).

The only analyses of the polypeptides of the invertebrate viruses available so far are for Gonometa virus, with four polypeptides (Longworth et al, 1973), and Nodamura virus which has one major and traces of two minor polypeptides.

All the RNA bacteriophages contain two or more polypeptides (Kaper, 1968; Bendis & Shapiro, 1970; Hohn & Hohn, 1970).

RNA virus assembly

In an infected cell, the virus-specific proteins and nucleic acid are synthesized separately and consequently must be brought together and assembled into mature viral particles. The mechanism of assembly of these particles from progeny RNA and protein is not known with any certainty and no work has yet been done on FCV in this direction. Most information is available on poliovirus (Phillips, 1969;

TABLE 1.3

COMPARATIVE PROPERTIES OF CAPSID PROTEINS

OF SMALL RNA-CONTAINING VIRUSES

Group	No. of Proteins	Molecular weight ($\times 10^{-6}$)	No. per capsid
Vertebrate Viruses			
Entero	4	36,28,26,8	60,60,60,30
Cardio	4	34,30,23,9	60,60,60,60
Calici	1	68	180
Human rhino	4	36,27,34,8	60,60,60,30
Equine rhino	NA	NA	NA
FMD	4	34,30,36,13·5	60,60,60,30
Invertebrate Viruses			
Gonometa	4	36·5,32,29,15	NA
Nodamura	1 major 2 minor	46, NA	180
Plant Viruses			
BMV	1	20	180
CMV	1	32 Or 24*	180
TYMV	1	20	180
NEPO	1	57	60
AMV	1 Or 2*	30 Or 24*	180,150,114,96
Bacterial Viruses			
R_{17}	2	13·7, 38	180, 1
GA	NA	NA	NA
$Q\beta$	3	14,35,39	NA
θCb_5	2	12,40	180, 1

Main sources of information : Brown & Hull (1973)

* = Information still in dispute

NA = Not available

Abbreviations as in Table 1.1

Jacobson & Baltimore, 1968). Jacobson & Baltimore (1968) suggested that the empty 74S particles are the precursors of the mature virus particles, and that in some way, still unknown, the RNA is incorporated later into the already formed capsid. However, such a mechanism is not applicable to RNA bacteriophages (Valentine et al, 1969) in which, although empty capsids are found, they are merely by-products and not essential steps on the pathway to the mature virus particle. Reconstruction experiments suggest that a maturation protein is involved at the early stage of the phage assembly.

Not much is known about the maturation of plant virus particles although some workers tend to favour a mechanism of spontaneous self-assembly and have been able to demonstrate such a process in vitro (Lebeurier et al, 1971). Absolute proof of self-assembly requires that purified nucleic acids and structural protein (s) be able to combine in vitro to yield particles which resemble the original virus in shape, size and stability as well as being infectious. This has been shown for cowpea chlorotic mottle and alfalfa mosaic viruses by using protein and a wide variety of polyanionic nucleating agents (Bancroft, Hiebert & Bracker, 1969; Lebeurier et al, 1971).

The present study

When compared with other picornaviruses, e.g. poliovirus, foot-and-mouth disease virus or some cardioviruses, very little work has been done on the molecular biology of caliciviruses. The aim of this study, therefore, was to extend our knowledge of the structure and assembly of the feline calicivirus particles. There were four main aims.

The first was to determine whether the viral capsid was constructed from subunits. This was investigated by exposing purified FCV virions sequentially to low pH, reducing agents and denaturing conditions and analysing the resulting components at each stage by rate zonal centrifugation, polyacrylamide gel electrophoresis and guanidine hydrochloride-agarose chromatography. The nature of a "naturally occurring" FCV subunit, which was discovered in this study, was also investigated in this way.

Secondly, the assembly of FCV particles from smaller protein components was investigated. The effect of the protein synthesis in FCV-infected cells was studied. At a period after infection when the rate of FCV-specific protein synthesis was maximum, and cellular protein synthesis was minimal, the assembly of the FCV virion was examined. This was achieved by exposing FCV-infected cells to high concentrations

of radioactive amino acids for very short periods so that newly synthesized viral protein was radiolabelled. The label was then removed and the fate of the virus-specific protein was followed from synthesis to final incorporation into complete viral particles. It was hoped to relate the intermediate components found in the assembly of virus to the subunits resulting from breakdown of whole particles.

The third aim was to investigate the antigenic determinants of the FCV virion and its subunits. Sera were prepared in rabbits to the virus and the major subviral component and were used in immunodiffusion to define the antigenic relationship of the whole virus and the subunit.

The fourth aim was to explore the possibility that the viral subunit, which was shown in the course of the third part of the study to be antigenically related to whole virus, might be used as a non-infectious vaccine against FCV infection. Cats were vaccinated with the purified subunit together with adjuvant and the effects of subsequent challenge with virulent FCV were observed.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Cells

Feline embryo (FE) cells of the FEA strain (Jarrett et al, 1973) between 12 and 34 passages from primary culture were used in this study.

FEA cells were grown as monolayers in Glasgow modified Eagle's Minimal Essential Medium (MEM) (MacPherson & Stoker, 1962) which was prepared at the Institute of Virology, University of Glasgow. Prior to use, the medium was supplemented with 10% foetal bovine serum (FBS) (Gibco Biocult Ltd.) and is referred to as EFC_{10}.

Cell growth

FEA cells ($1x10^7$) were seeded in 2·5 litre rotating bottles with 100ml of EFC_{10}. The bottles were flushed with 5% carbon dioxide in air and the cells were incubated at 37°C. The cells were usually confluent four or five days later and were removed from the glass after two rinses with versene (0·1% EDTA in PBS) followed by another rinse with trypsin-versene mixture containing 0·25% trypsin. The cells were resuspended in EFC_{10} medium.

For assaying virus infectivity by plaque assay, cells were grown in 5cm diameter polystyrene plates (Nunclon) with 4ml of medium and were maintained at 37°C in an incubator with an atmosphere of 5% carbon dioxide in air.

Viruses

The FCV used was the extra-large plaque (EP) type, strain G1, originally isolated in this laboratory from a kitten with lingual ulceration (Ormerod & Jarrett, 1978).

Virus clones were produced by three cycles of growing virus from single plaques in plates containing only one or two plaques. After the third cloning the virus suspension was dispensed in aliquots and stored at -70°C for future stock production.

Virus stocks

Virus stock suspensions were obtained by infection of confluent FEA cells in 2·5 litre roller bottles at a multiplicity of infection of 2 for 16 hours. The virus was harvested after cell debris was removed by centrifugation at 10,000 rpm for 10 minutes and the supernatant was stored in 2ml lots at -70°C

Virus infectivity assay

Estimation of viral infectivity was determined by counting the number of plaques formed by dilutions of virus in confluent monolayers overlaid with agar.

Monolayers of FEA cells in 5cm plates were drained of medium and infected with 0·1ml of serial dilutions of the virus using two plates per dilution. The plates were gently rocked at intervals throughout a 1 hour adsorption period at 37°C in an atmosphere of 5% carbon dioxide in air. The plates were then washed twice in MEM prior to addition of 4ml of overlay. The agar overlay consisted of 0·9% bacto-agar (DIFCO) in MEM with 1% FBS. The plates were further incubated for 48 hours before fixing and staining with 0·5% crystal violet in 10% formalin. Virus infectivity was expressed in plaque-forming units (pfu) per ml.

Neutralisation tests

Virus neutralising antibody was detected by a plaque reduction test. Neutralisation reactions were carried out in plastic microtitre plates (Cooke Laboratory Products). All sera were inactivated at 56°C for 30 minutes before use to destroy non-specific inhibitors of viral infectivity. Sera were diluted in twofold steps in Leibovitz (L15) medium (Gibco Biocult Ltd.) using 50μl diluters. An equal volume of FCV ($1x10^5$pfu/ml) was then added to each well.

After a reaction time of 1 hour at 37°C, 50μl was transferred from each well into a bottle containing 4·95ml of L15 medium thus diluting the reactants 100-fold and stopping further reaction. A 100μl sample per dilution was assayed for residual infectivity. The serum titre was taken as the highest dilution which caused 95% plaque reduction of the FCV by comparison with 2 virus controls incubated in the same microtitre plate.

Buffer solutions

Phosphate buffered saline (PBS: Dulbecco 'A') Twenty tablets of phosphate buffered saline (Oxoid Ltd.) were dissolved in 2,000ml of distilled water and autoclaved. The buffer was then dispensed in 200ml amounts and stored at 4°.

Tris-saline (TS) buffer A concentrated stock solution (TSx10) was made by dissolving NaCl (116·9g), tris (24·2g) and EDTA (6·7g) in 1900ml of distilled water. The pH was adjusted to 7·5 with 0·1N hydrochloric acid and the solution was made up to 2000ml with distilled water. The solution was dispensed in 200ml amounts, autoclaved and then stored at 4°C. Working stocks of TS buffer (pH 7·5) were prepared by diluting one volume of TSx10 in 9 volumes of distilled water.

Sodium phosphate buffers These were prepared as outlined in the eleventh edition of "Medical Microbiology" by Cruickshank (1970). Two stock solutions were made. A 0·2M solution of sodium dihydrogen phosphate was

prepared by dissolving 31·2g $NaH_2PO_4 \cdot 2H_2O$ in 1000ml of de-ionized water. A 0·2M solution of disodium hydrogen phosphate was made by dissolving 28·39g of Na_2HPO_4 or 71·7g of $Na_2HPO_4 \cdot 12H_2O$ in 1000ml of water. The solutions were then mixed in specific proportions according to the pH of the phosphate buffer required. The mixture was diluted to a total of 200ml and then stored at room temperature.

Radiolabelling of virus

Confluent FEA cells in roller bottles were washed in leucine-free MEM (Gibco Biocult Ltd.) and were inoculated with 1ml of virus suspension containing $1 \cdot 5 \times 10^8$ pfu/ml to which was added 18ml of leucine-free medium containing 1mCi of L(4,5-3H) leucine (40-60,000mCi/m.mol) (Radiochemical Centre, Amersham). After incubation at 37°C for 1 hour, 1ml of FBS was introduced into the bottle which was then incubated for a further 16 hours. Radiolabelled virus particles were harvested and stored as already described.

^{14}C-labelled FCV particles were prepared as above, but using 50μCi of L-(U-^{14}C) leucine (300mCi/m.mol) in 18ml leucine-free medium and 1ml of virus ($1 \cdot 5 \times 10^8$ pfu/ml) as inoculum.

A sample of ^{14}C-leucine labelled encephalomyocarditis (EMC) virus used for comparative studies was obtained from Dr David Frisby, Imperial Cancer Research Fund, London.

Radioactive sample preparation and counting

Samples of radiolabelled materials were mixed with 1ml of distilled water contained in polyethylene scintillation vials before adding 6ml of NE 260 scintillation fluid (Nuclear Enterprises, Edinburgh). Radioactivity, expressed in counts per minute (cpm), was determined in an Intertechnique SL40 liquid scintillation spectrometer.

In certain experiments where it was necessary to determine acid-precipitable counts, and in dual-labelling experiments involving ^3H and ^{14}C, radioactivity in samples was counted following precipitation with trichloracetic acid (TCA). Each sample was mixed with 0·5ml containing 100μg bovine serum albumin followed by 0·5ml of ice-cold 10% TCA. The mixture was allowed to stand at 4°C for 30 minutes and the precipitate was then collected by filtration on Whatman GF/C glassfibre paper. The filter was washed with 5% TCA and then ethanol. The filter paper was dried and then placed in a polythene vial. Radioactivity was estimated after adding 6ml of a toluene-base scintillation fluid. This fluid contained 10g of 2,5, diphenyloxazole (PPO) and 1g of 1,4-di-2(5-phenyloxazolyl-benze (POPOP) in 2·5 litres of toluene. Samples were

counted to an error of less than 1%. In double-labelling experiments relative amounts of ^{3}H and ^{14}C isotopes in each sample were determined after appropriate correction for background and spillover.

Guanidine hydrochloride-agarose chromatography

About 150ml of Sepharose CL-6B gel (Pharmacia Ltd.) was dissolved in 0·02M phosphate buffer, pH 6·8, mixed gently and allowed to settle at 4°C. The supernatant was decanted and the gel precipitate was washed three more times in buffer to remove fine particles. Guanidine hydrochloride (Sigma London Chemical Co.) was added to the gel until the refractive index was 1·4310 which was equivalent to a concentration of 6M. The gel was then poured into a K 15/90 column (Pharmacia) and allowed to settle overnight.

Two mg of each of the marker proteins (bovine serum albumin (BSA), ovalbumin and cytochrome C) and 1mg of blue dextran 2000 were dissolved in 1ml of the solubilizing buffer (8M guanidine hydrochloride, 0·01M diothiothreitol (DTT), and 2% 2-mercaptoethanol (2-ME))and were then incubated at 37°C for 3 hours before heating at 100°C for 2-3 minutes.

The mixture was then layered on top of the gel in the column and the proteins were eluted at a flow rate of 3ml per hour. Fractions of 1ml were collected using an LKB Ultrarac. The relative position of the void volume and the protein standards in the eluting volume were determined on a Unicam SP 800A ultraviolet spectrophotometer.

The above process was then repeated using a sample of the purified FCV labelled with ^{3}H-leucine and a 100µl sample per fraction was taken for radioactive estimation.

SDS - polyacrylamide gel electrophoresis

Two different methods for SDS-polyacrylamide gel electrophoresis (PAGE) were used in this study.

Method 1 This was a continuous phosphate buffered system described by Jarrett et al, (1973). The gels were mixed to give a final concentration of 7·5% - 10% acrylamide (BDH, electrophoretic grade), 0·375% bis-acrylamide, 0·1M sodium phosphate buffer, pH 7·4, 0·1% SDS and 0·5M urea. Ammonium persulphate and N,N,N,N-tetramethylethylenediamine (TEMED) were added to final concentrations of 0·07% and 0·125% respectively just before the gels were poured into siliconised 100mmx6mm glass tubes. A thin layer of water was added to keep a flat meniscus on the gels until polymerization was complete after which it was discarded.

The test samples, already in the solubilizing buffer which

contained 2% SDS, 50mM DTT, 2M urea, 10% (W/W) sucrose, 2% 2-ME and 0·001% bromophenol blue, were incubated at 37°C for 3-5 hours prior to heating at 100°C for 2-3 minutes. After cooling, the samples were gently layered on top of the gels held in a Shandon Southern electrophoresis tank, top and bottom compartments of which contained the same electrophoresis buffer (0·1M sodium phosphate, pH 7·4, 1% SDS and 0·5M urea).

The gels were run overnight at a constant current of 2·5mA per gel. The gels containing the marker proteins were stained with Coomassie blue for 1 hour and then destained for several hours in the destain solution containing 5% V/V methanol, 7·5% V/V acetic acid in water. The gels containing the test samples were frozen with powdered solid carbon dioxide and were sliced transversely into 1mm slices using a Mickle gel slicer. Each slice was placed in a scintillation vial containing 0·5ml NCS solubilizer (Nuclear Chicago) which was then sealed and left at 37°C overnight. Six ml of toluene-based scintillation fluid was added to each vial and the samples were counted for radioactivity.

Method 2 The second method was the discontinuous tris-buffered system described by Laemmli (1970) and was done in collaboration with James C. Neil. The method involves a stacking gel which concentrates the sample, and a separating gel. The separating gel was cast with a fixed concentration of acrylamide (7·5-15%) such that the ratio of the acrylamide to bis-acrylamide was 30:0·8. Other ingredients were 0·375M Tris-HCl buffer, pH 6·8, 0·1% SDS, 0·1% ammonium persulphate and 0·25% TEMED.

A layer of 5% ethanol was pipetted on to the gel surface and left until the separating gel polymerized. This recipe was used for slab gels and in each case enough space was left for a stacker gel (approximately 10mm in length) made up of 3-5% acrylamide: bis-acrylamide (30:0·8), 0·125M Tris-H6l, pH 6·8, 0·1% SDS, 0·1% ammonium persulphate and 0·025% TEMED. Perspex or teflon combs were used to prepare sample wells to the desired size in the stacker gel.

The solubilizing buffer used in this method contained 0·0625M Tris-HCl, pH 6·8, 2% SDS, 5% 2-ME, 10% glycerol and 0·001% bromophenol blue. This was made up as double strength and was added to an equal volume of sample before boiling for 3 minutes. The samples were layered on to the gels and run at a constant voltage of 40V during stacking which was increased to 100V later. Gels were run until the tracker dye was about 1cm from the end of the gel and were then removed for staining and autoradiography.

Staining of gels The gels were left overnight in a fix-stain solution of 0·0125% Coomassie blue in a 10:8:1 water methanol and acetic acid mixture and was followed by destaining in 5% v/v methanol, 7·5% v/v acetic acid in water.

Autoradiography

This was done by the method of Bonner & Laskey (1974). Gels were dried under vacuum at 80-100°C on to hard filter paper (Whatman grade 182). As it was necessary to perform fluorography in order to detect ^{3}H-labelled proteins, the gels were first dehydrated by soaking in dimethylsulphoxide (DMSO) then impregnated with PPO by soaking for 3 hours in a 22% solution of PPO in DMSO. The DMSO was removed by soaking in water and the gels were dried as described above. The dried gels were clamped in close contact with medical X-ray film (Kodak XH-1 or Kodirex) which had been pre-exposed to a short flash of light: this procedure makes the response of the film to radiography linear (Laskey & Mills, 1975). The films were exposed to the gels at -70°C for periods ranging from 3 days to 4 weeks depending on the amount of radioactivity in the sample. The films were developed in a 50% solution of bromophen (Ilford) for up to 5 minutes and fixed in Ilford Hypam rapid fixer.

Immunoprecipitation

Immunoprecipitation was used to precipitate virus-specific components from infected cell extracts using cat antiviral serum. The precipitate was then washed and analysed by PAGE and autoradiography.

Infected cells were lysed with 0·5ml of 0·5% NP40 at 4°C for 15 minutes and were then spun at 2000rpm for 10 minutes. The supernatant was further treated with 0·05ml of 5% deoxycholate (DOC) also at 4°C for 15 minutes and centrifuged at 10,000rpm for 10 minutes.

A volume of 10μl of antiserum was then added to 0·5ml of soluble antigen preparation and incubated overnight at 4°C. Fifty μl of protein A-bearing Staphylococcus aureus (prepared by James C. Neil) was added and the mixture was allowed to stand for another 1 hour at room temperature. The tertiary antigen-antibody-bacterial complexes were then harvested by low speed centrifugation (5000rpm for 10 minutes) and washed twice in lysis buffer and finally in TS. The antigen-antibody complexes were resuspended in 100μl of solubilizing buffer and then analysed by PAGE and autoradiography as described above.

CHAPTER 3

THE SUBUNIT STRUCTURE OF FCV PARTICLES

INTRODUCTION

Crick and Watson (1956) first proposed that the simpler viruses were built from smaller subunit structures bonded together by repeated use of the same bonding contacts characteristic of, and presumably determined by, the protein subunit itself; and that such a superstructure must be of cubic symmetry. This hypothesis has since been extensively confirmed by X-ray crystallography (Finch & Klug, 1959) and by electron microscopy using the negative staining technique of Horne & Nagington (1959). The latter authors concluded that the poliovirus capsid was icosahedral with 5:3:2 symmetry implying that the capsid has 60 identical structural subunits each about 60Å in diameter.

Several attempts to establish an acceptable architectural pattern for picornaviruses based on this novel concept were unsuccessful for a number of reasons. First, it soon became clear that the properties of 5:3:2 symmetry and 60Å periodicity provide insufficient information to define a 60 subunit structure: for example, turnip yellow mosaic virus with crystallographic properties similar to poliovirus had 32 rather than 60 capsomeres (Huxley & Zubay, 1960; Nixon & Gibb, 1960; Klug & Finch, 1960); and subsequent studies by electron microscopy appeared to favour 32 morphological subunits among picornaviruses (Mayor, 1964; Breese *et al*, 1965; Jamison, 1969). Secondly, the picornavirus shell is extremely compact and seems impermeable to the negative stains commonly used in electron microscopy so that very little surface detail is revealed. The third problem is the presence of several types of non-identical polypeptide chains in picornaviruses (Maizel, 1963; Rueckert, 1965; Rueckert & Duesberg, 1966; Jacobson & Baltimore, 1970).

Since the theory of virus structure was generally applied to capsids containing a single type of subunit, the presence of multiple chains in an apparently simple virus immediately became the most intriguing aspect of picornavirus structure. There were speculations that the virions might be composed of pentamers of one chain type and hexamers or trimers of another and that the protomers might consist of multiple polypeptides (Mayor, 1964).

Maizel (1963) was first to show by polyacrylamide gel electrophoresis that the poliovirus particle contained 4 virus capsid proteins (VP_{1-4}). Subsequently Coxsackie viruses (Crowell & Phillipson, 1971), cardioviruses (Rueckert *et al*, 1969) and rhinoviruses (Medapa *et al*, 1971; Korant *et al*, 1972) were also reported to have four distinct polypeptides present in equimolar amounts in each kind of

virion. The only exception is the calicivirus which has only one capsid polypeptide (Burroughs & Brown, 1974).

The results of virion polypeptide analysis encouraged more workers to carry out controlled particle dissociation with the intent of elucidating the architecture of picornaviruses. This idea met with a degree of success in certain viruses: for example, mengovirus and EMC virus under acid conditions were dissociated into 14S and 5S fragments (O'Callaghan, Mak & Colter, 1970). Mouse-Elberfeld (ME) virus has also been similarly disrupted into 14S and 5S subunit structures, both containing three major polypeptides (Dunker & Ruekert, 1971). This led the authors to suggest a structure with a triangulation number of one, containing 60 protomers (5S) arranged in clusters of 12 pentamers (14S). A similar model was suggested for foot-and-mouth disease virus where the dissociation appears to occur along boundaries of the icosahedral facet creating a trimer of protomers (12S) instead of pentamers (Talbot & Brown, 1972).

However, similar treatment of the enteroviruses, e.g. poliovirus, has not been quite so successful because of their acid-stable nature; in fact, poliovirus has not been dissected into subviral particles beyond the stage of the 75S empty capsids, although alkaline degradation suggests that both VP_2 and VP_4 can be sequentially removed from the capsid structure (Maizel et al, 1967; Katagiri et al, 1971).

Until now there was no evidence that caliciviruses could be dissociated into subunit particles. In the present study attempts were made to sequentially disrupt purified FCV particles, first, by acid treatment and then subjecting the resulting products to the action of reducing agents, until finally, the basic viral capsid proteins were produced. This procedure would not only extend our knowledge of the structure of FCV subunit particles but also increase the understanding of the possible ways in which the virions were constructed.

Also in the work described in this chapter, the effect of storage on purified FCV particles at different temperatures was examined and the results were compared to normal storage of virus working stocks.

MATERIALS & METHODS

Virus purification

After infecting FEA cells with FCV and incubating the cultures for 16 hours at 37°C the virus was harvested. Cellular debris was removed by centrifugation at 10,000rpm for 10 minutes and to the supernatant fluid was added an equal volume of cold saturated ammonium sulphate neutralised with NaOH. The mixture was maintained at 4°C for 3 hours and was then centrifuged at 5,000rpm for 5 minutes. The resulting precipitate was resuspended in 1·0ml of TS and the virus was then purified by either of two methods.

Method 1 The resuspended material was layered in a cellulose nitrate ultracentrifuge tube on top of a solution consisting of 1·5ml $CsCl_2$ of buoyant density 1·50g.cm^{-3}, 1·5ml 70% sucrose and 1·0ml 20% sucrose. The tube was then centrifuged in an SW 50.1 rotor at 36,000rpm for 2 hours. The light-scattering band which formed at the junctions of the $CsCl_2$ and the 70% sucrose layer was collected after puncturing the bottom of the tube. This material was then diluted in TS and layered on a 4·5ml column consisting of 1·5ml, 1·5ml and 1·0ml of $CsCl_2$ solutions of buoyant density 1·50, 1·30, and 1·15g.cm^{-3} respectively. This was then centrifuged in an SW 50.1 rotor at 40,000rpm for 16 hours. The viral material was recycled once more, and the gradients were fractioned using a Buchler fraction collector. A sample of each fraction was taken for radioactive estimation.

Method 11 This method was similar to the above except that instead of concentrating the virus-containing material by initial centrifugation, it was layered directly on a 20-70% linear sucrose gradient and centrifuged in an SW 50.1 rotor at 45,000rpm for 30 minutes. The light-scattering band was recovered and the viral components were subjected to further centrifugation in $CsCl_2$ gradients as described above. Virus infectivity assay was carried out on all the radioactively labelled sedimenting peaks.

Buoyant density measurement

The refractive index of every third fraction was measured using an Abbey refractometer (Bellingham & Stanley, London) and the buoyant density was calculated from a calibration curve in which refractive index values were plotted against their corresponding buoyant densities.

Electron microscopy

Using the loop drop method, purified viral materials were placed on a 400 mesh formvar-carbon coated copper grid. The grids were washed twice with ammonium acetate to remove any remaining $CsCl_2$. An equal volume of 3% phosphotungstic acid (PTA), pH 7·2, was applied to the grid which was then drained off. The grids were examined in an AEI EM 6B electron microscope by Dr Helen M. Laird.

Dual radioisotope labelling of virus

The dual labelling experiment was designed to simultaneously label both viral ribonucleic acid and proteins with radioisotopes to determine which of the viral sedimenting components contained RNA or proteins or both together.

A mixture of 1mCi of ^{3}H-leucine and 50µCi ^{14}C-uridine in 20ml of leucine-free medium containing $1·5x10^{8}$ pfu of FCV was used to infect a confluent 2·5 litre roller bottle of FEA cells. After 1 hour 1·0ml of FBS was added to the inoculum and the cells were incubated at 37°C for 16 hours. Harvesting of virus and clarification were done as described earlier.

Determination of sedimentation rate

This was done by rate zonal ultracentrifugation. Samples of 200-300µl of the test sample were layered on top of a 5ml 15-30% linear sucrose gradient prepared in an SW 50 tube. The tube was loaded on to an SW 50.1 rotor and centrifuged at various speeds depending on the sample under investigation. For example, the fast sedimenting FCV peak was centrifuged at 45,000rpm for 30 minutes while the slower sedimenting viral subunits were spun at 30,000rpm for 18-24 hours. The gradients were fractioned and analysed as described earlier.

Preparation of IgG and IgM markers

About 20ml of freshly prepared calf serum obtained from Dr C.O. Dawson was precipitated with an equal volume of saturated ammonium sulphate. The precipitate was resuspended in TS buffer and the protein was re-precipitated with 2 volumes of ice-cold absolute ethanol at -20°C overnight. The process of precipitation and resuspension was continued until the purity of the globulins was confirmed by scanning a sample of the preparation, diluted 1:100, at 280µm in an ultraviolet spectrophotometer. A volume of 250µl of a 1:10 dilution of the partially purified globulin was centrifuged on a 10-40% sucrose gradient at 30,000rpm for 18 hours in the SW 50.1 rotor. The gradient was fractioned and the optical density of each

fraction was estimated at 280μm. Both 19S IgM and 7S IgG appeared as well defined peaks and were then harvested and stored at -20°C.

Dissociation of FCV into 15S subunits

One volume of a purified preparation of ^{3}H-leucine-labelled FCV was mixed with 4 volumes of 0·05M phosphate buffer at pH 6·5, thus bringing the $CsCl_2$ concentration in the preparation to approximately 0·7M. The mixture was then incubated at 42°C for 1 hour. The virus subunit generated by this treatment was isolated on a 15-30% linear sucrose gradient by rate zonal ultracentrifugation at 45,000rpm for 9 hours. The sedimentation rate of the virus subunit was determined by using purified bovine IgM and IgG as markers. In another experiment, the 14S ^{14}C-labelled EMC virus subunit was used as marker. The marker was prepared by acid treatment of purified EMC virus in 0·02M phosphate buffer at pH 6·2 in the presence of 0·14M NaCl at 37°C for 1 hour.

Effect of pH on virus infectivity

A sample of 50μl of purified FCV peak 1 was diluted in 1·0ml of 0·05M phosphate buffer and the pH was adjusted to 6·0, 6·2, 6·4, 6·5, 6·6, 6·8, 7·0 and 7·2 respectively. The samples were distributed in glass bottles and maintained at 42°C for 1 hour and then allowed to cool to room temperature. Serial dilutions of the samples were made in TS buffer and assayed for infectivity.

Dissociation of the 15S component into 5S subunits

The remaining FCV 15S subunit prepared as described above was made up to 500μl with TS buffer and exposed to the action of reducing agents (10μl 2-ME and 50μl 0·1M DTT) at 42°C for 1 hour. The resulting 5S viral subunit was isolated on a linear 15-30% sucrose gradient by rate zonal ultracentrifugation at 30,000rpm for 20 hours.

Polypeptide analysis of the 5S subunit

The polypeptide composition of the 5S FCV subunit was determined by 6M guanidine hydrochloride agarose column chromatography.

FCV stability under storage

The stability of purified FCV stored at three different temperatures (4°C, -20°C and -70°C) was investigated.

A purified virus sample was made up to 24ml in TS buffer and a 10μl sample was taken and diluted for infectivity assay. The rest was dispensed in eight 1·0ml samples in glass bottles and incubated at the various temperatures.

A bottle, stored at each temperature, was withdrawn at weekly intervals and subjected to infectivity assay and rate zonal ultracentrifugation using ^{14}C-labelled 15S FCV subunit as marker.

RESULTS

Purification of feline calicivirus

Two methods of purification of virus were examined. Virus was labelled with ^{3}H-leucine, precipitated from culture fluid with ammonium sulphate and treated as described above.

Method 1 Only one large peak of viral protein material was obtained by this method (Fig.3.1). However, on further purification of the peak by isopycnic centrifugation in $CsCl_2$, two separate and well defined radioactive peaks were obtained which for convenience were called peak I and peak II (Fig. 3.2). In terms of radioactive counts peak II was approximately ten times larger than peak I.

Method 2 Direct analysis of the resuspended viral material following ammonium sulphate precipitation yielded two peaks (peak I & peak II) by rate zonal ultracentrifugation in sucrose gradients (Fig. 3.3). The peaks were separately pooled and further purified by two cycles of $CsCl_2$ centrifugation (Fig. 3.4). This method was more commonly used in this study because it yielded directly the two viral peaks required for analysis and was more rapid.

Definition of the viral protein peaks

A number of experiments were performed to define the nature of the two radioactive peaks.

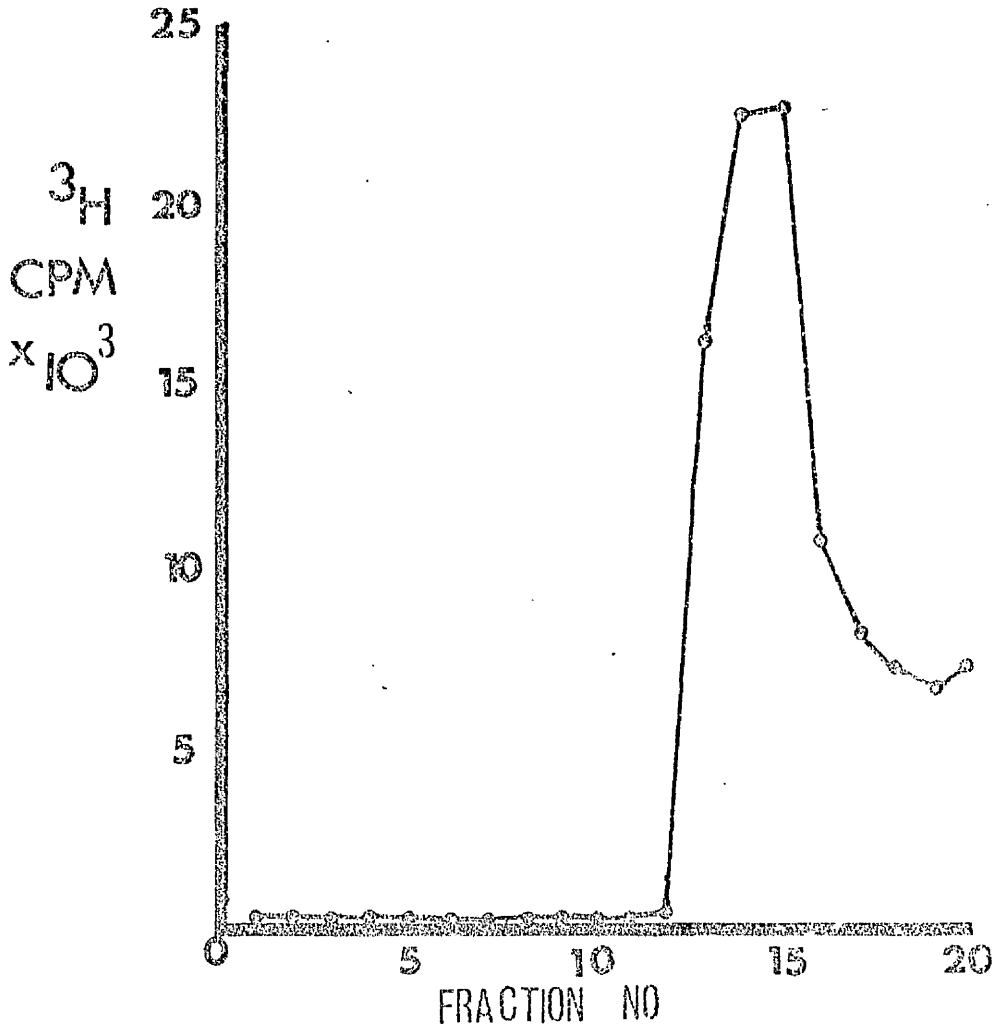
Buoyant density FCV has a reported buoyant density of 1·36- 1·39g.cm^{-3} (Newman et al, 1973; Studdert et al, 1970) and an estimate of 1·39g.cm^{-3} for peak I was obtained in this study (Fig. 3.4A). This compared with a value of 1·26g.cm^{-3} for peak II (Fig. 3.4B). These results suggested that peak I contained the complete virus.

Sedimentation rate Using purified 160S EMC virus as marker, peak I was found to have a sedimentation rate of 170S as shown in Fig. 3.5. This is within the range of 160-207S already published for vesicular exanthema virus of swine, another calicivirus (Wawrzkiewicz et al, 1968; Oglesby, Madin & Schaffer, 1971; Newman et al, 1973). By comparison, using 19S bovine immunoglobulin (IgM) as marker, peak II had a sedimentation value of 15S (Fig. 3.6).

Infectivity As shown in Fig. 3.7, virus infectivity assay revealed that about 99% of the infectivity was associated with peak I (Fig. 3.7A) compared with peak II (Fig. 3.7B).

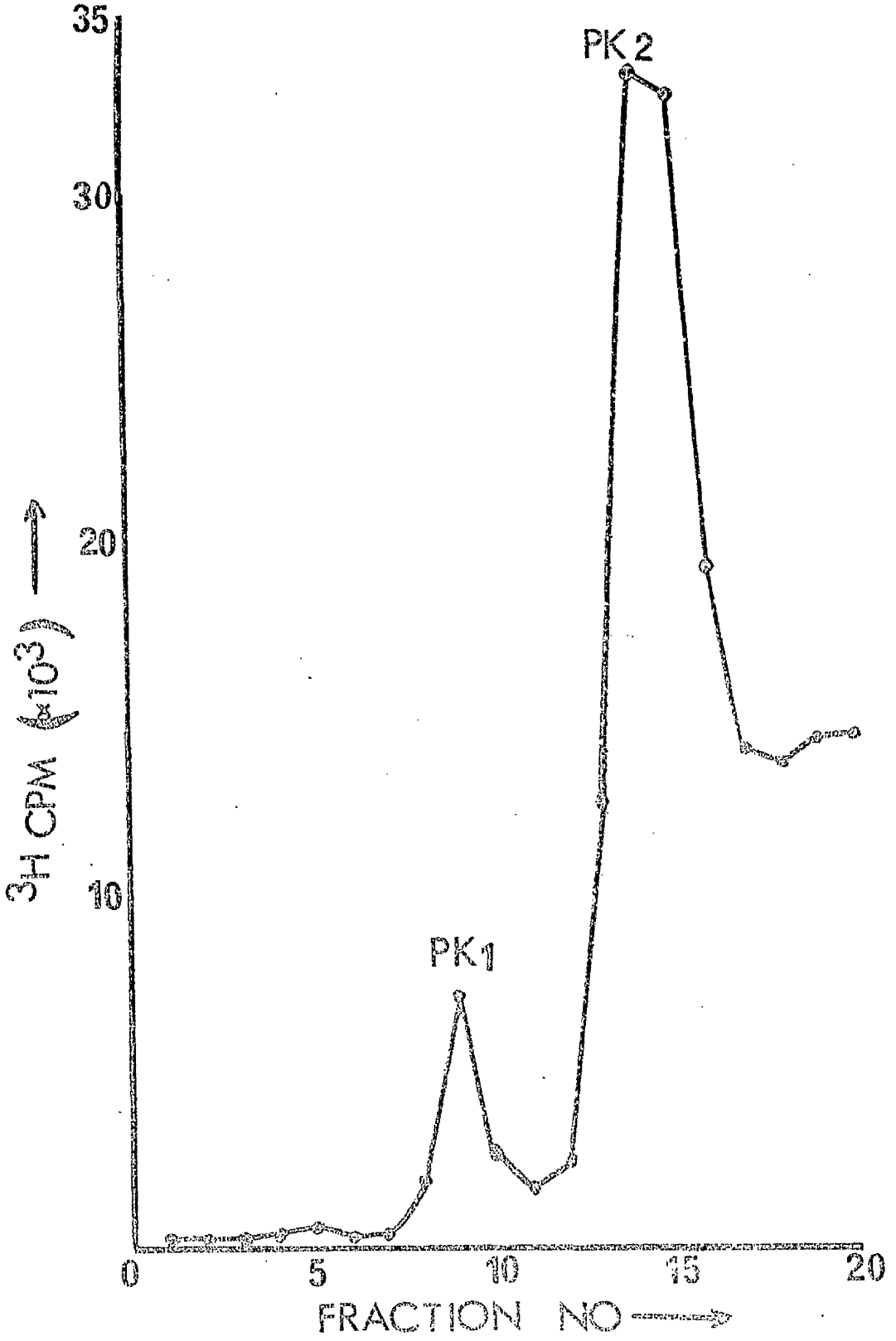
Viral RNA The result of the dual labelling experiment showed that viral proteins labelled with ^{3}H-leucine were found in both peaks I and II, whereas the ^{14}C from radiolabelled uridine was found only in peak I (Fig. 3.8). This suggested that peak I contained RNA and peak II did not.

Figure 3.1 Purification of FCV by Method 1:I



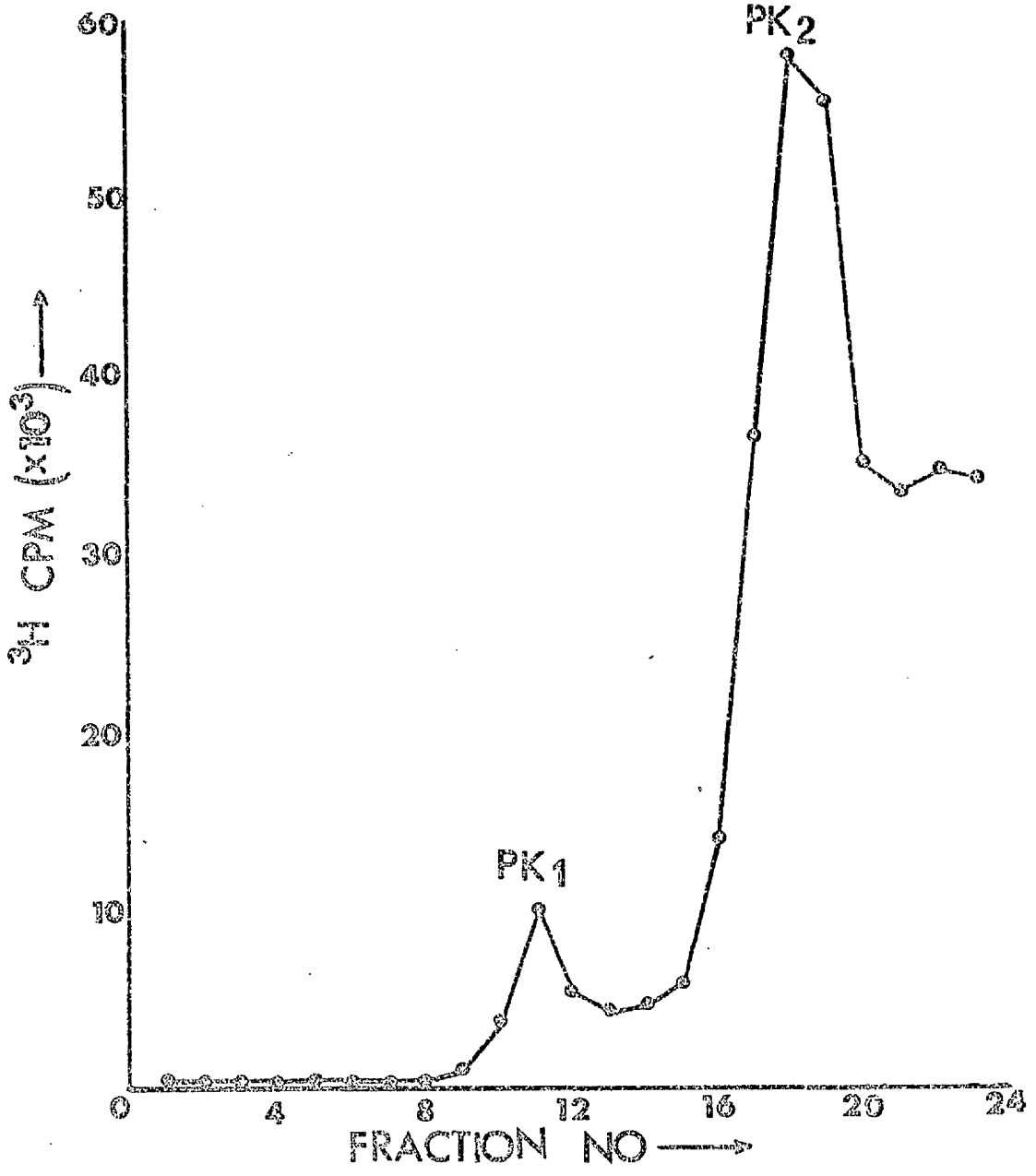
^{3}H-leucine labelled FCV was precipitated by ammonium sulphate and the resuspended precipitate was centrifuged as described above in the SW 50.1 rotor at 36,000rpm for 2 hours. Samples of 10μl from each fraction were assayed for radioactivity.

Figure 3.2 Purification of FCV by Method 1:II



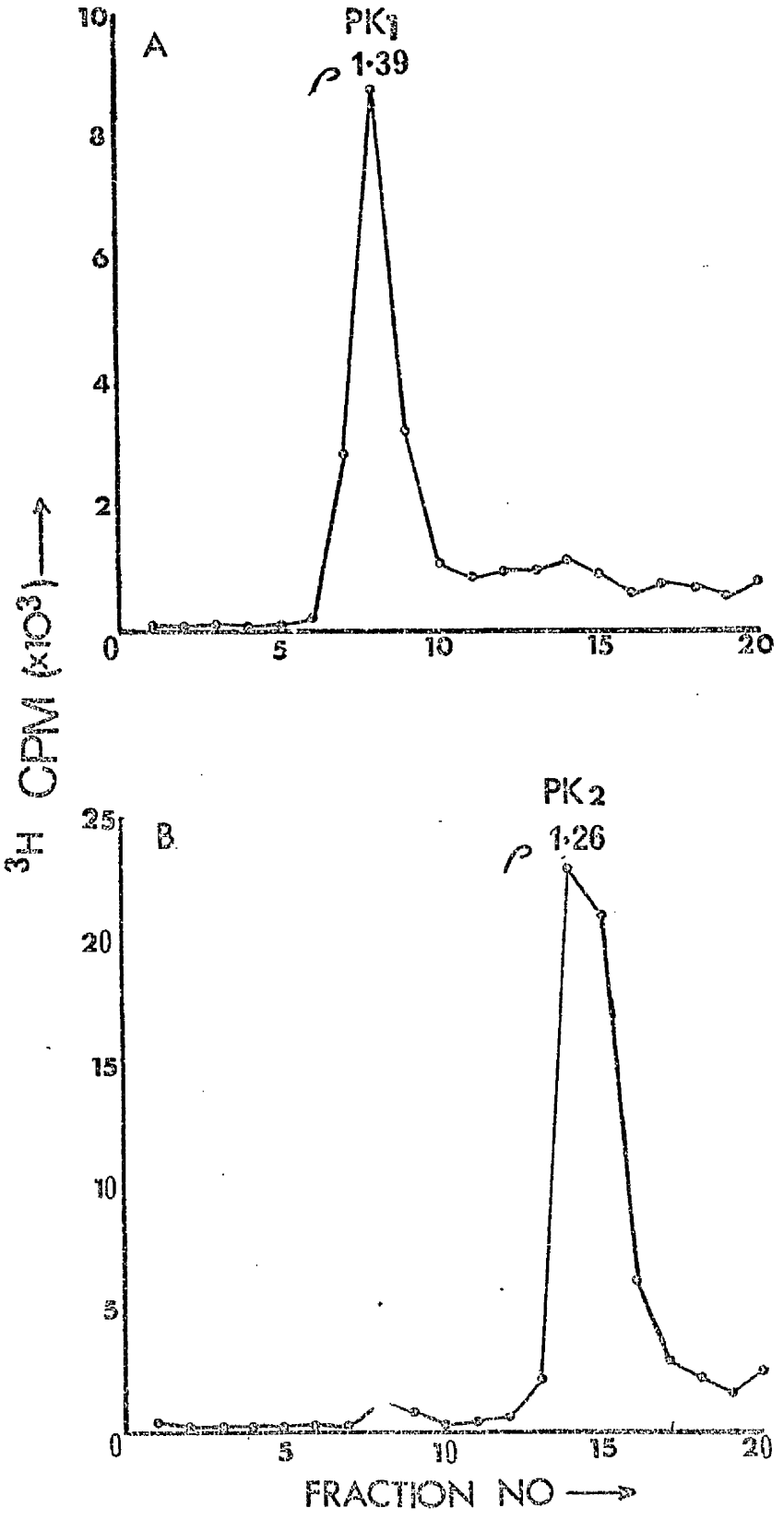
The peak shown in Fig. 3.1 was diluted in TS buffer and centrifuged on a $CsCl_2$ gradient in an SW 50.1 rotor at 40,000rpm for 16 hours. The radioactivity in 20μl samples of each fraction was determined.

Figure 3.3 Purification of FCV by Method 2:I



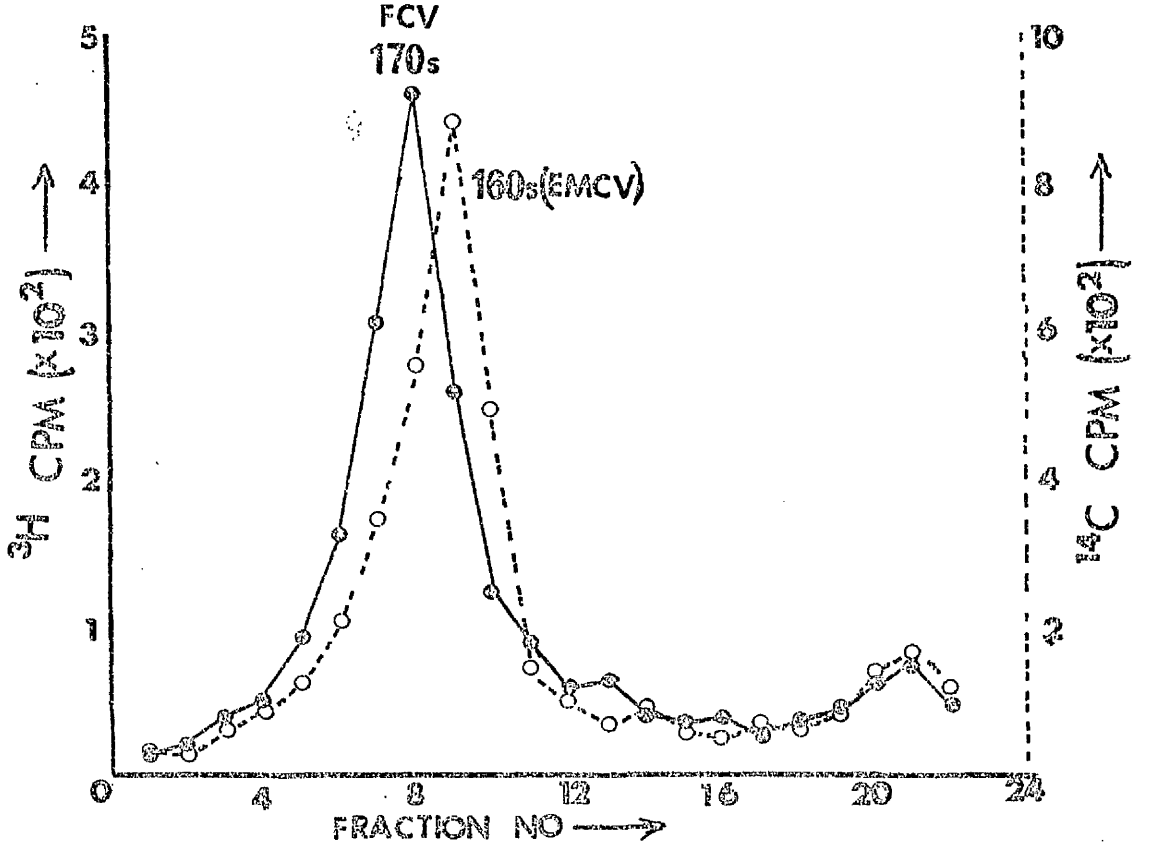
Preliminary purification of FCV by rate zonal ultracentrifugation in 20-70% sucrose gradient after ammonium sulphate concentration. The SW 50.1 was centrifuged at 45,000rpm for 30 minutes.

Figure 3.4 Purification of FCV by Method 2:II



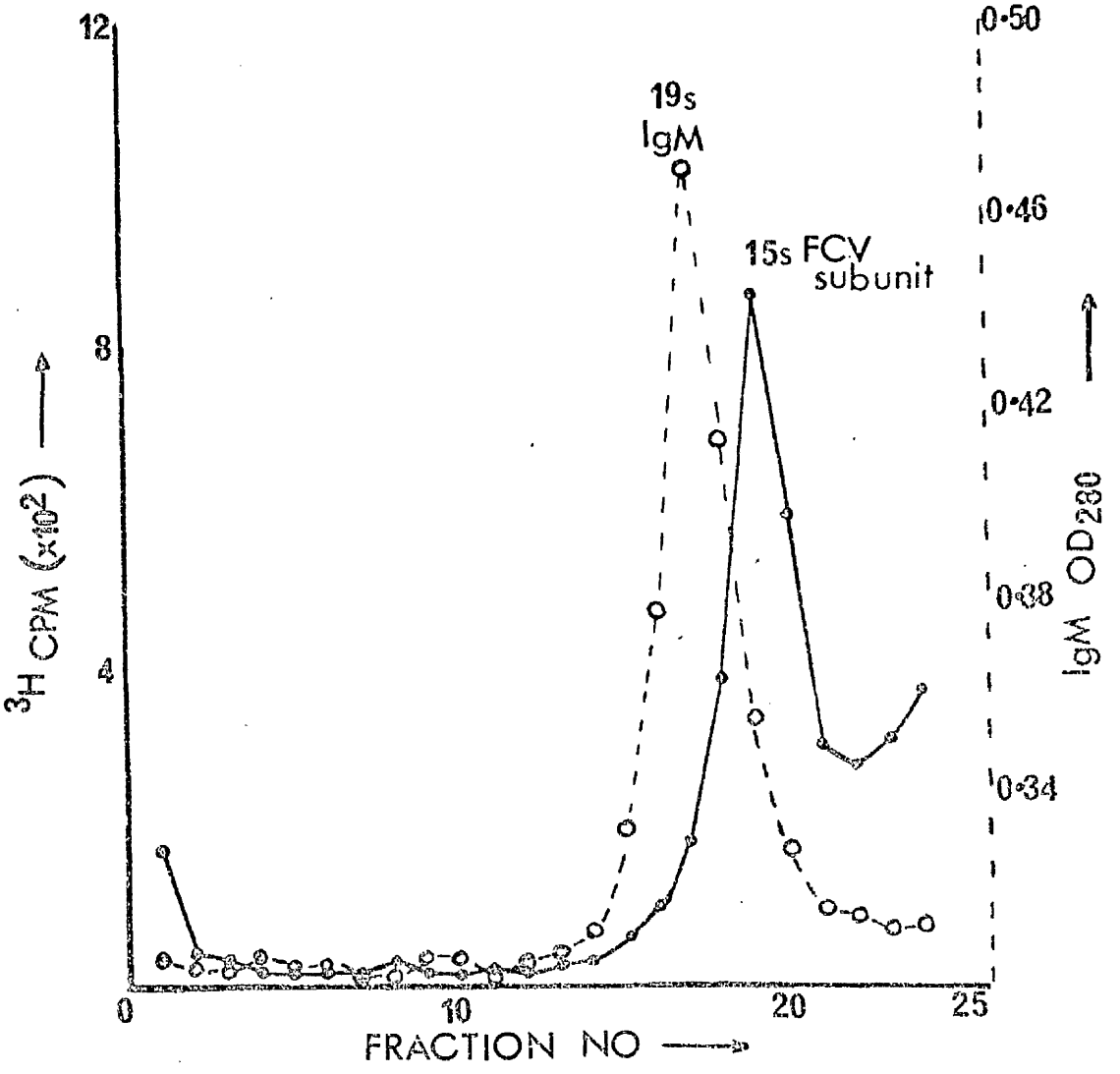
Recycling of peaks I and II shown in Fig. 3.3 on $CsCl_2$ gradients in the SW 50.1 rotor at 40,000rpm for 16 hours.

Figure 3.5 Determination of the sedimentation rate of FCV



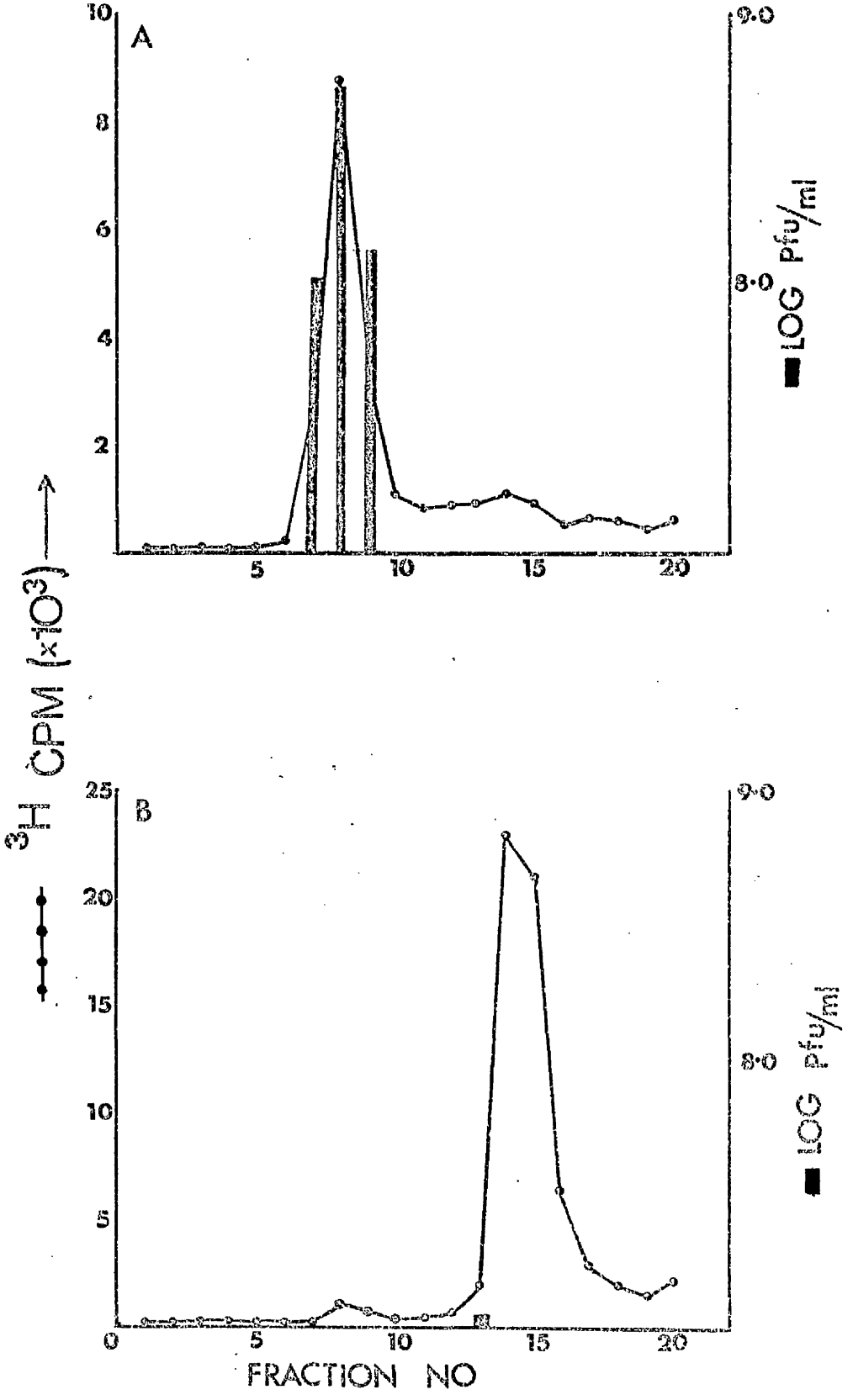
Sedimentation was in 15-30% sucrose gradient in the SW 50.1 rotor at 45,000rpm for 30 minutes.

Figure 3.6 Determination of the sedimentation rate of FCV subunit (peak II)



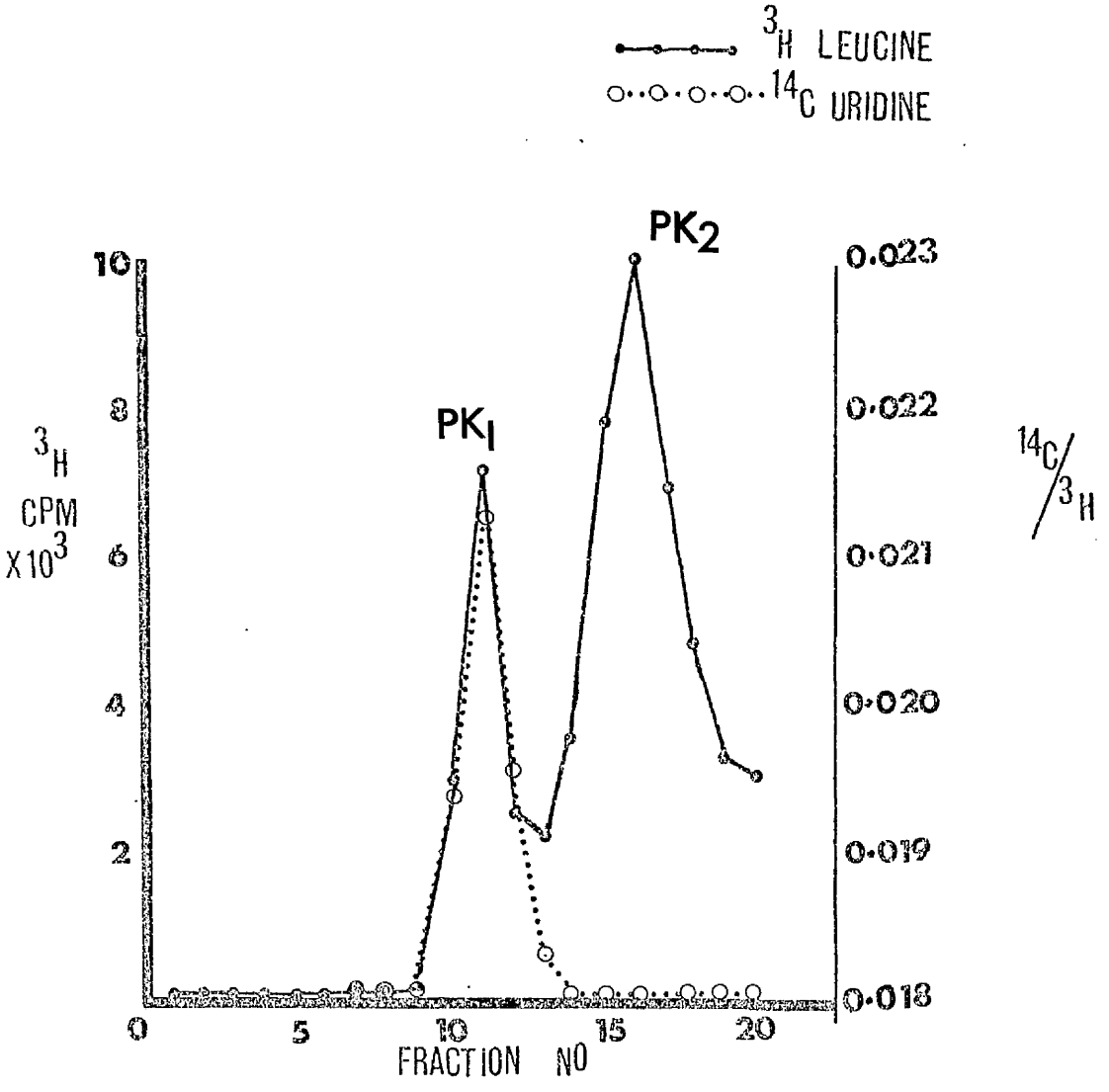
Sedimentation was in a 15-30% sucrose gradient in the SW 50.1 rotor at 45,000rpm for 5 hours.

Figure 3.7 Infectivity of peaks I and II



Centrifugation was as in Fig. 3.4A and B

Figure 3.8 Centrifugation of FCV labelled with ^{3}H-leucine and ^{14}C-uridine



The sample was centrifuged in $CsCl_2$ gradient in SW 50.1 rotor at 36,000rpm for 16 hours. Samples of 20μl from each fraction were assayed for radioactivity.

Electron microscopy Characteristic FCV particles were found in peak I after negative staining (Plate 3.1). By contrast, peak II contained a large mass of unidentified homogeneous material among which were a few virus particles (Plate 3.2).

All the above results confirmed that peak I contained the infectious virus. This posed the question of the nature of the material contained in peak II. This was investigated in two ways; first, by comparison of the molecular weight of the protein of peaks I and II; and secondly, by antigenic analysis as discussed in Chapter 5.

Polyacrylamide gel electrophoresis of viral components

One of the unique properties of FCV is that it has only one major capsid protein (Bachrach & Hess, 1973; Burroughs & Brown, 1974). Advantage of this feature was taken to analyse samples of peak I (virus) and peak II for their protein constituents by polyacrylamide gel electrophoresis and it was found that both contained a single major polypeptide of molecular weight 65,000 (Fig. 3.9).

Possible source of the 15S subunit in virus harvests

This result suggested that peak II contained virus-specific material, possibly viral subunits which were not incorporated into whole virus during virion assembly. Alternatively, peak II might be an artificial product resulting from: a) the long period of virus production (16 hours), since the reproductive cycle of the virus is completed by 6 hours; b) the effect of temperature (37°C) and pH (6·8) during incubation; or c) the viral subunits might be generated by centrifugation during purification. These points were investigated as follows.

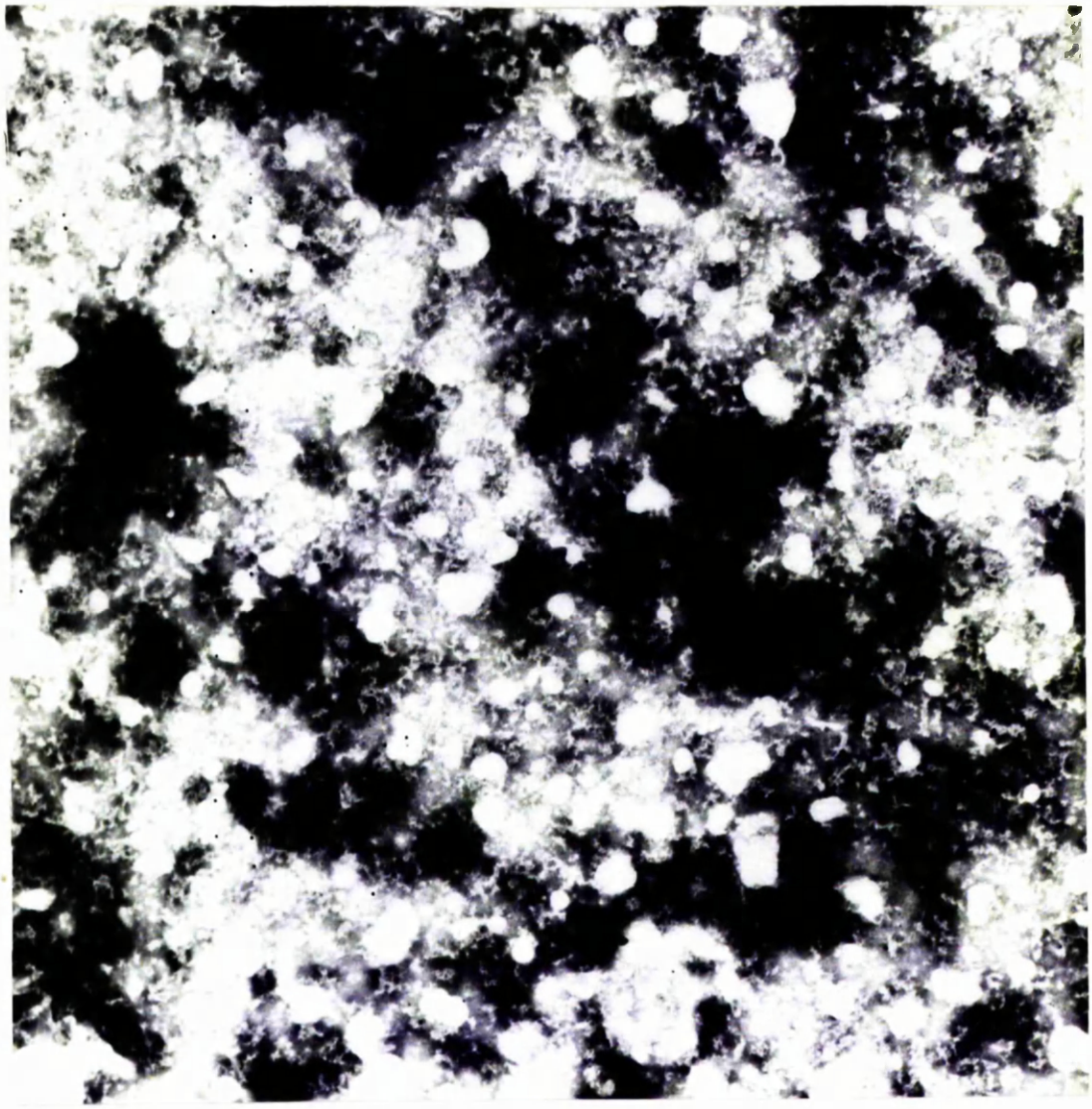
Effect of time on virus production FCV was harvested from FEA cells infected 6, 9 and 24 hours after infection and then analysed for peaks I and II. It was found that there was as much peak II in the 6 hour harvest as there was in the 9 hour and 24 hour harvests (Fig. 3.10). This meant that the time of virus production has no effect either on the yield or on the quantity of peak II produced.

Effect of temperature (37°C) and pH (6·8) In a reconstruction experiment the stability of purified peak I FCV added to EFC_{10} medium of pH 6·8 and incubated at 37°C for 16 hours was examined. These were the conditions under which the virus was normally produced. After incubation, the virus suspension was analysed on $CsCl_2$ gradients to detect if any peak II had been generated. The result (Fig. 3.11) showed that under these conditions peak I FCV remained stable.

Plate 3.1 Electronmicrograph of peak I

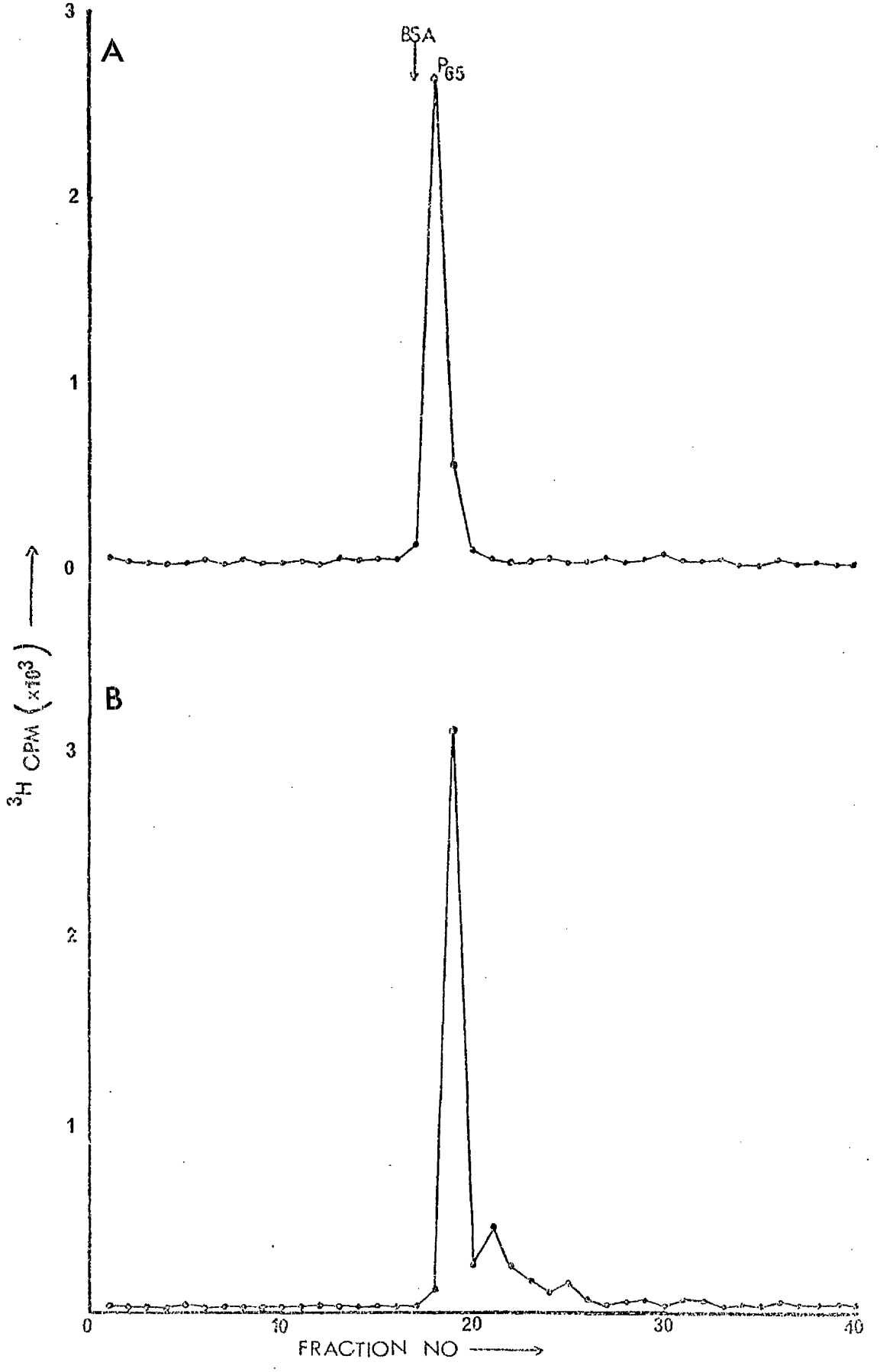
X 80,000

Plate 3.2 Electronmicrograph of peak II



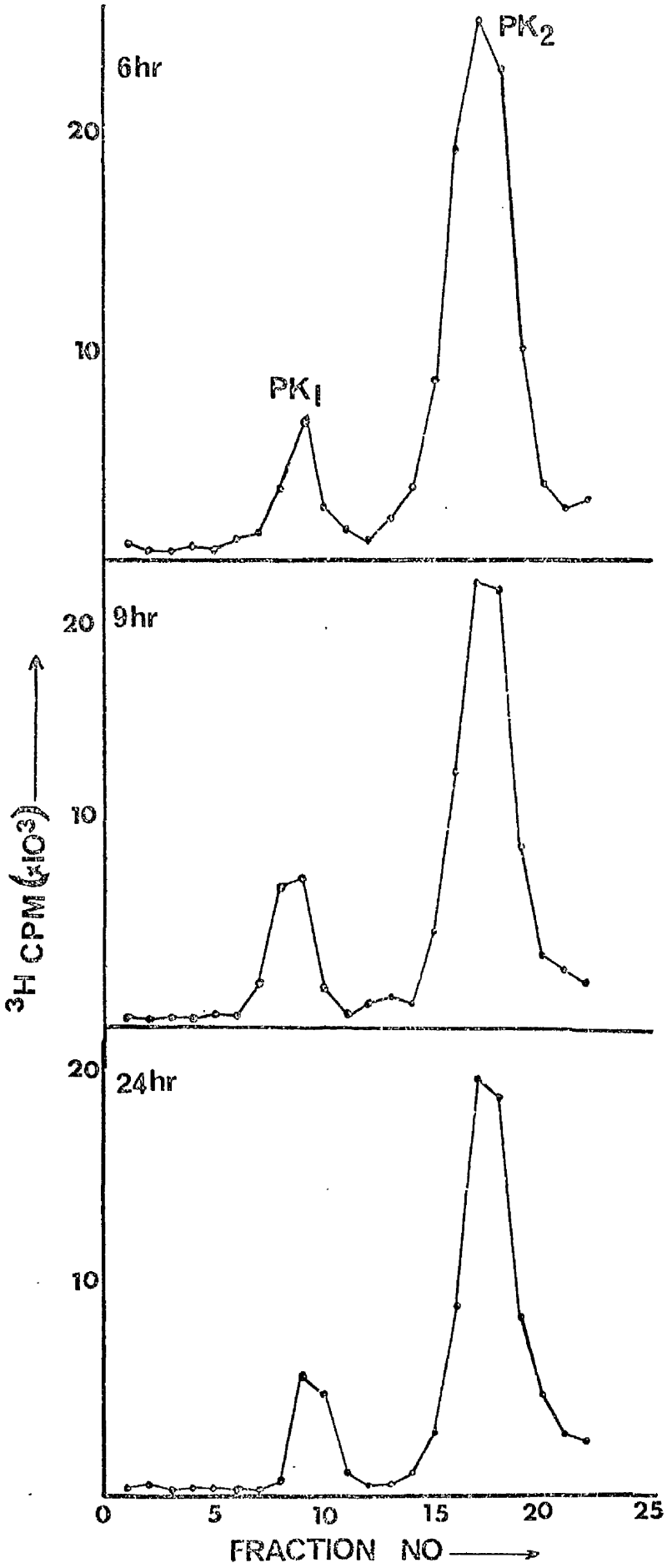
X 80,000

Figure 3.9 Polyacrylamide gel electrophoresis of FCV peaks I and II



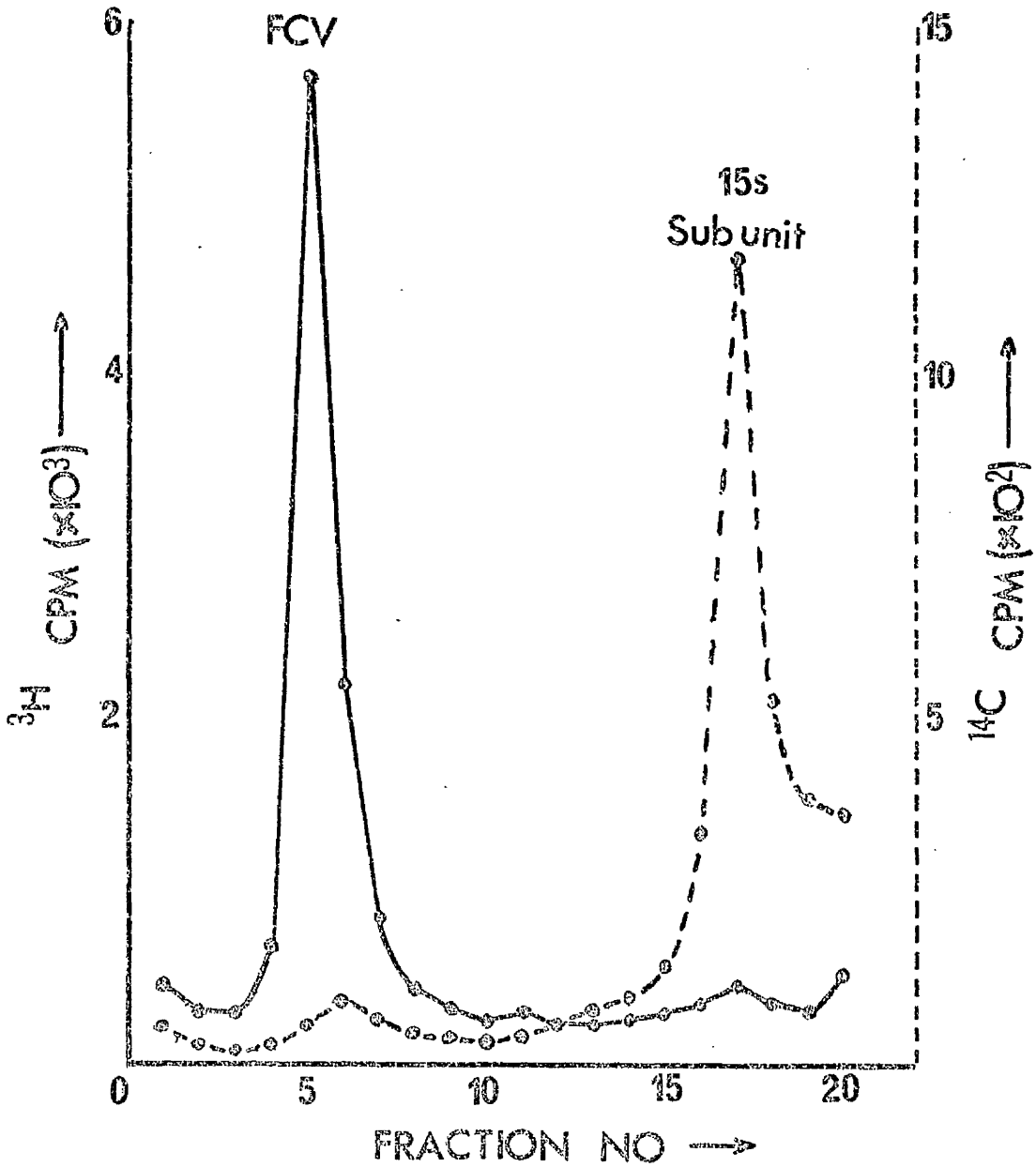
A = peak I B = peak II

Figure 3.10 Effect of time of incubation at 37°C on FCV production



Centrifugation was on $CsCl_2$ gradients in the SW 50.1 rotor at 40,000rpm for 16 hours. Samples of 20 µl from each fraction were assayed for radioactivity.

Figure 3.11 Effect of temperature and pH on FCV components



Sedimentation was in 15-30% sucrose gradient in the SW 50.1 rotor at 45,000rpm for 30 minutes. Samples of 25 µl from each fraction were assayed for radioactivity.

Effect of centrifugation Samples of a feline calicivirus preparation obtained following ammonium sulphate precipitation were centrifuged on $CsCl_2$ gradients in the SW 50.1 rotor at 36,000rpm for 3, 6 and 24 hours to see how centrifugation might effect the integrity of the virus. Figure 3.12 shows that centrifugation per se did not dissociate the virus into subunit structures.

From these results, it was concluded that the 15S components which constitute the so-called peak II are not artificial products but possibly unassembled viral proteins found in infected cells.

FCV particle dissociation

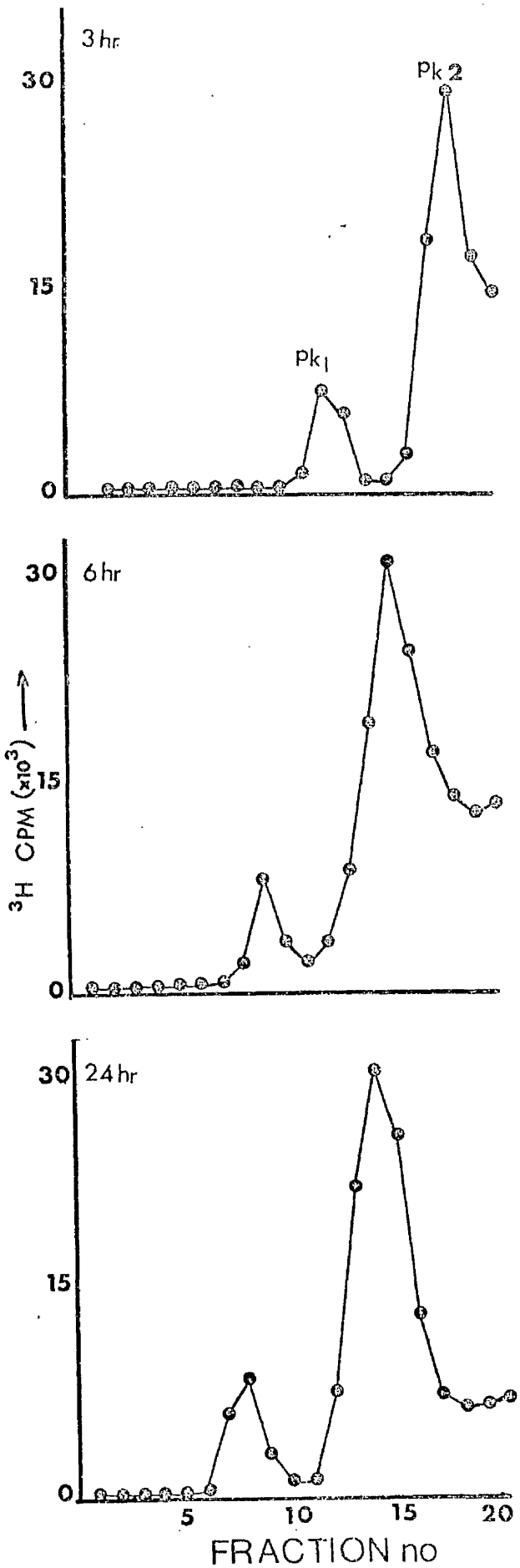
Generation of a 15S component Using bovine IgG and IgM (Fig. 3.13) or 14S EMC virus subunit (Fig. 3.14) as markers, it was found that acid treatment of FCV dissociated the virus into 15S subunit. Oglesby et al (1971) found that a 2M concentration of $CsCl_2$ was deleterious to the integrity of vesicular exanthema virus (a calicivirus), so that the presence of 0·7M $CsCl_2$ in this reaction, therefore, probably aided FCV dissociation. Furthermore, the artificial 15S subunit labelled with ^{14}C-leucine co-sedimented with the naturally produced subunit (peak II) labelled with ^{3}H-leucine in sucrose gradients by rate zonal ultra-centrifugation (Fig. 3.15) suggesting that both might be identical.

Generation of a 5S component When exposed to the action of reducing agents, such as 2-mercaptoethanol (2-ME) and DTT as was used in this study, the 15S viral subunits dissociated into smaller 5S subunits. This was observed for the "natural" (Fig. 3.16) and "viral" (Fig. 3.17) 15S subunits. This sequential breakdown of virus particles provides experimental support for the hypothesis that the calicivirus particle is held together by (at least) two different types of inter-subunit bonds.

Polypeptide analysis of the 5S subunit

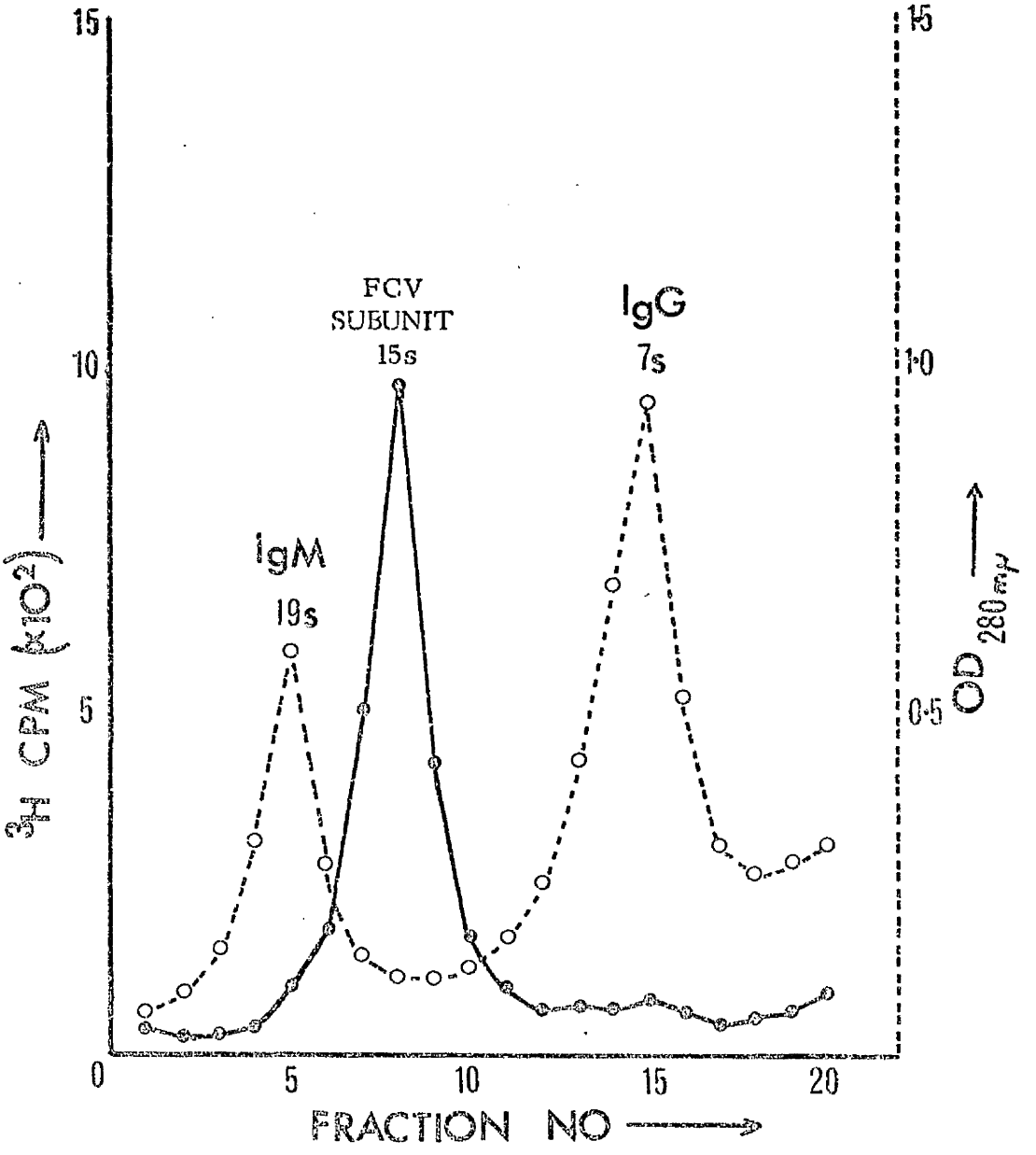
The polypeptide content of the 5S subunit was analysed by guanidine hydrochloride-agarose column chromatography and the result showed that it contained a single major polypeptide with a molecular weight of 65,000 (Fig. 3.18). Also seen was a minor polypeptide of molecular weight 13,500 daltons which might be similar to that found by Burroughs & Brown (1974) by polyacrylamide gel electrophoresis. Bachrach & Hess (1973) found a similar component in radiolabelled but not in stained gels and suggested that it could be either viral or a contaminant. However, detailed investigation in this study by poly-acrylamide gel electrophoresis and autoradiography seem to support the presence of a minor polypeptide in FCV (see Chapter 4).

Figure 3.12 Effect of centrifugation time on FCV



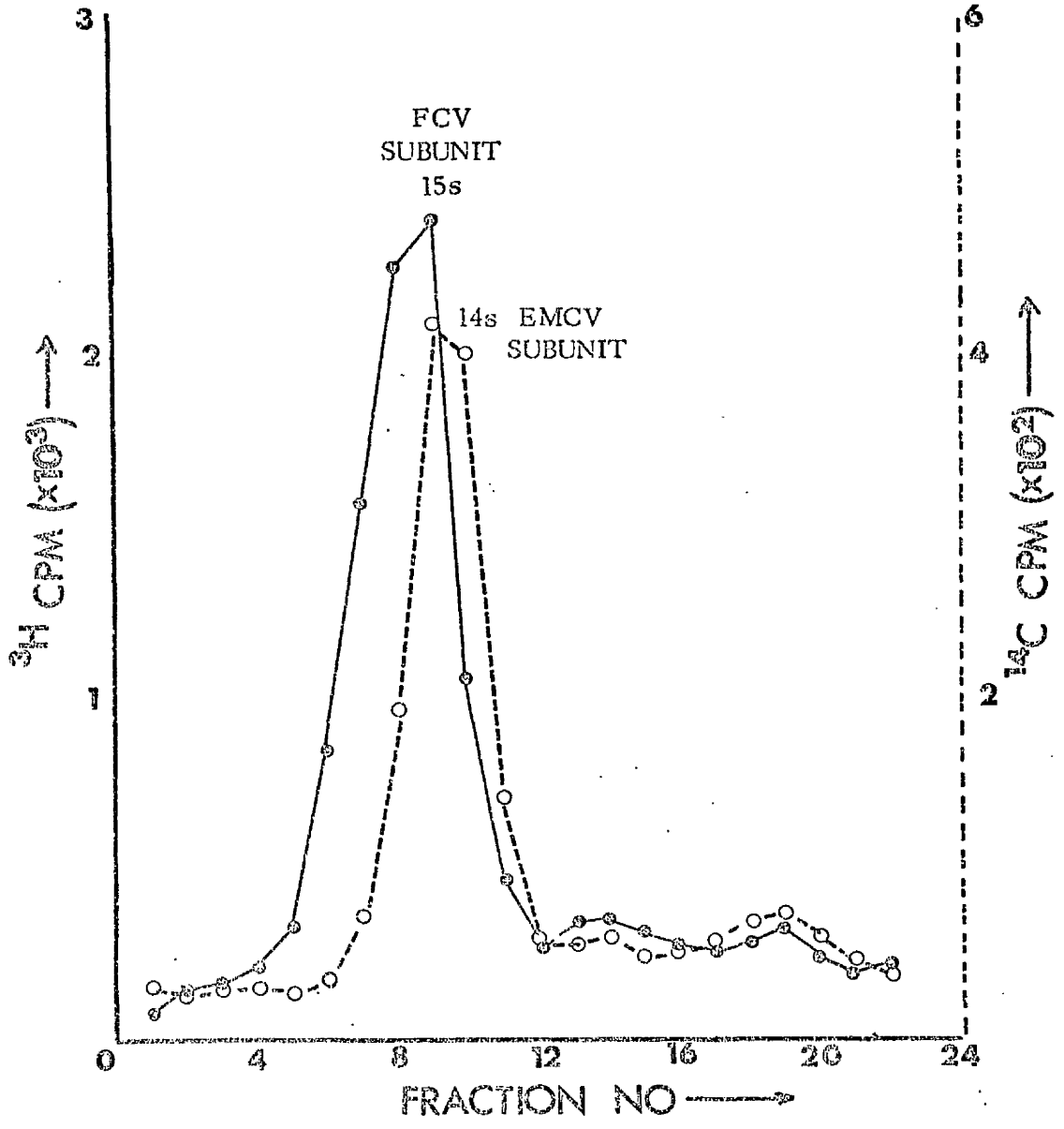
Samples of FCV preparation following ammonium sulphate precipitation were centrifuged in the SW 50.1 rotor at 40,000rpm for 3, 6 and 24 hours.

Figure 3.13 Sedimentation behaviour of a FCV subunit obtained by acid dissociation



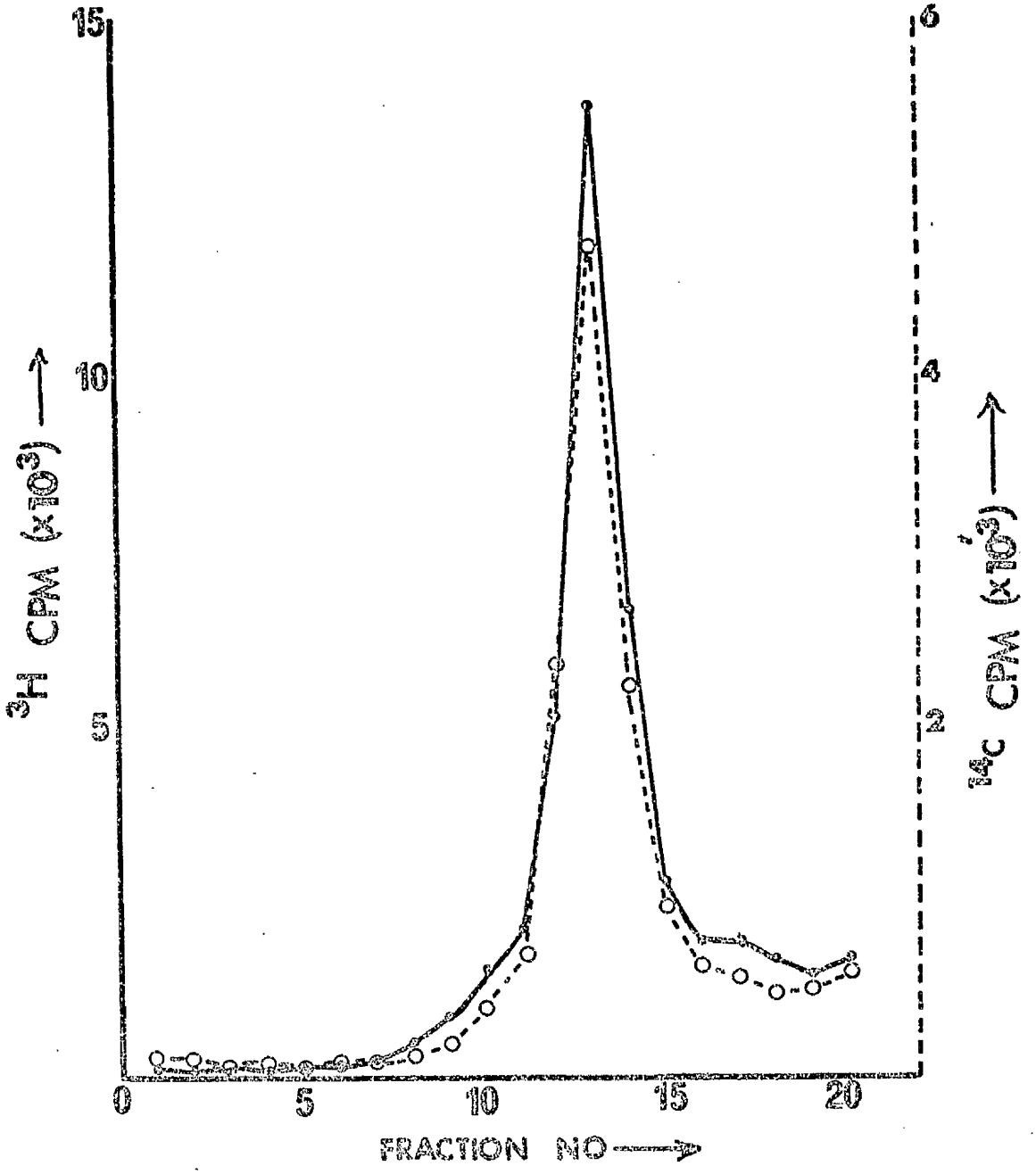
The sedimentation value of 15S FCV subunit was determined in a 15-30% linear sucrose gradient centrifuged at 45,000rpm for 9 hours in SW 50.1 rotor using bovine 19S IgM and 7S IgG as markers.

Figure 3.14 Sedimentation rate of the FCV subunit



^{14}C-labelled EMC virus was used as marker. The mixture was sedimented in 15-30% linear sucrose gradient at 30,000rpm for 20 hours. Samples of 20μl from each fraction were assayed for radioactivity.

Figure 3.15 Sedimentation behaviour of the natural and artificial 15S FCV subunit particles

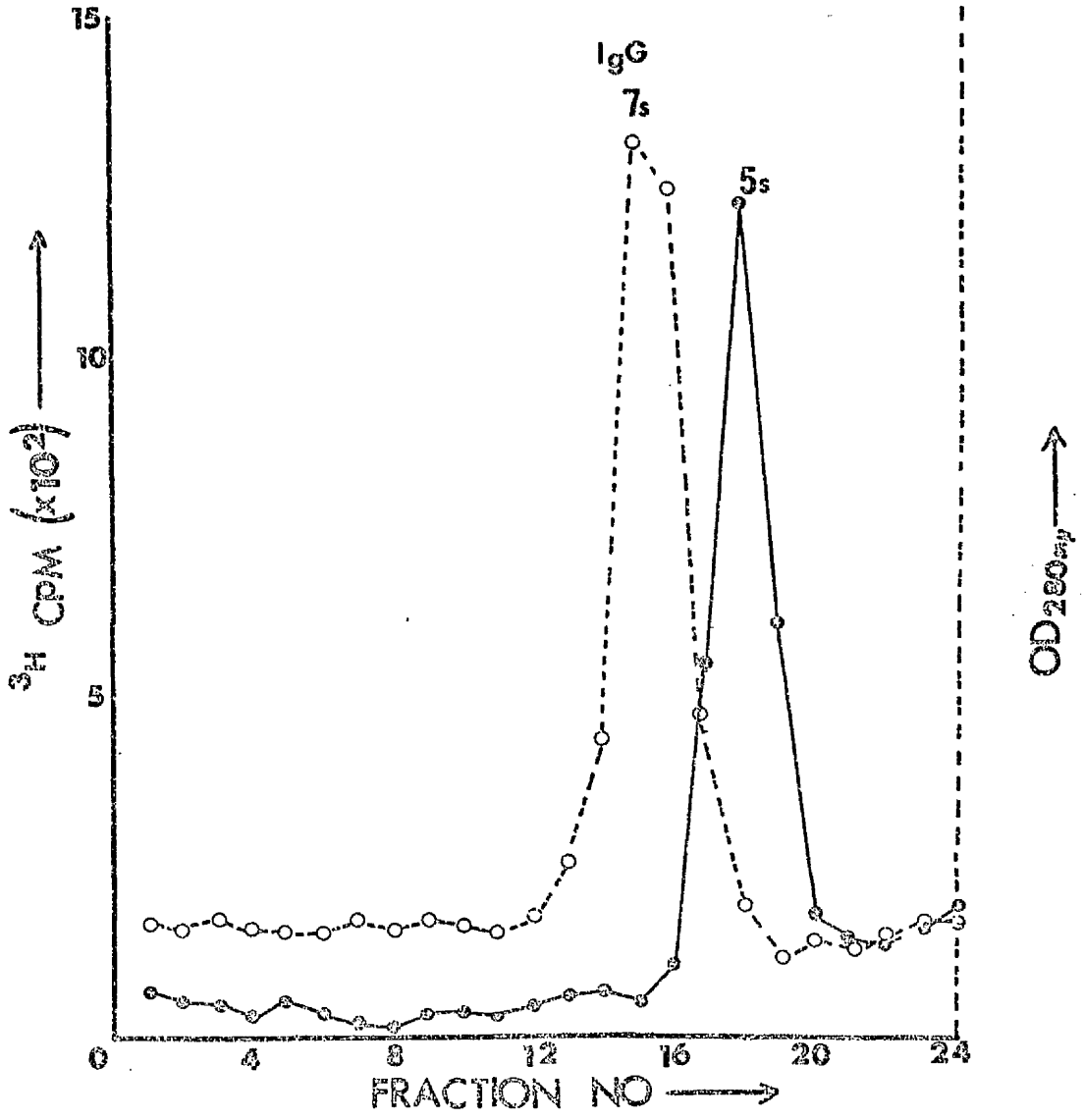


The samples were centrifuged in 15-30% sucrose gradient in SW 50.1 rotor at 30,000rpm for 16 hours. 20 µl samples from each fraction were assayed for radioactivity.

Natural 15S subunit (peak II) —•—•—•—

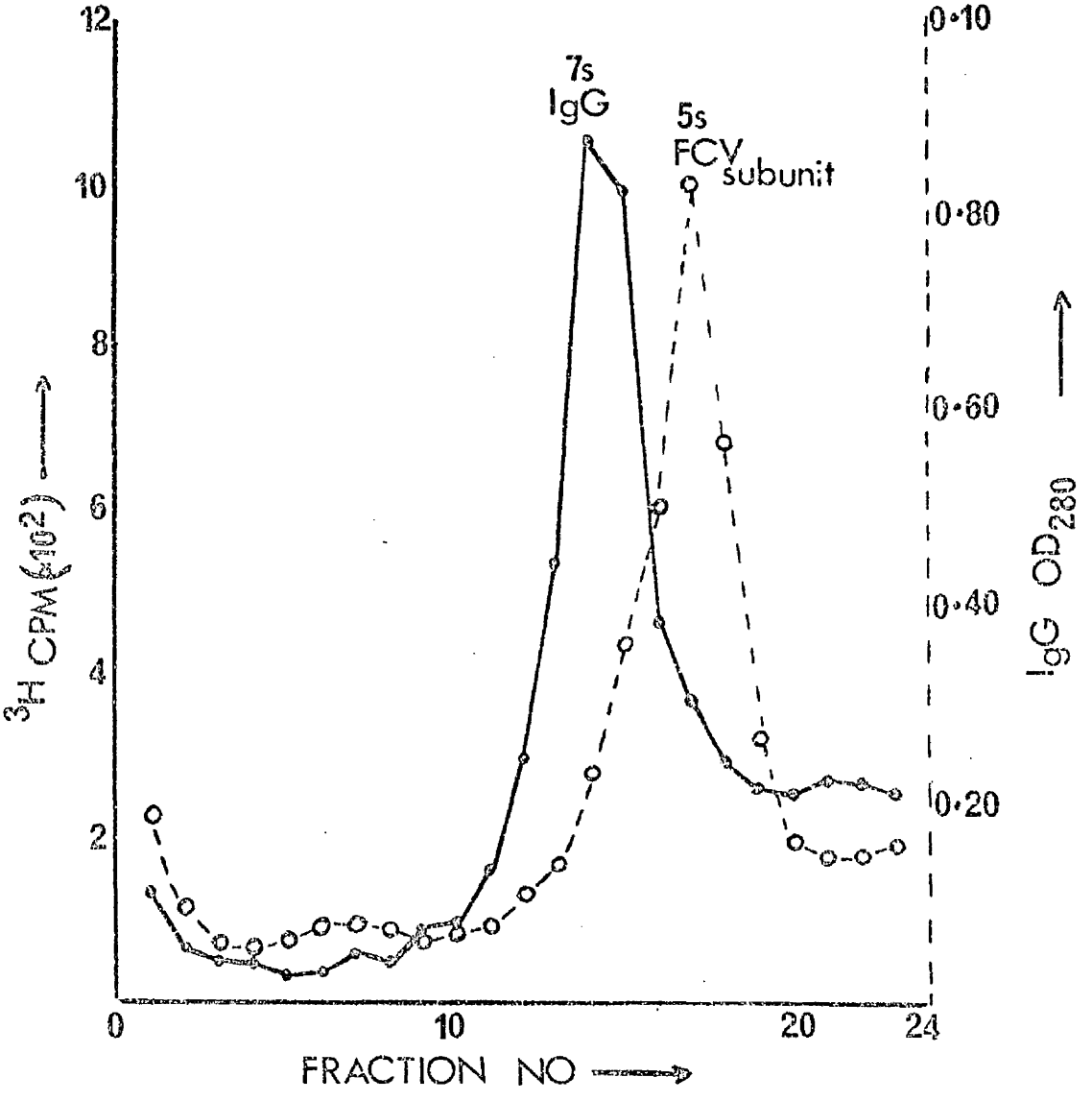
Dissociated 15S subunit --o--o--o--

Figure 3.16 Dissociation of the natural 15S subunit into a smaller 5S particle



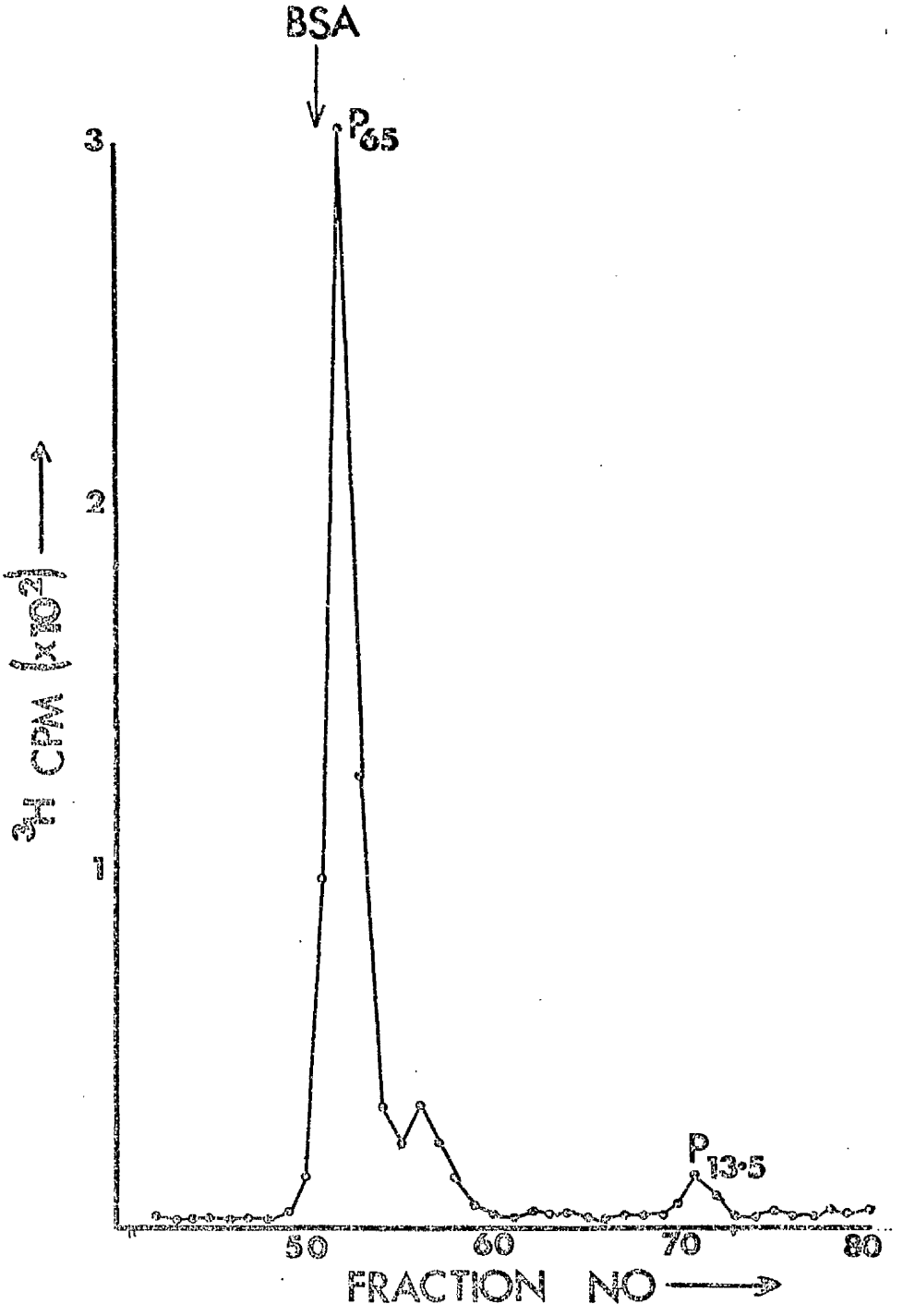
Sedimentation was in 15-30% sucrose gradient in the SW 50.1 rotor at 30,000rpm for 20 hours.

Figure 3.17 Dissociation of the viral 15S subunit into a 5S component



Centrifugation was as in Fig. 3.16.

Figure 3.18 Polypeptide composition of the 5S subunit



Analysis was by 6M guanidine hydrochloride agarose column chromatography.

Effect of varying pH on virus infectivity

This experiment showed that below pH 7·0 dissociation of virus occurred to such an extent that most infectivity was lost, whereas at pH 7·0 and above, the integrity of the virus as well as infectivity remained relatively stable. The result showed that the exposure of FCV to phosphate buffers of varying pH at 42°C for 1 hour resulted in the loss of infectivity of more than 3 logs at pH 6·5 and of about 4 logs at pH 6·0. Within the same period infectivity remained relatively stable in particles exposed to pH 6·8 - 7·2 (Fig. 3.19).

Stability of virus on storage

The result of this study showed that freezing at -70°C has a devastating effect on purified FCV. It was found that after three weeks of storage at -70°C (Fig. 3.20) almost all infectious virus had converted to the 15S virus subunit and infectivity had dropped by about four orders of magnitude (Fig. 3.23). The samples stored at -20°C remained appreciably unaffected for the first 3 weeks after which the virus rapidly dissociated (Fig. 3.21) and infectivity tailed off accordingly so that by six weeks infectivity had dropped by more than three log units (Fig. 3.23).

However, the best method of storage was at 4°C: even after 8 weeks virus dissociation (Fig. 3.22) and loss of infectivity (Fig. 3.23) was minimal at this temperature.

Figure 3.19 Effect of pH on FCV infectivity

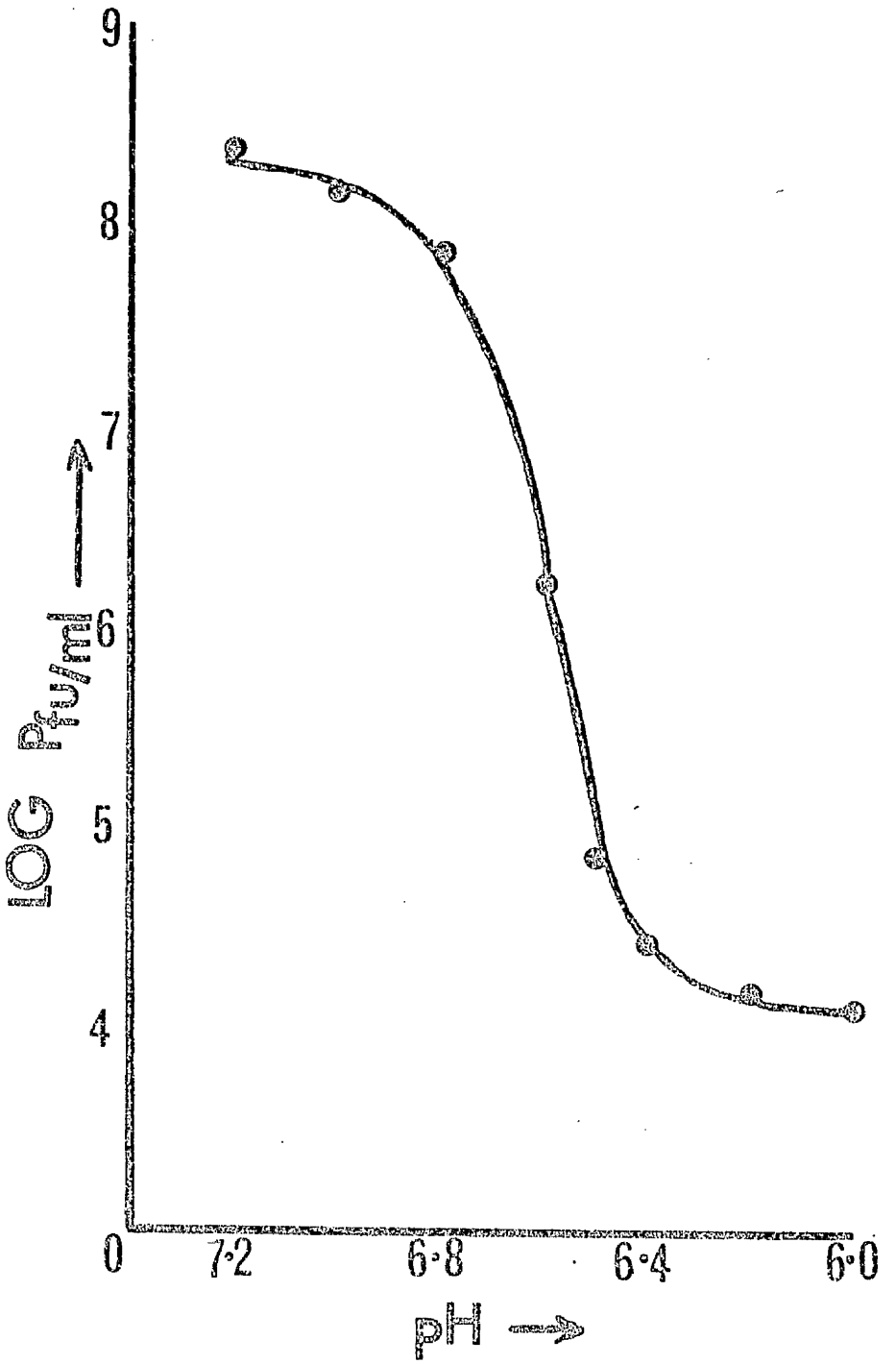
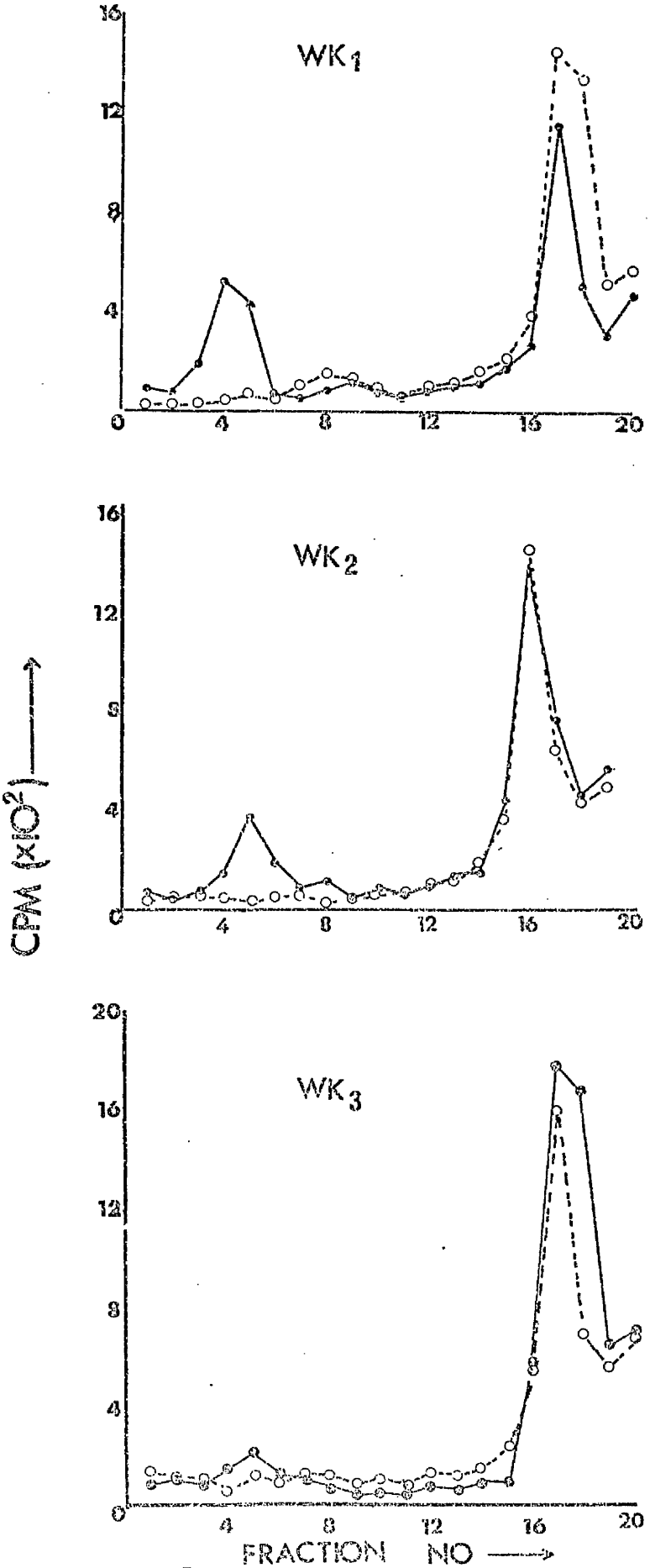


Figure 3.20 Effect of storage at -70°C on purified FCV



Purified ^{3}H-labelled FCV —●—●—●—●—

^{14}C-labelled peak II (marker) -o--o--o--o--

Figure 3.21 Effect of storage at -20°C on purified FCV particles

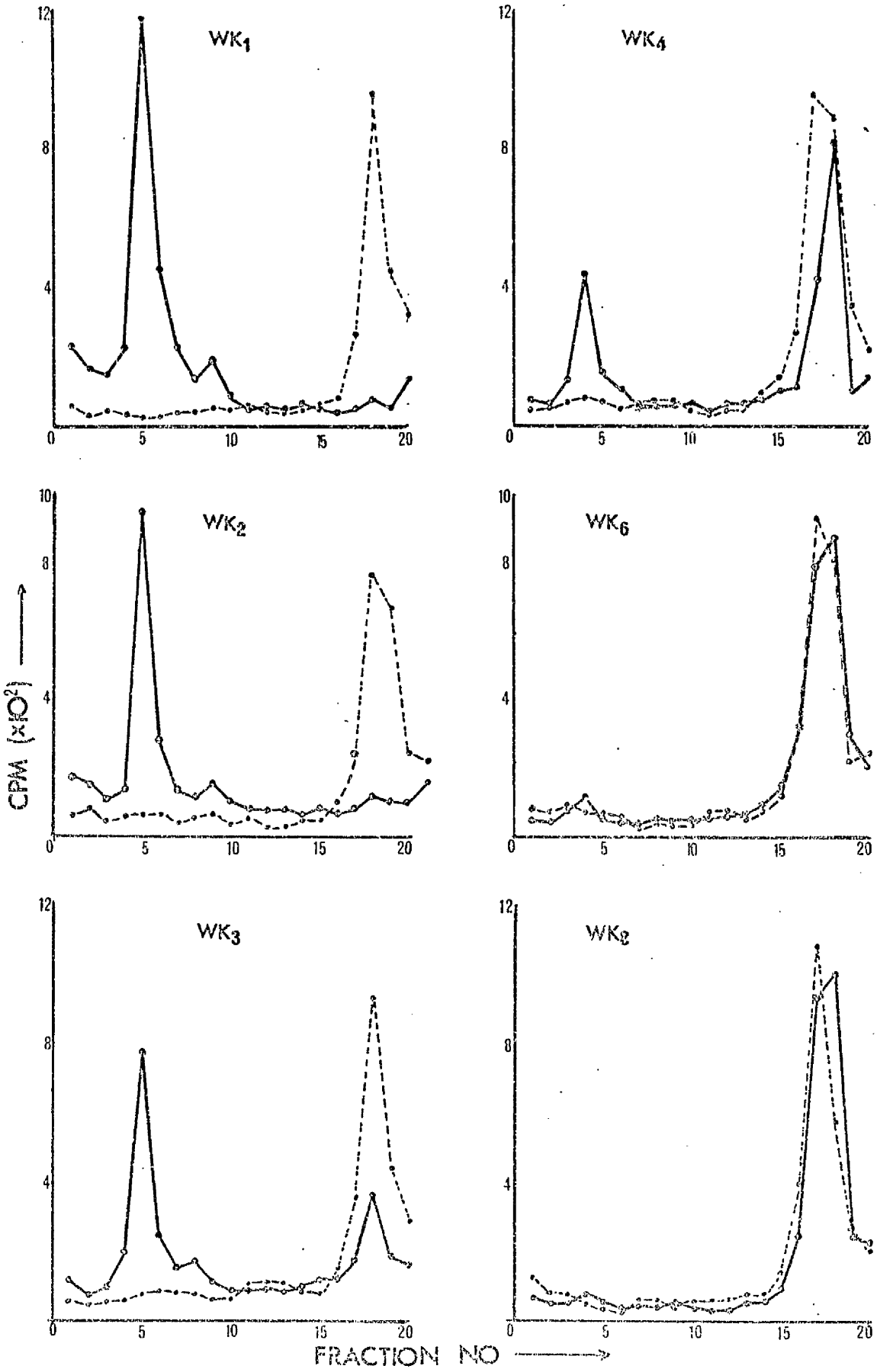


Figure 3.22 Effect of storage at 4°C on purified FCV

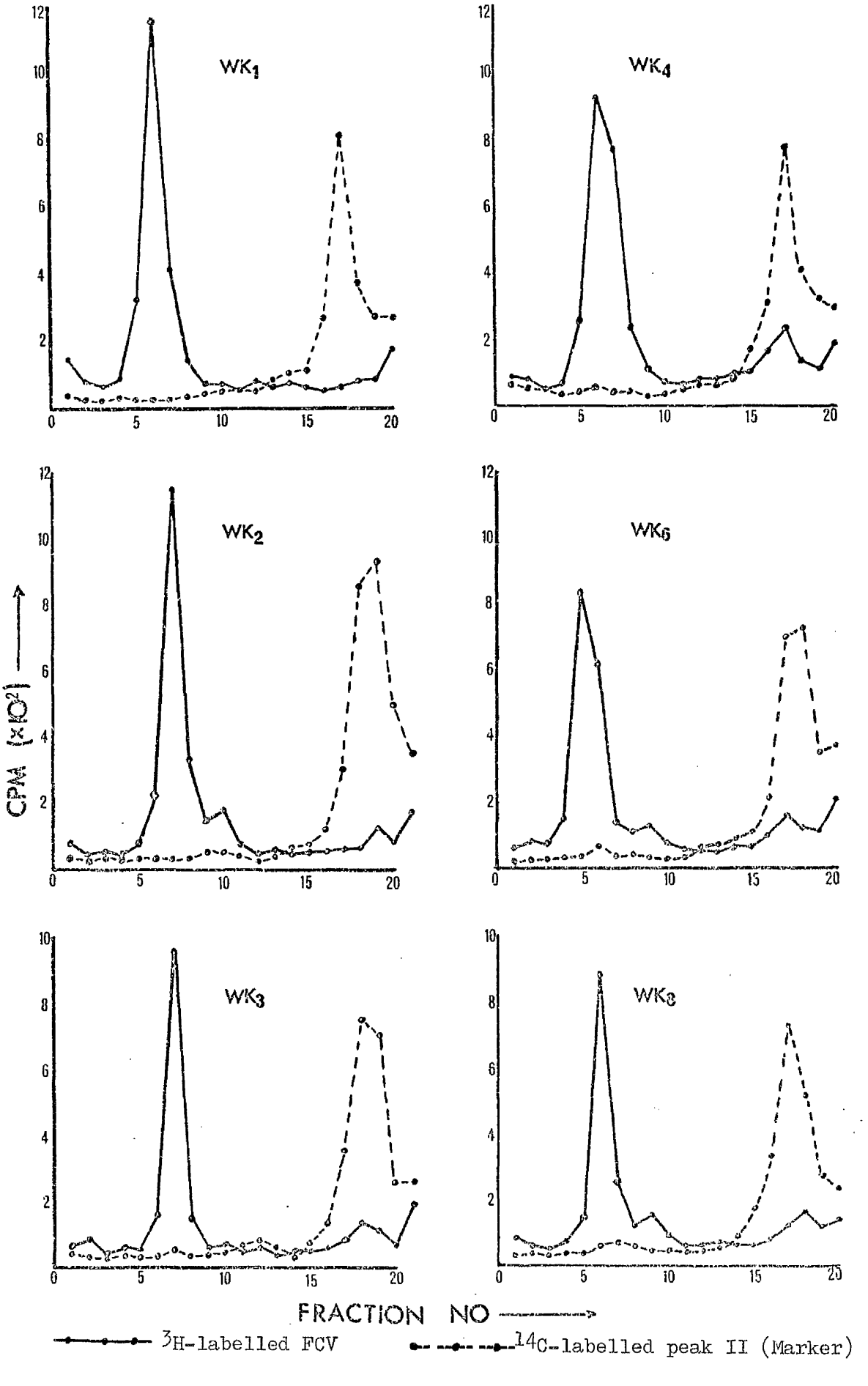
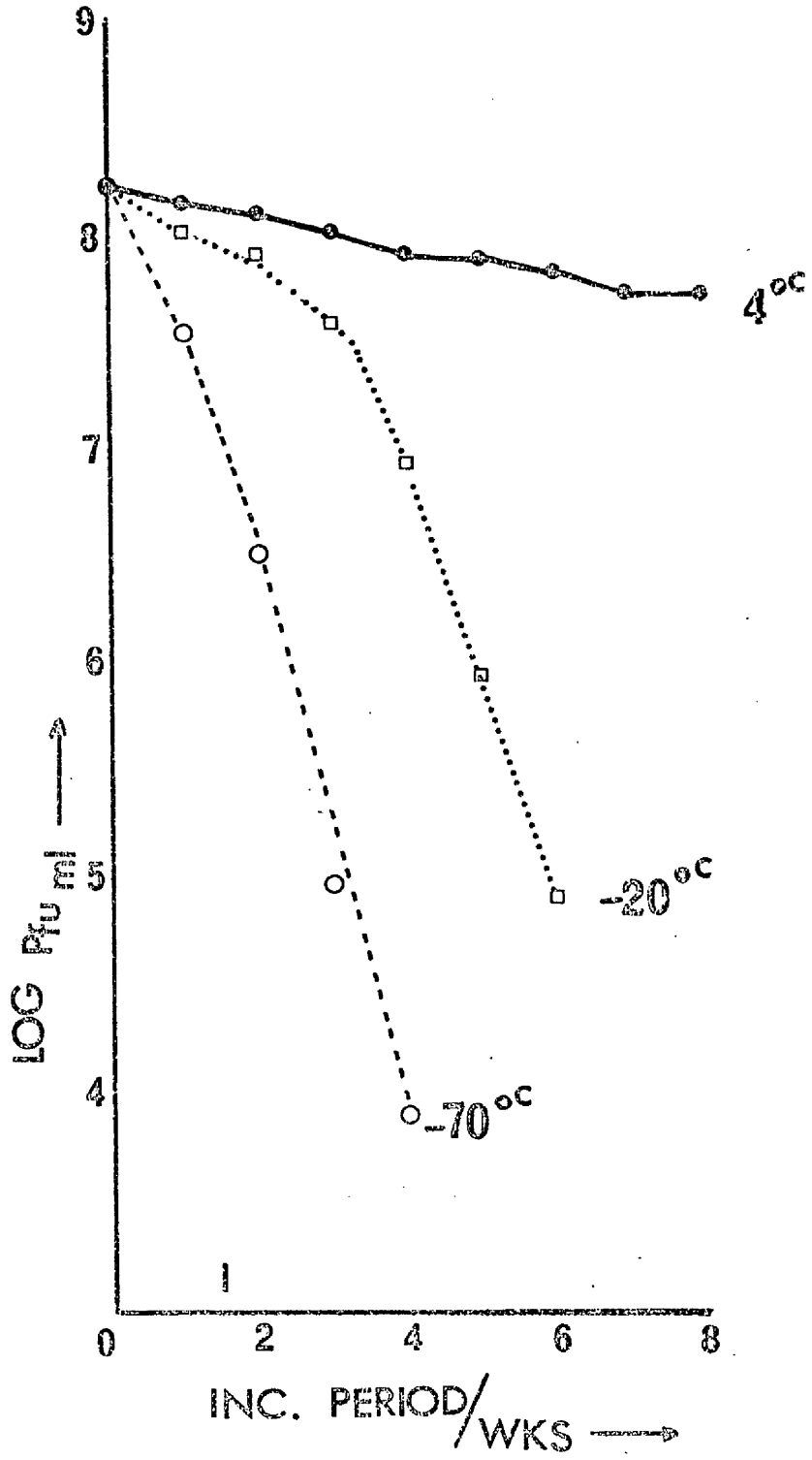


Figure 3.23 Effect of storage on the infectivity of purified FCV



DISCUSSION

In harvests of FCV, following several cycles of growth in FEA cells, two homogeneous populations of viral components with different buoyant densities were observed after centrifugation in $CsCl_2$ gradients. The first component, with a buoyant density of $1{\cdot}39 g.cm^{-3}$, contained RNA, was infectious and had a sedimentation value of 170S. This was the FCV particle. The second component, which appeared to contain ten times as much viral protein as the first, had a density of $1{\cdot}26 g.cm^{-3}$, did not contain RNA and was of low infectivity. It had a sedimentation value of 15S and was identified as a viral subunit.

Similar non-infectious virus-specific particles lacking nucleic acid and infectivity have been encountered during the purification of enteroviruses including poliovirus (Mayer et al, 1957; Scharff & Levintow, 1963), various Coxsackie viruses (Schmidt et al, 1963; Frommihagen, 1965) ECHO viruses (Halperen, 1964; Fabiyi et al, 1964), foot-and-mouth disease virus (Breese, 1968) and several other small RNA-containing plant and animal viruses (Kaper, 1960; Rhim, 1961; Longley & Leberman, 1966).

The non-infectious components of poliovirus (Penman, 1964), foot-and-mouth disease virus (Breese, 1968) and human rhinoviruses (Brown & Hull, 1973) contain empty viral procapsids which sediment at 75S. This is remarkably different from cardiovirus (Scraba & Colter, 1974) and equine rhinoviruses (Brown & Hull, 1973) preparations which lack this component, or FCV which has now been shown in this study to have a component sedimenting at 15S (rather than 75S) and containing viral subunits instead of procapsids.

Although it has been suggested by Jacobson and Baltimore (1968) that the 75S procapsid represent an intermediate structure in the morphogenesis of poliovirus, the lack of it in several other picornavirus systems tends to indicate a different morphogenic pattern from the polioviruses.

With reference to the above, the particle dissociation experiments were done to provide information on the various intermediate subunit structures that might be involved in FCV assembly; and it was found that under controlled conditions the virus dissociated into 15S and 5S subunit particles. Although as detailed above, FCV differs significantly in certain aspects from other picornaviruses, these results provide an area of congruence. For example, ME virus (Rueckert et al, 1969), Mengo and EMC viruses (O'Callaghan, Mak & Colter, 1970) have each been

dissociated into 14S and 5S subunit structures. Phillipson et al (1973) obtained 20S and 5S subunits after similar treatment of Coxsackie B_3 virus while foot-and-mouth disease virus has also been dissociated into 12S subunit components (Talbot & Brown, 1972). Poliovirus, by virtue of its acid stable property has not been dissociated beyond the stage of the 75S procapsid although there are reports that 14S and 5S poliovirus-related fragments can be demonstrated in infected cell extracts (Watanabe et al, 1967; Phillips et al, 1968).

Also in this study, it was found that between pH 6·0 and 6·5, in the presence of 0·05M phosphate buffer and 0·7M $CsCl_2$, the integrity of FCV suffered so much that more than 3 $\log_{10}$ units of virus infectivity was lost after incubation at 42°C for 1 hour. This result was in agreement with that of earlier workers (Zee and Hackett, 1967; Oglesby et al, 1971) who found that vesicular exanthema virus was not stabilized at 50°C by various salts and in fact, inactivation was observed when the virus was exposed to 2M $CsCl_2$. The presence of 0·7M CsCl in the FCV preparation therefore, may have had some influence in the dissociation of the virus. A similar virus preparation kept at pH 6·8, 7·0 and 7·2 remained relatively unaffected. It is presumed that the loss in infectivity was paralleled by breakdown of peak I into non-infectious 15S subunits.

These intermediate subunit particles are not only key fragments in deducing the organisation of the polypeptides in the capsid, they are also useful reference particles for evaluating the possible identity of biosynthetic intermediate subunits in infected extracts. Furthermore, they provide experimental evidence in support of the assumption that picornavirus, including FCV, particles are held together by two different intersubunit bonds: one type binding the 5S structures to form the 15S subunit and the other binding the 15S components together to form the viral shell. This hypothesis provides an integrating concept which suggests that assembly of the virion probably also occurs in two stages corresponding to the stepwise utilization of the two types of inter-subunit bonds. The architecture of FCV in relation to other picornaviruses is dealt with in more detail in Chapter 4.

During the course of this study, a purified ^{3}H-leucine labelled FCV was maintained at -70°C pending further analysis, but a few weeks later the virus was found to have lost infectivity and almost all of it had converted into the 15S subunit component. This discovery led to the study of FCV stability under storage at -70°C, -20°C and 4°C. The result showed that over a period of 8 weeks purified virus is best

preserved at 4°C. This result was in contrast to normal storage of virus stocks at -70°C. The presence of serum proteins in the storage medium (Eagle's medium containing 10% foetal bovine serum) acting as a cross-linking substance presumably helped to maintain the integrity of the virus under storage. By contrast, the purified FCV samples were stored in TS buffer which contained neither proteins nor other cross-linking reagents capable of preventing virus particle dissociation such as was noticed in the samples stored at -70°C and -20°C.

The idea of using cross-linking reagents to stabilize labile structure has been known for some time, for example, cross-linked Escherichia coli 30S ribosomal subunit retained their structural integrity with only a slight loss of biological activity (Bickle et al, 1972). Cross-linking protected virions of cowpea chlorotic mottle and brome mosaic viruses from disassembly upon dialysis against 1M NaCl at pH 7·5 (Bancroft & Smith, 1975). In a recent study of the behaviour of cross-linked and non-cross-linked purified San Miguel sealion calicivirions stored at 4°C over a period of 25 weeks, Schaffer and Soergel (1976) found that the integrity of the cross-linked sample remained stable whereas about half the non-cross-linked sample had already dissociated. In the interest of long term storage of purified FCV however, it would perhaps be best to consider using a cross-linking reagent such as dimethyl suberimidate although for short term purposes, storage at 4°C would be adequate as shown in this study.

CHAPTER 4

THE ASSEMBLY OF FELINE CALICIVIRUS PARTICLES

INTRODUCTION

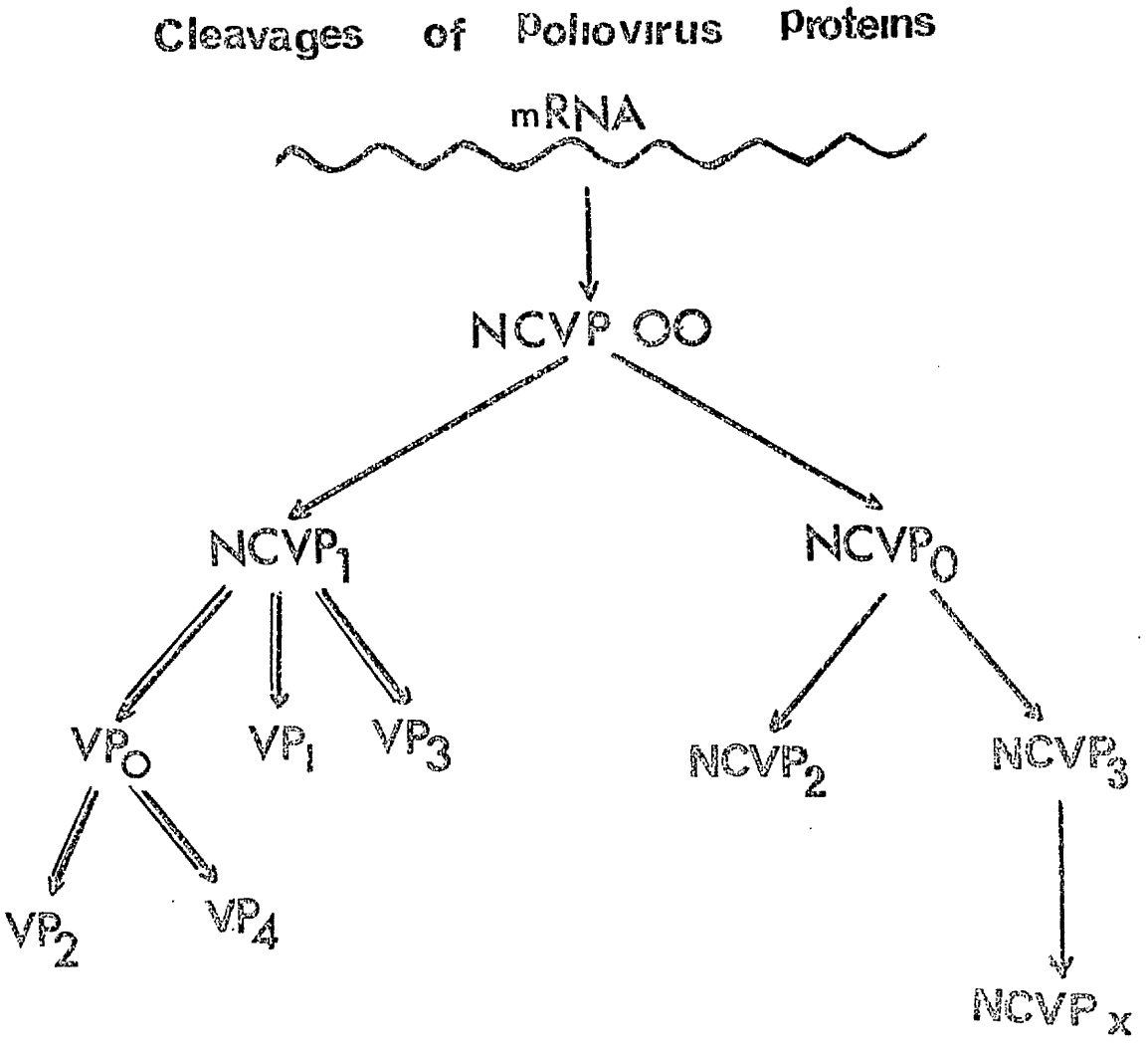
In the previous chapter, two viral subunit structures obtained by dissociation of FCV particles were described and were considered to be possible intermediates in the maturation pathway of virions of FCV.

Experimental evidence in support of this assumption has been provided by the results of studies on other small RNA viruses. For example, two capsid-related subunits sedimenting at about 6S and 14S have been identified in extracts of EMC virus-infected Krebs ascites tumour cells (Kerr *et al*, 1965). Similarly two poliovirus-related fragments sedimenting at 4S and 74S have been described in poliovirus infected cell extracts (Phillips *et al*, 1968), and kinetic labelling experiments *in vivo* (Penman, 1964; Jacobson & Baltimore, 1968a) suggest that these are sequential precursors of the mature virion because, under certain conditions *in vivo*, radioactive label from 74S empty procapsids can be chased into virions (Jacobson & Baltimore, 1968a). At the commencement of this study nothing was known about the assembly pathway of caliciviruses. The aim of the work described in this chapter was to examine the *in vivo* assembly of FCV.

Picornaviruses are thought to have monocistronic messenger RNA: that is, all the virus-specified proteins found in picornavirus virions and in infected cells arise by cleavage of a single polyprotein that represents the total information of the viral genome. Recent studies on the biosynthesis of poliovirus suggest that the viral RNA genome is translated into a single large polypeptide (non-capsid viral protein - NCVP-00) with a molecular weight of about 200,000-260,000 (Jacobson & Baltimore, 1968b; Kiehn & Holland, 1970) which is then cleaved to form a smaller chain (Maizel & Summers, 1968; Holland & Kiehn, 1968). This chain ($NCVP_1$), of molecular weight about 100,000, has been identified in extracts of cells infected with poliovirus (Jacobson *et al*, 1970; Cooper *et al*, 1970) or EMC virus (Butterworth *et al*, 1971).

There is evidence (Jacobson & Baltimore, 1968b; Summers & Maizel, 1968) to support the hypothesis that the $NCVP_1$ is the progenitor of procapsid proteins (Fig. 4.1). First, by the use of amino acid analogues which arrested the cleavage of the polyprotein, the procapsid proteins (VPo, VP1 & VP3) were not formed. Secondly, in pulse-chase experiments the loss of label from newly synthesised

Figure 4.1



This diagram shows the post-translational cleavage of poliovirus proteins. It is suggested that the viral RNA directs the synthesis of a single protein (NCVPoo) which is cleaved in steps to generate virus capsid proteins and several other non-capsid polypeptides.

Double arrows indicate cleavages for which evidence is strong and single arrows indicate events for which there is weaker or little experimental evidence (Jacobson and Baltimore, 1968b).

$NCVP_1$ was paralleled by a quantitative increase of radioactivity in the capsid proteins. This cleavage mechanism, therefore, appears to play an important role in the formation of most, if not all, virus-specific proteins of poliovirus and indeed all picornaviruses.

While picornaviruses exhibit translation of a monocistronic messenger RNA as a pre-requisite for replication, FCV appears to have a completely different strategy. Black and Brown (1975/76) found that during the replication of caliciviruses the only virus-specified polypeptide synthesized was the structural capsid protein (p65) and synthesis of this protein did not appear to involve either precursors or proteolytic cleavage. In their later work these authors were able to demonstrate the synthesis of two additional polypeptides of molecular weight 80,000 and 100,000 in cells infected with VEV or FCV (Black & Brown, 1977). Again, pulse-chase experiments failed to demonstrate any precursor-product relationship between the three polypeptides. The use of protease inhibitors further showed that the synthesis of p100, p80 and p65 was independent of each other.

Using the slab gel electrophoresis and autoradiography techniques experiments were carried out in this study to examine the sequence of polypeptide synthesis in FCV-infected cells with particular attention to the presence of any large molecular weight capsid protein precursors. These were designed as follows:

1) Total proteins (pre-existing and newly synthesised) present in the cells at 30 minute intervals throughout the growth cycle were analysed by polyacrylamide gel electrophoresis and staining of the gels.

2) Newly synthesised proteins were investigated by analysis of the polypeptides which became labelled with ^{3}H-leucine during each 30 minute interval.

3) Newly synthesised virus-specific proteins were selected by immuno-precipitation and were analysed by polyacrylamide gel electrophoresis and autoradiography.

The occurrence of possible larger virus-specific subunits was also examined by radiolabelling cells infected with FCV for a short period (the pulse) and subsequently incubating them in radioisotope-free medium (the chase). At intervals during the chase the appearance of subviral components was analysed by rate zonal ultracentrifugation.

MATERIALS AND METHODS

Protein synthesis in FCV-infected cells

Twelve 5cm polystyrene plates, each containing 4 sterile round coverslips of 10mm diameter, were seeded with 10^6 FEA cells and incubated overnight at 37°C. Six of the plates were kept as controls while the remainder were each infected with FCV at a multiplicity of about 100 for 30 minutes. The inoculum was then removed and the monolayers were washed twice with PBS and re-incubated in EFC_{10} at 37°C.

After 30 minutes, and at 30 minute intervals thereafter until 240 minutes post-infection, two infected and two uninfected coverslips were withdrawn into separate plates, incubated in leucine-free MEM for 5 minutes and labelled for 15 minutes with 100µl of leucine-free medium containing 10µCi of ^{3}H-leucine. The coverslips were then washed with, consecutively, 10% TCA, 5% TCA, distilled water and ethanol and were then left to dry. Each coverslip was placed in a scintillation vial with 6ml of toluene-based scintillation fluid and the radioactivity was determined. The amount of protein synthesised during each 15 minute period of labelling was taken as the average of the radioactive counts of the two coverslips used at each time point.

Simultaneously, plates containing FEA monolayer cells were infected as above and at the same intervals after infection one plate was withdrawn and the cells were lysed with 0·5ml of 0·5% NP 40 and 0·5% DOC. A sample of 50µl of each lysate was assayed for virus infectivity by the plaque assay method. The remaining lysate was divided into two parts: one was analysed for total (i.e. cellular and viral) proteins and the other for virus-specific proteins by immunoprecipitation using the method described in Chapter 2.

Pulse-chase experiments

After 30 minutes infection and a further incubation at 37°C for 150 minutes, the infected cells in 5cm plates were starved in leucine-free medium for 15 minutes and labelled with 200µCi/ml of ^{3}H-leucine for periods ranging from 5 seconds to 5 minutes. The cells were then incubated for 5, 15, 30 or 60 minutes in 5ml MEM containing 100-fold excess of unlabelled L- leucine (Gibco Biocult Ltd.).

The cells were lysed as before and 50µl of 15S and 5S FCV subunits labelled with ^{14}C-leucine was mixed with 200µl of cell

lysate and analysed by rate zonal ultracentrifuagtion in 15-30% sucrose gradients in the SW 50.1 rotor at 30,000rpm for 20 hours. In some experiments a pad of 0·5ml of $CsCl_2$ of density 1·5g.cm^{-3} was included at the bottom of the tube to collect any fast-sedimenting components (e.g. 170S whole virus). The gradients were fractioned and the TCA-precipitable radioactivity in each fraction determined.

Similarly, in Experiment 4, 600μCi of L - (^{35}S) methionine contained in 1ml of methionine-free Eagle's medium was used to pulse-label FEA cells infected as above. The infected cells were stored in methionine-free medium for 15 minutes prior to labelling for 5 seconds. The cells were then incubated for 30 seconds in 100-fold excess of unlabelled L-methionine. The cell lysate obtained was treated as described above.

Precursor-product relationship

FCV-infected cell extracts were prepared at 30 minute intervals over a period of 5 hours as described in Chapter 2. The extracts were each divided into two equal parts; one part was analysed by electrophoresis on low percentage (8%) polyacrylamide gels followed by autoradiography in order to detect any large molecular weight capsid protein precursors. The other was analysed on high percentage (12%) polyacrylamide gels to detect low molecular weight virus-induced polypeptides, especially the minor polypeptide of molecular weight 15,000 first described by Burroughs and Brown (1974), the presence of which in calicivirus particles is still a subject of controversy (Schaffer & Soergel, 1976).

RESULTS

Protein synthesis in FCV-infected cells

In several picornavirus-cell systems, host cell protein synthesis is severely inhibited in infected cells shortly after infection to such an extent that only virus-specific proteins are found later in the viral growth cycle. These can be analysed directly by polyacrylamide gel electrophoresis after a radioactive amino acid is added to the infected cells at the appropriate time.

Fig. 4.2 shows the rate of protein synthesis in FCV-infected and uninfected cells. The rate of cellular protein synthesis in the uninfected cells kept as controls remained constant over a period of 4 hours during which samples were taken for analysis.

By contrast, there was a dramatic fall in cellular protein synthesis within 30 minutes and up to 90 minutes after infection with FCV. This was followed by a sudden upsurge of protein synthesis, presumably virus-specified, which continued till the end of infection. Evidence suggesting that the newly synthesized protein was virus-specific was obtained from two sources. First, viral infectivity of cell lysates was monitored by plaque assay and it showed an initial decrease, followed by a gradual increase concomitant with the build-up of viral proteins in infected cells (Fig. 4.2). Figure 4.3 also shows the rate of protein synthesis in infected cells expressed as a proportion of the rate in uninfected cultures. Secondly, polypeptide analysis by gel electrophoresis and autoradiography revealed that the virus-induced polypeptide (p65) was not detectable in infected cells until about $2\frac{1}{2}$-$3\frac{1}{2}$ hours after infection and increased in amount thereafter (Figs. 4.4 and 4.5).

The significance of this result was that it showed the period during the life cycle of the virus when protein synthesis in the cell was minimized in favour of virus protein synthesis which was greatly increased. Analysis of virus-specific protein in subsequent studies was performed during the period when most of the protein being produced was virus-specific.

Subunit components involved in FCV assembly

Among the major viral proteins found in the cytoplasm of picornavirus-infected cells, for example, poliovirus, the large polypeptide $NCVP_1$ is unique in that it is highly labelled only if cells are harvested within about 15 minutes after addition of the radioactive amino acids. If the cells are harvested after 30 minutes

Figure 4.2 The rate of protein synthesis in FCV-infected cells

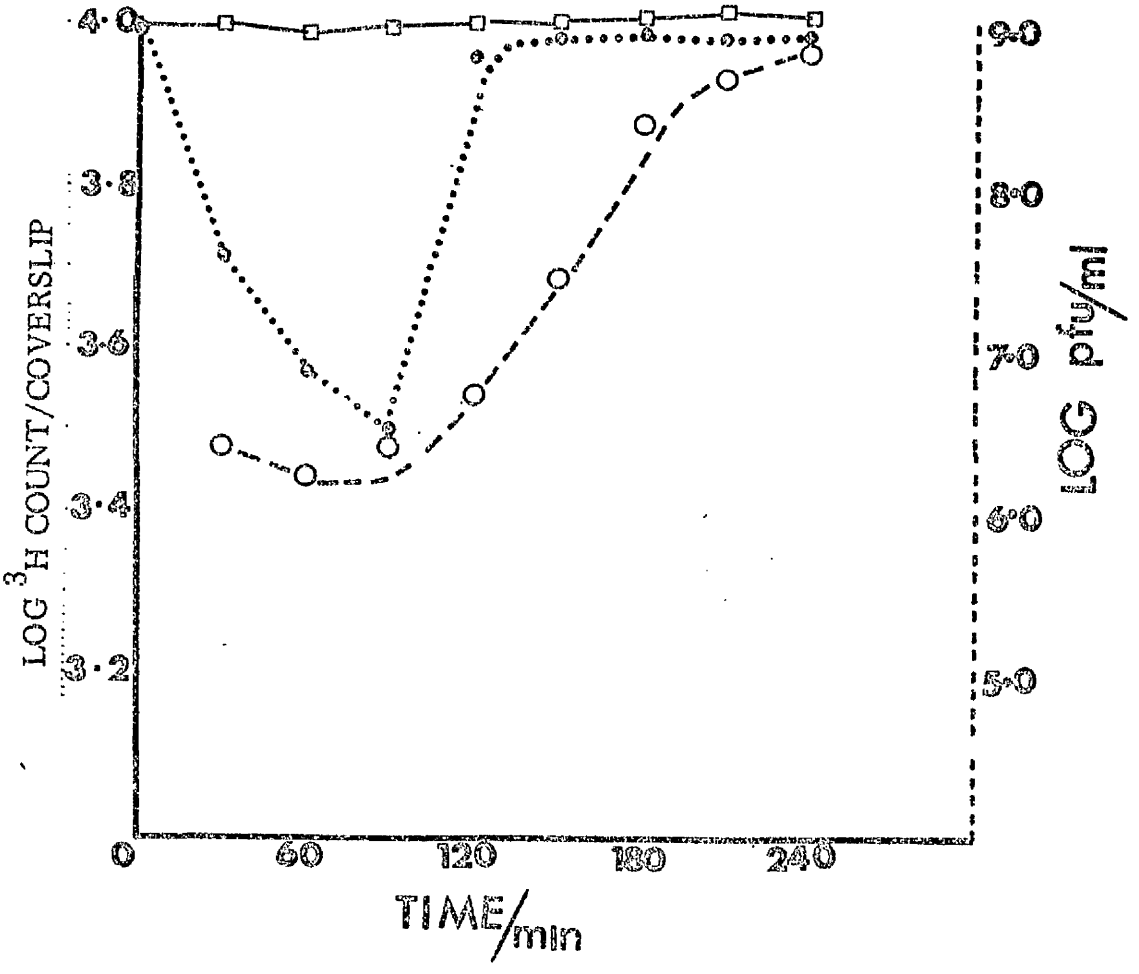
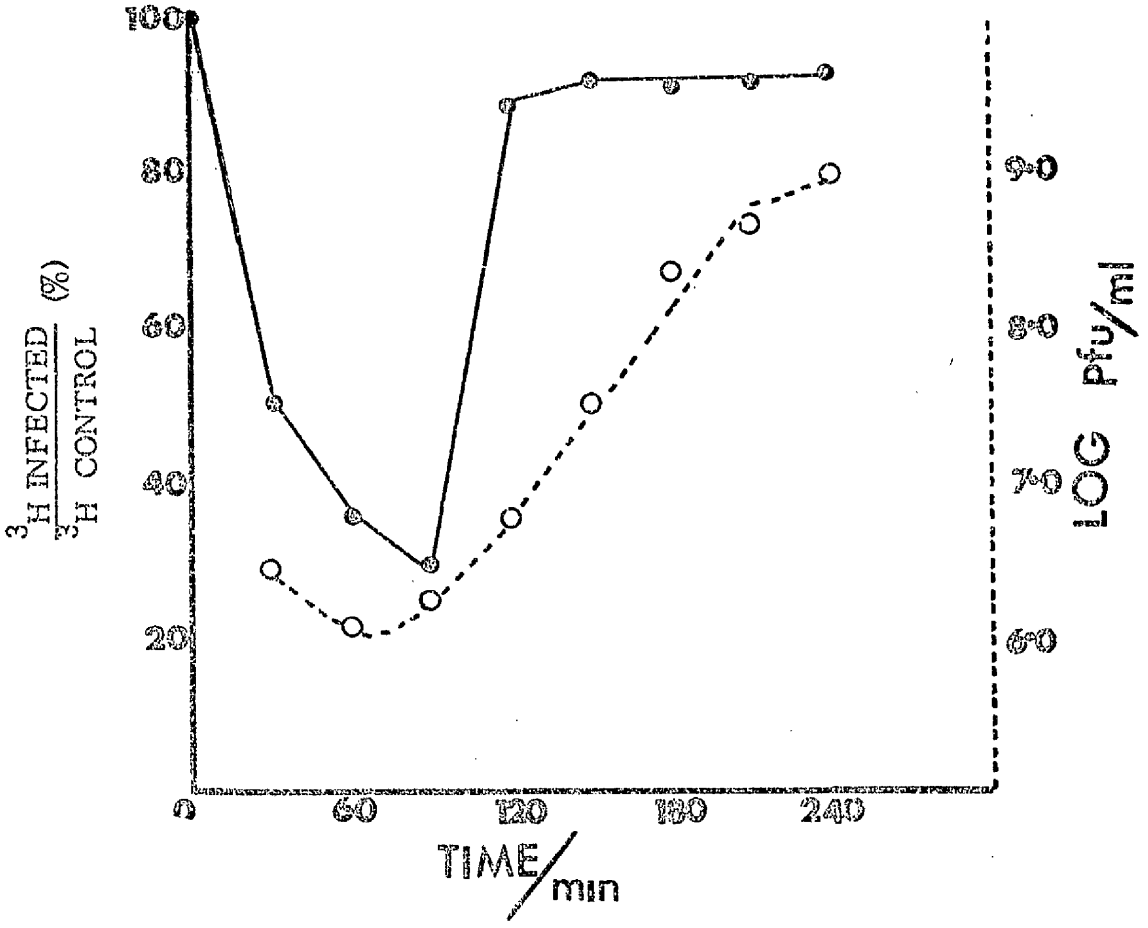


Figure 4.3 Rate of protein synthesis in FCV-infected cells expressed as a percentage of uninfected controls



—●—● Ratio of protein in FCV-infected cells expressed as a percentage of the uninfected cellular protein synthesis

-O-O- Virus infectivity

Figure 4.4 legend

FEA cells were infected with FCV. At 30 minute intervals throughout the virus growth cycle the cells were labelled with ^{3}H-leucine for a 15 minute period. Cell lysates were prepared and analysed by polyacrylamide gel electrophoresis on 10% gels. This figure shows the resulting gel stained with Coomassie blue.

Tracks A-K represent the total protein content (pre-existing and newly synthesised) of the cells during each 30 minute period.

A	Uninfected cell control
B	0 - 30 minutes
C	30 - 60 "
D	60 - 90 "
E	90 - 120 "
F	120 - 150 "
G	150 - 180 "
H	180 - 210 "
I	210 - 240 "
J	240 - 270 "
K	270 - 300 "

Numbers 1-5 represent the relative migration of standard proteins used as markers

1	β- galactosidase	(130,000)
2	phosphorylase a	(94,000)
3	catalase	(60,000)
4	alcoholdehydrogenase	(41,000)
5	carbonic anhydrase	(29,000)

The small arrow on the right indicates the position of the newly synthesised FCV capsid protein which in this analysis had an apparent molecular weight of about 68,000 daltons but was termed p65 for convenience. The p65 appeared first between 180 and 210 minutes post-infection.

Figure 4.4 Analysis of FCV-infected cell extract for protein by polyacrylamide gel electrophoresis

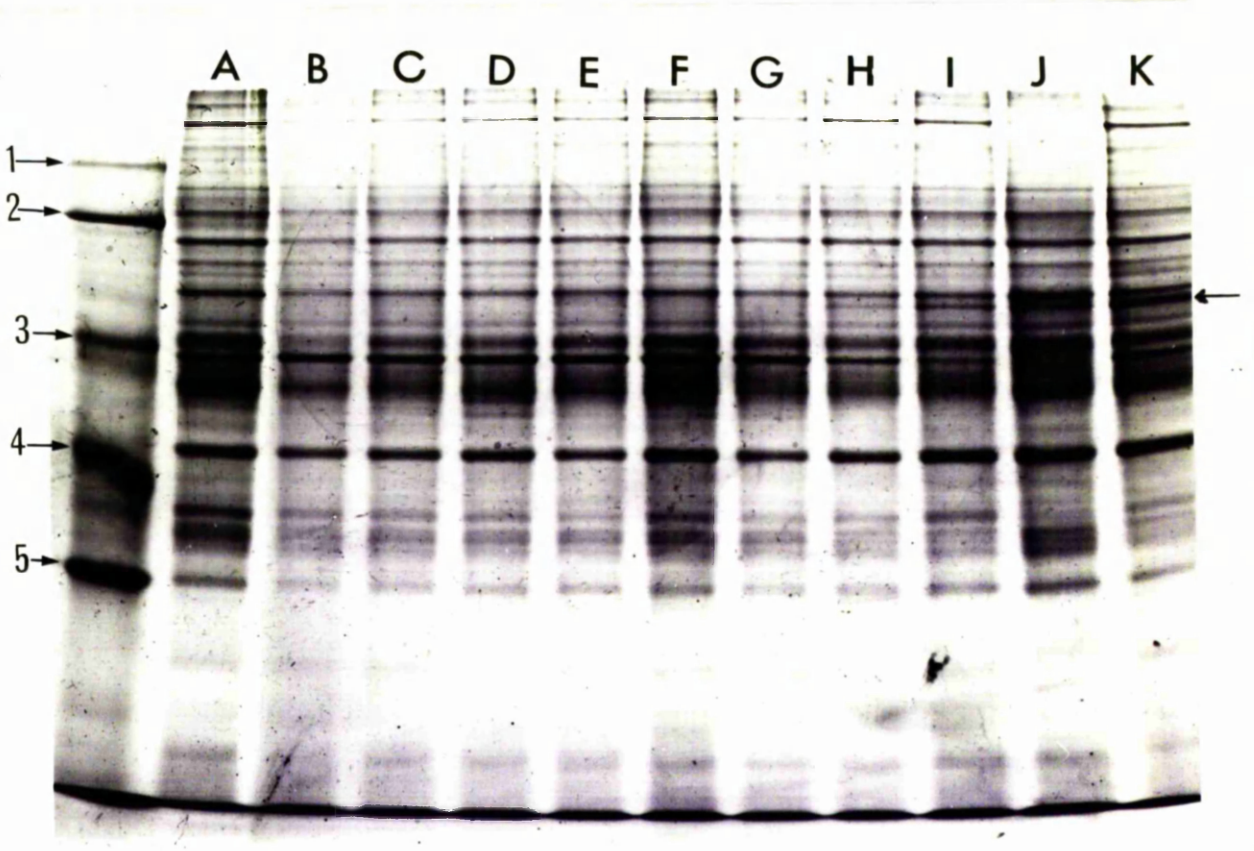


Figure 4.5 legend

This shows an autoradiograph of the same gel as in Fig. 4.4.

Tracks A-K represent the protein synthesised during each period of radiolabelling.

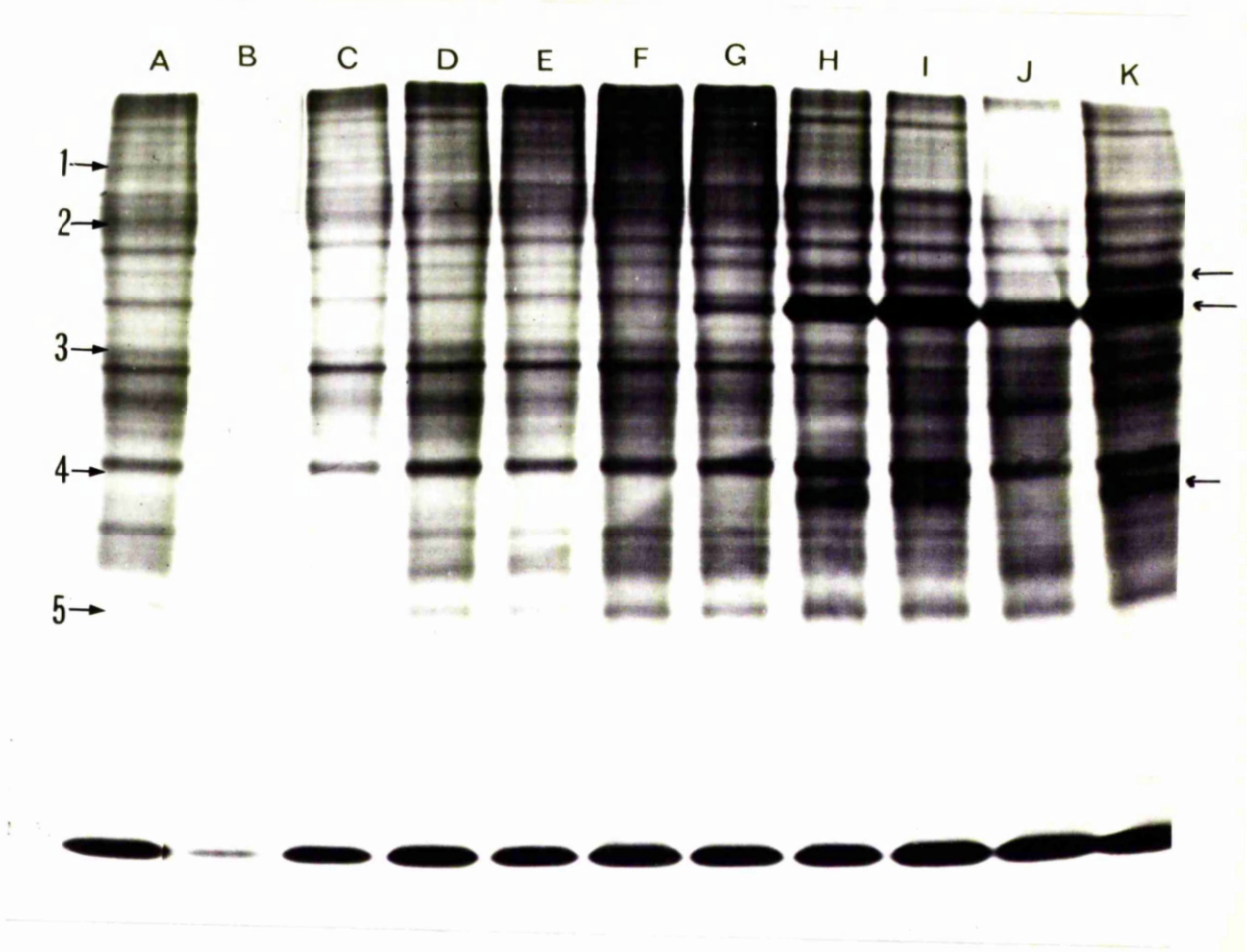
A	Uninfected cell control	
B	0 - 30	minutes
C	30 - 60	"
D	60 - 90	"
E	90 - 120	"
F	120 - 150	"
G	150 - 180	"
H	180 - 210	"
I	210 - 240	"
J	240 - 270	"
K	270 - 300	"

Numbers 1-5 represent the position of the marker proteins as in Figure 4.4.

The middle arrow on the right shows the position of FCV capsid protein (p65) which first appeared in infected cells at 150 minutes post-infection.

Top and bottom arrows indicate the presence of infected cells of two additional protein bands of molecular weight 80,000 and 40,000. Both protein bands appeared at 210 minutes post-infection and persisted until the end of the growth cycle at 5 hours.

Figure 4.5 Analysis of FCV-infected cell extract for newly synthesised protein by polyacrylamide gel electrophoresis



or after a pulse followed by a long chase, very little labelled $NCVP_1$ is seen in infected cell extracts. The disappearance of $NCVP_1$, however, is mirrored by an accumulation of counts in the region of the gel where the procapsid proteins (VP_0, VP_1 and VP_3) are found, suggesting that the polyprotein has been converted to specific procapsid proteins.

Using this pulse-chase method, attempts were made to find a polyprotein analogous to the poliovirus $NCVP_1$ in FCV-infected cell extracts. Four experiments were carried out.

Experiment 1 FCV-infected cells were pulse labelled for 5 minutes and then chased for 0, 5, 15, 30 and 60 minutes and the cell lysates were analysed on linear sucrose gradients by rate zonal ultracentrifugation using ^{14}C-leucine 5S and 15S FCV subunits as markers.

The result (Fig. 4.6) showed that both 15S and 5S subunit structures were present in FCV-infected cell extracts, thus confirming the belief that these subunit components might be intermediates in the assembly pathway of the virus.

Other interesting results observed included:

1) The preponderance and the non-disappearance of the 15S subunit in infected cells.

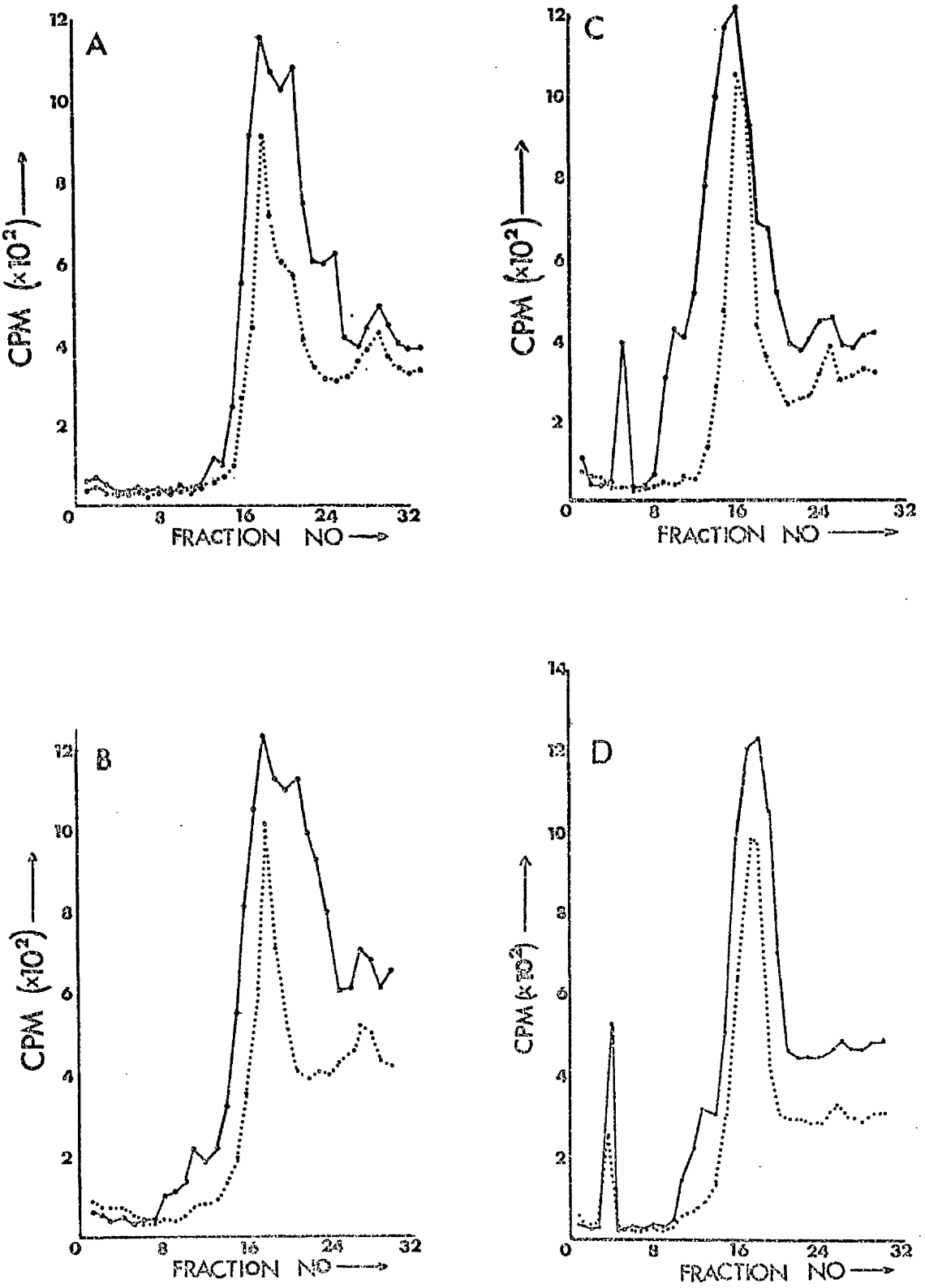
2) The probable existence of two or three other intermediates between the 5S and 15S subunit components.

3) The appearance of whole FCV particles in infected cells about 30 minutes after the initiation of viral protein synthesis (Fig. 4.6c).

Experiment 2 In this experiment, the pulse time of infected cells was shortened to 1 minute and no chase was carried out. Analysis of the harvested cell extracts (Fig. 4.7) showed that even within one minute of the initiation of viral protein synthesis, both the 15S and the 5S subunit components were already present in infected cells. Also the radioactive peaks between the 5S and the 15S components found in the previous experiment became more prominent. In addition, a new peak suspected to contain viral polypeptide (p65) was seen at the top of the gradient. Evidence confirming this view was obtained by centrifuging a purified p65 sample obtained by guanidine hydrochloride agarose chromatography with 5S and 15S virus subunit markers under similar conditions and it was found that the p65 did not migrate but remained at the top of the gradient (Fig. 4.8).

This result showed that FCV polypeptide could be demonstrated in infected cell extracts but it still remained unclear whether it

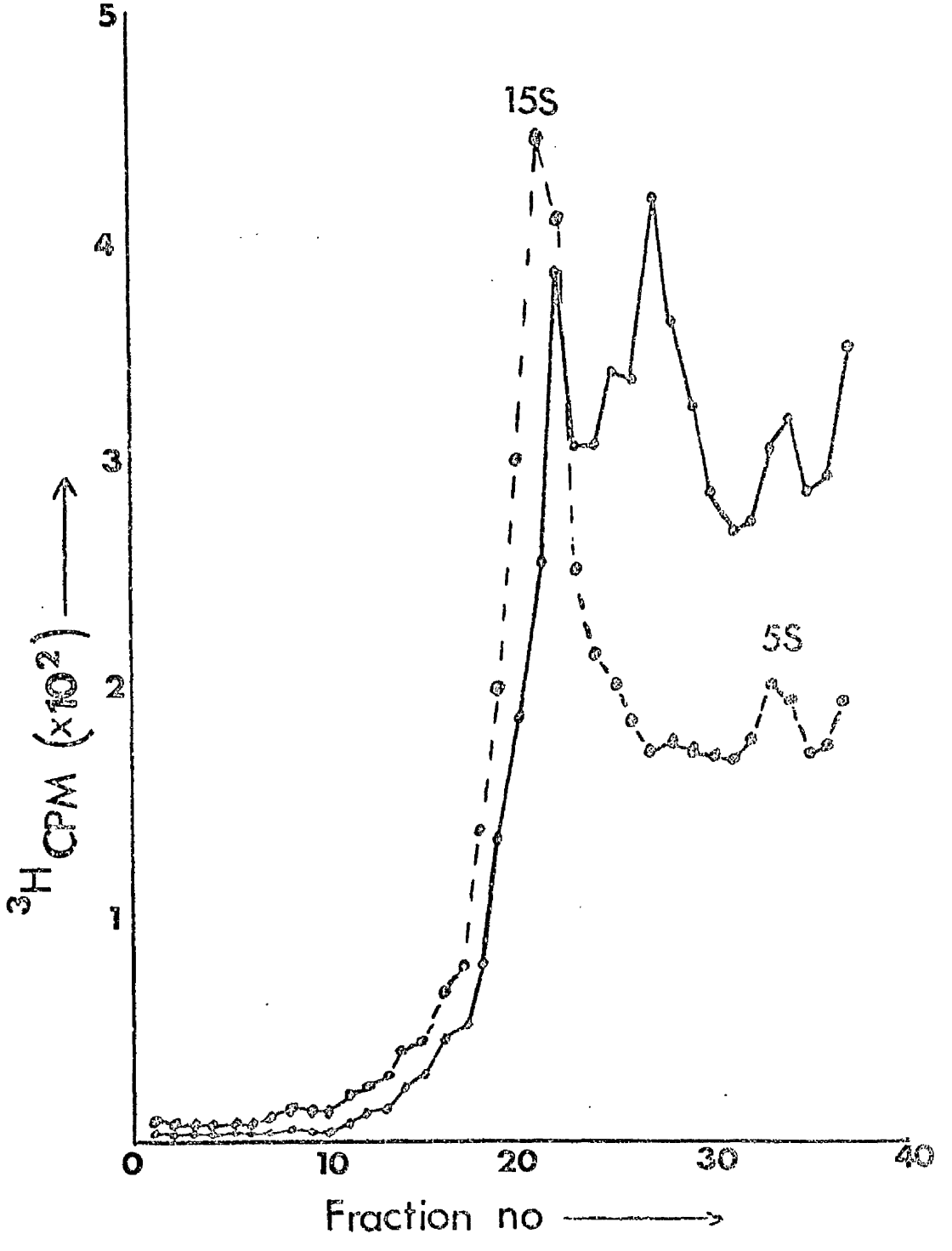
Figure 4.6 Viral subunits in FCV-infected cells -1



Infected cells were pulsed for 5 minutes (A) and chased for 15 minutes (B), 30 minutes (C) and 60 minutes (D).

Dotted lines represent 15S and 5S ^{14}C-labelled FCV subunit used as markers.

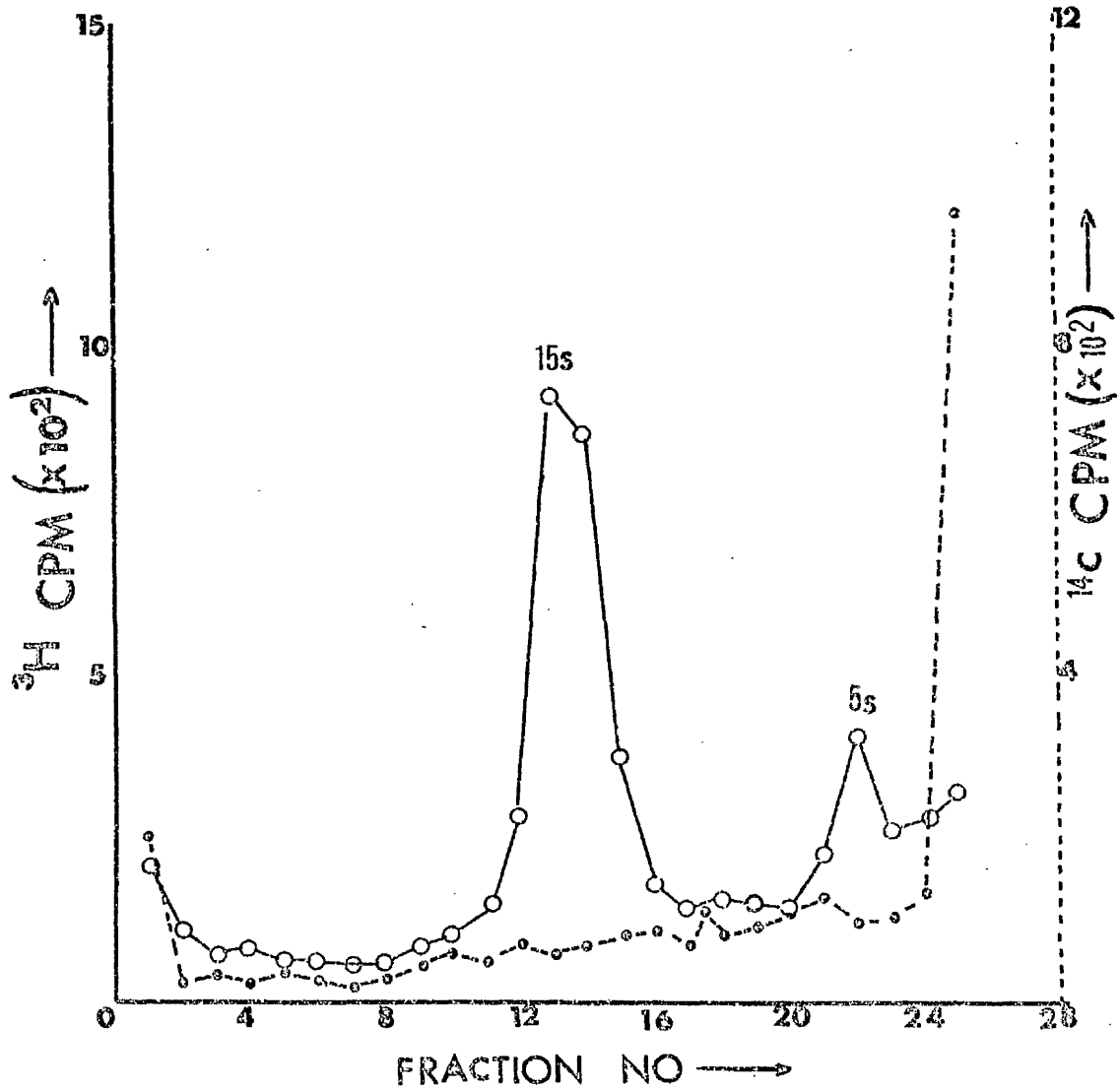
Figure 4.7 Viral subunits in FCV-infected cells - 2



—●—●—●—^{3}H-labelled viral proteins

--●-●-●-●-^{14}C-labelled 15S and 5S FCV subunit markers

Figure 4.8 Sedimentation behaviour of FCV major polypeptides in sucrose gradients



15S and 5S FCV subunit markers —o—o—o—

^{14}C-FCV polypeptide (p65) —•—•—•—

was a primary gene product or a breakdown component of the 15S virus subunit.

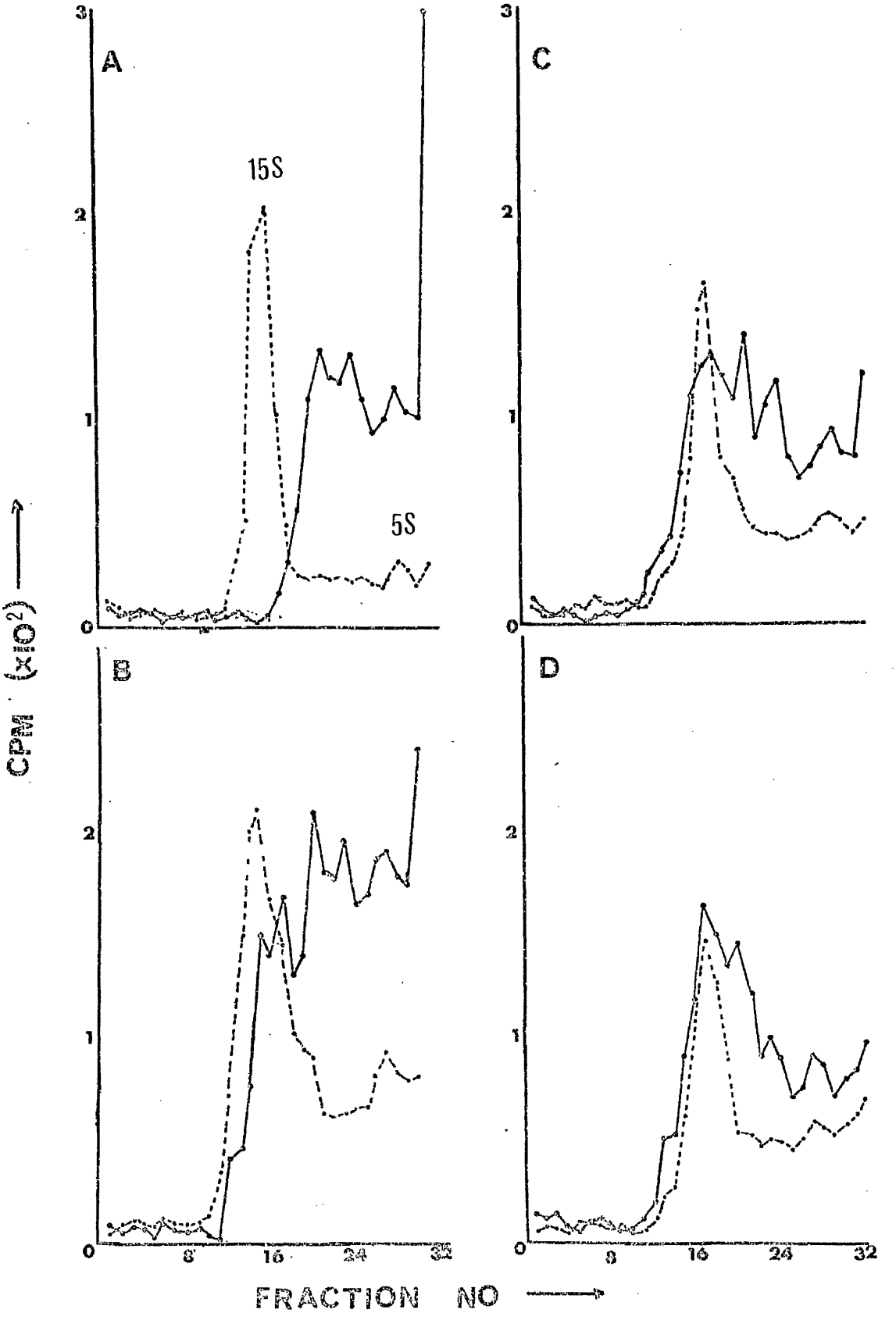
Experiment 3 In this experiment, the shortest possible pulse of radiolabel was used in an attempt to find if there was a precursor of the 15S component. FCV-infected cells, 200 minutes post-infection, were labelled for a period of 5 seconds and then chased for 30 seconds, 5, 15 or 30 minutes as described above and the extracts were analysed as before. The results of this study are shown in Fig. 4.9.

When pulse-labelled for 5 seconds with no chase (Fig. 4.9a) the most abundant viral component was the polypeptide (p65) which formed 26% of the total of newly synthesized viral proteins. Within this short period, other viral structures sedimenting at 5S, 9S and 11S were also observed in infected cell extracts. It was not clear whether these were direct primary gene products or were formed from the p65. However, a rapid depletion in the amount of p65 was reflected in a corresponding increase in the amount of 5S subunit component; and at the same time two other viral structures sedimenting at 13S and 15S appeared in infected cells, 30 seconds after the initiation of protein synthesis (Fig. 4.9b) Shortly after synthesis, the 9S component disappeared completely while the p65, 5S and 11S structures continued to decrease in amounts in favour of the 15S component which later became the most labelled viral structure in infected cells (Figs. 4.9c,d). After about 30 minutes from the initiation of viral protein synthesis, whole FCV particles appeared in infected cells (Fig. 4.9e).

The movement of radioactivity in infected cells was also considered by comparing the total radioactivity in each peak at each time point. The results, in Table 4.1 and Fig. 4.10, showed a continued migration of radioactivity from the p65 and into the various intermediates before culminating in the 15S peak. The involvement of the 5S, 9S, 11S and 13S subunits in this process not only showed that the 15S component was generated from smaller subunit particles but also implicated the 9S, 11S and 13S structures along with the 5S and 15S already clarified as possible intermediates in the assembly pathway of FCV particles.

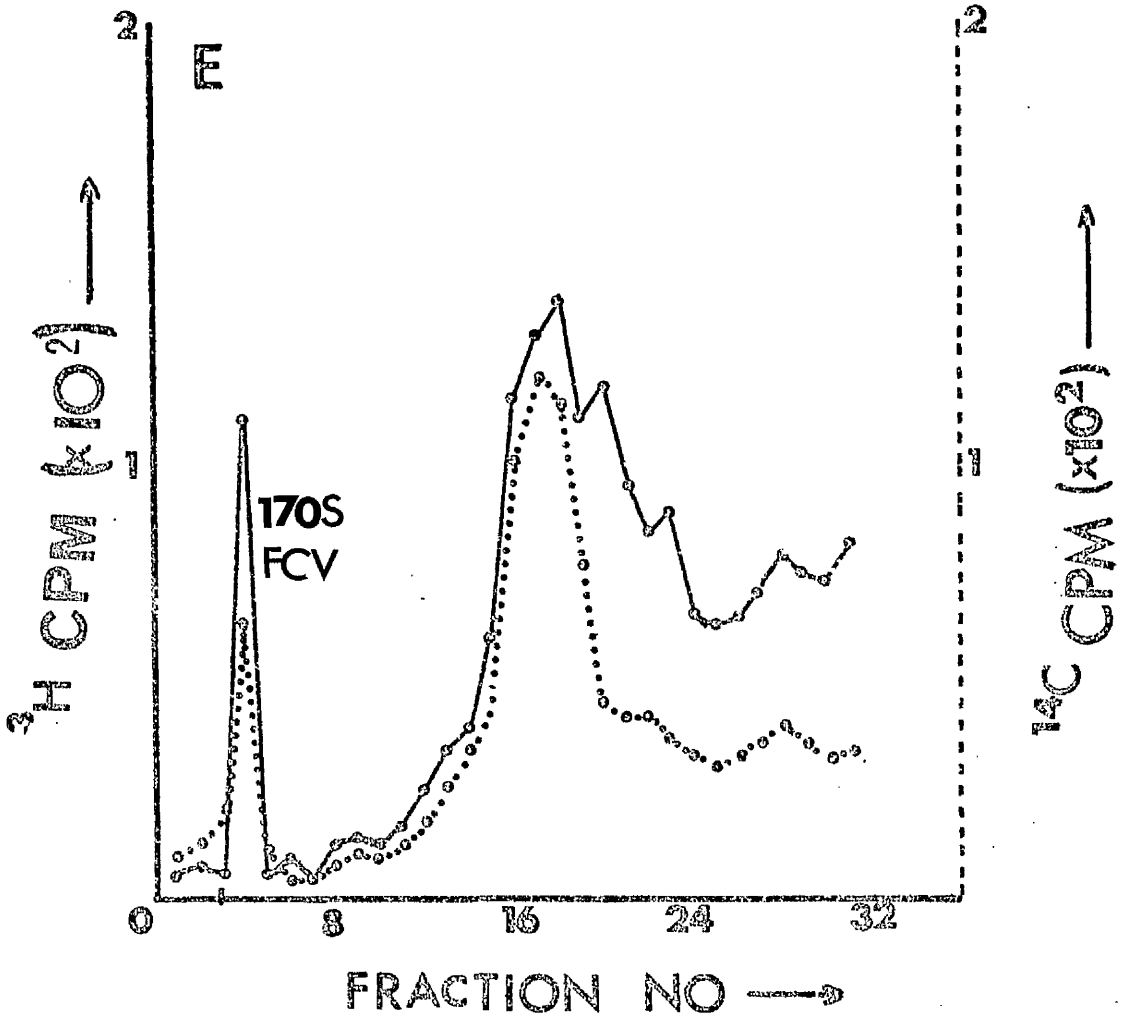
In this study, that is, after 30 minutes of protein synthesis (Table 4.1), the ratio of the 170S FCV particle to the 15S subunit component was 1:6. But in the 16 hour harvest, these structures were

Figure 4.9 Virus subunits in FCV-infected cells - 3



Infected cells were pulsed for 5 seconds (A) and chased for 30 seconds (B) 5 minutes (C); 15 minutes (D) and 30 minutes (E).
Dotted lines represent 15S and 5S subunit components used as markers.

Figure 4.9 (Contd.)



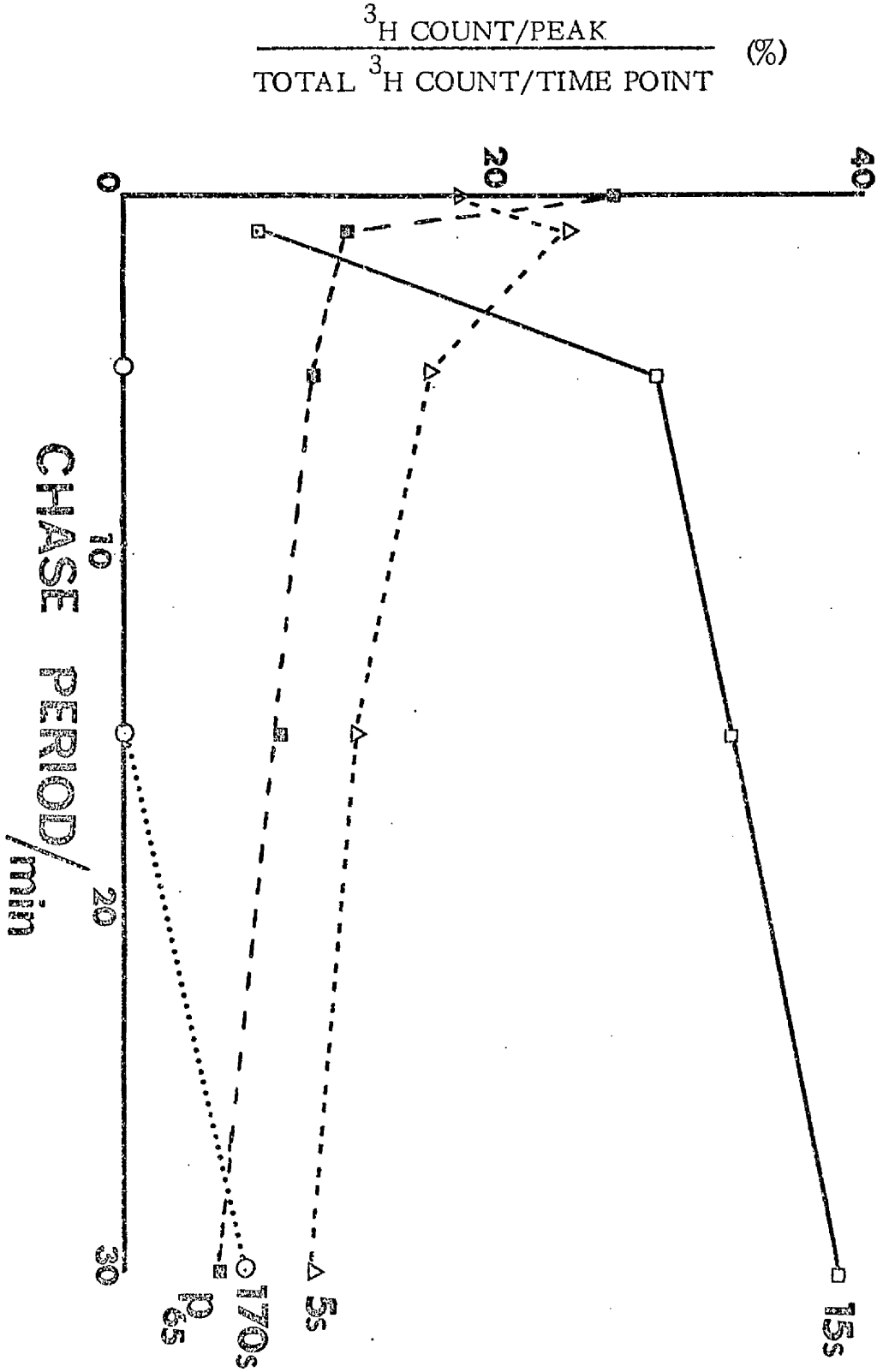
Conditions were as in Figure 4.9 A-D, but ^{14}C-leucine labelled 170S whole virus, 15S and 5S subunits were used as markers.

Table 4.1

Flow of ^{3}H-protein in FCV-infected cells

Chase Period	Proportion (%) of total ^{3}H-protein in each component						
	170S	15S	13S	11S	9S	5S	p65
0 seconds	0	0	0	23·8	19·9	18·0	26·4
30 seconds	0	7·2	14·5	17·3	17·6	24·0	12·0
5 minutes	0	28·4	17·3	15·6	0	16·5	10·5
15 minutes	0	32·9	20·5	13·6	0	12·7	8·2
30 minutes	6·5	38·5	12·9	9·9	0	10·2	5·6

Figure 4.10 Flow of ^{3}H-protein in FCV-infected cells



observed as peak I and peak II and the ratio increased to 1:10. This increase could be explained by the disappearance of the smaller subunit particles into the 15S peak.

Experiment 4 Before the 9S, 11S and 13S structures could be regarded as possible intermediates in FCV assembly, it was necessary to show that they were virus- rather than cell-specified. Accordingly, FCV-infected cells were pulse labelled with 600μCi of ^{35}S-methionine for 5 seconds and chased for 30 seconds (Fig. 4.11). Cell extracts were prepared as before and samples of each radioactive peak (p65, 5S, 11S, 13S and 15S) were analysed by slab gel electrophoresis and autoradiography.

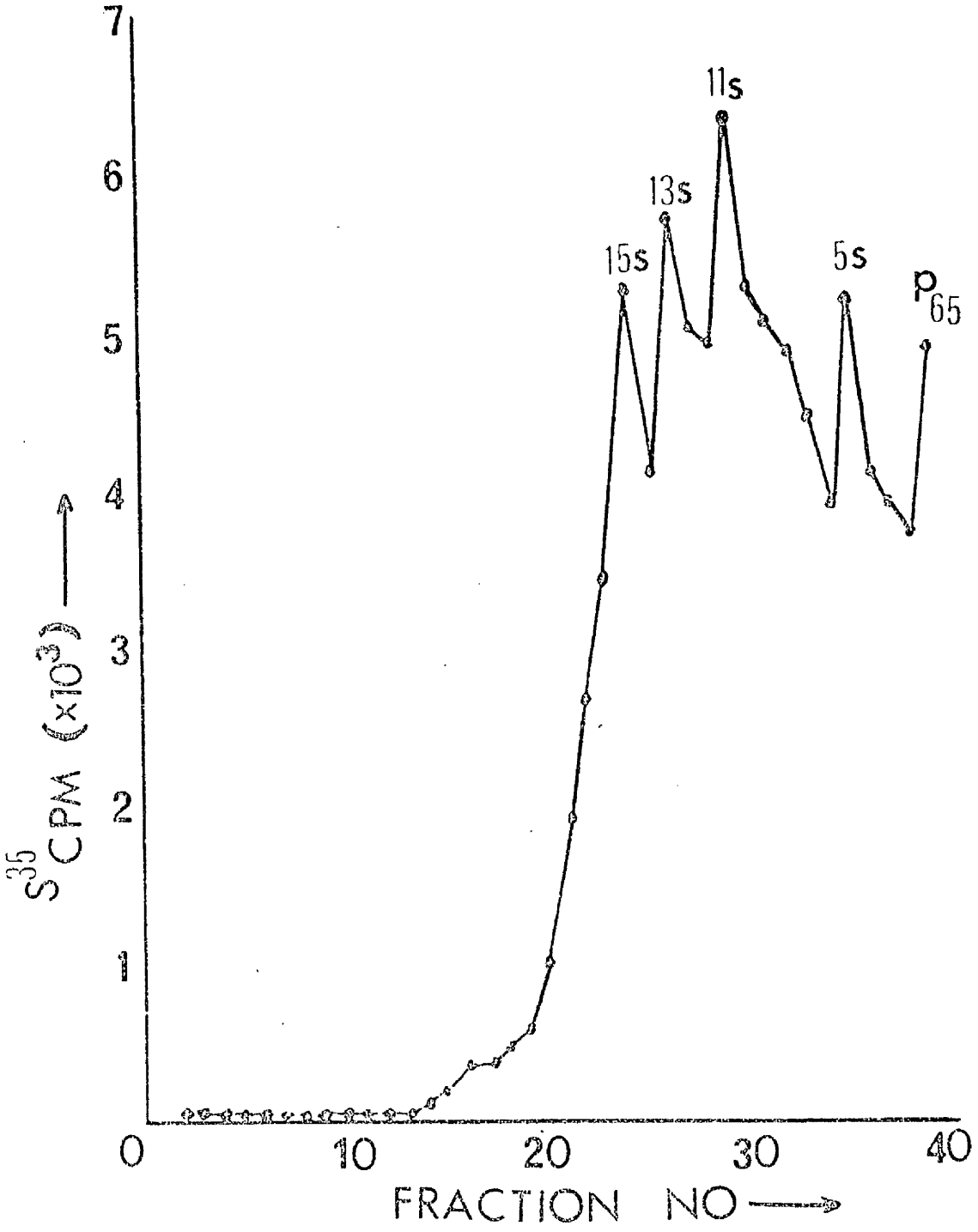
The result (Fig. 4.12) showed that all of the putative intermediates contained the major virus-induced polypeptide, p65.

Investigation of possible precursor-product relationship

High molecular weight proteins FCV-infected cells were analysed for specific virus-induced polypeptides. This was achieved by labelling the cells with ^{3}H-leucine at a time when most of the protein synthesis was virus-specific (Fig. 4.2), preparing cell lysates and selecting the virus-related polypeptides from these by precipitation with anti-viral serum. The serum used was from a specific pathogen free cat infected intranasally with FCV G1. It was anticipated that the cat would have been exposed to all of the antigenic determinants of proteins induced by the virus during its replication *in vivo*, so that the antiserum would precipitate not only capsid proteins but also any non-structural virus-specified polypeptides which appeared in infected cells.

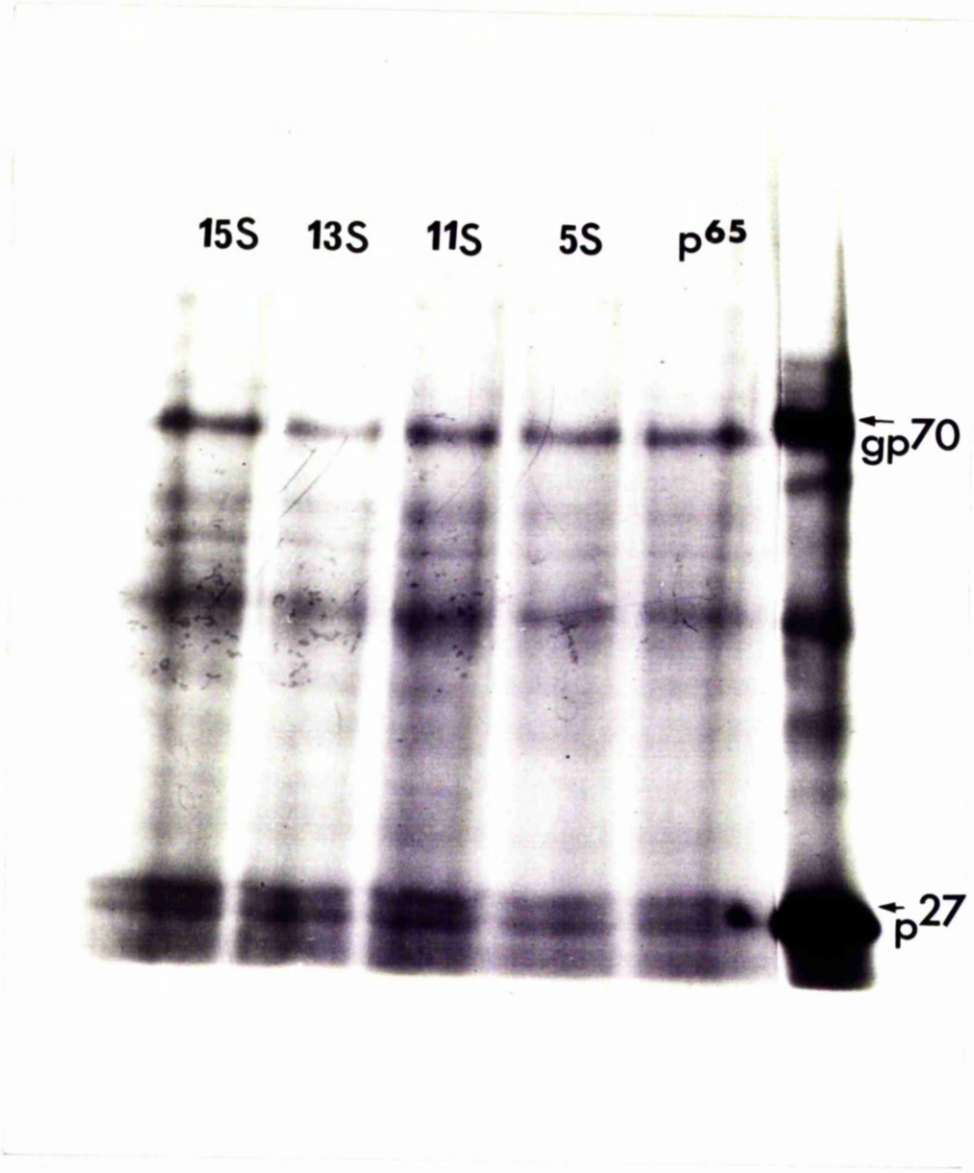
As shown in Figure 4.13, analysis of cell extracts for specific virus-induced polypeptides following immunoprecipitation with cat antiviral serum on low percentage (8%) polyacrylamide gels revealed no large capsid protein precursors in FCV-infected cells. Although traces of a virus-induced polypeptide of molecular weight 80,000 appeared transiently in infected cells between $2\frac{1}{2}$ - 3 hours after infection (track F and G in Fig. 4.13), the major virus polypeptide (p65) still remained the only virus-induced polypeptide found in FCV-infected cells. This result was in partial agreement with the observations of Black and Brown (1977) who found 3 polypeptides with molecular weights 100, 80 and 65x10^{3} in calicivirus-infected cells; but the result of the present study failed to support the presence of a polypeptide of molecular weight 100,000.

Figure 4.11 Isolation of viral subunits prior to virion assembly



At about 200 minutes post-infection, the cells were pulsed with ^{35}S-methionine for 5 seconds and chased for 30 seconds. The various intermediate subunit components were harvested on 15-30% sucrose gradient after centrifugation at 30,000rpm for 20 hours.

Figure 4.12 Polypeptide analysis of FCV subunit components



The various subunit components made prior to FCV assembly were analysed for viral capsid protein by PAGE and autoradiography. All subunit components had a capsid protein of molecular weight 68,000. Feline leukaemia virus glycoprotein (gp70) and protein (p27) were used as markers in track F.

Figure 4.13 legend

FEA cells were infected with FCV. At 30 minute intervals throughout the virus growth cycle, the cells were labelled with ^{3}H-leucine for a 15 minute period. Cell lysates were prepared and analysed by precipitation with cat antiviral serum followed by polyacrylamide gel electrophoresis and autoradiography. This figure represents the virus-induced polypeptides synthesised during each period of radiolabelling.

A	Uninfected cell control
B	0 - 30 minutes
C	30 - 60 "
D	60 - 90 "
E	90 - 120 "
F	120 - 150 "
G	150 - 180 "
H	180 - 210 "
I	210 - 240 "
J	240 - 270 "
K	270 - 300 "

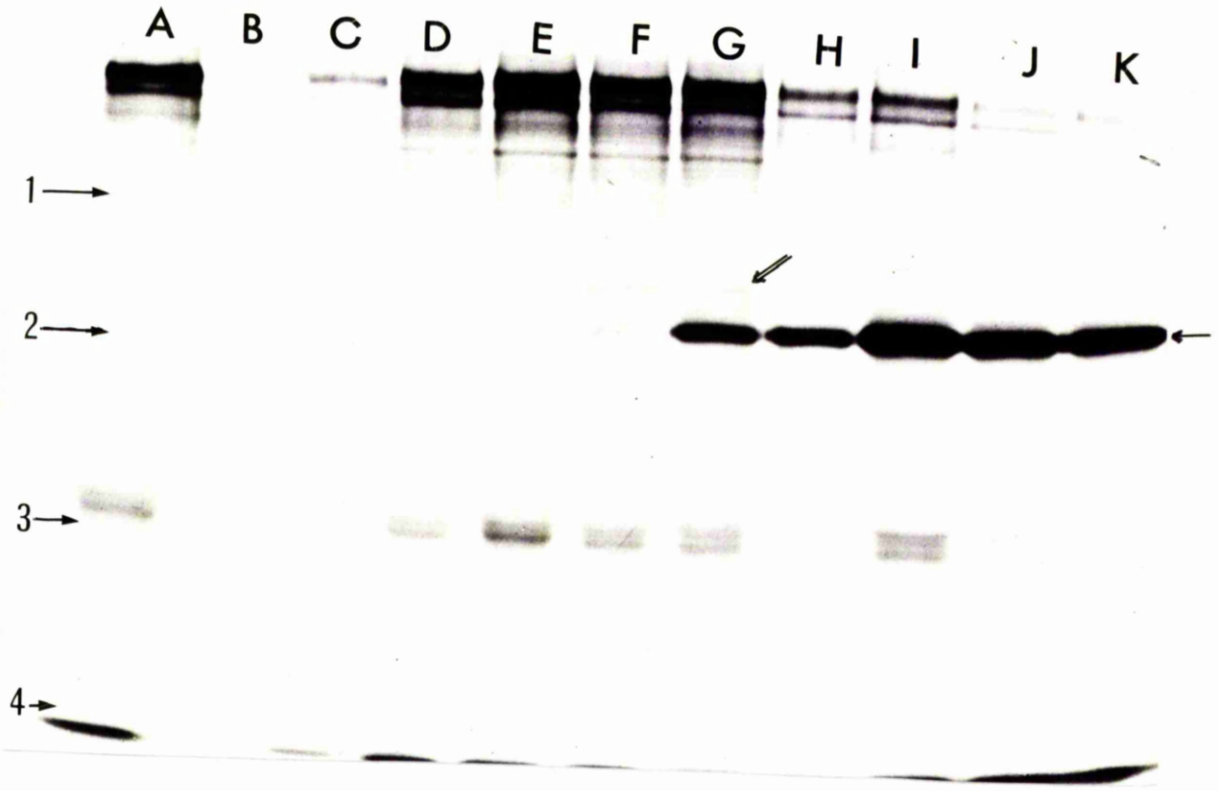
Numbers 1-4 represent the relative position of standard proteins used as markers.

1	phosphorylase a	(94,000)
2	catalase	(60,000)
3	alcoholdehydrogenase	(41,000)
4	carbonic anhydrase	(29,000)

Double arrows show the position of a high molecular weight protein (p80).

Single arrow shows the major capsid protein (p65).

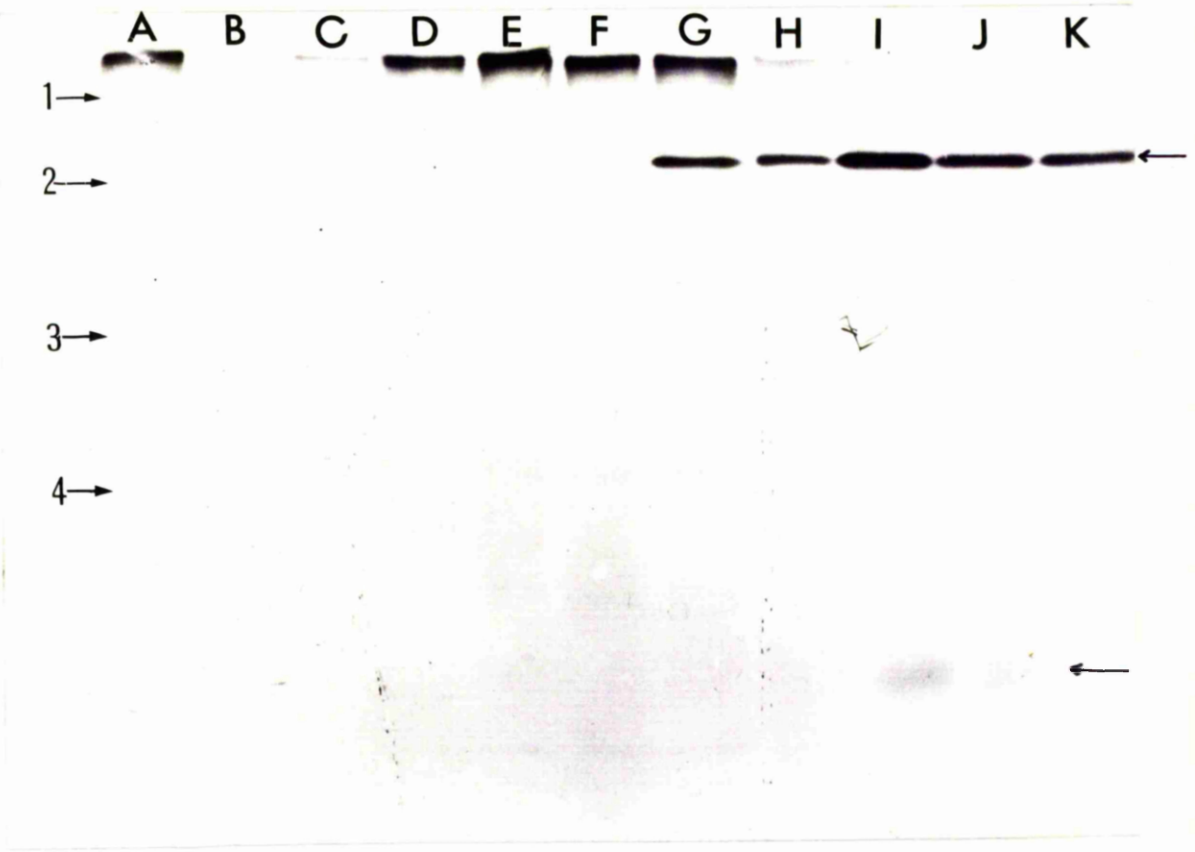
Figure 4.13 Immunoprecipitation and analysis of FCV-infected cell extract on 8% polyacrylamide gels



However, analysis of cell lysate for total (i.e. cellular and viral) newly synthesised proteins showed the presence of two polypeptides of molecular weights 80 and 40×10^3 daltons (Fig. 4.5, tracks H-K). Both p80 and p40 first appeared in infected cells at about $3\frac{1}{2}$ hours post-infection; that is, an hour after the detection of p65 in infected cells. Although these were highly labelled and did not decrease in amount after synthesis, their time of appearance in infected cells suggests that the synthesis of p80, p65 and p40 is independent.

Low molecular weight proteins Using the cat antiviral serum, a similar analysis of FCV-infected cell extracts on high percentage (12%) polyacrylamide gels was carried out to confirm the presence of the minor polypeptide in infected cells. The result (Fig. 4.14) showed traces of a polypeptide of molecular weight of about 13-15,000 daltons appearing in infected cells between $3\frac{1}{2}$ and $4\frac{1}{2}$ hours post-infection and disappearing thereafter.

Figure 4.14 Immunoprecipitation and analysis of FCV-infected cell extract on 12% polyacrylamide gels



Keys A-K and 1-4 are as in Fig. 4.13.
FCV-infected cell extracts were analysed on 12% polyacrylamide gels to detect the presence of any low molecular weight capsid protein.
Top arrow indicates p65.
Bottom arrow shows the presence of a minor capsid protein of about 14,000 molecular weight.

DISCUSSION

The aim of the present study was to gain further understanding of the assembly process of feline calicivirions and to compare this process with that in picornaviral systems.

The contention that 15S and 5S subunit particles were precursors in the maturation of FCV was reinforced by the following findings:

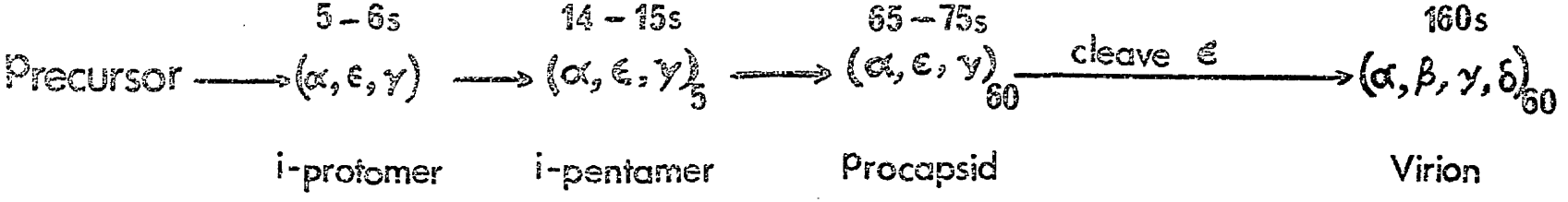
a) Whole virus particles, under controlled conditions (see Chapter 3), could be sequentially dissociated into 15S and 5S viral subunits.
b) Such intermediate (15S and 5S) particles could be detected in FCV-infected cells synthesizing only virus-induced polypeptides (Fig. 4.6).
c) The isolation of 11S and 13S components in infected cell extracts is not a unique feature of feline caliciviruses: a similar 13S subunit has been found to play an important role in the assembly of EMC virus (Fig. 4.16).
d) The kinetics of labelling of these intermediates and the subsequent assembly of the mature virion in infected cells is consistent with the role of these subunit structures as precursors (Fig. 4.10, Table 4.1).
e) The polypeptide composition of the 15S and 5S subunits, and indeed of the 11S and 13S components, is very similar to the capsid protein (p65) found in the mature virus particles (Fig. 4.12).

Despite the similarities in their dissociation and assembly properties (Figs. 4.15, 4.16 & 4.17) the mode of replication in FCV is basically different from that observed in picornaviruses. For example, replication in picornaviruses has been shown to involve post-translational cleavage of precursor proteins (Summers & Maizel, 1968; Holland & Kiehn, 1968; Jacobson & Baltimore, 1968) and in some instances, a polyprotein molecule thought to be the progenitor of capsid proteins has been demonstrated in picornavirus-infected cells (Jacobson & Baltimore, 1970; Holland & Kiehn, 1970).

By contrast, analysis of FCV-infected cell extracts by pulse-chase experiments failed to support the presence of any polyprotein precursor analogous to the $NCVP_1$ found in picornavirus-infected cells; instead, the most predominant viral structure found in infected cells was the 15S component, which, rather than being converted into other capsid protein, was itself constructed from smaller subunits and remained a permanent end product.

Figure 4.15

POLIOVIRION ASSEMBLY



Information from Rueckert, R.R. (1971)

Figure 4.16

EMC Virus Assembly & Dissociation

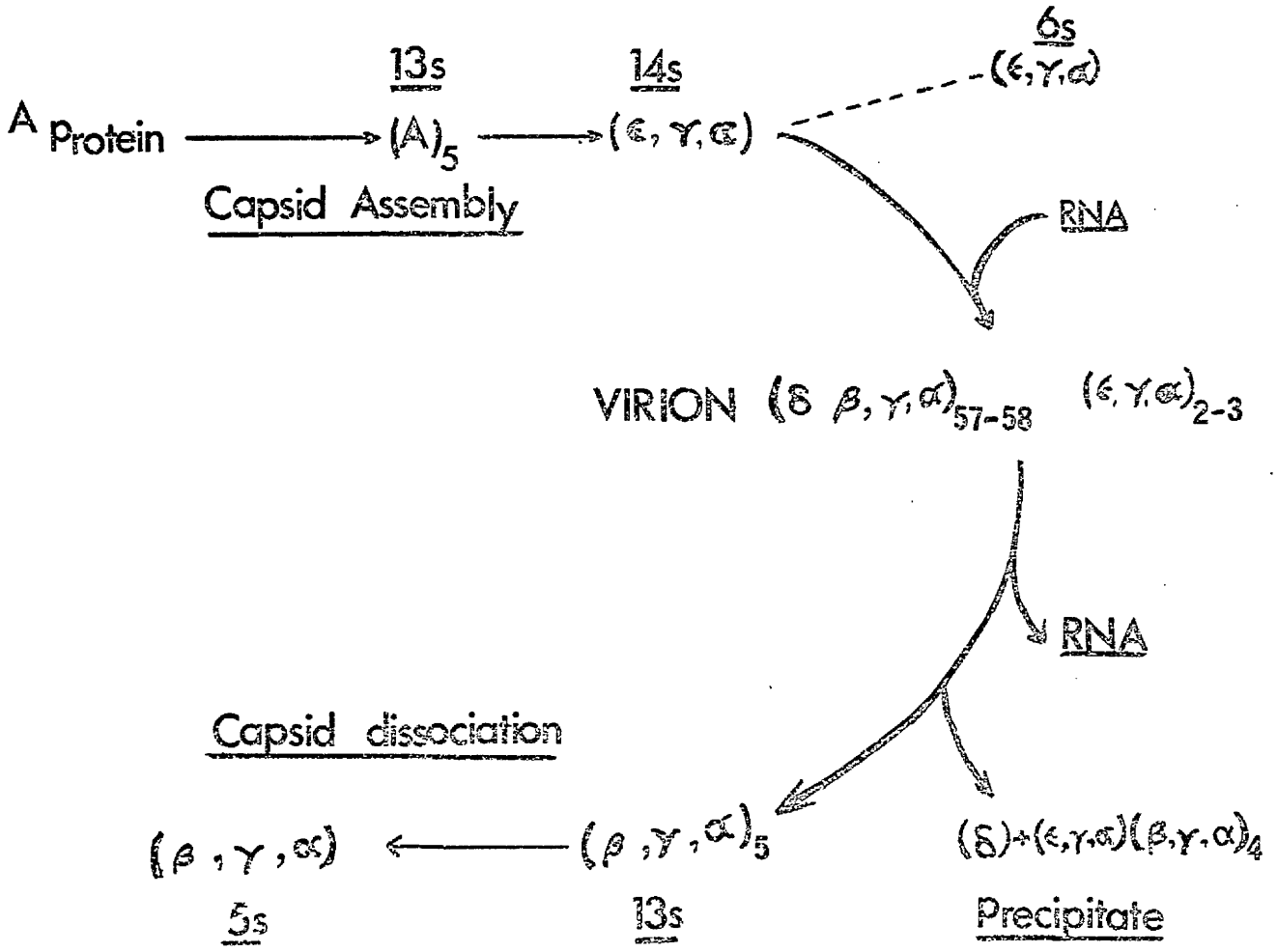
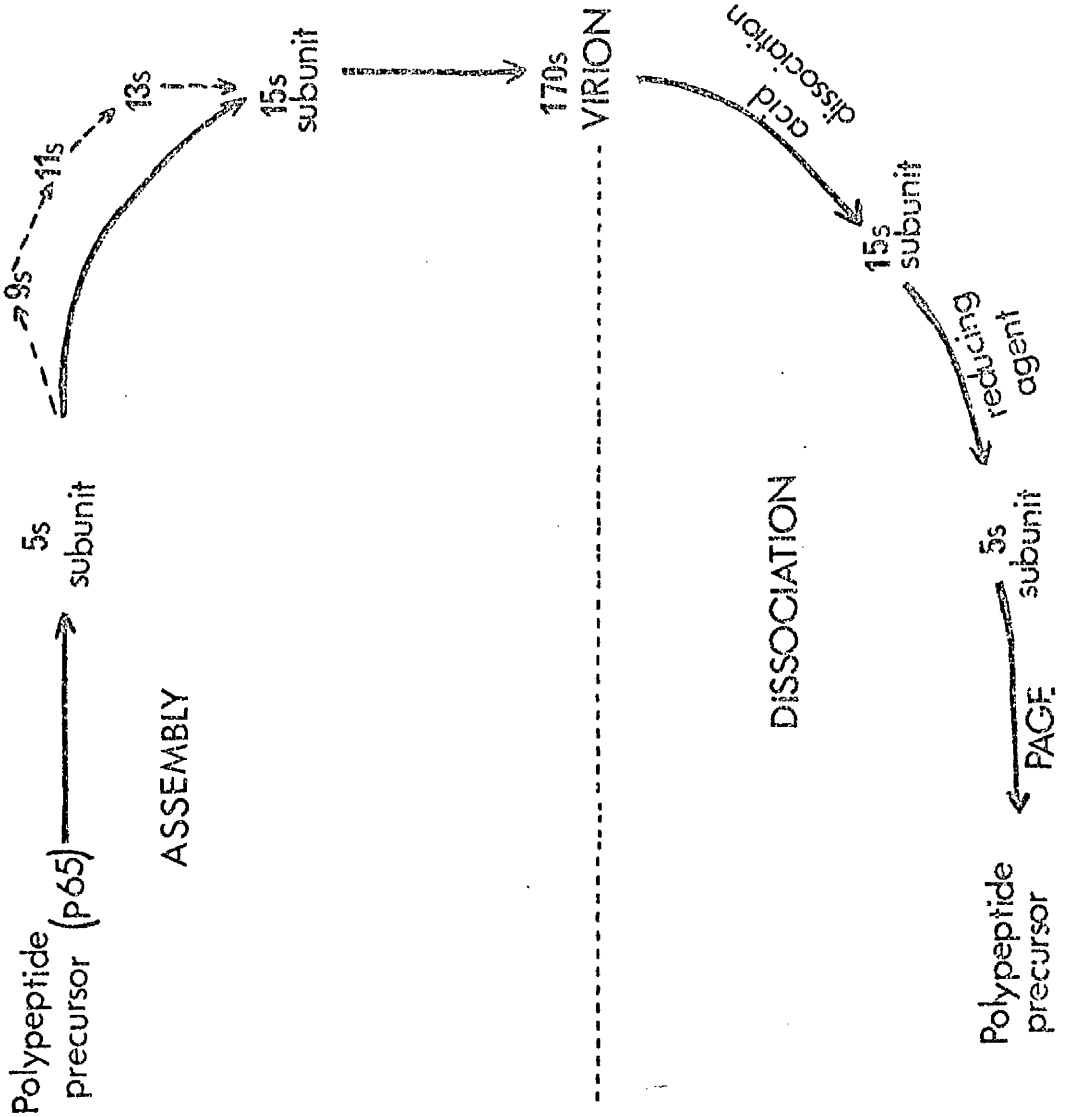


Figure 4.17 FCV dissociation and assembly



The first attempt to demonstrate large molecular weight protein precursors yielded only the capsid protein of the virus and no larger precursor polypeptides could be detected (Black & Brown, 1975/76). In later studies two additional FCV-induced polypeptides (p100 and p80) were reported in cell extracts although they bore no precursor-product relationship to the p65 (Black & Brown, 1977a).

Analysis of FCV-infected cell lysates in the present study by immunoprecipitation and autoradiography (Fig. 4.13) showed that apart from p80 found in traces for a short period, the only virus-induced polypeptide was the capsid protein, p65. Although both p80 and p65 appeared in infected cells at about 150 minutes post-infection, the p80 was present in submolar amounts and disappeared about 30 minutes after the initiation of synthesis. The p65, however, prominently increased in amount and became the only capsid protein identifiable in FCV-infected cells. From these results, it is doubtful if the p65 was a product of cleavage from the p80. Black & Brown (1977) however, suggested that the synthesis of p80 and p65 were independent of each other. One particular point of interest in this study is that neither p80 nor p40 were recognised by the cat antiviral serum when cell lysates were analysed by immunoprecipitation. This suggests that both p80 and p40 might be enzymes required for the replication and assembly of FCV particles in infected cells. If this assumption is correct, then two separate polypeptides of molecular weight 80×10^3 daltons can be identified in FCV-infected cells, the first appearing in infected cells at about $2\frac{1}{2}$ hours post-infection and disappearing 30 minutes later (Fig. 4.13, tracks F and G) and the other appearing at about $3\frac{1}{2}$ hours post-infection and persisting until the infection was stopped after 5 hours (Fig. 4.5, tracks H-K).

The presence of a minor polypeptide of molecular weight 15,000 in FCV particles was first described by Burroughs and Brown (1974). Recent studies by Schaffer and Soergel (1976) failed to support the presence of this polypeptide in caliciviruses. In Chapter 3, chromatographic analysis of the polypeptide of 5S subunit component obtained by sequential virus dissociation revealed the presence of a minor polypeptide of molecular weight 13,500 in addition to the major capsid protein, p65. In the study described in this Chapter a minor polypeptide of molecular weight about 14,000 was observed when FCV-infected cell extract was analysed by immunoprecipitation followed by electrophoresis on 12% polyacrylamide gels (Fig. 4.14). These results

show that a minor polypeptide of about 13-14,000 molecular weight is indeed present in FCV-infected cells.

In cells infected with typical picornaviruses a virion-size single-stranded RNA (Levintow, 1974) appears from which a giant polypeptide is translated before progressing by cleavage to form capsid proteins (Fig. 4.1). The detection of two virus-induced polypeptides in FCV-infected cells with no evidence of precursors or post-translational cleavage raised the question as to whether the polypeptides were synthesized from the single 37S viral RNA, as in picornaviruses, or from individual subgenomic mRNA molecules synthesized after infection. To date, internal initiation of protein synthesis has not been demonstrated in eukaryotic cells so that it seems likely that subgenomic RNAs are involved in the replication of FCV.

Evidence in support of this was obtained from the work of Ehresmann & Schaffer (1976) who found that multiple classes of RNA were synthesized in cells infected with caliciviruses. The predominant single-stranded RNA of molecular weight $2{\cdot}6 \times 10^6$ sedimenting at 36S was genome size. There was also a prominent 22S single-stranded RNA component with a molecular weight of $1{\cdot}1 \times 10^6$ and one or more double stranded, or partially double stranded, components. Black and Brown (1975/76) suggested that the 22S subgenomic mRNA was translated into the major polypeptide (p65). Thus it appears that calicivirus replication involves the synthesis of subgenomic mRNA which is then translated to provide the coat protein and possibly the other virus-induced polypeptides found in infected cells.

This mode of replication is similar to that described previously for Semliki Forest virus, a group A togavirus, and tobacco mosaic virus (TMV). In Semliki Forest virus-infected cells, single stranded 42S RNA is the virus genome and 26S "interjacent" RNA serves as mRNA for virion polypeptide synthesis; double stranded replicative forms appear to be heterogenous (Levin & Friedman, 1971; Pfefferkorn & Shapiro, 1974; Wengler & Wengler, 1976). In TMV-infected cells, the major virus-specified polypeptide synthesized is the coat protein (Jockusch, 1968; Gianinazzi, Vallee & Martin, 1969; Van Loon & Van Kammen, 1970; Doke, 1972). Two other large polypeptides were translated *in vitro* from the virus RNA whereas coat protein was translated from a separate subgenomic mRNA (Bruening et al, 1976; Higgins, Goodwin & Whitefield, 1976).

Caliciviruses were considered as provisional members of the family picornaviridae (Melnick et al, 1974; Fenner, 1976) at a period of incomplete knowledge of their molecular biology. Evidence is now accumulating to suggest that the caliciviruses belong to a new and separate family of viruses and as such should not be classified as picornaviruses. The major difference in the strategy of replication of the caliciviruses and picornaviruses described above provides an addition to an already long list of differences.

CHAPTER 5

ANTIGENS OF FELINE CALICIVIRUS

INTRODUCTION

Although extensive studies have been made on the serological relationships among feline caliciviruses (Povey, 1974; Povey & Ingersoll, 1975) the nature and number of antigenic determinants on the virus particles still remained undefined.

In Chapters 3 and 4, a naturally produced 15S subunit component encountered during virus harvest was described. This component, as well as the 170S virus particle contained the single major capsid protein of molecular weight 65,000. Furthermore, the amount of capsid protein in the subunit peak was found to be 10 times more than the virus peak. Subsequent studies by controlled virus dissociation and *in vivo* virus biosynthesis showed that the 15S subunit was an intermediate structure in the pathway of virion assembly.

The aim of the present study was to determine and compare the antigenic properties of the 15S subunit with the whole virus particles. This was done by raising antisera to FCV and 15S subunit antigens in rabbits and reacting the antisera with the homologous and heterologous antigens in immunodiffusion tests. Both antisera were also subjected to virus neutralization tests by the plaque reduction method.

MATERIALS AND METHODS

Preparation of antigens

FCV and 15S virus subunit components were purified from 600ml of infected cell culture fluid as described in Chapter 3. Each purified component was made up to 5ml with TS and then pelleted by centrifuging in an SW 50.1 rotor at 45,000rpm for 6 hours. The pellet was resuspended in 1ml of TS and then divided into two 0·5ml amounts: one aliquot was used for immunisation of rabbits and the other was stored in liquid nitrogen and was used later as a booster inoculum.

Preparation of sera

Two rabbits, one for each antigen, were used. Prior to inoculation, the rabbits were bled and the sera were screened for anti-FCV antibodies by a neutralization test. The sera contained no virus neutralising antibodies.

For immunisation, 0·5ml of antigen was mixed with an equal volume of Freund's complete adjuvant (Miles Laboratories) and the mixture was vigorously shaken until an emulsion had been formed. That the emulsions formed were satisfactory was shown by the non-dispersal of the emulsions when dropped on a water surface.

The antigens were then administered by intramuscular injections into each hindleg. Four weeks later, the remaining antigen stored in liquid nitrogen was similarly inoculated. Seven days after the second inoculation, the rabbits were again bled to harvest immune sera. Both pre-immune and immune sera were then subjected to neutralisation tests by the plaque reduction method.

Immunodiffusion tests

An adaptation of the double diffusion method of Ouchterlony (1948) was used. Two ml of molten 1% agarose (Miles Laboratories) in PBS at 44°C was carefully poured on to a clean microscope slide and allowed to solidify at room temperature. A basic hexagonal arrangement of six evenly spaced wells, 6mm apart, with a central well, was made in the gel. Samples of about 10µl of antigen or antisera were carefully dropped into the appropriate wells using a micro-pipette. The slides were incubated in humidified chambers at 4°C for 3-4 days and washed in PBS for another 2 days. Where staining was required, the gels were dried in an oven (56°C) overnight and stained with 0·11% amido black for 30-60 minutes before destaining in 7·5% acetic acid - 5% methanol. Precipitin lines were examined visually and where necessary with the aid of a hand lens.

Serum absorption

A volume of 25µl of rabbit antiviral serum was absorbed with 100µl of concentrated purified 15S virus subunit at 4°C for 1 hour. Both absorbed and unabsorbed sera were reacted with the virus antigen by immunodiffusion. The remainder of the absorbed serum along with the unabsorbed and anti-15S sera were used in neutralization tests. Similarly, both dissociated and natural 15S subunits were absorbed with excess 15S antiserum and then reacted with the unabsorbed antiserum by immunodiffusion.

RESULTS

Immune sera

Analysis of rabbit antiviral and anti-15S sera by virus neutralization showed that both had a titre of 40 (Table 5.1) indicating that the antigens used had a common determinant which evoked neutralizing antibody or alternatively, that 2 or more separate antigenic determinants were involved in neutralization.

Immunodiffusion tests

The antigenicity of whole FCV particles and the 15S subunit component was examined by immunodiffusion and the results are represented in Figs. 5.1 - 5.5. The reaction of anti-FCV and anti-15S antisera against the virus antigen (Fig. 5.1) showed that both antisera were reactive against FCV antigen. Only one determinant was observed on the 15S subunit compared to two on the whole virus particles; one of these showed a reaction of identity with the 15S determinant.

As expected, both anti-viral and anti-15S antisera showed a reaction of identity against a 15S antigen (Fig. 5.2). This common determinant was probably responsible for producing neutralizing antibodies in FCV and the 15S immune sera (Table 5.1).

Absorption of serum by the shared antigenic determinant

Immunodiffusion The absorption of the common antibody from antiviral serum with excess 15S antigen was performed to isolate the virus-specific antibody and to test whether it had virus neutralizing activity. The result of the reaction between absorbed and unabsorbed sera against virus antigen in immunodiffusion is shown in Fig. 5.3. This experiment further confirmed that the unabsorbed antibody was virus-specific.

Neutralization When subjected to virus neutralization assay, along with anti-FCV and anti-15S antisera, it was found that the virus-specific antibody had no neutralizing capability (Table 5.2).

It would appear, therefore, that the neutralizing activities of the viral and 15S antisera were specific functions of the shared antibody (Table 5.1).

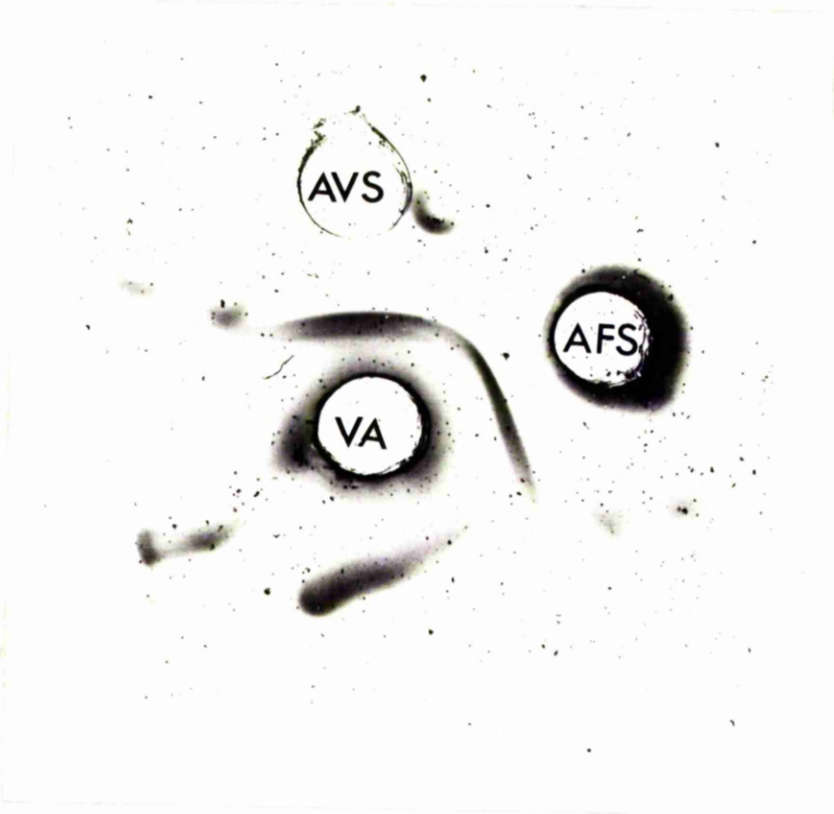
Table 5.1

The neutralizing activity of anti-FCV 170S and anti-15S antisera

Serum dilution	Cat anti-virus serum	Rabbit anti-virus serum	Rabbit anti-15S serum
Pre-immunisation	Number of plaques per plate at each serum dilution		
1:5	44	45	43
1:10	53	51	49
Post-immunisation			
1:5	0	0	0
1:10	0	0	0
1:20	2	0	2
1:40	5	3	9
1:80	25	21	27
1:160	34	29	35
1:320	40	38	44
1:640	51	49	52

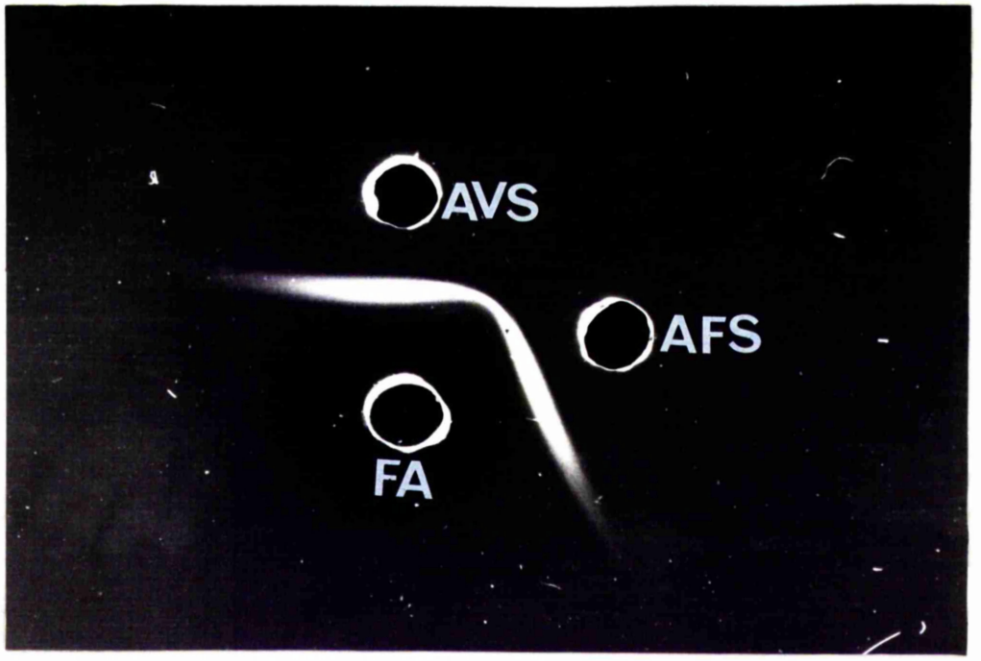
Control assay (no serum) = 56 plaques/plate

Figure 5.1 Immunodiffusion of anti-FCV and anti-15S antisera against virus antigen.



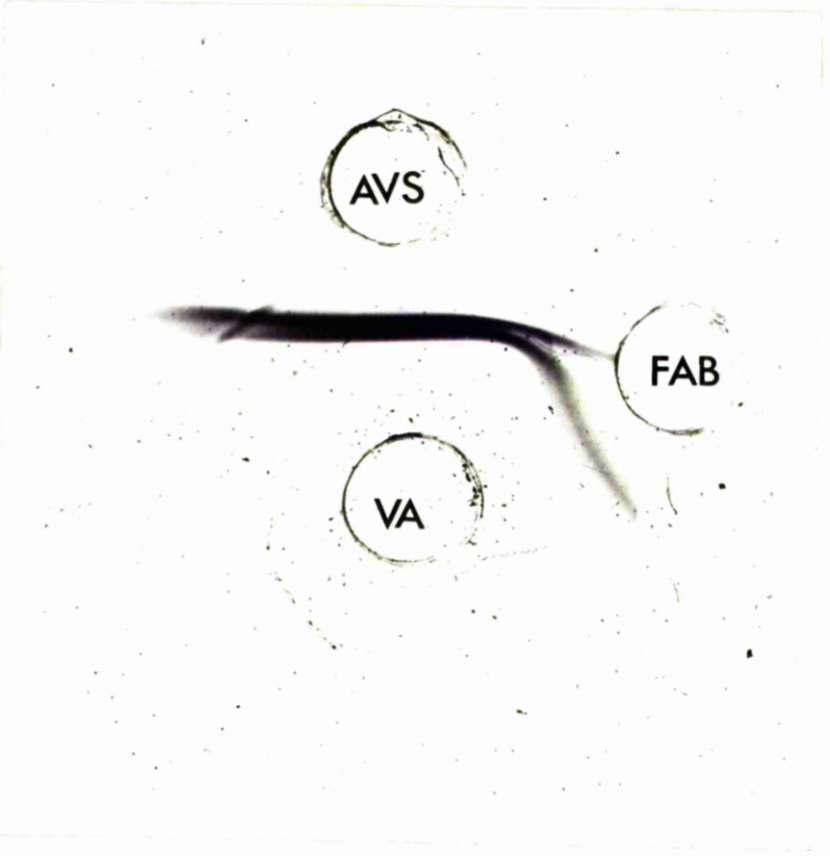
VA = virus antigen
AVS = antiviral serum
AFS = anti-15S serum

Figure 5.2 Immunodiffusion of anti-FCV and anti-15S antisera against 15S antigen



AVS = antiviral serum
FA = 15S antigen
AFS = anti-15S serum

Figure 5.3 Absorption of serum by the shared antigenic determinant



VA = virus antigen

AVS = antibiral serum

FAB = FCV absorbed serum

FCV antiserum was absorbed in excess 15S antigen

Table 5.2

Virus neutralization test of FCV absorbed serum

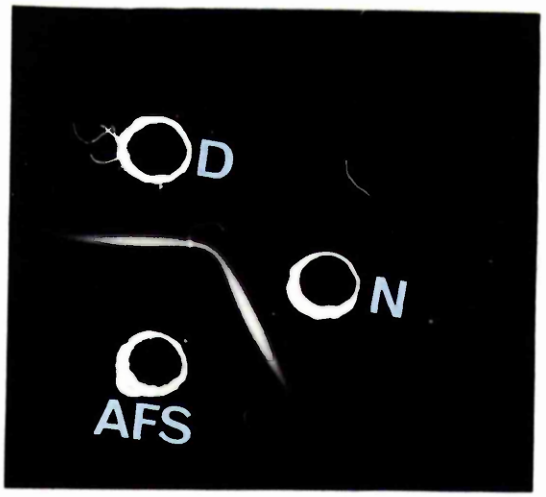
Serum dilution	Anti-FCV serum (Unabsorbed)	Anti-FCV serum (absorbed)	Anti-15S serum
	Number of plaques per plate		
1:5	0	45	0
1:10	0	49	0
1:20	0	48	1
1:40	4	51	7
1:80	27	54	29
1:160	35	52	37
Titre of serum:	40	5	40

Control assay (no serum) = 55 plaques

Comparison of the antigens of "natural" and "viral" 15S subunits

It was also observed that the naturally occurring 15S component (peak II) was antigenically identical to a corresponding component obtained by particle dissociation (Fig. 5.4). When each structure was absorbed with excess anti-15S antiserum, the absorbed serum failed to react against its homologous antigen (Fig. 5.5).

Figure 5.4 Immunodiffusion of viral 15S and peak II antigens against anti-15S antiserum

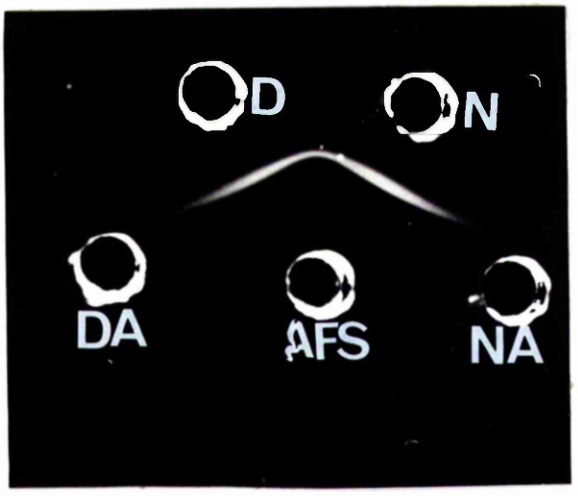


AFS = anti-15S serum

N = naturally produced 15S (peak II)

D = dissociated 15S antigen

Figure 5.5 Absorption of serum by dissociated and natural 15S subunit antigens



Immunodiffusion in 1% agarose gel.

AFS = anti-15S serum
N = natural 15S antigen
D = dissociated 15S antigen
DA = dissociated 15S antigen absorbed with anti-15S serum
NA = natural 15S antigen absorbed with anti-15S serum

DISCUSSION

Immunodiffusion tests were preformed to determine and compare the antigenicity of FCV particles and of the 15S subunit component. The results of this study revealed that 2 antigenic determinants were present on the surface of FCV particles (Fig. 5.1). The fact that only one was found on the 15S component was suggestive that the virus-specific determinant was formed by the characteristic arrangement of the subunit components to form capsids during virion assembly. The ability to demonstrate the virus-specific antibody by removing the common antibody from antivirus antiserum by a serum absorption test (Fig. 5.3) was confirmatory evidence that two determinants did exist on the surface of FCV particles. Since the virus-specific antibody was shown to lack neutralizing antibodies (Table 5.2) it was, therefore, relatively safe to assume that the neutralizing activities of the viral and 15S antisera were specific functions of their common determinants.

In a parallel electronmicroscopic study to this, Ormerod (1978) found that 5 hours after infection in FEA cells with the FCV G1 strain, newly assembled viral particles were found in large intracytoplasmic aggregates or crystals. The cells were examined by immunofluorescence using the rabbit sera described here and FITC-conjugated anti-rabbit globulin. On reaction with the anti-FCV serum, fluorescence was observed diffusely throughout the cytoplasm and also brightly in several cytoplasmic foci. These latter were assumed to represent the crystals of viral particles seen by electronmicroscopy. Using the anti-15S antiserum, only the diffuse fluorescence was observed. When the anti-FCV serum absorbed with 15S subunit, as was described in the present study, was used, the fluorescence was focal. The conclusion from these results was that the 15S subunit appears in the cytoplasm and when it is assembled into mature viral particle an antigen (the common antigen) is concentrated into the aggregates of particles. When the antibody to this antigen was absorbed from the antivirus serum, the antibody to the unique, virus-specific antigen remained. These results confirm the present findings.

It may be that the multiple antigenic determinants on the FCV particle which were revealed in the present study are relevant to the antigenic variety which is observed among FCV isolates (Povey, 1974). In spite of these antigenic differences the existence

of separate serotypes of FCV has not been established. Indeed there is considerable evidence for common antigenic determinants both from the results of neutralization (Povey, 1974) and cross-protection tests (Povey & Ingersoll, 1975). It will be important now to examine further strains of FCV by the methods reported here to find whether there is an antigenic determinant which is common to all FCV isolates (group-specific antigen); and whether this is the antigen shared between the FCV particle and the 15S subunit.

In foot-and-mouth disease virus (FMDV) where similar antigenic sites have been described (Rowlands et al, 1971) both sites have been shown to produce neutralizing antibodies although the mechanisms by which the two antibodies function appear to be different. One site is concerned with the production of an antibody which neutralizes virus by preventing its attachment to cells while the other is concerned with the prevention of uncoating of the virus RNA. In this respect, the antigenic determinants of FCV particles differ from those of FMDV in that only one determinant (the common determinant) produce virus neutralizing antibody. The function of the virus-specific determinant still remains undefined.

Another interesting aspect of this study was that the whole virus and the 15S subunit component both possessed common antigenic determinants for the production of neutralizing antibodies in the respective immune sera. The immediate implication of this result was that the 15S antigen might possibly be useful as vaccine against feline calicivirus disease in cats. A prototype vaccine based on this idea was prepared and tested in this study and is discussed in the next chapter.

CHAPTER 6

A FELINE CALICIVIRUS SUBUNIT VACCINE

INTRODUCTION

Since the original isolation of the virus by Fastier (1957) one factor of particular interest about feline caliciviruses has been the antigenic variation which exists between various strains. Preliminary attempts to classify the viruses serologically suggested that numerous distinct serotypes existed (Bittle et al, 1960; Bürki, 1965; Crandell, 1967). Subsequent cross-neutralization studies of 46 isolates, however, led Povey (1974) to suggest that there were in fact considerable antigenic relationships in the group and that the various isolates might be regarded as members of a single serotype. This study was later extended to in vivo situations for a total of 8 isolates by Povey and Ingersoll (1975) who postulated that such relationships might be reflected in cross-protection in cats infected with FCV.

A similar development occurred in attitudes towards vaccination against virus-induced respiratory disease in cats. Thus, at first it was considered that the antigenic diversity of FCV isolates, together with the poor immunogenicity of FHV, precluded the manufacture of an effective vaccine against these viruses. However, when the extent of cross-protection following infection with FCV became apparent, serious attempts were made to produce vaccines.

Due to the practical limitations of using all known antigenic strains in preparing a FCV vaccine, the F-9 strain, already known to be broadly antigenic and of low virulence (Kalunda et al, 1975), was used in several vaccine preparations either singly (Bittle & Rubic, 1976) or in combination with FHV and panleukopaenia virus vaccine (Bittle & Rubic, 1975; Davis & Beckenhauer, 1976). Kahn (1975), in a series of studies, showed that there was a distinctly better protection using a single intranasal dose of F-9 strain than using two intramuscular doses of the same immunizing antigen. The apparent immunologic advantages of intranasal vaccination for both FHV and FCV infections prompted development of a bivalent, single does, modified live virus vaccine, marketed commercially as "Felomune" (Norden). Other commercially available FHV-FCV combined vaccines now include "Feliflu" (C-Vet) and Katavac CH (Duphar) administered intramuscularly and intranasally respectively.

Live vaccines are commercially attractive owing to the comparatively small quantity of antigen which may be used. There are, of course, several potential problems associated with their use, including the possibility of persistence in, and excretion of virus by

the animal and the presence of extraneous infectious agents in the non-inactivated material. In fact, following the use of some live FCV-FHV vaccines in the field there have been indications that certain vaccinal viruses might not be completely attenuated (O. Jarrett - personal communication).

Such considerations prompted Povey and Wilson (1978) to compare inactivated FHV- FCV vaccines with live modified vaccines. They found that a killed vaccine given intramuscularly with adjuvant in two doses was marginally superior to the single dose of live modified vaccine described above.

Even killed vaccines have attendant side effects in that the chemicals used to inactivate the infectious RNA might cause unwanted reactions in animals or sometimes alter the protein responsible for activating the immune responses in such a way that protection is lessened.

There is an increasing conviction that vaccines could be made from the non-infectious components of viruses which are antigenically identical to the whole virus. In this way, the risks associated with the killed or attenuated virus vaccines can be averted. For example, of the three major proteins associated with the rabies virus envelope, only the glycoprotein was found on the external surface of the envelope and it has recently been shown to be responsible for inducing neutralizing antibodies in infected hosts (Dietzschold *et al*, 1978). When purified and used as vaccine, the glycoprotein vaccine had similar or better protective activity than a vaccine made from inactivated virus (Cox *et al*, 1977). Similarly, a vaccine made from purified non-infectious glycoprotein (haemagglutinin and neuraminidase) components of influenza virus has been found to be equally protective and safer than killed or live modified virus vaccines (Kilbourne, 1975).

In Chapter 3, the occurrence of a 15S subunit component encountered during the purification of FCV was described. This component was found to contain 10 times more protein than the virus peak in $CsCl_2$ gradients, and the work in Chapter 4 indicated that this 15S component was a major intermediate product during virion assembly. A further confirmation of this was that the same antigenic determinant was present on the subunit and the whole virus. In addition, both homologous antisera exhibited virus neutralizing activities as described in Chapter 5.

These findings suggested the use of the subunit as a vaccine. The present chapter concerns experiments in which cats were immunized with the purified non-infectious 15S subunit and were subsequently challenged with virulent FCV.

MATERIALS AND METHODS

Animals

Cats were obtained from the breeding colony of the University of Glasgow Department of Veterinary Pathology. Six one-year old cats were used.

Collection of samples for virus isolation

Live animals Oropharyngeal swabs of the cats were taken with the aid of a sterile cottonwool swab. The swab was broken into a glass bottle containing 2ml of EFC_2 (MEM with 2% foetal bovine serum) which was then sealed. In the laboratory the cottonwool was removed from the bottle, the medium was centrifuged at 2000rpm for 10 minutes and the top 1·0ml of fluid was removed and stored at -70°C prior to virus isolation.

At necropsy Samples of tissue were taken with sterile scissors into bottles and chopped into small pieces. Two ml of MEM were added and the suspension was agitated in a Whirlimix. The tissue suspension was centrifuged at 3,000rpm for 5 minutes and the supernatant was stored at -70°C for virus isolation.

Virus isolation

Samples of 100µl from each specimen were inoculated into each of two confluent FEA cell cultures and adsorption was allowed for 1 hour followed by inoculation with 2ml of EFC_{10}. The plates were incubated at 37°C and were examined daily until a CPE was observed, or 4 days had elapsed. At the end of 4 days, plates not showing CPE were regarded as negative.

Bacteriology

Oropharyngeal swabs from all experimental cats obtained before and after virus challenge were tested for the presence of bordetellae, β-haemolytic streptococci, haemophilic bacteria and pasteurellae. The swabs were each used to inoculate a 5% sheep blood agar plate, a 5% horse blood agar plate, a chocolate agar plate and a MacConkey agar plate. The sheep blood and MacConkey plates were incubated at 37°C aerobically, the horse blood plate anaerobically and the chocolate agar in an atmosphere enriched with CO_2 for up to five days if growth was not observed to occur before then. Gram-stained films were made from representative colonies resembling those produced by the bacteria and cultures were examined according to the

scheme given in "Manual for the Identification of Medical Bacteria" by Cowan and Steel.

Analysis of postmortem tissues A portion of lung from each cat was submitted for bacteriological examination in the course of which material was removed with routine sterile precautions from a bronchiole with a glass pipette and examined in the same way as the swabs.

Histopathology

Small pieces of lung tissue were taken and analysed by histopathology for microscopic lesions by Dr I.A.P. McCandlish. Intact lung lobes were fixed by instillation of 10% neutral buffered formol saline into the main bronchus followed by immersion of the lobes in an excess of fixative. After 48 hours, representative tissue blocks were selected, trimmed and transferred to fresh fixative for a further 24 hours. The tissue blocks were then processed for histological examination by dehydration through an alcohol-chloroform-xylene series followed by embedding in paraffin wax. Sections were cut at 4-6μm and stained with Mayer's haemalum and eosin.

Vaccination experiments

Cats Six cats were used in this study. Four of them (numbered 1, 2,3 and 4) were vaccinated while the others (5 and 6) were kept as controls.

Preparation of vaccine Six hundred ml of clarified culture fluid from FCV-infected cells was mixed with 20ml of a similar preparation labelled with ^{3}H-leucine (marker virus). The mixture was precipitated with an equal volume of saturated ammonium sulphate and centrifuged at 7,000rpm for 5 minutes. The precipitate was resuspended in 5ml of TS buffer and then centrifuged on a pad of $CsCl_2$ and sucrose gradients as described in Chapter 3. The peak of virus materials was harvested and recycled on $CsCl_2$ gradients in the SW 50.1 rotor at 40,000rpm for 16 hours. The virus and 15S subunit components obtained were separately pooled and recycled on $CsCl_2$ gradients as before. The 15S subunit component was then made up to 5ml with TS buffer and pelleted in the SW 50.1 rotor at 45,000rpm for 6 hours. The pellet was resuspended in 5ml TS and sonicated. The vaccine was then filtered through a 25nm Millipore filter to remove any virus particles which might still be trapped in the preparation. Of the 630,000cpm in the sample filtered, only 490,000cpm (77·7%)were recovered in the filtrate.

A sample of 50µl of the filtered sample was mixed with an equal volume of TS buffer and inoculated into a plate of confluent FEA cells at 37°C for 1 hour. After adsorption, 3ml of EFC_{10} medium was added and the plate was re-incubated for 4 days. The cells were examined each day for evidence of CPE but remained negative until the plate was discarded at the end of the fourth day.

Immunisation procedure The immunisation procedure was the same as for antiserum production in rabbits described in Chapter 5. Prior to immunisation, the cats were bled and the sera were screened for specific FCV antibodies by virus neutralization tests. The immunogen was prepared by mixing 0·5ml of the subunit vaccine with an equal volume of 1:10 dilution of Alhydrogel adjuvant (Miles Laboratories). An experiment was done to determine the dilution of the adjuvant to be used with the 15S antigen for immunisation. Two-fold dilutions of aluminium hydroxide (Alhydrogel; Miles Laboratories) prepared in distilled water and a 25µl sample of each was added to an equal volume of the ^{3}H-labelled 15S antigen preparation. The mixture was allowed to stand for 10 minutes at 4°C and was then centrifuged at 5,000rpm for 5 minutes. The precipitate was resuspended in 50µl of TS buffer. Radioactivity was estimated using NE 260 scintillation fluid and the amount of radioactive material precipitated by each dilution was expressed as a percentage of the radioactivity contained in a 25µl sample of 15S antigen used as control. As shown in Fig. 6.1, maximum precipitation of antigen was achieved using a 1:20 dilution of Alhydrogel.

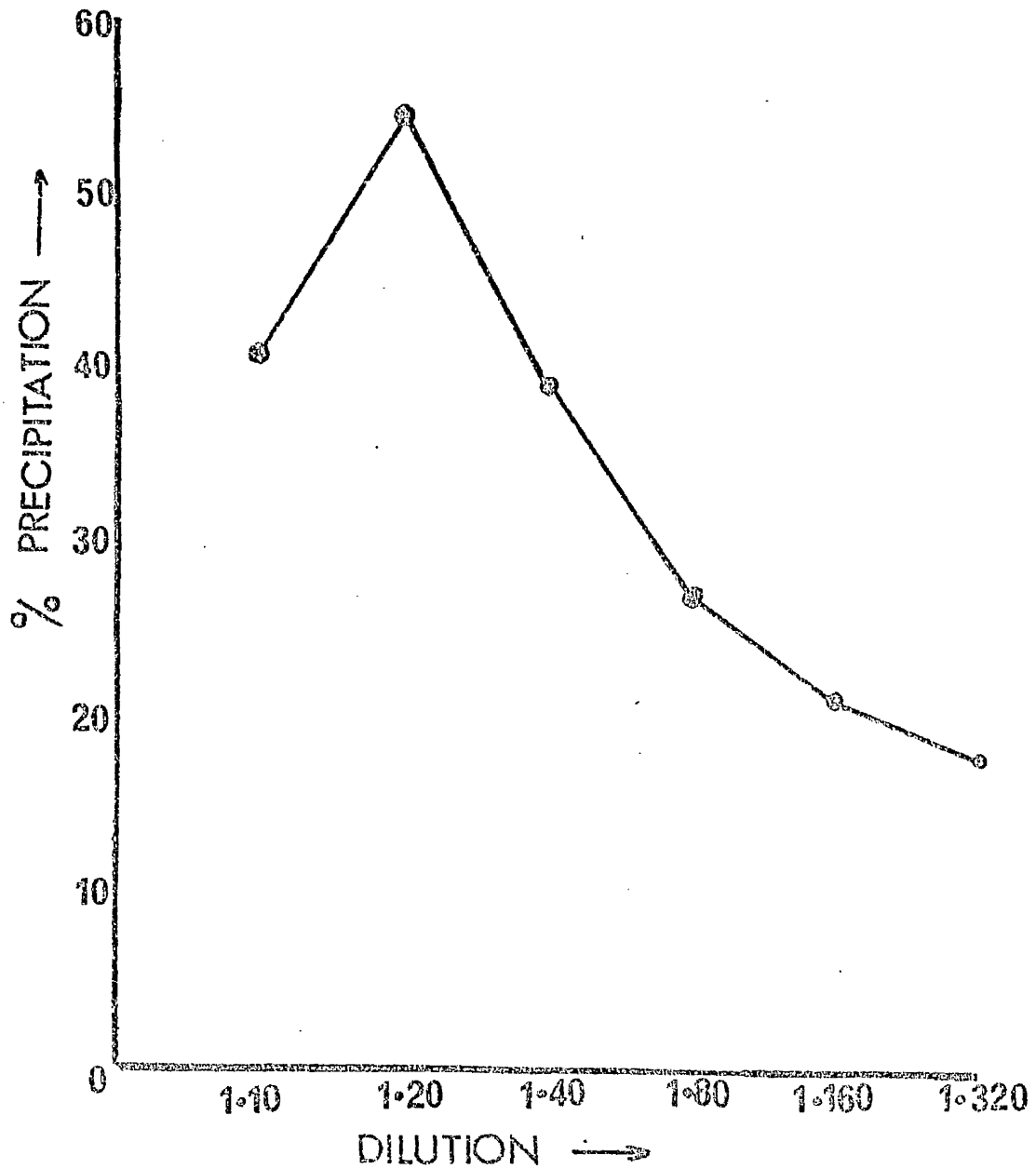
The vaccine was administered subcutaneously in a single dose in the neck region. A similar dose was administered 4 weeks later. Ten days after the booster dose the cats were challenged with $1X10^{6}$pfu of live FCV contained in 0·5ml of EFC_{2}. Using a pipette, virus inoculum was instilled in equal amounts into the nostrils.

The vaccinate and control cats were kept separately in rooms with a floor area of $10m^{2}$, about 30 feet apart. Any change in physical condition and demeanour could easily be observed.

The cats were examined daily for any clinical manifestations of FCV disease. Oropharyngeal swabs and rectal temperature of the cats were taken on day 0 and day 1 and every other day thereafter. Seven days after the virus challenge, two vaccinates (Cats 2 & 4) and one control (Cat 6) were sacrificed and the others were killed at 14 days following challenge.

The following organs or tissues were taken for virus isolation: tongue, tonsil turbinates, trachea, lungs, kidney, liver, spleen, retropharyngeal and bronchial lymph nodes. Both oropharyngeal swabs and lung tissues were taken for bacteriology but only lung was taken for histopathology.

Figure 6.1 Titration of Alhydrogel adjuvant



RESULTS

Virus isolations

Pre-inoculation oropharyngeal swabs and similar swabs taken from all 6 cats immediately before challenge were negative for FCV. Table 6.1 shows the results of attempts to isolate virus from the oropharynx of both vaccinates and control cats examined every other day during the period of virus challenge.

All the cats began to excrete infectious virus within 24 hours following inoculation. With the exception of one vaccinate (Cat 4) which excreted virus for 48 hours, subsequent oropharyngeal swabs taken from the others proved negative. By contrast, virus was recovered from swabs of the controls up to the time of necropsy. The pharynx was the most consistent source of virus in infected cats. Other workers have considered that the tonsils might be the site for continued virus multiplication in carrier cats which had been shown to excrete virus from the pharyngeal region for long periods (Povey et al, 1973).

Bacteriology

Swab samples taken for bacteriology failed to reveal the presence of any pasteurellae, bordatellae or -haemolytic streptococci each of which could influence the course of calicivirus infection in cats. Also, none of the plates inoculated with materials from the lung yielded any growth.

Serology

The results of neutralization tests on pre- and post-inoculation sera are shown in Table 6.2. Prior to virus challenge, antibody titres of about 40 were observed in the vaccinates while there was no change in titre of the unvaccinated control. The antibody titre of the control cat examined at necropsy on day 7 post-inoculation rose slightly to 10 while a dramatic increase in titre to 40 was observed in the other control cat killed on day 14. The titre of the vaccinates rose only slightly to about 80.

Clinical manifestations of FCV disease

Clinical signs of calicivirus disease in both the vaccinates and controls were monitored and the results are represented in Table 6.3. The vaccinates remained clinically normal throughout the period of virus challenge whereas the controls exhibited characteristic symptoms of FCV infection including a biphasic temperature rise (Fig. 6.2),

Table 6.1

FCV isolation from oropharyngeal swabs (post-infection)

Cat No	5	6	1	2	3	4
Days						
0	-	-	-	-	-	-
1	+	+	+	+	+	+
3	+	+	-	-	-	+
5	+	+	-	-	-	-
7	+	+	-	-	-	-
9	+				-	-
11	+				-	-
13	+				-	-
14	+				-	-

\+ = positive virus isolation

\- = negative

Cats 6, 1 and 2 were necropsied on day 7.
Cats 5, 3 and 4 were necropsied on day 14.

Table 6.2 Neutralizing activity of pre- and post-virus challenge sera

Pre-challenge sera

A	Controls		Vaccinates			
Cat No	5	6	1	2	3	4
Serum Dilution	Number of plaques per plate					
1/5	46	51	0	0	0	0
1/10	49	43	0	0	0	0
1/20	48	48	1	3	0	1
1/40	51	54	6	7	4	6
1/80	49	51	29	24	27	31
1/160	52	47	40	37	41	39
1/320	50	48	49	50	46	52

Post-challenge sera

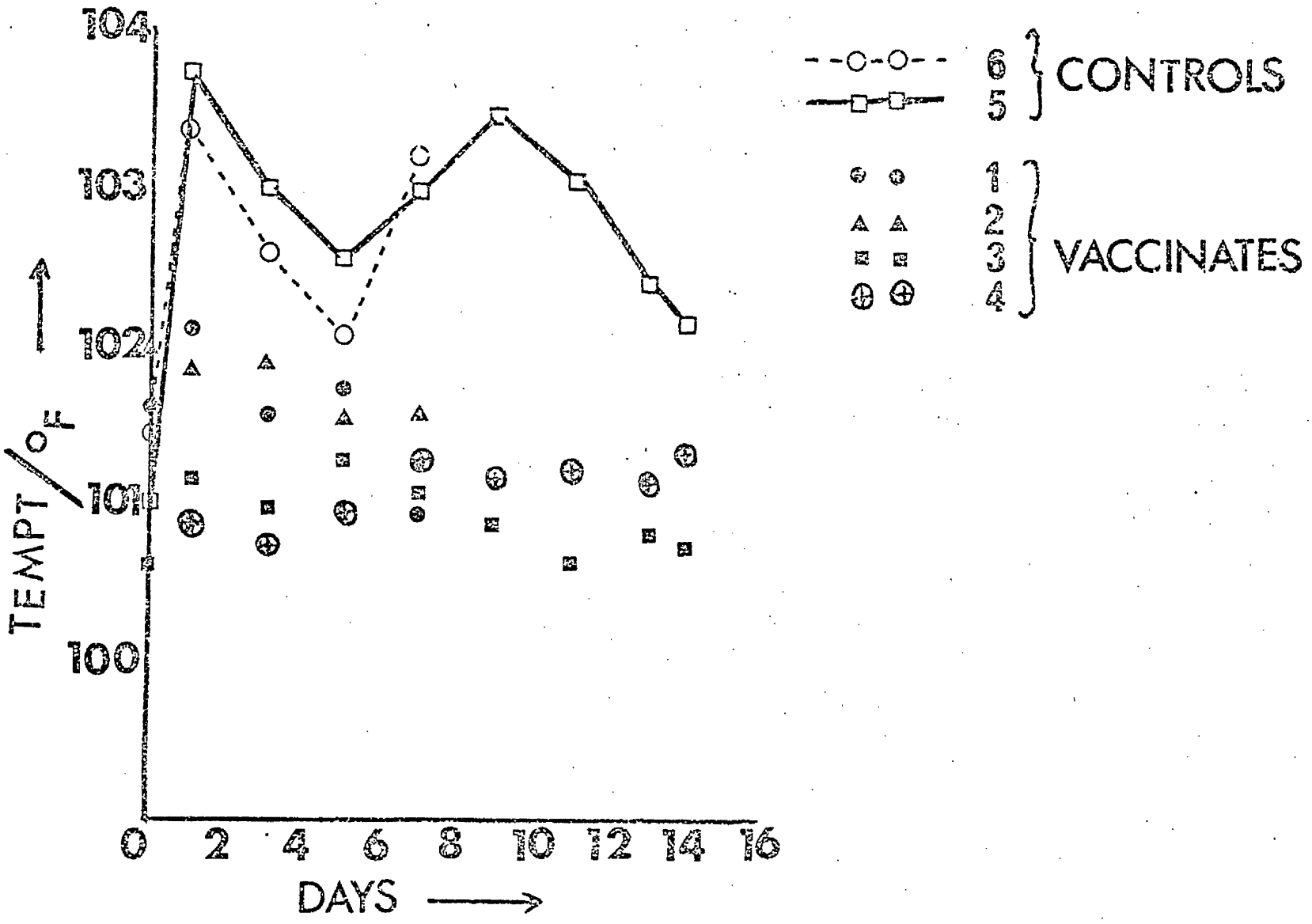
B	Controls		Vaccinates			
Cat no	5	6	1	2	3	4
Serum Dilution	Number of plaques per plate					
1/5	0	2	0	0	0	0
1/10	0	9	0	0	0	0
1/20	3	12	0	2	0	1
1/40	7	28	3	5	6	5
1/80	22	39	12	14	11	16
1/160	39	47	29	31	30	35
1/320	53	51	50	49	47	51

Number of plaques in control well = 58

Cats 6, 1 and 2 were necropsied on Day 7 after virus challenge

Cats 5, 3 and 4 were necropsied on Day 14

Figure 6.2 Pattern of rectal temperatures of vaccinate and control cats



a progressive loss of condition, depression and inappetance. The mucous membranes of the mouth at some stage appeared inflamed but ulcers did not develop.

Post mortem examination

Gross pathology All tissues and organs examined post mortem appeared normal except in one of the controls (Cat 6) autopsied on day 7 where the lungs appeared purulent.

Virus isolation Isolation of virus from post mortem tissues of vaccinates all proved negative (Table 6.4). In the unvaccinated cat (Cat 6), necropsied on day 7, virus was recovered from the tongue, tonsils and the lungs; whereas only the tonsilar tissues were still found to be harbouring infectious virus in the cat (Cat 5) necropsied on day 14. The increase in titre of virus neutralizing antibody in Cat 5 was probably responsible for the elimination of virus from possible areas of isolation, e.g. tongue or turbinates.

Histopathology The histopathological findings on the lung tissues of the cats is shown in Table 6.5. No abnormalities were detected in the lungs of Cat 2, one of the vaccinates, but the others (Cats 1, 3 and 4) showed mild bronchiolitis and alveolitis accompanied by cellular infiltration.

In the unvaccinated controls, histopathological lesions were prominent in Cat 5 involving focal alveolitis accompanied by extensive accumulations of macrophages in alveolar spaces. The alveolar walls were thickened due to mononuclear and polymorphic infilteration. Bronchiolitis and denudations of bronchiolar epithelium with peri-bronchial and perivascular lymphoid tissue were also prominent.

The cat necropsied on day 7 (Cat 6) with a low titre (10) of neutralizing antibody, in addition to the above lesions, had foci of proliferative interstitial pneumonia with hypertrophy and hyper-plasia of type II pneumocytes. Alveolitis was generalised and occasional bronchiolitis were found, especially in pneumonic areas.

Table 6.3 Clinical manifestation of FCV disease

Days Post virus Challenge	Controls		Vaccinates			
	5	6	1	2	3	4
0	Bright All food eaten 101·0°	101·4°	101·6°	Bright All food eaten 102·0°	100·6°	101·5°
1	¾ food eaten 103·7°	103·3°	102·1°	101·8°	101·2°	100·8°
3	⅔ food taken Dull 103·0°	102·6°	101·5°	Bright All food taken 101·9°	101·0°	100·7°
5	⅔ food eaten Reddish mucous membranes Dull 102·6°	Very dull 102·1°	101·7°	Bright All food taken 101·5°	101·8°	101·0°
7	⅓ food eaten Reddish & patchy mucous membranes 103·0°	103·2°	100·9°	Bright All food taken 101·5°	101·1°	101·3°
9	⅓ food eaten Still dull 103·4°				Bright 100·9°	All food taken 101·2°
11	¾ food taken 103·1°				Bright 100·6°	All food taken 101·3°
13	¾ food taken Appears bright 102·4°				Bright 100·8°	All food taken 101·2°
14	¾ food eaten 102·2°				Bright 100·7°	All food taken 101·4°

Cats 6, 1 and 2 necropsied on Day 7

Cats 5, 3 and 4 necropsied on Day 14

Table 6.4

FCV isolation from post mortem tissues

	Controls		Vaccinates			
	PM7	PM14	PM7		PM14	
Cat No	5	6	1	2	3	4
P.M. Tissues						
Tongue	+	-	-	-	-	-
Tonsil	+	+	-	-	-	-
Retropharyn. L.N.	-	-	-	-	-	-
Turbinates	-	-	-	-	-	-
Trachea	-	-	-	-	-	-
Lung	-	-	-	-	-	-
Spleen	-	-	-	-	-	-
Kidney	-	-	-	-	-	-
Bronchopharyn. Lymph node	-	-	-	-	-	-

\+ = Positive virus isolation

\- = No virus isolation

PM7 and PM14 = Cats necropsied on Day 7 and 14.

Table 6.5

FCV Subunit vaccine: Post mortem lesions in vaccinate and control cats

Cat Number	Lung pathology	Severity
Controls		
5	Alveolitis, with focal accumulation of macrophages and polymorphs in alveolar spaces. Severe bronchiolitis and epithelial denudation of the bronchioles	++
6	Proliferative interstitial pneumonia with thickening of alveolar walls by mononuclear cellular infiltration. Hypertrophy and hyperplasia of type II pneumocytes. Severe alveolitis and bronchiolitis. Prominent peribronchial and perivascular lymphocytic infilterations.	+++
Vaccinates		
1	Occasional focus of alveolitis and bronchiolitis. Peribronchial and perivascular lymphoid foci.	+
2	No abnormalities detected	-
3	Occasional focus of alveolitis and bronchiolitis	+
4	Alveolitis and bronchiolitis	+

DISCUSSION

The result of the vaccination experiment was interesting and encouraging. The vaccinated cats remained clinically normal throughout the period of virus challenge (Table 6.3; there were no significant pathological changes in one cat and in the others lesions were confined to mild bronchiolitis and alveolitis. By contrast, the unvaccinated cats exhibited clinical and pathological signs of infection associated with infection by this strain of FCV (E. Ormerod - personal communication). These included a biphasic temperature rise, progressive loss of condition, depression and anorexia. At necropsy, one of the controls had severe alveolitis and bronchiolitis and the other, in addition to these lesions had focal pneumonia. It would appear from these results that the vaccine protected against the effects of FCV infection. However, few experimental animals were used and until the vaccine is tested on a larger scale it is inappropriate to discuss its efficacy and duration of protection.

The present results were obtained with a vaccine prepared from purified 15S subunit which was filtered through a membrane with a pore size of 25 nanometres to remove any small quantities of contaminating infectious virus (see Plate 3.2). The reason for using partially purified material was to ensure that the antigen was well-defined and any biological effect which resulted from its use as an immunogen could be directly ascribed to it. In this way the experiment showed also that the 15S subunit acted as an antigen to evoke neutralizing antibodies, confirming the results in Chapter 5. Further experiments on the vaccine might profitably be done using filtered, crude, FCV-infected cell culture fluid to demonstrate that the expensive and time-consuming process of purification by centrifugation is unnecessary. However, it is likely that ultrafiltration would still be required as a final step in production.

An interesting question raised by the experiment reported here is to what extent the inactivated FCV vaccine described by Povey and Wilson (1978) relied on its presumed content of 15S subunit for its efficacy, in view of the fact, described in Chapter 3, that of the virus-specific components in viral harvests, the subunit (peak II) contains ten times as much viral protein as is in whole virus. It may be that by a process of filtration of the harvests, rather than chemical treatment, a superior immunogen is obtained. Further

experiments require to be carried out to answer these questions.

Feline calicivirus, therefore, joins influenza and rabies as examples of viruses from which a non-infectious subunit may be generated which acts as a vaccine against virulent virus challenge. The calicivirus vaccine is important not only for the use to which it might be put in cats, but in the context of a possible model for the development of future vaccines for other small RNA viruses of animals and man.

CHAPTER 7

GENERAL DISCUSSION

GENERAL DISCUSSION

The aims of this study were, to a large extent, accomplished. Substantial experimental evidence was provided to propose a scheme by which the virion of feline calicivirus is assembled from smaller subunits. By a study of the antigenic determinants of the virus and its major subunit, a non-infectious subunit vaccine was successfully developed. In addition, several observations were made which suggest avenues for further investigation of the molecular biology of FCV infection.

The purification of FCV as a preliminary step in studying its subunit structure yielded two homogeneous populations of viral components differing in density, sedimentation values, and not surprisingly, in content. The infective 170S component was identified as FCV while the slower sedimenting component with a density of $1{\cdot}26 g.cm^{-3}$ was shown to contain an aggregate of viral proteins sedimenting at 15S. Although slow sedimenting non-infective viral components have been associated with other small RNA-containing plant and animal viruses (Mayer *et al*, 1957; Kaper, 1960; Rhim *et al*, 1961; Schmidt *et al*, 1963; Halperen *et al*, 1964; Fabiyi *et al*, 1964; Planterose & Ryan, 1965; Graves *et al*, 1968; Breese, 1968), it is rather surprising how a similar component of FCV, in spite of its high protein content, has escaped detection until the present study.

One of the major attributes of the 15S subunit particle is the ability of its homologous antiserum to neutralize FCV infection as effectively as the antiviral serum in neutralization tests. When applied to *in vivo* situations, it was found that none of the cats vaccinated with the 15S antigen developed the calicivirus disease, whereas, the cats used as controls exhibited clinical signs of disease and one was found to be pneumonic at post mortem. Apart from its potency, the 15S prototype vaccine has the advantage of being cheaply produced and this could be a valuable asset in terms of commercial preparations.

The fact that viruses make use of subunit construction was first gleaned from the field of X-ray diffraction (Caspar, 1956; Caspar & Klug, 1962) and visualization of these subunits only became possible when the electron microscope technique of negative staining was applied to virus particles (Brenner & Horne, 1959). More recently it has been possible to study the subunit structure of F & MDV (Talbot & Brown, 1972) Mengo and EMC viruses (O'Callaghan, Mak & Colter, 1970; Lund *et al*, 1974) mouse-

elberfeld (ME) virus (Dunker & Rueckert, 1971) and rhinovirus 1A (McGregor & Rueckert, 1977) by controlled particle dissociation thus throwing some light on the mode of assembly of these viruses. Argueing on a similar basis, this thesis presents evidence that FCV is assembled in at least 2 stages and the supporting evidence is two-fold.

First, purified virus can be degraded by low pH into homogenous populations of smaller non-infective particles sedimenting at 15S which on exposure to reducing agents breaks down further into a 5S subunit. Each of these contains a polypeptide of molecular weight 65,000 daltons (Chapter 3) The second line of evidence was obtained by pulse-chase experiments of FCV-infected cells in which newly synthesized polypeptide is extremely rapidly converted into a 5S subunit which possibly through several unstable intermediates (9S, 11S & 13S) is incorporated within 30 seconds into a 15S component. Between 15 and 30 minutes after the initiation of polypeptide synthesis, complete and infectious feline calicivirions are formed in infected cells.

It should be noted that all of the work described in this thesis was done with one strain of FCV, the extra-large plaque type, G-1 (Ormerod & Jarrett, 1978). It is considered extremely unlikely that all FCV strains are not assembled in the same way; however, it would be useful in future work to compare the construction of several different FCV isolates.

Unlike poliovirus (Phillips & Fennell, 1973) EMC virus (McGregor, Hall & Rueckert, 1975) or foot-and-mouth-disease virus (Breese, 1968), the 75S procapsid is not an intermediate in the assembly process of FCV. No such structure is found in this study either by particle dissociation or in pulse-chase experiments of infected cells. The assembly of FCV, therefore, is more akin to the cardioviruses (Rueckert, 1971; McGregor, Hall & Rueckert, 1975) in which the capsid precursor proteins (5-6S) aggregate to form a pentameric 13S and later 14S subunits which then associate with the newly synthesized RNA to form a complete virion.

It will be helpful in future studies to estimate the molecular weight of the 5 and 15S subunit components to determine whether the 15S particle is a pentamer of the 5S component as in EMC virus (McGregor, Hall & Rueckert, 1975) ME virus (Dunker & Rueckert, 1971), or a trimer as in foot-and-mouth disease virus (Talbot & Brown, 1972). In this way it might be possible to deduce the number and the configurational arrangement of the subunit components in the mature virion.

Another subject for further experiments is the fact, discovered in this work, that there are (at least) two antigenic determinants on the FCV particle, one of which is shared with the 15S subunit while the other is unique to the whole virion. The common antigen appeared to be involved in the induction of virus neutralizing antibodies in cats and rabbits. It will be important to determine the biological significance of the other, unique antigen; and also to investigate how the serological cross-reactivity between different strains of FCV is related to the present findings.

It is relevant at this stage to return to the classification of FCV which is still controversial. Although the International Committee for the Nomenclature of Viruses (Wildy, 1971) classified the caliciviruses as members of the family picornaviridae, evidence is rapidly accumulating which suggests that caliciviruses belong to a new family of viruses. The reasons are as follows:

a) The morphology of caliciviruses is quite distinct, the particles having a fringe-like appearance and an easily recognisable pattern of staining (Zwillenberg & Burki, 1966; Almeida et al, 1968; Wawrzkiewicz et al, 1968). By contrast, the picornaviruses show little or no structural detail (Newman et al, 1973).
b) The diameter of 35-40nm is significantly greater than the values (24-30nm) obtained for other members.
c) Published sedimentation values for caliciviruses range from 160S to 207S (Wawrzkiewicz et al, 1968; Oglesby et al, 1971; Newman et al, 1973) and are greater than those for other picornaviruses (140S-160S, Newman et al, 1973).
d) Caliciviruses have lower (18%) RNA content (Burroughs et al, 1978) compared to about 30% found in other members of the picornavirus family (Rueckert, 1971).
e) Only one capsid protein is found in caliciviruses (Burroughs & Brown, 1974; Schaffer & Soergel, 1976) compared to 4 found in other members.
f) Typical picornaviruses show only virion size single stranded RNA (Levintow, 1974) whereas subgenomic RNA (multiple classes of RNA) has been identified in calicivirus-infected cell extracts (Ehresmann & Schaffer, 1977).

Cooper (1974) suggested that the genome strategy should be used as a major criterion in assessing the degree of relatedness among viruses and, on this basis, challenged the validity of the suggestion

that caliciviruses be removed from the family Picornaviridae. However, Black & Brown (1975/76) have since shown that the caliciviruses exhibit a strategy of replication which is completely different from the monocistronic polypeptide translation characteristic of other members of the picornaviridae. The present study has confirmed and extended these studies. No evidence was found of a large molecular weight precursor of the capsid polypeptide p65 in FCV-infected cells using a different, and more sensitive, method from that of Black & Brown (1975/76). Nevertheless, the present study did show that FCV, like some picornaviruses, is constructed in at least two stages from smaller protein subunits.

Although the caliciviruses are similar to the picornaviruses in certain aspects, these various significant differences appear to favour the suggestion (Burroughs & Brown, 1973; Newman et al, 1973; Ehresmann & Schaffer, 1977) that the caliciviruses be regarded as forming a new family, the Caliciviridae.

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