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ANTIGENIC STUDIES ON CORONAVIRUSES

by Shehab A. Yaseen

Thesis presented to the School of Graduate Studies
in partial fulfillment of the requirements for the
degree of Ph.D. in Microbiology and Immunology

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to Faeda

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LIST OF ABBREVIATIONS

AF	Ascitic fluid
AS	Antiserum
CAM	Chorioallantoic membrane
CF	Complement fixation
$CHCL_3$	Chloroform
CONV.	Convalescent serum
DOC or NaDOC	Sodium deoxycholate
FCS	Fetal calf serum
HCV/229E or 229E	Human coronavirus strain 229E
HEV	Hemagglutinating encephalomyelitis virus
IBV	Infectious bronchitis virus
LSC	Liquid scintillation counting
MHV	Mouse hepatitis virus
NP-40	Nonidet P40
PBS	Phosphate buffered saline
pfu	Plaque forming unit
PRO	Pronase
RNP	Ribonucleoprotein
SARK	Sarkosyl
SDS	Sodium dodecyl sulfate
SNF	Supernatant fluid
TART	Potassium tartrate gradient
TCID	Tissue culture infective dose
TGE	Transmissible gastroenteritis virus
TRYP.	Trypsin
TX-100	Triton X-100

229E/AS-H	Hyperimmune anti-229E antiserum absorbed by L132 host antigen.

GENERAL INTRODUCTION

In 1968, a morphologically distinct group of viruses, capable of causing illness in man and a number of animals, was recognized and because of the characteristic "corona"-like petals surrounding the virion, the proposal to name them coronaviruses was made (Tyrrell et al, 1968). In addition to the human strains, members of the group include the type species avian infectious bronchitis virus, mouse hepatitis virus, hemagglutinating encephalomyelitis virus of swine, transmissible gastroenteritis of swine, rat pneumotropic coronavirus, a calf diarrhea virus, a dog coronavirus, feline infectious peritonitis virus, and several others. In addition to the equine (Ditchfield, 1969) and simian isolates (Caul & Egglestone, 1979), viruses resembling coronavirus in morphology and morphogenesis have been described in association with different disease syndromes and in many animal species, suggesting that coronaviruses can cause disease or infection in most mammals and birds. It is probable that other strains specific for other animal species will emerge.

Historical Review

Coronaviruses are a large group of RNA containing agents. They infect a wide range of different species including man, pigs, mice, rats, cats, dogs, cows, horses, chickens, and turkeys. They are the cause of a variety of diseases which involve mainly the respiratory, digestive, and/or nervous systems. The specific disease manifestation is sometimes a characteristic of the species involved and the following is a brief summary of the clinical pattern of the accepted members of the group.

Human Coronaviruses (HCV)

The first human coronavirus reported to cause colds in human volunteers was code-named B814 and was obtained from a specimen collected in 1960 from a school boy suffering from a typical common cold (Tyrrell & Bynoe, 1965). Initial attempts to grow the virus in tissue culture failed but these workers were able to passage the virus recovered from the human upper respiratory tract in organ cultures of human embryonic trachea. The virus was subsequently examined by electron microscopy (Almeida & Tyrrell, 1967) and found to resemble the particles obtained from cultures of avian infectious bronchitis virus (IBV) (Berry et al, 1964). Thus, although the B814 agent could

be only partially characterized, it was concluded that a 'new' type of common cold virus had been propagated.

The first human agent of a similar type to be studied thoroughly had been isolated in 1962 from five Chicago medical students with upper respiratory illness (Hamre & Procknow, 1966). It was considered to be a separate agent due to its serological unrelatedness to other respiratory viruses known at the time and the clinical and epidemiological differences in the illness. The virus which was isolated from specimen 229E was chosen as the prototype strain, and it has been called 229E since then.

About that time, and in another laboratory, six viruses were isolated in organ cultures of human tracheal tissue from specimens which had been obtained from laboratory staff who were suffering from the common cold. The specimens were initially tested by a variety of other techniques but no viruses were recovered; they were then inoculated into organ cultures and passaged serially up to eight times. In the six positive series virus particles were observed in every passage following the second one. Like B814, these viruses were ether labile and failed to grow in cell culture, eggs, or in organ cultures of ferret trachea (McIntosh et al, 1967). Since then, more similar agents, LP and EVS, have been demonstrated by electron microscopy and were grown in human embryo tracheal organ

culture (Tyrrell et al, 1968).

Human coronavirus infections are most prevalent in the winter months and may occur in epidemic fashion, with the same strain being geographically widespread (Hamre & Beem, 1972; Kapikian et al, 1969; Hendley et al, 1972; Jackson & Muldoon, 1973). Recurrent epidemics of the same type do not seem to occur in sequential years. The epidemiological data available suggests that coronaviruses cause 3 to 4% of acute respiratory illnesses in humans and the clinical syndrome is usually that of a common cold. Based on serological evidence, it has been reported that only about one half of naturally occurring infections cause clinical illness (Kaye et al, 1971). Preliminary serologic investigations indicate that certain strains may infect children preferentially, whereas others are prevalent in adults.

The emergence of new epidemic strains with the temporary disappearance of older strains has been suggested and, in addition to human respiratory coronavirus strains, coronavirus-like agents causing other diseases have been reported. While more information must be obtained on these viruses before they can be included as official members of the coronavirus group, they are included in this summary.

Endemic Balkan nephropathy was first described in 1950 (Cookson & Reed, 1963) and reported to occur only in

adjacent areas of three Balkan countries, Yugoslavia, Bulgaria and Romania. About 20,000 cases were detected by Maselli et al (1970) from these three Balkan countries. As has been described with the other human coronaviruses, there was a tendency for familial clustering of the cases (Longsdon et al, 1972). Clinically the disease is marked by bilateral, symmetrical, progressive reduction in the size of the kidneys and tumors may develop in the proximal region of the urinary tract (Smith & Parker, 1970; Townley et al, 1970; Parker & Smith, 1973; Alston et al, 1974). The recovery of coronaviruses in gastro-intestinal outbreaks was first announced by Caul and Clarke (1975). However, though these enteric coronaviruses showed signs of growth in human intestinal organ culture by the increase in the number of virus particles, they were subsequently lost on passage (Caul & Clarke, 1975; Mathan et al, 1975; Caul & Egglestone, 1977).

Porcine Coronaviruses

Two different species of porcine coronaviruses have been described which cause differing disease syndromes and are easily distinguished serologically.

Hemagglutinating encephalomelytis virus (HEV): A disease causing encephalomyelitis in suckling pigs was first described in Canada by Alexander et al (1959). Richard and

Savan (1960) demonstrated that the disease was caused by a virus which was later isolated in cell culture (Greig et al, 1962). Two groups of workers then showed that the isolated hemagglutinating encephalomyelitis virus (HEV) belonged to the coronavirus group (Greig et al, 1971; Philip et al, 1971). Alexander et al (1959) described the infection as a disease affecting the nervous system of suckling pigs and suggested that it was not related to breed, husbandry or feeding practices, but that there was a marked age incidence. The disease was most common and severe in pigs under two weeks of age, while older suckling pigs were also affected but less severely. The mortality rate approached 100% within the very young litters but it was usually low in older litters (over three weeks of age). Since the original report, the disease has been found in the United States (Mengeling et al, 1972) and in many European countries (Cartwright et al, 1969; Gotink et al, 1969; Pensaert & Callebant, 1974).

Transmissible gastroenteritis virus (TGE): A disease called transmissible gastroenteritis was first described in the United States where it is still enzootic (Doyle & Hutchings, 1946; Haelterman, 1963). In 1963 it was reported in Japan (Harada et al, 1963) and several outbreaks of a highly infectious gastroenteritis caused by TGE were observed in Ontario (Ditchfield et al, 1967). Since the

disease has also been reported in many other countries it is now considered to be widespread throughout the world. (Gualandi, 1957; Knox, 1957; Godwin & Jennings, 1958; Pehl & Benndorf, 1960; Janowski & Golaszewski, 1961; Vishnaykov & Grosheva, 1961; Bratanovic et al, 1962; Eto et al, 1962; Huang & Lin, 1962; Adriansen et al, 1964; Aldasy et al, 1966).

Clinically the disease is characterized by watery diarrhea, vomiting, dehydration, and a high mortality in suckling pigs (100% among pigs less than one week old). TGE virus was shown by Tajima (1970) to be a member of the coronavirus group.

Murine Coronaviruses--Mouse Hepatitis Virus (MHV)

Murine encephalitis caused by a virus was first isolated by Cheever et al (1949). This virus was called the JHM virus. A virus causing hepatitis in mice was first recognized in a closed mouse colony by Gledhill and Andrewes (1951). The JHM virus was subsequently shown to be antigenically related to the mouse hepatitis virus strains discovered by Gledhill (Morris, 1959; Calisher & Rowe, 1966) and since that time all strains of mouse hepatitis virus (MHV) have been found to cause both hepatitis and encephalitis, although most are either primarily encephalitogenic or hepatitogenic.

Rat Coronaviruses (RCV)

A pneumonitis in newborn suckling rats and a symptomatic pulmonary infection in adults was described by Parker et al (1970). The virus agent was classified as a coronavirus on the basis of morphology and disease characteristics. A second group of rat coronaviruses, called sialodacryoadenitis viruses (SADV), were reported which infect rat salivary glands and could be transmitted from one rat to another by intranasal inoculation of a suspension of infected salivary glands (Jonas et al, 1969; Bhatt et al, 1972). No further studies have been reported to date.

Infectious Bronchitis Virus (IBV)

A highly contagious respiratory disease of chickens was described by Schalk and Hawn in 1931 and the infectious agent was first grown in eggs by Beaudette and Hudson in 1937. Initially two main types of virus were recognized; the Massachusetts type, of which the prototype strain was isolated by Van Roekel at the University of Massachusetts in 1941, and the Connecticut type, the prototype strain being isolated in Connecticut in 1951 (Jungherr et al, 1956). The Beaudette strain is now a completely egg-adapted non-pathogenic strain and generally considered to be of the 'Massachusetts type'. Later Hofstad (1958) reported three new strains (97, 104 and, 609), two of which were referred

to subsequently as Iowa 97 and Iowa 609. All these strains were found to be the cause of acute respiratory disease, tracheal rales and cough accompanied by a drop in egg production and a high mortality in young chicks (McDougall, 1968). Winterfield and Hitchner (1962) have reported two new virus isolates, designated the Gray and Holte Strain and these were found to be capable of producing nephrosis in experimental birds (Hirai & Shimakura, 1971). Other isolates continued to be isolated and appear to produce similar disease characteristics.

Transmissible Enteritis of Turkeys

Although "blue comb," a highly infectious enteritis of turkeys (TET) was recognized in the early 1950s, isolation and identification of the etiologic agent had eluded researchers. Even today there is a lack of agreement as to the cause. Workers (Deshmukh et al, 1969; Hofstad et al, 1969; Larsen, 1968) suggested that the etiologic agent was an enveloped virus, sensitive to heat and low pH. In 1971, Adams and Hofstad succeeded in propagating the blue comb agents in turkey embryos. Panigraphy et al (1973) first proposed these agents as belonging to the coronavirus group since he showed that coronavirus-like agents could be demonstrated consistently in "blue comb" positive preparations. The disease is characterized by acute intestinal disorder, dehydration, loss of weight, and high morbidity. Although turkeys of all ages appear to be susceptible, "blue comb"

generally occurs in young poults of one to six weeks of age. As in the case of TGE, "blue comb" carrier states persist after infection and the severity of the disease generally decreases with age at time of infection (King, 1975).

Bovine Coronavirus

A severe disease called neonatal calf diarrhea (NCDV) was first reported in the United States by Mebus and his associates (Mebus et al, 1972, 1973; Stair et al, 1972). They suggested one of the etiological agents was a coronavirus since an agent with coronavirus morphology was isolated from diarrheal feces of calves. The virus was isolated and propagated in a continuous bovine embryonic kidney cell line (Matumoto et al, 1970; Inaba et al, 1976). In addition to being identified in Canada (Acres et al, 1975; Morin et al, 1976), the neonatal calf diarrhea coronavirus (NCDV) has also been isolated in Great Britain (Woode et al, 1974), France (Wood & Bridger, 1978), and recently in Denmark (Wood & Bridger, 1978).

Canine Coronavirus (CCV)

During an epizootic of diarrheal disease in Germany a coronavirus-like agent was recovered from U. S. military

dogs (Binn et al, 1975). Experimental gastroenteritis in neonatal dogs and pups given infectious material was reported by Keenen et al (1976) and the virus was identified by electron microscopy as a coronavirus by Takeuchi et al (1976). In 1978 widespread outbreaks suddenly appeared in dogs throughout the United States. Dogs infected suffered from contagious vomiting and diarrhea, sometimes hemorrhagic. From stool and fresh intestinal specimens, several strains of a virus having the morphological features of coronaviruses were isolated in canine kidney cell cultures. Preliminary serological comparisons indicated that the 1978 isolates (Cornell) were very similar, if not identical to those recovered by Binn et al (1975). Lesions caused by this virus were similar to those caused by other gastroenteritis coronaviruses (calf diarrhea and transmissible gastroenteritis of pigs) (Appel et al, 1979; Pollock & Carmichael, 1979).

Feline Infections Peritonitis (FIP)

A viral agent causing peritonitis in cats was first described by Zook et al (1968). Ward et al (1968) reported the same findings and transmitted the virus experimentally. Feline infectious peritonitis (FIP) is a chronic progressive disease of domesticated and wild felines characterized by fever, emaciation, abdominal or thoracic exudation, or

granulomatous changes in a variety of organs and tissues. It is most typically seen in cats less than two years of age but it may occur at any age. Occurrence is usually sporadic but small epidemics have been reported (Povey, 1976).

In addition to the above, coronaviruses have also been isolated from foals located in an area of endemic foal diarrhea in the United States. Besides a profuse watery diarrhea and fever with extensive lymphatic involvement, a high mortality was reported (Bass & Sharpee, 1975).

Properties of Coronaviruses

Morphology

In 1968, Tyrrell et al grouped together viruses having a similar morphology. Although IBV was studied by electron microscopy as early as 1948 (Reagan et al, 1948; Reagan & Brueckner, 1952), it was not until the techniques of negative staining were applied that surface morphology was adequately characterized. Berry et al (1964) were the first to notice the difference in the morphology of IBV and that of the myxovirus group. A few years later, in studies of the properties of human coronavirus B814, Almeida and

Tyrrell (1967) described the distinct morphology of both B814 and strain 229E and noted their resemblance to IBV. McIntosh et al (1967), studying the coronaviruses isolated by organ culture (OC37, OC43 etc.) and mouse hepatitis virus (MHV), found that they had a similar morphology and suggested that they should be added to this morphologic group. It was at this point that the international committee suggested the family name "coronaviruses" for this group (Tyrrell et al, 1968). Coronaviruses then, are pleomorphic, 80-160 nm in diameter with large, widely-spaced, 12-24 nm long petals that form a characteristic "corona" around the particle. To date, morphology is still the main criterion for adding candidate members to this virus group.

Physical and Chemical Properties

Coronaviruses were considered to be RNA viruses on the basis of early studies because DNA inhibitors did not prevent a cytopathic effect (CPE) or infectious virus production (Bohl & Kumagi, 1965; Mallucci, 1965; Sheffy, 1965; Estola, 1966; Hamre & Procknow, 1966; McClurkin & Norman, 1967; McIntosh et al, 1967; Caletti et al, 1968; Harada et al, 1968; Tevethia & Cunningham, 1968; Witte et al, 1968; Bradburne, 1969; Greig & Girard, 1969; Kapikian et al, 1969; Pensaert, 1970). RNA has now been isolated and shown to be polyadenylated and infectious indicating

that it is of messenger (positive) polarity (Lomniczi, 1977; Macnaughton & Madge, 1977, 1978; Pocock, 1977; Schochetman et al, 1977; Tannock & Hierholzer, 1978). The RNA was found to be single stranded and of a high molecular weight ranging between 5.6 - 9.0 x 10^6 (Watkins et al, 1975; Lomniczi, 1977; Macnaughton & Madge, 1977, 1978; Schochetman et al, 1977; Tannock & Hierholzer, 1977). The RNA is associated with a small protein of 50,000 daltons molecular weight and is considered to be packaged as a single helical nucleoprotein (Garwes et al, 1976; Kennedy & Johson-Lussenburg, 1976; Macnaughton et al, 1977; Sturman & Holmes, 1977; Macnaughton et al, 1978).

Lipid solvents such as ether and chloroform markedly reduced the infectivity by disruption of coronavirus because of the essential lipid present in the envelope of the virus (Berry et al 1964; Nazerian & Cunningham, 1967; Kaye et al, 1970). Treatment with detergents such as SDS and DOC had a similar destructive effect, following which little infectivity or organized structure remained (Berry et al, 1964; Cartwright et al, 1965; Estola, 1966; Tevethia & Cunningham, 1968; Greig & Girard, 1969; Kaye et al, 1970; Hierholzer et al, 1972). Recently phospholipids and glycolipids resembling those of the host cell were reported to be present in the viral envelope (Pike & Garwes, 1977).

Coronaviruses contain several essential proteins. The number of polypeptides is now generally accepted to vary from 4 - 7 depending on the strain and the methods used

(Tyrrell et al, 1978). HCV and IBV have 6 or 7 polypeptides (Hierholzer et al, 1972; Hierholzer, 1976; Macnaughton & Madge, 1977), 5 polypeptides were reported in HEV (Pocock & Garwes, 1977), while both MHV and TGEV have 4 to 6 polypeptides (Garwes & Pocock, 1975; Sturman, 1977). The range of the molecular weights of these polypeptides also tends to vary and lies between 15,000 - 196,000 for HCV (Hierholzer et al, 1972; Hierholzer, 1976); 26,500 - 180,000 in the case of HEV (Pocock & Garwes, 1977); 33,000 - 130,000 for IBV (Macnaughton & Madge, 1977), 23,000 - 180,000 for MHV (Sturman 1977), and 28,000 - 200,000 for TGEV (Garwes & Pocock, 1975). Several peptides are glycosylated, probably the surface peptides (Hierholzer et al, 1972; Garwes & Pocock, 1975; Hierholzer, 1976; Macnaughton & Madge, 1977; Pocock & Garwes, 1977; Sturman & Holmes, 1977) and one, the 50,000 dalton protein, is the only major non-glycosylated component and has been identified as the nucleoprotein.

The sedimentation coefficient reported for HCV-229E was 378 - 400S and for HCV OC43 was 374 - 416S, while it was 330S for IBV, and 495S for TGE (Garwes & Pocock, 1976; Tyrrell et al, 1978). The pH of optimum stability of IBV and TGE is between pH 6.0 and pH 6.5, but there is conflicting or no evidence for the other viruses (Tyrrell et al, 1978). The heat stability of those viruses studied showed that they are rapidly inactivated at 56° while they

are slowly inactivated at 37° and moderately stable at 4° (Tyrrell et al, 1978). Coronaviruses are considerably unstable with common disinfectants and detergents (Tyrrell et al, 1978). The reported density of coronaviruses varies from 1.16 to 1.23 g/cm^3 in sucrose and 1.23 - 1.24 g/cm^3 in CsCl.

Antigenic Studies

Due to its early discovery, IBV was the first to be studied thoroughly and Woernle (1959) identified one precipitating antigen which developed in agar gel between infected allantoic fluid and immune chicken serum. Berry and Stokes (1968) studied 10 IBV isolates and identified a common precipitating line. An exclusive study of IBV-42 by Tevethia and Cunningham (1968) identified 3 antigens in crude allantoic and sonicated infected chorioallantoic membrane (CAM). Identical antigens were identified after ether treatment of partially purified IBV. These three antigens were different in physical and chemical properties from each other. These results paralleled the report of Chubb and Cunningham (1971) who found 3 antigens by using another ether-disrupted strain of IBV. However, they found only a single antigen in crude concentrated allantoic fluid and none in sonicated chorioallantoic membrane (CAM) (Chubb and Cunningham, 1971). A single

precipitation band was found by Hironao et al (1970) in both crude allantoic fluid and homogenized CAM. Bradburne (1969) reported 2 antigens in IBV-infected mouse brain.

Such detailed antigenic studies with the other coronaviruses have not so far been reported in the literature. The first published study on the precipitating antigens of TGE was the one by Philip et al (1971) as a brief statement that they had found a single antigen in TGE and HEV-infected cell lysates. Bohac et al (1975), using immunodiffusion tests to study TGE antigens, described a single precipitation line. Based on these methods, precipitating TGE antibodies were also found in milk whey from a sow vaccinated with inactivated TGE virus and in sera from sows and piglets either vaccinated or infected with the virus (Bohac & Derbyshire, 1976). Philip et al (1971) described a single precipitating antigen of HEV, however, a year later, Mengeling (1972) reported three HEV antigens. These antigens differed in their susceptibility to heat, ether, and chloroform. Bradburne (1970) described two antigens by using MHV3 and homologous hyperimmune serum. Recently antigens of bovine coronavirus strain LY-138 were studied in the agar gel precipitation test (Hajer & Storz, 1978) using hyperimmune serum prepared against purified intact virions, and four antigens were detected.

In spite of its importance in human infections, human coronaviruses have not been subjected to detailed antigenic study. This can be explained by the difficulty in propagating these viruses in a suitable tissue culture. Two antigens were described when 229E was tested with human convalescent sera (Bradburne, 1970). Using animal sera raised against HCV/229E preparations, the results were not clear enough for a reliable interpretation because of the profusion of anti-tissue lines which appeared in the reaction (McIntosh, 1969; Bradburne, 1970). Also precipitation lines were detectable only when the antigens were concentrated 10 to 20 times (Bradburne, 1970). Hierholzer, in 1976 described one antigen in agar gel immunodiffusion and immunoelectrophoresis by using purified 229E and its homologous rabbit antisera. Three antigens were detected by using suckling mouse brain grown HCV/OC43 (Hierholzer et al, 1972), while Bradburne (1970) identified four lines by using OC43 and its specific hyperimmune serum. One of these antigens was described as a host antigen (Hierholzer et al, 1972).

Coronaviruses are a large group with a complex viral structure and it might be expected that antigenic interrelationships within strains and between members of the group would exist. All the swine TGE strains were found to be related and no antigenic differences were reported (Lee et al, 1954; Sasahara et al, 1958; Bohl & Kumaga, 1965; Ristic et al, 1965; Cartwright, 1966). However, serological

variations between strains of the avian coronavirus IBV were first described by Jungherr et al (1956). Winterfield et al (1964) indicated that the Australian IBV isolates were related to the American isolates but that the relationship was entirely "one-way". In the United Kingdom, all the isolates were of the 'Massachusetts type' (Berry & Stokes, 1968; Dawson & Gough, 1971). Comparing New Zealand, Australian, and American IBV strains by neutralization tests, New Zealand's were different from the others, but a weak relationship did exist (Pohl, 1967; Lohr, 1976). Mouse hepatitis virus (MHV) strains were all interrelated either by complement fixation or neutralization tests (Gledhill & Niven, 1955; Dick et al, 1956; Morris, 1959; Starr & Pollard, 1959; Manaker et al, 1961; Calisher & Rowe, 1966; Morris et al, 1966). One report by Philip et al (1971) described an antigenic relationship between TGE and HEV by immunodiffusion but not by the neutralization test. No relationship between strains of IBV and other coronaviruses were reported. The widest cross-relationship yet described for coronaviruses was between MHV and other coronaviruses. MHV strains were related to the rat coronaviruses (RCV and SADV) by CF tests and not by neutralization (Parker et al, 1970; Bhatt et al, 1972). MHV and both HCV/OC38 and HCV/OC43 were also related (McIntosh et al, 1967). MHV-3 has also been shown to be related to strain 229E (Bradburne, 1970). McIntosh et al (1969) found

that IBV-42 was unrelated to the human and murine viruses and that 229E had very little, if any, relationship with the murine or other human strains tested by neutralization, CF, and fluorescent antibody tests. Using the same tests, McIntosh et al, (1969) found that three of the organ culture viruses were related to several strains of MHV. Relationships between OC43 and HEV were identified by hemagglutination inhibition, complement-fixation, and neutralization tests (Kaye et al, 1977). Pedersen et al's (1978) study of a few coronaviruses by direct and indirect fluorescent antibody procedures have divided those coronaviruses tested into two groups on the basis of their relationships. In the first were MHV-3, HEV-67N, NCDV and HCV/OC43 and in the second were FIP, TGE, HCV/229E and CCV.

The data from experiments examining the antigenic components of coronaviruses tend to be confusing. Part of this confusion can be attributed to the variety of techniques used to study various members of the group and the lack of a comprehensive study of these antigens in relation to their structural and biological functions. Some viral or viral-modified host proteins may not be good immunogens and, therefore, can only be detected by the concentration of both antigens and antisera. The known incorporation of host (Pike & Garwes, 1977) components into the infectious virion makes the characterization of such antigens a difficult task since any procedures designed to yield highly-purified antigens may

succeed simply in removing essential viral envelope along with non-virion host components and/or host impurities.

Since their original discovery which, in some cases was a few decades ago, relatively little information has been published on the antigenic relationship between coronaviruses. And because of the variations in the test systems, it has been difficult to assess their inter-relationships. Thus, as more has become known about coronaviruses as well as the fast increase in candidate members, the problems with the interpretation of their antigenic structure and relationships have not only increased but become an important issue with regard to the classification of these agents.

AIMS OF THE STUDY

1. To detect and identify the antigenic components of 229E and subsequently correlate them with the structure and function.

2. To correlate HCV 229E antigenic components with those of other animal coronaviruses and investigate these relationships in more detail by the isolation and characterization of the internal components of 229E, TGE, and HEV.

3. To extend the study by conducting a serological survey for coronavirus precipitins in the normal sera from a variety of animals including pigs, cats, dogs, and cows, using the immunodiffusion test.

MATERIALS AND METHODS

Viruses Used in the Study

HCV 229E

This virus was originally obtained from Dr. A. Z. Kapikian at the U.S. National Institutes of Health, Bethesda, Maryland. It was reported that it had been passed in WI-38 cells and, after an initial two passages in WI-38 cells in this laboratory, has since been cultivated in L132 cells.

Mouse Hepatitis Virus (MHV)

A semipurified suspension of this virus was received from Dr. K. Holmes of the Uniformed Services, University of the Health Sciences, Bethesda, Maryland.

Transmissible Gastroenteritis Virus of Pigs (TGE)

The Purdue strain, prepared in primary pig kidney cells, was supplied by Dr. G. Dulac of the Animal Disease Research Institute (ADRI), Ottawa, Ontario.

Hemagglutinating Encephalomyelitis Virus of Pigs (HEV)

This virus was also cultivated in our laboratory from an inoculum and primary pig kidney cells obtained from Dr. G. Dulac of the ADRI.

Infectious Bronchitis Virus of Chickens (IBV)

The unpurified virus, cultivated from infected chick embryos, was also obtained from the ADRI.

Cell Cultures

The type of cell used depended on the virus type and the experimental design. Each of the types used are outlined below.

L132 Cells

These human embryonic lung cells derived by Davis and Bolin (1960) were obtained from Mr. D. A. McLeod, Laboratory Centre for Disease Control (LCDC), National Health and Welfare, Ottawa, who in turn had obtained them from the American Type Culture Collection (ATCC).

The passage number from tissue of origin is unknown (Shannon & Macy, 1972) but our numbering system was a continuation of that used by LCDC. The cells had been passaged five times at a 1 to 2 ratio at LCDC however, upon arrival at our laboratory, they were passaged at 1 to 3 split ratios. The cells used in this study were limited to those between passage 20 and 40.

WI-38

This diploid cell strain, derived from human embryonic lung (Hayflick, 1965), was obtained from the American Type Culture Collection, Maryland, U.S.A.

Human Fetal Lung (HFL)

Diploid cell strains similar to human embryonic lung (WI-38) were obtained through the courtesy of Mr. P. A. Phipps and Mr. B. MacCulloch of the Ottawa Regional Virus Laboratory, Children's Hospital of Eastern Ontario, Ottawa. These strains were used while in phase II of their passage history (Hayflick & Moorhead, 1961; Hayflick, 1965).

All the above mentioned cell cultures were grown in Eagle's minimal essential medium (MEM, 'Auto-Pow', Flow Laboratories, Inc.) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin, 100 units/ml; streptomycin, 100 µg/ml, and neomycin, 50 µg/ml). Only the

L132 cell line was used throughout the project while WI-38 and HFL were used only in a small scale to compare with L132 cells.

All cell cultures were grown in 75 cm^2 disposable plastic flasks (Falcon Plastics, Inc. Flow Lab.) and used within 24 hours of reaching a confluent monolayer.

Primary Pig Kidney Cells

They were supplied as a cell suspension by Dr. G. Dulac from the ADRI. The growth medium was a modified Hank's lactalbumin hydrolysate (HBSS-LAH) (Greig et al, 1967; Dulac et al, 1977).

Virus Preparations

HCV/229E

Confluent monolayers of L132 cells were produced within 2 days of splitting and contained between 1.5 - 1.8 x 10^7 cells. Prior to infection, the growth medium was removed and the cells washed with phosphate buffered saline (PBS). The PBS was removed and the cells were incubated for 24 hours in maintenance medium (M-199, Flow Laboratories) without fetal calf serum (FCS). The cells were again washed with PBS and were left to drain for several minutes before

being inoculated with 0.5 ml of virus suspension having a titre of 10^7 - 10^8 pfu/ml. The virus was adsorbed for 60 minutes at room temperature during which time it was redistributed over the surface every 15 minutes. At the end of the adsorption time the cells received 10 - 12 ml of M199 without serum and were incubated at 33°C for 36 - 40 hours. After this time the cells were either frozen at -80°C or the contents were subjected to 3 cycles of freezing and thawing to release the virus from the cells.

For the preparation of labelled virus, the cells were infected as above and received 10 - 12 ml of MEM or M199 plus (5-^{3}H)-uridine (10 µCi/ml, New England Nuclear). Incubation and subsequent treatments were the same as those for unlabelled virus.

The virus production was determined by plaque assay (Kennedy & Johnson-Lussenburg, 1976) in monolayers of L132 cells in 75 cm^2 disposable culture flasks. Dilutions of the virus were prepared in cell growth medium at ice bath temperature. Inocula of 0.33 ml were added to well drained monolayers and allowed to adsorb at room temperature for 60 minutes again re-distributing the inoculum over the cell sheet at 15 minute intervals.

At the end of the adsorption period, 25 - 30 ml of the following overlay medium, at 40°C, was added to each flask: M199 plus 0.2% $NaHCO_3$; 5-bromodeoxyuridine (BUdR),

50 µg/ml; DEAE-dextran, 200 µg/ml; 2% FCS; antibiotics; and 0.6% Oxoid agar no. 1. After the solidification of the overlay, the flasks were incubated with the cell surface uppermost for 6 to 7 days at 33°C. The cell monolayer was then fixed with 4% formaldehyde in saline for at least one hour at 37°C. The agar was then removed and the cell sheet was stained with crystal violet to facilitate counting of the plaques.

TGE

This virus was prepared in primary pig kidney cells or in a pig kidney cell line after three passages at a 1 to 3 split ratio. Only the primary pig kidney was grown in "HBSS-LAH" solution; all others were done in MEM. The cell monolayer, prior to inoculation with the virus, was washed with PBS then left with MEM without FCS for 24 hours. The cells were again washed with PBS and inoculated with 5 ml of TGE diluted in MEM to a titre of 1.58×10^4 $TCID_{50}$. The virus was adsorbed at 37°C for 30 minutes. The monolayer was then covered with an additional 10 ml MEM without FCS and the flasks were incubated at 37°C for 48 hours. At the end of the incubation time a 90% cytopathic effect (CPE) had developed. This was typified by clusters and strings of large round cells, most of which were floating in the maintenance medium.

Labelled virus was prepared in the same way it had been prepared for HCV/229E.

HEV

The virus was prepared in the same way TGE had been using primary pig kidney cells. The cells were grown in Hanks balanced salt solution for the first passage but were then switched to MEM + 10% FCS in the subsequent passages. The cell monolayer was washed with PBS and left for 24 hours in MEM without FCS. After that, the cells were washed again with PBS and infected with 5 ml of HEV inoculum diluted in MEM to give the titre 3.17×10^4 $TCID_{50}$. The virus was adsorbed for 30 minutes at 37°C. Maintenance medium (10 ml of MEM without FCS) was added, and the infected cells were incubated at 37°C for 48 hours. Again the flasks not used were stored at -80°C while those to be used were freeze-thawed 3 times. Labelled virus was prepared in the same way as HCV/229E.

Concentration and Purification of 229E Virus

Clarification and Purification

The virus was prepared in L132 cells as described along with a few (5-^{3}H)-uridine labelled flasks as a marker

to locate the virus peak in the gradients. In this step the cells were subjected to 3 freeze-thaw cycles to release the cell-associated virus which has been estimated to be 75 - 90% (D. Kennedy, Ph.D. Thesis, 1977). The resulting mixture was clarified by centrifugation at low speed (2,000 rpm for 20 minutes) in an IEC PR6 centrifuge at 4°C. This procedure was designed to remove the large cell debris. Clarified whole culture lysates were centrifuged at 48,000 g for 60 minutes at 10°C in the Beckman Spinco SW25.2 over a cushion containing 7 ml of 65% w/v sucrose. After centrifugation, an opalescent band was visible at the interface. The band was collected in 4 ml per tube by either tube bottom puncture, or without puncture from the top of the tube. The cushioned virus band, containing a mixture of sucrose and maintenance medium, was further concentrated and washed free of sucrose by ultrafiltration using the XM300 membrane in an Amicon UF52 cell under nitrogen pressure in a cold room. Since the virus bands contained about 40% sucrose, successive washings with 0.001M phosphate buffer (PO_4) pH 7.2 (see appendix) were required to reduce the concentration to 2% or less.

Density Gradient Centrifugation:
Rate Zonal Centrifugation

Sucrose gradients were prepared in cellulose nitrate tubes supplied by Beckman for the SW25.2 rotor. Six step gradients were prepared using 9.3 ml each of 10, 15, 20, 25, 30, and 35% w/w sucrose (Schwartz-Mann, density gradient grade), for a total volume of 55.8 ml per tube. After formation, the step gradients were refrigerated overnight at 4°C to allow diffusion to linearity. The gradient shape was chosen so that virus sedimentation was not likely to be density-inhibited, yet a good separation from non-viral components could be expected (Anderson et al, 1966).

Gradients were overlayered with 2 ml of virus concentrate per tube and immediately run at 63,000 g for 90 minutes at 10°C after which 2 ml aliquots were again collected through either tube bottom puncture or from the top of the tube. The sucrose concentration in the fractions was determined in a refractometer (Erma Optical Works, Ltd., Tokyo). The position of the virus band was determined by the peak of (5-^{3}H)-uridine labelled virus as measured by liquid scintillation counting of portions of the above aliquots. Two liquid scintillation fluids were used; the first consisted of BBS-3, 100 ml; butyl-PBD, 8 gm (both from Beckman Instruments, Inc.); water, 50 ml and toluene to 1 litre (Newman, 1973), while the second, used for aliquots of less than 200 µl (Newman 1973) contained BBS-3, 40 ml;

PPO, 5 gm (Amersham/Searle); water, 20 ml and toluene to 1 litre.

Density Gradient Centrifugation:
Equilibrium Density Gradient Centrifugation

Steeper sucrose gradients were used for further purification and separation of the whole virus from the other contaminants. These were prepared manually, using 9/16 x $3\frac{1}{2}$ inch cellulose nitrate tubes obtained from Beckman for the SW41 rotor. Gradients were formed by layering 1.8 ml amounts of 6 sucrose solutions decreasing in concentration from 65% to 15% w/w sucrose by 10% steps and the gradients were left to diffuse to linearity before being used. Samples of 0.6 ml volume were applied to the gradients. Running conditions were variable, but usually were at 63,000 g for a minimum of 20 hours unless otherwise referred to in the text. Fractions (0.5 ml) were collected by either bottom puncture or from the top of the tube for subsequent analysis.

Sucrose solutions, with only a few exceptions, were prepared from a 66% w/w working stock and diluted according to a curve supplied by Beckman (reproduced in Griffith, 1975). Buffers, reducing agents, and other additives were used when required as a portion of the diluent and were only used for certain specific conditions as will be described.

Antigen Preparation

The following antigens were prepared and used throughout the study.

Host Antigen (L132 Cells)

The host antigen was the L132 cell line (L132) which was used to grow HCV/229E. After the formation of the monolayer, the cells were washed 2 times with PBS. Each time the PBS was left on the monolayer for 30 minutes. MEM without FCS was then added to the cells and left for 24 hours. The cells were again washed with PBS and finally covered with 5 ml 0.001 PO_4 buffer. The monolayer was then frozen and thawed 3 times at -20°C.

Virus-host Antigen (229E Infected Cells, L132)

Two types of virus-host (229E/L132) antigens were used. One type was prepared by infecting the cell monolayer as described above using the mixture as the antigen. The other was prepared by using only the cushioned band after it had been washed and concentrated by ultracentrifugation.

HCV/229E Virus Antigens

The virus used for antiserum preparation had been

concentrated on a sucrose cushion, then purified by rate zonal followed by equilibrium ultracentrifugation on the sucrose gradient consisting of 1.8 ml of 15, 25, 35, 45, 55, and 65% w/w. The labelled virus peak was collected as before. Then the washed virus was concentrated by pelleting at 100,000 g for 3 hours in a Spinco Type 50 rotor. The pellet was resuspended in 0.001 M PO_4 buffer. The virus was concentrated approximately 300 to 1 before being used as an antigen.

All virus samples (229E, TGE, etc.) were evaluated by electron microscopy, and protein estimation before and after purification.

229E-RNP Antigen

Ribonucleoprotein was isolated by the procedure described by Kennedy and Johnson-Lussenburg (1976).

HCV/229E labelled with (5-^{3}H)-uridine was initially concentrated on sucrose cushions, further concentrated by ultrafiltration and then purified by rate zonal ultracentrifugation as described above. The purified virus preparations with or without further equilibrium sucrose density gradient purification were treated with NP40 to release the internal component. The following stock solutions were used:

Tris-EDTA buffer was prepared as a stock 1 M in Tris ('Trizma' brand of tris-(hydroxymethyl)-aminomethane, Sigma Chemical Co.), 0.1 M in EDTA (disodium ethylenediaminetetracetate, A.C.S., Fisher Scientific) adjusted with HCl to give a pH of 7.2 at 1/100 dilution. Dithiothreitol (DTT, Sigma) was freshly prepared as a 10% w/v solution in deionized water and Nonidet P-40 (NP40, Shell Chemicals Co.) was similarly prepared as a 2.5% v/v solution. The disrupting solution used in the experiments contained 0.5% NP40, 2% v/v DTT, 1/50 of Tris-EDTA buffer prepared from the above stocks. To this solution was added an equal volume of freshly purified virus in 0.001 M phosphate buffer and the mixture was immediately layered onto preformed, precooled sucrose gradients, 25 - 65% w/w containing 0.4% DTT and 1/100 dilution of Tris-EDTA buffer. Untreated virus was mixed with an equal volume of 0.001M phosphate buffer and run in parallel on phosphate buffered sucrose gradients to serve as a control. The gradients were centrifuged in a Spinco SW41 rotor at either 37,000 rpm (170,000 g), for 21 hours at 10°C, or, at 30,000 rpm (120,000 g) for 40 hours. All virus preparations were a mixture of unlabelled and labelled virus so that peak virus and RNP levels could be determined. Gradients were fractionated as described above. Aliquots were taken for liquid scintillation counting (LSC) into the previously described cocktail.

TGE Antigen

This virus was purified using the same procedure as for the preparation of 229E antigen. Again the virus was concentrated by approximately 300 to 1 before being used as an antigen (TGE).

TGE-RNP Antigen

TGE-RNP was isolated in the same way as 229E-RNP.

HEV Antigen

This antigen was prepared in the same way as HCV/229E and TGE antigens.

HEV-RNP Antigen

The antigen was also prepared in the same way as TGE-RNP.

MHV Antigen

Mouse hepatitis virus (MHV) as a semi-purified concentrate and antisera prepared in rabbits were obtained from Dr. K. Holmes. Due to the necessity of using a strong antigen, MHV antigen was concentrated further by pelleting at 40,000 rpm for 4 hours and resuspending the pellet in 1/10th volume of 0.001 M PO_4 buffer.

IBV Antigen

IBV-containing allantoic fluid was clarified by low speed centrifugation (2,000 rpm for 30 minutes at 4°C), after which the supernatant was applied onto 65% w/v sucrose cushions and concentrated as before. The interface virus band was collected and placed in an ultrafiltration unit where it was washed several times in 0.001 M PO_4 buffer before concentrating.

Antisera and Ascitic Fluids

Preparation

Antisera and ascites were prepared against 229E-virus antigen (229E), 229E-infected L132 cell antigen (229E/L132), and L132 host antigen (L132) in guinea pigs

using a modification of the procedure described for the preparation of ascitic fluid in mice (Munoz, 1957; Sommerville, 1967). Male guinea pigs (Hartley/albino outbreed) weighing 400 - 500 gm were used. The animals received the first sensitizing dose by injecting 0.2 ml of the antigen in the footpad. Four days later, 1 ml of 50% Ag + 50% Complete Freund's Adjuvant (Grand Island Biological Company) was given subcutaneously and was followed two weeks later by 1 ml of antigen/adjuvant mixture given intramuscularly. This was again followed by the same injection in another two weeks. After this series of injections the ascitic fluid preparation was started with a 4 ml peritoneal injection (2 ml antigen and 2 ml Complete Freund's Adjuvant) two weeks after the last intramuscular injection. The animals continually received these injections every week for four weeks. Fifty percent of the guinea pigs started to develop ascitic fluid after the second intraperitoneal injection. Those that didn't were stimulated to do so by a third or fourth intraperitoneal injection. The fluid was drained when distention of the abdominal cavity was noticeable, usually every two days. This was accomplished, without anesthesia, using a 50 ml syringe equipped with an 18 gauge needle that was inserted into the lower left part of the abdomen. The fluid was then transferred to 50 ml Corning disposable centrifuge tubes (Canlab Ltd.) and left overnight at 4°C. The samples were then centrifuged at

3,000 rpm for 20 minutes. An aliquot of the supernatant fluid was titrated to determine the concentration of antibody, the remainder was stored at -20°C.

The animals were bled before the administration of the antigen and weekly after the antigen had been administered, by the heart puncture route. The blood was left to clot overnight at 4°C and the serum was isolated by centrifugation at 2,000 rpm for 20 minutes.

MHV antisera (MHV/AS) were prepared and supplied by Dr. K. Holmes.

Hyperimmune convalescent TGE antisera (TGE/AS) and convalescent HEV antisera (HEV/AS) were obtained through the courtesy of Dr. G. Dulac and IBV antisera (IBV/AS) prepared in chickens were obtained from Dr. E. Thomas, both of ADRI, Hull.

Normal bovine (COW/S), feline (CAT/S), and canine (DOG/S) sera were obtained from frozen stock samples stored in this department, through the courtesy of Dr. R. Fyson.

Concentration

Anti-229E sera (229E/AS), ascitic fluid (229E/AF), 229E convalescent sera (229E/CONV.), anti-L132 host sera (L132/AS), and anti 229E/L132 sera (229E/L132/AS) were concentrated by ultrafiltration or ammonium sulphate

precipitation.

Ultrafiltration: Concentration of serum and ascitic fluid by this method was done using a PM30 membrane in an Amicon ultrafiltration cell under nitrogen pressure in a cold room.

Ammonium sulphate precipitation: This method was used to precipitate out the gamma globulins from the serum and ascitic fluid. In this procedure one volume of saturated ammonium sulphate was added (drop-by-drop) to every two volumes of serum or ascitic fluid, the entire suspension being continually stirred. When the appropriate amount of ammonium sulphate had been added, the pH of the suspension was adjusted to 7.8 with 2N NaOH. The stirring of the suspension continued for an additional two to three hours to limit the amount of other serum components that are often trapped in the gamma globulin precipitate. The suspension was centrifuged at room temperature for 30 minutes at 1,400 g (about 3,000 rpm). The precipitate was washed two times in saline before being resuspended in borate-buffered saline (5 parts of borate buffer with 95 parts of saline, pH 7.8).

The ammonium sulphate was removed from the precipitate by dializing against borate-buffered saline for three to four days at 4°C. The dialysate was changed twice a day, each time checking for sulphate ions by adding a few drops of 2% barium chloride to a small aliquot of the dialysate. In the presence of sulphate ions a white precipitate of

barium sulphate is formed. The dialysis is complete when there is no more barium sulphate precipitated. After dialysis the suspension was centrifuged at 1,400 x g for 30 minutes at 4°C to remove some of the insoluble material formed during dialysis. The final slightly opalescent suspension was stored at -20°C.

Methods for Assaying Antigens and Antibodies

Neutralization

Two-fold serial dilutions of antiserum or ascitic fluid were prepared in saline. Aliquots of 0.5 ml of these fluids were mixed with 0.5 ml of a suspension of 229E in M-199 at a concentration of 504 pfu/ml. After one hour incubation at room temperature 0.33 ml of the mixture was added to an L132 cell monolayer in 75 cm^2 flasks. Two monolayers were used for each dilution. After one hour of adsorption, during which time the inoculum was frequently redistributed over the monolayer, the agar overlay previously described was added to the cells which were then incubated at 33°C for six to seven days to develop plaques. A virus control consisting of 0.5 ml virus diluent plus 0.5 ml saline was prepared from which a 0.33 ml sample was assayed. Also included was a cell control in which uninfected cell development

was monitored. The highest dilution of serum or ascitic fluids showing 50% plaque inhibition was considered the end point of the titration.

Immune Electron Microscopy

The EM was used here to study the extent of viral agglutination in the presence of either immune sera or ascitic fluid. Purified virus antigen, as prepared before, was used. Dilutions of anti-virus ascitic fluid (229E/AF; 1/10, 1/50) and anti-virus antiserum (229E/AS; 1/50, 1/100) were prepared in the wells of a micro titre plate. Ten microliters of each dilution were mixed with 10 µl of virus preparation and incubated at room temperature for 30 minutes. Grid preparation will be described later.

Protein Estimation of the Antigens

Two procedures for protein estimation were used. The first one was the procedure of Lowry as was described by Leggett Bailey (1962). The second is the one described by Marian M. Bradford (1976) for the quantitation of microgram quantities of protein. In both procedures bovine serum albumin was used as a standard solution. Either one procedure or the other was used, depending on the quantity of sample available.

Immunodiffusion

Antigen-antibody reaction was analyzed by the Ouchterlony double diffusion test (O. Ouchterlony, 1949). The technique used in our experiments was the one previously described by Johnson et al (1964). In their study cellulose acetate strips were found to give a more complex system of component lines as well as better separation and resolution than were obtained with agar.

The cellulose acetate strips (Gelman Instrument Company) were cut transversely into pieces and soaked by immersing them in buffer containing Tris, KCl, and azide. These strips were then put on a clean glass slide and the perspex template described by Johnson et al (1964) was placed at one end of the strip. The template was then slipped into a central position along the wet surface thereby eliminating the formation of air bubbles. The prepared slides were placed into small moisture chambers (square petri dishes, containing damp filter paper) which were in turn placed in a larger moisture chamber. Precipitation lines were allowed to develop over 40 to 48 hours at 23°C. Following incubation the templates were removed under running tap water and the cellulose acetate strips were removed and washed in physiological saline for a minimum of four hours followed by a ten minute wash in distilled water.

All immunodiffusion reactions were stained with thiazine red (Crowle, 1958) and direct enlargements made on F-3 Kodabromide paper (Eastman-Kodak Ltd.).

Besides the above mentioned buffer, others (0.001 M PO_4, Tris, "Borate-saline," and PBS) were tried and found to be as effective as the Tris, KCl, and azide buffer.

Absorption of 229E (Virus) Antisera by L132 (Host) Cell Antigen

During initial immunodiffusion experiments there appeared to be a large amount of host antibody in the immune fluids (antiserum and ascitic fluid) even after the extensive purification steps previously described for the purification of HCV/229E. Thus an attempt was made to absorb out the excess host antibody by mixing 1 ml of anti-virus serum with 4 ml of packed L132 cells. The mixture, with frequent shaking, was first incubated at 37°C for two hours and was then transferred to 4°C for 24 hours. At the end of the incubation, the mixture was centrifuged at 5,000 rpm for 30 minutes. The supernatant (absorbed serum) was removed and centrifuged at 4,000 rpm for 30 minutes to remove the remaining debris. When this absorbed serum was tested in immunodiffusion experiments, it appeared that host antigens had been contributed to the

serum. These antigens could not be removed by two cycles of ultracentrifugation at 75,000 g for either 90 minutes or four hours. Because of these 'new' antigens, sera absorbed in this manner had a limited use in our immunodiffusion analysis consequently absorption procedures were not carried out further.

Disruption of Antigens

Because of the delicate structure, the coronavirus disrupts spontaneously during the normal procedure of purification or storage at high temperatures, and virus RNP has been demonstrated even following storage of 229E at 4°C (Kennedy & Johnson-Lussenburg, 1976). In order to obtain a larger amount of viral components, several disrupting agents were tested. Following treatment with any of the agents described below, the resulting suspension was tested using the previously outlined immunodiffusion procedure.

Ether (Mallinckrodt Chemical Works)

An equal volume of ether and virus antigen were incubated for two hours at 37°C with occasional vigorous shaking. The two layers were allowed to separate and the

ether layer was removed by pasteur pipet. The residual ether was evaporated using a gentle stream of nitrogen or by exposure to air.

Chloroform (Fisher Scientific Company)

In this procedure 0.1 ml of chloroform was added to 0.1 ml of purified virus. The mixture was swirled gently for two hours at room temperature. The chloroform was then evaporated at 37°C (Kates et al, 1961; Eckert, 1966).

Trypsin (Grand Island Biological Company)

To 1 ml of purified virus, 0.1 ml of 2.5% trypsin was added, and the mixture was incubated at 37°C. At the end of two hours incubation, 0.05 ml of 5% soy bean trypsin inhibitor was added to stop the reaction.

Pronase (Calbiochem)

A 0.2 ml sample of purified virus was treated with 20 µg of pronase. The mixture was incubated at 37°C for six hours with intermittent agitation.

Triton-X-100 (Rohm & Haas Company)

A 10% TX-100 solution in distilled water was incubated with antigen suspension to a final detergent

concentration of 1%. The mixture was incubated at room temperature for two hours with frequent shaking.

Sodium Deoxycholate (Difco Laboratories)

An aqueous solution of 10% stock sodium deoxycholate was added to an antigen suspension to a final concentration of 1%. The mixture was then incubated for two hours at room temperature to complete the procedure.

Sarkosyl (Ciba-Geigy Foundation)

This was prepared in an aqueous solution to give a 20% concentration. It was incubated with the purified virus antigen. The mixture had the final concentration of 1% of Sarkosyl. It was incubated for two hours at room temperature with frequent shaking.

Electron Microscopic Preparations

Most investigations were done using the negative staining technique of Brenner and Horne (1959). A 2% aqueous solution of phosphotungstic acid adjusted to a final pH of 6.4 was found to give the best results with 229E and was also used for the other coronaviruses examined.

Specimens for electron microscopy were prepared by several methods; one was similar to that described by Howatson (1969). Using finely drawn pasteur pipettes, preparations were adsorbed for one minute directly onto carbon-stabilized formvar-coated grids. Excess sample was drained by touching filter paper to the edge of the grid. The grid was washed once or twice with distilled water, again draining the excess with filter paper. The staining solution was then applied to the grid, allowed to remain for 30 to 60 seconds, drained as above, and the grid was allowed to air dry.

In a second method the material to be examined was diluted in 0.001 M phosphate buffer and centrifuged at 100,000 g for two to three hours. The resulting pellets were resuspended in distilled water and a droplet was applied to the grid, mixed with stain in situ, the excess was drained off, and the grid allowed to air dry.

In a third method the grids were prepared using the agar diffusion method described by Anderson and Doane (1972). This method was found to produce acceptable specimens from a variety of solutions.

Once prepared, grids were examined immediately in the EM or stored in a desiccator until they could be screened. All examinations were done with a Philips EM300 electron microscope.

Analysis of Immunoprecipitates of Virus and Host Antigens

Initially we were interested in following the overall pattern of events during the 72 hour period following infection to detect the changes as they were reflected by the composition of the cell lysate (replicative events) and cell supernatant (released virus).

In the first experiment enough flasks (75 cm^2) of L132 cells were infected with 229E to provide 10 flasks at eight hour intervals for a total period of 72 hours. At each interval the supernatant fluid (SNF) was decanted from each of the 10 flasks and replaced with equal volumes of 0.001 M phosphate buffer. The monolayers were subjected to three cycles of freezing and thawing after which each group of samples was pooled to give a total of nine lysate and nine SNF samples. The samples were centrifuged at 2,000 rpm for 20 minutes to remove the large cell debris. They were then further clarified and concentrated by centrifugation onto sucrose cushions at 48,000 g for 60 minutes at 10°C in the Beckman Spinco SW 25.2 rotor. The samples were then pelleted by centrifugation at 100,000 g for three hours in a Spinco FA50 rotor. The pellet from the SNF samples was resuspended in 1 ml buffer while the lysate pellet was resuspended in 2 ml of 0.001 M PO_4 buffer. The resulting SNF samples were expected to contain mainly virus while the lysates probably

contained infected cell components and cell associated virus.

Each sample was divided in half and to each equal volumes of either anti-virus or anti-host ascitic fluid was added. Following incubation at room temperature for four hours and then at 4^{o} overnight, the antigen-antibody complexes were pelleted by centrifugation (6,500 g) in a FA50 rotor, for 30 minutes, washed twice with veronal buffer (pH 7.6), and the final pellet prepared for polyacrylamide gel electrophoresis (PAGE) by resuspending in 5% SDS followed by 12 hour dialysis against the modified electrophoresis buffer (SDS, 1 gm; glycine, 2.88 gm; urea, 30 gm (BIO-RAD Labs.); Trizma, 0.6 gm (Sigma) → 1 litre H_2O). The samples were then boiled for one to two minutes, before being applied to preformed gel (10% acrylamide). The gels were stained with Coomassie Blue to observe the protein bands.

Next, following essentially the same procedure, the proteins were labelled with C^{14} amino acids. These had been added at the time of infection.

In order to locate the C^{14}-labelled polypeptides, after the gels were stained with Coomassie Blue, they were treated with dimethyl sulfoxide for 40 minutes, changing it every 20 minutes. The gel with PPO/dimethyl sulfoxide (26.4 gm/120 ml) was incubated for 180 minutes, then washed, and incubated with water for 60 minutes before

finally being treated with 50% methanol for 30 minutes (Bonner & Laskey, 1974). It was then dried under vacuum and incubated in contact with x-ray film at -80°.

To study the glycosylated polypeptides, C^{14}-glucosamine was used. In these experiments the same experimental design used for the C^{14} amino acid experiments was used.

EXPERIMENTAL RESULTS

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PART I

Study of Antigens of Human Coronavirus 229E

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Growth of HCV/229E

The conditions chosen for the growth of 229E were those described by D. Kennedy (Ph.D. thesis, 1977) taking into consideration reports published by others in WI-38 cells (Hamre et al, 1967) and L132 culture (Bradburne & Tyrrell, 1969). The virus yields obtained were generally in the order of 10^7 to 10^8 pfu/ml when titrated after harvesting after three freeze-thaw cycles.

The studies on virus growth in either tube or flask cultures (Kennedy, Ph.D. thesis, 1977) indicated that virus titres reached a maximum at around 24 hours after infection. No increased yield of infectious virus was found after longer incubation, but this does not necessarily mean that there is no more virus production since human coronaviruses are temperature labile (Bradburne & Tyrrell, 1971; Bucknall et al, 1972).

Therefore, since the maximum quantity of virus antigen was required and infectivity was of secondary importance, to ensure the infection of most of the monolayer and the maximum production of virus, incubation was continued for 40 hours.

Purification of HCV/229E Antigen

Gradient purification techniques have proven adequate for the preparation of IBV (Bingham, 1975) and TGE (Garwes & Pocock, 1975) prior to their biochemical analysis.

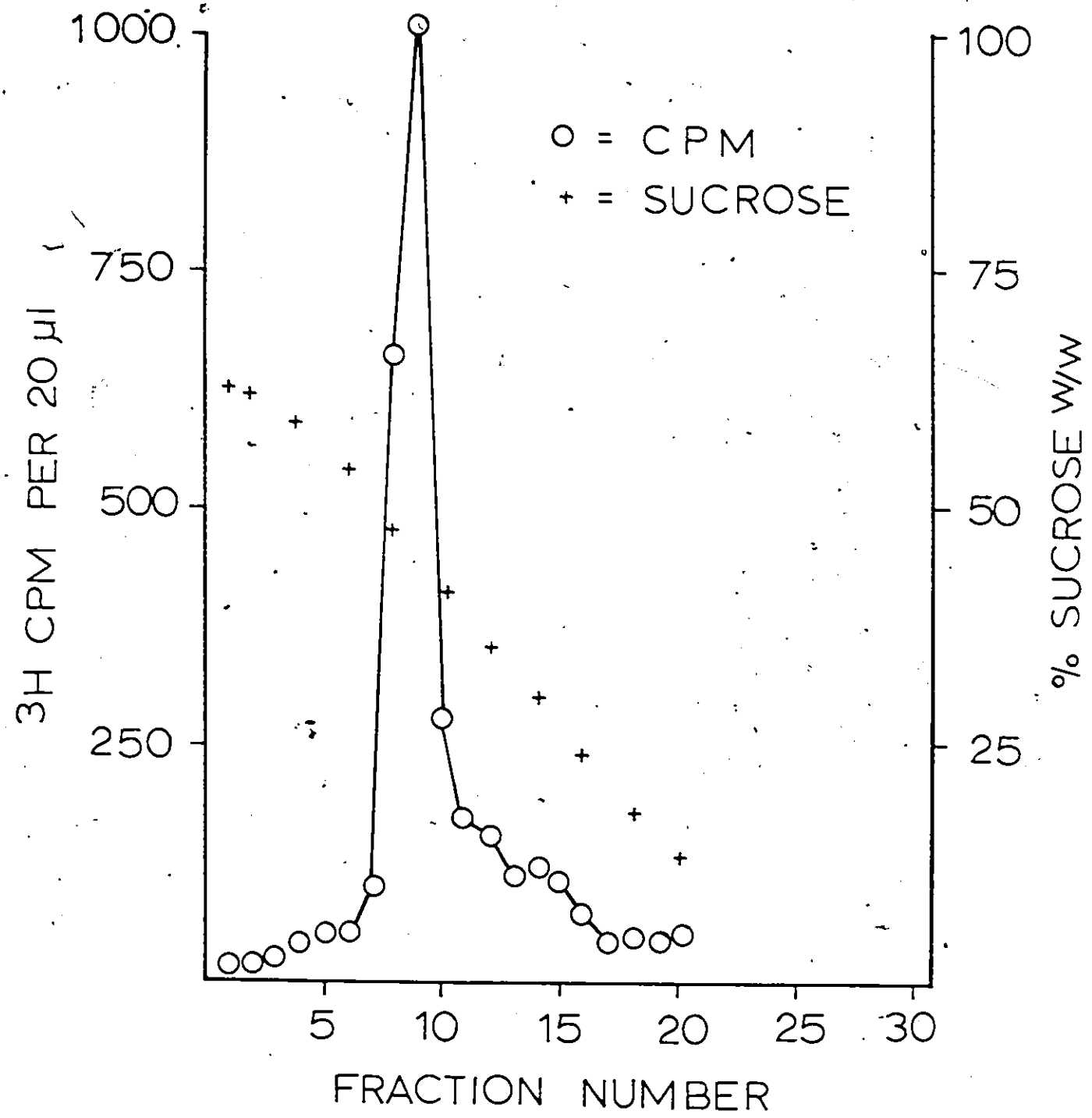
The incorporation of (5-^{3}H)-uridine specifically into the RNA of the virus during replication aids in both identification of the virus genome and location of the virus during the purification procedure. Using density gradient ultracentrifugation to separate the virus from material of different densities, it was found that the peak of radioactivity was located at a density of 1.18, and that this coincided with the peak of typical virions as detected by electron microscopy (Kennedy & Johnson-Lussenburg, 1976).

HCV/229E was purified by rate zonal and equilibrium density gradient ultracentrifugation on sucrose to the extent that was considered appropriate to be used as virus antigen. Figure 1 presents the profile of a typical experiment showing the sharp virus peak obtained by scintillation counting of the fractions collected. The pelleted and

Figure 1

Figure 1:- Final purification peak of (5-^{3}H)-uridine labelled HCV/229E obtained by ultracentrifugation to equilibrium on sucrose density gradients.

EQUILIBRIUM GRADIENT OF HCV.229E



resuspended virus obtained from the pooled peak fractions as a moderately clean preparation is shown in plate 1-a.

Stability of HCV/229E

Different investigators disagree regarding the stability of the various coronaviruses under different ionic conditions. This disagreement may reflect the use of different methods or strains of coronaviruses in their studies. At 4°, Cunningham and Stuart (1946) found that keeping IBV-Beaudette at pH 3.03 for 14 days, the infectious agent stayed fully active and no change in titre was seen. Using the same IBV strain, after 30 minutes at pH 3.0 and 4°C, Stinski and Cunningham (1969) found 92 to 96% virus survival, however, contrary to this, Hirai and Shimakura (1971) discovered a high loss of virus activity ($10^{7.2}$ to $10^{2.5}$) after 30 minutes at pH 3.05 and 4°C. The same controversy was seen with the other coronaviruses. In relation to TGE at pH 3.0 and 37°C, Cartwright et al (1965) found no change in titre after three hours while McClurkin and Norman (1966) found a 2 - 3 log loss after only one hour incubation at the same temperature.

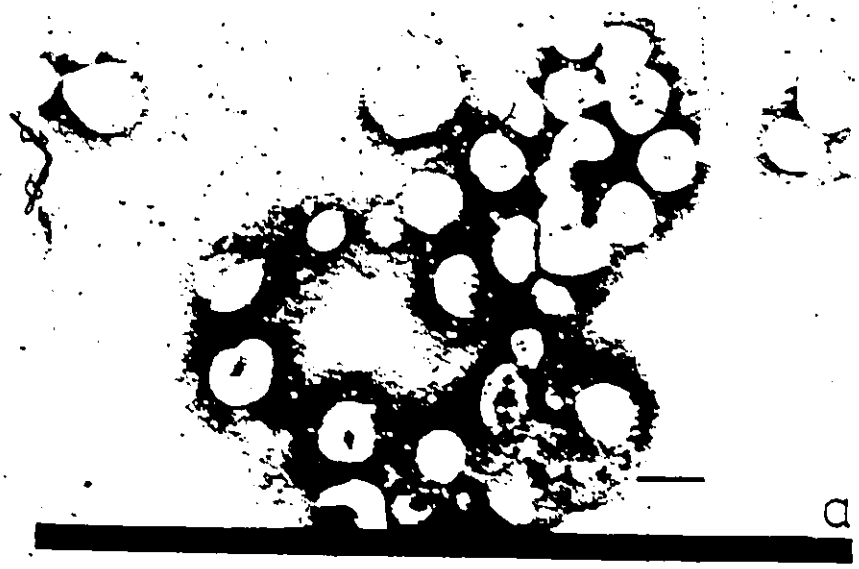
Different buffers at varying pH, some commonly used to store different viruses, were tested to discover their effect on the physical integrity of HCV/229E.

Plate 1

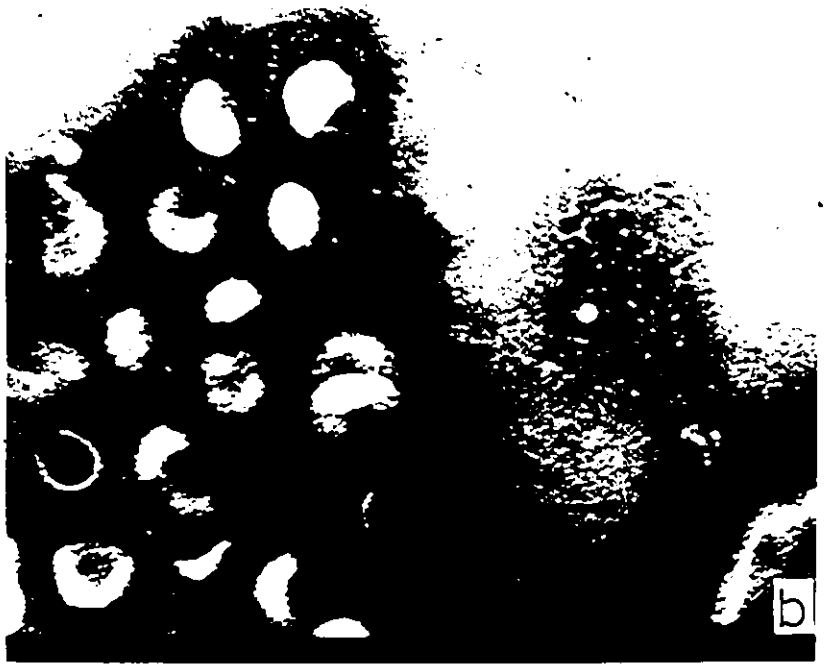
Plate 1:- Electron microscopy of HCV/229E preparations used for immunodiffusion experiments.

a) Purified HCV/229E antigen showing typical coronavirions with minimal amounts of membranous debris.

b) and c) Immune electron microscopy of aggregates of HCV/229E obtained with antiviral ascites fluid (229E/AF). (b) and virus antiserum (229E/AS) (c) demonstrating the similarity of virus-antibody interaction.



a



b

c

These experiments were designed to take advantage of the predicted change in density due to particle disruption after storage in a selected media, thus avoiding the lengthy and expensive plaque assay procedure for infectivity. In addition, it was important to correlate the release of specific labelled components such as RNP, with the disruption of the virus under the conditions of ultracentrifugation. It was reasoned that, as a result of virion disruption, there would be a shift in the density of the labelled components, and this shift would be detectable during and after equilibrium gradient ultracentrifugation. We were especially interested in the effects of phosphate ions because it had been found that virus preparations were more stable in 0.001M phosphate buffer than either saline or PBS. Also, because apparently 2% PTA at pH 6.4 was best for staining virions for electron microscopy, it was of interest to determine whether any change in particle integrity could be correlated to pH.

The virus preparations used in these experiments were labelled with (5-^{3}H)-uridine and were obtained after concentration by rate zonal ultracentrifugation in sucrose. Each sample was divided equally to the appropriate number of aliquots and applied immediately onto sucrose gradients prepared in the solutions to be tested. The results of these experiments are presented in figures 2, 3, and 4. In figure 2, the virus was applied on the sucrose gradients

Figure 2

Figure 2:- The results obtained after equilibrium sucrose gradient centrifugation of HCV/229E in water and in the presence of different concentrations of phosphate buffer at pH 7.2.

EFFECT OF DIFFERENT CONCENTRATIONS OF PO_4 BUFFER.

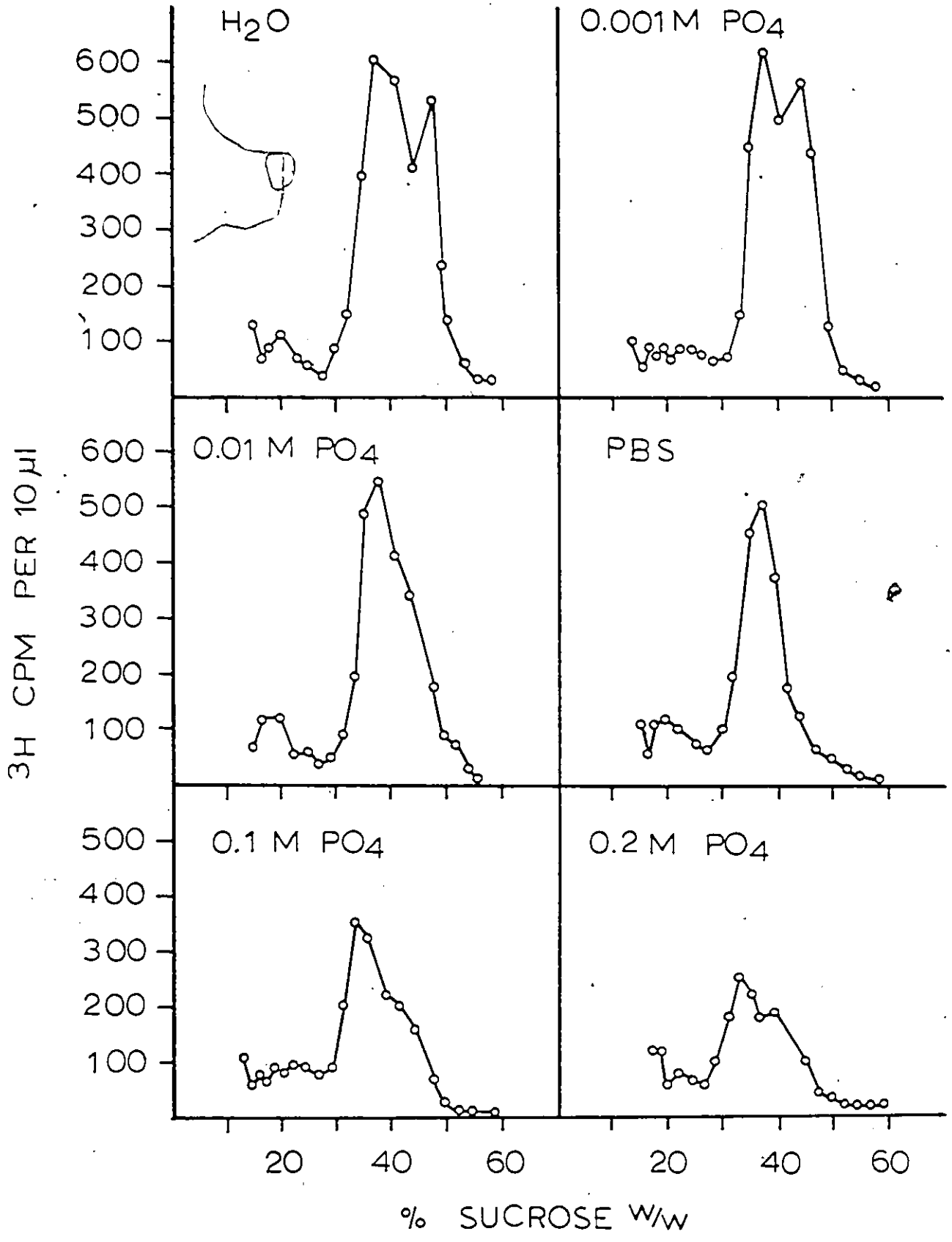


Figure 3

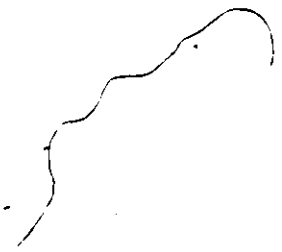


Figure 3:- The effect of prolonged incubation of HCV/229E in different ionic conditions indicated by the distribution of (5-^{3}H)-uridine label. Note the reduced height of the peaks in 0.001 M PO_4 buffer and PBS and the density shift in 0.1 M PO_4 buffer. The scale shows the density range of the peaks.

EFFECT OF PROLONGED INCUBATION ON 229E STABILITY IN DIFFERENT BUFFERS.

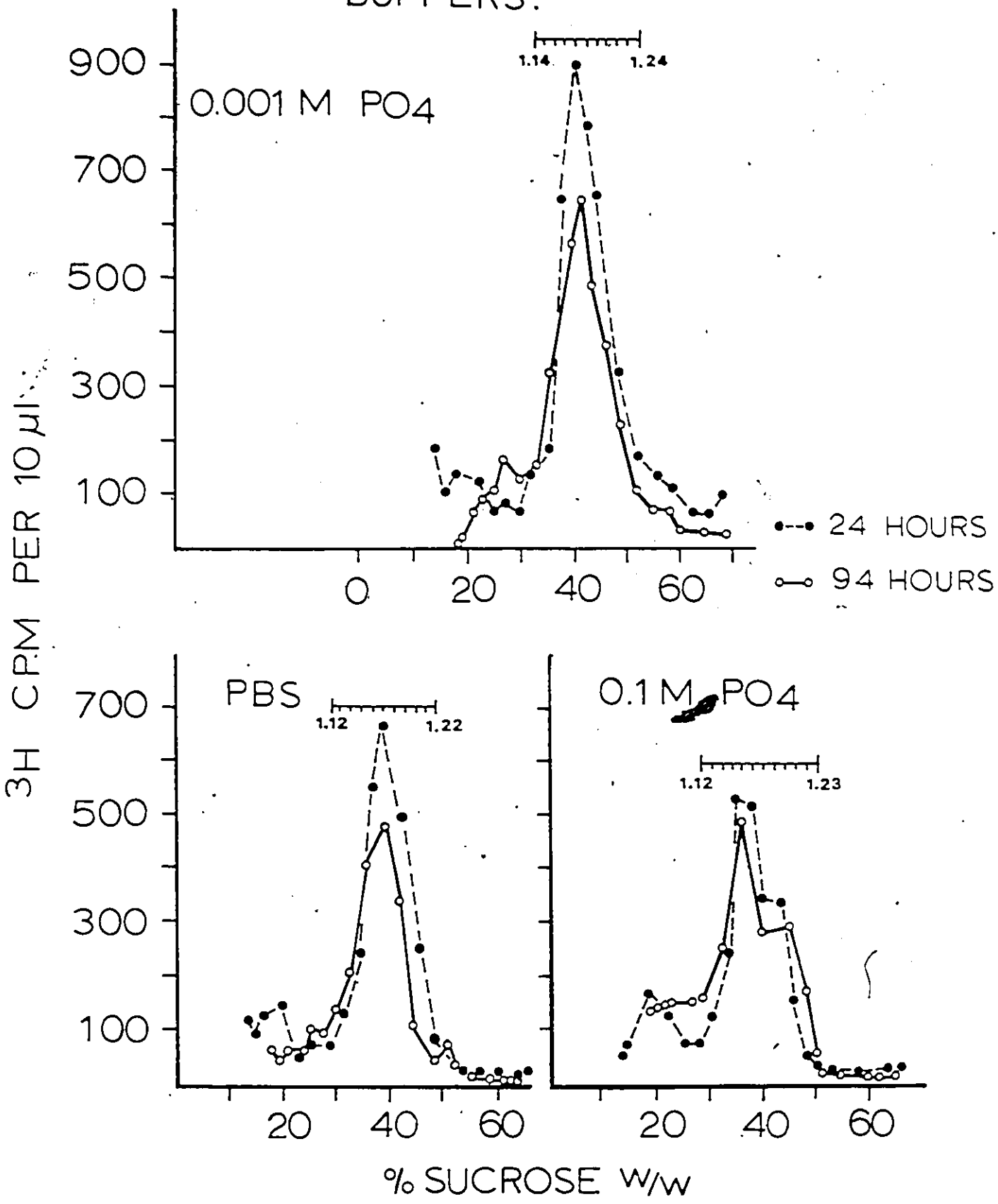


Figure 4

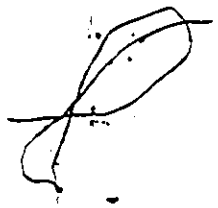
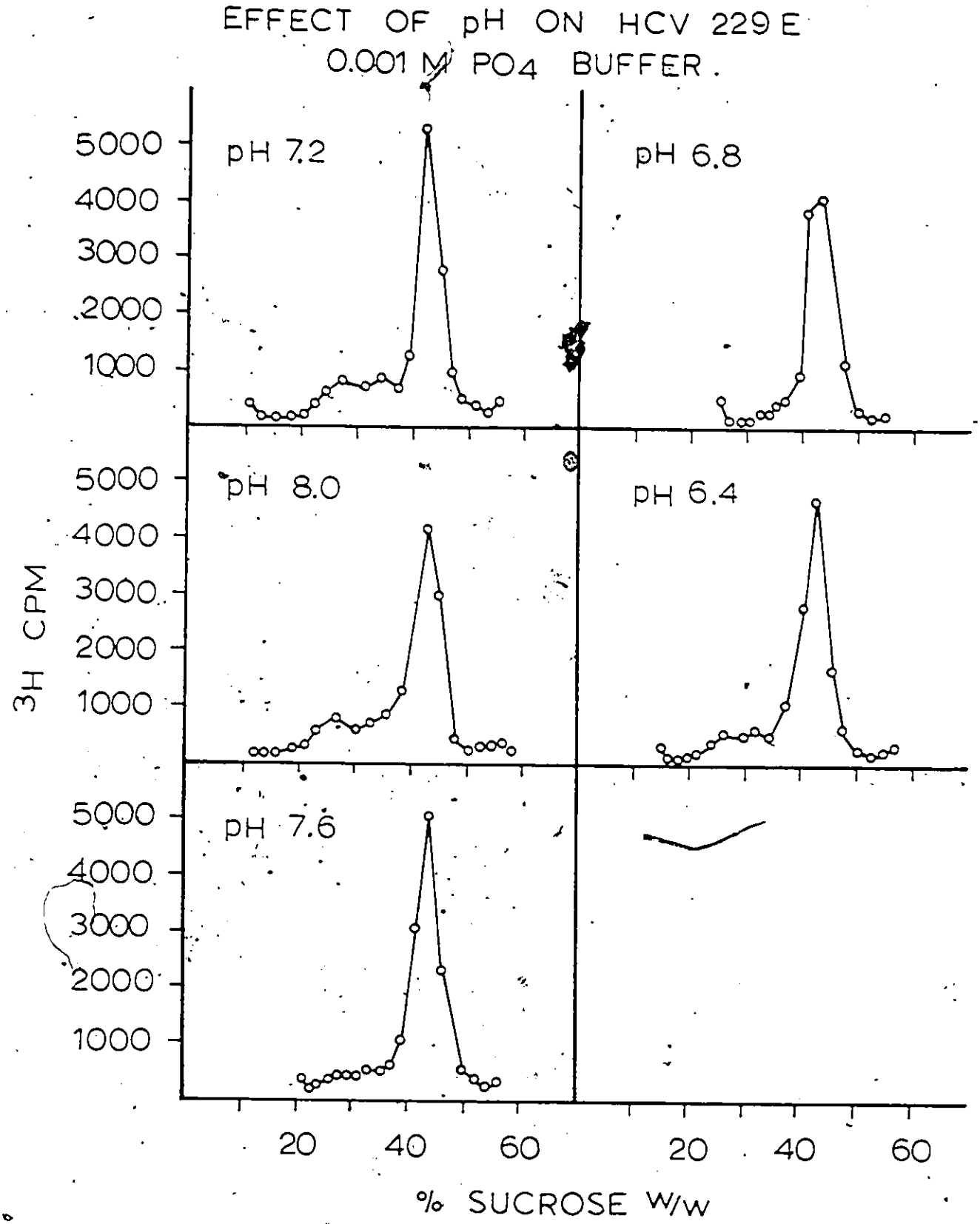


Figure 4:- The effect of pH on the storage of HCV/229E in .001M potassium phosphate buffer as indicated by the distribution of (5-^{3}H)-uridine label after equilibrium centrifugation on a sucrose density gradient.


EFFECT OF pH ON HCV 229 E
0.001 M PO4 BUFFER.
pH 7.2
pH 6.8
pH 8.0
pH 6.4
pH 7.6
5000
4000
3000
2000
1000
3H CPM
20
40
60
% SUCROSE W/W

made up with water alone or different concentrations of phosphate buffer at pH 7.2 and centrifuged to equilibrium (63,000 g for 24 hours). There was no significant difference evident in the profiles of the radioactive peaks when water and 0.001M phosphate buffer (PO_4) were used.

It was of interest to note that in both cases, there were two peaks of radioactivity, indicating the presence of two classes of particles with differing densities (1.17 and 1.21). These densities lie within the range reported for coronaviruses (1.16 - 1.23 or 36.6% - 50% sucrose) and may represent a mixed HCV/229E virus population. However, further work was not carried out to explore this finding and, to date, this has been the only occurrence of such double peaks in relatively equal proportions. However, the occurrence of mixed virus population (light and heavy particles) has been reported by other workers, notably for TGE (Leaton et al, 1971) and IBV (R. W. Bingham, personal communication).

The effect of the phosphate ion concentration on the virus integrity was indicated by the loss of the heavier peak (density of 1.21), the shifting of the major virus peak toward the lower sucrose concentration (densities of 1.16, 1.15, 1.14) and the reduction in the height of the peak by as much as 40 to 50%. The resulting virus peak in PBS which consists of 0.14M NaCl and 0.009M phosphate buffer was intermediate to those in solution with

0.1M PO_4 and 0.01M PO_4 and implied no additional effect by the NaCl ionic contribution. This shift in the virus peak was considered to be due to the PO_4 concentration and could be correlated with the decrease in the radioactivity counts, which may reflect the disruption of intact virus particles.

Prolonged incubation of HCV/229E at different phosphate concentrations was also examined. The results are presented in figure 3. After 24 hours incubation peaks were found at essentially similar densities within the range described for coronaviruses. However, the total amount of radioactivity decreased from 900 counts (0.001M PO_4) to approximately 700 counts (PBS) and 550 counts (0.1M PO_4) indicating a loss of virion RNA, most probably due to disruption. Furthermore, the profile of the radioactivity in the 0.1M PO_4 sample was quite different. It can be seen that there appeared to be a reduction in overall radioactivity of the major peak, however, a distinct shoulder indicated the presence of two populations of the major peak at a slightly lower density range than normal, possibly occurring as a result of the loss of surface projections. It has been reported that "bald" virus (OC43) has a density of 1.15 in potassium tartrate (Hierholzer et al, 1972). After 94 hours incubation there was no change in the profiles indicating no major shift in the density of the virus populations. However, there was

a continuing and pronounced loss of intact labelled virions in 0.001M PO_4 and PBS indicated by the reduced number of counts by as much as 30%. In 0.1M PO_4 the reduction in counts could not be regarded as significant and it is possible that under those conditions, the populations had stabilized to a certain extent. Further experiments to investigate this in correlation with morphology and infectivity studies should be of interest.

In the experiment described for the examination of a pH range between 6.4 - 8.0 using the 0.001M phosphate buffer, it can be seen that no significant physical disintegration occurred from slightly acid to slightly alkaline pHs after 94 hours centrifugation and the virus was found to be quite stable in that pH range (figure 4).

As a result of these experiments, it is felt that this method provides an effective means of evaluating the disruptive action of certain conditions on the virion and the preliminary information is a quick guide for further stability studies. Such studies, however, must be correlated with morphological and infectivity investigations. On the basis of these results, we conclude that the continued use of the 0.001M phosphate buffer or water, at a pH of 7.2 is justified and that the effect of the pH on the virus stability is not as great as had been previously thought.

Isolation of 229E-RNP

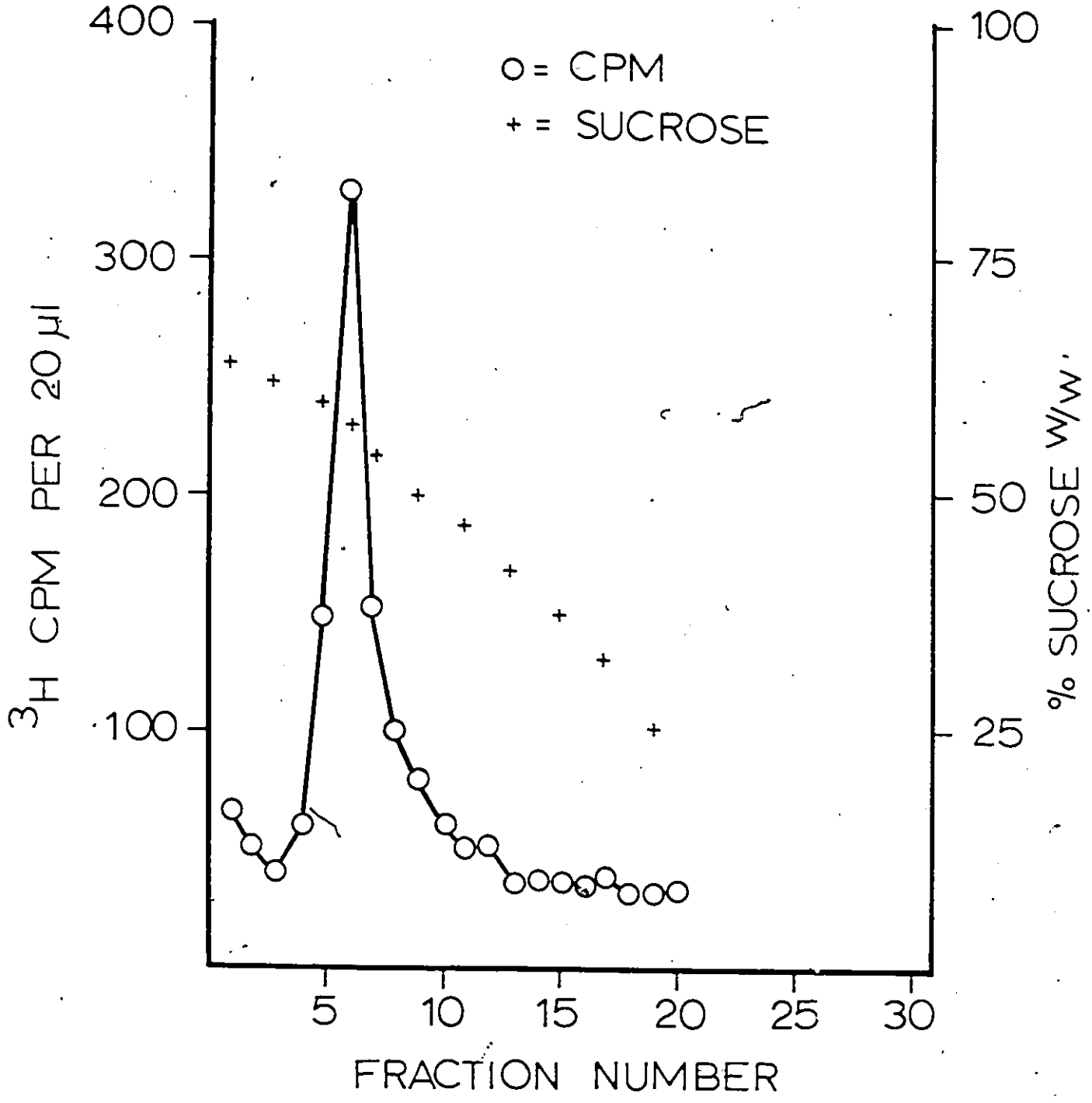
When HCV/229E was treated with NP40 according to the procedure described in the previous section, a clear difference could be seen between detergent-treated and untreated virus. Figure 5 shows the distribution of (5-^{3}H)-uridine label in the gradient of NP40-treated virus preparation. The peak in the NP40 tube corresponds to a density of 1.27, while the untreated virus in control gradients usually comes at a density around 1.18. In both cases there was a single sharp peak, indicative of a homogeneous uridine-containing species, which differs in the two situations. The 1.27 density component was identified as the internal component, a ribonucleoprotein with a linear helical configuration by biochemical and morphological methods (Kennedy & Johnson-Lussenburg, 1976). Both the RNP preparation obtained by NP40 treatment and the RNP obtained after spontaneous disruption during the virus purification (Kennedy & Johnson-Lussenburg, 1976) were used as RNP antigen in the immunodiffusion tests. Unfortunately there was not enough for active immunization and preparation of anti RNP serum.

Preparation of Analytical Antisera and Ascitic Fluids

The method of ascitic fluid preparation originally described for mice (Munoz, 1957) proved to be suitable for

Figure 5

Figure 5. Isolation of HCV/229E RNP following NP40 treatment of purified HCV/229E according to the method of Kennedy, 1977. Peak indicates location of (5-^{3}H)-uridine incorporated into the viral RNA of the RNP.


NP40 EQUILIBRIUM GRADIENT OF HCV. 229 E
O = CPM
+ = SUCROSE
400
300
200
100
100
75
50
25
3H CPM PER 20 µl
% SUCROSE W/W
5
10
15
20
25
30
FRACTION NUMBER

guinea pigs. The guinea pig was preferred for several reasons; 1) guinea pigs produce two to three times the volume of antibody-containing fluids compared to that produced by the mouse, and 2) the mouse was considered unsuitable due to the endemicity of mouse hepatitis virus (MHV) and the cross-relationships reported with HCV antigens (Bradburne, 1970). Also guinea pigs were found to be better animals for the preparation of anti-229E antibodies than rabbits, confirming the findings of others (Bradburne, 1970).

All the antigen preparations used, virus (229E), virus-host (229E/L132), and host (L132), in combination with Complete Freund's Adjuvant were able to stimulate the guinea pigs to produce large volumes of ascitic fluid (30 to 50 ml) every two days for a period of about a month. Blood was collected weekly from these same animals for the preparation of antisera.

Complete Freund's Adjuvant seems to have been the important factor for the production of immune ascitic fluids in that it created the "ascitic reaction," a phenomenon described by others (Sommerville, 1967; Stux et al, 1977), in those animals which were initially not responding.

The accumulation of the ascitic fluid can be seen by the marked distension of the abdomen of the guinea pigs which did not seem to interfere with the activity of the animals and their general health throughout the period.

At the end of about a month, ascitic fluid production had reduced and the abdominal area started to fill with a hard mass of fibrous tissue. The animals, at this stage, were bled to death and the resulting antisera were stored for testing. A further advantage to the use of ascitic fluid is that the procedure for collecting ascitic fluid was neither as painful nor as hazardous for the animals as heart puncture or any other blood collecting procedures. Anaesthesia was required only when collecting blood from the animals.

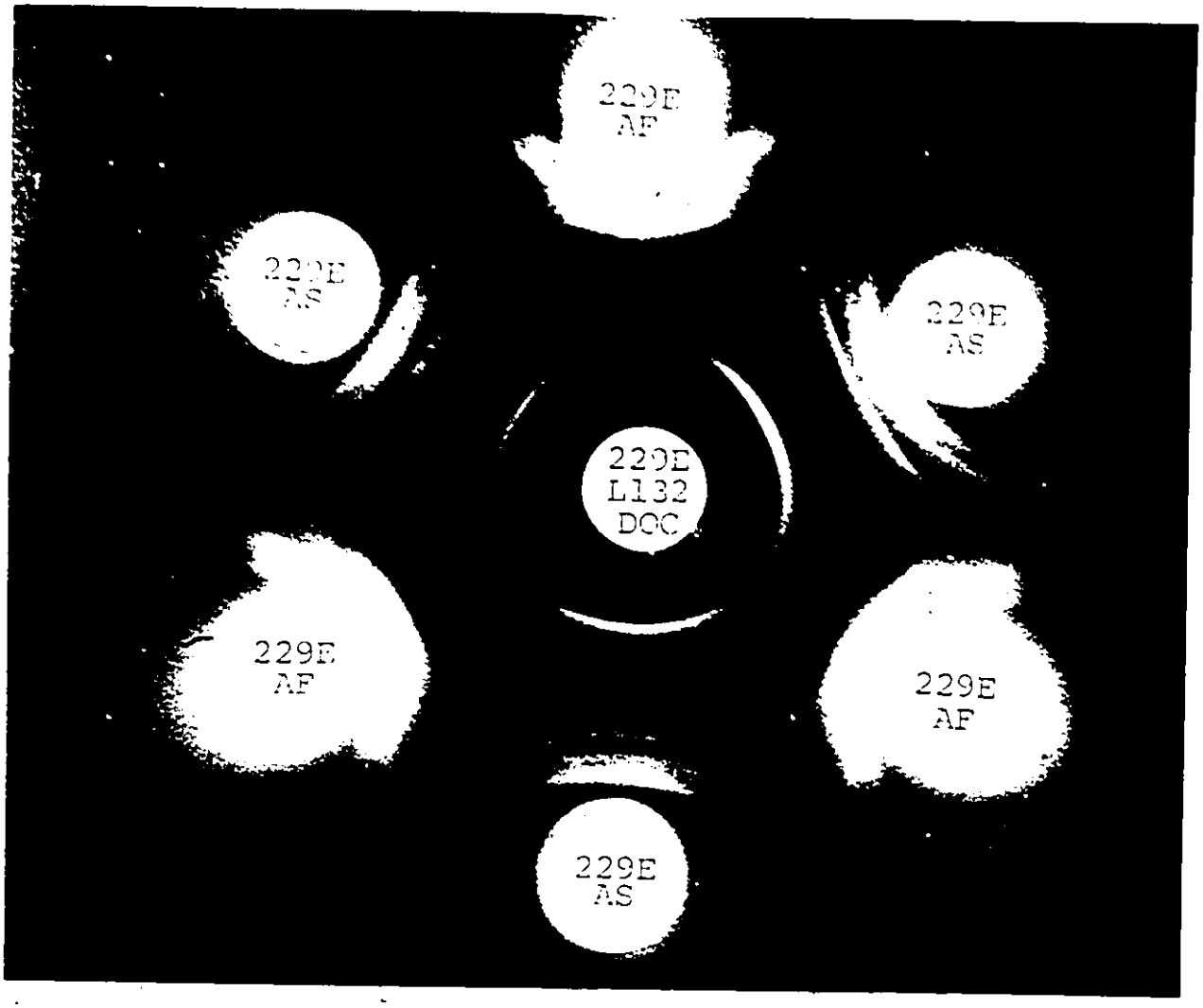
There was no loss in the quantity of ascitic fluid due to the presence of cells, a problem usually faced in the case of blood used to prepare antiserum where approximately half is removed by clotting to achieve the removal of red and white blood cells. Furthermore, the ascitic fluid was found to have as good an antibody content as the serum, and thus a larger volume was available for the necessary concentration.

Characterization of Analytical Antisera and Ascitic Fluids

Antibody preparations to be used for the identification of 229E antigens were evaluated by several methods; neutralization, complement fixation, immune electron microscopy, and immunodiffusion. Immunodiffusion reactions demonstrated an equal precipitating ability (plate 2) as

Plate 2

Plate 2:- Comparison of immunodiffusion reactions obtained with guinea pig ascitic fluid (229E/AF) and anti-serum (229E/AS) prepared against HCV/229E.


229E
AF
229E
AS
229E
AS
229E
L132
DOC
229E
AF
229E
AF
229E
AS

shown by the lines in the sodium deoxycholate (DOC) disrupted virus-host (229E/L132) antigen reaction between both the virus specific hyperimmune antiserum and ascites fluid. The same number of lines with similar intensities developed with both ascites and antiserum.

Both the antihost serum and ascitic fluid did not show neutralizing activity while the anti-229E and anti-229E/L132 ascites and serum gave similarly high titres (table 1).

On the other hand, the convalescent human sera had a much lower neutralizing titre than the hyperimmune sera and ascitic fluid. This finding was expected since the convalescent sera were positive specimens obtained by routine screening by complement fixation of sera from patients with clinical respiratory illness and reflected the presence of low levels of antibody due to natural coronavirus infection. These tests were performed through the courtesy of P. Phipps at the Regional Virus Laboratory at the Children's Hospital of Eastern Ontario, using a CF coronavirus antigen which we had prepared according to the method of Hamre and Beem (1972). Most of the sera had low complement fixation titre of 10, however a few had a titre of 20 while only one sample had a titre of 80. The specific antisera and ascitic fluid prepared in guinea pigs were also tested by complement fixation, however, the presence, in all samples, of host-reacting components made interpretation impossible.

TABLE 1

NEUTRALIZATION OF HCV/229E IN L132 CELLS (PLAQUE INHIBITION) BY GUINEA PIG ANTISERA, ASCITIC FLUIDS AND SELECTED HUMAN CONVALESCENT SERA

Immune Fluids	Neutralization Titre
Host (L132)	
Antiserum and ascitic fluid, control (L132/AS, L132/AF)	0
Virus-host	
Antiserum (229E/L132/AS)	5120*
Ascitic fluid (229E/L132/AF)	2560
Virus	
Antiserum (229E/AS)	5120
Ascitic Fluid (229E/AF)	2560
Convalescent Serum	
(229E/CONV. 1) (CF 10)	80
(229E/CONV. 2) (CF 20)	80
(229E/CONV. 3) (CF 80)	40

*All titres are expressed as the reciprocal of the end point dilution showing 50% plaque inhibition.

Immunoelectron microscopy served as a quick test for the evaluation of our antibody fluids in the early stages of antisera and ascitic fluid preparation. This method was used to evaluate agglutination by electron microscopic observation of clumps of purified HCV/229E virions in suspension after incubation with the homologous antiserum or ascitic fluid. Typical reactions can be seen in the micrographs presented in plates 1-b and 1-c (p. 57) using either antisera or ascitic fluid.

Identification of HCV/229E Antigens by Immunodiffusion

The aim of this study was to resolve three main problems: 1) Detection of all HCV/229E antigens, a problem made difficult because of the profusion of host lines (Bradburne, 1970). These lines complicate the interpretation of the results and the identification of the virus specific antigens. Human convalescent sera which, by definition, should not contain anti-host reacting components, were reported to give different results varying from two precipitation lines identified by Bradburne in agar gel immunodiffusion (1970), to only one consistently found in both immunodiffusion and immunoelectrophoresis (Hierholzer, 1976). The identification of putative host/virus antigens would not be achieved, using the convalescent sera.
2) Identification of all the virion antigenic components,

whether they are host or virus specific according to their relationships with the host, virus-host, and the virus antibodies. 3) Correlation of the HCV/229E antigens with the structure of the virus.

All the antigens (229E, 229E/L132, L132, and 229E/RNP) used in the immunodiffusion reactions to identify HCV/229E antigenic components were tested for protein content (Lowry et al, 1951) (table 2). Relatively large quantities of protein were removed from the crude virus sample (virus-host) through the cycles of purification in order to obtain a clean virus, and more than two-thirds of the virus protein was removed in the process of isolation of the RNP antigen. All the antigens except the RNP were used for the immunization of guinea pigs by the procedures described in the previous section. No antibodies were prepared for the RNP due to 1) its low protein content, 2) the long process of purification required in order to get enough RNP to be used as antigen, 3) the availability of convalescent human serum, and, 4) the importance of using limited RNP preparations as a defined antigen in the analytical immunodiffusion experiments. Extra- and intracellular virus from whole cell preparations were used as the virion antigens even though cleaner samples could have been obtained from the extracellular SNF. However, the small amount of virus in the extracellular virus preparation was not enough

TABLE 2

ESTIMATED PROTEIN CONTENT OF PREPARATIONS USED IN THE ANTIGENIC STUDIES OF HCV/229E USING THE METHOD OF LOWRY ET AL, 1951

Antigen Preparation	Protein Concentration (µg/ml)
Uninfected Host (L132)	3000
Crude Virus-host Preparations (229E/L132)	3400
Semi-purified Virus, Cushioned (229E/L132)	2950
Purified Virus (HCV/229E)	260 - 320
Ribonucleoprotein (229E/RNP)	90

for our immunodiffusion experiments since less than 30% of the virus was found in the extracellular supernatant fluids.

In our experiments, all the immunodiffusion reactions were set up in duplicate. The duplicate reactions presented in plate 3 demonstrate the consistency of the micro-immunodiffusion technique in cellulose acetate. Our immuno-diffusion studies in cellulose acetate (Johnson et al, 1964) have revealed a complex and unusual pattern of different identities between the homologous and heterologous antigen/antibody reactions using sera prepared against normal host cells (L132), virus-infected cells (229E/L132), and purified virus concentrate (229E) and to date none of the preimmunization normal guinea pig sera used in our work has given a precipitation line in immunodiffusion with our V, VH or H antigens.

Preliminary immunodiffusion tests were performed with convalescent sera with three different complement fixation titres (10, 20, 80) to evaluate their reaction with virion antigens. Both intact and DOC-disrupted HCV/229E antigen preparations were used and the results are shown in plate 4-a. Two precipitation lines developed with the disrupted components whereas only one line formed close to the well of the intact virus due to its slower diffusion in the reaction. An interesting point here is that the intensity of the reaction lines was not related to the CF

Plate 3

Plate 3:- Duplicate immunodiffusion reactions demonstrating the reproducibility of the technique.

Both 229E/AF and L132/AF are able to precipitate at least three 229E/L132/DOC and L132/DOC components. An interesting observation here is the apparent reaction seen between 229E intact antigen and 229E/L132/DOC antigen. The reaction line however has fused across the 229E well with L132/AF and 229E/AF in this reaction with 229E/L132/DOC. Reactions like this are sometimes encountered in the cellulose acetate immunodiffusion tests and are considered to be the result of unbalanced reacting participants.

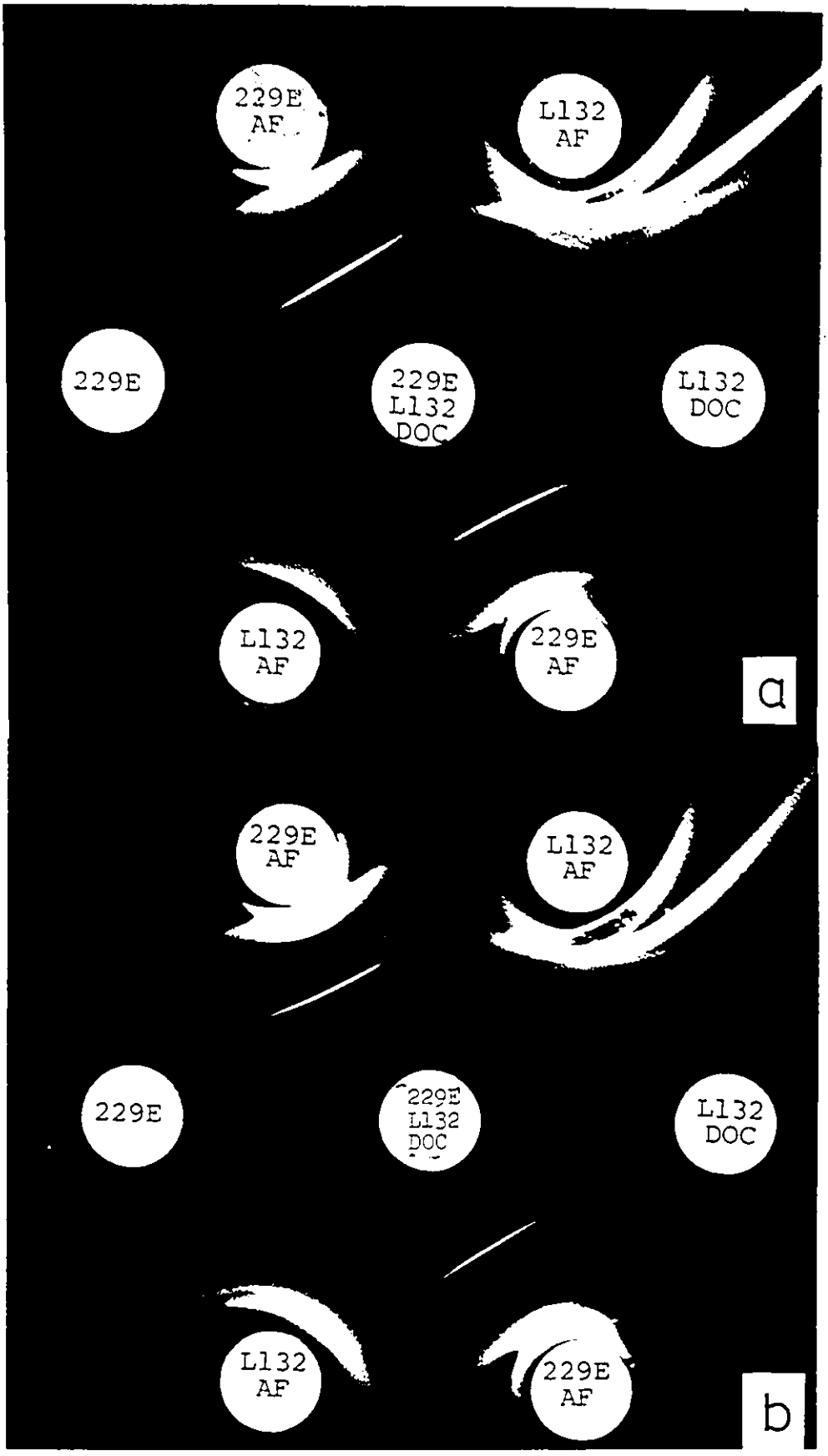

229E AF
L132 AF
229E
229E L132 DOC
L132 DOC
L132 AF
229E AF
a
229E AF
L132 AF
229E
229E L132 DOC
L132 DOC
L132 AF
229E AF
b

Plate 4

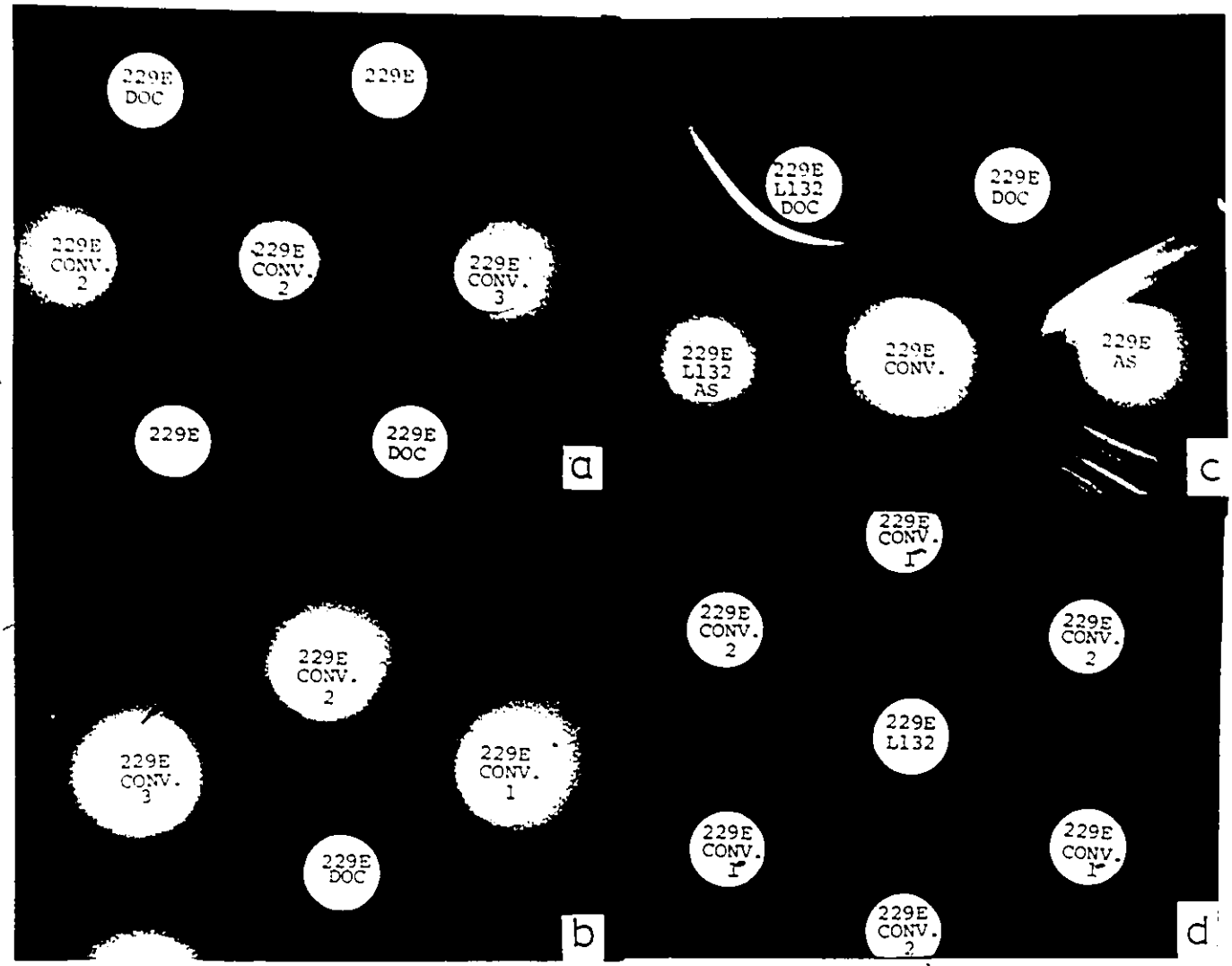
Plate 4:- Demonstration of the reaction characteristics of the different convalescent sera containing HCV/229E precipitins.

a) Two weak precipitation lines can be seen between the DOC-disrupted virus preparation (229E/DOC) and the convalescent sera, while only one very weak reaction line can be seen close to the undisrupted 229E well.

b) In this reaction two clear lines have developed between human sera 1, 2, and 3 and 229E/DOC antigen.

c) This reaction clearly demonstrates that 229E/CONV. serum and 229E/L132/AS detect a component in 229E/DOC which is also present in 229E-L132/DOC.

d) A very faint precipitation reaction (arrowed) is barely detectable between the virus host soluble antigen and two different human sera (1' and 2). These sera had a CF titre of 20.


229E DOC
229E
229E CONV. 2
229E CONV. 2
229E CONV. 3
229E
229E DOC
a
229E L132 DOC
229E DOC
229E L132 AS
229E CONV.
229E AS
c
229E CONV. 1
229E CONV. 2
229E CONV. 2
229E CONV. 2
229E CONV. 3
229E CONV. 1
229E L132
229E DOC
229E CONV. 1
229E CONV. 1
b
229E CONV. 2
d

titre of the antiserum. The CF 10, 20, and 80 convalescent sera (229E/CONV. 1, 2, and 3) developed equally good reactions with the disrupted virus (plate 4-a and 4-b).

The specificity of the convalescent serum reactions was demonstrated by the precipitation line identifying the serum reacting component an antivirus antibody in the reaction of identity with purified, DOC-disrupted virus (plate 4-c).

In further immunodiffusion reactions, the convalescent sera reacted with both 229E and 229E/L132 antigens while no reaction was identified with the host antigen (plate 5-a): These reactions therefore confirmed the specificity of the convalescent human serum permitting its use in other tests to identify specific virus components.

Preliminary reactions, using ascitic fluids with host (L132/AF), virus-host (229E/L132/AF), and virus (229E/AF) specificities were next carried out using the pattern demonstrated in plate 5-b. As many as 7 components were identified between the sucrose-purified DOC-disrupted virus (229E) antigen and 229E virus ascitic fluid and one of these (arrowed) is detected only by the virus ascitic fluid. Fewer and weaker precipitation lines have developed between 229E purified by a tartrate gradient instead of sucrose, and itwas thought to be due to the instability of the virus in this salt. Such instability had been previously observed (C. M. Johnson-Lussenburg, unpublished results).

Plate 5

Plate 5:- Further characterization of the antigen and antiserum preparations for use in the analytical immunodiffusion reactions.

a) Confirmation of the specificity of the human convalescent serum after pooling and concentration. Two 229E precipitation lines are identified by the reactions between 229E intact antigen and 229E concentrated convalescent serum pool. One of these was also identified by the reaction between 229E/L132 antigen and 229E convalescent. No reaction has developed between the 229E/CONV. sera and the host antigen. The blurred areas to the left of each L132 well illustrate a technical problem which sometimes occurs during the setting up of the reaction chambers. Because the perspex template is slipped gently over the surface of the wet cellulose acetate which had been placed on a glass slide, any burrs or rough edges on the bottom of the template will permanently scratch the surface of the cellulose acetate resulting in blurrs or marks on the final stained strip which appears in the print.

b) A reaction to compare two virus preparations, both of which were disrupted by DOC prior to testing, one was prepared by purification on tartrate gradients (229E/TART./DOC) and the other by the standard

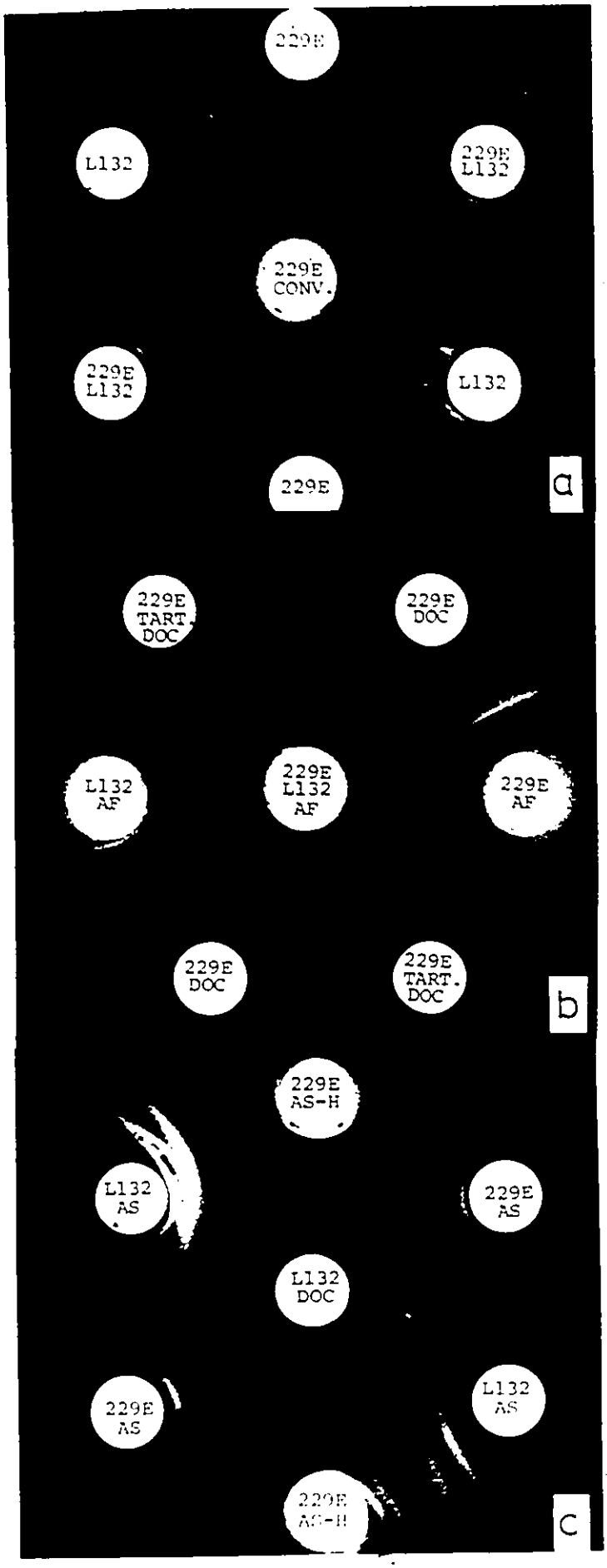
Plate 5 continued:-

sucrose gradient procedure (229E/DOC). Seven antigens can be detected between 229E/DOC and 229E/AF and some of these are identified by 229E/L132/AF. Fewer lines developed with 229E/TART./DOC compared to the 229E/DOC reaction.

c) Result of absorption of 229E antiserum to remove host reacting antibodies. No precipitation lines between 229E/AS-H and the L132/DOC antigen have developed indicating that the host precipitins have been removed and no longer react with DOC-treated host (L132/DOC) antigen. However, it can be seen that the host antiserum (L132/AS), in addition to precipitating components in the L132/DOC, reacted strongly with components in the absorbed serum (229E/AS-H). This reaction indicates that the absorption procedure has contributed host antigens to the serum. These antigens could not be completely removed by differential centrifugation (two cycles at 75,000 g for 90 minutes and 4 hours). Not all the lines identified between 229E/AS-H and L132/AS fused with those between L132/DOC and L132/AS due to the difference in host antigen preparations.

Plate 5 continued:-

The 229E/AS-H sample was not treated with any disrupting agents while the L132/DOC sample had been treated with DOC.


229E
L132
229E L132
229E CONV.
229E L132
L132
229E
a
229E TART. DOC
229E DOC
L132 AF
229E L132 AF
229E AF
229E DOC
229E TART. DOC
b
229E AS-H
L132 AS
229E AS
L132 DOC
229E AS
L132 AS
229E AS-H
c

On the basis of this reaction, it is clear that most of the viral antigenic components carry some host specificity, either as a necessary part of the intact virion or as a non-specific absorbed contaminant.

Because of the complications caused by the presence of host antibodies in hyperimmune antisera and ascitic fluids identified by their reaction with the purified preparations of the virus, their removal was attempted by absorption of the antisera with host antigen (L132). The results shown in the reaction presented in plate 5-c demonstrate that the host precipitins have been removed. But, the absorption procedure has contributed host antigens to the serum as indicated by the reaction between host antiserum (L132/AS) and absorbed virus antiserum (229E/AS-H) and these could not be removed by extensive ultracentrifugation (2 cycles at 75,000 g for 90 minutes and 4 hours). For this reason, this absorbed antiserum had limited potential in comparative analytical reactions, but was a valuable tool for the identification of virion antigens as demonstrated in plates 6-a and 6-b. Unfortunately, any specific host antigens incorporated into the virions would not be detected since the appropriate antibody would have been removed by absorption.

Evaluation of different virus disrupting agents:

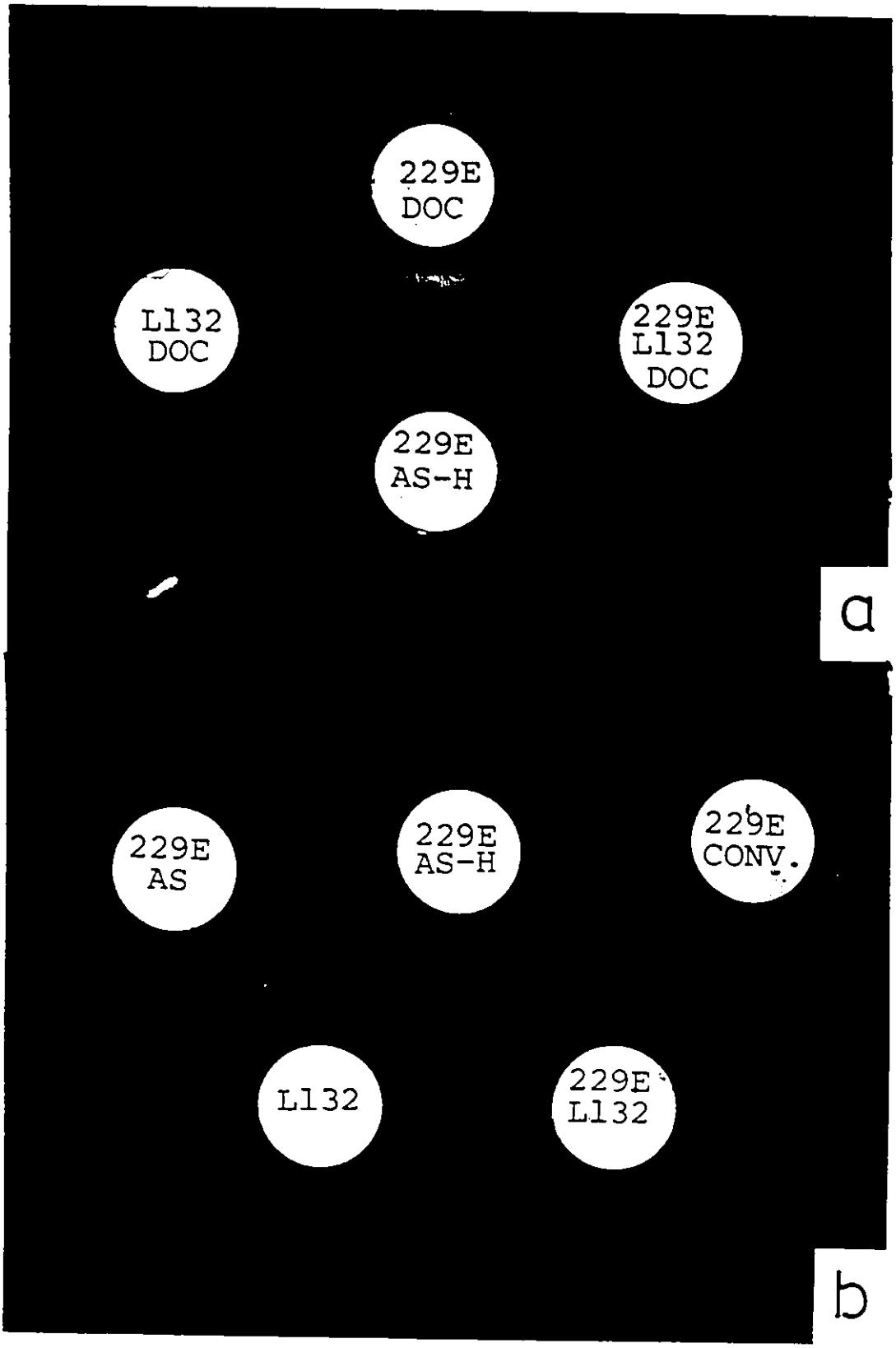
In our preliminary immunodiffusion reactions we had relied

Plate 6

Plate 6:- Identification of HCV/229E viral antigens using the absorbed serum.

a) Two virus components are identified by 229E/AS-H absorbed sera and the 229E/DOC antigen. No reaction can be seen with the host due to the removal of the host antibodies by the process of absorption.

b) Again no reaction has occurred between the host antigen (L132) and the absorbed serum while one of the 229E/L132 antigens is identified as a virus specific component. The relationship to the conv. reacting component is not demonstrated probably due to the low levels of antigen available. One to two lines have developed between the virus antiserum (229E/AS) and the absorbed virus antiserum (229E/AS-H), which are identified as host antigens contributed to the absorbed serum and detectable by host antibodies present in the virus specific antiserum.


229E
DOC
L132
DOC
229E
L132
DOC
229E
AS-H
a
229E
AS
229E
AS-H
229E
CONV.
L132
229E
L132
b

on the use of sodium deoxycholate (DOC) for virus disruption and release of antigenic components for several reasons. First, it had proven to be reliable and valuable in similar studies on the antigens of influenza virus (Johnson & Westwood, 1971), a virus having a chemical structure close to that of the coronaviruses and, second, in earlier EM studies on the disruption of HCV/229E virions to release identifiable structural components, NaDOC was the most promising. However, once the basic immunodiffusion reacting patterns had been established, it was essential to evaluate other disrupting agents with respect to their effects on the coronavirus antigens.

The reaction patterns were designed to: 1) Detect and identify the antigens released by the treatment, 2) compare these components with those released by DOC disruption, and, 3) identify specific viral component(s) using the human convalescent antiserum. The disrupting agents were chosen on the basis of their characteristic abilities and the historical context. Thus, since coronaviruses are membraned viruses, containing essentially lipids, ether and chloroform were selected, as much for their lipid solvent properties as for their historical importance in the study of influenza virus antigens. The reactions presented in plate 7 demonstrate the effect of lipid extraction on HCV/229E.

Plate 7

Plate 7:- Evaluation of HCV/229E disrupting agents: ether and chloroform.

a) Detection of the antigens released by virus treated with ether using 229E, 229E/L132, and L132 antisera. It can be seen that three virus structural components are detected, of which one is identified as host (L132) due to the reaction of identity with the host, one is clearly virus because it shows a reaction of non-identity with the host line and it was identified by both 229E and 229E/L132 antisera. The third virus component has only been detected by the 229E/AS and not 229E/L132/AS.

b) In this comparison reaction, there are two major 229E/DOC reacting components. One of these components is present in the 229E/ETHER reaction but in reduced amounts. In addition ether treatment has produced a reacting complex, possibly consisting of 1-2 bands, which was not detected in the 229E/DOC reaction. No clear reaction can be seen with the human serum, however, one to two lines are seen between 229E/CONV. and 229E/DOC. Less intense reacting components were identified in the undisrupted virus compared to both DOC and ether-disrupted 229E.

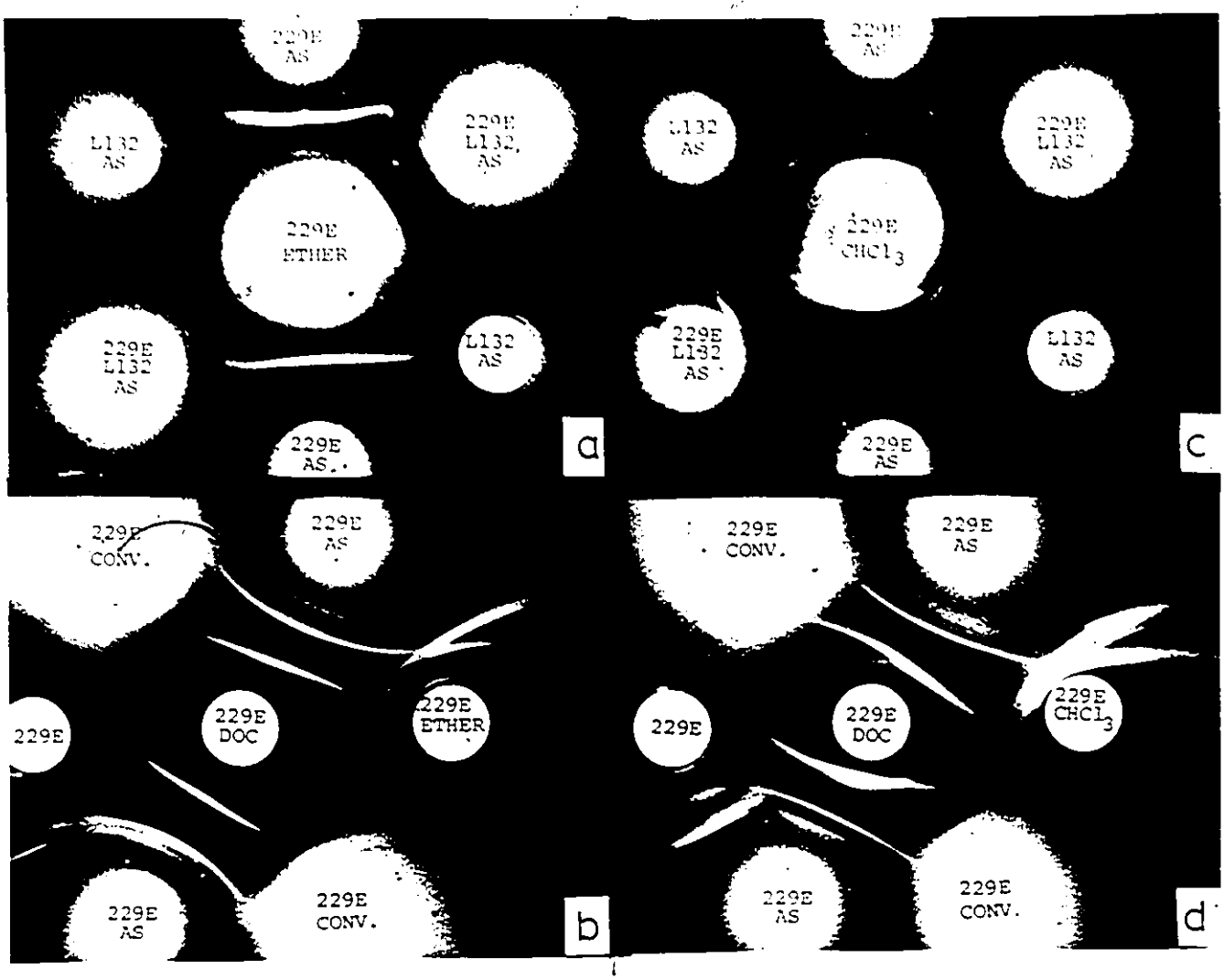
c) The reactions of virus disrupted by chloroform treatment are not sufficiently clear to warrant

Plate 7 continued:-

detailed analysis.

d) In this comparison reaction, a pattern somewhat similar to ether treatment is revealed except the patterns of identity are not clear. Five components are identified by chloroform treated virus. Human convalescent serum again reacted only with the DOC-disrupted preparation.

In plate 7-b there is an apparent reaction between two antigens (229E/DOC and 229E/ETHER) which, as discussed earlier (plate 3), is a result of an unbalanced reaction between the 229E/AS and 229E/CONV sera, and the 229E/DOC antigen and 229E/ETHER antigen. A similar reaction is seen in plate 7-d between the two antigens 229E/DOC and 229E/$CHCl_3$.


229E AS
L132 AS
229E L132 AS
229E ETHER
229E L132 AS
L132 AS
a
229E AS
229E CONV.
229E AS
229E
229E DOC
229E ETHER
229E AS
229E CONV.
b
229E AS
L132 AS
229E L132 AS
229E CHCl3
229E L132 AS
L132 AS
c
229E AS
229E CONV.
229E AS
229E
229E DOC
229E CHCl3
229E AS
229E CONV.
d

Ether treatment of purified virus preparations produced three precipitating antigens (plate 7-a). Two of these components were detected by antiserum against the virus or virus-host preparations and were virus (229E) antigens, and one by the anti-host component (L132) was common to all reactions making a total of 3 antigens detected. The identification of the virus component by the virus antiserum preparation and not by the virus-host antiserum was probably due to the presence of a higher concentration of the appropriate antibody in the virus antiserum than in the virus-host antiserum as a result of the original immunizing preparation. A second viral component was identified as having virus specificity only, because of the reaction of non-identity with the host antiserum.

In the reaction comparing the components released by ether with those by DOC and the intact virus (plate 7-b), it is apparent that both agents produce similar components which are identified by lines of identity. Additional components were detected and these differed depending on the mechanisms of action of these two disrupting agents. The human convalescent serum did not appear to react with the ether-released virus antigens while one to two lines were detected between human serum and DOC-disrupted viral preparation indicating the loss of some antigens due to the ether treatment. Both ether and NaDOC disruption provided a larger spectrum of viral antigens for analysis

than the intact non-disrupted viral preparations.

Essentially similar results were obtained with chloroform disruption (plate 7-c&d). However, the resolution of the precipitation lines and overall quality of the reaction was not as good. On the basis of these reactions, it was considered that DOC treatment was preferred but that ether disruption released new viral antigens of interesting potential which should be investigated further.

Two proteolytic enzymes, trypsin and pronase, were also evaluated as disrupting agents and the results can be seen in plates 8-a, 8-b, 8-c, and 8-d. Following the digestion of virus with trypsin, only two clear lines can be seen and both show a host identity (plate 8-a). No virus specific lines were detected in these reactions. In the comparative reaction (plate 8-b), none of the three lines resulting from trypsin disruption showed a relationship to those resulting from DOC disruption. The latter released six reacting components. Three to four antigens were detected between DOC-disrupted 229E and human convalescent serum (229E/CONV.). In most of the reactions using human sera, a precipition line developed between the serum wells containing human serum and virus, virus-host, and host antisera or ascitic fluid. This reaction (arrowed, plate 8-b) resulted because the host component of the virus was derived from a human cell line (L132) and contains antigens which are also present in human sera. Thus, antiserum

Plate 8

Plate 8:- Evaluation of the HCV/229E disrupting agents: trypsin and pronase.

a) Following the digestion of virus with trypsin, only two clear lines can be seen and both show a host identity.

b) In this comparative reaction between trypsin and DOC disruption, three components are detected after trypsin disruption, but none show a relationship to the six reacting components released by DOC. In this reaction at least three 229E precipitation lines have developed with the 229E human convalescent sera.

c) One, possibly two, reacting components are released after 229E/Pronase disruption.

d) In this comparative reaction three to four lines were seen between 229E/Pronase and 229E/AS and probably only one was related to the DOC-disrupted virus. Three were seen between 229E/CONV. and 229E/DOC. In plate 8-b there is an apparent reaction between two antigens (229E/DOC and 229E/TRYP) which, as discussed in plate 3, is a result of the imbalance reaction of the 229E/AS and 229E/CONV. serum, and, 229E/DOC

Plate 8 continued:-

antigen and 229E/TRYP. A similar result is seen in plate 8-d between the antigen 229E/DOC and 229E/PRO.

229E AS
L132 AS
229E L132 AS
229E TRYP.
229E L132 AS
L132 AS
229E AS
a
229E CONV.
229E AS
229E
229E DOC
229E TRYP.
229E AS
229E CONV.
b
229E AS
229E CONV.
229E DOC
229E PRO.
229E AS
d

against those cellular host components may react with the common antigens present in human serum or tissue extracts. That this is not a virus specific reaction is indicated by the reaction of non-identity between the two precipitation lines (arrowed).

Pronase as the other proteolytic enzyme released as many as three to four lines (plates 8-c and 8-d). Several of these were related to the components resulting from DOC disruption (plate 8-d).

On the basis of these results, neither trypsin nor pronase appeared to be satisfactory agents for the release of the complete spectrum of virus antigens for further studies.

Finally, two detergents, commonly used to release antigenic components were tested in our immunodiffusion system. These were Triton X-100 and Sarkosyl. In the reactions presented in plate 9, it can be readily seen that Triton X-100 disruption released at least one extra antigen not detected after DOC treatment (plate 9-a). In the second reaction (plate 9-b), it was evident that Sarkosyl was not as effective as either DOC or Triton X-100 since fewer (two) precipitation antigens were detected. Of the two Sarkosyl-released antigens, one was identified as the internal 'RNP' antigen in the reaction shown in plate 9-c. However, again, it was evident that Sarkosyl was not an agent of choice for the detection of the complete

Plate 9

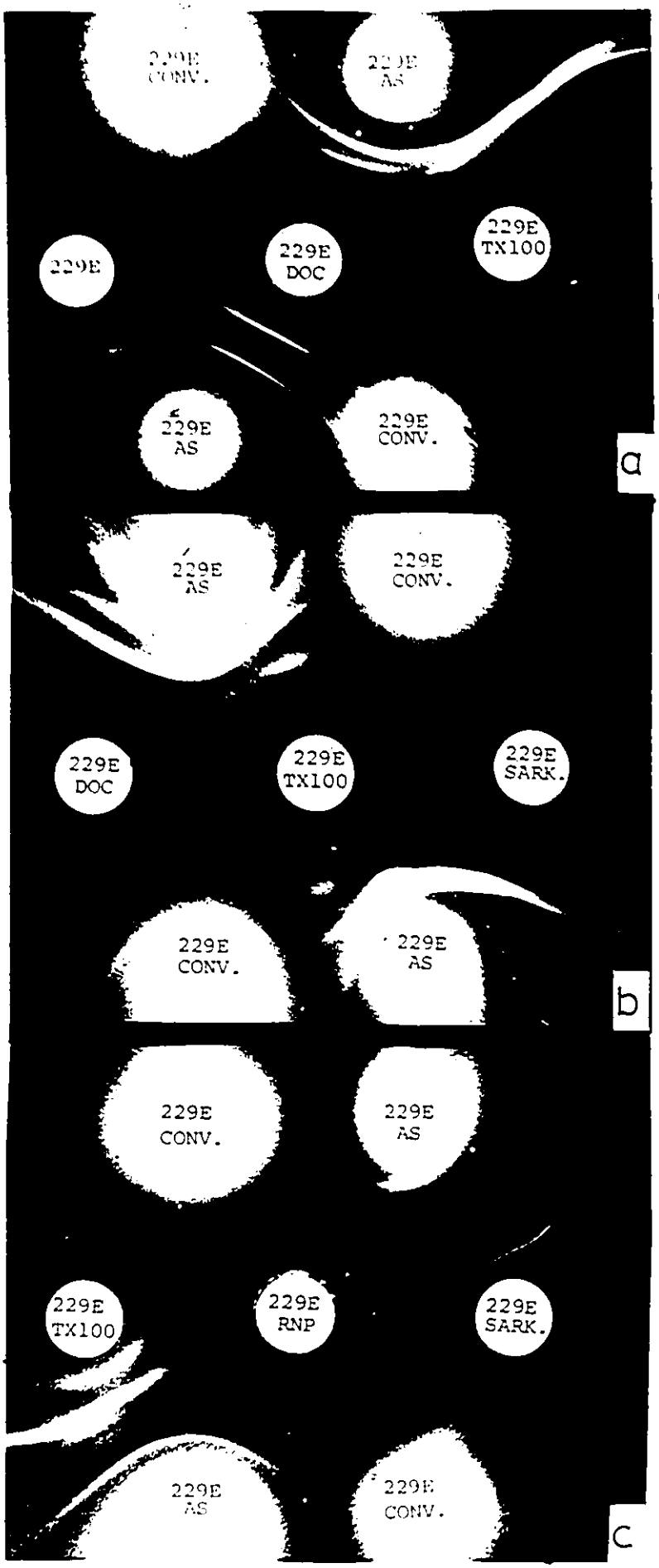
Plate 9:- Evaluation of HCV/229E disrupting agents: Triton X-100 and Sarkosyl.

a) At least five precipitation lines can be seen in the 229E/TX-100 homologous reaction with 229E/AS. Four of these (two major and two minor) are present in the 229E/DOC-disrupted preparation as shown by the reactions of identity. At least one more line was detected only in TX-100 disrupted virus specimens (arrowed).

b) Using Sarkosyl, only a few components are detected in comparison to those produced by the DOC or TX-100 disruption of virus preparations.

c) In this plate one of the antigens detected in the 229E/SARK and 229E/AS reaction showed a relationship to 229E/RNP by the reaction of identity with the 229E/RNP reaction with 229E/AS (arrowed). Similarly one of the antigens produced by TX-100 disruption was found to be an RNP antigen due to the identity indicated by the precipitation line between 229E/RNP and 229E/AS.

In both 9-b and 9-c, 229E disrupted by TX-100 and DOC showed a wide range of lines with the 229E/AS.


229E CONV.
229E AS
229E
229E DOC
229E TX100
229E AS
229E CONV.
a
229E AS
229E CONV.
229E DOC
229E TX100
229E SARK.
229E CONV.
229E AS
b
229E CONV.
229E AS
229E TX100
229E RNP
229E SARK.
229E AS
229E CONV.
c

spectrum of virus antigens.

In further reactions exploring the effects of Triton X-100, two virus antigens with host specificities (229E/L132) and four without (229E) were identified (plate 10-a). At least six precipitating components were identified by both antivirus immune serum and ascitic fluid (plate 10-b) and at least one was identified as virus specific by the reaction of identity with the convalescent serum reaction.

These foregoing results have shown that both TX-100 and DOC were the most promising agents of disruption by exposing the most virus-reacting components. Throughout the remainder of this work, only DOC or Triton X-100 was used, and special attention was always given to the possible occurrence of non-specific reaction artefacts, created as a result of the detergent interaction with serum proteins. In all cases, if a hazy zone or halo was evident surrounding the central well and common to all reaction areas, the band was not included as a precipitin line, and if occurring alone, was considered to be negative.

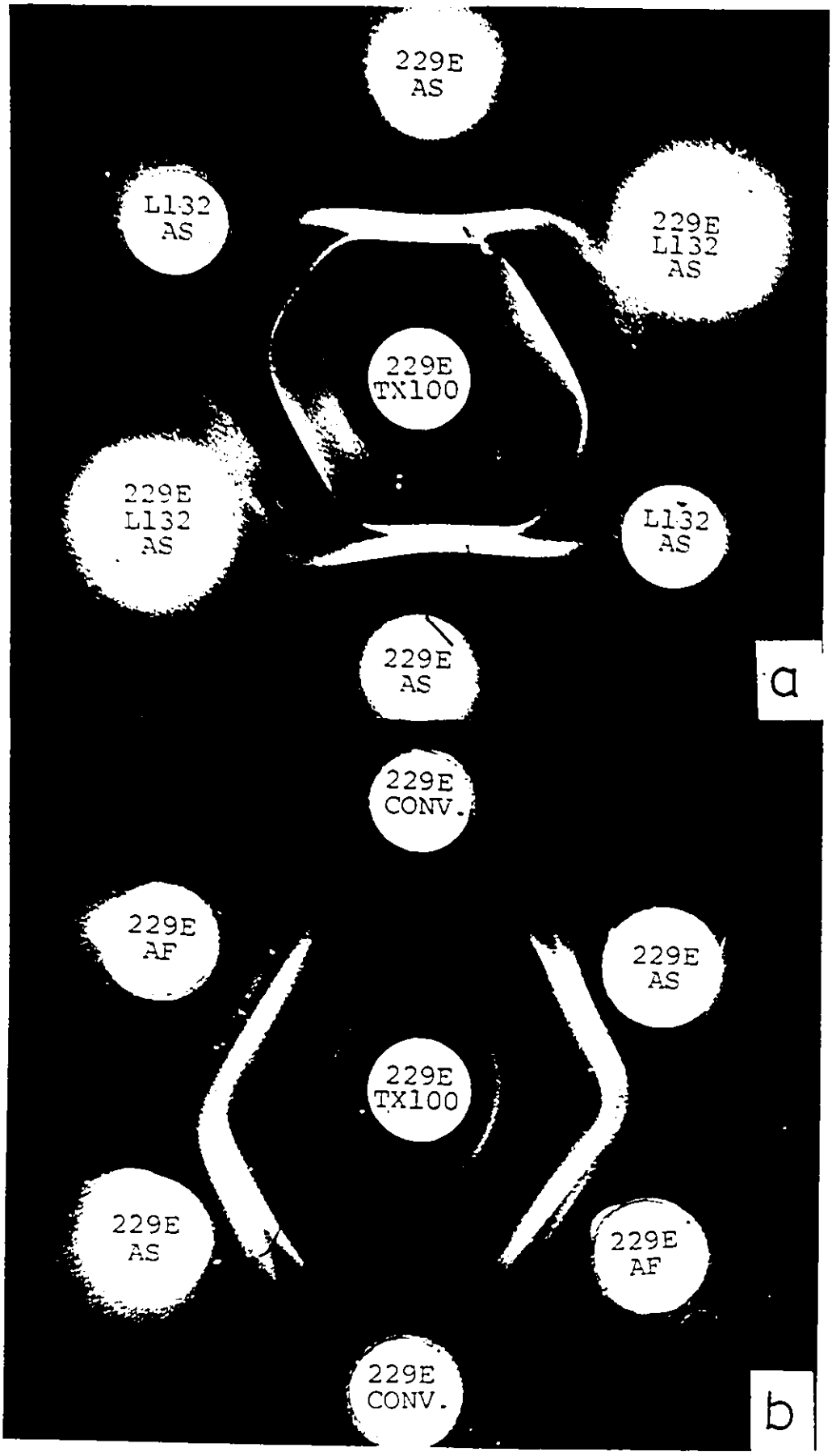
Identification of the HCV/229E internal component antigen (RNP): After the detection and identification of the spectrum of HCV/229E antigens, we moved to the next problem in which these antigens were studied in relation to the structure of the virus. The internal component was the most appropriate one of these structural components

Plate 10

Plate 10:- Further evaluation of HCV/229E disrupting agents: Triton X-100.

a) Two host antigens identified by their reaction with host antiserum (L132/AS). Two virus (229E) antigens are identified by the reaction of non-identity with the host. Another extra two virus components are detected, one in the reaction with 229E/AS only, and the other one by both 229E/AS and 229E/L132/AS. The specificity of these latter components is not clearly demonstrated in this reaction.

b) Reactions comparing the 229E antiserum, ascitic fluid, and convalescent sera. At least six antigens are detected by both 229E/AS and 229E/AF of which one was identified as virus specific by the convalescent sera in the reactions of identity.


229E
AS
L132
AS
229E
L132
AS
229E
TX100
229E
L132
AS
L132
AS
229E
AS
a
229E
CONV.
229E
AF
229E
AS
229E
TX100
229E
AS
229E
AF
229E
CONV.
b

at this stage because it was important to establish the identity of this antigen within the complete HCV/229E antigenic spectrum before it could be possible to investigate the role of the RNP (internal component) with respect to the coronavirus group serological cross reactions. It was considered that the RNP antigen was very likely a common group antigen and therefore of importance to the classification of coronaviruses.

Further studies on the antigenic identification and characterization of this antigen were aided by the fact that successful methods for the isolation of internal RNP were already established (Kennedy & Johnson-Lussenburg, 1976).

The results of the immunodiffusion reactions used to identify the internal component (RNP) are presented in plates 11-a, 11-b, 11-c, and 11-d. RNP antigen was identified in both virus (229E) and virus-host (229E/L132) antigenic material by the reactions of identity detected by virus specific antiserum (229E/AS) between these antigens and the isolated RNP reaction, which indicated that this component was present in the virus and virus-host preparations (plates 11-a). In some of the reactions (plate 11-c), two RNP-reacting components were detected. On the basis of the physical characteristics of the precipitation reaction (i.e. location, intensity, etc.), it was thought that one was the major and the other was the minor constituent of the RNP, the latter possibly a breakdown

Plate 11

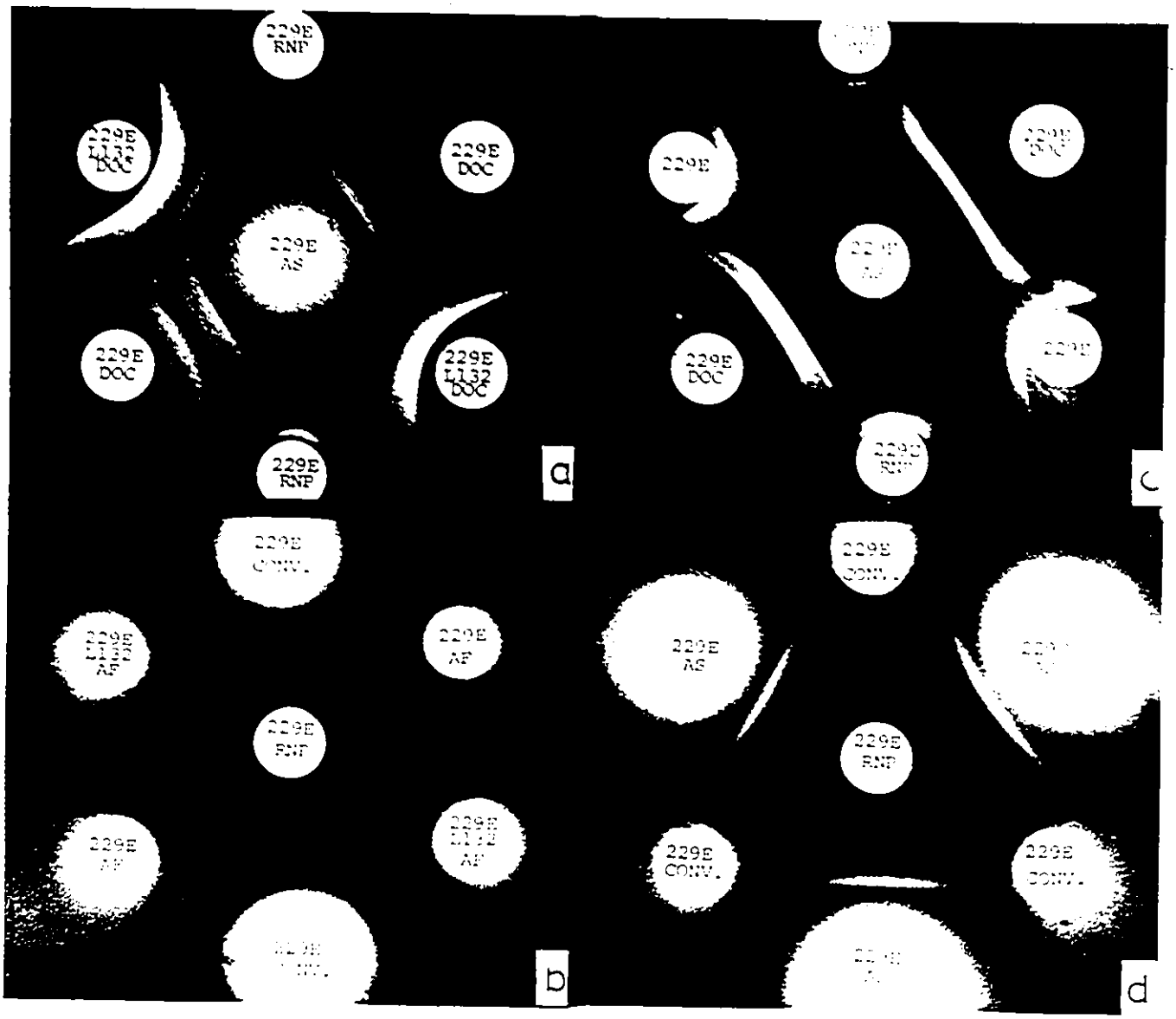
Plate 11:- Identification of the HCV/229E internal component (RNP) antigen.

a) The RNP antigen detected by the reaction of RNP and 229E/AS shown to be present in the 229E/DOC and 229E/L132/DOC preparation by the line of identity between the RNP and those two antigens (arrowed).

b) A continuous faint precipitation line (arrowed) shared between the convalescent serum (229E/CONV.) and both 229E and 229E/L132 specific immune ascitic fluid serves to identify the convalescent antibody as anti-229E internal component.

c) Two RNP reacting components are detected by the specific antivirus serum (229E/AS). One of these components is apparently detectable in both intact and disrupted virus, while the second was detected only in the disrupted virion (229E/DOC).

d) In this reaction pattern using 229E convalescent and specific sera and the 229E/RNP, two RNP lines can be seen. They are not clear enough for interpretation, however the outer line appears to be bending toward the 229E/CONV. well which would indicate a relationship between it and 229E/RNP.


229E RNP
229E DOC
229E AS
229E L132 DOC
229E CONV.
229E AF
229E L132 AF
a
b
c
d

product of the former or a contamination of a host component.

In the next reactions, the RNP antigen was used to determine the identity of the reacting antibody in the 229E convalescent sera. A continuous precipitation line shared between the 229E AF, 229E L132 AF, and convalescent human serum can be seen in plate 11-b, which serves to identify this convalescent antibody as anti-229E internal component. The 229E convalescent specificity was confirmed by using a different reaction pattern including anti-virus serum instead of ascitic fluid (plate 11-d).

Immunoprecipitation studies of HCV/229E by polyacrylamide gel electrophoresis: The enumeration, identification, and determination of the functions of the various polypeptides made in coronavirus 229E-infected cells are essential to the understanding of the virus-host cell interaction. The major difficulty encountered in such a study is the identification as virus or host specificity of the polypeptides made in the infected cells.

Experiments involving immunoprecipitation of radiolabelled virus components with specific antisera, followed by polyacrylamide gel analysis using the standard SDS system (Bonner & Laskey, 1974; Bingham, 1975; Garwes & Pocock, 1975) were undertaken in an attempt to correlate host, virus-host, and virus antigens with the polypeptides developed during replication.

Preliminary work demonstrated that there was no technical difficulty in detecting and identifying virus specific polypeptide antigens. By labelling virus antigens with ^{14}C-glucosamine, it was possible to confirm the reported finding that the NP (50,000 molecular weight) was the only non-glycosylated antigen. However, using either virus or host analytical antisera, it was not possible to distinguish any major differences between the polypeptides precipitated due to the large numbers present. Furthermore it was found that host antiserum was capable of precipitating the radiolabelled NP polypeptide from crude infected lysate or SNF preparations but not from purified virus preparations. To further complicate the picture, it was found that the host antiserum also precipitated polypeptides from unlabelled, uninfected lysate controls which had the same migration characteristics as the NP polypeptide (50,000 molecular weight).

Therefore, it was apparent that the host contribution and role in coronavirus replication, while considered to be of vital importance, could not be readily distinguished at this stage without suppressing host-related metabolism in the infected cell. Because further investigation in this direction would divert attention from the major aim, i.e. to fully characterize the internal component antigenically, no further PAGE immunoprecipitation was carried out at this time.

The significance of these results remains to be determined. However, in view of the virus-host shared specificities demonstrated in the immunodiffusion reactions, it was not a totally unexpected finding.

Summary

As a result of these studies which have been concerned mainly with the antigenic structure of HCV/229E, we can draw the following conclusions.

Phosphate buffer, at a concentration of 0.001M, or water, were good diluents for the storage and preparation of the virus. The pH range of 6.4 to 8.0 was found to be tolerable with the buffer described within the period of 94 hours.

The procedure of producing ascitic fluid in guinea pigs was useful for the preparation of a high quality and quantity of immune fluids. The combination of anti-host (L132/AS), anti-virus-host (229E/L132/AS), anti-virus sera (229E/AS), and ascitic fluids as well as the convalescent human sera (229E/Conv.) were useful tools in the identification and characterization of 229E antigens, whether of virus or host-derived specificity.

On the basis of our immunodiffusion reactions, as many as 6-7 viral structural antigens were identified by using both hyperimmune serum or ascitic fluid in combination

with the human convalescent sera. Both NaDOC and TX-100, in the concentrations used, were the best disrupting agents for the production of HCV/229E antigens. Of the total viral antigens, two to four, as represented by two to four lines in immunodiffusion, were identified as specific virus antigens due to the non-identity of these lines with the host lines (antigens) and the identification of those antigens by the human convalescent sera. One antigen was found to be ribonucleoprotain antigen. The rest of the antigens showed a host relationship. These reactions with the host demonstrated by some of the antigens are an indication of the presence of host components, possibly modified during viral infection and integrated into the virion.

EXPERIMENTAL RESULTS

PART II

Antigenic Relationships Between Coronaviruses

EXPERIMENTAL RESULTS

Part II

Antigenic Relationships Between Coronaviruses

New candidate members of the coronavirus group are continuously proposed on the basis of the typical morphology of the family. Expansion of the group, however, only adds more problems to those still unresolved within the already existing members, even though some members of the group have been in circulation for long periods of time.

The major problem in assigning membership within the group has been concerned with the lack of adequately defined group criteria. Information has recently been accumulating pertaining to certain physical properties of the virion, the nucleic acid structure and function (s.s., infectious (messenger RNA), the biochemical characteristics of the structural components, and the site of viral replication. However, there has been much conflicting information concerning the antigenic relationships not only within but between species. Varying degrees of antigenic relatedness have been reported between different coronaviruses, and these have depended to a large extent on the nature of the serological test used (Robb & Bond, 1978; R. W. Bingham, Personal Communication).

The intent of this work was, first to fully characterize the antigens of one strain (HCV/229E) followed by an in-depth exploration of the relationships of it with other

coronaviruses, using the immunodiffusion reaction which would allow a detailed interpretation with identification of common reacting components. Of particular interest was the possibility that the internal component might possess group characteristics, as has been found for most viruses. The viruses chosen for this study were readily available, but also represented the best known strains of the group, i.e., TGE, HEV, MHV, and the type species, IBV. Both hyperimmune and convalescent sera were obtained for these studies. And finally, it was intended, that, in the event that an antigen common to the various strains was detected and identified, a survey of normal serum samples from a variety of animals would be undertaken to determine the prevalence of anti-229E antibodies in the respective populations. The results of these studies and their implications are presented in this section.

Growth of Transmissible Gastroenteritis Coronavirus (TGE)

A Purdue strain of this virus was used in our study. The virus originally obtained from Dr. Lambert, Ames, Iowa, was obtained through the courtesy of Dr. G. Dulac. The virus was grown in primary pig kidney cells without serum or bovine serum albumin.

The nature and extent of the CPE the virus produced was clear and extensive and generally the monolayers were 50% destroyed within 24 to 36 hours from the first appearance of CPE, at which time virus was harvested.

Preparation of Transmissible Gastro-enteritis Virus Antigens (TGE)

The procedure described for the preparation of HCV/229E antigen was followed for the purification and preparation of TGE antigen. Viral RNA was labelled with (5-^{3}H)-uridine to provide a tracer throughout the purification. Centrifugation through sucrose gradients resulted in a homogeneous virus peak after the equilibrium gradient centrifugation (figure 6). Electron microscopy of the virus peak demonstrated typical coronavirus morphology (plate 12). Non-viral material in the electron micrograph of a negatively stained preparation of the purified virus was also detectable, however, it constituted a small proportion of the total material present. Such non-viral material could consist of normal host components, "possibly membrane," which were attached to some of the virus particles as a result of the budding coronavirus release from the cell. As shown in figure 6, the peak of radioactivity occurs in the zone of 47 to 48% w/w sucrose having a density equivalent of 1.210 to 1.215. These virus peaks were collected, pelleted, and resuspended in buffer to be used as TGE antigen. A concentration factor of 300 times, starting from 300 ml of crude virus suspension and ending with 1 ml of concentrated and purified virus was obtained.

Preparation of Transmissible Gastro-enteritis Virus Ribonucleoprotein (TGE-RNP)

The procedure described for the isolation of 229E-RNP by NP40 treatment was used for the isolation of TGE-RNP. After equilibrium density gradient purification, TGE labelled

Figure 6

Figure 6:- Purification of TGE virus by sucrose density gradient of (5-^{3}H)-uridine labelled virus preparation.

EQUILIBRIUM GRADIENT OF TGE.

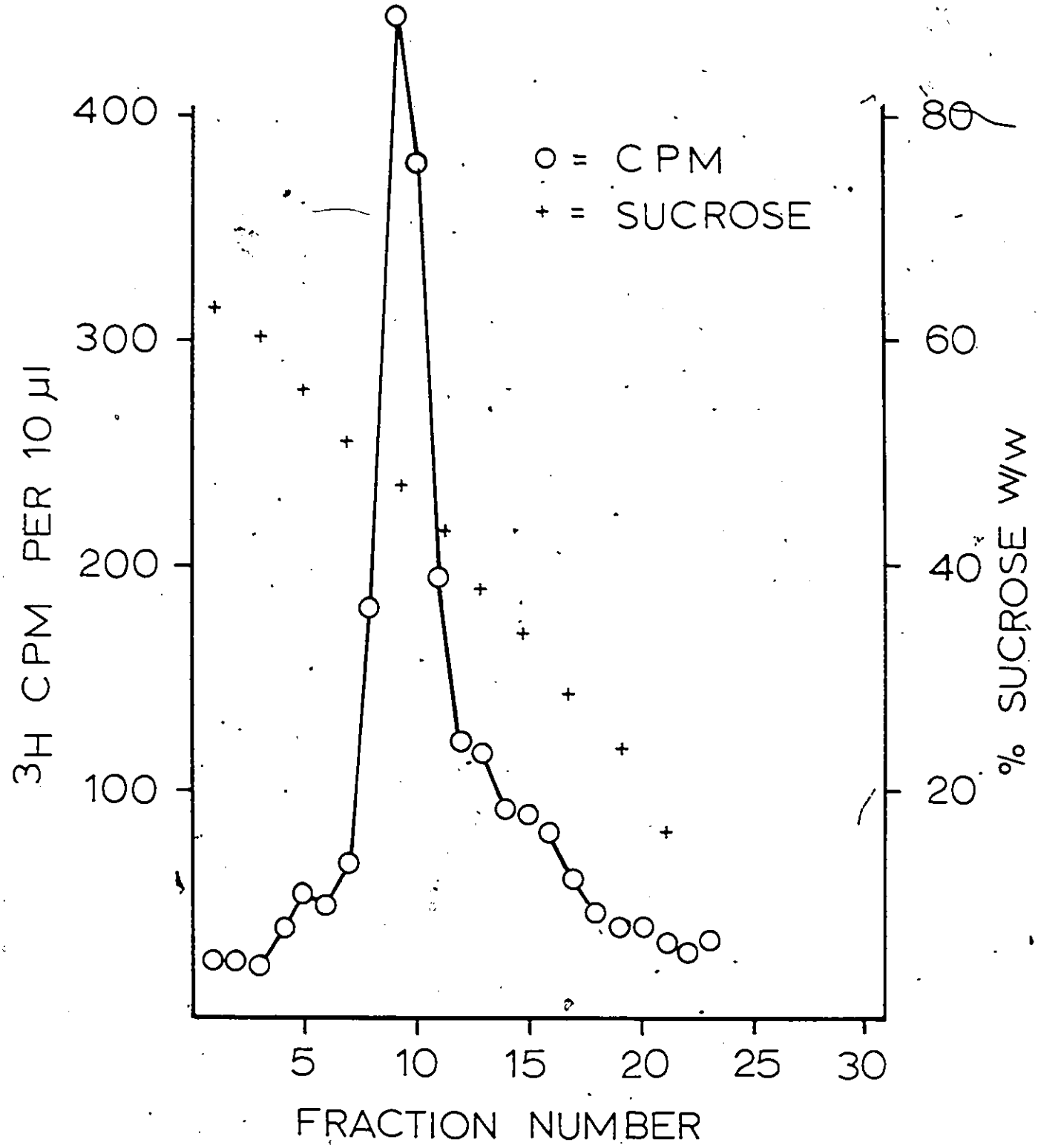


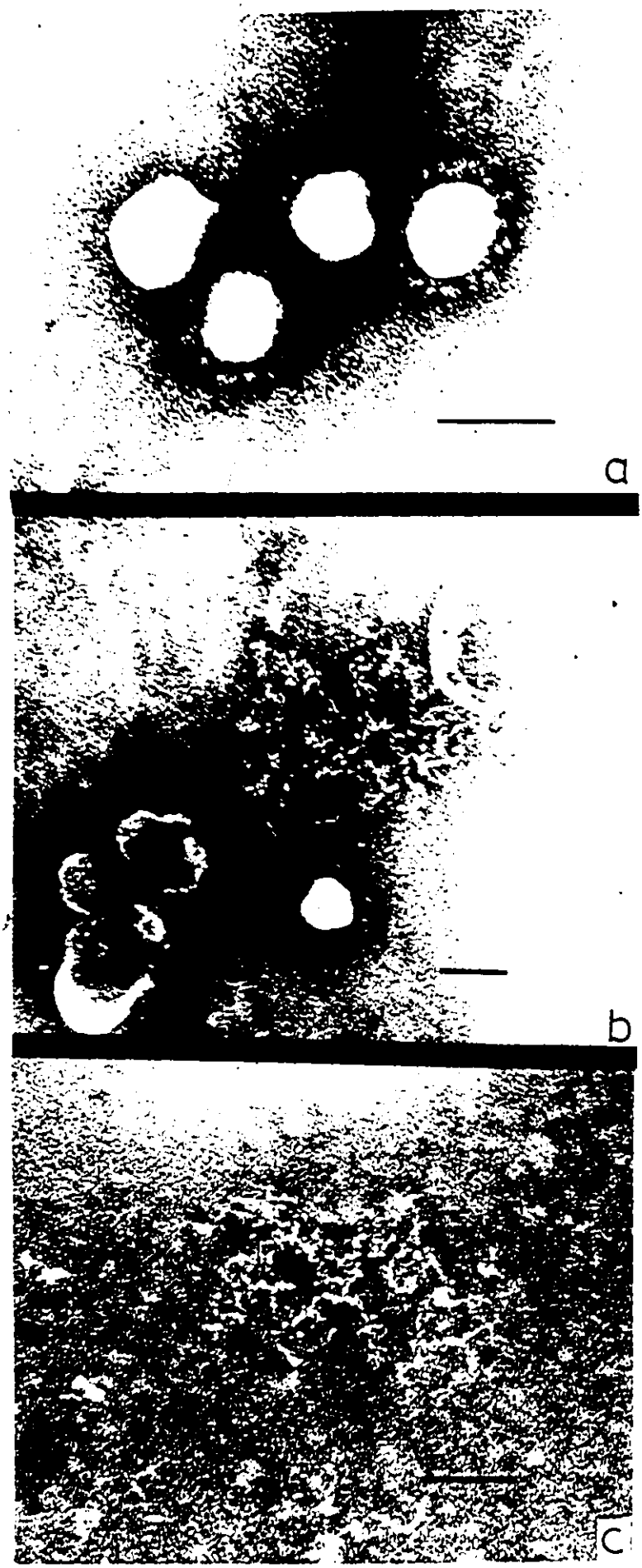
Plate 12

Plate 12:- Morphology of TGE.

a) Appearance of TGE virus after equilibrium sucrose density purification with corona-like projections surrounding the virus particles which give it the typical morphology of the group.

b) Morphology of coronavirus internal component as a result of spontaneous disruption of the virus during the purification procedure. The RNP structure seems to be attached to the fringe fragment of the virus.

c) Morphology of the coronavirus internal component isolated by NP40 treatment of purified TGE virus preparation. These specimens were all negatively stained with 2% PTA at pH 6.4 and, in the micrographs, the bar represents 100 nm.


a
b
c

with 3H-uridine was incubated either with distilled water and used as a control or with the NP40 mixture for the isolation of the internal component. The radioactive label of NP40-treated TGE consistently was found at a different sucrose concentration than untreated controls (figure 7) and had a buoyant density of 1.29.

Growth of Hemagglutinating Encephalomyelitis Virus (HEV)

The strain of HEV-2 used here was originally isolated by Dr. Greig (Greig et al, 1962) and we in turn obtained it from Dr. G. Dulac of the ADRI. The virus was used after almost complete destruction of the primary pig kidney cell monolayer, within 24 hours of inoculation. Virus titres were obtained by titration on IB-RS-2 cells, a continuous line of pig cells.

Preparation of Hemagglutinating Encephalomyelitis Virus Antigens (HEV)

^{3}H-uridine-labelled HEV was purified in a similar manner to TGE and HCV/229E. The purification resulted in one sharp virus peak of the labelled HEV, occurring in the zone of 1.20 to 1.21 density in the equilibrium sucrose gradients (figure 8). Typical coronaviruses were seen in the EM preparations of the material from the peak fractions (plate 13).

Figure 7

Figure 7:- Purification of RNP from TGE-purified virus by treatment with the non-ionic detergent NP40.

EQUILIBRIUM GRADIENTS

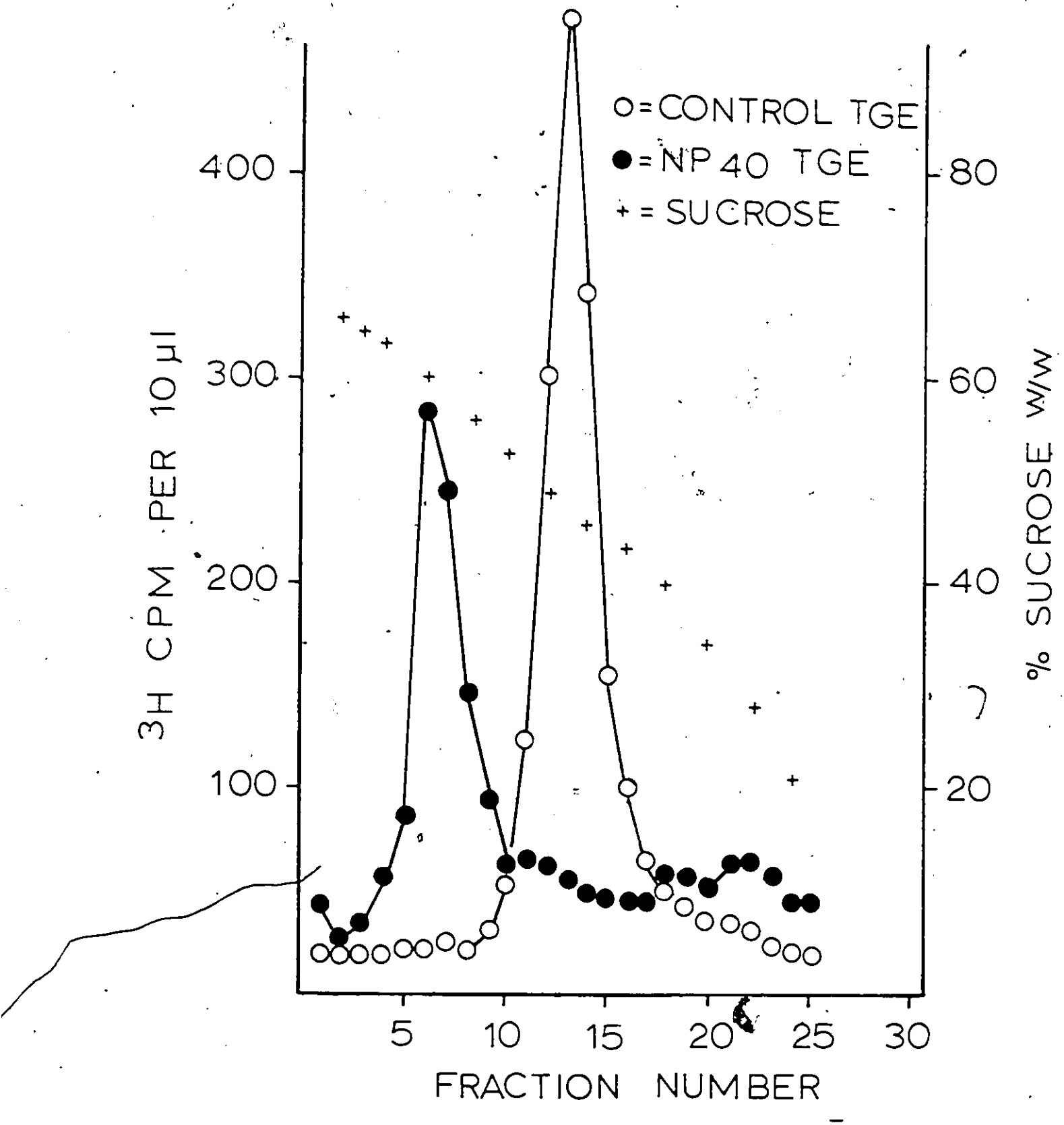


Figure 8

Figure 8:- Purification of HEV virus by sucrose density gradient ultracentrifugation of (5-^{3}H)-uridine labelled virus.

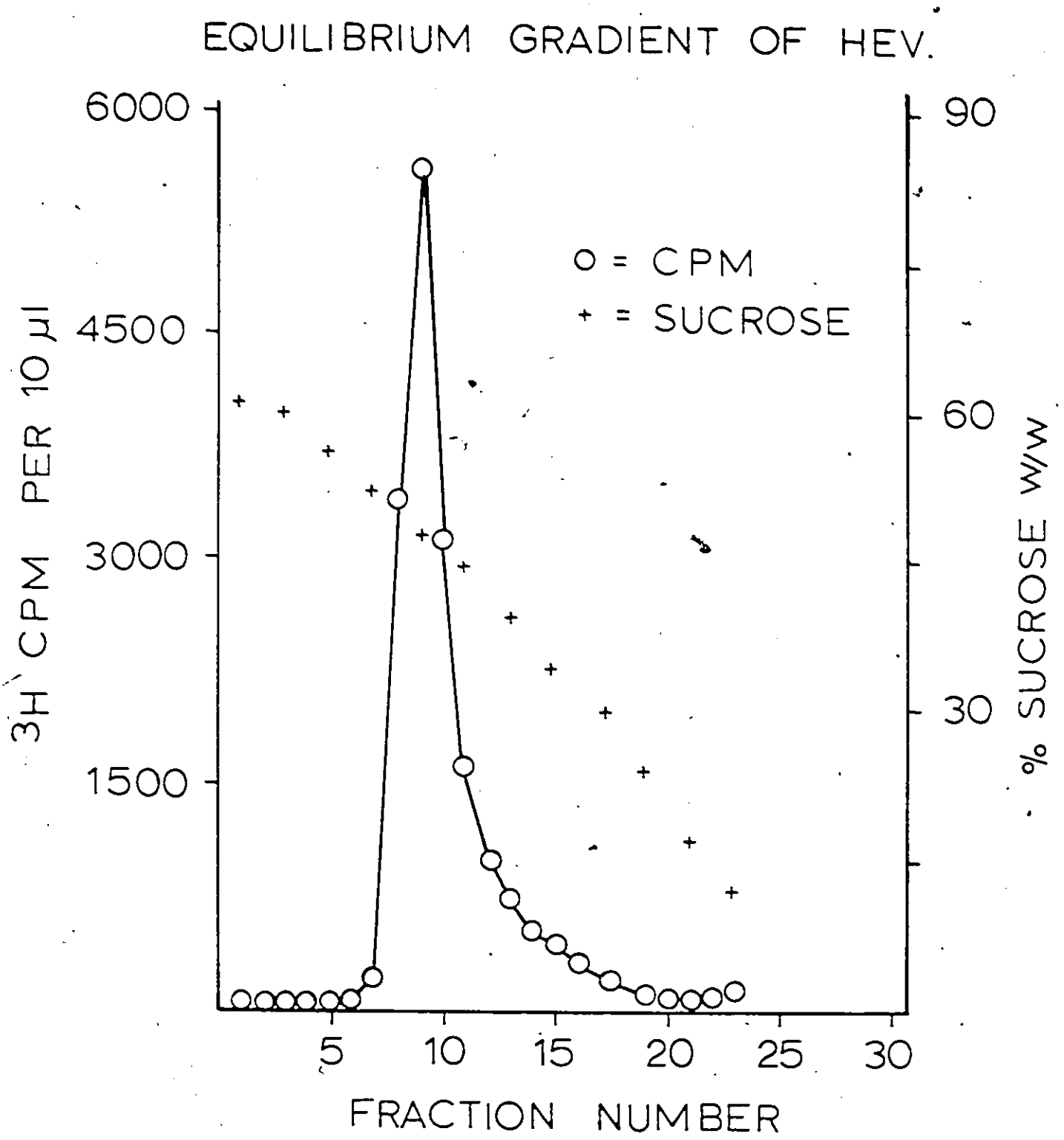

EQUILIBRIUM GRADIENT OF HEV.
6000
4500
3000
1500
90
60
30
O = CPM
+ = SUCROSE
3H CPM PER 10 µl
% SUCROSE W/W
5
10
15
20
25
30
FRACTION NUMBER

Plate 13

Plate 13:- Morphology of HEV.

a) Morphology of HEV virus particles obtained following equilibrium density gradient purification. Again, the characteristic virus morphology is readily apparent.

b) Electron microscope preparations illustrating the morphology of the RNP components obtained following NP40 treatment of purified HEV virus preparations.

c) Enlarged area, inset above. Specimens were negatively stained for electron microscopy with 2% PTA at pH 6.4. The bar represents 100 nm..


a
b
c

Preparation of Hemagglutinating Encephalomyelitis Virus Ribonucleoprotein (HEV-RNP)

When samples of NP40-treated, (5-^{3}H)-uridine-labelled HEV were centrifuged through linear sucrose gradients, a single peak of radioactivity was observed sedimenting at a sucrose concentration of 58.5% (w/w) which is equivalent to a density of 1.27. The virus control peak was found at a sucrose concentration of 47% (w/w) which equals a density of 1.21 (figure 9). Treatment of HEV with NP40 therefore liberated an internal component sedimenting at a different density from the control virus. The internal component was found to contain RNA and a reduced amount of protein as revealed by protein estimation (table 3). As with other antigens, HEV-RNP in the pooled peak fractions was pelleted and resuspended in buffer for use in immunodiffusion reactions. Using polyacrylamide gel electrophoresis, both TGE and HEV nucleoproteins were found to have migration characteristics similar to those of the NP polypeptide of HCV/229E (50,000 molecular weight).

Preparation of Mouse Hepatitis Virus Antigens (MHV)

The virus preparation used in our study was received as semipurified suspension through the courtesy of Dr. K. Holmes (see Materials and Methods). Due to the weak reaction of this preparation in the immunodiffusion experiments, it was concentrated further by ultracentrifugation. Unfortunately only small quantities were available and therefore most of the analytical evaluations depended on the heterologous antiserum reaction.

Figure 9

Figure 9:- Isolation of HEV-RNP internal component by the treatment of purified virus with the detergent NP40.

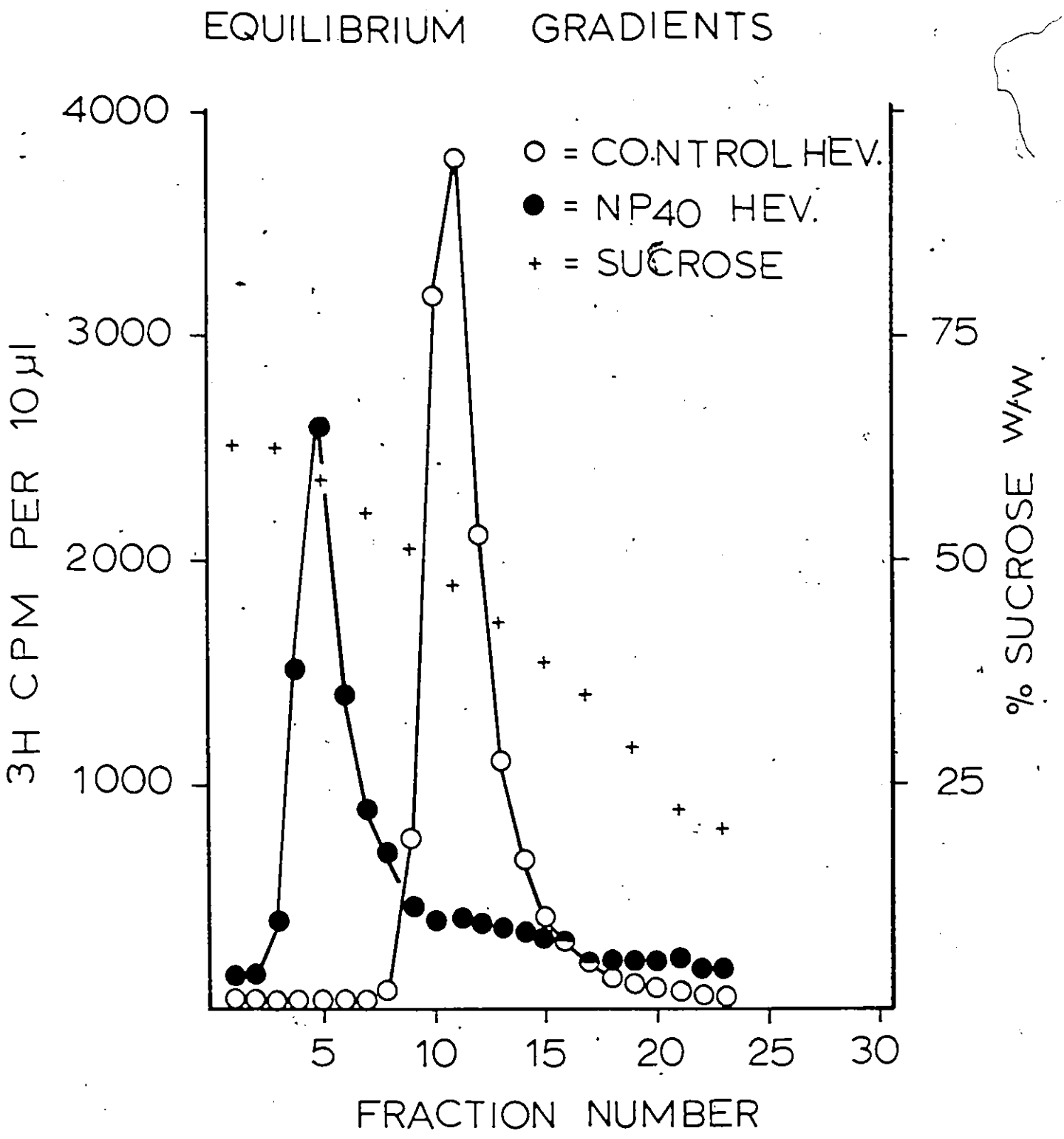

EQUILIBRIUM GRADIENTS
4000
3000
2000
1000
3H CPM PER 10 µl
75
50
25
% SUCROSE W/W
○ = CONTROL HEV.
● = NP40 HEV.
+ = SUCROSE
5
10
15
20
25
30
FRACTION NUMBER

TABLE 3

PROTEIN CONTENT OF THE DIFFERENT PREPARATIONS USED IN STUDIES OF THE ANTIGENIC RELATIONSHIPS BETWEEN THE DIFFERENT CORONAVIRUSES

Antigen Preparation	Protein Content (µg/ml)
TGE Virus	145
TGE-RNP	50
HEV Virus	190
HEV-RNP	65
IBV Virus	2900
MHV Virus	ND*

*Not done due to the small sample available.

Preparation of Infectious Bronchitis Virus Antigens (IBV)

Semi-purified and concentrated virus was obtained from infected allantoic fluid which was provided by Dr. E. Thomas of ADRI. As with mouse hepatitis virus, further purification in sucrose gradients was not possible because the virus was not labelled with a radioactive tracer. Typical virus particles were identified in both MHV and IBV preparations by electron microscopy.

Protein Estimations

The protein content of the antigens used in this part of the study was determined by the Coomassie blue procedure described by Bradford (1976) (table 3). Approximately 65% of virus protein was removed in the process of RNP preparation.

Electron Microscopic Examination of the RNP Preparations

TGE-RNP: Grids for the electron microscope examination of purified virus and the isolated RNP preparations were prepared as described earlier. EM examination of the virus control preparation revealed different viral structures. Besides the typical virus seen in the appropriate density region of the control gradient, disrupted virions

with different amorphous matrices were also seen, some of which were similar to the RNP structure described for 229E-RNP (Kennedy & Johnson-Lussenburg, 1976), (plate 12-a). Many particles appeared to have lost only their corona-like projections and some particles could be found with minimal morphological damage. Complete fragments of the envelope with typical projections could also be seen in some of the grids. Some of these envelopes appeared to be associated with the internal structure in the process of its release (plate 12-b). These RNP structures occurred during density gradient centrifugation as a result of spontaneous virus disruption. They were collected as separate peaks found at higher densities and used as RNP antigen in some immuno-diffusion reactions. Plate 12-c shows a typical morphology of TGE-RNP structure isolated after NP40 treatment of TGE. These TGE-RNP structures have a similar morphology to that described for 229E-RNP (Kennedy & Johnson-Lussenburg, 1976). These structures were fairly discrete tangles of threads with diameters of approximately 10 nm. The total length was impossible to measure as the path of the linear structure cannot be followed with certainty. However, the morphology of these strands was consistent with a continuous structure for quite a distance.

HEV-RNP: Structures like those described in the TGE-RNP were seen in HEV preparations. Clear and typical

corona-like particles were present in the virus control (plate 12-a). Different stages of disruption were seen in the virus control and some of these have the typical RNP structure. The disrupted preparations and similar ones described with 229E and TGE lend support to the reported fragility of coronaviruses. Beside the rounded typical coronavirus particles, pleomorphic virions and envelope fragments with the corona-like projection were also seen. Again thread-like RNP structures appeared to be released from the surrounding envelopes and these threads had diameters in the neighbourhood of 10 nm. RNP isolated by NP40 appeared as a thread-like structure, approximately 10 nm in diameter (plates 13-b and 13-c) similar to that described for 229E and TGE-RNP. These results demonstrated that the three coronavirus RNPs (229E, TGE, and HEV) could be prepared by either spontaneous disruption or NP40 treatment of the virus.

The fact that both spontaneous disruption and detergent treatment produced a particle of similar density and appearance suggests that this is a relatively stable internal component. Contrary to the findings of other workers (Garwes et al, 1976; Pocock & Garwes, 1977), our results indicated that the procedure of NP40 treatment originally used for the isolation of 229E/RNP could be successfully applied to TGE and HEV.

The demonstration of a linear coronavirus RNP structure for the three coronaviruses studied, 229E, TGE,

and HEV suggested that the coronavirus group nucleocapsid might be considered to be a linear structure.

Immunodiffusion Analysis of Coronavirus Relationships

In our earlier studies concerned with detecting the complete spectrum of antigenic components of the human coronavirus strain 229E, it was found that fewer precipitin lines resulted using undisrupted virus preparations. This finding was interpreted to mean that the immunodiffusion reactions involved the virion surface antigenic components since the internal antigens had not been released by specific pretreatment. Thus, it was of importance to distinguish, if possible, whether putative cross reactions between coronavirus strains would involve surface antigens, internal antigens released by disruption, or both. Our first series of comparative immunodiffusion reactions were designed to detect and identify virion surface cross reacting components by analysing the immunodiffusion precipitation lines of homologous and heterologous reactions first to establish the quality of the homologous reaction, second to detect the occurrence of heterologous reactions, and finally, to determine the relationships of the participants in both the heterologous and homologous reactions through their patterns of identity, i.e., a linkage or fusion indicating shared components, a crossover, or non-fusion

indicating differing components and a partial fusion or spur, indicating a slight antigenic difference or partial identity of antigens.

In these and the following comparative immunodiffusion reactions, the antisera used, in addition to the 229E antisera and ascitic fluid described in the preceding section, were obtained as either field-convalescent or hyperimmune-convalescent sera from the natural host of the coronavirus infection (i.e., swine and chickens) with the exception of the MHV antiserum which was obtained from hyperimmune rabbits. In the case of both field-convalescent and hyperimmune-convalescent, there should be no precipitins against the virion-incorporated host antigens, the precipitin lines being thus identified as virion antigen specific.

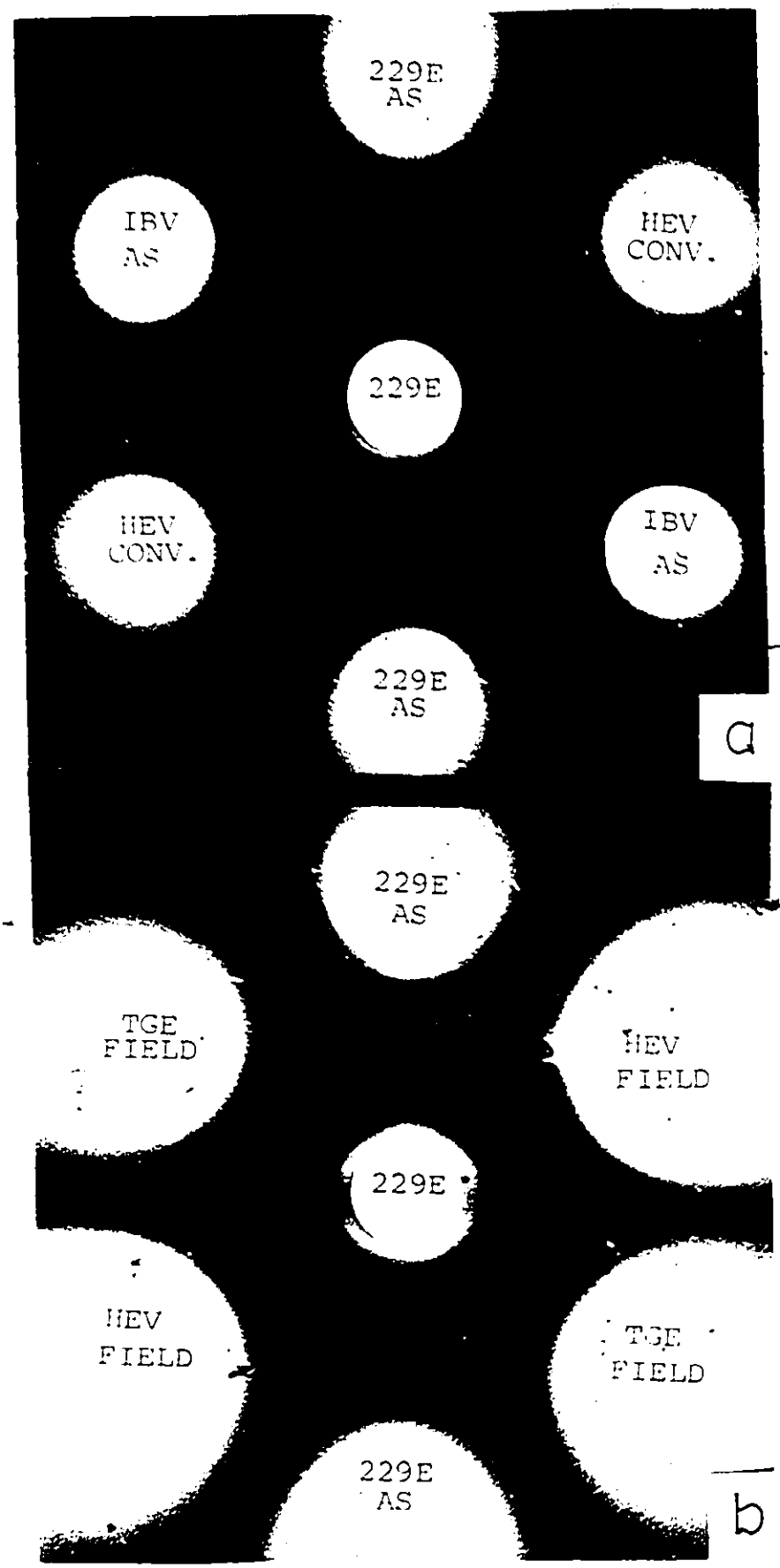
Comparison of homologous and heterologous reactions using undisrupted coronaviruses: The first analysis involved the homologous and heterologous reactions of undisrupted HCV/229E using TGE, HEV, and IBV antisera. The results are presented in plate 14. In the intact HCV/229E homologous reactions (plates 14-a and 14-b), either one or two precipitation lines can be seen.

Plate 14

Plate 14:- Homologous and heterologous immunodiffusion reactions of undisrupted human coronavirus/229E.

a) In addition to the homologous reaction, there is a weak cross reaction between IBV/AS and 229E antigen which indicates the existence of an antigen common to 229E and IBV. The identity of this with one of the 229E detectable components is demonstrated by the weak line of identity between the two (arrowed).

b) Neither TGE nor HEV field convalescent sera appear to contain a related 229E undisrupted (surface?) component in this reaction. However, at least one reacting component can be seen in the homologous 229E reactions.


229E
AS
IBV
AS
HEV
CONV.
229E
HEV
CONV.
IBV
AS
229E
AS
a
229E
AS
TGE
FIELD
HEV
FIELD
229E
HEV
FIELD
TGE
FIELD
229E
AS
b

There was a faint but discrete precipitation line formed with IBV antiserum and HCV/229E which indicated the possible existence of a related antigen. Furthermore, this component seems to be related to only one of the HCV/229E antigens because of the weak pattern of identity with one precipitation line in the homologous reaction (plate 14-a, arrowed). The other sera used in these analyses, HEV hyperimmune convalescent (HEV/CONV.) and field convalescent (HEV/FIELD), and TGE field convalescent (TGE/FIELD) did not develop any lines with HCV/229E intact antigen. However, the absence of a reaction cannot be taken as absolute in this context, being possibly because levels of precipitating antibody are beneath the optimum requirement for the line formation. These experiments were restricted to the reaction of surface antigenic components and thus were capable of identifying only a few of the relationships between intact HCV/229E and other coronaviruses. However, these reactions served to indicate that a complex system of cross relationships could be detected and analyzed. Furthermore, this analysis provided the first evidence of any relationship (other than morphological) between IBV and any other coronavirus. This relationship was further examined and confirmed as shown in the other reactions presented later.

In the following reactions extending this comparison, when intact TGE and HEV virions were used as the antigen,

Plate 15

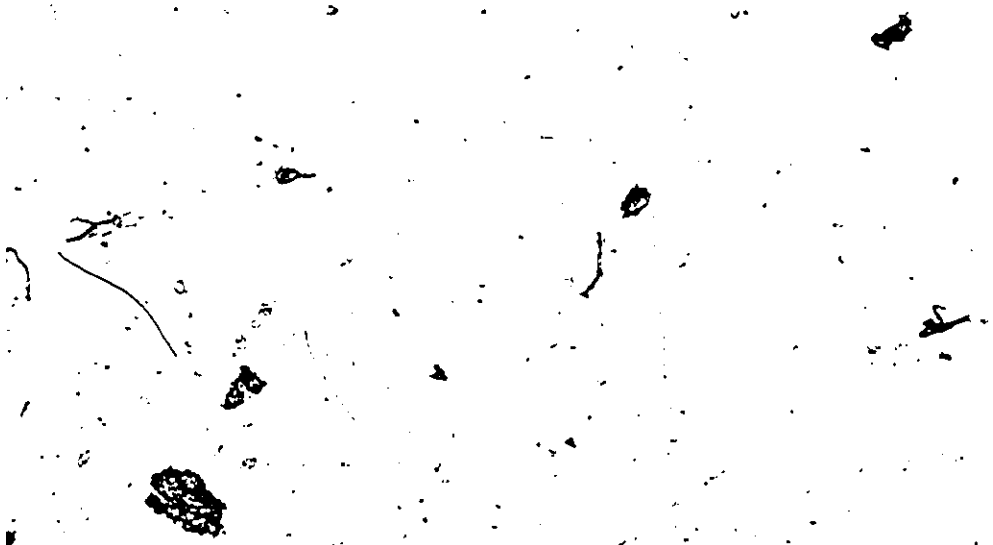
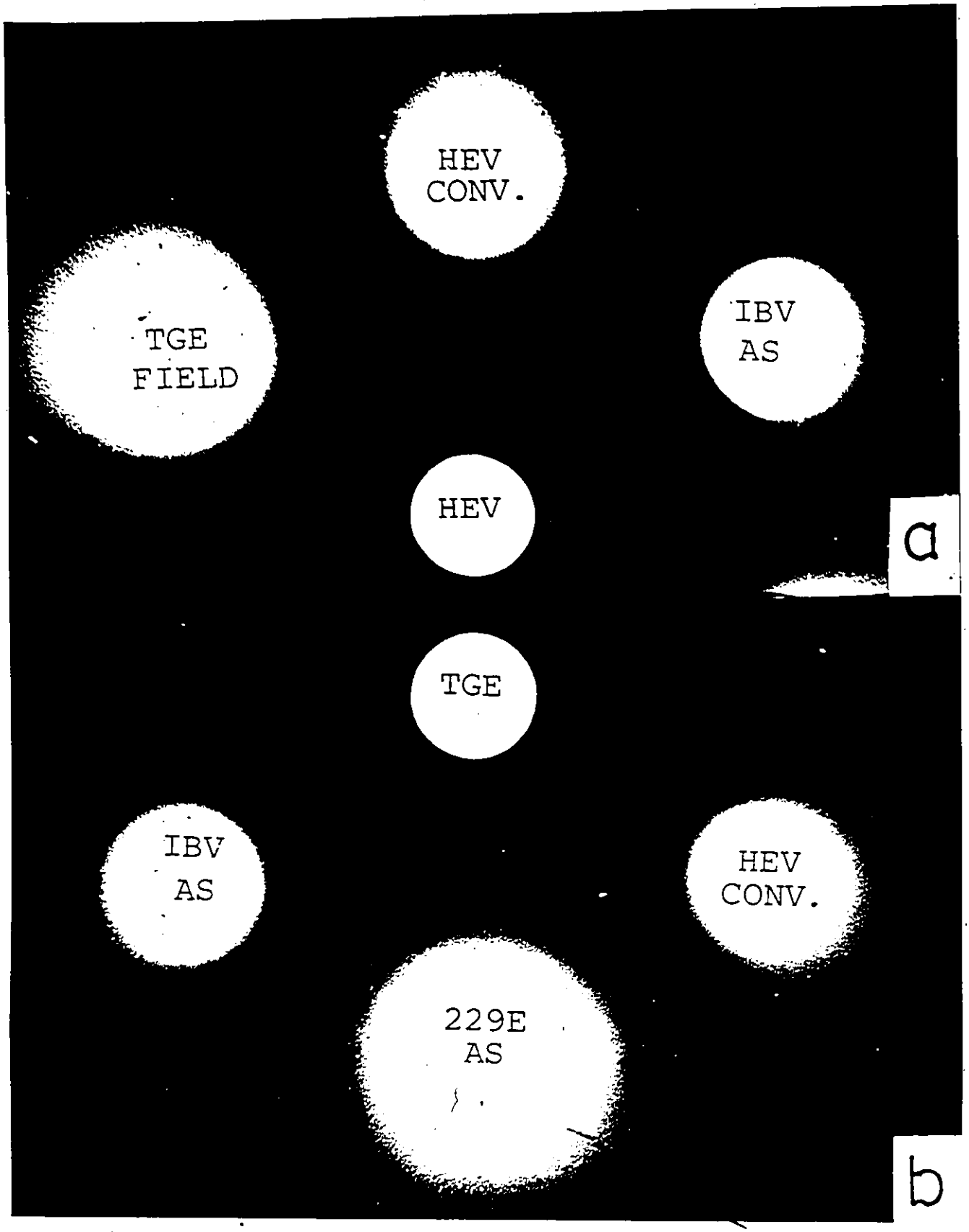


Plate 15:- Homologous and heterologous immunodiffusion reactions of undisrupted hemagglutinating encephalomyelitis (HEV) and transmissible gastroenteritis (TGE) coronaviruses.

a). The reaction of identity between the homologous HEV/CONV.-HEV reaction and the heterologous IBV/AS-HEV reaction indicates the possible existence of a common antigen (arrowed 1). TGE/FIELD, on the other hand, precipitated a partially related component as can be seen by the line of partial identity (arrowed 2).

b) The heterologous reaction between undisrupted TGE and 229E antiserum is clearly evident but there is no cross reaction with the other antisera, IBV and HEV/CONV.


HEV
CONV.
TGE
FIELD
IBV
AS
HEV
a
TGE
IBV
AS
HEV
CONV.
229E
AS
b

clear heterologous reactions were obtained in addition to the homologous reaction. One HEV precipitation line was identified in the homologous reaction (plate 15-a), and it was further noted that there was present, in the HEV virion, a component which was related to both HEV hyperimmune convalescent (HEV/CONV.) and IBV antiserum (IBV/AS). The component detected by TGE/FIELD serum was only partially related to the component detected by both HEV and IBV sera which indicated a slight antigenic difference or a partial identity of antigens (plate 15-a). In plate 15-b, only 229E/AS precipitated a related TGE component while neither IBV/AS nor HEV/CONV. were able to do so. This implied a complex one-way relationship between TGE and HEV virus strains, in which TGE antisera were capable of detecting certain HEV antigens but not the converse, TGE thus possessing extra antigenic components not present in HEV. This finding, however, could be simply due to the level of reacting antibody present in the different systems as mentioned above. But, it was also necessary to consider the possibility that the pigs used for preparing TGE hyperimmune convalescent sera had previous exposure to HEV. Although this was unlikely because of the precautions taken (G. Dulac, personal communication) to ensure that the pigs were free of specific pathogens, the possibility cannot be ruled out. Further analysis of undisrupted HEV was carried out in other immunodiffusion patterns (plates

16-a and 16-b). A reaction of identity between homologous reactions and the heterologous MHV/AS line served to identify an antigen shared by MHV and HEV viruses. A weak antigenic component was detected by the 229E antiserum (plate 16-a) but not by the 229E ascitic fluid (plate 16-b), however both the homologous (HEV/CONV.) as well as the heterologous (229E/CONV.) sera recognized similar antigens as represented by the line of identity formed by the two precipitation lines (plate 16-b). These findings were somewhat inconsistent but were considered to be due to the fact that undisrupted virus antigen was being used and it was impossible to control or evaluate the development of antigens due to spontaneous breakdown. Such products could be either detectable antigenically or disappear due to their small size.

The heterologous reaction between 229E and IBV/AS was investigated in more detail using the IBV antigen. The IBV component identified by its homologous serum was also detected by the 229E convalescent sera as can be seen by a line of identity between those two (plate 16-c, arrowed). This reaction thus indicates the presence of related antigens in IBV and HCV/229E. An unexpected observation was the absence of reaction between IBV antigen and hyperimmune 229E/AS. Such a development required more investigation. As shown in a further reaction presented in plate 17-a, a relationship between the component identified in the IBV homologous reaction and that identified by the HCV/229E

Plate 16

Plate 16:- Homologous and heterologous immunodiffusion reactions of undisrupted hemagglutinating encephalomyelitis (HEV) and infectious bronchitis (IBV) coronaviruses.

a) The component identified by the HEV homologous reaction was similar to the one identified by MHV/AS as demonstrated by the line of identity (arrowed).

b) The 229E/CONV. sera included in this reaction has detected a component similar to the one identified by the HEV homologous reaction as indicated by the line of identity (arrowed).

c) There is a line of identity developed between the heterologous 229E/CONV./IBV reaction and the homologous undisrupted IBV reaction, identifying the existence of related antigens. 229E/AS failed to identify the same component in this reaction for reasons which are, at present, unclear.

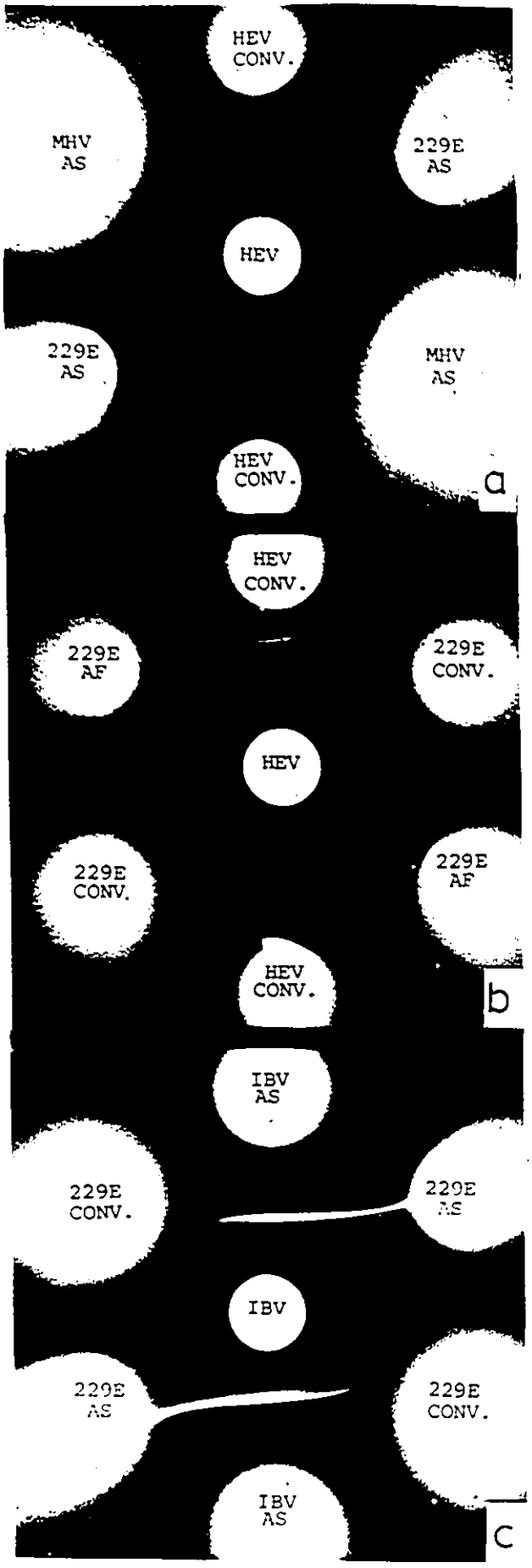
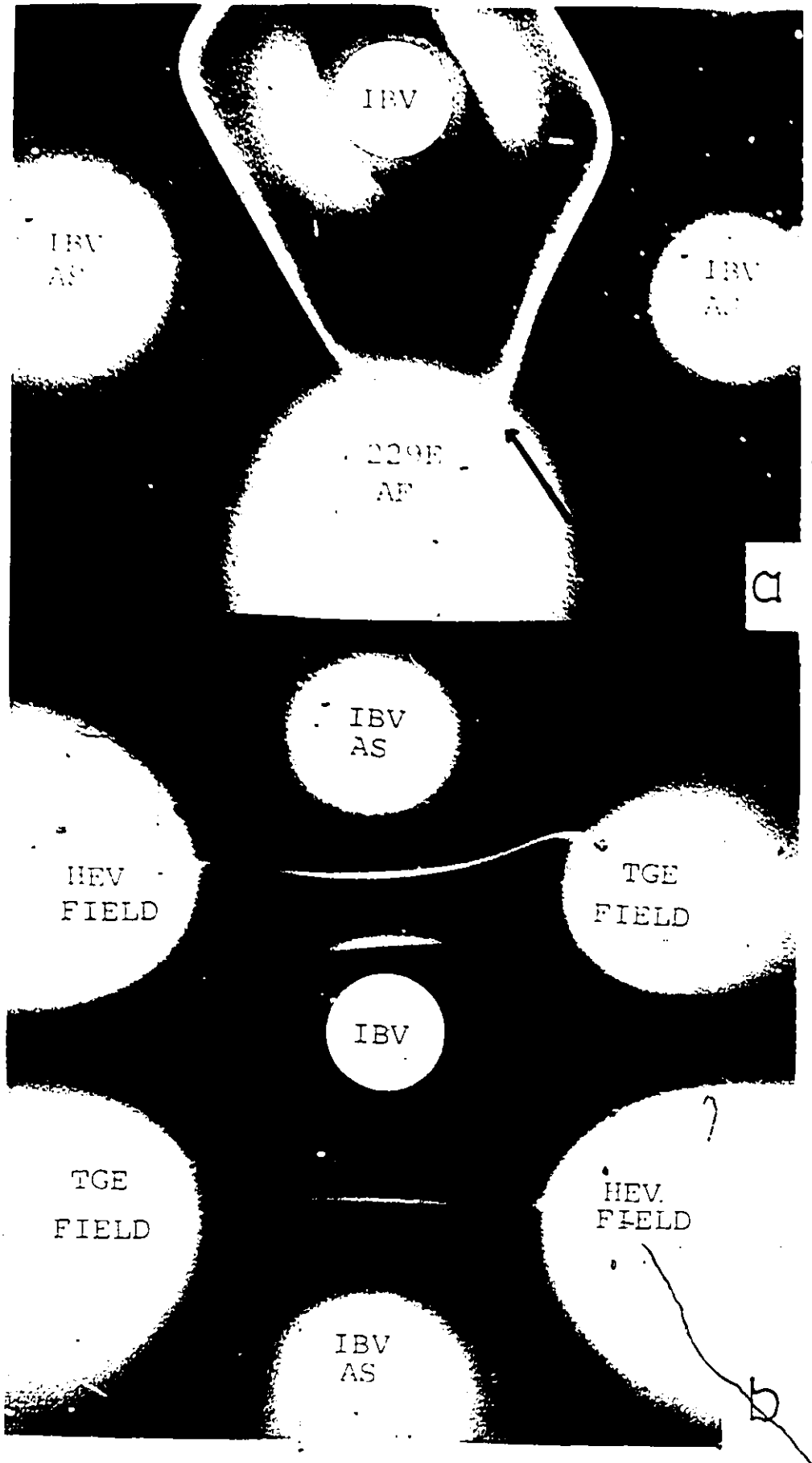

HEV CONV.
MHV AS
229E AS
HEV
229E AS
MHV AS
HEV CONV.
a
HEV CONV.
229E AF
229E CONV.
HEV
229E CONV.
229E AF
HEV CONV.
b
IBV AS
229E CONV.
229E AS
IBV
229E AS
229E CONV.
IBV AS
c

Plate 17

Plate 17:- Homologous and heterologous immunodiffusion reactions of undisrupted infectious bronchitis coronavirus (IBV).

a) Both IBV/AS and 229E/AF are recognizing closely related antigens as represented by the fusion of the precipitation lines detected by IBV and 229E antibodies (arrowed).

b) One of the two homologous IBV reactions formed a reaction of identity with both TGE and HEV field sera indicating that the three antisera are recognizing a very closely related antigen.


IBV
IBV
AS
IBV
AS
229E
AF
a
IBV
AS
HEV
FIELD
TGE
FIELD
IBV
TGE
FIELD
HEV.
FIELD
IBV
AS
b

ascitic fluid (AF) was demonstrated by the pattern of identity (arrowed). It was therefore considered that the apparent lack of reaction was due to the extremely low level of reacting IBV-related antibodies present in the HCV/229E/AS. Relationships were also established between the IBV undisrupted antigen and both TGE and HEV (plate 17-b). In similar reactions, the presence of cross reacting components in IBV, HEV, and MHV was indicated but since only limited quantities of MHV antigen were available, the identity of the reacting component could not be further studied. In addition to the reaction identified between IBV intact antigen and both TGE and HEV sera, a line of fusion also developed between IBV and TGE and HEV field sera and it was found to be related to the IBV component detected in the homologous reaction as can be seen by the line of identity (plate 17-b, arrowed).

These reactions served to establish the basis for examining in more detail the relationships between coronavirus strains. However, further interpretation of all these reactions depended on the precise identification of the reacting components. As mentioned above, this could not be achieved with non-disrupted virion preparations

because it was not possible to control non-specific degradation of the antigens. In order to obtain a more complete picture of the cross reacting patterns and relationships, a series of reactions were carried out using disrupted virus preparations.

Due to the related chemical structures of coronaviruses, the detergents TX-100 and NaDOC, found to be the best as described earlier, with 229E, were used with the coronaviruses included in this study. These results are presented in the following section.

The antigenic relationships between disrupted coronaviruses: The relationships of HCV/229E antigens released by DOC (229E/DOC) as demonstrated by the homologous and heterologous reactions are presented in the different patterns of immunodiffusion in plate 18. In the 229E homologous reactions, four precipitation lines were resolved (plates 18-b, 18-c, and 18-f). Two of these components were detected by MHV antiserum (plates 18-b and 18-c) indicating the presence of similar antigens in mouse hepatitis virus. One of these was related to TGE (plate 18-f) as shown by the line of identity between the homologous reaction and the antisera of both MHV and TGE. A precipitation line of identity was also described between the reactions of 229E/DOC with TGE and with HEV conv. sera (plate 18-a), IBV and HEV field sera (plate 18-d), as well as TGE field

Plate 18

Plate 18:- Homologous and heterologous immunodiffusion reactions of DOC-disrupted human coronavirus 229E (HCV/229E).

a) Both TGE antisera have weakly recognized at least one 229E antigen, however the reaction lines of TGE/AS/1 is suspiciously like a non-specific reaction and shows no identity with the HEV/CONV.-229E/DOC reaction. The reaction of TGE/AS/2 has formed a weak reaction of identity with the HEV conv. line and serves to identify a common antigen participant.

b) The fusion patterns of the line of the homologous 229E reaction and the HEV/CONV. and MHV/AS heterologous reactions demonstrate the presence of shared reacting components.

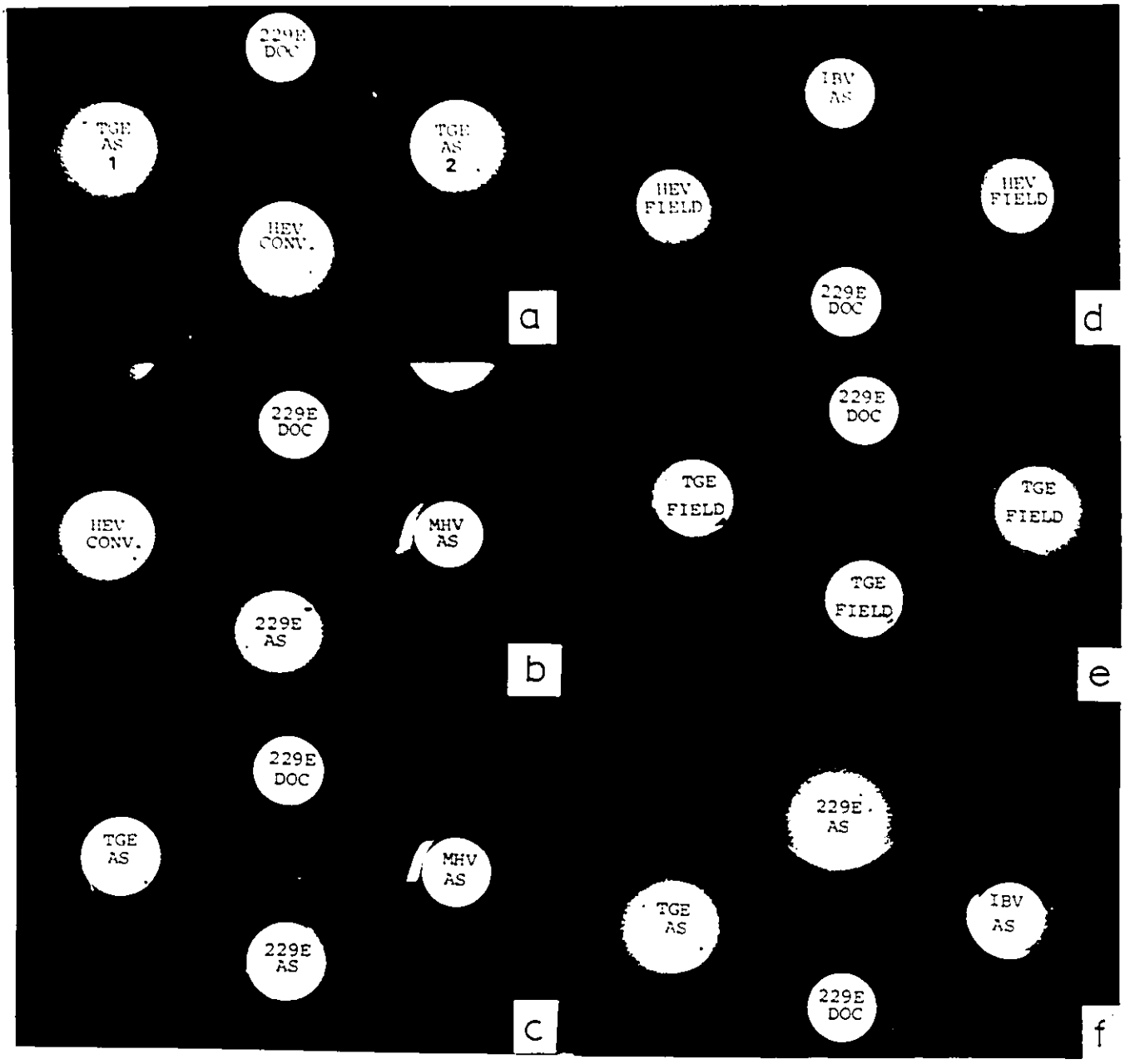
c) Essentially the same reaction as b) with TGE substituted for HEV, in which a stronger pattern of identity has developed between the shared component demonstrated by the cross reactions of MHV. There is no reaction evident between TGE/AS and 229E/DOC.

d) Weak reactions demonstrating the presence of a component in 229E recognized by both HEV/FIELD and IBV (arrowed) and an additional 229E component detected by HEV/FIELD.

Plate 18 continued:-

e) The reaction between different TGE/FIELD sera and 229E antigen, demonstrating the detection of a similar antigenic component.

f) It can be seen that the TGE antiserum reacts with only one related 229E component and that IBV/AS detected a partially related component.


229E DOC
TGE AS 1
TGE AS 2
HEV CONV.
a
229E DOC
HEV CONV.
MHV AS
229E AS
b
229E DOC
TGE AS
MHV AS
229E AS
c
IBV AS
HEV FIELD
HEV FIELD
229E DOC
d
229E DOC
TGE FIELD
TGE FIELD
TGE FIELD
e
229E AS
TGE AS
IBV AS
229E DOC
f

sera (plate 18-e). The hazy line seen between 229E/DOC and HEV/CONV. (plate 18-b) is not clear enough to indicate a relationship between the homologous and heterologous reactions and TGE/AS (plate 18-c) showed only partial identity (arrowed) to the precipitation line developed by both 229E/AS and MHV/AS. Since, in other reactions, a 229E component was identified by HEV and TGE sera as well as 229E/AS (plates 18-a and 18-f) we therefore considered that, on the basis of these reactions, there are two related coronavirus antigenic components common to HCV/229E and MHV, and one of these is also related to TGE, HEV, and IBV. The precipitation line of TGE/AS/1 could possibly be a non-specific line and therefore was not considered when determining relationships between coronaviruses.

However, the interpretation of these reactions was limited in scope because only the HCV/229E antigens had been involved. It was necessary to carry on this study in more detail using reactions of other DOC-released coronavirus antigens to clarify the basis of the relationships. The homologous and heterologous immunodiffusion reactions of the two swine coronaviruses disrupted with DOC (TGE/DOC and HEV/DOC) are presented in plate 19. One TGE antigen was identified in the homologous reaction of this virus (plates 19-a and 19-b). Similar components were identified by 229E/AS (plate 19-a) and HEV/CONV. and MHV/AS (plate 19-b) as demonstrated by the reaction of identity. This

Plate 19

Plate 19:- Homologous and heterologous immunodiffusion reactions of DOC-disrupted TGE and HEV swine coronaviruses.

a) The component demonstrated by the TGE/AS : TGE/DOC reaction is also related to those detected by 229E/AS and IBV/AS, although there is a slight antigenic difference indicated between 229E and IBV by the spur of partial identity.

b) The TGE/AS demonstrates a single reacting component in TGE/DOC, but the HEV/CONV. apparently distinguishes two lines of which one is shared by MHV. Furthermore, the linkages between the HEV and MHV reaction are stronger than between the TGE and MHV.

c) 229E convalescent human serum reacts strongly with an HEV component, whereas the 229E/AS and ascitic fluid apparently do not. The reasons for this reaction are unclear, however they could reflect problems with reaction conditions (antigen or antibody excesses) which are sometimes encountered.

d) Two to three components are detected in the homologous system and two of them are similar to those detected by MHV/AS, though one cross reacts to a much lesser extent. The minor reacting component detected by both MHV/AS and HEV/CONV. is also

Plate 19 continued:-

detected by IBV/AS, identifying an HEV antigen common to all three viruses. Another component detected by MHV partially reacted with one identified by the homologous reaction (arrowed).

a
b
c
d

Plate 20

Plate 20: Homologous and heterologous immunodiffusion reactions of DOC-disrupted hemagglutinating encephalomyelitis (HEV), transmissible gastroenteritis (TGE), and mouse hepatitis (MHV) coronaviruses.

a) Four antigens are detected in the HEV homologous reaction; one developed a faint line of relationship to 229E and TGE heterologous reactions. These two sera have also developed a second precipitation line, close to the respective antiserum wells but no lines of identity are demonstrated.

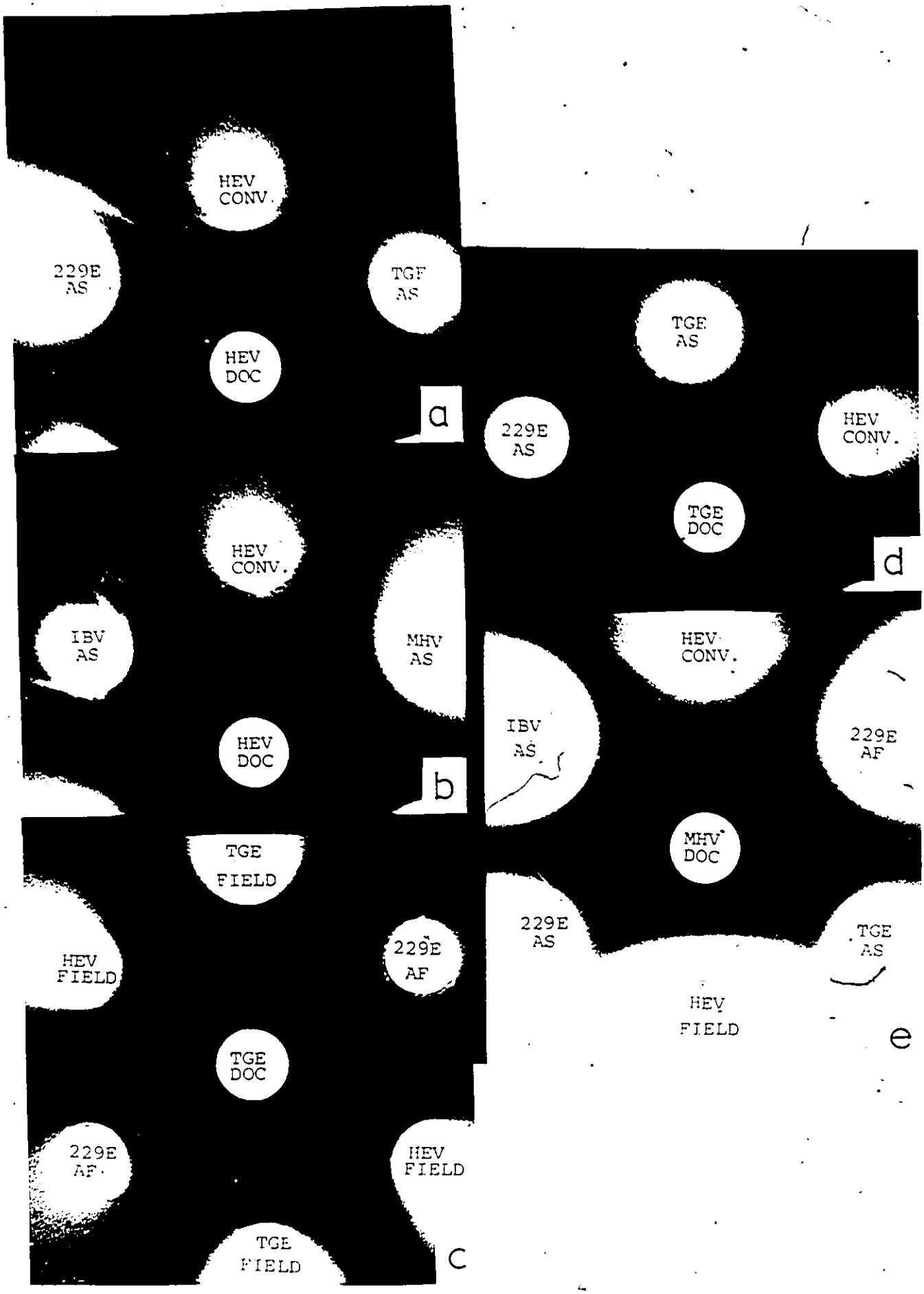
b) Again four lines are seen in the HEV homologous reaction and one developed a line of identity to MHV and IBV heterologous reactions. IBV/AS has recognised two more components in its reaction with HEV/DOC.

c) A continuous faint (arrowed) line demonstrating the relationships between three coronaviruses, HEV, TGE, and 229E, has developed in the TGE/DOC homologous and heterologous system.

d) The faint continuous line (arrowed) seen in this reaction between homologous and heterologous reacting components further confirms previous relationships. HEV convalescent serum has recognized an extra component in the TGE/DOC antigen.

Plate 20 continued:-

e) A released MHV antigen has been weakly recognized by HEV and TGE antibodies, however, further details of the relationships are not demonstrated.


HEV CONV.
229E AS
TGE AS
HEV DOC
a
HEV CONV.
IBV AS
MHV AS
HEV DOC
b
TGE FIELD
HEV FIELD
229E AF
TGE DOC
229E AF
HEV FIELD
TGE FIELD
c
TGE AS
229E AS
HEV CONV.
TGE DOC
d
HEV CONV.
IBV AS
229E AF
MHV DOC
229E AS
TGE AS
HEV FIELD
e

component was partially related (as represented by the line of partial identity) to the one identified by IBV/AS (plate 19-a, arrowed). In other reactions (plates 20-c and 20-d), the TGE component was closely related to those detected by HEV/FIELD and 229E/AF (plate 20-c) as well as 229E/AS and HEV/CONV. The HEV reactions, on the other hand, show a homologous as well as heterologous reaction as demonstrated in plates 19-c and 19-d and plates 20-a and 20-b. As many as four lines were detected in the homologous reactions (plate 19-d; plates 20-a and 20-b) and at least one of these lines showed a common reaction with TGE, IBV, 229E, and MHV precipitating sera. An interesting observation is that both MHV/AS (plate 19-d) and IBV/AS (plate 20-b) recognized several of the HEV-related antigens with varying degrees of identity. HEV/DOC in one case reacted with the 229E convalescent serum but no reaction was evident with both hyperimmune fluids (ascitic fluid and antiserum) of that virus (plate 19-c). The reasons for this reaction are unclear, however, it could reflect problems with reaction conditions (antigen or antibody excesses) which are sometimes encountered.

The interrelationship between the field convalescent and immunized convalescent sera of the two pig coronaviruses raises two possibilities: 1) That under field conditions, the swine have been exposed to both HEV and TGE viruses, or, 2) the possibility that both HEV and TGE have some kind of antigenic relationship. These are not mutually exclusive.

The reactions of MHV/DOC antigen with HEV/CONV. and TGE/AS indicated the presence of low levels of related antigens. No other heterologous reactions could be detected between IBV and HCV/229E antibodies, however, this was a very weak reaction and it is probable that the reaction proportions were inadequate.

The antigenic relationships of IBV with the other coronaviruses which were identified earlier using intact IBV antigen, were investigated in more detail by using DOC-disrupted IBV antigen. Two reacting components were seen in the homologous reaction (plate 21-c) and one similar component was precipitated by all sera and ascitic fluids used in this study (plate 21-a, 21-b, and 21-c). This common antigen was identified by the line of identity between these sera.

After establishing the reaction patterns using DOC-disrupted coronavirus antigens, our work was extended to explore the relationships using antigen preparations disrupted by TX-100. Representative reactions are presented in plates 22 and 23 and serve to confirm our initial results. In the homologous 229E reactions, 3 to 4 antigens were detected (plate 22-a). In plate 22-b, a weak precipitation line developed between IBV/AS and 229E/TX100 disrupted antigen. In the reaction presented in plate 22-a, a closely related component was identified by the three reacting sera: TGE/FIELD, HEV/FIELD, and 229E/AS as

Plate 21

Plate 21:- Homologous and heterologous immunodiffusion reactions of DOC-disrupted infectious bronchitis coronavirus (IBV).

a) The three 229E immune sera, 229E antisera, 229E convalescent sera, and 229E ascitic fluid, have recognized a similar antigen in IBV/DOC.

b) Antigens similar to an IBV component are recognized in weak heterologous reactions (arrowed) with HEV and MHV antisera.

c) One of the two IBV antigens identified in the homologous reaction is related to the one recognized in the heterologous TGE reactions (reaction of identity, arrowed).

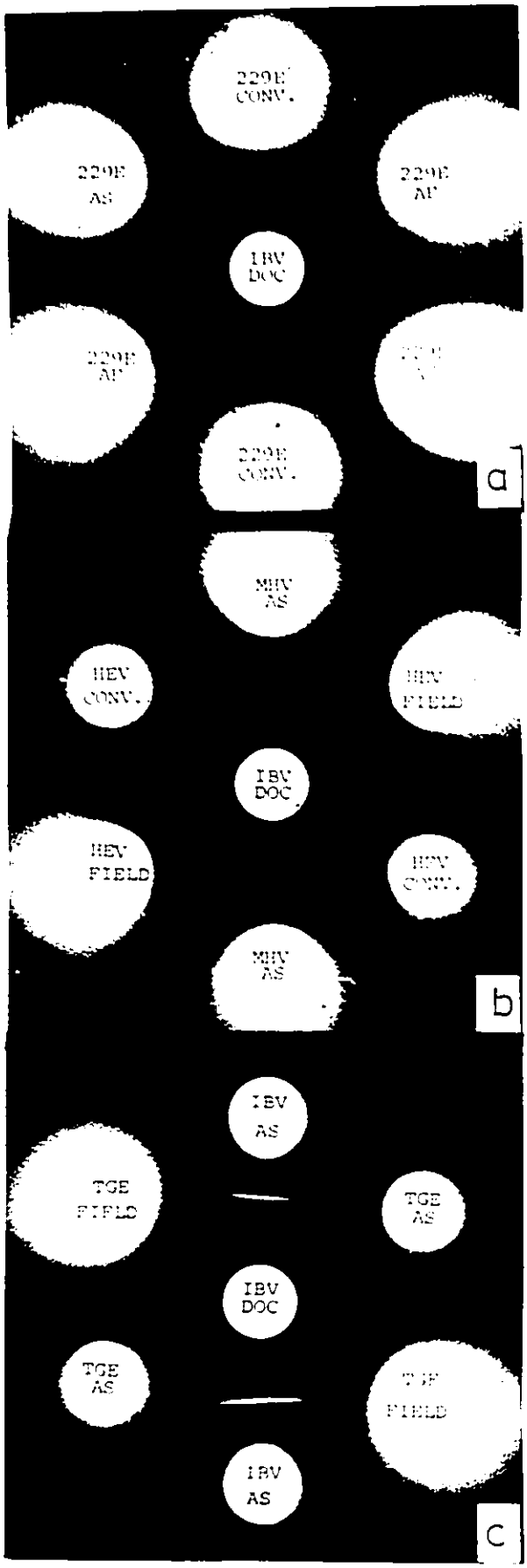

229E CONV.
229E AS
229E AP
IBV DOC
229E AP
229E CONV.
a
MHV AS
HEV CONV.
HEV FIELD
IBV DOC
HEV FIELD
HEV CONV.
MHV AS
b
IBV AS
TGE FIELD
TGE AS
IBV DOC
TGE AS
TGE FIELD
IBV AS
c

Plate 22

Plate 22:- Homologous and heterologous immunodiffusion reactions of TX-100-disrupted human coronavirus 229E (HCV/229E) and infectious bronchitis coronavirus (IBV).

a) Three to four reacting components are demonstrated in the 229E homologous reaction and one of these components was closely related to those identified by both TGE and HEV field sera (reaction of identity, arrowed).

b) A weak heterologous reaction with IBV/AS (arrowed) has developed but no relationship can be seen with MHV and HEV.

c) At least two IBV antigens are detected in the homologous reaction and none have been recognized by TGE antibodies.

d) Two antigens are demonstrated in this IBV homologous reaction, and only one antigen has been recognized by the TGE reaction (arrowed).

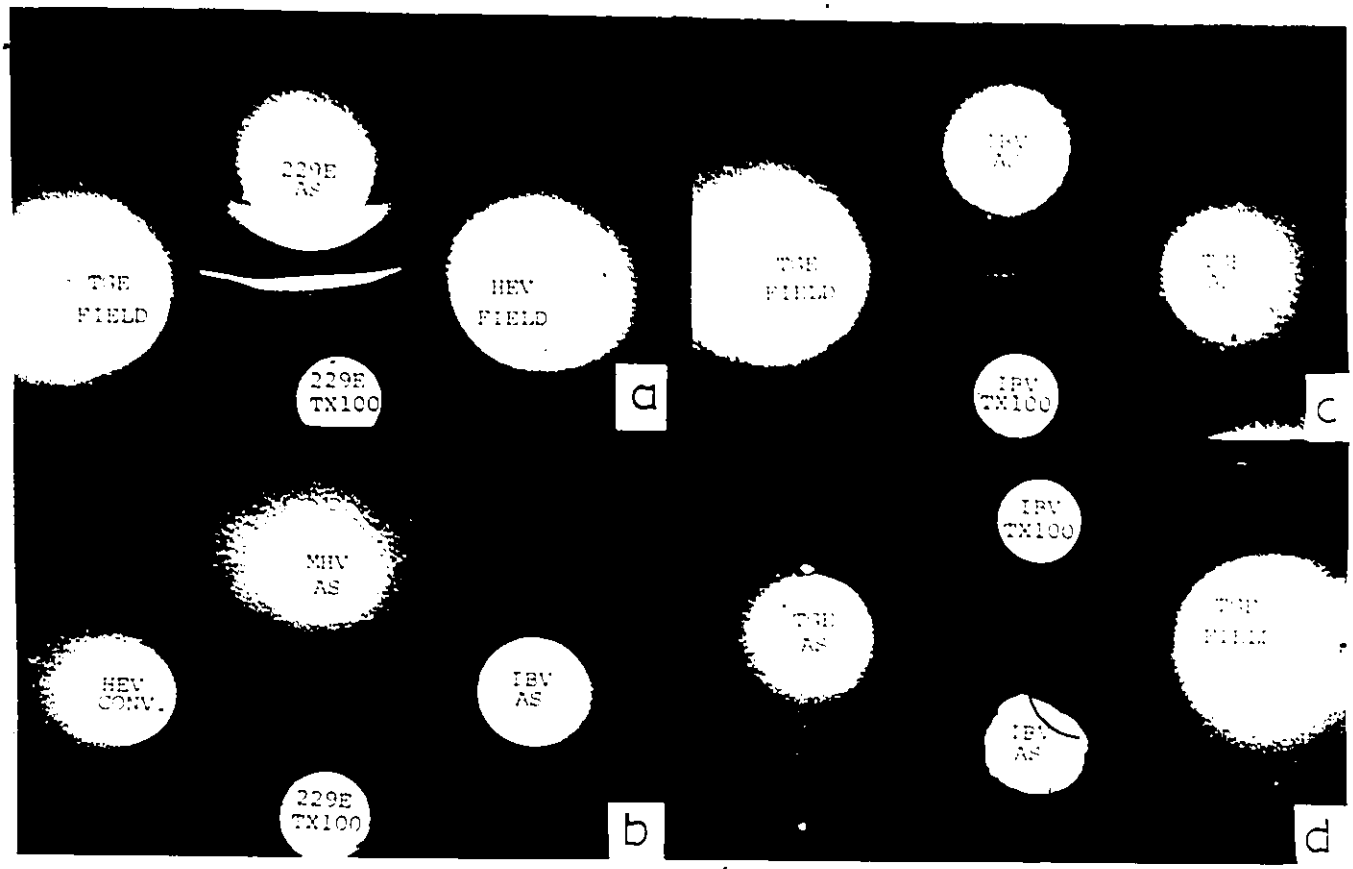

229E
AS
T3E
FIELD
MHV
FIELD
229E
TX100
a
MHV
AS
MHV
CONV.
IBV
AS
229E
TX100
b
T3E
FIELD
IBV
TX100
c
IBV
TX100
T3E
AS
IBV
AS
d

Plate 23

Plate 23:- Homologous and heterologous immunodiffusion reactions of TX-100 and DOC-disrupted coronaviruses.

a) One of the two antigens identified by HEV homologous reactions developed an identity with MHV heterologous reactions. In addition, the MHV heterologous system has recognized a component partially related to HEV. 229E/AS developed a very weak line of reaction with HEV/TX-100.

b) Two components are detected in the MHV/TX-100 homologous reaction; one is a strong and the other is a weakly reacting component (arrowed 1). Probably a third antigen (arrowed 3) can be seen very close to the MHV/AS well. No heterologous reactions can be seen because of the low concentration of the MHV antigen.

c) MHV antibodies have recognized three HEV antigenic components released by DOC but none of the 229E or TGE components released by TX-100.

d) MHV/AS developed weak reactions with three IBV preparations: IBV, IBV/DOC, and IBV/TX-100, however the patterns of identity are not sufficiently clear for further interpretation.

a
b
c

represented by the line of identity between them. The absence of a relationship between HEV/CONV. (plate 22-b) and 229E/TX-100 was considered due mainly to imbalances in the reaction conditions. These reactions were capable of identifying a relationship between 229E/TX-100 antigen and the different sera (HEV, TGE, and IBV). The absence of a relationship with MHV/AS was probably because the serum was of a low titre.

The reactions with IBV antigen disrupted by TX-100 (plates 22-c and 22-d) identified two reacting components in the homologous reaction. One of these components was related to the one detected by TGE/AS (plate 22-d).

The reactions presented in plate 23 have been selected to illustrate some of the complications inherent in these analyses. In plate 23-a, using HEV disrupted by TX-100 as the antigen, two reacting components were detected in the homologous as well as heterologous reactions. Also one of the two HEV-reacting components show a line of identity with one of the components identified by the MHV heterologous reaction.

However, in plates 23-b, 23-c, and 23-d, when more details of MHV relationships were sought, using different reaction patterns and reagents, it became evident that the antigen concentration of MHV disrupted by TX-100 was too low to permit detection in heterologous reactions (plate 23-b). And, using the MHV antiserum, it was apparent that the

strongest relationship was between MHV and HEV/DOC antigens (plate 23-c) and a weaker reaction developed with IBV antigens (plate 23-d). Whether the use of TX-100 as the disrupting agent for 229E and TGE was responsible for the loss of the reaction in plate 23-c requires further analysis, especially since it is not clear whether the middle precipitation line is bending towards the other wells or not.

Taken together, the reactions with disrupted virus preparations presented so far all indicated the presence of at least one common antigen reacting in most of the immunodiffusion systems. Since those reactions with intact viruses did not demonstrate a similar situation, it was a logical consequence to determine whether, in fact, the internal component of the coronavirus was indeed the common reacting component and thus could be a group specific antigen. The final series of analytical immunodiffusion reactions was designed to investigate this possibility.

Comparative Reactions of the Coronavirus Internal Component (RNP)

The nature of the relationships described between the different coronaviruses and the identification of a common reacting antigen were studied in this phase by using the internal component isolated from the three coronaviruses, HEV/229E, TGE, and HEV, and the homologous and heterologous antisera and ascitic fluid of the

coronaviruses described previously. Representative analytical immunodiffusion reactions are presented in plates 24, 25, 26, and 27. The close relationship between 229E virus and host antigen was established and described in the earlier part of this study where it was also learned that in the polyacrylamide gel immunoelectrophoresis study of the relationships between host and virus, the nucleoprotein of the RNP was precipitated by both host and viral antibodies. In immunodiffusion reactions, it was found that one of the two RNP components was precipitated by the host antisera (plate 24-a). It was not clear whether the second component was a host contaminant or a virus-modified host component specifically incorporated in the RNP especially since, as shown in plate 24-b, it could not be entirely removed during further purification. In other immunodiffusion reactions included in plate 24, a component related to 229E internal components was precipitated by MHV, HEV, TGE, and IBV sera. In plate 24-c, the homologous precipitation line occurring between 229E-RNP and 229E/AS fused into a reaction of identity with the MHV/AS versus 229E/RNP line demonstrating the identity of 229E-RNP with an MHV antigen, probably the MHV-RNP. In this reaction, the faint hazy band bending into the area between the HEV/CONV. and the 229E-RNP cannot be interpreted and it may represent an identity with the HEV-RNP. In plate 24-d, the reaction demonstrated the identity of the 229E-RNP with a TGE antigen by the precipitation line between TGE/AS and 229E-RNP. A similar but much

weaker reaction with TGE/FIELD was suggested by the bending of the 229E/AS. 229E-RNP reaction toward the TGE/FIELD well (arrowed). A similar reaction in plate 24-f served to identify the antigenic component in HEV/CONV. with 229E-RNP. In plate 24-e, a faint precipitation line between IBV/AS and 229E-RNP demonstrated the relationship between the 229E-RNP antigen and an IBV component although it has not been firmly identified by a pattern of fusion with the neighbouring homologous reaction.

Both TGE and HEV isolated RNPs showed one precipitation line in the homologous reaction which was also recognized by IBV/AS and 229E/AS (plates 25-a, 25-b, 26). In the reaction shown in plate 26 and plate 27-a, the TGE-RNP antigen was detected by both TGE/AS and IBV/AS. However, no reaction could be detected between the 229E/CONV. (anti-229E-RNP) serum and MHV/AS. This might be due to the fact that both 229E/CONV. and MHV/AS are very weakly reacting sera. However, since only HCV/229E hyperimmune antiserum was capable of weakly recognizing the TGE-RNP preparation, it was considered that the degree of identity between HCV/229E and TGE-RNP was quite low. It was evident that TGE-RNP was recognized by both TGE/CONV. and HEV/CONV. and the reactions (plate 27-b) formed a clear-cut pattern of identity. This meant that both sera recognized a closely related antigen (TGE-RNP and a 229E antigen, probably RNP), indicating both viruses have a similar internal component.

Plate 24

Plate 24:- Homologous and heterologous immunodiffusion reactions of the internal component of human coronavirus 229E (229E/RNP).

a) This reaction illustrates the test used to evaluate the RNP preparation. It can be seen that there is a trace of host-reacting component present in the 229E/RNP preparation (arrowed).

b) In this preparation of RNP, there is essentially no host component detectable as can be seen by the absence of reaction between the RNP antigen and the host antiserum. But the RNP line is bending toward the host well.

c) The homologous precipitation line occurring between 229E/RNP and 229E/AS fuses into a reaction of identity with the MHV/AS line versus 229E/RNP which demonstrates the identity of 229E/RNP with an MHV antigen, probably the MHV/RNP. A faint hazy band (arrowed) appears to bend into the area between the HEV/CONV. and the 229E/RNP, indicating a further relationship with a HEV component (probably HEV/RNP).

d) This reaction demonstrates the identity of the 229E/RNP with a TGE reacting component by the precipitin line between TGE/AS and 229E/RNP. A similar but much weaker reaction with TGE/FIELD is suggested by the bending of the 229E/AS : 229E/RNP reaction toward

Plate 24 continued:-

the TGE/FIELD well (arrowed).

e) A clear precipitation line between IBV/AS and 229E/RNP demonstrates the relationship between the 229E/RNP antigen and an IBV component although it has not been firmly identified by a pattern of fusion with the neighbouring homologous reaction.

f) This reaction serves to identify the 229E/RNP with the reacting component recognized by the HEV reactions.

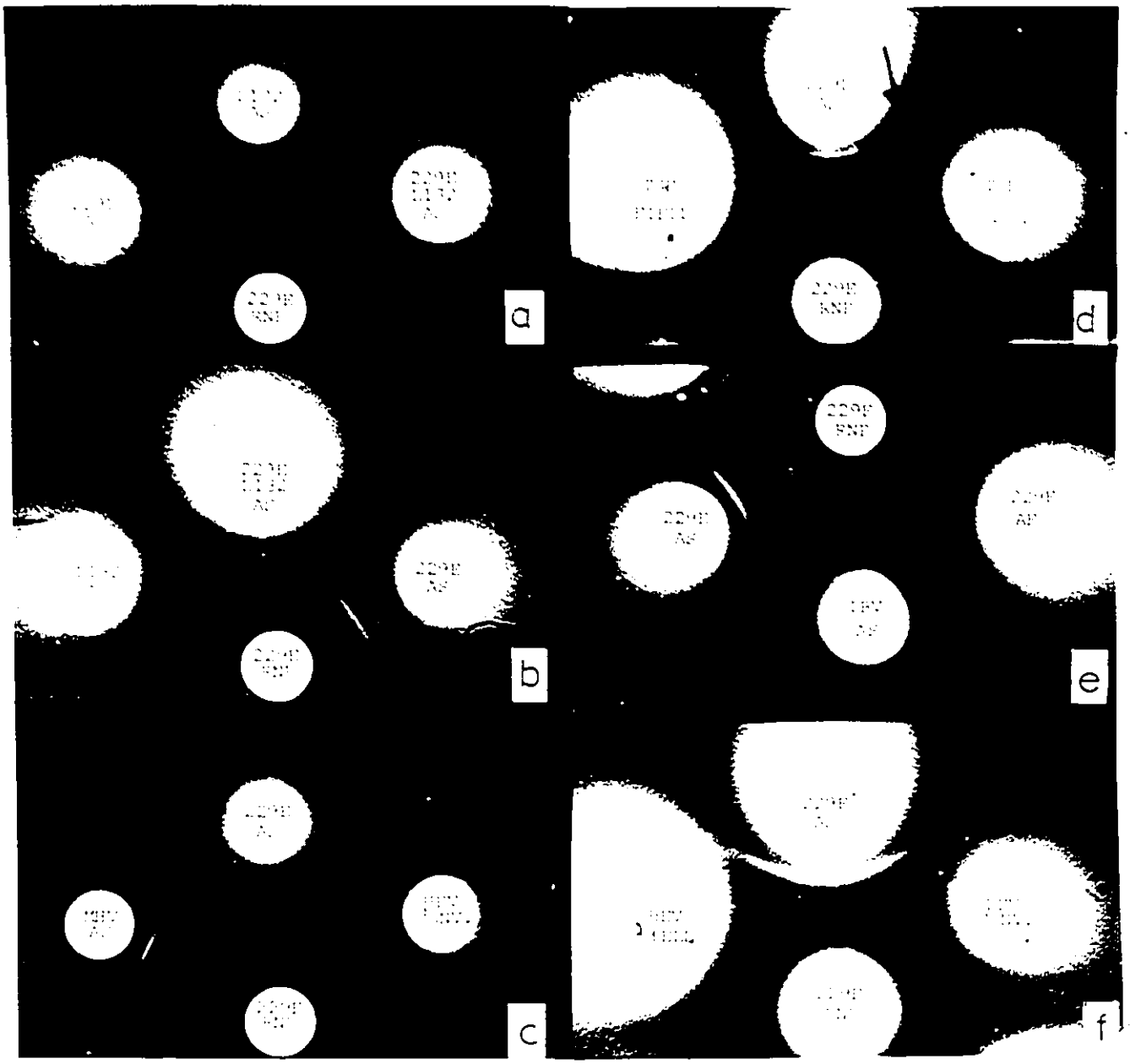

a
b
c
d
e
f

Plate 25

Plate 25:- Homologous and heterologous immunodiffusion reactions of the internal component of hemagglutinating encephalomyelitis coronavirus (HEV-RNP).

a) The homologous reaction of HEV-RNP with HEV/CONV. can be seen. A related HEV-RNP antigen is also recognized in this reaction by the 229E/AS, however, patterns of identity have not developed.

b) A weak reaction line has developed between HEV-RNP and the hyperimmune IBV/AS which seems to be bending toward HEV/AS. The homologous reaction (HEV-RNP:HEV/CONV.) is too weak to provide further information.

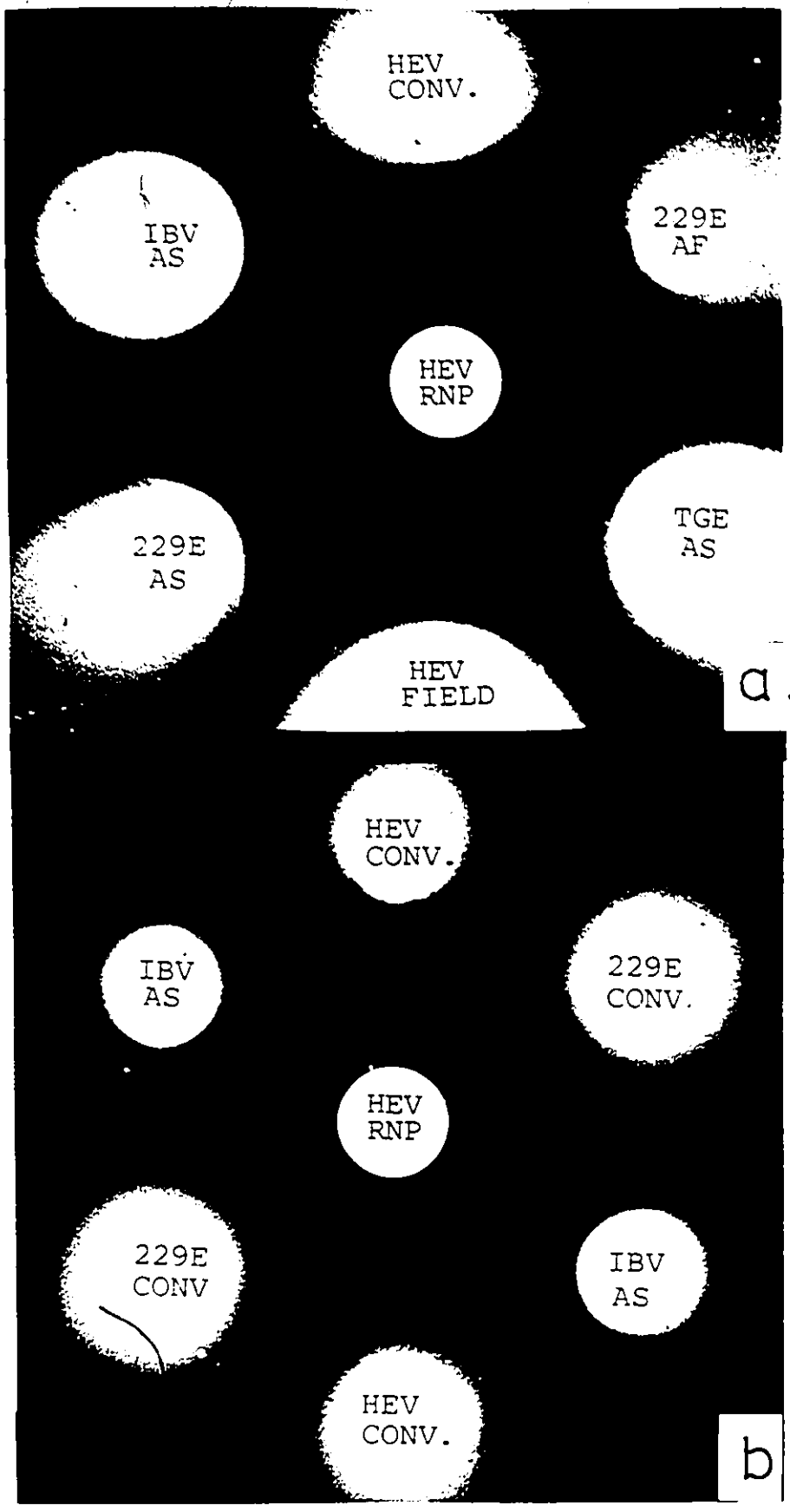
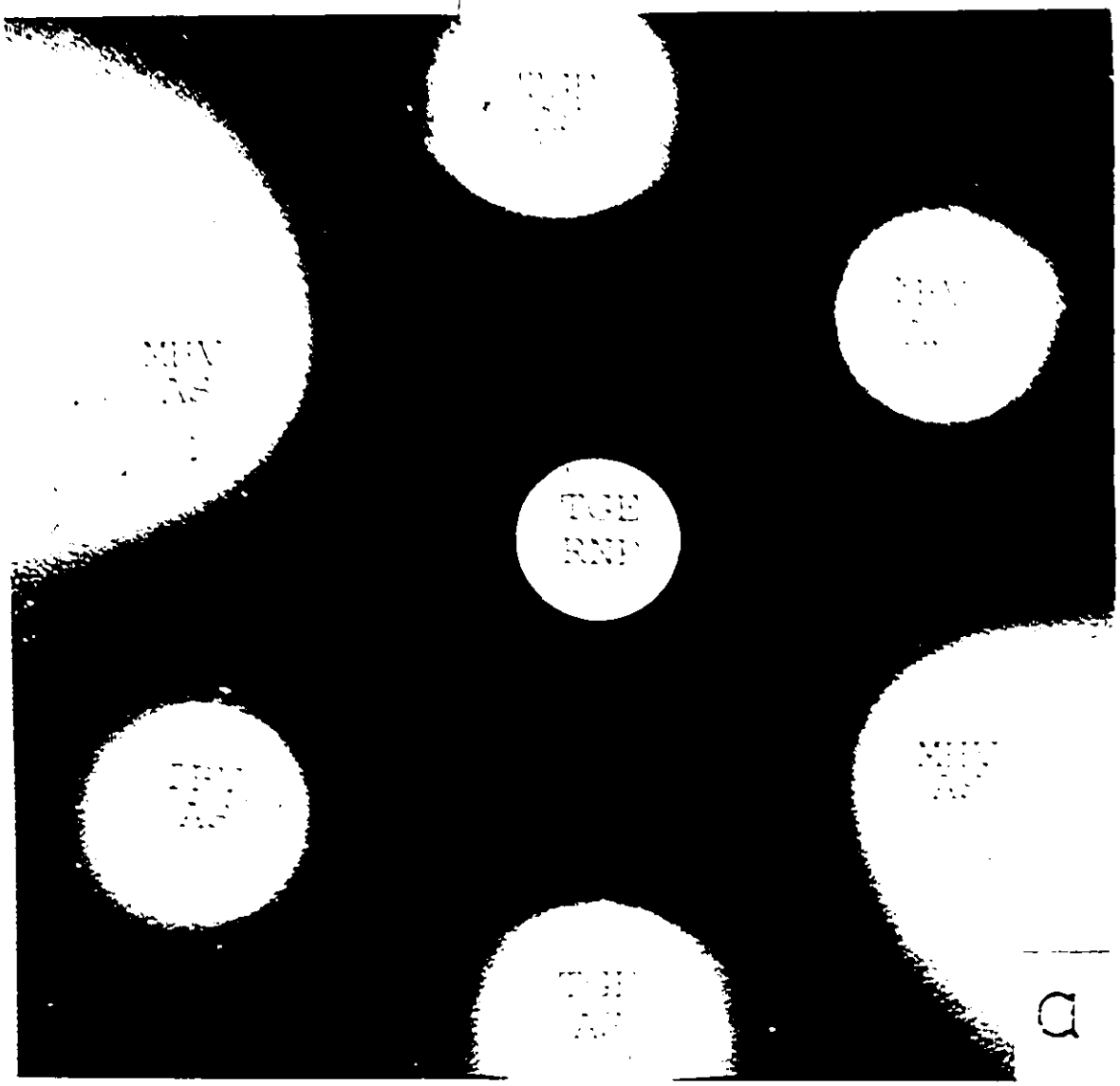

HEV
CONV.
IBV
AS
229E
AF
HEV
RNP
229E
AS
TGE
AS
HEV
FIELD
a.
HEV
CONV.
IBV
AS
229E
CONV.
HEV
RNP
229E
CONV
IBV
AS
HEV
CONV.
b

Plate 26

Plate 26:- Homologous and heterologous immunodiffusion reaction of the internal component of transmissible gastroenteritis coronavirus (TGE-RNP).

a) One precipitation line has developed in the TGE-RNP homologous reaction. A related TGE-RNP antigen is also identified by the heterologous reaction with IBV/AS demonstrating a very closely related component in IBV, probably IBV-RNP.



a

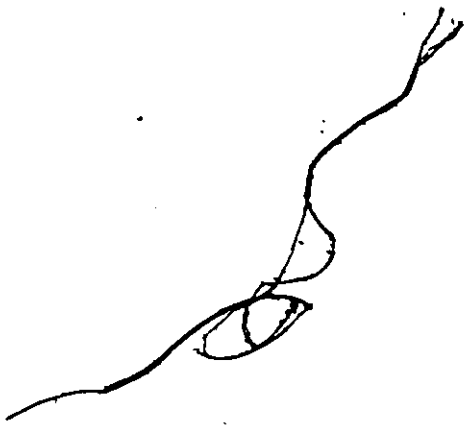
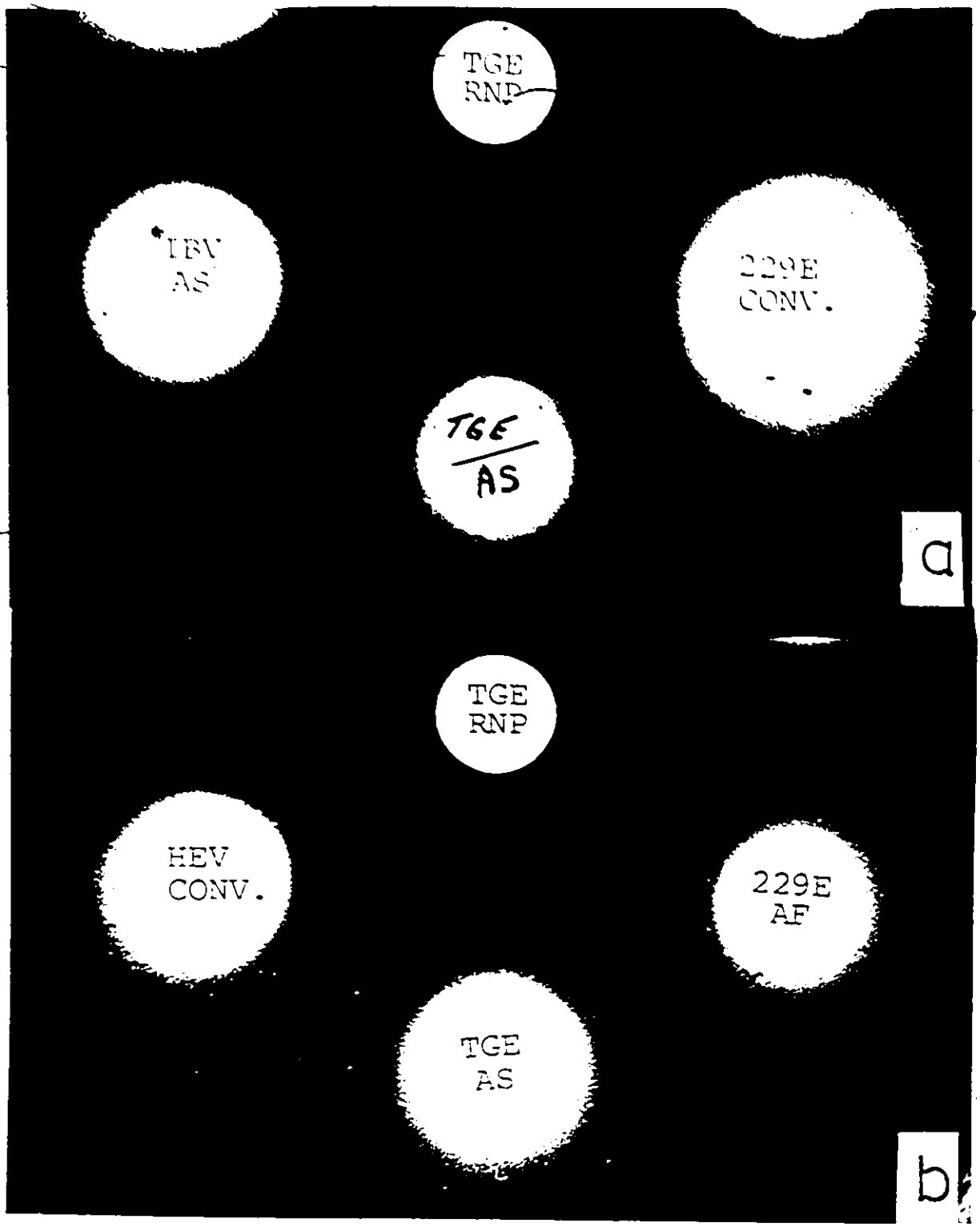


Plate 27

Plate 27:- Homologous and heterologous immunodiffusion reactions of the internal component of transmissible gastroenteritis (TGE-RNP).

a) The TGE-RNP antigen is recognized by both the TGE/AS and IBV/AS, however, no reaction has occurred with the 229E/CONV. serum. This might be due to the fact that the 229E/CONV. is a very weakly reacting serum.

b) The TGE-RNP antigen which is recognized by TGE/AS seems to be closely related to the one identified by HEV/CONV. as demonstrated by a clear-cut pattern of identity (arrowed).


TGE
RNP
TBV
AS
229E
CONV.
TGE
AS
a
TGE
RNP
HEV
CONV.
229E
AF
TGE
AS
b

While these reactions serve to demonstrate a close antigenic relationship between the TGE-RNP and the HEV-RNP, and that there is a common antigenic component shared by IBV, 229E and TGE, and HEV-RNP, it was not possible to identify those antigens with MHV components due to the weakness of the MHV antisera. Because the existence of such MHV-shared components was demonstrated in the previous reactions, it was felt that the problem of demonstrating the relationship will prove to be dependent on the concentration of the reacting components in their respective reactions.

While further investigation to confirm and extend these observations needs to be done, on the basis of the immunodiffusion analyses to date, we believe that the coronavirus RNP can be regarded as a group specific antigen. However, further attention must also be given to the secondary reacting components which showed either additional common reactions or partial identities since they may indicate a means of subdividing within the group. This cannot be done conclusively without the isolation of the other virus components (virus envelope, virus projections, etc.) and their extensive study using this analytical immunodiffusion system.

Serological Screening of Normal Sera by Immunodiffusion

Different animal sera were tested for anti-229E antibodies by using 229E antigen disrupted by NaDOC. The following are the results of the individual sera tested.

Swine: TGE and/or HEV positive sera from 61 pigs tested by neutralization was supplied by the Animal Disease Research Institute (ADRI). These animals were free of any symptoms caused by either virus (TGE, HEV). These sera were referred to as field sera and were screened by immunodiffusion using 229E/DOC. Sixty of the 61 sera (98%) developed precipitin lines, between one and three being identified in the normal TGE/Field (positive for TGE antigen), while one to two lines being identified in the normal HEV/Field sera (positive sera for HEV antigen). These results are not surprising because these sera were positive for HEV and TGE antigens and those two viruses were found to be related to 229E in our antigenic studies mentioned before. These findings served as a good control for the validity of the immunodiffusion reactions and the use of 229E/DOC antigen.

Canine: A number of normal dog sera were tested for antibodies against 229E antigenic components. Results of the immunodiffusion experiment and the quality of the lines resulting from the total of 237 sera are recorded in table 4. The

TABLE 4

THE RESULTS OF THE IMMUNODIFFUSION TEST OF NORMAL CANINE SERA AGAINST SODIUM DEOXYCHOLATE-DISRUPTED 229E/AG

Quality of the Immuno-diffusion Reaction*	Number of the Lines Described in the Reaction
No Reaction	124
1 Distinct Precipitin Line (+1)	31
2 Distinct Precipitin Lines (+2)	10
1 Precipitin Line and 1 Hazy Line (+1±1)	22
2 Precipitin Lines and 1 Hazy Line (+2±1)	3
2 Hazy Lines Only	1
1 Hazy Line Only	46
Total	237
Total Positive	66
Total Considered Negative	171

*See also plate 28-a to 28-d.

immunodiffusion reactions were presented in plate 28.

Of all the sera tested, the largest number, 124, were negative. Some of the reactions were interpreted as negative because of the poor unresolved quality of the reaction line which could have resulted from the detergent used to disrupt the antigen. Out of the 237 dog sera tested, 66 were clearly positive. As shown in plate 28-d, a weak reaction of identity could be demonstrated with the homologous 229E reaction.

Feline: Normal cat sera were screened with DOC-disrupted 229E antigen in immunodiffusion reactions for 229E or related antibodies. Immunodiffusion lines with similar qualities to those resulting with the dog sera were seen (plate 29, table 5). Of all the sera tested (49), only one appeared to be negative with no reaction detectable between the cat serum and the 229E antigen. However, of the remaining 48, only 15 were undoubtedly positive while the remainder were unresolved because of the poor quality of the reaction. And of the 15 positive sera, one developed two precipitation reaction lines. It must be again emphasized that our interpretation was perhaps overcautious because of the possibility of non-specific reactions which could not be identified because of limited amounts of sera.

Plate 28

Plate 28:- Some of the reactions of normal canine sera tested against 229E antigen.

a) A positive reaction between 229E/DOC and canine serum S2. There is a second hazy line which has developed between the antigen and all the canine sera. These hazy lines were considered to be negative because they may be the result of a non-specific reaction.

b) A positive reaction of a normal canine serum (S/11) and 229E/DOC antigen. The rest of the sera are considered negative.

c) One positive line developed with 229E/DOC antigen and DOG/S/15. The rest of the sera show no reaction with 229E antigen.

d) The specificity of the reaction of the canine sera is suggested in this reaction by the bending of the homologous reaction line toward each of the canine serum wells. This is an extremely weak indication.

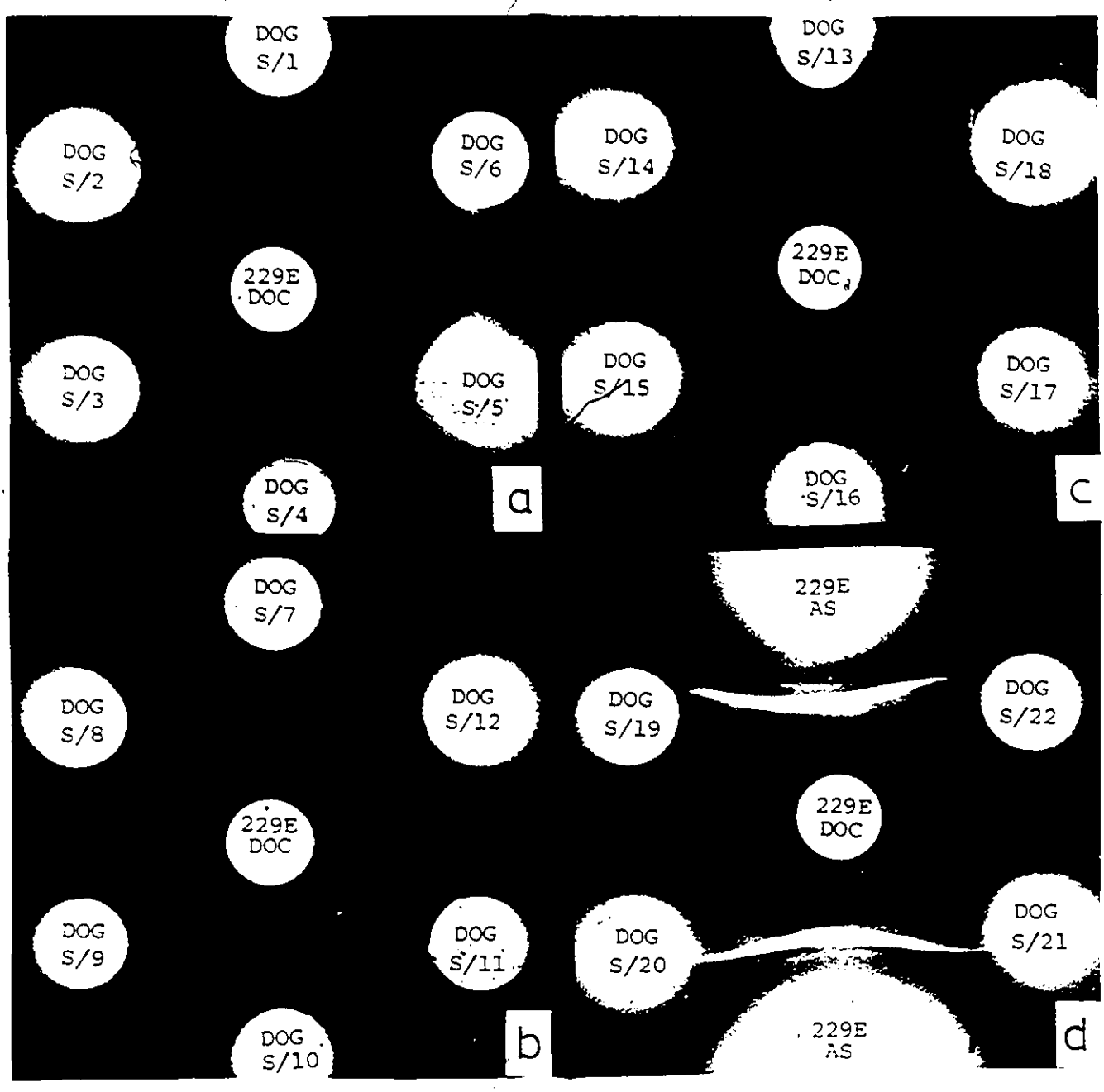

DOG S/1
DOG S/2
DOG S/3
DOG S/4
DOG S/7
DOG S/8
DOG S/9
DOG S/10
229E DOC
229E DOC
DOG S/6
DOG S/5
DOG S/12
DOG S/11
DOG S/14
DOG S/15
DOG S/19
DOG S/20
a
b
DOG S/13
DOG S/18
DOG S/17
DOG S/16
229E AS
DOG S/22
DOG S/21
229E AS
229E DOC
c
d

Plate 29

Plate 29:- Representative reactions obtained when screening specimens of normal feline sera against DOC-disrupted 229E antigen by the immunodiffusion test.

a) and b) Representative immunodiffusion reactions between normal feline sera and 229E/DOC antigen. Two sera in a) (S1 and S2) and in b) (S12 and S9) are considered positive. The rest were inconclusive or negative.

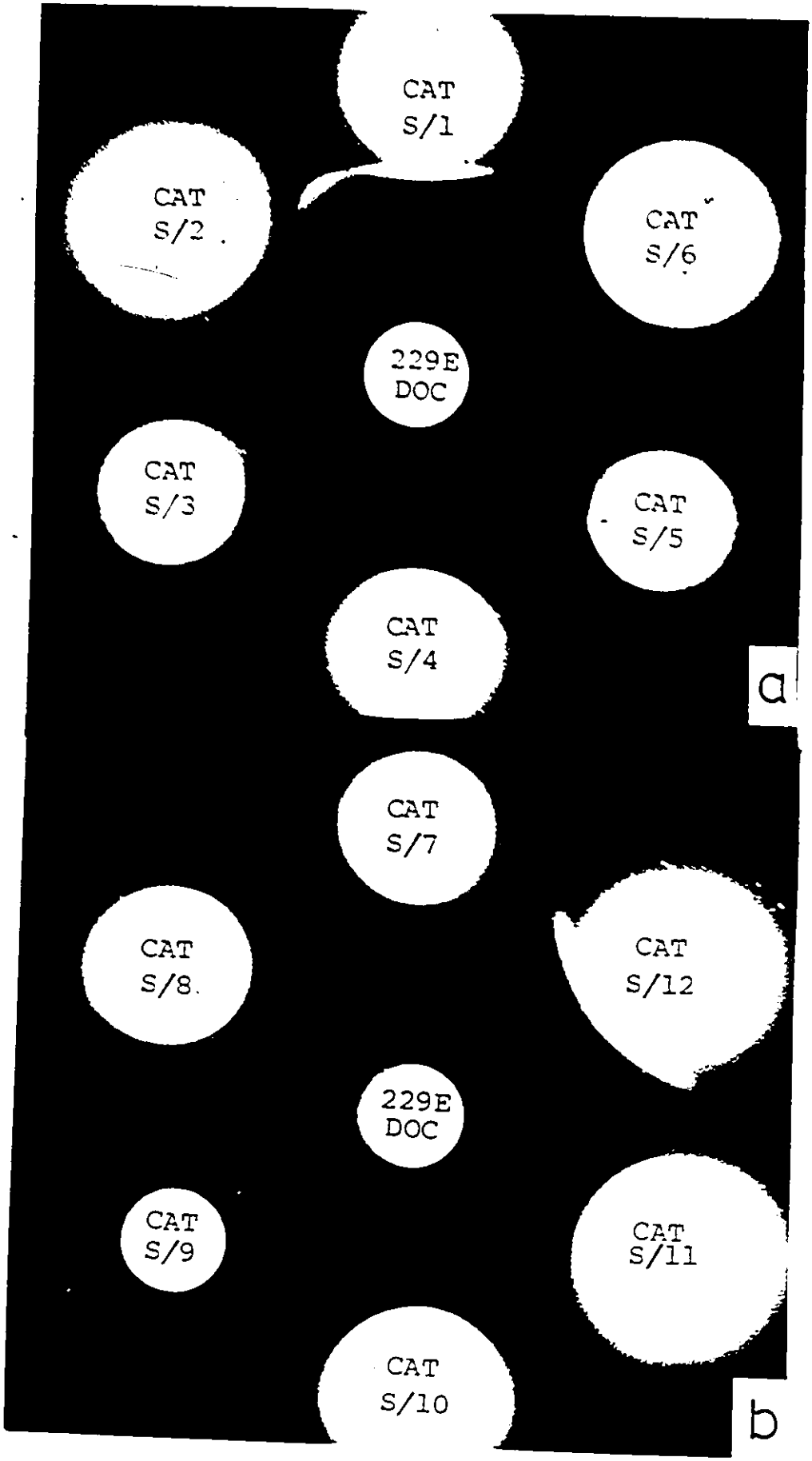

CAT
S/1
CAT
S/2
CAT
S/6
229E
DOC
CAT
S/3
CAT
S/5
CAT
S/4
a
CAT
S/7
CAT
S/8
CAT
S/12
229E
DOC
CAT
S/9
CAT
S/11
CAT
S/10
b

TABLE 5

THE RESULTS OF THE IMMUNODIFFUSION TEST OF NORMAL FELINE SERA AGAINST SODIUM DEOXYCHOLATE-DISRUPTED 229E AG

Quality of the Immuno-diffusion Reaction*	Number of the Lines Described in the Reaction
No reaction	1
1 Distinct Precipitation Line (+1)	5
1 Precipitation Line and 1 Hazy Line (+1±1)	9
2 Precipitation Lines and 1 Hazy Line (+2±1)	1
2 Hazy Lines Only	4
1 Hazy Line Only	29
Total	49
Total Positive	15
Total Considered Negative	34

*See also plate 29-a and 29-b.

Bovine: The normal sera of these animals were included as a part of the general screening test conducted for the relationship between 229E Ag and the different normal animal sera. Eighty-seven serum samples were tested against 229E antigen disrupted by DOC. Among the positive reactions, 15 gave two precipitation lines and 12 serum samples gave one positive line of reaction for a total of 31% positive. Twenty of the sera tested were completely negative in our immunodiffusion reactions (table 6, plate 30) and the remainder were considered negative due to the quality of the reaction.

The detection of anti-229E antibodies in the serum of normal animals might be expected if, 1) coronaviral infections are indeed as widely distributed in nature as has been proposed, and, 2) there is a group specific antigen common to all strains of coronavirus, regardless of the susceptible species. Since coronaviral infections of dogs, cats, and cows have been widely reported and our immunodiffusion analyses described earlier have indicated the existence of a group specific antigen, the results of this limited serological survey, taken together with the reactions of the positive pig sera, lend further support to the conclusion that there is a coronavirus group specific antigen.

TABLE 6

SCREENING OF THE NORMAL BOVINE SERA AGAINST 229E AG DISRUPTED BY DOC

Quality of the Immuno-diffusion Reaction*	Number of the Lines Described in the Reaction
No Reaction	20
2 Distinct Precipitation Lines (+2)	15
1 Precipitation Line and 1 Hazy Line (+1±1)	12
2 Hazy Lines Only	12
1 Hazy Line Only	28
Total	87
Total Positive	27
Total Considered Negative	60

*See also plate 30-a, 30-b, and 30-c.

Plate 30

Plate 30:- Some of the reactions resulting from the normal bovine sera and 229E/DOC antigen.

a) A hazy zone circling the inner well containing 229E/DOC antigen was considered to be negative because of the possibility that it might be non-specific. A weak definite positive line close to the well of sera no. 5 can be seen (arrowed).

b) Two continuous hazy lines between 229E/DOC and all the normal cow sera used in the reaction were also considered negative. A weak positive line close to the normal cow sera well no: 11 can be seen.

c) The specificity of the reaction is suggested by the identity of the line identified by the normal cow sera and the specific anti-229E ascitic fluid (229E/AF).

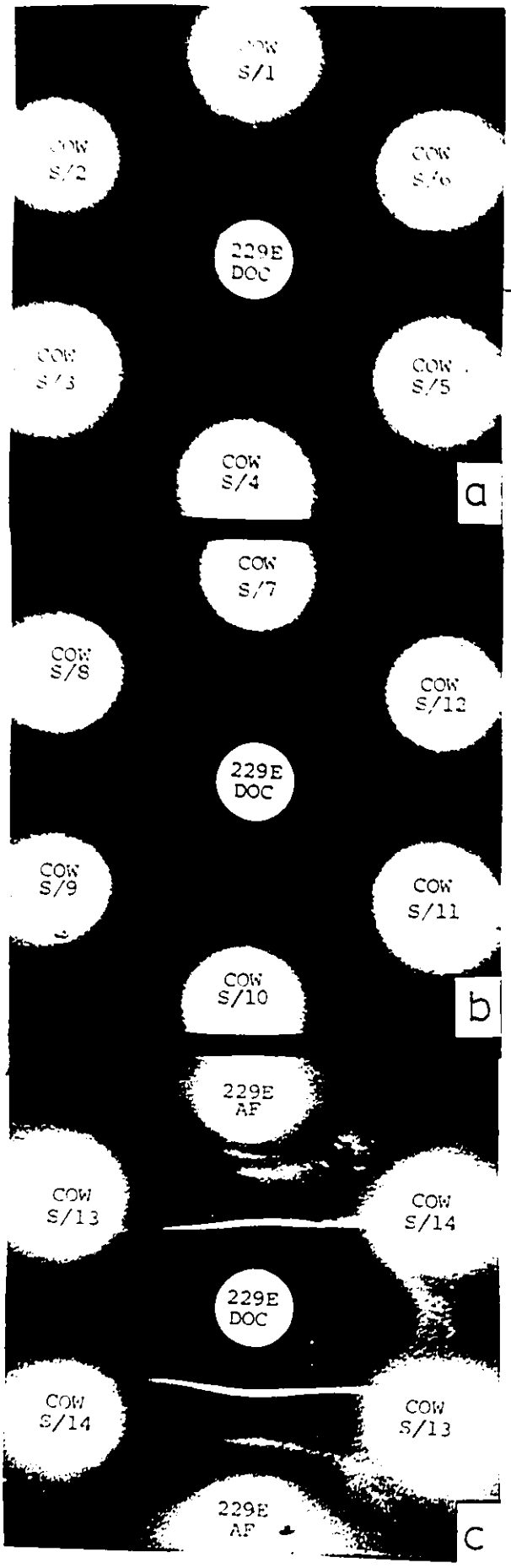

COW S/1
COW S/2
COW S/6
229E DOC
COW S/3
COW S/5
COW S/4
a
COW S/7
COW S/8
COW S/12
229E DOC
COW S/9
COW S/11
COW S/10
b
229E AF
COW S/13
COW S/14
229E DOC
COW S/14
COW S/13
229E AF
c

TABLE 7

RESULTS OF SCREENING TEST OF DIFFERENT ANIMAL SERA WITH HCV/229E ANTIGEN

Animal Sera	No. of Sera Tested	No. of Positives	%
Swine*	61	60	98
Feline**	49	15	31
Bovine**	87	27	31
Canine**	237	66	28

*These sera were all neutralization positive (against TGE and/or HEV) field sera obtained after routine testing by the ADRI.

**These specimens were obtained from normal populations and had no recent clinical history to correlate with the serological findings.

Summary

1) The internal components of the three different coronaviruses, HCV/229E, TGE, and HEV which have slightly different densities (229E-RNP = 1.27, TGE-RNP = 1.29, and HEV-RNP = 1.27), have several features in common. First, these RNPs can be isolated from preparations of purified virus by either spontaneous disruption of these fragile coronaviruses or by NP40 treatment. Second, these coronavirus RNPs have been found to have a similar morphology.

2) The coronaviruses 229E, TGE, HEV, MHV, and IBV are antigenically related to each other as demonstrated by the immunodiffusion tests. The basis of this relationship is the presence of a common group specific antigen between these viruses. This common group antigen was found to be the internal component. Other secondary relationships were also described and cannot be identified until the other viral components have been isolated.

3) Antibodies against human coronavirus 229E were detected in the sera of swine (98%), feline (31%), bovine (31%), and canine (28%). The specificity of these findings was confirmed by the identity with the specific 229E homologous reactions and support the suggestion of a common group coronavirus antigen.

GENERAL DISCUSSION AND CONCLUSION

This study was directed towards detecting and identifying the antigenic structures of coronaviruses by first characterizing in detail the antigens of a representative strain and then comparing these antigens with those of other viruses in the group. The human coronavirus 229E (HCV/229E) grown in L132 human embryo lung cells was chosen as the most appropriate model for the detailed antigenic analysis. In this study the first problem we encountered was the reportedly weak antigenic property of HCV/229E (Bradburne, 1970) and the relatively large quantities of specific antibodies required throughout the study. The animal of choice for raising antibodies against HEV/229E was the guinea pig (Kennedy and Johnson-Lussenburg, Personal Communication). The guinea pig however, does not yield large quantities of serum. Therefore, in order to minimize the amount of immunizing material (purified virus, etc.) we attempted to induce the production of ascites fluids in immunized animals according to established procedures reported for production of mouse antibodies (Munoz, 1957). These attempts proved to be successful and adequate quantities of ascites fluids containing specific antibodies were obtained. Throughout this antigenic analysis, both immune

ascitic fluids and sera, usually concentrated, have been used and, on the basis of this experience, we can recommend the ascitic fluid method as a valuable technique for the production of large amounts of antibody. These immune reagents then become the tools for the detection of the complete spectrum of antigens used for their production.

Our antigenic study of coronaviruses was conducted by using the continuous flow microimmunodiffusion test in cellulose acetate (Johnson et al, 1964). We excluded the use of immunodiffusion in agar gel since HCV/229E virions (80-160 nm in diameter) are too large to diffuse through gels whose pore size has been calculated to be 60 to 100 nm in diameter (Ackers & Steere, 1962). Cellulose acetate has a high structural uniformity and physiochemical inertness while different batches and sources of agar and agarose present widely variable characteristics. Also, agar has a negative charge which prevents its use with basic dyes and basic antigens. Other advantages of cellulose acetate are that it requires no preparation for use other than wetting, it can be employed with both acidic and basic antigens, is also compatible with a wider variety of buffers than can be tolerated by agar or agarose, and is the most economical of reactants.

Using different patterns of reactions and the "complete spectrum" analytical immune preparations, as many as seven viral structural antigens were detected. Of these, only two could be demonstrated using the human convalescent sera and were by definition identified as virus specific structural antigens.

In comparative reactions using analytical and convalescent sera, 2 to 4 antigens, as represented by 2 to 4 lines in immunodiffusion, were identified as virus specific antigens due to their non-identity with the host reactions. By using a defined antigen in the form of the isolated internal ribonucleoprotein in further comparative immunodiffusion analysis, at least one virion component was identified as a ribonucleoprotein antigen. The remaining virus antigens showed a host relationship. The reactions with the host demonstrated by the purified virus antigens are an indication of the presence of host components possibly modified during viral infection and integrated into the virion.

The role of the host antigens in coronavirus replication is of interest due to the restricted range of species susceptibility to this virus which has been suggested to imply some specific functional requirement for virus replication. However, the identification of the putative host antigens and their contribution to virus replication require further study before confirmation of this suggestion is provided.

It should be emphasized, at this point, that the successful demonstration of 6 to 7 virus structural antigens was dependent not only on the quality of the analytical antibody preparations but also on the "detectability" of the virion antigens. This characteristic must be considered as a function of the concentration and diffusion properties of these antigens. As we have demonstrated, whether the virus preparations were disrupted or not was of prime importance with regard to the number of precipitin lines which developed. Furthermore, the

nature of the disrupting agent was also found to be of importance. Of the disrupting agents tested, sodium deoxycholate and Triton X-100 and to a lesser extent the lipid solvents ether and chloroform are the agents of choice.

Because of the failure in previous studies to find multiple virion antigens in unfreated purified coronavirus preparations (Berry & Stokes, 1968; Bradburne, 1970; Hironao et al, 1970; Kaye et al, 1970; Chubb & Cumming, 1971; Hierholzer, 1976), it was suggested that such antigens are not produced in large quantities as they are in some other virus groups. But on the basis of our studies, we have been able to demonstrate enough viral antigens to be consistent with the size and complexity of the virion. Indeed, in the light of our findings, the "soluble antigens" which were reported by others for untreated virus preparations may represent components of spontaneously disrupted whole virion particles capable of diffusion in agar with subsequent precipitation by antibody.

Once the initial spectrum of HCV/229E antigens was established, our study was extended to include other coronaviruses namely the two swine viruses TGE and HEV, and the avian coronavirus IBV as well as the murine coronavirus MHV.

The cross-reactions between the different coronaviruses as reported by other workers accompanied by the serological technique used to identify that relationship are summarized in table 8. In Bradburne's study of HCV/229E, LP, B814, MHVS, and IBV (1970), IBV was found to be unique

among the others in that it was not related to any of these viruses while MHV was related to most of the coronaviruses studied. McIntosh et al (1969), using seven organ culture (OC) viruses as well as 229E, MHV, and IBV found that only three of the OC viruses were related to several strains of MHV. Antigenic studies reported by Kaye and Dowdle (1969) showed the identity of strain OC38 and OC43 and the one-way relationship of those strains with MHV. Recently Kaye and his group (1977) reported a relationship between OC43 and HEV virus. More recent was the antigenic study of several coronaviruses by fluorescent antibody procedures (Pedersen et al, 1978). These workers found their test viruses fell into two groups on the basis of their interrelationships. In the first group were MHV3, HEV-67N, NCDCV, and OC43. The second was FIP, TGE, and CCV.

This profusion of cross-reactions as summarized in table 8, does not provide a common dimension for defining coronaviruses as a group according to their antigenic character. There are several reasons which can be suggested for this situation. 1) Different immunological methods with variable sensitivity were used. 2) Many of the cross-reactions were obtained using the complement fixation test "the results of which using coronavirus antigens were seldom constant or repeatable" (McIntosh et al, 1969; Bradburne, 1970). 3) The molecular characteristics of the antigenic components were not adequately identified nor established

TABLE 8

SUMMARY OF THE ANTIGENIC RELATIONSHIPS BETWEEN CORONAVIRUSES AS PUBLISHED BY OTHER WORKERS

Sera Against: Virus or Antigen	229E	LP	B814	OC43	OC38	MHV	IBV	HEV	RCV	SDAV	TGE	CD	FIP	CCV
229E	H	+N,C,G	-	+C		+C,G	-				+FA		+FA	
LP	+N,C,G	H	-	+C		-	-							
B814	-	-	H	+C		+C	-							
OC43	+N,C	+C,G	+C	H	+C,HI,N	+C,FA	-	+FA,C,N,HI			-	+FA	-	-
OC38				+C,HI,N	H	+C								
MHV	+C,G	+C,G	+C	+HI,C,FA	+C,HI	H	-	+FA,C	+C,N	+C,N	-	+FA	-	-
IBV	-	-	-	-		-	H							
HEV	-			+FA,HI,C,N		+FA		H			+G	+FA	-	-
RCV						+C,N			H					
SDAV						+C,N				H				
TGEV	+FA			-		-		+G	-		H	-	+FA	+FA,N
CD	-			+FA		+FA		+FA			-	H	-	-
FIP	+FA			-		-		-			+FA	-	H	+FA
CCV	-			-		-		-			+N	-	-	H

Table 8 Continued:-

H = Homologous Reaction; N = Neutralization; C = Complement Fixation;
G = Gel-Diffusion; HI = Hemagglutination-Inhibition;
FA= Fluorescent Antibody.

due mainly to constraints imposed by technical problems.

The general summary of our results with 229E, TGE, HEV, MHV, and IBV (table 9) reveals that our approach has been more successful in providing such a common relationship to the extent that we have proposed that the internal component of coronaviruses is antigenically related. It might be further suggested that superimposed on this fundamental shared component lie the more superficial inter-relationships provided by the membrane or other antigens of the viruses. These, then, would be the necessary antigens which confer intergroup specificities. Indeed, on the basis of some of our findings, several strains seemed to be more clearly related, as indicated by the number of lines showing reactions of identity, i.e., 229E and TGE; MHV and HEV. However, a further classification must depend on the isolation of surface and other virus components with subsequent analysis of their antigenic characteristics and relationships within the group.

As discussed in the previous section each animal species supports at least one coronaviral infection, the agent being highly species specific (calf diarrhea coronavirus, canine coronavirus, and feline infectious peritonitis coronavirus). A further extension of our study was made by screening the normal sera of such animal populations against HCV/229E antigen for the presence of coronaviral antibodies. The number of positives which were detected correlated closely to the number which could be expected (20 - 30%) as

TABLE 9

SUMMARY OF ANTIGENIC RELATIONSHIPS BETWEEN CORONAVIRUSES

Antiserum	Antigen	HCV/229E				TGE				HEV				MHV			IBV		
		Intact	Disrupted			Intact	Disrupted			Intact	Disrupted			Intact	Disrupted		Intact	Disrupted	
			NaDoc	TX-100	RNP*		NaDoc	TX-100	RNP**		NaDoc	TX-100	RNP**		NaDoc	TX-100		NaDoc	TX-100
HCV/229E	Antiserum	3#	7	7	2	1	3		2	1	2	4	1				1	1	1
	Ascites Fluid	3	6	7	2		1	1	1			1	1				1	1	1
	Convalescent	2	4	3	2		3	±		1	1	1					1	1	1
TGE	Antiserum	1	3	1	1	2	2	1	1	2	2	2	1	1	1	2	1	1	1
	Field		3	1			1			1	1						1	2	
HEV	Convalescent		2		1	1	3	2	1	1	4	2	1		1	±	1	2	
	Field		2	1			1			1	1		1				1	2	
MHV		1	2	1	1	1	2	1		1	3	2		1	1	3	1	2	1
IBV		1	2	1	1	1	3	1	1	1	3	1	1	1			2	3	3

Maximum number of precipitin lines obtained in immunodiffusion reactions. _ = homologous reactions.

* RNP isolated by NP-40 procedure. ** RNP obtained after spontaneous disruption and/or NP-40 procedure. Blank spaces indicate no reaction.

a reflection of the reported prevalence of natural coronaviral infections (Bradburne & Somerset, 1972; Reynolds et al, 1977; Horzinek & Osterhaus, 1979).

Because it is unlikely that HCV/229E could infect these animals (suggesting a hitherto unreported distribution of this virus among other animal species), these results lend support to our suggestion of a putative common coronavirus antigen. If such is the case, then the dog, cat, and cow coronaviruses should be included in the established list of coronaviruses in circulation.

Therefore, in summary, we conclude:

1) That HCV/229E is a complex virus consisting of six to seven antigenic components of which one has been identified as being internal component and do not contain host material while the remainder show varying degrees of host relationships.

2) That the RNP antigen which was detected should be regarded as a group antigen common to all coronaviruses and that its presence should serve to qualify candidate viruses to membership in the coronavirus group.

APPENDIX I

Genus: Coronavirus

Avian infectious bronchitis virus (Type species) (Massachusetts, Connecticut, Iowa-97, Iowa-609, Gray, Holte, Clark-333).

Canine coronavirus (1-71).

Feline infectious peritonitis (FIP).

Hemagglutinating encephalomyelitis virus of pigs (HEV-1, HEV-2, 2063/68, 67N).

Human coronaviruses (B814, 229E, OC strains, LP, EVS).

Mouse hepatitis viruses (JHM, MHV-1, MHV[PRI], MHV-3, MHV-S, A-59).

Neonatal calf diarrhea (LY-138).

Rat coronavirus (81900).

Sialodacryoadentis virus of rats (681).

Transmissible enteritis of turkeys (Bluecomb).

Transmissible gastroenteritis virus of swine (Purdue, New York II, SH, FS216/64, TO, Ckp, V-52, 67-1, DL).

APPENDIX II

Glossary

Analytical antiserum: Immune serum containing antibodies produced in response to artificial immunization with a known specific antigen or group of antigens or in response to natural or artificial infection and which have been characterized by standard immunodiffusion reactions.

Convalescent sera: Sera collected after recovery from a natural or artificially acquired coronavirus infection.

Field serum: Serum specimen obtained from normal populations which have no recent clinical history to correlate with the serological findings.

Host antigen(s): A collective term to include all antigens detected by immunodiffusion tests which have a host relationship.

Hyperimmune antisera: Serum obtained after hyperimmunization of an animal by an antigen or group of antigens.

Purified virions: Virus suspensions after concentration and purification by differential and sucrose density gradient centrifugation.

Reaction of identity: When two populations of antisera are compared by immunodiffusion with respect to one antigen or a mixture of antigens, the two different antisera can produce a reaction of identity although they may contain antibodies of entirely different specificity. This reaction is represented by a continuous line of identity which indicates a relationship between reacting antibodies and antigens. A reaction of identity may also occur between two populations of antigens reacting with an antibody. A reaction of identity between a homologous and a heterologous antiserum against an antigen or mixture of antigens has been interpreted as identifying heterologous antigens.

Reaction of non-identity: The pattern formed when two unrelated components react in an immunodiffusion test. In these reactions the precipitation bands form independently and cross over each other indifferently.

Reaction of partial identity: A slight spur formation between two antisera reacting against a complex mixture of antigens has been interpreted as "a slight antigenic

difference" or partial identity of antigens. It is also produced when partially related antigens are compared using a single antiserum. The individual bands extending beyond the point of crossing are acutely curved and distinctly fainter before the point of crossing.

Soluble antigens: Those antigens capable of migration through the cellulose acetate strips which can be detected by specific antisera.

Specific virus antigens: Those viral antigens which show non-identity with the host by immunodiffusion.

Surface antigens: Antigenic components at the surface of the intact virion detectable by immunodiffusion.

Viral structural antigens: Those antigenic components incorporated into the mature virus particle which may be detected by disruption of the virus.

APPENDIX III

1. Immunodiffusion buffer:

Tris	0.05M
KCl	0.1 M
Sodium azide	0.2%

Adjust pH with 1N HCl to 7.6
Make up to 1 litre in distilled water.

2. Thiazine Red (stain for cellulose acetate):

Thiazine Red	0.1%
Acetic acid (Glacial)	1.0%

Thiazine red is dissolved in boiling water (distilled). The mixture is allowed to cool before acetic acid is added to a final concentration of 1.0%. The stain is then filtered using a 0.45μ nalgene filter unit.

3. Veronal buffer:

5,5-diethyl barbituric acid (A)	5.75g
Sodium 5,5-diethyl barbiturate (B)	3.75g
Sodium Chloride (C)	85.00g
Distilled water	2,000ml

The barbituric acid (A) was dissolved in 500 ml of hot distilled water, and allowed to cool. Sodium barbiturate (B) and sodium chloride (C) were dissolved in one litre of distilled water. The barbituric acid (A) was added to this solution (B+C) and then diluted to a total volume of two litres.

4. Polyacrylamide gel preparation:

a) Acrylamide 30%
Bis-acrylamide 0.8%

Both were dissolved in distilled water, filtered, and kept at 4°C in dark bottles (8 weeks shelf life).

b) Trizma 36.3g
1N HCl 48.0 ml
Temed 0.46ml

Made to 100 ml with distilled water. Filtered through 0.45µ nalgene filter and stored at 4°C (8 weeks shelf life).

c) SDS 0.8g
Urea 24 g

These were dissolved in 100 ml distilled water, filtered and stored at room temperature.

d) Ammonium persulfate 0.28%

Dissolved in distilled water, filtered, and stored at 4°C for a maximum of 6 weeks.

The gel was prepared by mixing together 18 ml of a) and 6.75 ml of b) and 6.75 ml of c) and 9 ml of distilled water and 13.5 ml of d).

5. PAGE electrophoresis buffer:

SDS	1 g
Tris	3.0g
Glycine	14.4g
Urea	30.0g

Make to 1 litre in distilled water and filter. Add 0.05% 2-mercaptoacetic (0.5 ml) immediately prior to use.

6. SDS dialysis buffer (This buffer was used to dialyze the sample following SDS disruption):

SDS	1 g
Tris (Trizma)	0.6g
Glycine	2.88g
Urea	30 g

Add distilled water to one litre, and then filter.

7. Polyacrylamide gel stain:

Coomassie blue (0.2%)	100ml
Methanol	100ml
Acetic acid (Glacial)	15ml

The gel is removed from the apparatus and fixed in 12.5% tri-chloroacetic acid for 30 minutes. It is then rinsed 5 to 6 times in tap water, then stained with Coomassie blue overnight.

8. Polyacrylamide gel electrophoresis de-stain procedure:

Acetic acid (Glacial)	7.5%
Methanol	50 %

These were diluted in distilled water.
The procedure took approximately 48 hours during which time several changes of the de-stain were needed. The gel was then stored in 10% acetic acid.

9. The X-Ray film detection for labelled proteins and nucleic acids in polyacrylamide gels:

a) Soak gel in 20 volumes (approximately 600 ml) of dimethyl sulfoxide for 20 minutes.

b) Transfer to another 20 volumes dimethyl sulfoxide for 20 minutes.

c) Immerse gel in 4 volumes PPO/dimethyl sulfoxide (26.4 gm/120ml) for 180 minutes.

d) Immerse gel in water for 60 minutes.

e) Soak gel in 50% methanol for 30 minutes (to shrink gel and make it less fragile).

f) Dry on slab under vacuum.

g) Incubate in contact with X-ray film for 5 to 10 days at -80°C.

10. Phosphate buffer (1M):

a) Sodium phosphate (Na_2HPO_4) 95.1g

Dissolve this salt in 500 ml distilled water.

b) Potassium phosphate (KH_2PO_4) 44.9g

Dissolve this salt in 500 ml distilled water.

Mix both solution a) and solution b) then autoclave to prepare 0.001M PO_4 buffer solution in sterile distilled water.

11. Complement fixation antigen (Hamre & Beem, 1972):

HCV/229E infected L132 cells were used to prepare the antigen as follows:

a) Freeze-thaw the flasks and contents 3 times to release the cell associated virus.

b) The resulting mixture was clarified by centrifugation at 2000 rpm for 20 minutes at 4°C in an IEC PR6 centrifuge.

c) The virus suspension was homogenized by using a glass homogenizer.

d) The homogenized suspension was clarified by centrifugation at 500 rpm for 5 minutes at 4°C.

e) This virus suspension (antigen) was then dispensed into small vials.

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