SOME SURFACE PROPERTIES OF ENTEROVIRUSES

A BIOPHYSICAL, BIOCHEMICAL AND SEROLOGICAL STUDY OF SELECTED MEMBERS OF THE ENTEROVIRUSES.

by

M. S. SMITH.

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S U M M A R Y.

The purpose of this investigation was to study the biophysical and biochemical properties of some of the enteroviruses. Previous work in this laboratory on several members of this large sub-group of the Picornaviruses had emphasised the importance of a more detailed examination of some of the surface properties of representative strains of enteroviruses, by currently available techniques.

1. Physico-chemical studies on ecbovirus SA 1, the first enteric cytopathogenic bovine orphan virus to be isolated in South Africa, revealed that the properties of this virus were those of a typical enterovirus. The diameter of the virus, determined by three different methods, was 27 to 29.9 mμ. The virus was resistant to ether and pH 2.8, infectivity was stabilized to heat at 50° for 30 min. by the presence of molar concentrations of divalent cations, and infectious particles contained RNA as their genetic determinant, the content of which was calculated to be 28%.

2. The presence of a haemagglutinin in ecbovirus SA 1 was demonstrated for the first time. Virus propagated in primary cultures of calf kidney cells was found to be capable of haemagglutinating red cells from vervet and rhesus monkeys, and from sheep umbilical cords. In the presence of 140 mM $CaCl_2$ the titre of the haemagglutinin for vervet and rhesus monkey red cells was considerably enhanced, and in addition virus preparations were able to haemagglutinate horse red cells. Haemagglutination occurred only when the reactants were incubated at 4°, but not at $20-22^{o}$ or 37°. The hydrogen ion concentration was not critical, but optimal agglutination occurred at about pH 6. These observations led to a critical reappraisal of existing criteria for the classification of bovine enteroviruses.

3. The properties of the haemagglutinin and the infectious particle of ecbovirus SA 1 suggested that the surface of the virus particle was responsible for haemagglutination and that the haemagglutinin was inseparable from intact virus particles. This was demonstrated by the fact that the haemagglutinin and the infectious particle have identical electrophoretic mobilities at two different hydrogen ion concentrations, and the observation that particles of lower density than the infectious particles, the "coreless" particles, have the ability to haemagglutinate vervet monkey red cells.

4. A further physical property of the surface of ecbovirus SA 1 was determined, namely the isoelectric point. This was achieved by submitting virus preparations to sucrose density gradient zone electrophoresis in succinate/NaOH buffers of ionic strength 0.1. This method was shown to be suitable for the purpose by results obtained when the isoelectric point of a protein of known isoelectric point, human serum albumin, was determined by this method. The isoelectric point of ecbovirus SA 1 was confirmed by two further methods: by elution of virus from columns of agar spheres which acted as a weak cation exchanger, and by submitting the virus to isoelectric focussing in "artificial" pH gradients. The isoelectric point of ecbovirus SA 1 was found to be pH 4.40.

5. Knowledge of this constant was used in the concentration and purification of preparations of ecbovirus SA 1. At the isoelectric point virus was precipitated from suspensions using a minimal amount of polyethylene glycol as a precipitant. The precipitate was submitted to sucrose density gradient zone electrophoresis at the isoelectric point and purified virus was recovered from the position of the original virus band in the zone electrophoresis column. It is suggested that precipitation

of virus from suspension with polyethylene glycol may be a means of determining the isoelectric point of viruses. The pH at which most of the virus is removed from suspension after addition of a minimal amount of polyethylene glycol would be the isoelectric point of a virus which is normally soluble at its isoelectric point.

It was found furthermore that virus contained in large volumes of culture fluid could be concentrated by submitting the fluid to multimembrane electrodecantation at pH 7.2, and purified by submitting the fluid to multimembrane electrodecantation at pH 4.40, the isoelectric point of the virus. It is suggested that the technique of multi-membrane electrodecantation could be of considerable value in purifying and concentrating, from large volumes of culture fluid, attenuated viruses as for instance in live poliovirus vaccines.

6. The isoelectric points of several other enteroviruses were determined. While only a limited number of viruses was studied, it was found that the viruses could be classed into two main groups on the basis of these measurements. The first group consisted of enteroviruses which had an isoelectric point at, or very close to, pH 4.4 and contained ecbovirus SA 1, CBO virus and a mouse-adapted strain of MEF_1 poliovirus. The second group consisted of enteroviruses having isoelectric points of pH 4.65 to pH 4.81 and contained the original strain of MEF_1 poliovirus. Coxsackieviruses B 1, 3 and 4, and the "control" virus, reovirus type 1. Coxsackievirus A 9 had a higher isoelectric point of pH 5.33. It was noted that the three viruses which had isoelectric points at or near pH 4.4, were not pathogenic for man, including the mouse-adapted strain of MEF_1 poliovirus which had lost its neurovirulence for monkeys in the course of passage in

suckling mouse brains. It is thus suggested that an isoelectric point at or near pH 4.4 may be a further genetic marker of polioviruses which might allow the laboratory differentiation between virulent and attenuated poliovirus strains.

7. These studies showed that Coxsackievirus B 1 and B 3 preparations propagated in secondary cultures of monkey kidney cells have two electrophoretic infectious virus components. The two components of each virus revealed that they were distinct entities which were separable on the basis of their surface properties. It is suggested that the "fast" electrophoretic components consist of complete infectious particles, while the "slow" electrophoretic components are aggregates of non-infectious particles entrapping within their matrices a number of the infectious particles. These observations were correlated with those made by other investigators who have separated from preparations of Coxsackieviruses two distinct antigens, C and D, by gel precipitin and complement fixation tests. It is proposed that the "fast" electrophoretic components of Coxsackieviruses B 1 and B 3 correspond to the infectious D antigen, while the "slow" electrophoretic components consist of aggregates of non-infectious C antigen and infectious D antigen.

8. In the course of attempts to characterize the "slow" and "fast" components of Coxsackieviruses B 1 and B 3 it was observed that preincubation of viruses with trypsin remarkably increased the net negative charges of both components. The possible significance of these observations is discussed in relation to naturally occurring enterovirus infections of man, where virus, in the course of its passage through the duodenum and jejunum, is submitted to relatively high concentrations of trypsin before infecting man through the Peyer's patches.

1. INTRODUCTION.

1.1. ENTEROVIRUSES.

The causative agent of poliomyelitis was shown to be transferable to monkeys by Landsteiner & Popper (1909) who isolated the virus from the spinal cord of a fatal human case of the disease, and thereafter the use of monkeys as experimental animals led to the isolation of a number of strains of virus from human cases of poliomyelitis. It later became evident, when vaccinated monkeys were challenged with the different strains, and from experiments in which infectivity for monkeys was neutralized by pooled sera from the immunized animals, that these strains were not all antigenically related. Using the results obtained from such experiments Kessel & Pait (1949) and Bodian, Morgan & Howe (1949) were able to differentiate strains of poliovirus into 3 serological types. These three were recognised officially by the Committee on Typing of the National Foundation for Infantile Paralysis (1951), and standard conditions for the typing of poliovirus strains were laid down at the same time. The 3 prototype strains recognised were (1) Brunhilde, (2) Lansing and (3) Leon.

Faecal specimens collected during an outbreak of poliomyelitis in the summer of 1947 in New York were examined by Dalldorf & Sickles (1948) for evidence of mouse-adapted viruses. From the faeces of two children early in the disease an agent was isolated which induced paralysis in suckling mice and hamsters. This paralysis was associated with destructive lesions of the skeletal muscles, while the central nervous system remained unaffected. The paralysis could only be induced in suckling mice by intracerebral injection of animals less

than 12 days old. The virus was not neutralized by immune sera to 4 strains of poliovirus. The isolation of several more of these agents from cases of poliomyelitis-like disease soon followed. These hitherto unrecognised viruses, pathogenic for suckling mice and hamsters, and associated with "poliomyelitis", were named "Coxsackie viruses" by Dalldorf (1949) as the first recognised human cases were resident in the New York village of that name. The morbid anatomy of Coxsackievirus infections in mice suggested to Dalldorf (1950) the separation of these viruses into two groups. In group A - infected mice there was a generalised destruction of striated muscle, and these viruses were easily isolated and established in mice. In group B - infected mice similar lesions appeared in the striated muscle but they were focal and less extensive. The Group B viruses were not so readily adaptable to mice.

The demonstration by Enders, Weller & Robbins (1949) that the Lansing strain of poliomyelitis virus could be grown *in vitro* in non-nervous tissue fragments of the human embryo, demonstrating cytopathogenicity, paved the way for new great advances in animal virology. Using developments of this primitive tissue culture method Robbins, Enders, Weller & Florentino (1951) isolated a cytopathogenic strain of Coxsackievirus and an unidentified virus from 2 patients diagnosed as having "non-paralytic poliomyelitis". The cytopathogenicity of these 2 viruses in cultures of monkey kidney fibroblasts was unaffected by poliovirus antisera. Several more of these agents were isolated from patients diagnosed as having non-paralytic poliomyelitis, suggesting that hitherto unrecognised enteric viruses were prevalent (Melnick, 1954). Melnick called these viruses, which were apparently not associated with disease, "orphan" viruses.

The Committee on the ECHO viruses (1955) was convened to elucidate the status of these viruses. Multiple antigenic types existed, and were isolated from patients with aseptic meningitis as well as from healthy children in different parts of the world. These viruses were classified as the "enteric cytopathogenic human orphan (ECHO) group". The following properties were shared by these viruses: (i) they were cytopathogenic for monkey and human cells in culture, (ii) they were not neutralised by pools of the 3 poliovirus type antisera, (iii) they were not neutralised by Coxsackievirus antisera and failed to induce disease in infant mice, (iv) they were not related to other viruses recovered from the alimentary tract by inoculation of primate cell cultures (e.g. herpes, influenza, mumps and adenoviruses), and (v) they were neutralised by human gamma-globulin and by some individual human sera, suggesting that human infections occurred. The Committee (1955) further stated that a virus would be removed from this group if it were ever identified as the etiologic agent of a clinically distinct disease. Hsiung & Melnick (1957) divided these echoviruses into 2 groups on the basis of plaque morphology and growth in cultures of rhesus and patas monkey kidney cells.

Because the Coxsackieviruses, echoviruses and polioviruses shared pathogenicity, size, ether resistance, seasonal incidence, and epidemiologic pattern, the Committee on the Enteroviruses (1957) (formerly the Committee on the Echoviruses) believed that these three groups should be considered members of a single family of human enteroviruses. The Committee, while recognizing that some of the echoviruses caused disease in man, did not remove them from the echovirus group as they were responsible for syndromes which could be caused by a

variety of agents and could not be held directly responsible for a particular disease.

Wallis & Melnick (1962) demonstrated that the human enteroviruses were protected against damage by heating to 50° for 1 hr by molar solutions of cations, particularly Ca^{++} and Mg^{++}. This property of cationic stabilization was added to the three known properties of enteroviruses, namely a particle size of approximately 28 mμ diameter, an RNA genetic apparatus or core, and resistance to inactivation by ether (Melnick, 1962).

The Picornavirus group of small RNA viruses was defined in 1963 by an international committee (Melnick <u>et al</u>., 1963). This group contained viruses that were small (15 to 30 mμ diameter), not inactivated by ether, contained RNA cores, and the members of the group studied all had cubic symmetry. Two possible groupings were recognised: picornaviruses of human origin and those of lower animals. While recognizing the importance of those viruses of lower animal origins, and the possibility of subdivisions in this group, the committee was mainly concerned with the human picornaviruses. These were divided into three subgroups: enteroviruses, rhinoviruses and unclassified viruses. The enteroviruses were retained as a group, sharing the properties already described. The rhinoviruses (Tyrrell & Chanock, 1963), while not clearly defined at that time, differed from the enteroviruses in being isolated in embryonic human kidney cells and human diploid cells, as opposed to cultures of primary monkey kidney cells or heteroploid human cell lines (e.g. Hela cells). Unlike the enteroviruses, these viruses were unstable at low pH. Further studies by Mayor (1964) showed that picornaviruses other than poliovirus, namely echoviruses, types 4, 11 and 24, exhibited a polyhedral structure with icosahydral symmetry,

the capsid consisting of 32 capsomeres.

The most recent system of classification of the picornaviruses (Melnick & McCombs, 1966) recognises a further distinction between the enteroviruses and the rhinoviruses. The rhinoviruses have a buoyant density of 1.4 g/cm^3, while the enteroviruses have a buoyant density of 1.34 g/cm^3, in caesium chloride. This system further recognises the existence of multiple-type picornaviruses which infect the lower animals, consisting of enteroviruses, rhinoviruses, and a third "miscellaneous" group.

Poliovirus remained for a long time the "guinea pig" of the realm of the animal viruses. This can be attributed to the fact that this virus was the first animal virus obtained in crystalline form (Schwerdt & Schäffer, 1955) its remarkable stability, and its relative simplicity of form and composition. Consequently much of the information concerning the replication and composition of the RNA-containing viruses, and in particular the enteroviruses, has been assimilated from experimental data gained from investigations on this virus.

The ultraviolet absorption spectrum of poliovirus was that of a nucleoprotein, from which the nucleic acid content was calculated at approximately 22% (Schwerdt & Schäffer, 1955). Estimations of the RNA content of virus particles as determined by the orcinol colour reaction for pentoses indicated that the RNA content of particles was 24%, no DNA being detectable. A further estimate of the RNA content, employing the molar N/P ratio, resulted in a value of approximately 30% RNA. The molecular weight of this RNA was estimated as being approximately 2 x 10^6, and consisted of a single-stranded molecule with approximately 6,000 nucleotides, while the molecular weight of the intact particle was estimated as being 6.7 x 10^6 (Schäffer, 1963).

Crick and Watson (1956) drew attention to the observed fact that almost all small viruses were either rods or spheres, and suggested that a small virus contained identical subunits, packed together in a regular manner. On this basis the number of subunits in a rod-shaped virus was probably unrestricted, but for a spherical virus the number was likely to be a multiple of 12. Three types of cubic point-group symmetry was possible for such spherical viruses: tetrahedral (2:3), octahedral (4:3:2) and icosahedral (5:3:2) symmetry which respectively implied 12, 24 and 60 identical subunits. Finch & Klug (1959), in x-ray diffraction studies on crystals of purified poliovirus, found intensity distributions in the same relation to each other as the symmetry axes of the icosahedral point-group, 5:3:2. Thus, according to Crick & Watson (1956) poliovirus was built up of 60 structurally equivalent asymmetric units, although the x-ray data did not reveal the actual arrangement of these subunits.

By electron microscopic studies of negatively-stained preparations of poliovirus Horne & Nagington (1959) were able to demonstrate the presence on the surface of particles of about 20-30 "knobs". Klug & Caspar (1960) were able to reconcile this observation with the previous data obtained by x-ray diffraction studies by remarking that such representations represented only half the view of the particle, and that as a result the observation might resolve the 60 asymetric subunits indicated by the results from x-ray diffraction studies.

Terms for viral structures were provided by Lwoff, Anderson & Jacob (1959) who stated: "The viral infective system, the virion, may be considered as a clathrate type of compound in which the genetic component is enclosed in a coat or capsid formed of subunits or capsomeres".

Horne & Wildy (1961) pointed out that Finch & Klug (1959) had been thinking in terms of asymmetrical protein subunits packed to provide each other with an identical environment, and that 60 subunits were required by them to cover entirely the surface of poliovirus particles, but that electron photomicrographs had shown this was not so. Thus, they stated, for capsids of cubic symmetry the capsomeres were not equivalent to the subunits of Crick & Watson (1956). They proposed, as a solution to the problem of structural units and capsomeres, that the symmetrical capsomeres were built from a number of subunits held together in the form of a faggot.

Mayor (1964) contributed further to the knowledge of the external morphology of enteroviruses by studies of poliovirus type 1 and echoviruses types 4, 11 and 24 using a negative staining technique. She found that these viruses were identical in exhibiting icosahedral symmetry and a count of 32 capsomeres.

It was generally thought that the capsids of enteroviruses were built up of identical protein subunits. However, Maizel (1963), on subjecting the capsid protein poliovirus type 1, to electrophoresis, was able to detect at least 4 electrophoretically distinct components. This was followed by a report from Ruekert & Duesberg (1966) that the capsid protein of mouse encephalitis ME virus had two major electrophoretic components, the slower component furthermore appearing to be an unresolved mixture of two components. That these proteins were derived from a single genotype was beyond doubt as all possible mutant or companion viruses were removed by careful clonal purification. As an explanation for this phenomenon they suggested the possibility of these components arising from a single precursor chain by cleavage of the peptide. This cleavage had to be specific

due to the relative electrophoretic homogeneity of the components. That the capsid protein of enteroviruses is derived from a single large precursor polypeptide molecule by cleavage has since been proposed by other investigators and will be discussed in more detail in a later paragraph on the maturation of virus particles in infected cells.

The entrace of enteroviruses into susceptible cells, replication of the viruses within host cells, and release of mature particles from infected cells have been largely studied using the poliovirus/Hela cell system. The initial attachment rate of human enteroviruses to susceptible cells is relatively insensitive to temperature and requires the presence of cations, notably divalent cations. The initial attachment, which is probably electrostatic, is known to be specific as human enteroviruses generally only attach to cells of primate origin (Holland & McLaren, 1961). While cells from many primate organs are known to be insusceptible to human enteroviral infection _in vivo_, cells from such organs grown _in vitro_ have been shown to be susceptible to these viruses. Kunin (1964) has proposed three possible explanations for this phenomenon. (1) a small population of virus-sensitive, relatively undifferentiated cells already existing in the tissue grow abundantly _in vitro_ and form the virus-sensitive population; (2) receptors are actually present _in vivo_, but are in some way masked, incomplete, or inaccessible to virus until the cells are dispersed and grown _in vitro_; (3) receptors are synthesised _de novo_ in culture by cells previously lacking this property.

Holland (1964) has found that all susceptible primate cells released a virus-binding receptor-like material which was not detectable in insusceptible non-primate cells. These receptors were protein, or contained protein and most were associated with insoluble lipoproteins of

the microsomal membranes. Zajac & Crowell (1965b) resolved further the receptors for enteroviruses on Hela cells. Receptors for Coxsackievirus B 3 were inactivated by treatment of cells with chymotrypsin, while the receptors for poliovirus type 1 were inactivated by treatment with trypsin. Treated cells regained their enterovirus receptor activity after incubation in culture medium. Furthermore, by pretreating Hela cells with the enzymes prior to disruption and fractionation, intracellular receptors could not be found, indicating that the human enterovirus receptors existed only on the surface of the cells.

Mandel (1962) studied the entry of adsorbed poliovirus into Hela cells using specific antiserum to the virus. He proposed that virus entered the cell by pinocytosis, the externally adsorbed virus being surrounded by invagination of the cell membrane which eventually pinched off. The envelope surrounding the virus particle was then shed, exposing the particle to the cellular cytoplasm. A temperature-dependant process occurred that allowed virus to move from the cell surface to a point within the cell where antiserum could not penetrate, since at temperatures of less than 2° the infectivity could still be neutralised at the cell-surface by antiserum. Virus could be detected at all stages as an infective centre and could be recovered in all but the last stage (when released into the cytoplasm) by treatment of the cells with sodium dodecyl sulphate. At this stage the capsid was weakened and the RNA was released into the cytoplasm. Complete infective virus, as such, could no longer be recovered. This is termed the "eclipse phase". It appeared that the receptor remained associated with the virus particle and in fact was responsible for the weakening of the capsid once the particle reached the cytoplasm of

the invaded cell (Holland, 1964).

Once the RNA has been released, synthesis of viral protein and RNA commences almost immediately. In Hela cells poliovirus RNA synthesis begins within 30 min of infection (Baltimore, Girard & Darnell, 1966). Host cell DNA is not involved in this process. Simon (1961) demonstrated that DNA is not necessary for the growth of all RNA-containing viruses. Furthermore, the normal RNA and protein synthesis of infected cells is inhibited by enterovirus infection as was demonstrated for poliovirus in Hela cells (Salzman, Lockart & Sebring, 1959) and mouse encephalitis virus in L cells (Hausen & Verwoerd, 1963).

As enteroviral RNA synthesis does thus not depend on pre-existing cytoplasmic mediators, the invading viral RNA must mediate its own reproduction. The enzymes necessary for replication, RNA polymerases, must be encoded by the invading RNA (Baltimore & Franklin, 1963). A ribonuclease-resistant RNA, which was apparently triple-stranded, was found to exist in poliovirus-infected Hela cells by Bishop, Summers & Levintow (1965). They suggested that this was in fact template RNA, or the "replicative form" of the viral RNA. Studies by Baltimore <u>et al</u> (1966) confirmed the existence of such templates and concluded that they were formed during the early stage of infection, being in fact the first RNA molecules synthesised.

Ribonucleic acid molecules with the sedimentation behaviour of viral RNA are formed within 30-60 min of infection and their synthesis continues logarithmically for about 3 hr, after which the rate becomes linear (Baltimore <u>et al</u>., 1966). Up to 2 hr following poliovirus infection of Hela cells, none of the RNA which is

synthesised enters viral particles but is found in polyribosomes. About 30 min later tha RNA begins to be enclosed in virus particles (Baltimore et al., 1966).

Protein which constitutes the capsid of the progeny poliovirus particles is synthesised from the pre-existing intracellular free amino acid pool of the host cell (Darnell & Levintow, 1960). In infected Hela cells the existing polysomes disappear on invasion by poliovirus and are replaced with characteristic, larger polysomes for viral protein assembly. Immunological testing has shown these large polysomes to be the site of identifiable virus protein (Scharff, Shatkin & Levintow, 1963). Thegenetic information which determines the nature of the virus-specific protein is contained in the viral RNA. An RNA molecule can be coated with viral protein within 5 min of the synthesis of the RNA molecule, 3.75 hr after infection of Hela cells with poliovirus. At this stage sufficient viral coat-protein precursors exist in a pool in the infected cell for the coating of all existing and subsequently-formed RNA molecules. The synthesis of these precursor molecules begins at about the same time as the synthesis of the viral RNA (Baltimore et al., 1966).

The process of encapsulation of progeny RNA molecules is termed "maturation". From recent reports it appears that the three "procapsid" proteins of poliovirus are not primary gene products, but are formed by cleavage of a single large precursor polypeptide molecule (Jacobson & Baltimore, 1968). Holland & Kiehn (1968) similarly reported evidence for the specific cleavage of single large proteins into smaller functional units. They demonstrated a cleavage similar to that proposed by Jacobson & Baltimore (1968), of a large precursor protein in the case of poliovirus types 1 and 2. In

addition to this primary cleavage they found that, for mengovirus and Coxsackievirus B 1, a second distinct process of protein cleavage occurred during maturation of particles of these viruses.

The manner in which progeny virus is released from infected cells is not well understood. While the observed phenomenon of cell lysis due to viral cytopathogenicity undoubtedly releases the bulk of progeny virus from cells, it is thought that individual particles may be released from cells through cell membranes.

The possibility of non-primates acting as a reservoir for human enteroviruses is a subject which has received much attention. As early as 1910, Flexner & Lewis (1910) reported the presence of a neutralising substance to poliovirus in the sera of "normal" sheep, but Bartell & Klein (1955) found no specific neutralizing antibody to the virus in these animals. Subsequent results published by other investigators (e.g. Luginbuhl, 1958) revealed the presence of neutralising substances to a number of human enteroviruses in bovine sera, although these could not be shown to be true antibodies. Koprowski (1958) reported the isolation of a type 1 poliovirus from the faeces of a healthy calf, whose serum developed a rise in titre of "antibodies" to type 1 poliovirus at the time of isolation. The possibility of a laboratory contaminant being responsible for this observation, however, was not discounted.

It soon became recognised that inhibitors to many viruses were present in the sera of normal animals. Some of these non-specific inhibitors were heat-labile and could be inactivated by heating the sera to 56° for 30 min. Others, such as the Francis type inhibitors found in the sera of normal animals by Turner, Kipps, Polson and van den Ende (1961) to CBO virus were heat stable. Such

non-specific inhibitors could even be associated with the gamma-globulin fraction of the sera, as was demonstrated by Takemoto & Habel (1959) in the instance of a non-antibody inhibitor to type 1 poliovirus found in the sera of horses.

The first finite report on the isolation of human enteroviruses from non-primates was by Lundgren, Clapper and Sanchez (1968) who isolated 164 human enteroviruses from nose, throat and rectal swabs of beagle dogs in monkey kidney and Hela cell cultures. These consisted of Coxsackievirus B1, B3 and B5, and echovirus 6 strains. While the presence of neutralising antibodies, in low titre, to Coxsackieviruses B3 and B5 was detected in some dog sera, there was no correlation between the isolated viruses and the serum antibody titres. A possible reason for the failure to isolate these enteroviruses from other "domestic" animals may well be that isolation attempts were made in non-primate cells in culture, as for example when Gelfand (1961) used cultures of dog kidney cells. As Holland (1964) demonstrated, only susceptible primate cells produce receptors for the human enteroviruses.

As a result of the earlier reports on "antibodies" to human enteroviruses in the sera of non-primates, present trends in virology have led to a search in the lower animals for viruses related to human enteroviruses. The use of tissue culture methods has greatly facilitated this search. Many agents, primarily from faeces, have been isolated, which are generally not associated with disease in the animals from which they were derived, and hence are "orphans". Thus many new groups of animal enteroviruses have become recognised. Following on the original recommendations of the Committee on the echoviruses (1955), these groups have been named according to

the animals from which they were isolated. As a result animal enteroviral groups have emerged, designated ECMO (simian), ECBO (bovine), ECPO (porcine), ECCO (feline), ECAO (avian), viruses.

1.2. BOVINE ENTEROVIRUSES.

The first enteric bovine orphan viruses, 8 strains of a cytopathogenic agent, were isolated in cultures of calf kidney cells from the faeces of supposedly healthy cattle by Kunin & Minuse (1957). The viruses grew in cultures of monkey kidney cells but not in Hela cells. Antibodies to the viruses were found in 90% of the herd from which the 8 strains were isolated. While the strains were serologically related, they did not resemble the other human or bovine enteric agents recognised at that time (e.g. bovine adenoviruses). The term "ECBO" was tentatively suggested for these viruses.

Further reports of virus isolations from cattle followed. Moll and Finlayson (1957) isolated a virus cytopathogenic for bovine kidney cells from faecal samples of cows and calves which were febrile. Klein and Early (1957) isolated strains of viruses from healthy cattle in calf kidney tissue culture, but McFerran (1962) suggested that these might have been latent viruses in the calf kidney cells used. While most bovine enterovirus isolations have been made from "healthy" cattle and exhibited no pathogenicity on being transferred to further "healthy" animals, a number of isolations were made from animals which exhibited a clinical illness at the time of isolation, for example from cattle with respiratory disease (Moll & Davis, 1959), respiratory and enteric diseases (Dinter & Bakos, 1961), mucosal disease (Huck, 1961), diarrhoea and bronchopneumonia (Bontschev, St. Christov

& Andrev, 1963) and mucosal or respiratory disease and reproductive disorders (Huck & Cartwright, 1964). A similarity thus exists between these viruses and those of the echovirus group of the human enteroviruses.

The observation by Luginbuhl (1958), who isolated 26 enteric cytopathogenic agents from 111 "healthy" cattle, that the age of the cattle, and not the particular herd, was the more important association, again paralleled observations made on human enteroviruses in that there is a higher incidence of infection in young individuals. Most of Luginbuhl's isolations were made from cattle of less than 1 year of age.

Reports of virus isolations indicate the world-wide distribution of bovine enteroviruses. Isolations in the U.S.A. include those of Kunin & Minuse (1957), Klein and Earley (1957), Moll & Finlayson (1957), Luginbuhl (1958), Moll & Davis (1959), Moscovici, La Placa, Maisel and Kempe (1961) and Cliver & Bohl (1962). From the United Kingdom have come reports by McFerran (1958), Huck (1961) and Huck & Cartwright (1964), from Germany by Bögel & Mussgay (1960), Bürki (1962a), Liess and Höpken (1962) and Falk (1964), from Japan by Inaba et al., (1962a) and Yamada (1965a), from Sweden by Dinter & Bakos (1961), from Italy by La Placa, Portolani, Lamieri & Maioli (1964), from Bulgaria by Bontschev, St. Christov and Andrev (1963) and from Australia by Spradbrow (1964).

Just as the search for polioviruses in humans showing symptoms of "poliomyelitis" led to the recognition of the human Coxsackie- and echo-viruses, so the search for polioviruses in cattle led to the identification of the bovine counterparts of human enteroviruses. McFerran (1962) has proposed the following seven criteria for characterising bovine enteroviruses: (1) they are of small size (20-40 mμ); (2) they are not inactivated

by ethyl ether; (3) they have the capacity to multiply in the alimentary tract of cattle; (4) there is absence of a relationship to other groups of viruses recoverable from the alimentary tract, such as adenoviruses, reoviruses and myxoviruses; (5) if cytopathogenic, they have the ability to cause a cytopathic effect in bovine cells in culture; (6) they are not inactivated by sodium desoxycholate; (7) they have a core of ribonucleic acid. Of all the hundreds of bovine enteric virus strains isolated, very few have been tested for these specific properties, but it is probable that many of them will be assigned to the bovine enterovirus group (Bittle, 1967).

Relationships between viruses in this group have been established. McFerran (1958) found that the 112 agents he had isolated in N. Ireland fell into at least 3 serological types on the basis of cross-neutralisation tests performed in tissue culture. One of these types, VG(5) 27, appeared to be serologically identical with the prototype LCR4 strain isolated by Kunin & Minuse (1958) in America. This discovery led to the realisation that there were serologic interrelationships between viruses subsequently isolated from bovines in many parts of the world.

Most investigators have found that 2 main serotypes exist among the isolates studied by them (Cliver & Bohl, 1962; Liess & Höpken, 1962; La Placa, Portolani & Lamieri, 1965; Yamada, 1965). The first serious attempt at classification within the bovine enterovirus group was made by La Placa (1963) and La Placa _et al._, (1965) on the basis of the results of cross-neutralisation tests. Two main groups were proposed. The viruses contained in the first group were antigenically closely related and all had the ability to haemagglutinate rhesus monkey erythrocytes.

The viruses of the second group showed less uniform antigenic relationships and could be divided into subgroups. The viruses contained in this second major serological group were unable to haemagglutinate rhesus monkey erythrocytes.

While most of the bovine enteroviral strains which have been isolated appear to be true orphans, the possibility that several may be the aetiologic agents of disease in cattle cannot be discounted. The virus of foot-and-mouth disease is included in the bovine enteroviral group, while the F 266a serotype of Huck and Cartwright (1964) accounted for 75% of the viruses isolated from cases of infertility in cattle. That these viruses are widespread in cattle is beyond doubt. Agents have been isolated from "healthy" cattle belonging to all the herds so far examined. In most instances animals not excreting virus possess antibodies to the agents excreted by the members of the herd which do. This is particularly true for the older animals in the herds.

Although the significance in nature of most of the members of the bovine enteroviral group is not known, any information on members of this group can only lead to a better understanding of the group as a whole. Accordingly, the present study was undertaken on the first ecbovirus isolated in South Africa.

1.3. ECBOVIRUS SA 1.

The first isolation of an ecbovirus in South Africa was made by Alexander, Weiss & de Lange (1958), cited by de Lange (1959). While investigating the experimental infection of bovines with the viruses associated with Lumpy Skin Disease, a cytopathogenic agent was isolated

from the faeces of one of these bovines in lamb testis cell cultures. The virus was found to be able to multiply and cause cytopathic effect equally well in cells of lamb kidney and testis, and in calf kidney cells. De Lange (1959) suggested that this virus was an ecbovirus "like those of Kunin & Minuse (1958)".

Physico-chemical studies made on this virus by Polson & Kipps (1965) and Oellermann, Els & Verwoerd (1967) demonstrated that the virus was very similar to other enteroviruses, both human and bovine, on which comparable studies had been made. Serological studies by Verwoerd, Oellermann, Broekman & Weiss (1967) demonstrated that thevirus belonged to the Weybridge serotype 134 of Huck & Cartwright (1964).

Studies reported here demonstrate further relationships between this virus and the bovine and human groups of enteroviruses. The identification and characterisation of a haemagglutinin associated with the virus surface is described. A further surface property of the virus, namely the isoelectric point, was determined, and isoelectric studies were extended to other enteroviruses.

2. PROPAGATION, TITRATION AND CONCENTRATION OF INFECTIOUS VIRUS.

It was convenient to propagate echovirus SA1 in primary cultures of calf kidney cells.

2.1. MEDIA.

2.1.1. Hanks' lactalbumin growth medium.

Stock solution A.

NaCl	160.0 g
KCl	8.0 g
$MgSO_4.7H_2O$	4.0 g

dissolved in approximately 800 ml glass-distilled water

$CaCl_2$	3.8 g

dissolved in approximately 100 ml glass-distilled water

Once dissolved, the two were combined and made up to 1 litre with distilled water. Two millilitres of chloroform were added to the solution as a preservative and the solution was stored in a glass-stopped bottle at room temperature.

Stock solution B.

$Na_2HPO_4.2H_2O$	1.52 g
KH_2PO_4	1.20 g
Glucose	20.0 g

dissolved in approximately 800 ml glass-distilled water.

100 ml 0.4% phenol red, made up as follows, was added:

Phenol red 0.4 g
dissolved in 60 ml N/20 NaOH solution by heating.
Cooled, made up to 100 ml with distilled water.

Two millilitres ofchloroform were added as a preservative and the solution was stored in a glass-stoppered bottle at room temperature.

For use:

Stock solution A	250 ml
Stock solution B	250 ml

Distilled water up to 5 litres.

Autoclaved at a pressure of 10 lb per sq. in. for 10 min. to remove the chloroform. Cooled; then was added:

Lactalbumin hydrolysate	25 g
Penicillin	500,000 units
Streptomycin sulphate	0.5 g
$NaHCO_3$	1.75 g

Pressure filtered through a Seitz filter and dispensed in bottles.

2.1.2. Hanks' lactalbumin maintenance medium.

To 100 ml of the above growth medium was added 2 ml of pressure-filtered 5% (w/v in distilled water) bicarbonate solution.

2.1.3. Sera.

Calf and fowl sera were added to the growth and maintenance media to provide growth factors for the cells. Growth and maintenance media for calf kidney cells in culture contained 10% (v/v) and 2% (v/v) calf serum, respectively. The galactose medium used to maintain chick embryo cells in the agar-cell suspensions used in the titration of infectious virus contained a final concentration of 5% (v/v) fowl serum.

Blood was collected from animals and allowed to clot. Fowl blood was centrifuged immediately after it had clotted but calf blood was left for approximately 16 hr at 4° before it was centrifuged. The clotted blood was centrifuged at 2,000 rpm for 40 min., the supernatant serum was decanted and passed through a Seitz filter. The filtrate was dispensed in bottles and

non-specific heat labile inhibitors were inactivated by placing these bottles in a 56° waterbath for 30 min. The sera were stored at 4° (fowl) and -20° (calf).

2.1.4. Trypsin solution.

Trypsin base solution.

Solution A.

NaCl	80 g
KCl	4 g

dissolved and made up to 500 ml with glass-distilled water. One ml chloroform was added as preservative and the solution was stored in a glass-stoppered bottle.

Solution B.

This solution was identical to Hanks' stock solution B.

For use:

Solution A	100 ml
Solution B	100 ml

Distilled water up to 2 litres.
Dispensed in 95 ml amounts and autoclaved.
Stored at 4°.

Trypsin stock solution.

Five grams trypsin (Difco, 1/250) added all at once to 95 ml trypsin base solution. Dissolved as rapidly as possible at 37° with continuous stirring. Sterilised by passing through a Seitz filter, dispensed in 5 ml amounts, and stored at -20°.

Trypsin solution for use:

Trypsin base solution	95 ml
Trypsin stock solution	5 ml
5% (w/v) bicarbonate solution	2 ml

warmed to 37°.

2.1.5. Galactose medium.

Double-strength medium.

NaCl	16.0 g
KCl	1.0 g
Galactose	12.0 g
NH_4Cl	0.1 g
$MgCl_2 \cdot 6H_2O$	10.15 g
0.4% phenol red solution	0.75 ml
Lactalbumin hydrolysate	10.0 g

Dissolved separately by heating, cooled, and pooled.

$CaCl_2$	0.5 g
$NaH_2PO_4 \cdot H_2O$	0.25 g

Dissolved separately in a little distilled water and added to above.

Inositol	0.2 g
Glutamine	0.2 g
Glutamic acid	0.6 g
Methionine	0.2 g
Arginine hydrochloride	0.5 g

All added directly into the above solution.

Biotin	0.002 g
Folic acid	0.002 g
Nicotinamide	0.002 g
Calcium pantothenate	0.002 g
Pyridoxine hydrochloride	0.002 g
Thiamine hydrochloride	0.002 g
Riboflavin	0.002 g

All added directly into the above solution.

Made up to 1 litre with distilled water and the pH adjusted to 7.2 - 7.5 with N/3 NaOH solution. Sterilised by passing through a Seitz filter and stored at 4°.

Double-strength galactose medium + 10% fowl serum.

To 100 ml double-strength galactose medium was added 10 ml fowl serum and the medium was re-filtered.

Single-strength glalactose medium + 5% fowl serum.

Double-strength galactose medium + 10% fowl serum was diluted with an equal volume of sterile, glass-distilled water.

2.2. PROPAGATION OF ECBOVIRUS SA 1.

The strain of ecbovirus SA 1 was obtained from the Veterinary Research Institute, Onderstepoort, in the form of infectious culture fluid from infected foetal lamb kidney cell cultures. The passage level of the virus in these cells was not known.

The virus was propagated in monolayers of primary cultures of calf kidney cells in these laboratories and used in experiments at the eighteenth passage level in these cells.

To obtain dispersed cells from tissue fragments, a method of trypsinization based on the methods of Rappaport (1956) and Youngner (1954) was employed. Kidneys from freshly-slaughtered calves, embedded in the surrounding fat, were collected from the local abattoir. In the laboratory the fatty tissue and the capsules were removed aseptically. Cortical segments were chopped up into small pieces in a sterile beaker and washed in 95 ml trypsin base solution. The fluid was decanted and 100 ml trypsin solution was added. A sterile magnet was added to the beaker placed in a 37° waterbath over a magnetic stirrer. Trypsinization was

allowed to proceed for 1 to 2 hours.

Cell suspensions were filtered through sterile gauze and the filtrate was centrifuged gently at 1,000 rpm for 5 mins. The cells were washed in approximately 40 ml Hanks' lactalbumin growth medium containing 10% (v/v) calf serum and the packed cell volume of the cells was determined. The cells were resuspended in Hanks' lactalbumin growth medium containing 10% calf serum so that 500 ml of the medium contained 1 ml of packed cells.

The diluted cell suspension was dispensed in 50 ml amounts into 20 oz medical flat bottles which were incubated at 37° in a horizontal position. When the cell monolayers were confluent in 2 - 4 days, the fluid in each bottle was replaced with an equal volume of Hanks' lactalbumin maintenance medium containing 2% (v/v) calf serum. One millilitre of culture fluid, containing approximately 10^{7} plaque-forming units (pfu) of ecbovirus SA 1 per ml was inoculated into each of these bottles.

The infected culture fluid was harvested from these bottles when the monolayers had been completely destroyed by viral action. The cytopathic effect was generally observed within 24 hrs of inoculation. The harvested fluid was stored at 4° until required, and usually contained between 10^{7} and 10^{8} pfu per ml.

2.3. TITRATION OF ECBOVIRUS SA 1.

Infectivity of ecbovirus SA 1 was titrated by the plaque-counting method in chick embryo cell suspensions in agar. The method employed was that of Cooper (1961) with a slight modification of the

relative concentrations of Ca^{++} and Mg^{++} ions in the galactose medium (Russell, 1965). This consisted of increasing the $MgCl_2.6H_2O$ concentration from 0.4 g/l to 10.15 g/l, and decreasing the $CaCl_2$ concentration from 2.0 to 0.5 g/l.

Twelve-day chick embryos were harvested, the viscera removed, and the embryos chopped up in a sterile beaker and washed once in trypsin base solution. The solution was discarded and replaced with 100 ml trypsin solution, the embryo fragments being trypsinized for 30 min. in a 37° waterbath over a magnetic stirrer. The cell suspension was filtered through sterile gauze and the filtrate centrifuged at 1,000 rpm for 5 mins. The cells were washed once in single-strength galactose medium + 5% fowl serum and counted in a Spencer bright line haemocytometer. The cells were suspended in single-strength galactose medium + 5% fowl serum to a concentration of 1 to 2 x 10^{7} per ml.

At this stage a further modification of Cooper's (1961) method was introduced to enable the rapid production of large numbers of plates of cell suspensions. For example, to produce 100 plates, 200 ml double-strength galactose medium + 10% fowl serum was prewarmed to 45° and mixed with an equal volume of 1.2% (w/v) Ionagar No. 2 (Oxoid) in distilled water premelted and held at 45° in a waterbath. To this was added 100 ml of the cell suspension. To ensure a homogeneous suspension, this mixture was swirled several times. As Cooper's agar base layer in the plates was found to be unnecessary, 5 ml aliquots of this suspension were dispensed directly into each of 100 sterile 6 cm. glass petri dishes, using an automatic dispenser. The agar suspensions were allowed to gel, then 0.1 ml of a tenfold virus dilution

in single-strength galactose medium + 5% fowl serum was overlaid. In preliminary experiments no difference was found in the number of plaques produced by any virus dilution using this technique as opposed to incorporating the virus dilution in the suspension before it was allowed to gel. The plates were incubated in a humidified incubator at 37° for 3 days after which approximately 2 ml neutral red stain was overlaid. The neutral red stain was made up as follows:

Phosphate buffered saline (PBS):

$Na_2HPO_4.H_2O$	1.15 g
NaCl	8 g
KCl	0.2 g
KH_2PO_4	0.2 g

Made up to 800 ml with glass-distilled water, the pH being adjusted to pH 6.5 with N/1 solutions of HCl or NaOH.

Stain:

Neutral red	0.04 g
Glucose	1.5 g

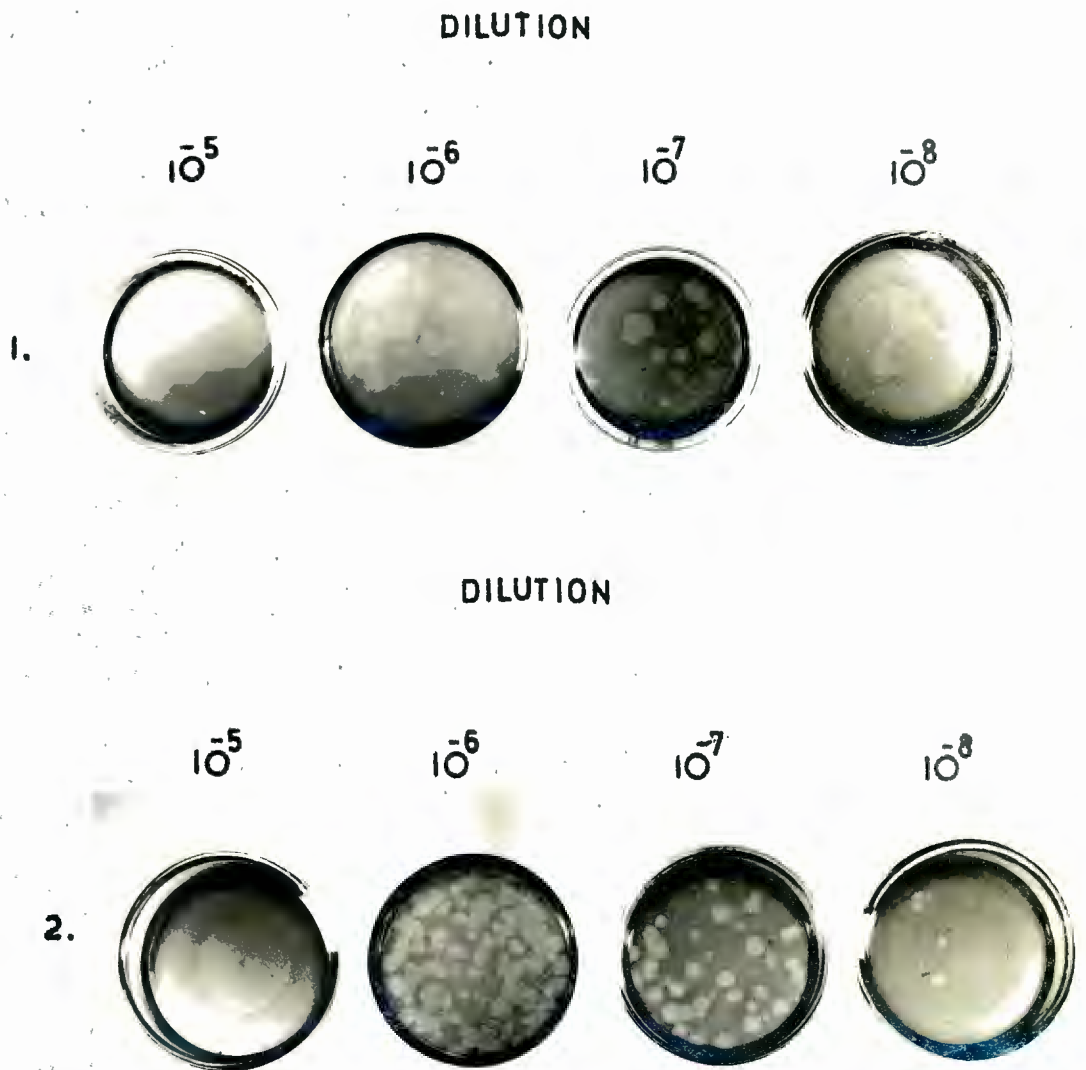
Dissolved in 100 ml PBS and filtered through filter paper.

After further incubation at 37° for 3 hrs., the stain was removed and the plaques counted. Plaques were visible as unstained areas against the red-stained background of cells which had taken up the vital dye. Titration results were reliably reproducible.

It was found by experimentation that each chick embryo provided sufficient cells for approximately 5 plates.

Figure 1 is a photographic record of two different virus titrations using this method.

Figure 1. Titration of ecbovirus SA 1 by the plaque method in suspensions in agar of chick embryo cells. Two separate titrations are shown.



2.4. TECHNIQUES TO CONCENTRATE ECBOVIRUS SA 1.

2.4.1. Differential ultracentrifugation.

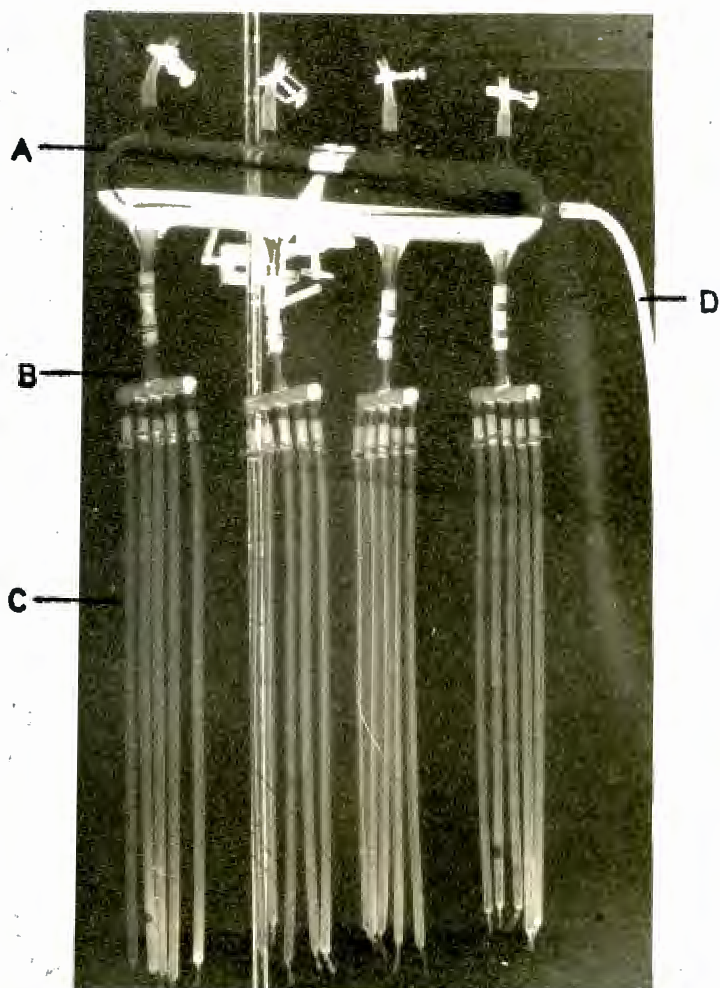
The virus in relatively small volumes of infected culture fluid (less than 100 ml) was concentrated and partially purified by differential ultracentrifugation in the No. 40 rotor in a Spinco model L preparative ultracentrifuge (Beckman Scientific Instruments, California). Centrifugation at 10,000 rpm for 10 mins resulted in a "clarified" virus suspension. The infectious particles were concentrated into a pellet by centrifugation at 30,000 rpm for 90 mins. This pellet was resuspended in a minimal volume of suspending medium, depending on the nature of the experiment, and clarified once more by centrifugation at 10,000 rpm for 10 mins. to remove denatured protein. Such preparations are referred to in the text as preparations prepared by differential ultracentrifugation.

2.4.2. Pressure dialysis.

Pressure dialysis is merely dialysis performed under pressure to speed up the process of dialysis, coupled with pervaporation. The process of pervaporation cools the solution thereby stabilizing the virus in the solution. To concentrate large volumes of culture fluid (1 litre or more) virus suspensions were first concentrated by pressure dialysis in cellophane dialysis tubing which removed water and salts, before undergoing a cycle of differential ultracentrifugation to separate the virus from the cell debris, proteins and peptides which had been concentrated with the virus.

To achieve this a glass manifold system was designed which could be easily dismantled for cleaning (see Fig. 2).

Figure 2. Apparatus used to concentrate suspensions of ecbovirus SA 1 by pressure dialysis.



A 7 cm diameter glass tube with inlet and outlet tubes
B small manifolds, each with 5 outlet tubes to which the dialysis sacs are attached (see Fig. 3)
C dialysis sacs
D pressure applied through this tube to the manifold system.

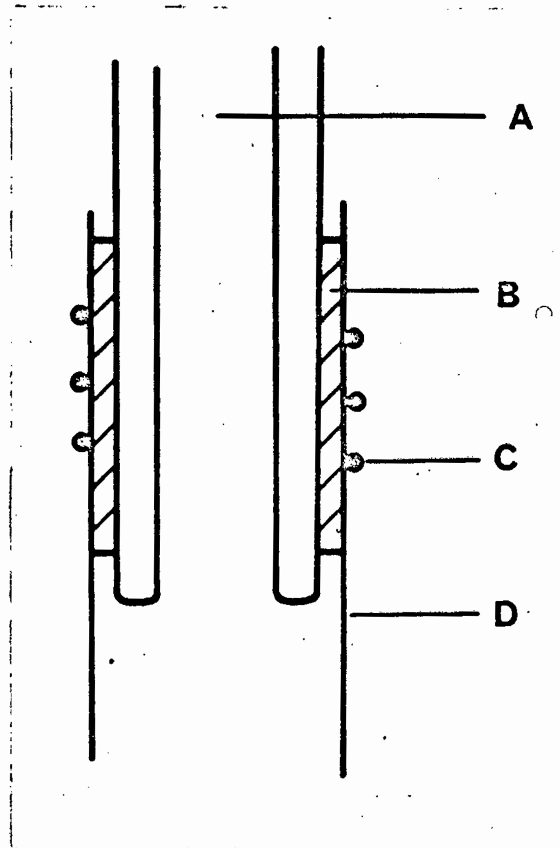
The large storage manifold consisted of a glass tube of 7 cm diameter sealed at one end and drawn out at the other. Along the top of this tube were attached 4 glass inlet tubes, and 4 corresponding outlet tubes were attached opposite these. To each of these outlet tubes was attached, by means of rubber latex tubing, a further manifold having 5 outlets made of glass of 5 mm internal bore. Over these outlets were slipped short sleeves of rubber latex tubing. Lengths of dialysis tubing (size 8 Union Carbide Corporation, Chicago, Ill) were wetted and slipped over these sleeves, being bound in position with cord, (see Fig. 3). The lengths of dialysis tubing were sealed by knotting and turning the end of the tubing back on itself and binding with cotton thread.

Four separate samples of approximately 200 ml amounts could be individually concentrated simultaneously by introducing them directly into the 4 smaller manifolds through the large reservoir manifold. For larger volumes the entire body of the apparatus was filled with fluid. In either instance all openings were sealed and a positive pressure of 10 lb per sq. in. was applied to the reservoir manifold. Three litres of culture fluid could be concentrated to approximately 50 ml within 24 hrs. It if was required to concentrate a larger volume of culture fluid, the fluid in the apparatus was periodically topped up whenever the main manifold had been emptied due to the pressure dialysis.

Volumes of infectious culture fluid of 1 - 5 litres were regularly reduced to 20 - 25 ml. The percentage recovery of infectious particles was never less than 50% of the original total infectivity, the highest recovery achieved being 87% of the total infectivity.

A particular advantage of this method was that the dialysis tubing could be used several times before it

Figure 3. Diagram of the method of attachment of the dialysis sacs to the small outlet manifold tubes.



A 5 mm internal bore glass outlet tube from the small manifold

B rubber latex sleeve

C cord used to bind dialysis tubing to manifold outlet tube

D dialysis tubing

was finally discarded. To recover the concentrated sample, the last few centimetres of each tube containing the concentrate was cut off. After washing, the sacs could be tied off and used once more. They were discarded only after continuous use resulted in the shortening of the sacs to such an extent that insufficient surface area was available for efficient pressure dialysis to occur.

3. IDENTIFICATION OF A HAEMAGGLUTININ OF ECBOVIRUS SA 1.

3.1. INTRODUCTION.

The ability of red blood cells to be agglutinated by a virus was first demonstrated, independently, by Hirst (1941) and McLelland & Hare (1941) who observed that chick red cells were agglutinated by amniotic and allantoic fluids from fertile eggs infected with strains of influenza virus. Subsequent work led to the establishment of the myxovirus group of animal viruses by Andrewes et al., (1955) as it was found that these viruses adsorbed to and eluted from a mucin-like substance on the red cell surface in a similar manner.

It is now known that haemagglutination (HA) occurs also with viruses of the arbovirus group, poxviruses, polyoma virus, reoviruses, and many enteroviruses but the elution of adsorbed virus from red cells appears to be a non-enzymic process, unlike the enzymic elution mechanism of the myxoviruses.

Applications of this phenomenon in animal virology include the use of suitable red cell systems for the rapid titration of viruses even though the method is not as sensitive as infectivity titrations since it has been calculated that one haemagglutinating unit (HAU) of influenza virus is equivalent to approximately 10^6 EID_{50} (egg infectious dose $_{50}$) (Waterson, 1961). By adsorbing haemagglutinating viruses to red cells, centrifuging these cells out of suspension, and eluting the adsorbed viruses in a salt solution, viruses in culture fluids may be purified from host cellular debris. The haemagglutination inhibition reaction provides a method for the rapid serological identification of viral strains.

The technique of haemadsorption, whereby red blood cells adsorb to the surface of infected cells is a useful technique for detecting the presence of haemagglutinating viruses within infected cells. Furthermore it is possible to enumerate individual virus particles by using an excess of red cells, and microscopically counting the red cell dimers in the resulting suspension.

The first report of enterovirus haemagglutination was made by Goldfield, Srihongse & Fox (1957) who found that several echoviruses and Coxsackievirus B3 agglutinated human type 'O' red cells and eluted from them in a non-enzymic manner. Lahelle (1958) confirmed the ability of echovirus 6 strains to agglutinate human type 'O' red cells and estimated that 1 HAU was equivalent to approximately 10^6 to 10^7 infectious dose $_{50}$ (ID_{50}). It is now known that most Coxsackievirus and echovirus strains have the ability to haemagglutinate human red cells. Factors which were previously known to affect the haemagglutinating capacity of other viruses such as age of the donor animal, pH of diluent and the temperature at which the red cells were allowed to settle, were shown by Kern & Rosen (1964) also to apply to those enteroviruses which could cause haemagglutination.

Experiments defining the mechanism of enteroviral haemagglutination and the chemical nature of the receptors have been reported from several laboratories. Philipson and Choppin (1960) found that sulphydryl reagents, such as p-chloromercuribenzoate (PCMB) prevented haemagglutination. Viruses treated with PCMB failed to adsorb to red cells. This inactivation was reversed by treatment of virus with thiol compounds. A sulphydryl group on the virus particle was thus implicated. Lerner <u>et al</u> (1966)

found that aldoses inhibited the haemagglutination reaction by attaching readily to red blood cells, but not to viruses, thus blocking the reactive sites. The fewer the number of carbon atoms in thechain, the broader was this blocking capacity. The carbonyl group of the aldoses was thus implicated as the reactive site in these blocking mechanisms. It was proposed that enteroviral haemagglutination involved the binding of a carbonyl group of a terminal sugar from an oligosaccharide side chain on the virus capsid to a specific receptor on the red cell surface. Philipson _et al_ (1964) have purified the receptor for haemagglutinating enteroviruses on human red blood cells and analysed it chemically. They found that it contained approximately 31% lipid, 60% protein, and 9% carbohydrate. Lerner & Miranda (1968) have confirmed the predominantly protein nature of the red blood cell receptor. This reactive site was sensitive to the action of trypsin and chymotrypsin but not the action of several other proteases, carbohydrases and sodium borohydride ($NaBH_4$). The carbohydrate moiety of the receptor (as described by Philipson _et al_)(1964) is thus not essential for adsorption of the virus, or is too well protected to be affected by the action of carbohydrases and the reducing agent $NaBH_4$. Of particular significance in all these studies is the fact that they act as a model for early virus-host cell interaction.

Haemagglutination due to bovine enteroviruses was first reported by Moscovici & Maisel (1958) who isolated 11 ecbovirus strains from apparently healthy calves in cultures of rhesus monkey kidney cells. Of these, 5 strains agglutinated bovine red cells at temperatures of 5° to 8° but not at room temperature or at 37°, while

another 3 strains agglutinated guinea pig red cells, also at this temperature. Elution followed rapidly on warming to room temperature. Prior to the publication of this report scant attention had been paid to the possibility of haemagglutinins existing for bovine enteroviruses, which is not surprising since the first report of haemagglutination due to human enteroviruses was only published in 1957. Since that time, however, more attention has been focussed on bovine enteroviral haemagglutinins. While several authors reported success with HA experiments, others failed to detect haemagglutinins associated with the viruses they had isolated. Verwoerd et al (1967) were unable to identify a haemagglutinin for ecbovirus SA 1.

In the study reported here this virus was re-examined for the presence of a haemagglutinin.

3.2. MATERIALS AND METHODS.

3.2.1. Red blood cell system.

Red blood cells were collected from various animal species into twice the volume of Alsever's solution. The species used were goose, chick, chick embryo, guinea pig, mouse, sheep, sheep cord, human type 'O', horse, vervet monkey and rhesus monkey red cells. Freshly-collected cells were stored at 4° being used, except where otherwise stated, within a week after collection.

Cells were washed twice in the diluent to be used in a particular experiment, being sedimented after each wash by centrifugation at 1,000 rpm for 10 min. in a refregerated centrifuge. They were resuspended in the diluent (see below) to a concentration of 1% (v/v).

3.2.2. Diluents.

The diluents used were 0.8% (w/v) sodium chloride (normal saline) of pH 6.5; phosphate-buffered saline (PBS) of pH 7.2; and a solution of calcium chloride in distilled water, of pH 6.2. The PBS solution consisted of equal volumes of 133 mM Na_2HPO_4 and KH_2PO_4 solutions mixed with an equal volume of normal saline. The $CaCl_2$ solution was, unlike the solution employed by Inaba et al. (1962b), a 140 mM solution in distilled water, as it was found that a 100 mM solution caused lysis of the red cells.

3.2.3. Temperature.

Virus-red cell systems were incubated at 4°, 20° or 37°.

3.2.4. Procedure.

Haemagglutination tests were conducted in 18 x 14.5 cm. standard perspex plates with wells, being read by the "Pattern" method of sedimented red cells. Into each well was dispensed 0.5 ml diluent. Doubling dilutions of virus suspensions were made in 0.5 ml amounts from left to right in these wells, the excess 0.5 ml at the end of each series being discarded. A volume of 0.5 ml of red blood cell suspension was added to each well, red cell controls were put up, and the cells were allowed to sediment at the different temperatures. Fluid from cultures of calf kidney cells which had not been infected with echovirus SA 1 was used in controls for each experiment. Titres were expressed as the reciprocal of the highest dilution showing complete haemagglutination.

3.2.5. Haemagglutination inhibition tests.

Haemagglutination inhibition (HI) tests were performed with antisera prepared in both roosters and rabbits by injections of virus in mineral oil adjuvant. The adjuvant used consisted of a mixture of Arlacel and Drakeol 6-VB in a ration of 1 : 9. This was emulsified with an equal volume of culture fluid having an infectivity titre of 2 x 10^8 pfu per ml.

From each of three mature roosters 10 ml blood was collected. The serum was separated from these samples, sterilised by filtration through a Seitz filter and stored at 4°, being the control pre-immune serum. The roosters were injected intramuscularly with 1 ml virus-adjuvant emulsion. Similar injections were administered at the following intervals: 4, 3, 7, 4 and 3 days. Seven days after the last injections an inoculum of 2 ml of infected culture fluid was administered intramuscularly to each rooster. The roosters were bled to death 10 days later. The blood was allowed to clot at 20° and the sera were separated by centrifugation, pooled and sterilised by filtration through a Seitz filter. Immune and pre-immune sera were heated to 56° for 30 mins. to inactivate heat-labile non-specific inhibitors.

Blood was similarly collected from each of 3 healthy mature rabbits before a course of immunizing inoculations in order to provide control pre-immune sera. The rabbits were immunized as follows: two injections of 5 ml virus-adjuvant emulsion were administered intramuscularly in the hindquarter with 7 days between injections. Nine days later 5 ml infected culture fluid was administered intravenously to each rabbit via a large vein in the ear. A second similar intravenous inoculation was given 10 days later. The rabbits were bled to death under anaesthesia

14 days later, the blood was allowed to clot, the serum was separated and sterilised and heat-labile inhibitors were removed by heating to 56° for 30 mins.

The HI test was conducted as follows: virus-infected culture fluid was diluted with $CaCl_2$ solution to contain 16 HAU per 0.4 ml. Doubling dilutions of sera from rooster and rabbit, both pre-immune and immune, were made in duplicate in perspex plates with $CaCl_2$ solution as diluent, in volumes of 0.4 ml., from 1/2 to 1/2048 dilutions. One row of each duplicate set was used for the HI test, the other acting as a serum control. To each HI test well was then added 0.2 ml virus suspension while 0.2 ml $CaCl_2$ solution was added to each of the serum control wells. The systems were incubated at 37° for 30 min. To each of the wells was then added 0.4 ml of a 1% (v/v) suspension of vervet monkey red cells in $CaCl_2$ solution. The systems were incubated at 4° for 4 hrs., following which the results were read.

3.3. RESULTS.

A summary of the results of haemagglutination experiments using different species of red cells, 3 different suspending media, and two different incubation temperatures is presented in Table 1. While it is evident that HA occurred when sheep cord red cells were employed, better results were obtained with horse, vervet- and rhesus monkey red cells, with $CaCl_2$ solution as the suspending medium. Haemagglutination was obtained only when the systems were incubated at 4° and not at 37°. As the highest HA titres were consistently obtained using vervet monkey red cells, these,

cells were employed to determine the optimal conditions for haemagglutination by ecbovirus SA 1. A photographic record of the first successful HA result, in which horse red cells suspended in $CaCl_2$ were agglutinated by ecbovirus SA 1, is presented in Fig. 4.

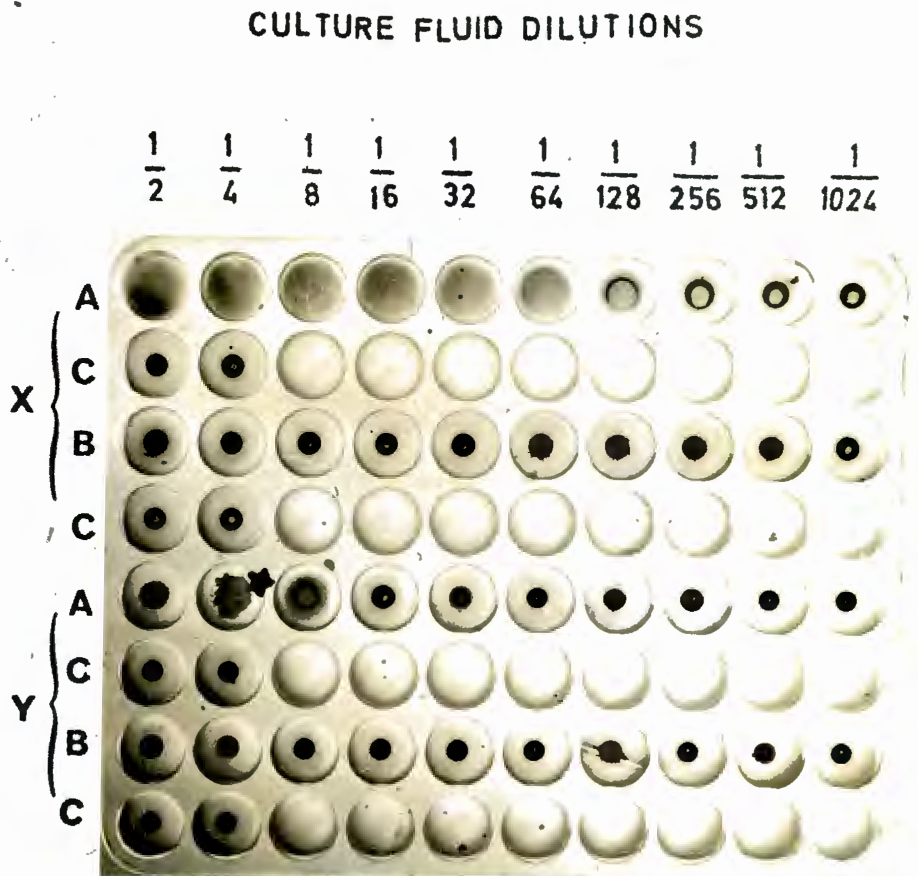
Table 1. Haemagglutination of red cells in various suspending media. (- indicates no HA).

Red blood cells	Suspending medium				Virus titre
	NaCl or PBS at pH 7.0		$CaCl_2$ at pH 6.2		pfu/ml
	4°	37°	4°	37°	
Goose, Chick, chick embryo, guinea pig, mouse, sheep, human 'O'	-	-	-	-	2×10^9
Sheep cord	8	-	8	-	2×10^9
Horse	-	-	128	-	1×10^8
Rhesus monkey	16	-	256	-	9×10^7
Vervet monkey	16	-	512	-	9×10^7

To determine the optimum hydrogen ion concentration for the reaction, the pH of the $CaCl_2$ solution was adjusted by means of 100 mM HCl and 50 mM tris solutions, to pH values from pH 5.5 to pH 8.0. The results of one such experiment, presented in Table 2, indicated that the pH requirement was not exacting, maximum agglutination occurring between pH 5.5 and pH 6.6. As the pH of a 140 mM $CaCl_2$ solution was always found to lie between pH 5.9 and pH 6.2, such unbuffered solutions were used in subsequent experiments.

Storage of vervet monkey red blood cells in Alsever's solution for prolonged periods did not affect their haem-

Figure 4. Haemagglutination of horse red blood cells in PBS and $CaCl_2$ solutions by a preparation of echovirus SA 1 having an infectivity titre of 1×10^8 pfu/ml. Incubated at 4°.



X red cells suspended in $CaCl_2$ solution

Y red cells suspended in PBS.

A infected culture fluid
B culture fluid from non-infected cells
C red blood cell controls

★ This particular figure was chosen to demonstrate the type of artifact encountered in these experiments.

agglutinability. The results of two experiments in which red cells that had been stored at 4° in Alsever's solution for varying periods were used in haemagglutination experiments, are presented in Table 3.

Table 2. Effect of the hydrogen ion concentration on the ability of ecbovirus SA 1 to haemagglutinate vervet monkey red cells suspended in $CaCl_2$ solution. Culture fluid containing 1×10^8 pfu/ml was used as haemagglutinin.

pH	HA titre
5.5	128
5.85	128
6.1	128
6.3	128
6.6	128
7.0	32
7.4	32
8.0	32

Table 3. Effect of storage of vervet monkey red blood cells at 4° in Alsever's solution on their agglutinability by ecbovirus SA 1.

Experiment 1		Experiment 2	
Days stored	HA titre	Days stored	HA titre
0	64	0	128
2	64	90	128
7	64	131	128
41	64		
42	64		
48	64		

Spontaneous elution of the virus from red cells occurred on leaving agglutinated systems at room temperature (20° to 24°) for 30 to 60 min., observed by the

fact that the red cells in such systems sedimented to form buttons at the bottoms of the wells. If such eluted systems were agitated and reincubated at 4°, haemagglutination occurred once more. This procedure could be repeated again before the HA titre dropped to a value of less than half that of the original.

Haemagglutination was inhibited by preincubation of virus with immune sera prepared to the virus in both roosters and rabbits. The results of haemagglutination-inhibition experiments, presented in Table 4, show that haemagglutination by 8 HAUs of ecbovirus SA 1 was inhibited by rooster and rabbit immune sera to titres of 128 and 256 respectively. While pre-immune sera exhibited no inhibitory activity, serum controls indicated that a haemagglutinin was present in both rabbit and rooster pre-immune sera to a titre of 16. This latter observation could have had no adverse effect on the experimental results obtained due to the dilution of the sera used in the haemagglutination-inhibition tests.

Table 4. Inhibition of HA by immune sera to the virus prepared in roosters and rabbits. 8 HAUs ecbovirus SA 1 were used. +

Sera	HI titre		HA titre of serum controls	
	pre-immune	immune	pre-immune	immune
Rooster	0	128	16	4
Rabbit	0	256	16	0

\+ Control titrations of haemagglutinins present in the sera for vervet monkey red cells are included.

3.4. DISCUSSION.

It has been demonstrated that a haemagglutinin was present in culture fluid from infected calf kidney cells. No agglutination of red blood cells was observed by control culture fluid from "normal" calf kidney cell cultures. The importance of the choice of the red cell system was demonstrated.

Since the first report of bovine enteroviral haemagglutinins made by Moscovici & Maisel (1958), when bovine or guinea pig red cells were agglutinated by several of their virus strains, most attempts at bovine enteroviral haemagglutination have been made with these red cells and those of rhesus monkeys, sheep, pigs, fowls and humans of the 'O' blood type. Of all these cell species, only rhesus monkey red cells were agglutinated by ecbovirus SA 1. A large panel of red cells was not always used by investigators of bovine enteroviral haemagglutinins. While Kunin & Minuse (1958), using chick red cells only, were unable to identify a haemagglutinin for their prototype LCR4 virus, it was later shown by Inaba et al.(1962b) that this virus agglutinated horse and sheep red cells as did BF 1 virus, although the two viruses were antigenically distinct from each other. Verwoerd et al.(1967) did not state what red cell species were examined by them in their attmpt to identify a haemagglutinin for ecbovirus SA 1 but it is likely that they used only a few.

The age of the donor animal is of importance. Before the use of $CaCl_2$ solutions as diluents in these experiments, the first successful HA result was obtained when sheep cord red cells were employed. Nowhere in the literature are there reports of sheep cord cells being used in HA tests but Rosen & Kern (1961) reported that

optimal titres were obtained for Coxsackie B viruses using red cells obtained from the umbilical cords of new-born human infants, only partial haemagglutination being obtained with adult human type 'O' red cells. In the present study no haemagglutination at all was obtained when adult sheep red cells were used. Furthermore, the titre obtained when using sheep cord red cells was not elevated when $CaCl_2$ solution was used as the suspending medium. This suggested that the surface of embryonic cells differed from that of the cells obtained from mature animals since cations (Ca^{++}) enhanced the haemagglutination of adult rhesus and vervet monkey red cells and allowed haemagglutination of horse red cells which were not agglutinable in the absence of these cations. Due to this presumed difference in the cell surface of the embryonic sheep red cells, cations were not necessary to enhance the HA reaction.

The observation that spontaneous elution of virus from red cells occurred on standing the agglutinated systems at "room temperatures" (approximately 20 - 24°) and that such cells were re-agglutinable on agitation and reincubation at 4°, parallels those made by Inaba et al. (1962b) and Yamada (1965b). A similar observation for human enteroviruses was made by Podoplekin (1964) with echoviruses. This type of reaction is strongly indicative of a non-enzymic elution mechanism, unlike the enzymic elution mechanism manifest by the myxoviruses.

A further indication of the non-enzymic nature of elution from red cells by ecbovirus SA 1 was provided by the observation that vervet monkey red cells were haemagglutinated by the virus with no loss in titre after they had been stored at 4° in Alsever's solution for at least 131 days, and that the virus eluted from these

cells in the same manner that it eluted from freshly-collected cells. Had a viral enzyme been necessary for elution of the virus from a specific site on the red cell surface, it is unlikely that such a site would have remained unaltered during the long storage period. This suggested that the reaction was of a physical rather than chemical nature, being mediated by electrostatic forces.

The ability of immune sera to the virus to inhibit haemagglutination, but not pre-immune sera, demonstrated the specificity of the reaction.

4. RELATIONSHIP BETWEEN THE VIRUS AND THE HAEMAGGLUTININ.

4.1. INTRODUCTION.

Hirst (1942) and Burnet (1942), investigating strains of influenza virus and Newcastle disease virus respectively, first suggested that the haemagglutinins of these viruses were the infectious virus particles themselves. It has since been shown that the haem-agglutinating activity of the myxoviruses is vested in the surface of the virus, being at the terminal position of the projections at the surface of the particle (Laver, 1964). The haemagglutinin is a function of the entire surface of the particle. The haemagglutinin itself is not a subunit of these viruses as the subunit instrumental in attaching to red cells is monovalent and cannot thus bridge red cells. This subunit or site on the surface of the particle which can attach to agglutinable cells has thus been more appropriately called haemadsorbin (Fazekas de St. Groth & Webster, 1964).

Studies on the haemagglutinins of other virus groups have revealed that the haemagglutinin may not necessarily be the infectious particle. This was first recognised for the group of organisms now known as the Bedsoniae and no longer considered to be viruses. In this group of organisms the haemagglutinin was shown to consist of complexes of lecithin and nucleo-protein, believed to be incomplete inclusion body material or degredation products (Anderson, 1959). While it was first believed that the haemagglutinins of the arbovirus group were associated with the infectious particle (Anderson, 1959), it has since been demonstrated by Mussgay & Rott (1964), Stevens & Schlesinger (1965) and

Parker (1966) that, in the case of those arboviruses examined by them, a further haemagglutinating component was present in viral preparations which was separable from the infectious particle.

Adenovirus, polyoma virus, reovirus and enterovirus haemagglutination is apparently a property associated directly with the infectious virus particle. Since the first reports of enterovirus haemagglutination, human and bovine, the virus particle has been implicated as the haemagglutinin.

In order to contribute further to the knowledge in this field, some of the chemical and physical properties of the infectious particle of ecbovirus SA 1 were compared with the corresponding chemical and physical properties of its haemagglutinin. The results suggested that the haemagglutinin was a property of the intact viral surface, and was not dependant on whether the particle was infectious or not.

4.2. CHEMICAL PROPERTIES.

4.2.1. Treatment of the virus with diethyl ether.

The sensitivity of the infectious particle and the haemagglutinin to diethyl ether was examined by the method of Andrewes & Horstmann (1949). Anaesthetic ether (Natal Cane By-products, Pty. Ltd., Natal) was added to infectious culture fluid to a volume of 20%. The mixture was shaken by hand and incubated at 4° for 20 hrs., being shaken periodically during incubation. Following incubation the ether was removed under reduced pressure and infectivity and haemagglutination were titrated. Infectious culture fluid which underwent the same treatment but with no ether added, served as a control.

Results of such an experiment presented in Table 5 demonstrate that neither the infectivity nor the haemagglutinin of ecbovirus SA 1 was adversely affected by this treatment.

Table 5. Effect of 20% (v/v) diethyl ether on the infectivity of, and haemagglutination by ecbovirus SA 1 after incubation at 4° for 20 hrs.

Sample	HAU/ml	pfu/ml
Ether-treated	32	4×10^7
Control	32	4×10^7

4.2.2. Treatment of the virus with trypsin.

Into each of two Bijou bottles was pipetted 2 ml virus-infected culture fluid. To one of these was added 4 mg trypsin (Difco; 1/250) the other acting as a control. Both bottles were incubated at 37° for 30 mins. Following incubation the culture fluid was removed from the Bijou bottles, made up to 10 ml with chilled $CaCl_2$ solution to prevent further trypsin activity, and the virus was collected in a pellet by centrifugation, at 5 - 6°, at 30,000 rpm for 30 mins. The supernatant fluids were decanted and held for trypsin control tests. These were performed by diluting the supernatant fluids 1/10 with PBS pH 7.2, placing droplets of these dilutions on a photographic plate, and incubating the plate at 37° for 30 mins. On examination of the photographic plate after incubation it was observed that the gelatine supportingthe photographic emulsion had been digested, leaving clear areas where drops of the trypsin supernatant fluid had been placed. No such clear areas

occurred on the photographic plate where drops of the control supernatant fluid had been placed. These observations proved that the trypsin preparation was biologically active.

The pellets recovered after ultracentrifugation were each resuspended in 2 ml 0.14 M $CaCl_2$ solution and the infectivity and haemagglutinin content of these suspensions was titrated.

The results of these titrations (Table 6) show that neither the infectivity nor the haemagglutinin of ecbovirus SA 1 was adversely affected by incubation with trypsin at a concentration of 2 mg/ml for 30 mins.

Table 6. Effect of incubation at 37° for 30 min. with 2 mg/ml trypsin on the infectivity and haemagglutinin of ecbovirus SA 1.

Sample	HAU/ml	pfu/ml
Trypsin-treated	16	2×10^7
Control	16	2×10^7

4.3. PHYSICAL PROPERTIES.

4.3.1. Thermostability.

Two-millilitre aliquots of virus infected culture fluid were incubated at different temperatures for 30 mins., then titrated for infectivity and haemagglutinin content. The original infectivity titre of this culture fluid was 2×10^8 pfu/ml while the haemagglutinin titre was 64.

Mean results of this experiment, which was performed in duplicate, are presented in Table 7. It was observed that while the infectivity of the virus was destroyed on

heating infectious culture fluid to 56°, the haemagglutinin was stable at this temperature for 30 mins.

Table 7. Effect of heating at different temperatures for 30 mins on the infectivity and haemagglutinin of ecbovirus SA 1.

Temperature	HAU/ml	pfu/ml
4°	64	2×10^8
37°	64	2×10^8
45°	64	2×10^8
56°	64	1×10^3

4.3.2. Buoyant density.

4.3.2.1. Materials and methods.

Buoyant density determinations of the infectious particle and haemagglutinin of ecbovirus SA 1 were made in preformed density gradients of CsCl using the technique described by Polson & Levitt (1963).

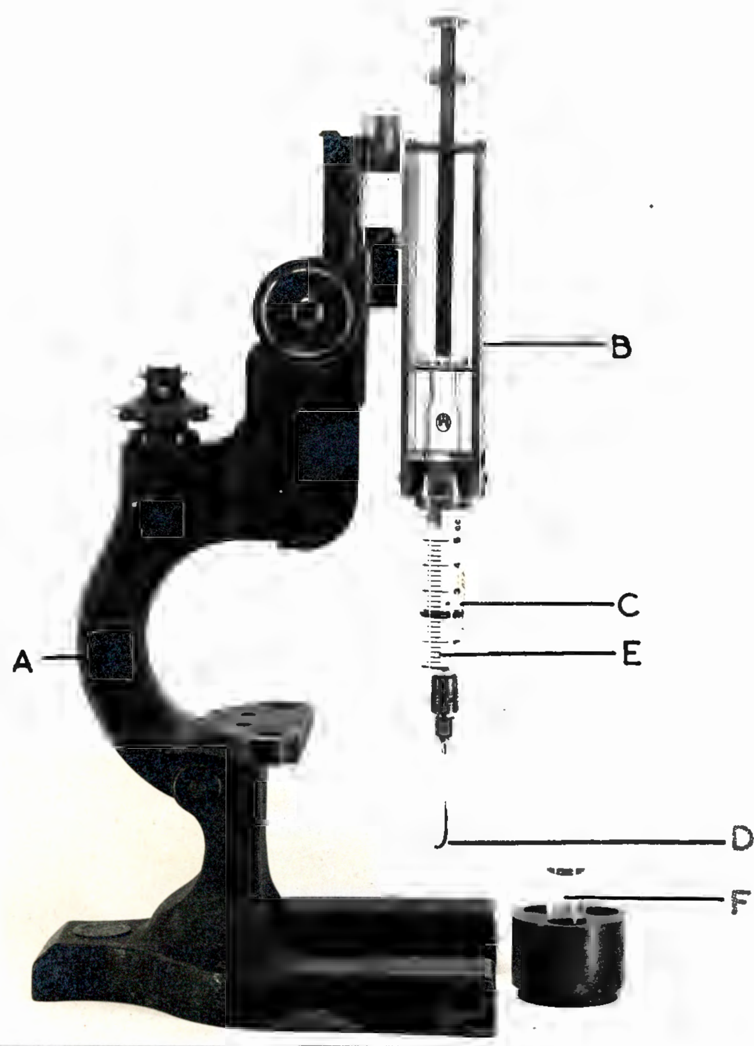
Infectious culture fluid, concentrated by pressure dialysis or used immediately after harvesting from infected calf kidney cells showing cytopathic effect, was partially purified and concentrated by differential ultracentrifugation, and the resulting virus pellets were resuspended in 0.5 ml 66 mM phosphate buffer pH 7.0 for density determinations on the infectious particle, or $CaCl_2$ solution for density determinations of the haemagglutinin. Eleven CsCl solutions ranging in CsCl concentration from 0% to 60% (w/v) were prepared in volumes of 1 ml in phosphate buffer (or $CaCl_2$ solution). The virus suspension in 0.5 ml phosphate buffer (or $CaCl_2$ solution) was mixed with 0.5 ml 60% (w/v) CsCl solution

in phosphate buffer (or $CaCl_2$ solution), resulting in a virus suspension in 30% (w/v) CsCl. Using the syringe holder described by Polson & Levitt (1963) illustrated in Fig. 5, a gradient of CsCl was formed in a 5 ml syringe by drawing up 0.4 ml amounts of each CsCl dilution, beginning with the least dense solution and 0.4 ml of the solution containing the virus being placed in the centre of the gradient. The pre-formed gradient was carefully expressed into a 1½ x 2" cellulose nitrate Spinco centrifuge tube and allowed to stand at 4° for 30 mins. to allow for inequalities to be ruled out. The tube containing the gradient was then centrifuged at 30,000 rpm for 180 min. at 4° in a swing-out SW-39 rotor of the Spinco preparative ultracentrifuge.

Following centrifugation, droplets were collected from these gradients by "bottom puncture" of the cellulose nitrate tubes. A controlled release of droplets from the tubes was obtained in the following manner. A 5-ml hypodermic syringe was fitted into the syringe holder and extended fully. To this was fitted a needle which in turn pierced a rubber stopper which fitted neatly into the top of the centrifuge tube. The bottom of the centrifuge tube was pierced with a 27-gauge needle and droplets were collected through this needle by slowly screwing down the syringe plunger, the air entering the tube causing a controlled displacement of fluid (see Fig. 6). Aliquots collected in this way each consisted of 4 to 6 droplets.

The refractive indices of these fractions were measured in an Abbé refractometer (Hilger; Sciex Scientific Instruments, England). The infectivity titres of the fractions collected after centrifugation in phosphate buffer were determined, while the haemagglutination titres of those obtained after centri-

Figure 5. Device for forming CsCl density gradients.



A stand

B screw-operated syringe holder

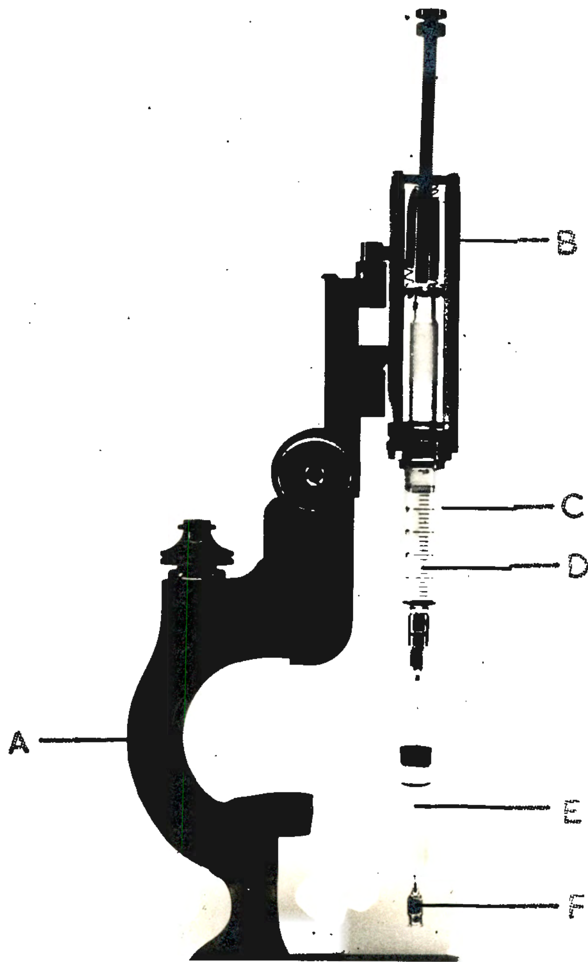
C 5-ml syringe

D 17-gauge needle with end bent

E part of CsCl gradient still in syringe

F part of CsCl gradient expressed into Spinco cellulose nitrate centrifuge tube

Figure 6. The apparatus used in sampling CsCl density gradients. By rotating the screw-operated plunger, air was slowly forced into the tube containing the gradient, resulting in the controlled displacement of CsCl solution which was collected through the 27-gauge needle.



A stand

B screw-operated syringe holder

C 5-ml syringe

D air

E CsCl density gradient after centrifugation

F 27-gauge needle

fugation in $CaCl_2$ solution were determined.

Standard curves were constructed of densities against refractive indices, the densities of standard CsCl solutions in phosphate buffer or $CaCl_2$ being determined pyknometrically. From these curves the densities of the aliquots were determined.

4.3.2.2. Results.

Standard curves, constructed as described above, are shown in Figs. 7 and 8 respectively, for CsCl in phosphate buffer and $CaCl_2$ solution.

In Table 8 the results of an experiment designed to determine the buoyant density in CsCl of the infectious particle of ecbovirus SA 1 are presented. In this experiment culture fluid concentrated by pressure dialysis and having an infectivity titre of 2 x 10^9 pfu/ml was used. The curve shown in Fig. 9 was constructed by plotting the infectivity of the aliquots recovered against their densities. The infectious particle was found tohave a buoyant density in CsCl of 1.335 g/cc.

Table 8. Results obtained on centrifuging ecbovirus SA 1 in a preformed gradient of CsCl at 30,000 rpm for 180 mins. CsCl solutions were prepared in 66 mM phosphata buffer, pH 7.0

Sample	Refractive index	Density g/cc	Infectivity pfu/ml x 10^6
1	1.3702	1.370	0.1
2.	1.3699	1.367	0.1
3	1.3681	1.350	30
4	1.3669	1.335	300
5.	1.3662	1.330	170
6	1.3645	1.317	10
7	1.3626	1.295	4.5
8	1.3617	1.284	2.6
9	1.3602	1.270	2.7
10	1.3586	1.225	3.8
11	1.3578	1.247	3
12	1.3573	1.242	0.5

Figure 7. Standard density curve for CsCl in phosphate buffer.

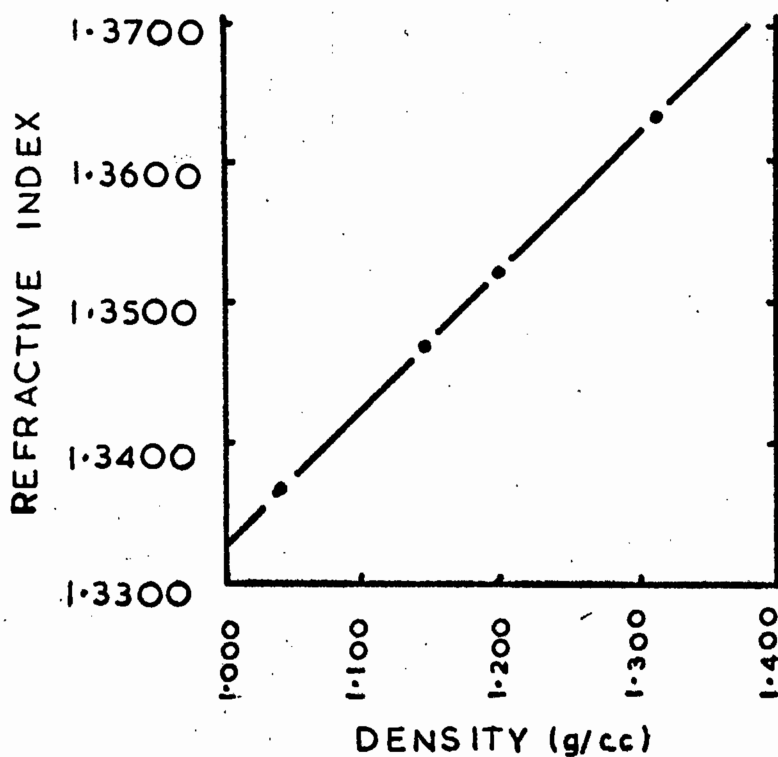
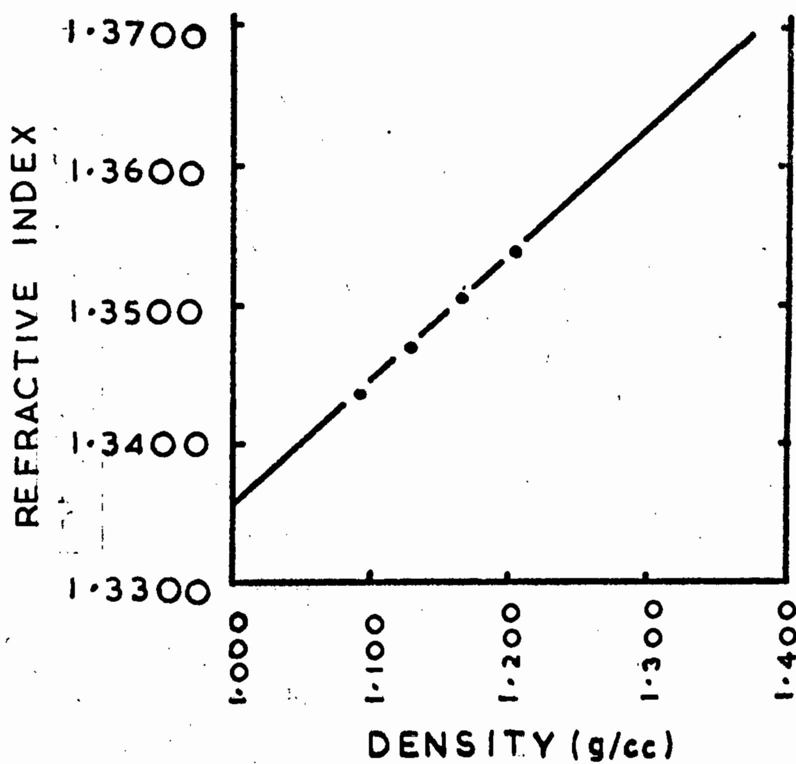


Figure 8. Standard density curve for CsCl in 140 mM $CaCl_2$ solution.



Results of an experiment designed to determine the buoyant density in CsCl of the haemagglutinin of ecbovirus SA 1 are tabulated in Table 9. In this experiment freshly-harvested culture fluid having an infectivity titre of 3×10^7 pfu/ml was used.

Table 9. Results of centrifugation of ecbovirus SA 1 in a preformed gradient of CsCl at 30,000 rpm for 180 mins. CsCl solutions were prepared in 140 mM $CaCl_2$ solution, pH 6.2.

Sample	Refractive index	Density g/cc	haemagglutinin HAU/ml.
1	1.3712	1.390	10
2	1.3697	1.374	10
3	1.3682	1.357	10
4	1.3660	1.334	160
5	1.3649	1.321	80
6	1.3634	1.305	80
7	1.3630	1.301	80
8	1.3597	1.265	40
9	1.3580	1.247	10
10	1.3570	1.236	<10
11	1.3538	1.202	<10

On plotting the haemagglutinin titres of aliquots collected in this experiment against their densities (Fig. 10) it is seen that two haemagglutinating components existed in this virus preparation. This result was confirmed in several similar experiments. The major haemagglutinating component had a buoyant density in CsCl of 1.334 g/cc, which was the same as the density of the infectious particle. The minor component had a buoyant density of approximately 1.30 g/cc.

Figure 9. CsCl density gradient plot of the distribution of infectious virus.

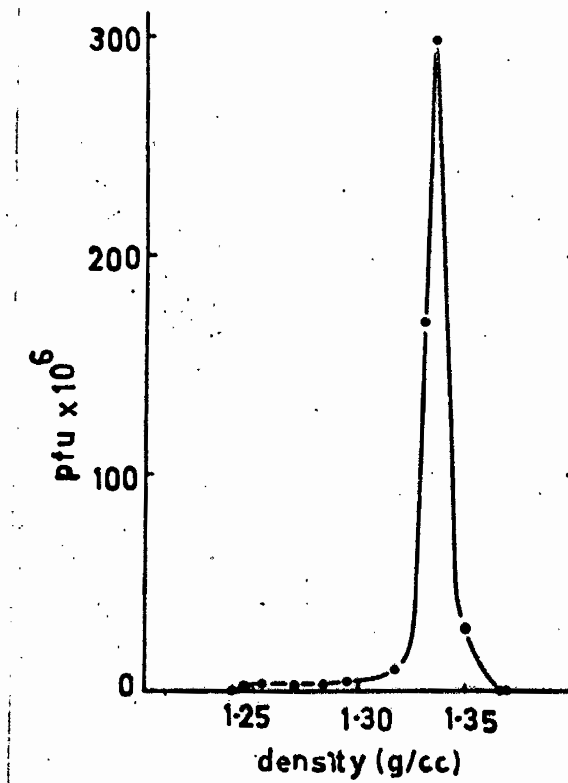
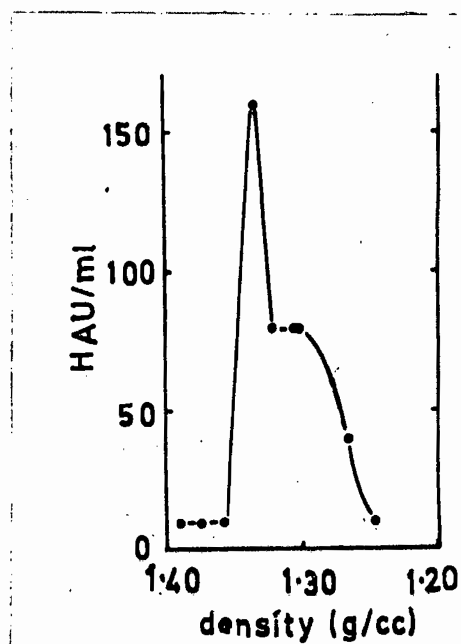


Figure 10. CsCl density gradient plot of the distribution of haemagglutinating activity.



4.3.3. Electrophoretic mobility.

4.3.3.1. Materials and methods.

The migration of ecbovirus SA 1 in an electric field was studied by zone electrophoresis in sucrose density gradients using the method and apparatus of Polson and Russell (1968). The migration of the infectious particle and the haemagglutinin was measured in relation to the migration of an internal reference substance, phenol red. R_{ϕ} values (van Regenmortel, 1968) for the infectious particle and the haemagglutinin were calculated by comparing the distance of migration of the particles to the distance migrated by phenol red which was included in the samples subjected to zone electrophoresis. Zone electrophoresis experiments were conducted in buffers of two different hydrogen ion concentrations, namely pH 8.6 and pH 4.65.

In experiments performed at pH 8.6 the borate buffer of Polson & Russell (1968) was used. The final molar concentration of this buffer was:

H_3BO_3	0.035 M
NaOH	0.0175 M
HCl	0.0075 M
NaCl	0.073 M, the ionic strength

of this buffer being approximately 0.1

In experiments conducted at Ph 4.65 a succinic acid-sodium hydroxide buffer, of ionic strength 0.1 was used. The final molar concentration of this buffer was:

succinic acid	0.004725 M
NaOH	0.004725 M
NaCl	0.073 M

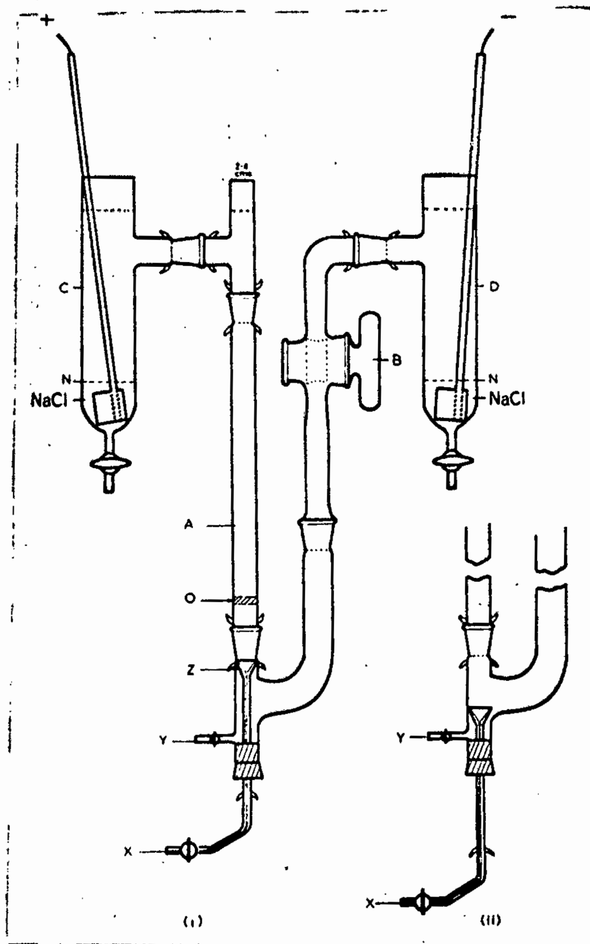
To 200 ml of double-strength buffer was added 160 g sucrose and the volume of this solution was made up to 400 ml. In the case of borate buffer solutions,

the pH of this 40% sucrose solution had to be adjusted to pH 8.6 by addition of approximately 7.5 ml N/1 NaOH solution due to the complexing of borate ions and sucrose which caused lowering of the pH. No such adjustment of pH was required in the case of succinic acid-NaOH buffers. The sucrose solution and the buffer, which had identical ionic strengths, were sterilised by boiling.

The apparatus (Fig. 11) was thoroughly cleaned and sterilised by filling with boiling distilled water. The water was drained off and sterilebuffer was added from the bottom through the tube Y, the large stopcock (B) being open and the collecting funnel (Z) being in the lowered position (see Fig. 11(ii)). The 40% sucrose solution was then similarly added through Y until it had reached the ground-glass joint in the right hand side column below the stopcock. Buffer was added to the left hand electrode vessel (C) forcing the sugar up the right hand limb until the interface between the buffer and 40% sucrose solution in the left hand side limb (A) was just above the gpound - glass joint in this limb. The collecting funnel (Z) was then raised to the position shown in Fig. 11 (i) and secured in this position and the stopcock B was closed. Excess 40% sucrose solution in the left hand limb was run off through the collecting funnel and the gradient was allowed to enter the electrophoresis column (A) through the capillary X.

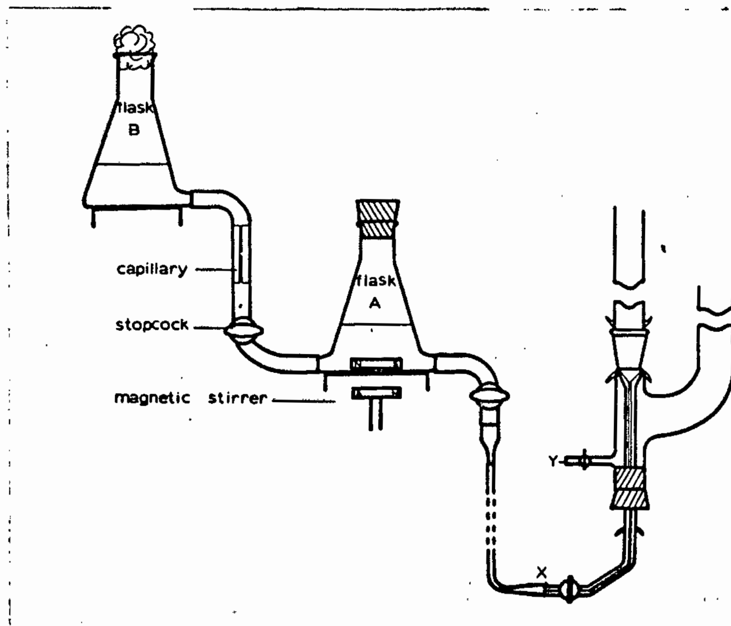
The gradient-forming device (Fig. 12) consisted of two flasks, A and B, joined together by a length of capillary tubing. To flask A was added 150 ml buffer and the neck of this flask was sealed with a rubber stopper. To flask B was added 150 ml 40% sucrose solution and the neck of this flask was not sealed but plugged with cotton wool. A sterile magnet was added to flask A which was placed over a magnetic stirrer.

Figure 11. Apparatus for zone electrophoresis.



A= electrophoresis column. B= large stopcock. C & D= buffer reservoirs (electrode vessels). X= capillary through which gradient and virus added to the column. Y= aperture used for the initial filling of the column. Z= collecting funnel. O= position of virus band in column before submitting to zone electrophoresis.

Figure 12. Apparatus for forming a sucrose density gradient in the zone electrophoresis column.



Flask A was connected by means of a length of rubber capillary tubing with a Luer-Lok attachment to the capillary tube leading into the electrophoresis column at X. The magnetic stirrer was switched on, the stopcock between the two flasks of the gradient-forming device was opened, and the resulting increasing density gradient flowing from flask A flowed into the electrophoresis column from the bottom. Due to the neck of flask A being sealed, fluid leaving this flask was replaced with 40% sucrose solution from flask B, the gradient resulting in the electrophoresis column being logarithmic. When all the sucrose solution in flask B had run into the mixing flask (A) the stopcock at X was closed and the rubber capillary tubing from the gradient-forming device was disconnected.

Approximately 5 ml of the lower, most concentrated part of the gradient was run out of the electrophoresis column through the collecting funnel, ensuring that the concentration of the sucrose solution at the bottom of the column was at most 35%.

Ecbovirus SA 1, concentrated from infectious culture fluid by pressure dialysis or used as unconcentrated material, was subjected to a cycle of differential ultracentrifugation. The resulting pellet was drained and resuspended in approximately 3 ml buffer containing approximately 37% sucrose. A few grains of the reference substance, phenol red, were added to the suspension which was clarified by light centrifugation. The supernatant suspension was introduced into the zone electrophoresis column through the capillary X, employing the screw-operated syringe holder (Fig. 5) and a 5-ml hypodermic syringe so that the suspension was forced gently into the column. Using a 20-ml syringe, approximately 15 ml 40% sucrose solution was added to the column through X,

pushing the phenol red-pigmented virus-containing band up the column ahead of it. The collecting funnel (Z) was lowered to the position shown in Fig. 11 (ii).

The reversible silver-silver chloride electrodes were placed in the electrode vessels (C and D) and covered with a saturated solution of NaCl. The large stopcock (B) was carefully opened and the position of the sample band was stabilised by adding buffer to the electrode vessels to balance the band. The apparatus was allowed to stand for 2 - 3 hrs. in order for diffusion to form a density gradient across the virus band. A direct current of 20 mA was applied to the column, the voltage gradient being 3 - 4 V/cm. Experiments were performed at room temperatures ($20^{o}$to 24°), a stream of air from a fan being directed onto the apparatus to assist in heat dissipation. The electric current was passed through the apparatus for periods of 16 - 24 hrs.

At the completion of a zone electrophoresis experiment the current was switched off, the large stopcock (B) was closed, and the collecting funnel was raised to the upper position. Samples of 1 cm column length were collected through the collecting funnel and titrated for infectivity and haemagglutinin. The distance migrated by the phenol red band was measured as the distance between the midpoints of the position of the original band in the column before the current was applied, and the phenol red band after zone electrophoresis. Similarly, the distance migrated by the virus band was measured as the distance between the midpoint of the original band and the midpoint of the sample(s) containing the highest concentration of infectivity or haemagglutinin.

4.3.3.2. Results.

The results of an experiment performed at pH 8.6 are plotted in Fig. 13. The infectivity and haemagglutinin titres of the samples collected after electrophoresis were plotted against their corresponding positions in the zone electrophoresis column. In this particular experiment the phenol red migrated a distance of 16.5 cm in 24 hrs. while the maximum infectivity and haemagglutinin titres were found to be in a position 8 cm from their original position. This corresponded to an R_ϕ value of 0.485 for both the infectious particle and the haemagglutinin.

The results of a zone electrophoresis experiment performed in buffer of pH 4.65 are plotted in Fig. 14. While the phenol red migrated 10.75 cm in 16 hrs, the greatest proportion of infectious particles and the haemagglutinin migrated 2 cm, corresponding to an R_ϕ value of 0.195 for both.

4.3.4. The haemagglutinin to infectious particle ratio.

Various preparations of culture fluid harvested from calf kidney cells infected with ecbovirus SA 1 were tested for their ability to agglutinate vervet monkey red cells suspended in $CaCl_2$ solution and the infectivity of these preparations was determined concurrently. One of these preparations was culture fluid which had been concentrated by pressure dialysis.

The results obtained, as presented in Table 10, were plotted in Fig. 15.

Figure 13. Gradient zone electrophoresis of ecbovirus SA 1 at pH 8.6 for 24 hr.
Cross-hatching indicates the haemagglutinin.
Ø red = phenol red.

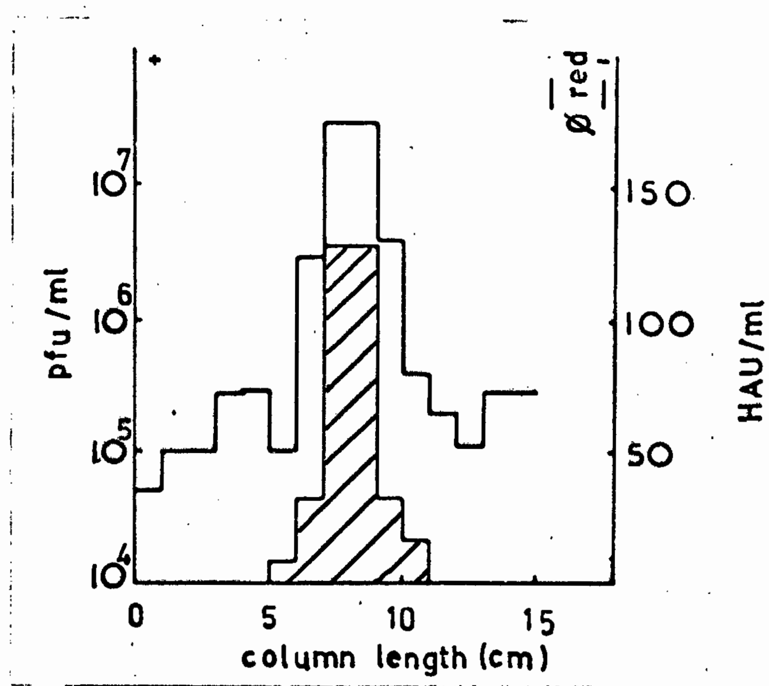


Figure 14. Gradient zone electrophoresis of ecbovirus SA 1 at pH 4.65 for 16 hr.
Cross-hatching indicates the haemagglutinin.
Ø red = phenol red.

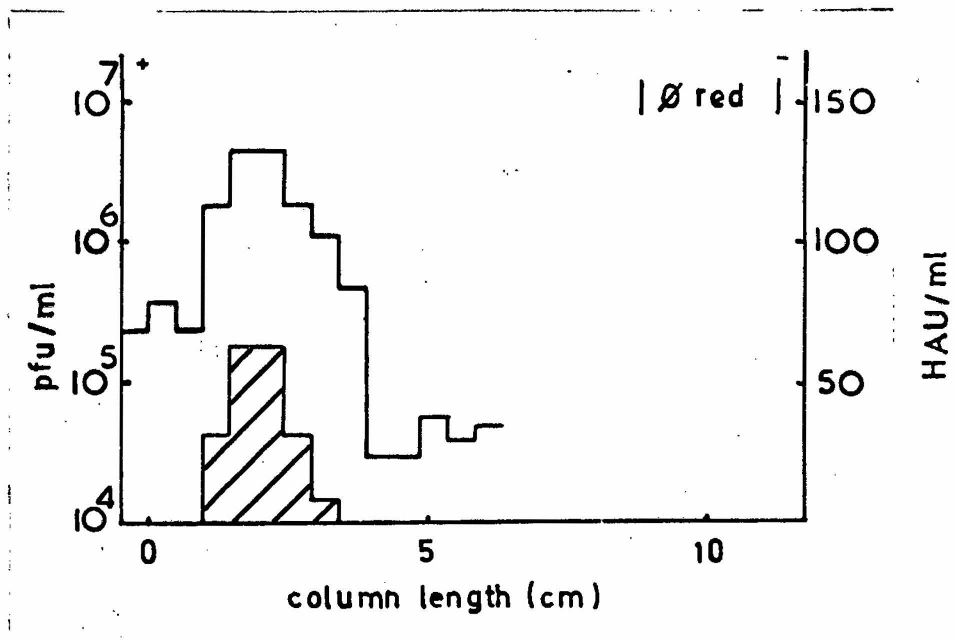


Table 10. Relationship between the titres of infectivity and haemagglutinin of 4 different preparations of ecbovirus SA 1.

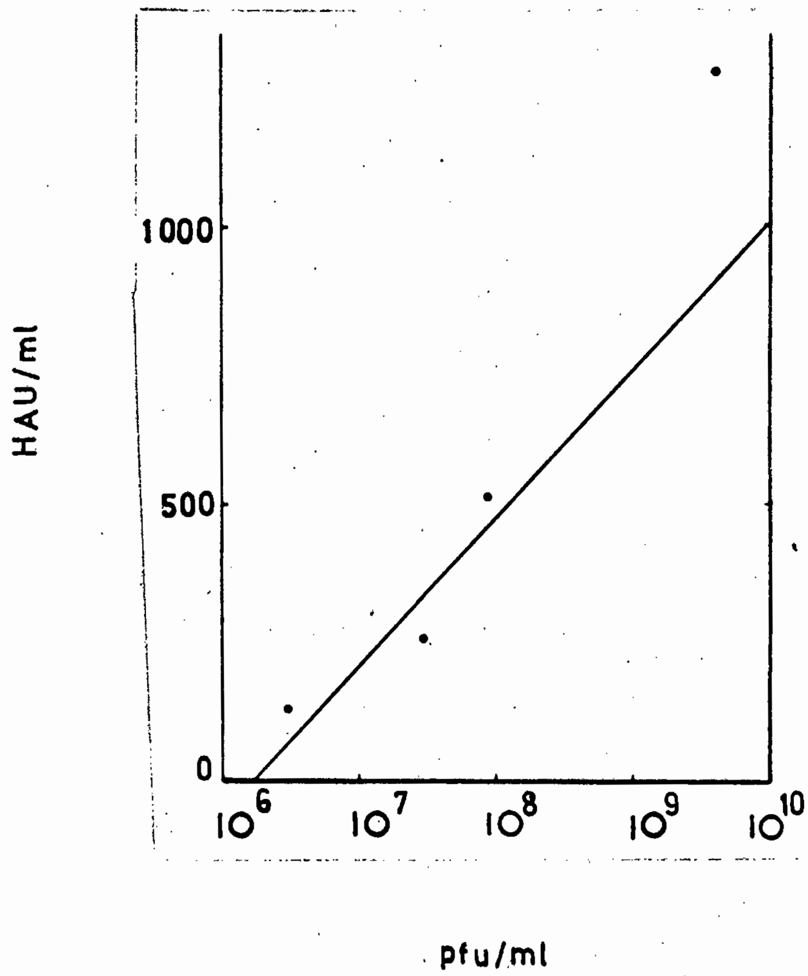
Preparation	Infectivity pfu/ml	Haemagglutinin HAU/ml
1	9×10^7	512
2	3×10^7	256
3	3×10^6	128
4	4×10^9	1280

The approximate linear relationship resulting from this plot showed that there was a constant relationship between the infectious particles and the haemagglutinin, one haemagglutinating unit (HAU) being approximately equal to 10^6 pfu.

4.4. DISCUSSION.

As the lipid solvent, ethyl ether, did not affect the infectivity of ecbovirus SA 1 this was suggestive evidence that this virus contained no essential lipid in its structure, the absence of lipid being a prerequisite for the inclusion of a virus in the enterovirus group (Cabasso, 1965). The fact that the haemagglutinin was not sensitive to the action of ethyl ether indicated that it did not exist as free lipid material, or a lipid-containing complex, which was released from infected cells together with the virus. Furthermore, as the haemagglutinin was not affected by trypsin treatment, this was evidence that it did not exist as free protein, apart from virus nucleoprotein which may have been released on lysis of infected cells. These observations on the chemical nature of the haemagglutinin suggested that it was thus inseparable from the infectious

Figure 15. The relationship between the number of infectious particles and haemagglutinating units in preparations of echovirus SA 1.



particle of this virus by these chemical means.

The observation that heating to 56° for 30 min destroyed the infectivity but not the haemagglutinin has two possible explanations. The haemagglutinin may not be associated with the infectious particle and may not be sensitive to this treatment as is the latter. Alternatively, the haemagglutinin may in fact be the viral capsid which remained intact during this treatment while the nucleic acid core of the virus was inactivated, thus destroying infectivity. Ribonucleic acid inactivation is associated with a low heat of entropy, which led Woese (1960) to assume that heat inactivation of animal viruses below 60°, especially those having an RNA core, occurred primarily through damage to the nucleic acid. This being so, the alternative explanation is more plausible, and the capsid of ecbovirus SA 1 is implicated in the HA reaction. Analogous results were obtained by Inaba et al. (1962b) and Yamada (1965b) with other bovine enteroviruses.

While isopycnic density gradient centrifugation studies in CsCl revealed the presence of 2 haemagglutinating components, one of which had the same buoyant density as the infectious particle, this did not necessarily mean that there existed, apart from the viral capsid, a further haemagglutinating component in virus preparations derived from cultures of calf kidney cells. Halperen, Eggers & Tamm (1964), investigating echovirus 12, observed that two haemagglutinating peaks emerged from density gradient centrifugation in CsCl, having buoyant densities of 1.33 and 1.29 g/cc. By correlating these observations with electron microscopic studies, they were able to demonstrate that the particles of buoyant density 1.29 g/cc represented non-infectious empty capsids lacking nucleic acid but still

possessing the characteristic structure of the complete virus. Frommhagen (1965), in density gradient studies on Coxsackievirus B5, was similarly able to demonstrate the presence of less dense particles which had high titres of complement fixing antigen but relatively little infectivity, and contained little or no RNA. These "coreless" particles do indeed exist in preparations of ecbovirus SA 1 as may be seen in electron micrographs of the virus presented in the following chapter (5.2.3.). Furthermore, Oellermann _et al_. (1968), while unable to detect a haemagglutinin for ecbovirus SA 1, were able to detect two ultra-violet-absorbing peaks, the more dense of which consisted of infectious particles, the less dense, presumably thus being these "coreless" particles.

The most decisive evidence for the identity of the infectious particle and the haemagglutinin was presented in the results obtained for the density gradient zone electrophoresis experiments. Parker (1966), Mussgay & Rott (1964) and Stevens & Schlesinger (1965) respectively demonstrated by buoyant density determinations that Wesselsbron virus, Sindbis virus and Dengue type 2 virus preparations respectively contained 2 haemagglutinating components, one of which always had the same buoyant density as the infectious component. Furthermore, Parker (1966) observed that 2 haemagglutinating components could be identified for Wesselsbron virus in zone electrophoresis experiments, only one of these being associated with the infectious particle. In the results reported here a single haemagglutinating component, having the same electrophoretic mobility as the infectious particle, was observed in experiments conducted with buffers at two pH values. As the electrophoretic mobility of such a particle in sus-

pension depends solely on the surface charge of the particle, this could only mean that the haemagglutinin of ecbovirus SA 1 is, in fact, the viral capsid.

The demonstration that a linear relationship existed between infectious particles and haemagglutinating units was further evidence for the validity of this hypothesis. The highest point on the curve (Fig. 12) appears to be exceedingly errant. On the other hand it must not be forgotten that haemagglutination titrations, which rely on doubling dilutions of virus, are particularly susceptible to error. An observed end-point one dilution below the expected end-point will result in 50% drop in titre, while an end-point one dilution above the expected end-point will result in a 100% increase in titre. Also, as the recovery of infectivity on subjecting infectious culture fluid to pressure dialysis was never greater than a theoretical 87%, it is evident that this method of concentration resulted in the loss of some of the infectivity. This loss could have been due to the adsorption of virus to the cellophane dialysis tubing or it may have been due to the destruction of the infectivity of the nucleic acid moiety of these particles, resulting in the observed "excess" of "coreless" haemagglutinating units in the highest point of Fig. 12.

5. SOME PHYSICO-CHEMICAL PROPERTIES OF ECBOVIRUS SA1.

5.1. INTRODUCTION.

The early taxonomic systems used for the classification of animal viruses were based on biological characteristics such as the host range, type of tissue affected, the host-response, and the type of lesion produced (Melnick & McCombs, 1966). This approach to virus classification has virtually become redundant. With the advent of more sophisticated tissue and cell culture methods and the application of modern biochemical, biophysical and immunological techniques to virology, a new approach to viral taxonomy and nomenclature has evolved.

Major considerations now involved in virus classification include the type of nucleic acid; size, shape and structure of the particle; the presence or absence of essential lipids; and the stability of the infectious particle under the influence of various applied external conditions.

The ability of these techniques to resolve information as to, for instance, the size and shape of virus particles has supplied information which is not only of academic interest for viral taxonomy, but which has wide application in the field of animal (as well as plant, insect and bacteriophage) virology. While the property of stabilisation to heating by cations, particularly the divalent cations Ca^{++} and Mg^{++}, of enteroviruses provided another criterion for inclusion of a virus in the human enteroviral group (Wallis & Melnick, 1962), Melnick (1962) pointed out that this property of enteroviruses was of considerable importance in the preparation of attenuated

poliovirus vaccines. Heating vaccine virus suspensions to 50° for 1 hr in the presence of molar $MgCl_2$ resulted in no loss of infectivity of a vaccine strain of polio-virus but contaminating simian viruses such as the vacuo-lating SV-40 virus could not withstand such treatment and were destroyed.

Knowledge of the electrophoretic mobility of viruses has resulted in the separation of such viruses from con-taminating proteins with the result that highly specific immune sera to the viruses have been prepared for immuno-logical idenficiation purposes.

Knowledge of the physical properties of the individual components of animal viruses, for instance the sedimentation coefficients of viral nucleic acids, has revealed much of the existing knowledge concerning entry of viruses into host cells, replication of the individual virus components within these cells, and the release of infectious particles from the infected cells.

Comparative physical studies on viruses which have been attenuated and strains before attenuation have revealed that physical changes occur in the composition of these viruses during the course of attenuation. Mayr, Strohmaier & Lorenz (1961) suggested that the protein coat of Teschen virus became modified during the course of attenuation and Russell (1968) and Parker, Wouters & Smith (1969) suggested that similar changes in the surface properties of African horsesickness virus and Wesselsbron virus respectively occurred on attenuation of these viruses.

The infectious particle of ecbovirus SA 1 was examined using some of these chemical and physical techniques, the results being reported here. Emerging from these studies

was a "new" physical constant based on the surface properties of the virus, namely, the isoelectric point.

5.2. SIZE OF THE VIRUS.

5.2.1. Size as determined by ultrafiltration.

5.2.1.1. Introduction.

The technique by which the size of virus particles may be determined by ultrafiltration is one of the earliest techniques which was used to determine this property. Viruses, in fact, were first recognised as "filterable agents" which were able to pass through Chamberlan filters and Berkefeld candles which retained bacteria, fungi and their spores. Sophistication was lent to this technique by the development by Elford (1931) and Bauer & Hughes (1935) of collodion membrane filters containing pores of uniform size which could be readily calibrated. By determining the number of infectious virus particles which passed through a graded series of these membranes under standard conditions, it became possible to estimate closely the size of the particles by extrapolation.

Elford's term "gradacol membranes" means in effect a graded coagulation of collodion (cellulose nitrate) on a horizontal surface whereby membranes of graded pore size are formed. This resulted from his discovery that amyl alcohol and acetone were mutually antagonistic in their ability to dissolve cellulose nitrate. Individually together with ethyl alcohol and ether, either of these two solvents could dissolve cellulose nitrate, but if both were present in certain ratios the cellulose nitrate coagulated. A mixture of all 4 of these solvents and collodion, in a ratio which dissolved the collodion com-

pletely, if placed in an open tray so that the more volatile solvents evaporated more readily than the less volatile solvents resulting in a change in the ratio of amyl alcohol to acetone, caused the coagulation of cellulose nitrate in the form of an "ultragel" structure. Elford further discovered that the pore size of the membranes could be controlled by adding to the solution varying amounts of acetic acnd and water. Addition of water resulted in membranes of larger pore size; addition of acetic acid resulted in membranes of smaller pore diameter.

In this study, ecbovirus SA 1 was filtered through membranes of known average pore diameter (APD) and the size of the particle was estimated by determining the theoretical diameter of an aperture through which it would just pass,

5.2.1.2. Materials and methods.

Analytical grade organic solvents, excepting primary amyl alcohol, were further dried to remove any contaminating water. Ether was dried over metallic sodium, acetone over anhydrous potassium carbonate and absolute alcohol over calcium oxide. To 125 g absolute alcohol was added 75 g Parlodion (Mallinckrodt Chemical Works, U.S.A.) in a 2.5 litre dark glass bottle fitted with a plastic screw cap. The Parlodion was left to swell at 22° for 24 hrs. and 375 g ether was added. The mixture was shaken periodically until all the Parlodion had dissolved and 575 g acetone was added. The mixture was shaken in a mechanical shaker for 2 hrs., 287 ml primary amyl alcohol was added, and the stock collodion solution was shaken for a further 2 hr before it was stored in a dark cupboard for 2 to 3 weeks.

The stock collodion solution was diluted with an equal volume of a mixture consisting of 1 part ethanol and 9 parts ether (by weight). This final stock mixture was divided into 200 ml aliquots for the preparation of the collodion membranes. Acetic acid was added in varying amounts (0.5 ml to 2.5 ml) to these aliquots before the organic solvents were allowed to evaporate to give membranes of APD 450 mμ to 24 mμ.

The organic solvents were allowed to evaporate from such mixtures from a glass cell in a room which was thermostatically controlled at 20° and whose relative humidity was kept at 70%. The glass cell consisted of two pieces of optically true glass 7 mm thick and 50 cm square. A circular section 40 cm in diameter was cut out of one square piece and the remainder was cemented on top of the uncut square with egg white, thus forming a well. The dell was raised above a levelling table on 4 rubber stoppers, ensuring an even distribution of temperature about the cell. A paper cylinder, 45 cm in diameter and 20 cm high, supported on 3 rubber stoppers, was placed around the perimeter of the cell to ensure even evaporation of solvents from the cell.

The solvents were allowed to evaporate for 90 mins after which membranes were floated free from the glass by carefully adding distilled water to the cell from the edge. The membranes were washed with a 5% (v/v) ethanol solution in distilled water twice daily for 7 days, and for a further 7 days in distailled water with daily changes. From areas of uniform opacity of washed membranes were cut, by means of a steel punch discs of sizes suitable for use in the membrane filter holders. These membrane discs were stabilized to shrinking by steaming in suitable glass containers, and

were stored in a 0.01% (w/v) solution of merthiolate in distilled water.

The average pore diameters of the membranes were calculated from the rate of flow of water through the membranes, their water content and their thickness. The pore size of the membranes was calculated from the equation

$$r = 2\ell\sqrt{\frac{2V\eta}{pw}}$$

where r = radius of pore (cm)

ℓ = thickness of membrane (assumed equal to the length of the pores; in cm)

V = rate of flow (ml/sec)

η = specific viscosity (CGS units)

w = water content of 1 sq cm membrane

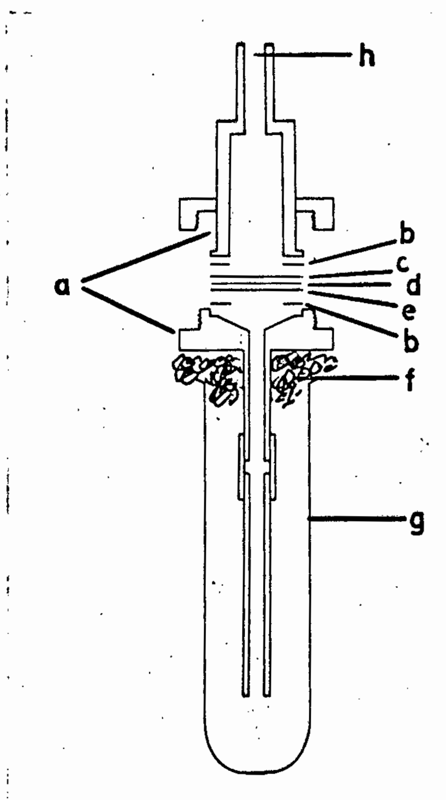
p = pressure producing flow (dynes/cm^2).

This equation, based on Poiseuille's law, was used by Elford (1931). The thickness of each membrane was measured with a micrometer screw gauge, the membranes being held for this purpose between two microscope coverslips. The rate of flow of water through the membranes was measured using the apparatus of Elford (1931). The water content of the membranes was determined by weighing membranes before and after drying at 90° for 24 hrs.

Virus-infected culture fluid was prepared for ultrafiltration (UF) by centrifugation to clarify the fluid which was then passed through a gradacol membrane of APD 334 mμ in order to remove particular matter of low density which may not have been removed by centrifugation.

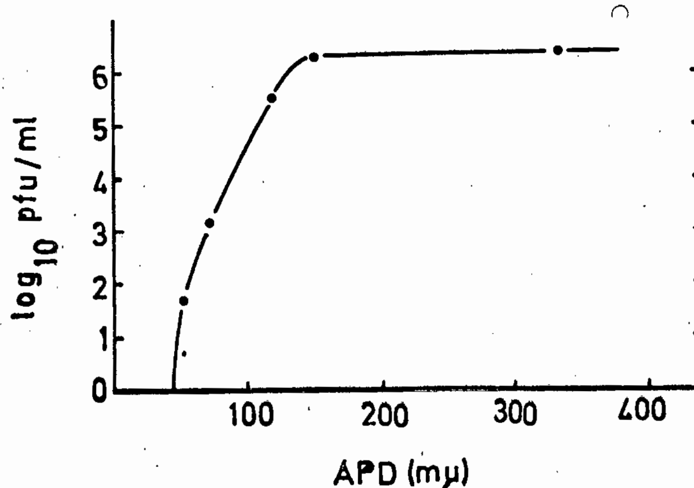
Membrane filter holders, made of stainless steel (Fig. 16), were prepared in the following manner. The filter holders were sterilised by autoclaving at 10 lb for 15 min in the assembled form but without the rubber

Figure 16. Diagram of a filter holder for supporting gradacol membranes.



a = stainless steel filter holder; b = rubber gaskets; c = gradacol membrane; d = supporting filter paper disc; e = stainless steel disc with perforations, to support the filter paper; f = cotton wool; g = boiling tube, for collecting the filtrate; h = supply of filtered air at a pressure of 10 lb per sq in.

Figure 17. Results of an ultrafiltration experiment on ecbovirus SA 1. Plot of average pore diameter (APD) against infectivity. By extrapolation, the limiting APD for ecbovirus SA 1 is seen to be 44 mμ.



gaskets, filter papers or membranes. The gaskets, filter papers and membranes were added aseptically to the autoclaved holders after they had been boiled in distilled water. The assembled filters, each containing a membrane of different APD, were placed in a stand supplied by a manifold which in turn was attached to a source of filtered air at a pressure of 10 lb per square inch. Five ml aliquots of Panmede (Paines & Byrne, Ltd., England) broth (5% w/v in phosphate buffer pH 7.5) were passed through each filter to neutralize the charges on the surfaces of the membranes, the filtrate being discarded. Five ml aliquots of the virus suspension were placed in the reservoir of each filter holder and forced through the membranes by applying a pressure of 10 lb per square inch. The filtrates were collected and titrated for infectivity.

5.2.1.3. Results.

The results obtained on filtering a suspension of ecbovirus SA 1 through membranes having APDs of 150 to 24 mμ are presented in Table 11. From a plot of these results (Fig. 17) a value of 44mμ was obtained as the limiting APD by extrapolation. Bauer & Hughes (1935) found it necessary to apply correction factors of 0.5 to 0.66 to proteins whose sizes were estimated by this method. A correction factor based on recent data of particle sizes by electron microscopic studies, i.e. 0.68 (Parker, 1966) was used in these studies. By applying this value a particle diameter of 29.9 mμ was calculated for ecbovirus SA 1.

Table 11. Ultrafiltration of a suspension of ecbovirus SA 1 through a series of gradacol membranes.

Membrane APD mμ	Titre of filtrate pfu/ml
334	2.5×10^7
150	2.0×10^7
119	3.5×10^6
73	1.5×10^4
52	5.0×10^2
40	0
24	0

5.2.2. Determination of particle size from sedimentation and buoyant density data.

5.2.2.1. Introduction.

The sedimentation coefficient is a fundamental property of colloid particles and is the sedimentation velocity per unit centrifugal field. The theory of sedimentation in the analytical ultracentrifuge is the subject matter of several textbooks and will thus receive the barest attention in this introduction. The fundamental equation

$$\frac{dx}{dt} = Sr\omega^2$$

where dx is the distance of sedimentation in time dt, r the distance, ω the angular velocity and S a constant for a specific particle, is integrated and the equation

$$S = \frac{2.303 \Delta \log X}{t\omega^2} \quad \ldots\ldots\ldots\ldots 1$$

is obtained. In this equation

S = sedimentation coefficient (cm/sec)

t = time (sec)

Δlog X = the logarithm of the distance sedimented by the

particle in time t

ω = angular velocity (radians/sec) = rev/sec x 2π

For details of the derivation of this equation, see Elias (1961).

To standardize results, sedimentation coefficients are generally related to sedimentation in water at a temperature of 20°, the designation for this coefficient thus being $S_{20,w}$.

Once known, the sedimentation coefficient may be used in a number of ways. Primarily it may be used to identify and distinguish different colloids. Estimations as to the size, shape, molecular weight and degree of hydration of particles may be made on combining the $S_{20,w}$ in suitable equations. For instance, by combining this equation with Stoke's law, the diameter of spherical particles may be estimated from the following equation (Pollard, 1953):

$$S = \frac{2r^2(1 - Vo\rho)}{9Vo\eta} \quad \text{............ 2}$$

or

$$r = \sqrt{\frac{9S\eta_{20}}{2\Delta\rho}} \quad \text{............ 3}$$

where r = radius of the particle
η_{20} = absolute viscosity of water at 20° = 0.01005 poise
$\Delta\rho$ = difference in densities of the particle and water.

Thus, if the buoyant density and sedimentation coefficient of a spherical virus are known, the radius, and hence diameter, may be estimated. This relationship was applied in order to estimate the diameter of ecbovirus SA 1.

5.2.2.2. Materials and methods.

Isopycnic density gradient centrifugation studies performed in order to determine the buoyant density of ecbovirus SA 1 have been described in the previous chapter (4.3.2.).

Sedimentation studies on the virus were performed in a Spinco model E analytical ultracentrifuge (Beckman Scientific Instruments, U.S.A). The virus suspension was concentrated by pressure dialysis of culture fluid followed by differential centrifugation. The pellet resulting from centrifugation at 30,000 rpm for 90mins was reconstituted in a sucrose/borate buffer solution and purified further by zone electrophoresis. Virus-containing aliquots from the ZE column were dialysed overnight against borate buffer pH 8.6 at 4° to remove sugar. Virus in the dialysate was concentrated by centrifugation at 30,000 rpm for 90 min, the pellet being resuspended in 0.5 ml borate buffer diluted 1/10 with normal saline. This suspension was examined in an Ephantis synthetic boundary cell in the analytical ultracentrifuge at a rotor velocity of 15,220 rpm at 20°, photographs of the sedimenting boundary being taken every 8 min using the Schlieren optical system.

5.2.2.3. Results.

Results reported in the previous chapter (4.3.2.2.) for the buoyant density of ecbovirus SA 1 namely 1.335 g/cc were used in the calculations of particle size.

A single sedimenting boundary was observed in the Schlieren diagrams obtained on analytical centrifugation (Fig. 18), indicating the presence in the sample of mono-

Figure 18. Pattern of sedimentation of ecbovirus SA 1 in a synthetic boundary cell in the Spinco model E analytical ultracentrifuge. Photographs were taken at 8 min intervals. Rotor velocity was 15 220 rpm.

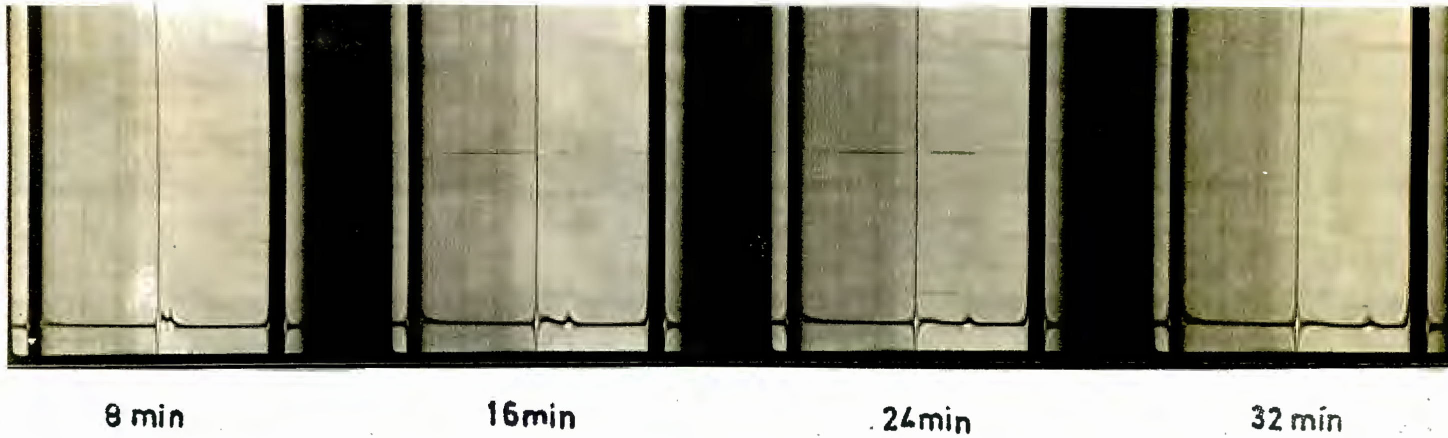
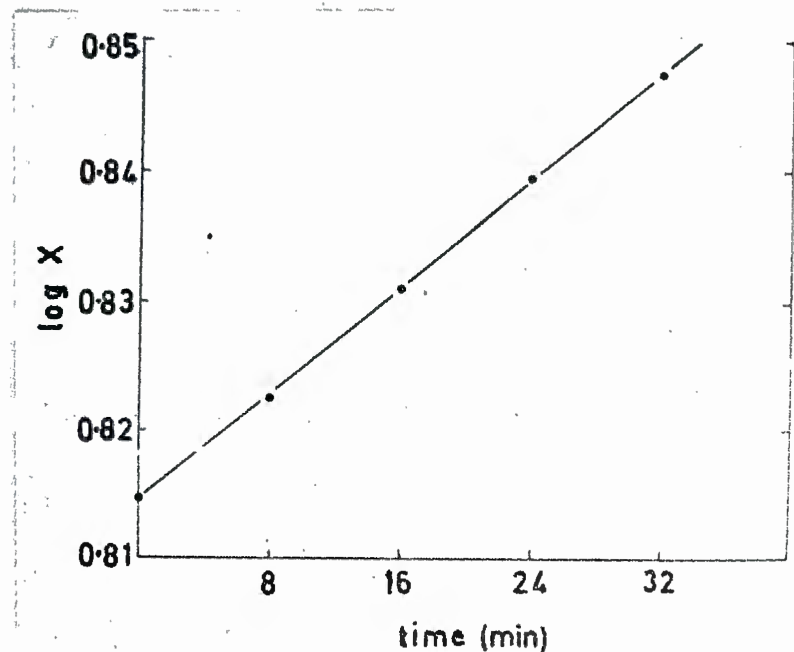


Figure 19. Ultracentrifugation of ecbovirus SA 1. Plot of the logarithm of the distance of the sedimenting boundary from the centre of rotation (X) against time in minutes.



disperse material only. The distance of the Schlieren peak from thecentre of rotation, X, was plotted using a microcomparator for 4 consecutive photographs. When log X was plotted against time (Fig. 19), the resulting straight line showed that sedimentation had been uniform. Time of centrifugation was 1920 sec, log X was 0.0329, and rev/sec were 253.7. By substituting these experimental values in equation 1, and correcting for the viscosity of the solvent at 20°, the $S_{20,w}$ of ecbovirus SA 1 was found to be 157.6.

By substituting the values for buoyant density (1.335 g/cc) and sedimentation coefficient (157.6) in equation 3, the radius of ecbovirus SA 1 was estimated to be 14.5 mμ, the diameter of the particle thus being 29 mμ.

5.2.3. Size determined by electron microscopy.

5.2.3.1. Introduction.

The electron microscope has become a very important tool for the virologist. In recent years, more sophisticated techniques have made possible observations of the intricate structure of most viruses examined, as well as the sequence of, and sites of virus replication in host cells. One of the techniques developed was that of negative staining (Brenner & Horne, 1959). At neutral pH, salts of phosphotungstate and uranyl acetate do not combine readily with proteins or nucleic acids, but on drying form an electron-opaque background in which objects stand out in detail. This technique was used to examine the size and morphology of ecbovirus SA 1.

5.2.3.2. Materials and methods.

The virus preparation used in this particular investigation was the one used in sedimentation studies

reported in the previous section (5.2.2.2.), which had revealed a single sedimenting boundary using the Schlieren optical system.

The stain used was a 2% (w/v) solution of phosphotungstic acid in phosphate buffer pH 7.2. A drop of virus suspension was mixed with a drop of stain on a glass slide. A droplet of this mixture was transferred to a carbon-coated microscope grid and the liquid was allowed to evaporate. The grid was examined in a Siemens Elmiskop model 1A electron microscope.

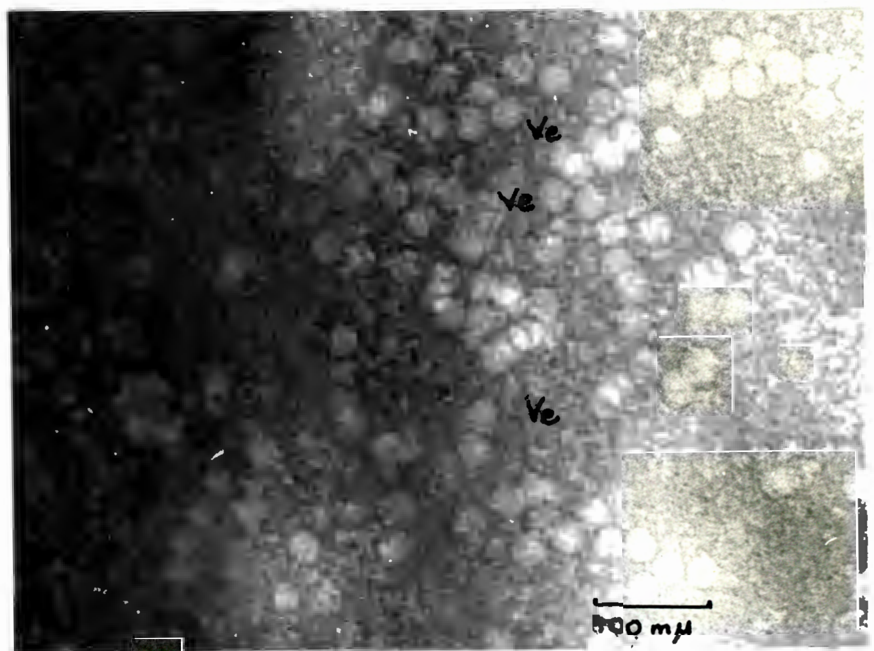
5.2.3.3. Results.

Electron photomicrographs of ecbovirus SA 1 (Fig. 20) revealed the presence of particles of uniform shape and diameter, the diameter being approximately 27 mμ. Closer examination of these particles revealed that they appeared to have an hexagonal shape which is consistent for viruses exhibiting icosahedral symmetry (Almeida & Ham, 1965). Several "empty" particles were visible in the fields seen by the fact that the stain penetrated these particles. These empty particles were presumably present in too small a proportion to have been recognised as a separately sedimenting peak in centrifugation diagrams.

5.2.4. DISCUSSION.

The three estimations of the particle size of ecbovirus SA 1 (namely 29.9, 29 and 27 mμ) using three different methods were in remarkable agreement. All three methods are subject to considerable experimental error and the estimations of size made by ultrafiltration

Figure 20. Electron photomicrograph of ecbovirus SA 1. Ve are "empty" virus particles. (X 180,000).



and sedimentation-buoyant density data cannot be considered to be absolute values as these two methods only apply to spherical particles. Furthermore, as particles studied by electron microscopy are examined in a high vacuum, they may lose many of their structural features and water of hydration (Pollard, 1953).

As the results were in such close agreement, however, it may be safely assumed that the true diameter of the virus particles was in the region of 27 to 30 mμ, well within the range (20 to 40 mμ) suggested by McFerran (1962) as being one of the criteria for inclusion of a virus in the bovine enterovirus group. This particle diameter is similarly of the same order as that of the human enteroviruses which have a diameter of approximately 28 mμ (Melnick, 1962).

5.3. STABILITY OF THE VIRUS.

5.3.1. pH Stability.

The ability of ecbovirus SA 1 to withstand high hydrogen ion concentrations was examined by the method described by Ketler, Hamparian & Hilleman (1962). Virus-containing culture fluid was diluted 1/10 with Eagle's medium and the pH of thissolution was adjusted to pH 2.8 using molar solutions of tris and HCl. This preparation was allowed to stand for 3 hr at 4° along with control samples which contained no tris-HCl but an equal amount of Eagle's medium. Titration results of this experiment, conducted in duplicate, showed that exposure to pH 2.8 for 3 hr had no effect on the infectivity of the virus (Table 12).

Table 12. Stability of ecbovirus SA 1 at low pH for 3 hr.

pH of suspending medium.	Titre pfu/ml
7.2	2×10^6
	6×10^6
2.8	3×10^6
	4×10^6

5.3.2. Virus stabilisation to heat by cations.

The stabilisation of infectivity to heat treatment by cations was examined by the method of Wallis & Melnick (1962). Virus-containing culture fluid was diluted tenfold with Hanks' LA solution. Stock solutions of 2M $CaCl_2$, 2M $MgCl_2$, and 4M NaCl were prepared in distilled water. To aliquots of the virus suspension were added equal amounts of these stock salt solutions, and phosphate buffered saline pH 7.2 was added to a fourth, control sample. Duplicates for each treatment were incubated at 50° and at 4° for 60 min, following which the infectivity of the samples was titrated.

From the results in Table 13 it is evident that, while stabilisation in each instance was never complete, the cations nevertheless had a considerable protecting effect. Thermostability was best preserved by divalent cations, the protection increasing in the order Na^{+}, Ca^{++} and Mg^{++}.

Table 13. Effect ofcations on the thermostability of ecbovirus SA 1 at 50° for 60 min.

Diluent	Temperature °	Titre pfu/ml
$CaCl_2$ (M/1)	50	3×10^3
	4	3×10^4
$MgCl_2$ (M/1)	50	7×10^3
	4	3×10^4
NaCl (2M)	50	1×10^3
	4	2×10^4
PBS	50	7×10^2
	4	4×10^4

5.3.3. DISCUSSION.

The virus was found to be resistant to low pH, an observation consistent with observations made on other enteroviruses. This resistance of picorna-viruses to low pH is a criterion for their inclusion in the enterovirus, as opposed to rhinovirus,group (Melnick & McCombs, 1966).

It has been demonstrated in section 4.3.1. that while the infectivity of ecbovirus SA 1 was heat labile on heating at 56° for 30 min the haemagglutinin was relatively stable to such treatment. The results here indicate that this thermolability is prevented to some degree by molar solutions of divalent cations or 2M solutions of NaCl. Few reports exist in the literature of similar cationic stabilisation of bovine enteroviruses to heating. Spradbrow (1964) and Yamada (1965a) reported that their bovine enteroviral isolates were protedted at 50° for 30 min by molar $MgCl_2$ solutions.

It is thus possible that this property of the human enteroviruses also applies to bovine enteroviruses.

5.4. THE NUCLEIC ACID.

5.4.1. Introduction.

The primary criterion for animal virus classification occurs at the nucleic acid level and depends on whether a virus contains DNA or RNA, irrespective of whether the nucleic acid core of the virus is double- or single-stranded.

Infectious nucleic acid was first extracted from animal viruses by Colter, Bird & Brown (1957) and Colter, Bird, Moyer & Brown (1957) who respectively extracted infectious RNA from cells infected with Mengo virus, and polio- and West Nile viruses. To show that the infectivity was not due to residual virus it was demonstrated that the RNA was sensitive to ribonuclease, was precipitated by alcohol or molar NaCl at 4°, was not neutralised by specific antiviral gamma-globulin and showed characteristic ultraviolet spectra with absorption maxima at 260 $m\mu$.

The type of nucleic acidcontained by a virus may be determined in other ways. Salzman (1960) demonstrated that 5-fluorodeoxyuridine (FUDR) could be used to distinguish the nucleic acid type of a virus in a simple technique. He found that concentrations as low as 10^{-6}M FUDR prevented the formation of viruses containing DNA while not affecting the formation in infected cells of viruses containing RNA.

The nucleic acid type of ecbovirus SA 1 was determined by extracting the acid from intact virus particles and incubating it together with ribonuclease. The RNA content of the virion was estimated.

5.4.2. Materials and Methods.

The method employed for extraction of infectious nucleic acid was that of Gierer & Schramm (1956) as modified by Naudé (1965). Cold phenol was used to dissociate the nucleoprotein capsid of the virus and bentonite was added to the virus suspension in order to inactivate ribonucleases (Fraenkel-Conrat, Singer & Tsugita, 1961).

A slurry of bentonite was prepared in 100 mM acetate buffer pH 6.05. Freshly-distilled ether and phenol were saturated with 20 mM phosphate buffer in $5x10^{-4}$M versene, pH 7.2 and chilled to 4°. Echovirus SA 1 was concentrated and partially purified by differential ultracentrifugation. The resulting viral pellet was suspended in 32 ml phosphate buffer in $5x10^{-4}$M versene in a glass-stoppered bottle in an ice bath. To this was added 320 mg bentonite and 32 ml phosphate-saturated phenol. The mixture was vigorously shaken for 5 min., and centrifuged at 10,000 rpm for 30 min. at 4° in a SW 25 rotor in a Spinco preparative ultracentrifuge. The aqueous layer was removed and transferred to a second glass-stoppered bottle standing in ice. An equal volume of phosphate buffer-saturated phenol was added, the mixture was again shaken vigorously for 5 min. and centrifuged at 10 000 rpm for 2 min. This latter treatment was repeated twice and the supernatant was transferred to a further glass-stoppered bottle in an ice bath and shaken 5 times with an equal volume of phosphate buffer-saturated ether. Residual ether was removed under negative pressure. The final 'RNA' preparation was snap-frozen and stored at -70°.

The infectivity of such preparations was assayed by the plaque method using monolayers of calf kidney cells prepared according to the method of Porterfield (1959). Dilutions of 'RNA' were made in trypsin base solution in

ice. The plates containing the monolayers were washed with trypsin base solution, 0.1 ml of 'RNA' dilution was overlayed and allowed to absorb at 20° for 20 min. The plates were washed again with trypsin base solution before the overlay was added, and they were incubated at 37°. Ribonuclease (Seravac Laboratories, Cape Town) and rooster immune serum controls were prepared by pre-incubating 0.5 ml aliquots of 'RNA' with 0.5 ml amounts of 1% ribonuclease or rooster immune serum at 22° for 60 min. before titration.

The 'RNA' content of the virus particles was estimated by the method of Reichmann (1965) which is based on the sedimentation coefficients of "empty" and "full" particles.

$$X = \%\ RNA = ({}^{S}F/S_{E} - 1)\ ({}^{S}F/S_{E} + 1) \times 100 \ \ldots\ldots\ 4.$$

where ${}^{S}F = {}^{S}20,w$ of full particles

${}^{S}E = {}^{S}20,w$ of empty particles.

As the sedimentation coefficient of the empty particles was not known, this value was estimated from the known data on the empty particles; namely, the buoyant density and diameter of these particles, by applying equation 3 (5.2.2.1.).

5.4.3. Results.

Results of duplicate titrations in plates of calf kidney cell monolayers, (Table 14) revealed the presence of an infectious component which was completely inactivated by ribonuclease, but only slightly by specific immune serum to the virus.

Table 14. Titration of an 'RNA' preparation, prepared by phenol extract of ecbovirus SA 1, in plates of calf kidney cell monolayers.

Sample	Titre pfu/ml
'RNA' preparation	9×10^2
	6×10^2
'RNA' preparation plus ribonuclease	0
	0
'RNA' preparation plus immune serum	6×10^1
	4×10^1

By applying equation 3 (5.2.2.1.), substituting the values of 1.300 g/cc for buoyant density and 29 mμ diameter for the empty virus particles, the $S_{20,w}$ of these particles was estimated to be 139.3. Using this value and the value of 157.6 obtained as the sedimentation coefficient of full particles, the percent 'RNA' of ecbovirus SA 1 was found to be 28% (w/w) on application of equation 4 above.

5.44 Discussion.

As ribonuclease inhibited the plaque-forming ability of the 'RNA' preparation, this suggested that the nucleic acid core of ecbovirus SA 1 did in fact consist of ribonucleic acid. While pre-incubation of this preparation with immune serum to the virus resulted in a 90% decrease in infectivity, this did not necessarily mean that the preparation contained intact virus particles. The sera of animals is known to contain nucleases and it is quite possible that a trace of ribonuclease remained in this rooster serum after heat 'inactivation'.

The results obtained by Reichmann (1965) for estimations of the 'RNA' content of viruses were in good agreement with those obtained by chemical analysis. Only plant viruses were used in his experiments. He emphasised that the following 4 criteria had to be satisfied before his equation could be applied to a virus. The virus (a) must have only protein and 'RNA'; (b) must have no residual 'RNA' in the empty particles; (c) must have had identical original composition of full and empty particles, and (c) the full and empty particles must have the same shape and degree of hydration. The preparations of ecbovirus SA 1 examined in this series of experiments appeared to have fulfilled all these conditions.

5.5. THE ISOELECTRIC POINT

5.5.1. Introduction

The isoelectric point of a protein, the pH at which it does not migrate in an electric field, is a fundamental physical property of the protein. It ranks in importance with other physical properties such as molecular weight, sedimentation coefficient, partial specific volume, diffusion constant, and other properties. Apart from having no mobility in an electric field, proteins at their isoelectric point (IEP) tend to form aggregates leading to a high degree of turbidity and a maximum ease of precipitation by alcohol or other lyophilic reagents. Lloyd (1926) summarised the properties of proteins in the isoelectric state as follows :

(1) the lowering of the surface tension of water is at a maximum; (2) the viscosity is at a minimum; (3) turbity is at a maximum; (4) precipitability by alcohol or acetone is at a maximum; (5) the osmotic pressure is at a minimum; (6) the conductivity is at a minimum.

Proteins are ampholytes. At pH values above their IEPs they have a net negative charge, while below their IEPs they carry a net positive charge. Thus, with pure proteins, a reliable method of determining the IEP is by ascertaining the pH at which the protein does not alter the pH of an unbuffered solution, nor combines with either H^+ or OH^- ions (Lloyd, 1926).

All the methods of determining the IEPs of proteins are based on the 6 attributes of proteins at their IEP as stated above by Lloyd. The two methods most commonly used are based on electrophoretic mobility and turbidimetric determinations.

The IEP, as a physical property of viruses, has been virtually ignored. Electrophoretic studies on viruses have on the whole been directed towards purification of the viruses concerned, or the determination of the electrophoretic mobility at a single buffer pH (usually pH 8.6) and ionic strength (usually 0.1). However, various electrophoretic techniques for the determination of virus IEPs are available. The simplest method is that of microelectrophoresis using the Northrop-Kunitz type of apparatus whereby the migration of inert particles coated with virus may be measured optically using a travelling microscope (Abramson, Moyer & Gorin, 1942). Using this method, Sharp, Taylor, Beard & Beard (1942) estimated the IEP of rabbit papilloma virus protein to be at approximately pH 5.0, and Oster (1951) determined that the IEPs of strains of tobacco mosaic virus (TMV) were in a range of pH 3.91 and pH 4.90. The moving boundary method of Tiselius similarly relies on optical observations. The motion of macromolecules in an electric field is followed by optical methods which

register the position and shape of the boundary between the solvent and the solution. Using the Tiselius electrophoresis apparatus, Miller & Price (1946) determined the IEP of southern bean mosaic virus to be at pH 5.50, MacDonald, Price & Lauffer (1949) found the IEP of the same virus to be at pH 5.9, and Semancik (1966) found that the IEP of bean pod mottle mosaic virus was at pH 5.4.

These two methods, however, have severe limitations. In the first instance, a pure, concentrated virus sample is a prerequisite for optical measurements of a moving boundary in the Tiselius apparatus, conditions which are not easily fulfilled when this technique is applied to animal viruses. Attempts have been made at sampling the Tiselius apparatus by hand in order to collect serial samples for the titration of biological activity, thus overcoming the limitations of optical measurements by allowing the use of relatively impure viral preparations (Bourdillon & Lennette, 1940; Randrup, 1953). However, in sampling the narrow Tiselius cell by hand it is doubtful whether interruption of layers sampled is avoidable. In the instance of the microelectrophoresis method, Abramson et al (1942) have demonstrated that the IEPs of pure proteins were elevated as much as 0.5 pH units over values obtained by other means.

The problem of sampling a free electrophoresis column is largely overcome by using the technique of density gradient zone electrophoresis (ZE). This method has the added advantage that relatively impure and dilute virus suspensions may be examined. Employing this technique, Aach (1963) was able to determine the IEPs of coliphage X 174 mutants, using an apparatus modified from that of Cramer & Svensson (1961). In a sucrose density gradient, a uniform migration velocity over the whole path of migration can be guaranteed because of the

constancy of the product: conductivity x viscosity (Matheka & Geiss, 1965). This observation that the mobility of viruses is not altered by the sucrose gradient for a given voltage gradient was confirmed empirically by Ball (1966).

No matter the technique used to determine the IEP, this particular physical property of many viruses may never be determined, since these viruses are acid-labile, and do not exist as intact particles at pH values at or near their IEPs. For this reason, Bourdillon (1940) was not able to determine the IEP of influenza A virus as the virus was destroyed below pH 5.6.

As echovirus SA 1 was found to be stable down to a pH value of at least pH 2.8, it was decided to investigate the mobility of this virus in an electric field by the technique of density gradient zoneelectrophoresis in order to determine the IEP of the virus. The apparatus of Polson & Deeks (1962) was employed and the IEP of a protein of known IEP, namely human serum albumin, was first determined in order to test the validity of the method.

5.5.2. Materials and Methods.

Succinic acid/NaOH buffer systems in 70 mM NaCl, having a total ionic strength of 0.1 (Kekwick, 1940), were used throughout these experiments. By varying the relative amounts of succinic acid and NaOH in these buffers, experiments could be performed in a range of pH from below pH 4.0 to pH 6.0 (Polson & Largier, 1960).

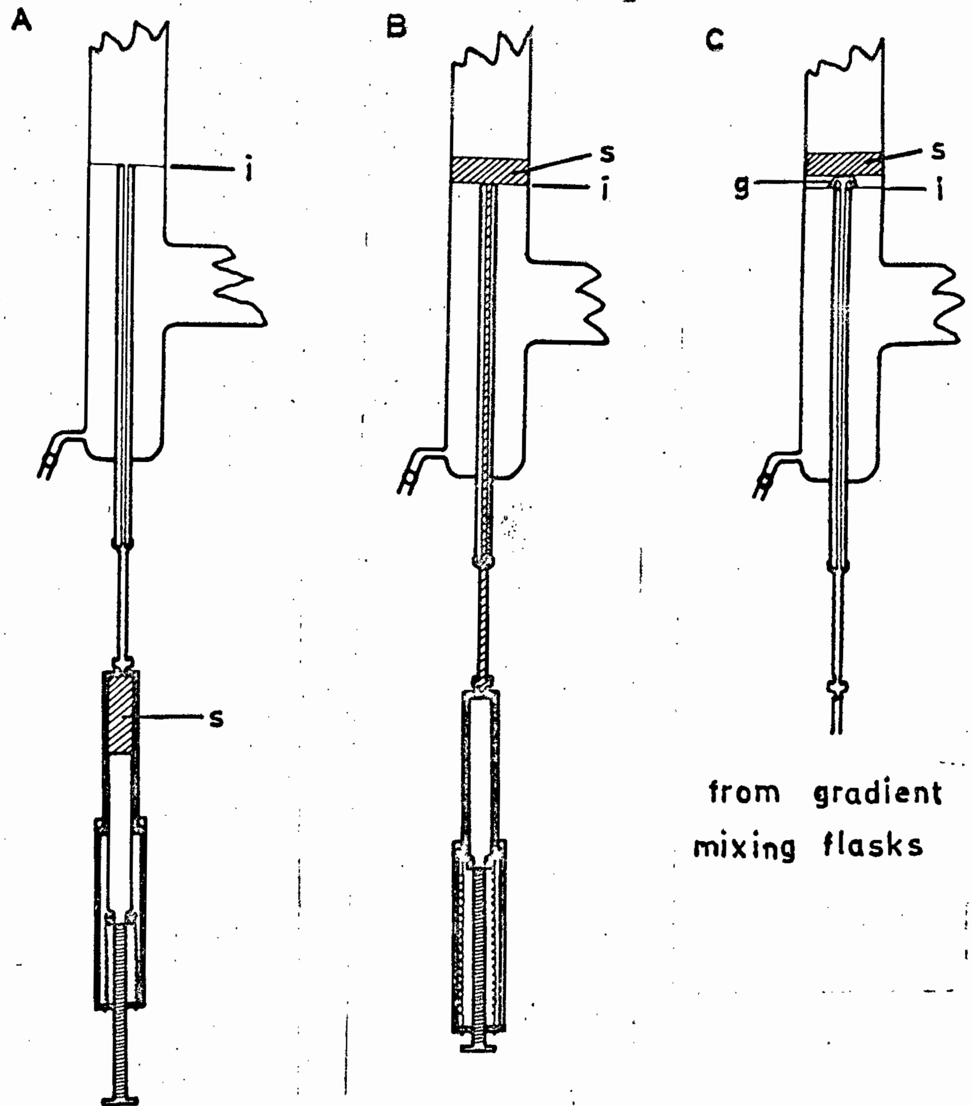
The human serum albumin (HSA) used was a 99% pure preparation (Lister Institute, Elstree, Herts., U.K.)

Echovirus SA 1 preparations were derived from infected culture fluid which was concentrated and partially purified by differential ultracentrifugation.

The apparatus described by Polson & Deeks (1962) was used. As it was not known in which direction the HSA/virus would migrate at a particular buffer pH value, the mode of operation of the apparatus was modified so that the sample was introduced into the electrophoresis column in approximately the middle of the density gradient. This was accomplished by stopping the inflow of the sucrose density gradient into the electrophoresis column when approximately half of the gradient had entered the column. The following approximate 4 ml of the sucrose gradient flowing from the mixing flasks was collected in a sterile Bijou bottle.

The sample which was to be subjected to electrophoresis was suspended in this solution. In the case of electrophoresis of HSA, the protein was added to a concentration of 0.1% (w/v). When virus was submitted to zone electrophoresis, the pellet resulting from differential ultracentrifugation was resuspended in the recovered portion of the gradient which suspension was then clarified by centrifugation at 10,000 rpm for 10 min. To the HSA or virus preparation a few grains of phenol red were added to act as an internal reference substance by means of which R_{ϕ} values could be calculated (van Regenmortel, 1968). By use of a 5 ml syringe held in the syringe holder with screw advance (Fig. 5), 2.5 ml of this preparation was introduced into the ZE column through the capillary tube. The remainder of the HSA or virus preparation was placed on the bench next to the ZE apparatus for control titrations. The sucrose gradient-forming device was reconnected to the capillary tube and the gradient was

Figure 21. Introduction of sample to be submitted to gradient zone electrophoresis in the centre of the sucrose density gradient.



i = interface between 40% sucrose solution (below) and sucrose gradient already run into ZE column

s = sample introduced

g = "mushroom effect" due to the remainder of the sucrose density gradient entering the ZE column, pushing the phenol red-pigmented virus-containing zone before it. The "mushroom effect" was due to the refraction of light by the more dense sucrose solution entering the column under the influence of gravity, then sinking to the base of the gradient.

The remainder of the zone electrophoresis apparatus, not shown in these figures, is identical with the apparatus of Polson & Russell (1968), and may be seen in Fig. 11.

very carefully allowed to continue to pass into the ZE column, pushing the phenol red stained preparation ahead of it (see Figs. 21a to 21c). When the gradient had run to completion, the preparation was thus visible as a yellow-stained band 1 cm column length in the centre of the ZE column. The apparatus stood at room temperature for at least 2 hr. to allow for diffusion of sucrose to form a continuous density gradient through the introduced preparations. Silver/silver chloride electrodes in saturated NaCl solution were introduced into the electrode vessels and a current of 20 mA was passed through the apparatus at a potential of 2-3 v/cm for 15 to 17 hr. To assist in the dissipation of heat generated by the current, a current of air from an electric fan was directed on to the apparatus for the duration of the experiment.

On completion of the electrophoresis, the position of the phenol red band was noted and 1/2 or 1 cm column length samples were collected through the capillary tube. The HSA content of such samples were measured by their ability to absorb light of 280 mμ wavelength in a Unicam SP 500 spectrophotometer. Virus content of such samples was titrated in agar-chick embryo cell suspensions. R_{ϕ} values for the migration of the HSA/virus were calculated for results of experiments conducted at different buffer pH values. From this data, pH/mobility (in terms of R_{ϕ} values) curves were constructed for HSA and echovirus SA 1.

5.5.3. Results.

Electrophoresis of human serum albumin was performed in buffers of 6 different hydrogen ion concentrations, ranging from pH 4.30 to pH 5.15. The results of these experiments are presented in Table 15. The pH/mobility curve constructed from these results (Fig. 22) intersected the pH-axis at pH 4.72.

When ecbovirus SA 1 was submitted to zone electrophoresis in buffers of 5 different hydrogen iron concentrations, ranging from pH 3.90 to pH 5.00, the results in Table 16 were obtained. The pH-mobility curve resulting from these observations dissected the pH-axis at pH 4.40 (Fig. 23).

Table 15. Isoelectric point determination of human serum albumin by zone electrophoresis in succinate buffers, ionic strength 0.1.

Buffer pH	Distance migrated by protein to anode (cm)	Distance migrated by phenol red to anode (cm)	R_{ϕ} Value
4.30	-2.23	+10.0	-0.223
4.63	-0.5	+10.0	-0.05
4.68	-0.1	+10.0	-0.01
4.80	+0.8	+10.0	+0.08
4.85	+1.5	+10.0	+0.15
5.15	+1.73	+10.0	+0.173

Figure 22. Determination of the isoelectric point of human serum albumin by zone electrophoresis in succinate buffers, ionic strength 0.1, of varying hydrogen ion concentration.

pH - mobility curve.

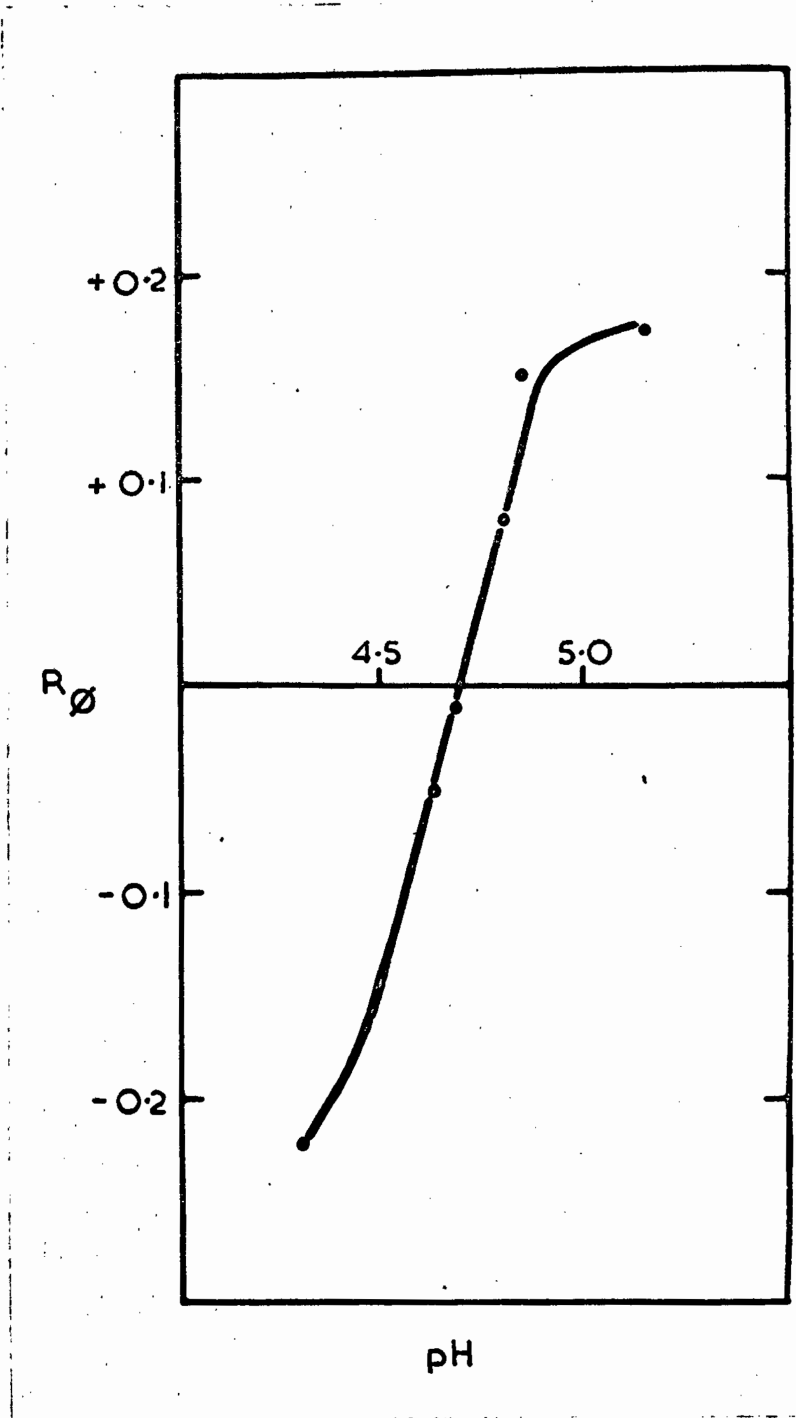


Figure 23. Determination of the isoelectric point of ecbovirus SA 1 by zone electrophoresis in succinate buffers, ionic strength 0.1, of varying hydrogen ion concentration.

pH - mobility curve.

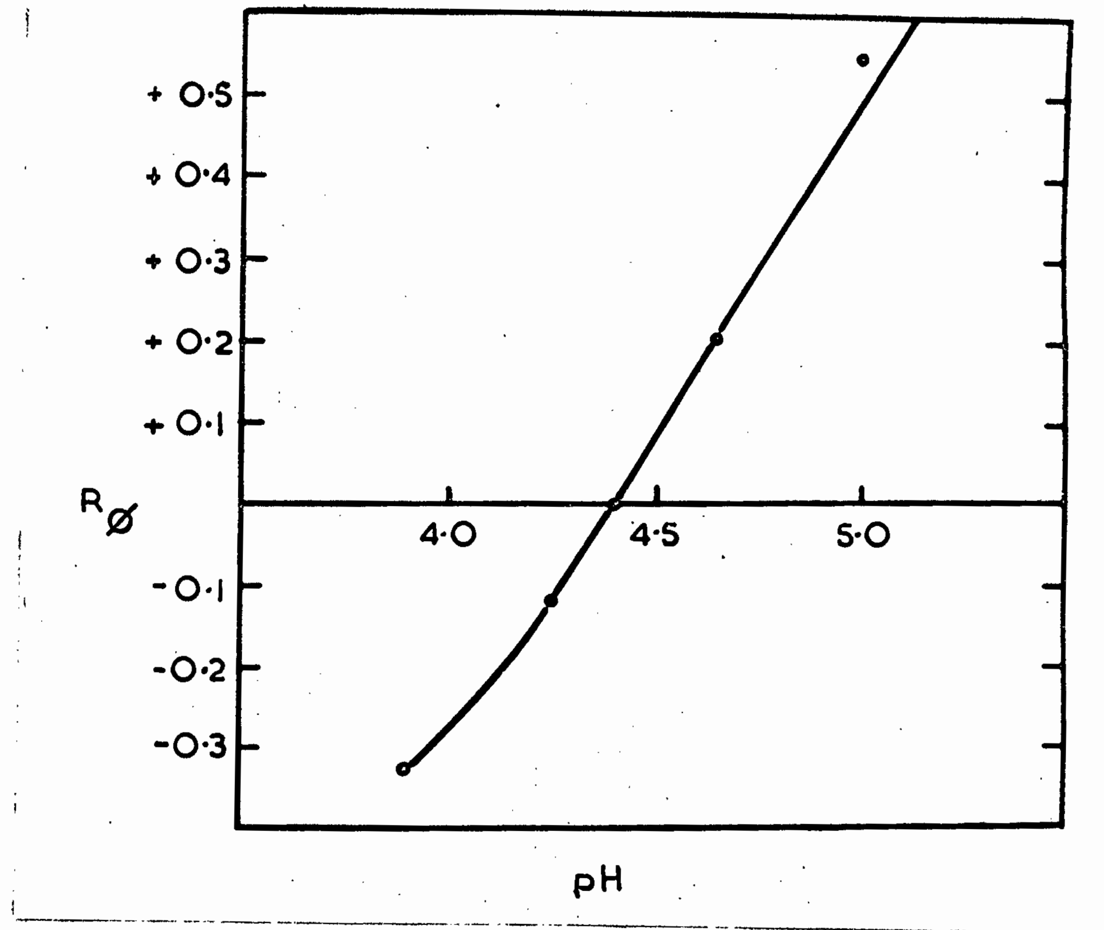


Table 16. Isoelectric point determination of ecbovirus SA 1 by zone electrophoresis in succinate buffers, ionic strength 0.1.

Buffer pH	Distance migrated by virus to anode (cm)	Distance migrated by phenol red to anode (cm)	R_ϕ Value
3.90	-3.5	+11.375	-0.308
4.25	-1.25	+11.625	-0.108
4.40	0.0	+11.0	0.0
4.66	+2.0	+10.75	+0.186
5.00	+5.5	+11.0	+0.50

5.5.4 Confirmation of the isoelectric point of ecbovirus SA 1 by ion exchange chromatography

5.5.4.1. Introduction.

Ion exchange chromatography was first introduced during the early stages of the United States atomic energy programme, in order to separate nuclear fission products such as La, Sm, Eu, Y, Ce, Pr, Nd and Pm (CIBA Review, 1966). Most of the early ion exchangers were developed to separate small molecular weight substances, and consisted of columns packed with particles of synthetic cation exchange resin. The separation of amino acids on these columns by Moore & Stein (1951) led to the development of the automatic amino acid analyser. With the development of ion exchangers using derivatives of cellulose (Peterson & Sober, 1956) it became possible to separate larger proteins and viruses, as a more favourable, hydrophilic environment was created by these polysaccharide structures for the more labile proteins and viruses. Using carboxymethyl cellulose columns, Lampson & Tytell (1965) were able to determine the isoelectric points of haemoglobin preparations in phosphate buffers, as they found that the elution pH of a protein was a function of

the isoelectric point.

Agar, a polysaccharide preparation from seaweeds, consists of two compoments: (i) agarose, a neutral linear galactose polymer consisting of alternate residues of β-D-galactopyranose and 3, 6-anhydro-α- L-galactopyranose; (ii) agaropectin, which consists of the same polysaccharides, but with the addition of pyruvic acid, glucuronic acid and sulphate groups. (Araki, 1959). The presence of these sulphate groups confers a charge on the agaropectin molecule, thus making agar preparations unsuitable for use in molecular sieve chromatography, as many substances are strongly adsorbed to agaropectin (Hjertén, 1962). Ordinary agar preparations should, thus, be capable of acting as cation exchangers. Ragetli & Weintraub (1966) have synthesised agar derivatives such as trimethylaminoacethydrazide-agar, pyridinium acethydrazide-agar and diethylaminoethyl-agar for use in gel electrophoresis in which a reduced endosomotic flow was required, but these derivatives were not used in attempts at ion exchange chromatography.

Experiments were conducted to determine the cation-exchanging potential of an agar preparation, at the same time employing the basic principles of Lampson & Tytell (1965) in an attempt to confirm the isoelectric point of ecbovirus SA 1.

5.5.4.2. Materials and methods.

The agar preparation used in these experiments was Ionagar No.1 (Oxoid, England). The method of Hjertén (1964) was employed to produce uniform-sized spheres of agar for chromatography. The method, which relies on the principle of a "water in oil" emulsion, was used to prepare 12% (w/v) Ionagar spheres.

A 12% (w/v) agar suspension was boiled in a 1 litre round-bottomed flask until the agar had dissolved. The organic liquid phase (430 ml toluene, 170 ml carbon tetrachloride, and 25 g Emulphor-L1 as stabilizer), was pre-warmed in a water bath to approximately 50° and added to the agar solution. The suspension was immediately stirred with a high-speed stirrer (approximately 1 500 rpm) for 1 min. then cooled rapidly, while still stirring, by placing the flask in a water bath supplied with a continuous stream of cold water. After about 5 min. stirring the agar spheres had gelled and the agitation was stopped. The suspension was filtered on a Buchner funnel to remove most of the organic phase and washed several times with methanol until all the toluene and carbon tetracholoride had been washed free from the spheres. The spheres were washed thoroughly on the funnel with distilled water. The spheres used were able to pass through a sieve of size 100-mesh, but were retained by a sieve of size 200-mesh.

A glass column (1 cm x 15 cm) was packed with these spheres and washed with distilled water for approximately 16 hr. Before use, the column was equilibrated by passing through it the appropriate buffer solution for at least 24 hr. The buffer system used was that of McIlvaine, consisting of solutions of 5 mM citric acid and 10 mM Na_2HPO_4. By varying the relative amounts of these two solutions, experiments were conducted from pH 4.40 to pH 6.9. As the gel strength of agarose, and hence agar, is labile at acid pH (Hickson & Polson, 1968), it was considered inadvisable to conduct experiments below pH 4.40.

The void volume of the column was determined before each experiment by passing through the column a suspension of formalinised <u>Escherischia coli</u> in the appropriate

buffer, recording the emergence of the suspension from the column by means of its light absorption in a Uvicord recorder (LKB Produkter, Sweden).

Infected culture fluid was clarified by centrifugation at 10 000 rpm for 10 min. and 0.4 ml of the supernatant fluid was layered on top of the column. This was eluted with the appropriate buffer for the particular experiment, a volume equivalent to the void volume of the column being discarded before 0.2 ml aliquots were collected for infectivity titrations. Satisfactory information was provided in titrating alternate samples.

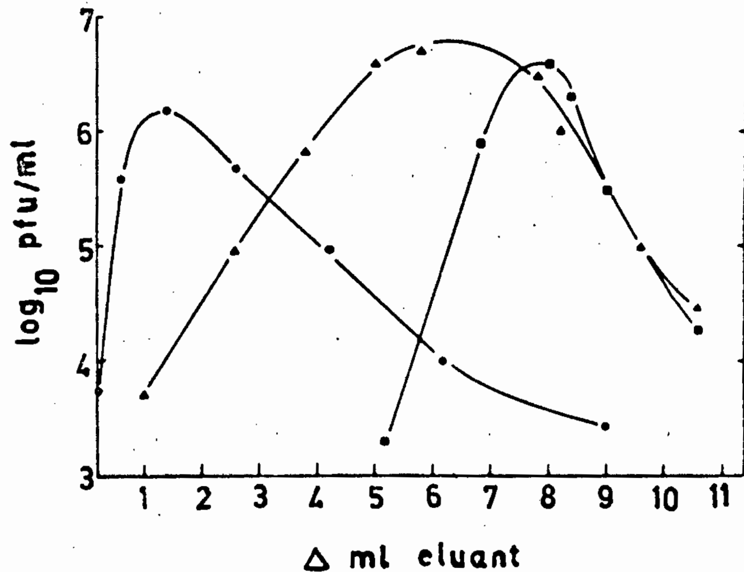
5.5.4.3. Results.

The results of a number of experiments are presented in Fig. 24. The infectivity of the aliquots was plotted against the difference between the eluant volume and the void volume of the column, i.e. eluant volumes emerging after the front. It is seen that in buffers of pH values greater than pH 4.5 the infectious virus emerged soon after the front. While only one experimental result for buffers of pH above pH 4.5 is presented in Fig. 24, eight further experiments, performed in duplicate, with eluting buffers of pH higher than pH 4.5 showed that the distribution of the recovered virus in the effluant was virtually identical with that shown in Fig. 24. At pH 4.5 the virus emerged from the column in a broad zone, while at pH 4.4 it was considerably retarded.

5.5.4.4. Discussion.

Due to the presence of the sulphated polysaccharide agaropectin in ordinary preparations of agar, these preparations should have cation exchanging properties. Above their isoelectric points proteins are anionic (i.e. they bear a net negative charge) and the effect of a cation exchanger would be negligible at pH values above

Figure 24. Elution diagrams of ecbovirus SA 1 from a column of Ionagar No. 1 spheres (12%) using citrate buffers as eluants. The void volume of the column was 2.8 ml ± 0.02 ml.



● — ● buffers of pH 4.6 to pH 6.1

▲ — ▲ buffer of pH 4.5 0

■ — ■ buffer of pH 4.40

Δ ml eluant = volume of eluant (ml) - void volume of column (ml).

the isoelectric point of a protein. At and below the isoelectric point of a protein the net surface charge changes from negative to positive. Thus it is to be expected that ecbovirus SA 1 would be retained by a cation exchanger at and below its isoelectric point. The results of these experiments show that this phenomenon was observed with ecbovirus SA 1 on a column of Ionagar spheres. The transition state for the virus appeared to occur at pH 4.5 in McIlvaine's buffer. Lampson & Tytell (1965) found that, by using ion exchange chromatography to determine the pH at which haemoglobins were eluted from columns, isoelectric points so obtained were consistently higher (0.4 to 0.6 pH units) than IEPs determined electrophoretically. In the present experiments, however, the observations were the opposite of those of Lampson & Tytell (1965), in that the isoelectric point of ecbovirus SA 1 is 0.1 pH unit higher by this method than the electrophoretically determined value. While Lampson & Tytell (1965) determined the pH at which proteins were eluted from the cation exchanger carboxymethyl cellulose, in the present experiments the pH at which virus was adsorbed was determined, which accounts for the discrepancy.

Exclusion chromatography could not have influenced the results reported here, as the concentration of Ionagar (12%) was too high for the infectious particles to have been able to penetrate the gel matrix.

It thus appears that the phenomenon of ion exchange did occur on spheres of Ionagar No. 1, although the agaropectin moiety of the agar carried insufficient cationic groups to retain completely the virus particles at pH values below their isoelectric point.

5.5.5. CONFIRMATION OF THE ISOELECTRIC POINT OF ECBOVIRUS SA 1 BY ELECTROFOCUSSING.

5.5.5.1. Introduction.

While the technique of "isoelectric focussing", i.e. the electrophoresis of proteins in a pH gradient, has only recently come into general use, the principles involved in this procedure have been recognised for some time. Electrophoretic separation based not on ionic mobilities, but on differences in the IEP, thus being limited to ampholytes, was first attempted by Williams & Waterman (1929) who used a multi-compartmented cell, electrolyte pH values increasing from compartment to compartment from the anode to the cathode.

Kolin (1954) revived interest in this type of electrophoresis. He employed two buffer systems, a more dense acid buffer with the density being conferred by the addition of sucrose, and a less dense basic buffer containing no sucrose. By introducing the material to be examined at the interface of these two buffer systems he was able to obtain steep pH and density gradients in this region. He found, on passing an electric current through this interfacial region, that a concentration of ampholyte occurred at its IEP when the current flowed in the direction of increasing pH, whereas a diminution in the ampholyte concentration at a point corresponding to the isoelectric pH was produced by a current flowing in the reverse direction. The same principle was applied to paper electrophoresis by Hoch & Barr (1955) who formed a pH gradient on the paper strip by wetting the ends of the strip with two phosphate buffers of

differing hydrogenion concentrations. While this method proved useful for the analysis of very dilute solutions, it was unable to resolve proteins which had very similar isoelectric points, e.g. haemoglobins A and F.

These pH gradients were called "artificial" pH gradients by Svensson (1961). Svensson pioneered electrophoresis in what he termed "natural" pH gradients in which mixtures of peptides were used as carrier ampholytes in a sucrose density gradient. In an electric field these ampholytes were repelled by both electrodes (the cathode being basic, the anode acidic) and each ampholyte species collected at the place in the gradient where the pH was equal to the IEP of that species. A stable pH gradient was thus formed in which proteins, on being introduced into the system, collected at the point in the pH gradient which was their IEP. As this collection of proteins at one place in the gradient was caused by the electric field, the term "isoelectric focussing" was adopted for this technique. As the range of peptides readily available for forming such a natural gradient was limited and a prerequisite of such an ampholyte was that it be a good conducter at its IEP and have a good buffering capacity at this pH, the technique was limited in its application, for limited pH ranges, until the development of synthetic ampholytes (Vesterberg & Svensson, 1966) which were a mixture of low-molecular aliphatic polyamino-poly-carboxylic acids. These are now available commercially (LKB-Produkter, Sweden) and the technique is gaining rapid popularity for the resolution of protein mixtures.

As a general rule, the IEPs of proteins are higher the lower the ionic strength. Isoelectric points obtained by isoelectric focussing in an ampholyte medium always refer to almost zero ionic strength. Thus proteins that undergo irreversible denaturation on removal of their natural salt medium can never be examined by this technique, e.g. serum lipoproteins, and many viruses. While this technique is of undoubted use in identifying proteins it is of little value for determining IEPs of proteins if the data so gained is to be applied in further experimentation on those proteins in the presence of ions. This is especially the case for the work reported in the next chapter of this thesis where knowledge of the IEP of ecbovirus SA 1 was utilised in purification and concentration procedures.

Ecbovirus SA 1 was submitted to density gradient zone electrophoresis in an "artificial" pH gradient in an attempt to verify the IEP of the virus obtained by other techniques. Experiments were first conducted to determine the stability of such an artificial pH gradient

5.5.5.2. Materials and methods.

The density gradient zone electrophoresis apparatus of Polson & Deeks (1962) was again used in these experiments (see Section 5.5.2.)

The buffers used in these experiments were of acetic acid and sodium acetate in distilled water, having a final ionic strength of 0.1. For each experiment, two buffers were prepared from stock acetic acid and sodium acetate solutions, a more acid one which also contained 40% (w/v) sucrose and a less acid one containing no sucrose. Gradients were formed using the mixing flasks described by Polson & Russell (1968) (see Section 4.3.3.1.)

the pH of the gradients decreasing as the density of the sucrose gradient increased.

Several experiments were conducted with only these buffer systems present in the apparatus to determine the stability of such artificial pH gradients. Further experiments were conducted, introducing 0.2% (w/v) each of human serum albumin (as used previously) and bovine plasma albumin (Armour, Fraction V) into the centre of the gradient by the technique described in a previous section (5.5.2.). Experiments were also attempted in which samples of ecbovirus SA 1 were introduced into the centre of the gradient or were incorporated in the whole length of the gradient in the ZE column. The latter was accomplished by adding virus which had been partially purified and concentrated by differential ultracentrifugation to the lower mixing flask of the gradient forming device (i.e. the flask which contained the buffer of higher pH and no sucrose).

5.5.5.3. Results.

The results of two experiments performed to establish the stability of artificial pH gradients are presented in Figs. 25 and 26. For the results presented in Fig. 25, the initial buffers were of pH 4.15 (plus 40% sucrose) and pH 5.25. This resulted in an effective pH gradient from pH 4.60 to pH 5.00 after a current of 20 mA had been passed through the ZE apparatus for 16 hr. For the results presented in Fig. 26, the initial buffers were of pH 3.86 (plus 40% sugar) and pH 5.18, resulting in an effective pH gradient of pH 4.40 to pH 4.80.

Zone electrophoresis of a mixture of human serum albumin (HSA) and bovine plasma albumin (BPA), using buffers of pH 4.00 and 5.17 to form an artificial pH gradient, for 41 hr resulted in the distribution of

Figure 25. Stability of an "artificial" pH gradient of buffers consisting of acetic acid and sodium acetate, ionic strength 0.1. "Starting" buffers used to form the gradient had pH values of 4.15 and 5.25. Duration of electrophoresis was 16 hr; the current was 20 mA.

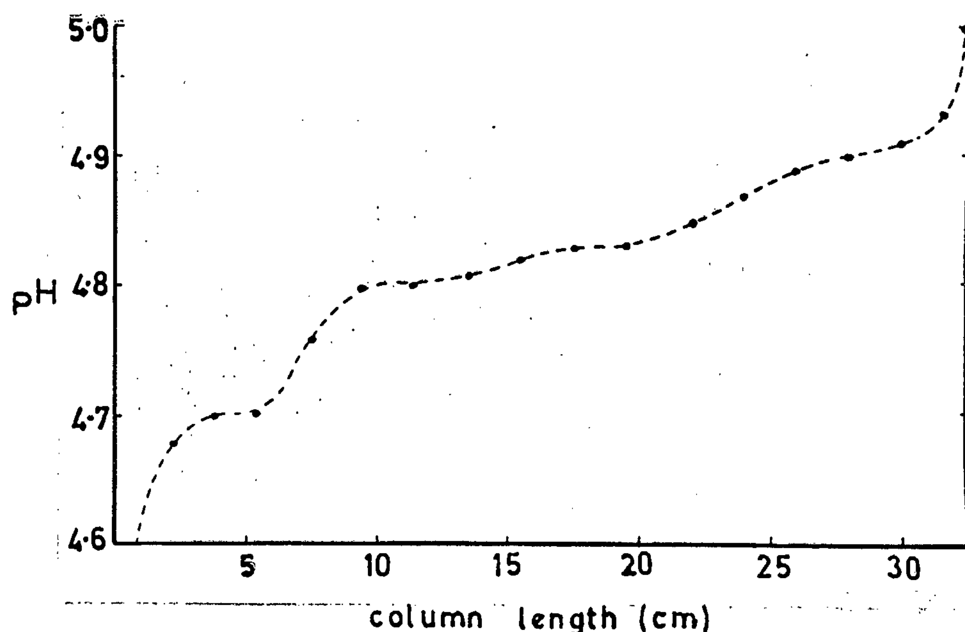
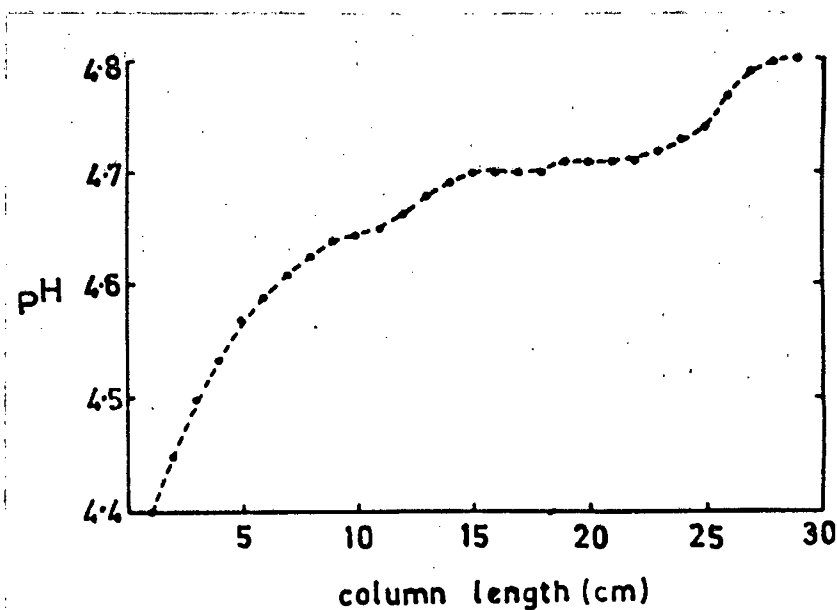


Figure 26. Stability of an "artificial" pH gradient of buffers consisting of acetic acid and sodium acetate, ionic strength 0.1. "Starting" buffers used to form the gradient had pH values of 3.86 and 5.18. Duration of electrophoresis was 16 hr; the current was 20 mA.



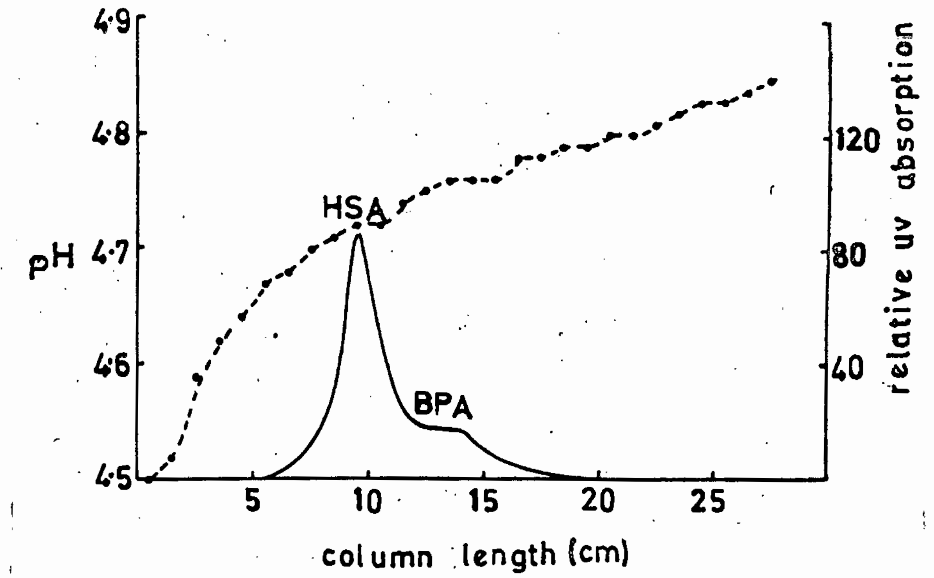
these proteins in the pH gradient plotted in Fig. 27. The protein concentration along the length of the ZE column was determined by collecting the aliquots via a Uvicord recorder. The peak of major ultraviolet light absorption, which consisted of the HSA, was concentrated at pH 4.72, while a smaller absorbing peak, consisting of BPA, existed at pH 4.76. The peaks were identifiable due to the fact that HSA absorbs light of the wavelength of the Uvicord recorder (2537 Å) to a greater extent than does BPA (Potgieter, G.M., personal communication).

When ecbovirus SA 1 was added to the mixing flask of the gradient forming device, the pH values of the buffers used to form the gradient were pH 3.70 and pH 4.90. Electrophoresis in the resulting gradient was continued for 42 hr. and the results presented in Fig. 28 were observed. While infectious virus was detectable over a considerable length of the ZE column (more than 13 cm), the maximum infectivity was concentrated at pH 4.41.

5.5.5.4. Discussion.

It was suggested by Svensson (1961) that artificial pH gradients could be successful if the proper buffers were chosen. Most favourable buffer systems would be those containing the same ion species, e.g. two acetate buffers with pHs including the IEPs of the proteins. The difference between these buffers would be a certain concentration of excess acetic acid, which is very slightly ionised, and thus would be an almost stationary constituent. Such a pH gradient would be stable for this reason if the salt concentration remained constant due to a sufficient buffer reservoir.

Figure 27. Distribution of two proteins, human serum albumin and bovine plasma albumin, after electrophoresis in an "artificial" pH gradient for 41 hr with a current of 20 mA. Acetate buffer, ionic strength 0.1.

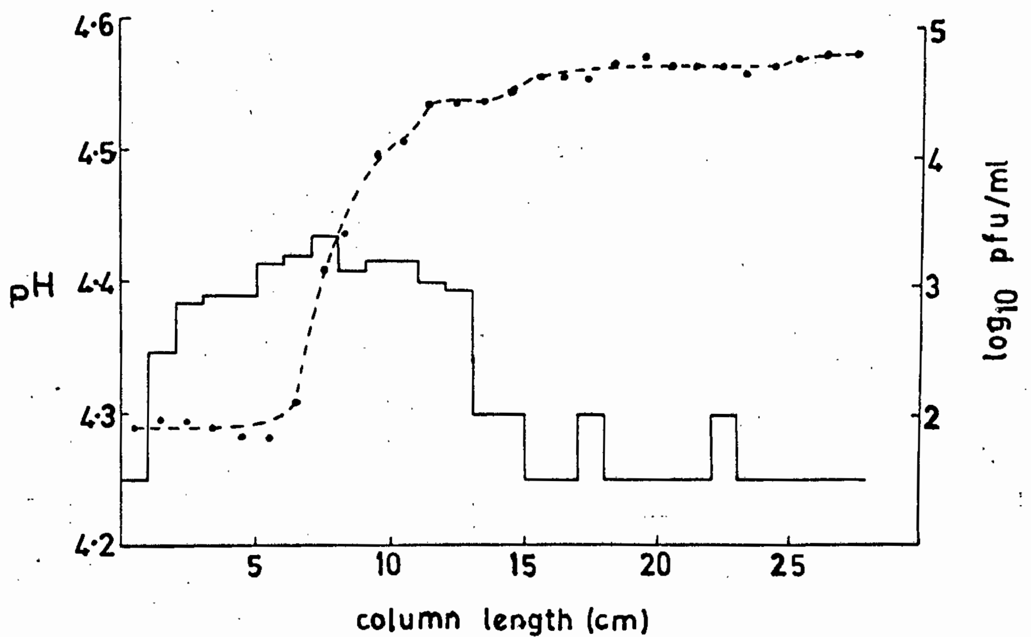


HSA = human serum albumin

BPA = bovine plasma albumin

- - - - = pH gradient.

Figure 28. Distribution of infectious ecbovirus SA 1 after electrophoresis in an "artificial" pH gradient for 42 hr with a current of 20 mA. Acetate buffer, ionic strength 0.1.



This suggestion of Svensson (1961) has been shown by the results of the present experiments to be valid. Human serum albumin was focussed at pH 4.72 which corresponded to the value obtained for its IEP by density gradient ZE at different buffer pH values. While bovine plasma albumin was identifiable with an IEP at pH 4.76, the ultraviolet-absorbing peak of this protein was lower than expected, indicating some denaturation of the protein. Furthermore, the "flattening" of the pH gradient in the region of these protein peaks indicated that the proteins had a buffering action at their isoelectric points. These plateaux in the regions of protein concentration were observed in a number of similar experiments. The observation suggests a new method for the detection of protein peaks in artificial pH gradients, providing the protein concentration is sufficiently high to exert such a buffering effect.

The IEP value obtained by this technique for ecbovirus SA 1 (pH 4.41) was in close agreement with the values obtained by other methods (pH 4.40 and pH 4.5). Unlike the observations made for the two albumins, this virus did not have a buffering capacity at its IEP as was demonstrated by the steepness of the pH gradient in this region. This is presumably because the virus was present in too low a concentration to exert such an effect.

The acetate buffer systems used lend themselves ideally to the IEP determinations of viruses, like ecbovirus SA 1, which have isoelectric points at or near pH 4.4, as these buffers have buffering capabilities over a range of pH from pH 3.8 to pH 5.6 (Polson & Largier, 1960).

5.6. Discussion.

The physico-chemical data derived from the examination of ecbovirus SA 1 in the experiments reported here show that this virus fulfills all the criteria for

inclusion in the enteroviral group of the picornaviruses.

The theoretical value for the ribonucleic acid content of this virus (28%) obtained by the method of Reichman (1965) is in close agreement with the value of approximately 30% obtained experimentally by Oellermann <u>et al</u>. (1967). Furthermore it is in close agreement with the theoretical and empirical values determined by Schwerdt & Schaffer (1955) for poliomelitis virus, which were 24% to 32%. It thus appears that an RNA content of this order (25 to 30%) is yet another constant of the enteroviruses, which should receive further investigation.

The "new" physical property emerging from these studies, the isoelectric point, is a property which should be approached with some caution. The IEP of a virus is of necessity a feature of the surface of the particle which, for some viruses at least, is dependent on pre-existing host cell material (Wecker, 1957; Mussgay, 1964). A difference in electrophoretic mobility for Wesselsbron virus (a group B arbovirus) propagated in different host cells was demonstrated by Parker, Wouters & Smith (1969). Darnell & Levintow (1960) demonstrated that poliovirus protein which was incorporated into viral particles was not synthesised until virus maturation began and that this protein was not pre-existing host cell protein, but was synthesised from the free amino acid pool of the host cell. Furthermore, Polson & Deeks (1962) found that the electrophoretic mobilities of a number of enteroviruses were not altered by propagation of these viruses in different cell systems. That mutants of viruses could arise which have surface properties differing from those of the reference strains is, however, quite possible. Indeed, Breeze & Thorne (1966) found that the large (r) and small (r^+) plaque-forming encephalomyocarditis (EMC)

virus infectious particles could be separated by zone electrophoresis at pH 8.8, indicating a difference in the surface properties of the two types. Goodheart (1965), on performing equilibrium centrifugation in CsCl on suspensions of EMC virus found minor density components for this virus which had greater buoyant densities than the major density component. A fluctuation test indicated that the increased density was due to a mutational change, although he was not able to obtain evidence as to the mechanism of the mutation.

Despite this difference in the physical properties of enteroviruses due to mutational changes, it is most likely that wild type strains of the same virus of the same passage level have identical surface properties, and hence identical isoelectric points regardless of the host cell used for propagation of the virus. The alteration in the surface properties of enteroviruses on attenuation of these particles will receive attention in a following section of this thesis.

6. APPLICATION OF THE KNOWLEDGE OF THE ISOELECTRIC POINT IN VIROLOGY.

6.1. INTRODUCTION.

If the isoelectric point of an enterovirus is a physical constant of that virus, knowledge of the constant may be of use in the further taxonomy of viruses belonging to the enterovirus group. Studies by Polson & Deeks (1962) on the electrophoretic mobilities of human enteroviruses showed that these viruses could be grouped into three main classes on the basis of results obtained from zone electrophoresis experiments at pH 8.6. It is reasonable to expect that similar relationships exist between these viruses with regard to their isoelectric points.

Knowledge of this physical "constant" could be of considerable value in the purification and concentration of these viruses. It could be employed in the so-called "isoelectric precipitation" of viruses from suspensions in which they are held as it is known that proteins have their lowest solubility in the isoelectric state. This often coincides with maximum "turbidity" (Lloyd, 1926). Furthermore, if the isoelectric point of a protein (or virus) is known, the technique of multimembrane electrodecantation may be applied to large volumes of virus suspensions to purify and concentrate the virus concerned (Polson & Largier, 1960).

6.2. CONCENTRATION OF VIRUS BY PRECIPITATION AT THE ISOELECTRIC POINT.

6.2.1 INTRODUCTION.

The precipitation from solution of ampholytes at their isoelectric points is a phenomenon which has long been known, and is analogous to the principle of "salting out" of ampholytes from solution by the addition of salts to the solution which neutralize the surface charges of the ampholytes thus causing them to agglutinate. It has in fact often been impossible to determine the isoelectric points (IEPs) of viruses by electrophoretic means because the viruses precipitate from solution at or near their IEPs (Sharp, Taylor, Beard & Beard, 1942; Putman, 1950). This principle has been used to determine the IEPs of viruses since Oster (1946) determined the pH of suspensions of tomato bushy stunt virus at which the "point of maximum coagulation" of the viruses occurred.

So-called "isoelectric precipitation" has been used on a number of occasions as a step in the concentration of infectious virus particles. A number of plant viruses have been concentrated in this manner (Oster, 1951; Gordon & Price, 1953; Ginoza & Atkinson, 1955; Atabekov et al., 1968). Behrens & Barker (1936) concentrated and partially purified suspensions of rabies virus in this way, whole poliomyelitis virus was precipitated from suspension at pH values ranging between 4.0 and 4.6 by a number of investigators (Bourdillon & Moore, 1942; Racker, 1942; Polson & Selzer, 1954; Bachrach & Schwerdt, 1954). Brandt, Neal, Owens & Jensen (1963) and Stone & Hess (1965) were able to precipitate from solution and concentrate preparations of adenovirus and African swine fever virus, respectively, by employing this principle.

Proteins are least soluble at their IEPs, and, while adjustment of the pH of the suspending medium to the isoelectric point of a protein may not be sufficient to cause coagulation of the molecules, the addition of relatively small amounts of a protein precipitant often causes coagulation and precipitation to occur. This principle was made use of by Schwerdt & Schaffer (1955) to precipitate poliomyelitis virus from infectious culture fluid at pH 4.0 by the addition of methyl alcohol.

Linear polymers have been used with much success for the precipitation of proteins from solution at pH values of the suspending medium not necessarily at or near the IEPs of the proteins concerned. Using varying concentrations of polyethylene glycol (PEG) Polson _et al_. (1964) were able to selectively precipitate proteins from mixtures, notably the proteins contained in whole serum, by varying the pH of the solvent. In virology, this principle was successfully employed by Charney _et al_. (1961) who precipitated poliomyelitis virus from suspension using nucleic acids as the precipitant. Best results were obtained with a "highly polymerised" yeast nucleic acid preparation below pH 4.5. Polyethylene glycols have been used to precipitate various plant viruses (Herbert, 1963; van Kammen, 1967; Clark, 1968) and neurotropic African horse-sickness virus (Polson & Deeks, 1963) from suspension.

The experiments described hereunder were designed to illustrate the advantage of the knowledge of the IEP of a virus, ecbovirus SA 1, which is normally soluble at its isoelectric point in order that the virus might be precipitated from suspensions and concentrated at this pH by using a minimal amount of precipitant. Precipitation of the virus from suspension with PEG was followed by zone electrophoresis to further purify the preparation

which was examined for purity in the electron microscope.

6.2.2. Materials and Methods.

Polyethylene glycol (Shell Chemicals; mol wt 6 000) was used in these experiments. The PEG was pulverised to a fine powder using a mortar and pestle before it was used.

Precipitation experiments were performed at two different buffer hydrogen ion concentrations. Infected culture fluid, pH 7.2, was used unaltered in one series of experiments. In another series of experiments, the culture fluid was adjusted to pH 4.4 using 2M solutions of acetate buffer (2M glacial acetic acid and 2 M sodium acetate) at pH 3.80.

To 30 ml aliquots of these fluids pulverized PEG was added to concentrations between 0% and 20% (w/v) for pH 7.2 culture fluid, and 0% and 10% (w/v) for culture fluid which had been adjusted to pH 4.4. After thorough mixing, the suspensions were allowed to stand at 22° for 60 min. The precipitates resulting from this treatment were collected by centrifugation at 2000 rpm for 30 min at 22° and resuspended in 1 ml PBS pH 7.2. The precipitates and the supernatant fluids were retained for titrations of infectivity.

6.2.3. Results.

The results of these experiments are presented in Table 17 and in Fig. 29. From these results it was evident that 10% (w/v) PEG or more was required to precipitate most of the infectious particles from suspension at pH 7.2, while only 3% (w/v) PEG was required for the optimal precipitation of infectious particles from suspension at pH 4.4. Accordingly, experiments were designed to concentrate the virus by precipitation with

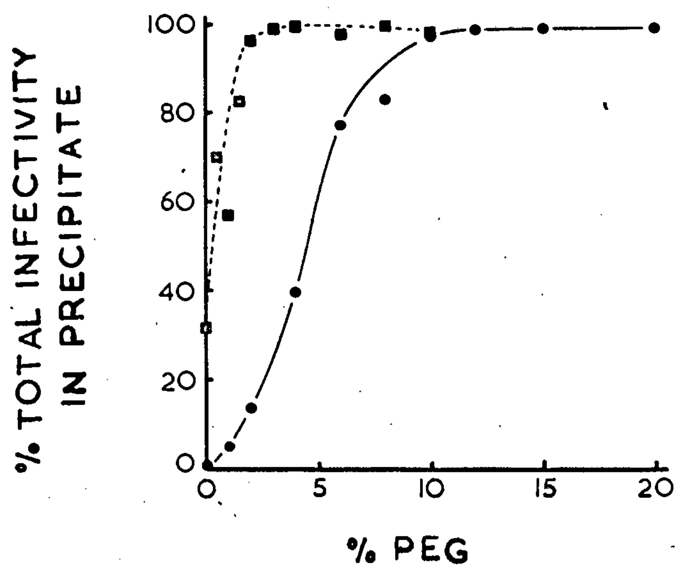
Table 17. Titration of infectivity in pfu/ml in the precipitate and supernatant fluid after precipitation of ecbovirus SA 1 from 30 ml suspensions of virus at pH 7.2 and pH 4.4 by increasing concentrations of polyethylene glycol. Precipitates redissolved in 1 ml P.B.S.

pH	% PEG (w/v)	Total infectivity (pfu) Precipitate	Supernatant	% infectivity in precipitate
7.2	0	3×10^5	3×10^7	1
	1	1×10^6	18×10^6	5
	2	1×10^6	6×10^6	14
	4	6×10^6	9×10^6	40
	6	2×10^7	6×10^6	77
	8	1×10^7	21×10^5	83
	10	1×10^7	3×10^5	97
	12	2×10^7	3×10^5	99
	15	2×10^7	3×10^5	99
	20	2×10^7	3×10^5	99
4.4	0	7×10^6	15×10^6	32
	0.5	7×10^6	3×10^6	70
	1	6×10^6	45×10^5	57
	1.5	1×10^7	21×10^5	83
	2	2×10^7	75×10^4	96
	3	3×10^7	3×10^5	99
	4	15×10^6	3×10^4	99.8
	6	7×10^6	18×10^4	98
	8	15×10^6	6×10^4	99.6
	10	5×10^6	12×10^4	98

Figure 29. Precipitation of echovirus SA 1 by polyethylene glycol (PEG) from suspensions at pH 7.2 and pH 4.4. The percentage of total infectivity recovered in the precipitate is plotted against the PEG concentration in each instance.

■---■ virus suspension at pH 4.4

●——● virus suspension at pH 7.2



with 3% PEG at pH 4.4 followed by purification of the precipitate by zone electrophoresis at this hydrogen ion concentration. Such an experiment is described below.

To approximately 1500 ml infectious tissue culture fluid, adjusted to pH 4.4 by the addition of 2 M acetate buffer pH 3.80, was added pulverised PEG to a final concentration of 3% (w/v). The resulting precipitate was collected by light centrifugation as described above (6.2.2.) and resuspended in 20 ml 0.85% NaCl. This suspension was clarified by centrifugation at 10 000 rpm for 10 min. Virus in the supernatant was centrifuged at 30 000 rpm for 90 min., and the resulting pellet was resuspended in approximately 3 ml succinate/sucrose solution pH 4.4 which had been recovered from a sucrose density gradient in a zone electrophoresis column after approximately half the density gradient had entered the column (see 5.5.2.). The suspension was clarified and introduced into the zone electrophoresis column as before (see Fig. 21), and forced up the column by the remainder of the sucrose density gradient.

A current of 20 mA was passed through the apparatus for 16 hr, after which the column was photographed (Fig. 30). Aliquots 1, 2 and 3 (Fig. 30), each of 1 cm column length, were diluted to 10 ml with distilled water and centrifuged at 30 000 rpm for 90 min. The resulting pellets were resuspended in 10 ml distilled water and subjected to a cycle of differential ultracentrifugation. The final pellets were each resuspended in 4 drops of their supernatant fluids and examined after negative staining with phosphotungstic acid in the electron microscope.

Figure 30. Zone electrophoresis column following electrophoresis of ecbovirus SA 1 which had been precipitated by 3% (w/v) PEG at pH 4.40. The resuspended precipitate was subjected to zone electrophoresis for 16 hr in a buffer of pH 4.40.

0	position of virus before electrophoresis.
1, 2 & 3	three fractions collected after electrophoresis.

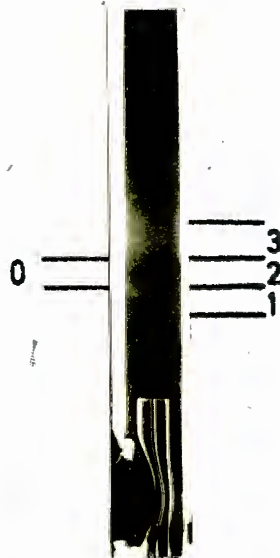
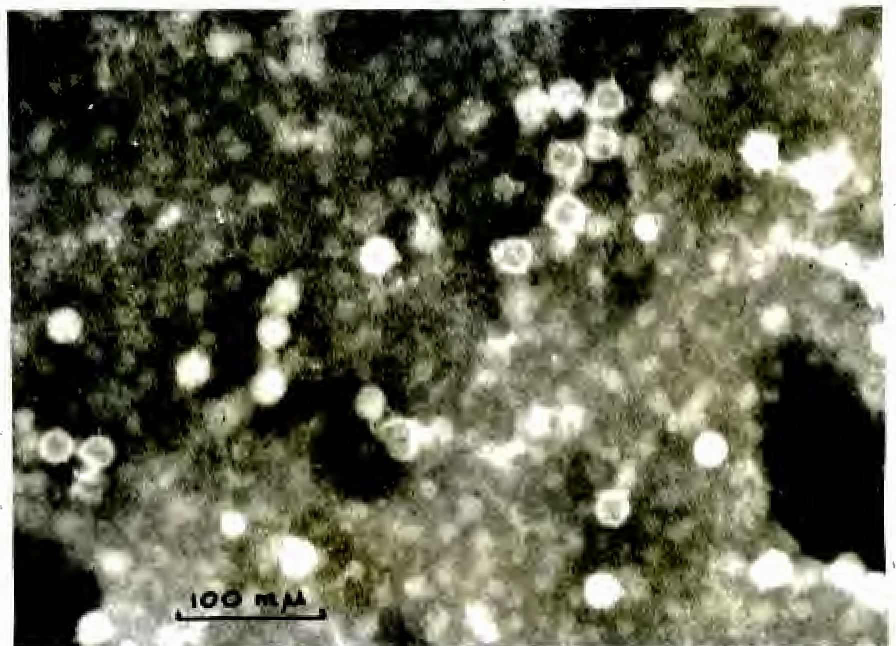


Figure 31. An electron photomicrograph of material from fraction 2 in Fig. 30. Note the high proportion of apparently "coreless" particles in the field. (x 187 000).



On grids supporting material from aliquots 1 and 3 occasional virus particles were seen after careful examination, together with much amorphous material. In aliquot 2, however, a large number of particles of uniform shape and size, similar to particles observed after concentration and purification by other methods (5.2.3.), were observed (Fig. 31). A higher proportion of these particles than those observed previously (5.2.3.3.) were found to be "coreless", as was seen by the stain having penetrated the capsids of these particles.

6.2.4. DISCUSSION.

While the value of these observations may be limited because precipitation experiments were not performed over a comprehensive pH range, it is nevertheless evident that at the isoelectric point ecbovirus SA 1 was far more susceptible to precipitation from solution by PEG than it was at physiological hydrogen ion concentrations. While, at concentrations of PEG greater than 3% (w/v), even more of the infectivity of a preparation was removed from suspension, this did not coincide with a greater recovery of infectivity in the precipitate. It was, in fact, observed that the infectivity of the recovered precipitates decreased slightly as the concentration of PEG increased, indicating that under these conditions the virus was either not as stable as it was in the absence of polyethylene glycol, or, more likely, stable aggregates of infectious particles were formed. On the other hand, due to the abnormally high proportion of "coreless" particles observed in the electron microscope, it was probable that some physical damage to the virus had resulted under the experimental conditions. As the virus underwent several cycles of differential ultracentrifugation, it is possible that this physical damage was caused by the shearing force resulting from a different density of the viral RNA as compared with

the density of the enveloping viral protein as suggested by Black, Reddy & Reichmann (1967) to explain the damage to wound tumour virus during density gradient centrifugation, and that the physical damage to ecbovirus SA 1 did not occur under the conditions of virus precipitation.

This technique may be of considerable use as a preliminary step in the concentration of viruses, either plant or animal, which are soluble at their isoelectric points. Brinton & Lauffer (1959) mentioned that the spherical plant viruses were all soluble at their isoelectric points, presumably because of hydration. Herbert (1963) found that the rod-shaped plant viruses, wheat mosaic virus and tobacco mosaic virus, were optimally precipitated by 2% PEG in 100 mM phosphate buffer pH 7.5 and 300 mM NaCl, respectively, (although he did not use buffers of pH other than pH 7.5). A higher concentration of PEG was needed for the precipitation of the spherical plant viruses tobacco ringspot virus and bean pod mottle virus, namely 8% PEG in 300 mM NaCl. These results emphasized further the solubility of spherical viruses. As plant viruses are present in infected plant tissues in far greater concentrations than are animal viruses in infected animal tissues, it is conceivable that very little PEG would suffice for the precipitation of these viruses from solution at their IEPs.

As ecbovirus SA 1, a "spherical" animal virus, is soluble at its isoelectric point, it is not impossible that spherical animal viruses, like spherical plant viruses, are normally soluble at their isoelectric points. The use of relatively small concentrations of PEG should, however, suffice to precipitate such viruses at their isoelectric points.

6.3. PURIFICATION AND CONCENTRATION OF A VIRUS BY MULTI-MEMBRANE ELECTRODECANTATION.

6.3.1. INTRODUCTION.

When an electric current is passed through a protein solution the molecules will migrate to one or other of the electrodes, provided that they are not at their isoelectric point. If the electrodes are separated from one another and the protein solution by two semipermeable membranes, the proteins attracted to the electrodes will tend to concentrate at the surface of these membranes. This results in dense zones of high protein concentration at these surfaces which tend to move downwards to the bottom of the vessel under the influence of gravity, where they become concentrated.

Practical application was made of this phenomenon by Pauli and his coworkers (cited by Svensson, 1948) in the concentration and purification of rubber latices. Gutfreund (1943) applied the principle to the separation of a protein mixture (ovalbumin and haemoglobin) into its electrically homogeneous constituents by electrodialysing the mixture at the IEP of haemoglobin, resulting in the concentration of ovalbumin at the bottom of the container. This process he called "electrodecantation".

This electrodecantation process could be accelerated by introducing a number of membranes between the electrodes (Stamberger & Schmidt, cited by Svensson, 1948) thus reducing the distance the material (in this instance rubber latex) had to migrate before being impeded by and concentrated at a membrane surface. Polson (1953) developed a multi-membrane electrodecantation (M.M.E.D.) apparatus suitable for the separation and concentration of proteins from large volumes of fluid. This apparatus allowed for the circulation of chilled buffer in compartments between the separation cells to prevent overheating

and to remove electrolytic by-products which might influence the pH. By sloping the electrodes towards each other at the top a temperature gradient, resulting in a density gradient, was established through the separation cells, thus further minimizing convection currents in these compartments. Furthermore, by inclining the apparatus to the horizontal, a more rapid separation of proteins was achieved.

A multi-membrane electrodecantation apparatus was used here in attempts to purify and concentrate ecbovirus SA 1 from suspensions in culture fluid from infected calf kidney cells. Experiments, which were exploratory in nature to determine the feasibility of the method for concentrating and purifying virus from large volumes of fluid, were performed at two buffer hydrogen ion concentrations, pH 7.2 and pH 4.4.

6.3.2. Materials and Methods.

The apparatus used was that designed by Polson & Largier (1960) having separation cells of different widths and hence different capacities. Only the smallest separation cell (capacity approximately 200 ml, containing 10 membranes) was used in each experiment (Fig. 32).

Volumes of infectious culture fluid, each of 200 ml, were dialysed for 16 hr against 5 litres of 13.3 mM phosphate buffer pH 7.2 and 100 mM acetate buffer pH 4.40 at 4°. The dialysates were collected in sterile bottles for M.M.E.D. experiments. Sufficient buffer solution, 30 litres each of 13.3 mM phosphate buffer pH 7.2 and 100 mM acetate buffer pH 4.40, was prepared to fill the apparatus and the buffer circulatory reservoir for each experiment.

Figure 32. Diagram of multi-membrane electrodecantation apparatus.

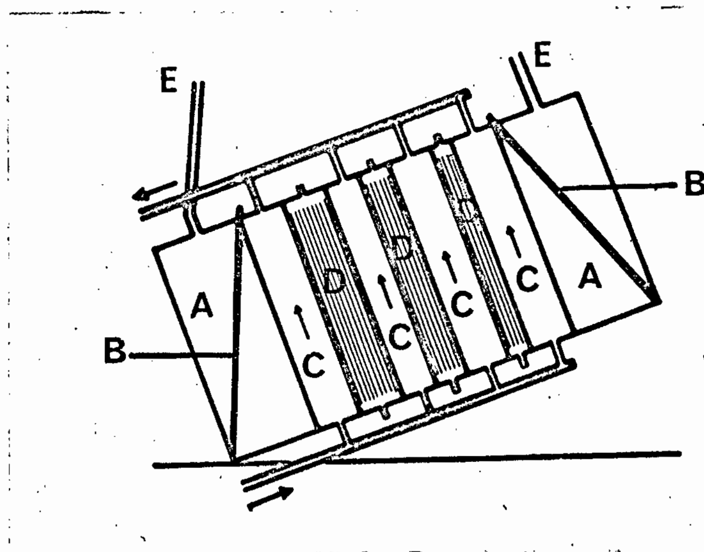
A. electrode vessels

B. platinum electrodes

C. compartments between the separation compartments (D) for circulation of chilled buffer. Arrows indicate direction of flow of buffer.

D. three separation compartments containing cellophane membranes separated by strips of mica.

E. tubes from electrode vessels to avoid spilling of buffer when the apparatus is held at an angle (as in diagram).



The apparatus and the buffer reservoir was filled with pH 7.2 phosphate buffer and chilled buffer was circulated until the temperature of the apparatus had been lowered to 10°. The smallest separation cell was drained of phosphate buffer, rinsed with 70% (v/v) ethanol and sterile phosphate buffer, and the 200 ml culture fluid which had been dialysed against pH 7.2 phosphate buffer was introduced into this cell from the bottom. Excess buffer in the tube through which the dialysate was introduced was run off and discarded. A current of 2.8 A (at a potential of 250 V) was passed through the apparatus for 75 min, the electrodes being reversed after 40 min. At the completion of the electrodecantation, three fractions were collected from the separation cell. The first fraction had a volume of approximately 20 ml, the second a volume of approximately 30 ml, and the third fraction, the remaining volume of dialysate, was approximately 150 ml.

The apparatus was drained of pH 7.2 phosphate buffer, rinsed with pH 4.40 acetate buffer, and filled with the latter buffer, which, as before, was circulated until the temperature of the apparatus had reached 10°. The separation cell was washed with 70% ethanol and rinsed with sterile acetate buffer and the pH 4.4. acetate buffer dialysate was added from the bottom. Electrodecantation proceeded for 80 min. with a current of 1 A, the potential difference across the platinum electrodes being 280 V. The electrodes were reversed every 20 min. Following electrodecantation three fractions were collected from the separation cell as above.

The infectivity of these fractions and control aliquots of the two dialysates were titrated in chick embryo cells suspended in agar.

6.3.3. Results.

The results of the infectivity titrations of the various fractions collected after multi-membrane electrodecantation in the two buffer systems are presented in Table 18. After electrodecantation at pH 7.2 the lower 20 ml fluid in the separation cell had an infectivity titre 17 times greater than the infectivity titre of the 150 ml fluid in the top of the cell and 5 times greater than the infectivity titre of the original dialysate, showing that a good concentration had been achieved after only 75 min. of electrodecantation at this pH.

After electrodecantation at pH 4.4, the infectivity titres of all three fractions was virtually identical (bottom 3×10^5; middle 2×10^5; top 2×10^5) although at least half of the original infectivity of the dialysate (6×10^5) had been lost. It was observed that, following multi-membrane electrodecantation at pH 4.4, nearly all the phenol red which had been in the original culture fluid was concentrated in the lower 20 ml fractions, indicating that electrodecantation had occurred.

Table 18. Concentration and purification of ecbovirus SA 1 by submitting to multi-membrane electrodecantation at pH 7.2 and pH 4.4. Fractions collected from the electrodecantation cell were assayed for infectivity.

Buffer	Sample	Volume (ml)	Infectivity (pfu/ml)
13.3 mM phosphate buffer pH 7.2	Dialysate	200	1×10^7
	Bottom	20	5×10^7
	Middle	30	1×10^7
	Top	150	3×10^6
100 mM acetate buffer, pH 4.40	Dialysate	200	6×10^5
	Bottom	20	3×10^5
	Middle	30	2×10^5
	Top	150	2×10^5

6.3.4. Discussion.

Multi-membrane electrodecantation has been applied to the concentration of viruses in only one other instance. Polson (1953) submitted the MEF_1 strain of poliovirus to multi-membrane electrodecantation at pH 8.7 and pH 6.0. He found that the virus particles were concentrated at the bottom of the separation cell as was evinced not so much by the infectivity of the fluid at the bottom of the cell as by the disappearance of infectivity from the supernatant fluid.

The results presented in Table 18 revealed that ecbovirus SA 1, when submitted to multi-membrane electrodecantation at a pH above its isoelectric point, behaved as MEF_1 poliovirus did in being concentrated at the bottom of the separation cell. At the isoelectric point the virus remained in suspension, the infectivity of the fractions throughout the cell being virtually identical. These preliminary results indicated the feasibility of applying the technique of multi-membrane electrodecantation to the concentration and purification of viruses which are in solution at their IEPs such as ecbovirus SA 1, by first removing contamination proteins from solution by electrodecanting at the IEP of the virus followed by concentration of the virus by electrodecanting the supernatant fluid at a pH well above the IEP of the virus.

A further advantage of this method is that it is not necessary to remove cell debris from the fluid prior to electrodecantation, as most of the debris remains adsorbed to the membranes leaving a concentrate and supernatant fluid relatively free of cell fragments (Polson, 1953).

6.4. CLASSIFICATION OF ENTEROVIRUSES.

6.4.1. Introduction.

Different strains of poliovirus have been precipitated from suspensions at pH values remarkably similar to one another. Racker (1942) precipitated an untyped strain of poliovirus from suspension at pH 4.3. The SK strain of poliovirus was precipitated at pH 4.6 (Bourdillon & Moore, 1942), the Lansing strain at pH 4.5 (Bachrach & Schwerdt, 1954), the MEF_1 strain at pH 4.3 (Polson & Selzer, 1954) and the Saukett strain, with the aid of yeast nucleic acid as precipitant, at and below pH 4.5 (Charney _et al_., 1961).

The value obtained for the isoelectric point of ecbovirus SA 1 in succinate buffer of ionic strength 0.1 (i.e. pH 4.40), indicated that there was a close relationship between this virus and various strains of poliovirus with regard to their precipitability near the isoelectric point.

It was decided to investigate the isoelectric points of a number of enteroviruses by density gradient zone electrophoresis in succinate/NaOH buffers of ionic strength 0.1 by the method used to determine the IEP of ecbovirus SA 1 to determine whether isoelectric relationships existed for this group of viruses. The isoelectric point of reovirus 1 (formerly echovirus 10) was determined at the same time as a representative from a viral group other than the enteroviruses.

6.4.2. Materials and Methods.

The apparatus and buffer systems used in these experiments were those as previously described for the determination of the isoelectric point of ecbovirus SA 1 by zone electrophoresis in a sucrose density gradient (5.5.2.).

The following viruses were examined. Cytopathogenic bovine orphan virus (CBO virus), a virus which had been isolated in chick embryos from a calf which died of lumpy skin disease (van den Ende, Don & Kipps, 1948). After 16 passages in chick embryos the virus had been propagated in chick embryo cell monolayers in tissue culture. While the virus was thought to be a bovine enterovirus (Kipps, Turner & Polson, 1961), Andrewes (1964) suggested that it may in fact be a chick enterovirus.

Two variants of the MEF_1 strain of poliovirus were examined. One of these was the mouse-adapted strain of Selzer, Sacks & van den Ende (1952) which had been passaged 218 times in suckling mouse brains, the other being the original strain which had not been adapted to mice and which had been stored at -20° since 1951.

A coxsackievirus B 3 strain, isolated and identified by Naudé, Selzer & Kipps (1958) was similarly examined. Since the original isolation this virus strain had undergone less than 10 passages in suckling mice.

The other viruses examined were obtained from Dr. T. Johnsson of the State Bacteriological Laboratory, Stockholm, Sweden. These were strains of coxsackievirus B 1 and B 4, and reovirus type 1.

Virus stocks were prepared as follows. CBO virus was propagated in chick embryo fibroblast monolayers. These cells were obtained from 11-12 day old chick embryos by trypsinization of tissue segments as described in the chapter on tissue culture (2.3.). Monolayers were grown in 20 oz. medical flat bottles, the growth medium being Hanks' LA growth medium containing 5% (v/v) calf serum, and the maintenance medium for these cells being Hanks' LA maintenance medium with 5% (v/v) fowl serum.

The infectivity of CBO virus preparations was titrated in the chick embryo cell suspensions already described.

All other viruses used were propagated in monolayers of secondary vervet monkey kidney cells. Kidneys were removed aseptically from freshly-sacrificed monkeys and cortical segments were subjected to trypsinization, as were calf kidney segments (2.2.). To obtain the maximum number of cells from the kidneys, the trypsin solution was replaced with fresh trypsin solution every 30 min. three to four times during the digestion. Cells were filtered through sterile gauze, collected by light centrifugation and resuspended, after washing, in Hanks' LA growth medium (5% calf serum) at the rate of 0.1 ml packed cell volume for each 50 ml of growth medium and 50 ml amounts of this cell suspension were dispensed into 20 oz. medical flat bottles. On incubating these bottles at 37° in a horizontal position, cell monolayers were generally confluent after 5 days, when the medium was replaced with an equal volume of Hanks' LA maintenance medium which contained 2% (v/v) fowl serum. Secondary monkey kidney cell cultures were prepared from these primary cell cultures as follows. The maintenance medium in the bottles was replaced with an equal volume of the following trypsin-versene solution:-

NaCl	...	40	g
KCl	...	2	g
Glucose	...	5	g
$NaHCO_3$	...	2.9	g
Trypsin (Difco, 1/250)		2.5	g
Sodium versenate		1	g
Phenol red (0.4% solution)		2.5	ml
Distilled water		to 500	ml

The solution was filtered through a Seitz filter and dispensed for use in sterile McCartney bottles. For use, this stock was diluted 10 times with sterile glass-distilled water.

The primary cell cultures were incubated with this solution at 37° for 20-30 min. after which time the cells had been removed from the glass. The cells were collected by light centrifugation, washed in Hanks' LA growth medium (as used for the primary cell cultures), and resuspended to twice the original volume in this growth medium. The cell suspensions were dispensed in 1 ml amounts into sterile roller tubes which were incubated at 37° in a stationary position at a slight angle from the horizontal. After 1-2 days the secondary monkey kidney cells had grown to confluent monolayers in the tubes and the medium was replaced with an equal volume of maintenance medium (as was used for the primary monkey kidney cell cultures) and reincubated at 37° in a roller drum. Virus was propagated in these tubes by the inoculation of 0.1 ml virus suspension, containing approximately 1 x 10^6 tissue culture infective dose $_{50}$ ($TCID_{50}$) per ml, into each tube. When the monolayers had been completely destroyed by viral action after further incubation in a roller drum at 37°, which usually occurred within 24 hr. the medium was removed aseptically from the tubes and stored until use at 4°. The infectivity of these virus preparations was titrated in similar secondary monkey kidney cell monolayers grown in tubes. Ten-fold dilutions of virus suspensions were made in maintenance medium and 0.1 ml of each dilution was inoculated into each of 4-6 tubes. Tubes were examined daily for cytopathic effect caused by the virus. The tubes were discarded the day following the last day on which cytopathic effect had been observed in the series. The titres of the samples were calculated by the method

of Reed & Muench (1938) and was expressed as $TCID_{50}$.

Zone electrophoresis experiments were performed, as described for echovirus SA 1 (5.5.2.), with these virus preparations. Twenty ml aliquots of infectious culture fluid were partially purified and concentrated by a single cycle of differential ultracentrifugation. The resulting viral pellets were resuspended in a portion of the ZE column density gradient of appropriate pH and introduced into the centre of the gradient in the column. After being submitted to zone electrophoresis for 15-17 hr. with a current of 20 mA passing through the apparatus, phenol red acting as an internal reference substance, samples were collected from the zone electrophoresis column for infectivity titrations.

6.4.3. Results.

The results of 5 zone electrophoresis experiments with the mouse-adapted MEF_1 strain of poliovirus at buffer pH values ranging from pH 4.18 to pH 4.68 are presented in Table 19.

The pH-R_{ϕ} curve plotted from these data (Fig. 33) showed that the IEP of this virus was at pH 4.39. Similarly, the results of 5 experiments with the original strain of MEF 1 poliovirus (Table 20) showed that this strain of the virus had an IE^P at pH 4.79 (Fig. 34).

When CBO virus was submitted to zone electrophoresis at 6 different buffer pH's, the results presented in Table 21 were obtained. By plotting the pH against the R_{ϕ} values, it could be seen (Fig. 35) that the IEP of this virus was at pH 4.45.

The results of zone electrophoresis experiments with Coxsackievirus B 1, presented in Table 22, demonstrated that two infectious components existed for this

Table 19. Determination of the isoelectric point of the mouse-adapted strain of MEF_1 poliovirus.

pH	Distance migrated by virus to anode. (cm)	Distance migrated by phenol red to anode. (cm)	R_ϕ value
4.18	-1.75	+12.0	-0.146
4.27	-1.25	+12.0	-0.104
4.39	0.0	+10.75	0.0
4.51	+1.0	+11.0	+0.091
4.68	+1.75	+12.0	+0.146

Figure 33. R_ϕ -pH diagram from which the isoelectric point of the mouse-adapted strain of MEF_1 poliovirus was determined. The curve cuts the pH-axis at pH 4.39, which is the iso-electric point of this virus.

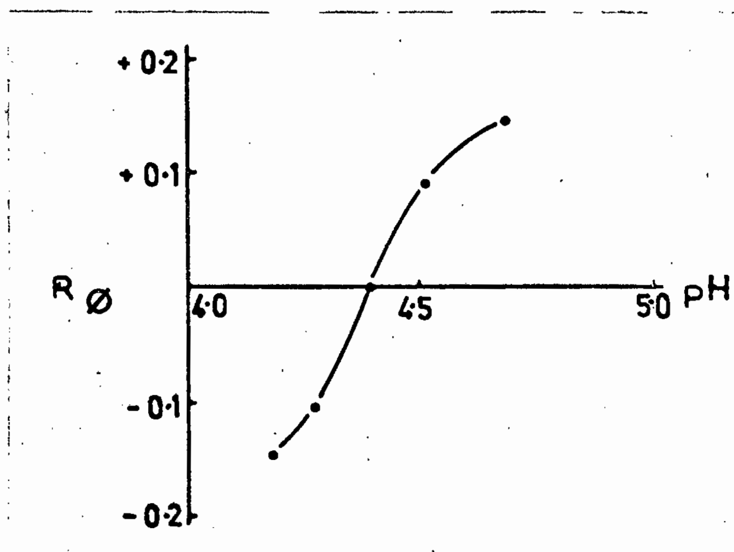


Table 20. Determination of the isoelectric point of the original strain of MEF_1 poliovirus.

pH	Distance migrated by virus to anode. (cm)	Distance migrated by phenol red to anode. (cm)	R_ϕ value
4.29	-4.65	+12.0	-0.387
4.49	-2.5	+11.75	-0.21
4.69	-0.5	+11.0	-0.045
5.00	+1.0	+11.25	+0.089
5.26	+3.0	+12.5	+0.24

Figure 34. R_ϕ-pH diagram from which the isoelectric point of the native strain of MEF_1 poliovirus was determined. The curve cuts the pH-axis at pH 4.79, which is the isoelectric point of this virus.

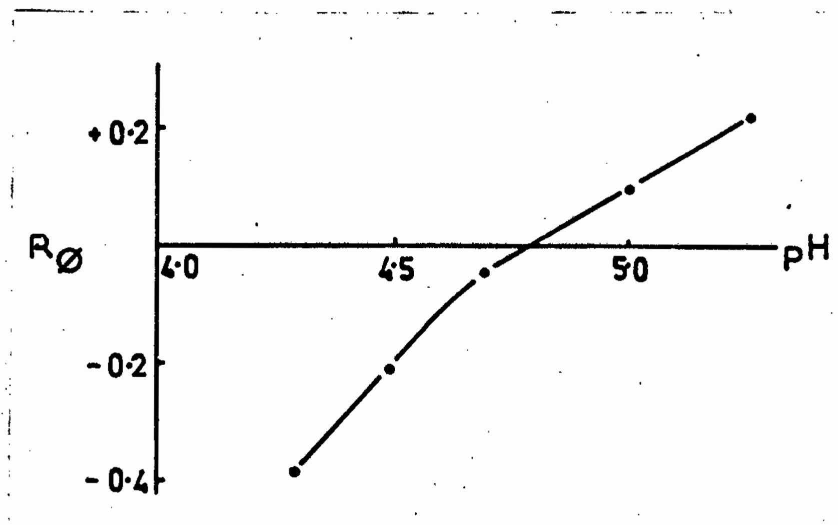
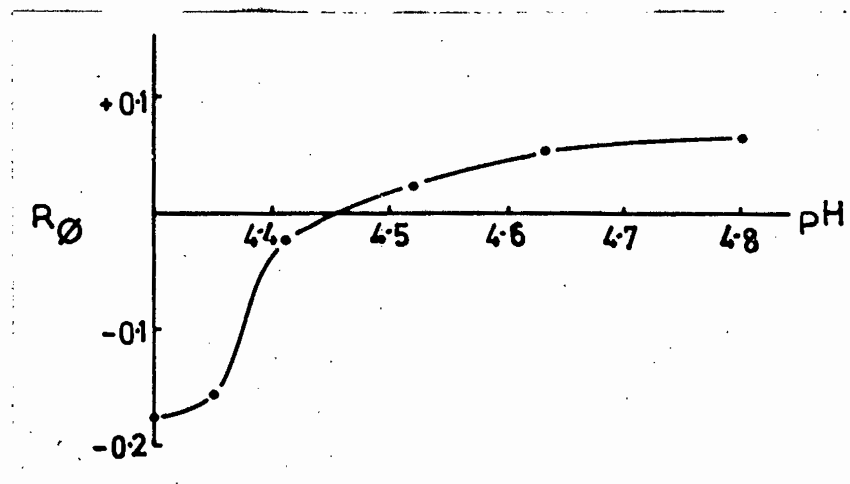


Table 21. Determination of the isoelectric point of CBO virus.

pH	Distance migrated by virus to anode. (cm)	Distance migrated by phenol red to anode. (cm)	R_ϕ value
4.30	-1.75	+10.0	-0.175
4.35	-1.875	+12.0	-0.156
4.41	-0.25	+10.75	-0.023
4.52	+0.25	+10.75	+0.023
4.63	+0.5	+ 9.25	+0.054
4.80	+0.75	+11.25	+0.066

Figure 35. R_ϕ -pH diagram from which the isoelectric point of cytopathogenic bovine orphan (CBO) virus was determined. The curve cuts the pH-axis at pH4.45, which is the isoelectric point of this virus.



virus propagated in secondary cultures of monkey kidney cells. By plotting pH against R_ϕ values (Fig. 36) it was seen that the "slow" component had an isoelectric point at pH 5.14 while the "fast" component had an isoelectric point at pH 4.65.

A similar observation was made for Coxsackievirus B 3. The results of several zone electrophoresis experiments with this virus are presented in Table 23. From the resulting pH-R_ϕ curve (Fig. 37) it could be seen that the IEP of the "slow" component of this virus was at pH 5.42, while the IEP of the "fast" component was at pH 4.82. Of particular interest was the observation that at and below pH 4.58 only one infectious electrophoretic component was detected.

Zone electrophoresis experiments with Coxsackievirus B 4, however, revealed the presence of a single infectious component (Table 24; Fig. 38). This component had an IEP at pH 4.65.

When Coxsackievirus A 9 was submitted to electrophoresis at a number of buffer pH's ranging from pH 4.56 to pH 6.02, the results presented in Table 25 were obtained. The single component was found to have an IEP at pH 5.33, as may be seen from Fig. 39.

The results of zone electrophoresis experiments with reovirus type 1 are presented in Table 26. When a pH-R_ϕ diagram was constructed for this virus (Fig. 40) it could be seen to have an isoelectric point at pH 4.65.

Table 22. Determination of the isoelectric point of Coxsackievirus B 1.

pH	Distance migrated by virus to anode. (cm) "slow"	; "fast"	Distance migrated by phenol red to anode. (cm)	R_{ϕ} values "slow" ; "fast"
4.30	-3.0	; -0.5	+10.5	-0.286;-0.047
4.74	-2.4	; +0.25	+10.375	-0.231;+0.024
4.95	-1.4	; +1.6	+11.25	-0.124;+0.142
5.10	+0.275	; +3.325	+11.25	+0.024;+0.295
5.16	0.0	; +3.5	+11.0	0.0 ;+0.318
5.23	+0.75	; +4.25	+11.5	+0.065;+0.369
5.97	+2.6	; +6.25	+11.25	+0.232;+0.555

Figure 36. R_{ϕ}-pH diagram from which the isoelectric point of Coxsackievirus B1 was determined Two infectious components were present for this virus, having isoelectric points at pH 5.14 ("slow") and pH 4.65 ("fast").

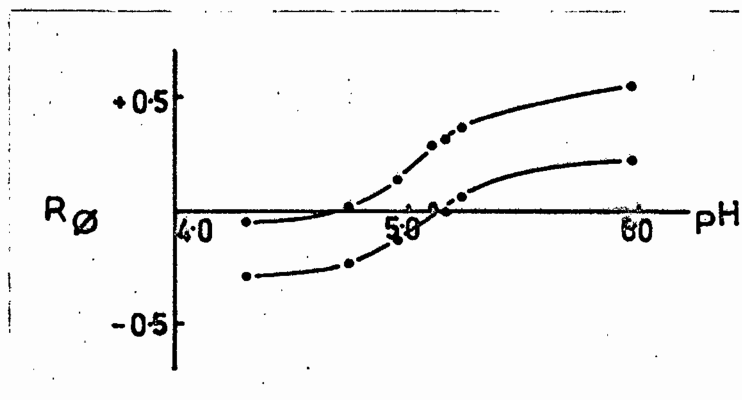


Table 23. Determination of the isoelectric point of Coxsackievirus B3.

pH	Distance migrated by virus to anode. (cm) "slow"	;	"fast"	Distance migrated by phenol red to anode. (cm)	R_ϕ values "slow"	;	"fast"
4.58	-1.25	;	-1.25	+10.75	-0.116	;	-0.116
4.82	-1.65	;	0.0	+12.25	-0.134	;	0.0
4.92	-2.0	;	+1.0	+11.0	-0.182	;	+0.091
5.32	0.0	;	+3.0	+12.5	0.0	;	+0.24

Figure 37. R_ϕ-pH diagram from which the isoelectric point of Coxsackievirus B3 was determined. Two infectious components were present in preparations of this virus, having isoelectric points at pH 5.42 ("slow") and 4.82 ("fast"). Note that at pH 4.58 only one infectious component existed.

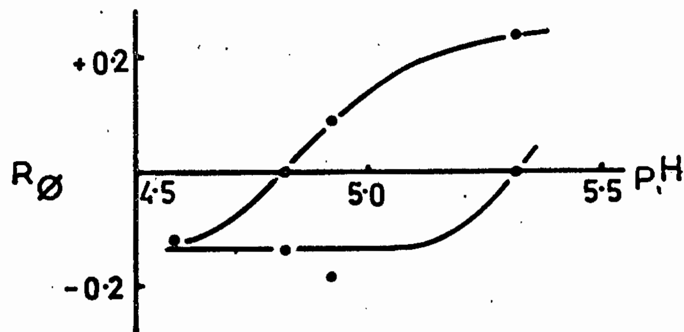


Table 24. Determination of the isoelectric point of Coxsackievirus B 4.

pH	Distance migrated by virus to anode. (cm)	Distance migrated by phenol red to anode. (cm)	R_{ϕ} value
4.37	-2.5	+11.5	-0.217
4.51	-0.55	+11.5	-0.048
4.61	-0.025	+13.0	-0.019
4.74	+0.425	+11.5	+0.037
4.90	+1.03	+11.0	+0.094

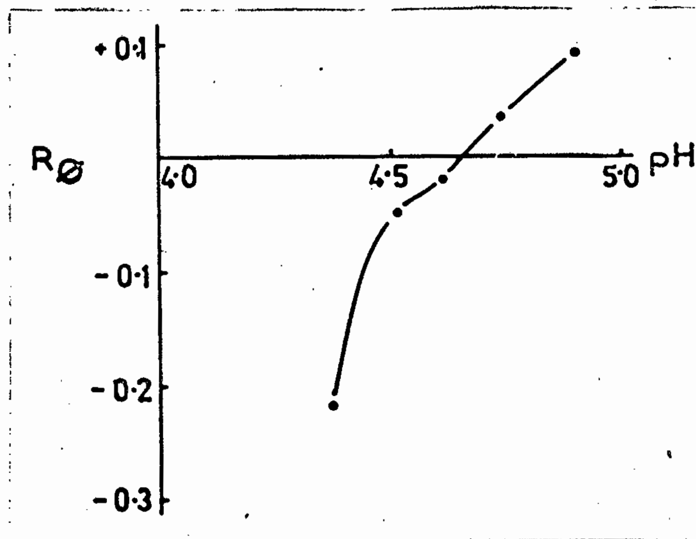


Figure 38. R_{ϕ}-pH diagram from which the isoelectric point of Coxsackievirus B4 was determined. The curve cuts the pH-axis at pH 4.65, which is the isoelectric point of this virus.

Table 25. Determination of the isoelectric point of Coxsackievirus A9.

pH	Distance migrated by virus to anode. (cm)	Distance migrated by phenol red to anode. (cm)	R_ϕ value
4.56	-2.75	+10.5	-0.262
4.90	-2.25	+11.0	-0.205
5.15	-1.5	+10.25	-0.146
5.32	-0.125	+11.0	-0.011
5.42	+0.375	+11.5	+0.033
5.92	+0.5	+11.25	+0.044
6.02	+0.5	+10.0	+0.05

Figure 39. R_ϕ-pH diagram from which the isoelectric point of Coxsackievirus A9 was determined The curve cuts the pH-axis at pH 5.33, which is the isoelectric point of this virus.

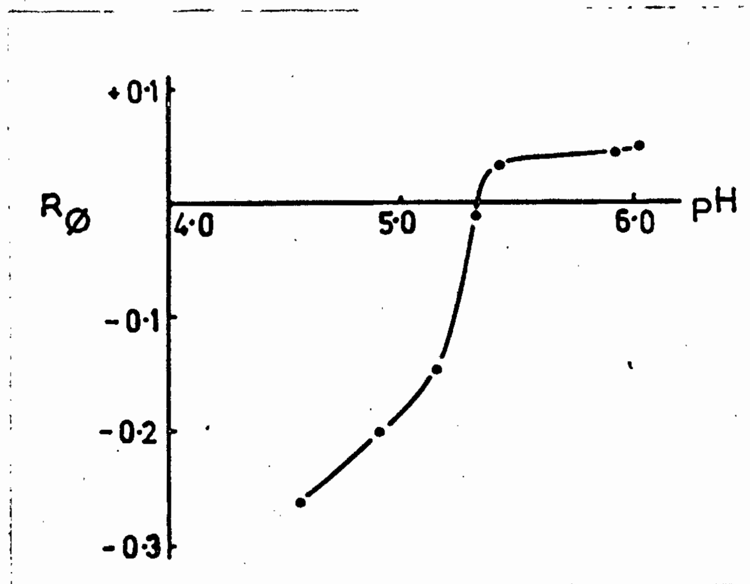
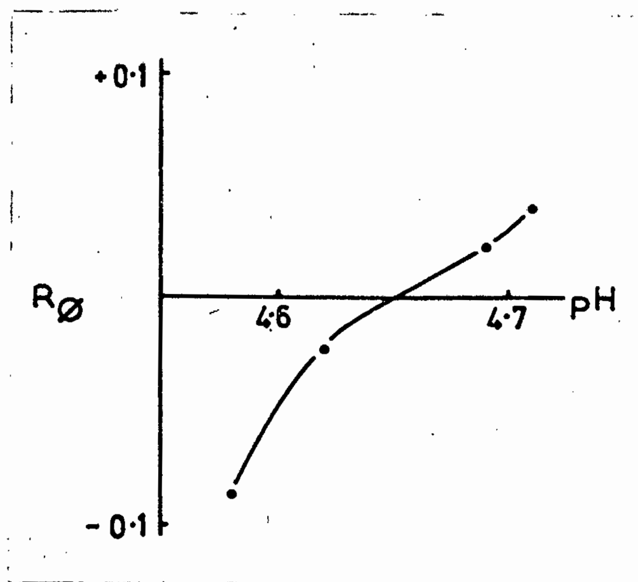


Table 26. Determination of the isoelectric point of reovirus type 1.

pH	Distance migrated by virus to anode. (cm)	Distance migrated by phenol red to anode. (cm)	R_{ϕ} value
4.58	-1.0	+11.5	-0.087
4.62	-0.25	+11.0	-0.023
4.69	+0.25	+11.0	+0.023
4.71	+0.5	+12.25	+0.041.

Figure 40. R_{ϕ}-pH diagram from which the isoelectric point of reovirus type 1 was determined. This virus was examined as an example of a virus from a non-enterovirus group. The curve cuts the pH-axis at pH 4.65, which is the isoelectric point of this virus.



6.4.3.1. Further characterization of Coxsackieviruses B 1 and B 3.

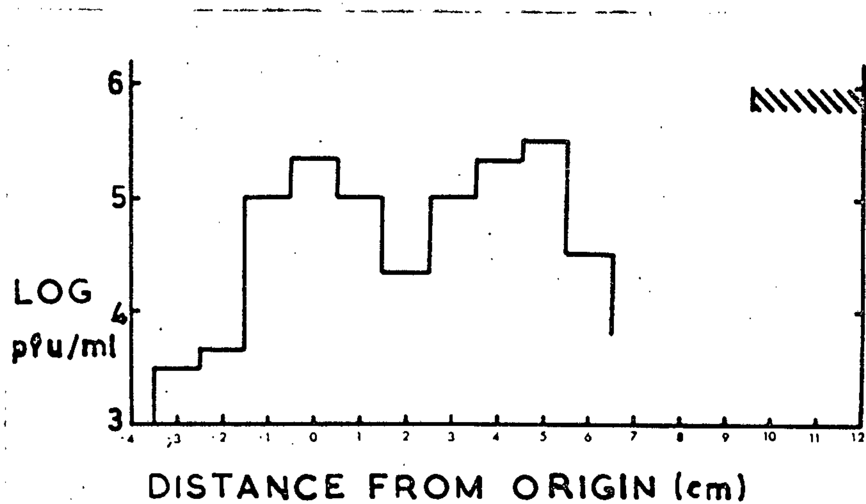
It was suspected that the slower components of these two viruses resulted from some of the infectious particles being absorbed to cellular debris. This was suggested from results obtained on submitting Coxsackievirus B3 to density gradient zone electrophoresis at different buffer pH values where the "slow" component was always associated with an opalescent band visible in the ZE column to the naked eye, presumably caused by light scattering due to large particles. For this reason the two electrophoretic components of these viruses were examined electrophoretically after they had been treated in a number of ways, and the components of Coxsackievirus B 1 were examined for their ability to give rise to one or both components after propagation in cultured cells.

6.4.3.1.1. Coxsackievirus B 1.

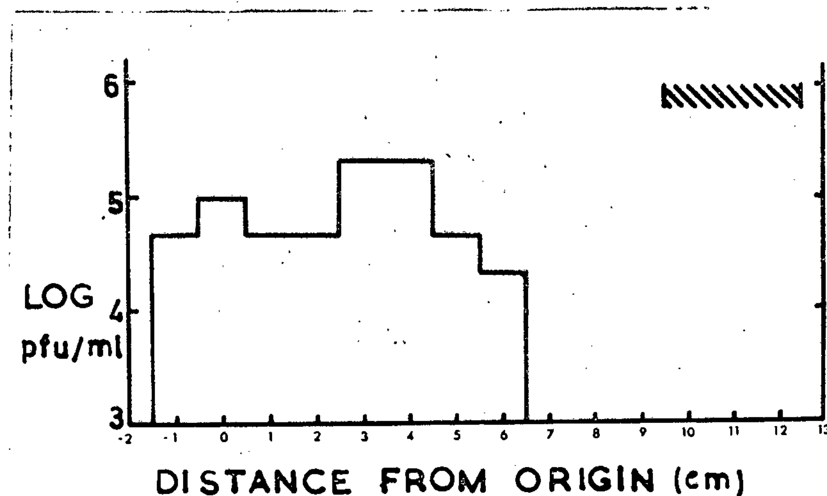
Virus from positions in the zone electrophoresis column representing the "slow" and "fast" components was inoculated separately into tubes of secondary monkey kidney cells. The culture fluids from these tubes were harvested separately after the monolayers had been destroyed by cytopathic effect. Infectious particles from these two preparations were submitted to zone electrophoresis. The results of these experiments (Fig. 41 a and b) show that both types of infectious components were once more produced by each of the two mobility components. Furthermore, neutralization tests on the two components separated by zone electrophoresis, using immune serum prepared in mice to Coxsackievirus B 1, showed that both components were neutralized to the same extent, as was an unfractionated native virus suspension, by the antiserum (Table 27). The immune serum was prepared to the prototype strain of Coxsackievirus B 1 and used in routine diagnostic investigations for typing this virus.

Figure 41. From positions in the density gradient zone electrophoresis column representing the "slow" and "fast" components of Coxsackievirus B1, material was inoculated into a number of tubes containing secondary cultures of monkey kidney cells. Harvested infectious culture fluids were examined separately by zone electrophoresis.

a. Electrophoresis at pH 5.18 of virus derived from the "slow" component.



b. Electrophoresis at pH 5.16 of virus derived from the "fast" component.



Cross-hatched areas represent the position of the phenol red bands following density gradient zone electrophoresis.

Table 27. Neutralization of the two electrophoretic components of Coxsackievirus B 1 by immune serum prepared in mice to native prototype preparations of the virus.

	Component of IEP pH 4.56	Component of IEP pH 5.16	Unfractionated native virus
Virus titre $\log_{10}$ $TCID_{50}$	3.4	3.8	5.1
Titre after incubation with immune serum $\log_{10}$ $TCID_{50}$	2.000	2.4	3.7
Drop in titre due to immune serum $\log_{10}$ $TCID_{50}$	1.4	1.4	1.4

A preparation of Coxsackievirus B 1 was incubated with 2 mg/ml lecithinase-C (Sigma Chemical Co., U.S.A.) at 37° for 30 min. The virus was centrifuged at 30 000 rpm for 90 min. after this treatment and the pellet was resuspended in a portion of a ZE gradient at pH 5.50 and submitted to electrophoresis. Aliquots were collected from the ZE column and titrated for infectivity. As may be seen from Fig. 42 which is a plot of the R_{ϕ} values thus obtained superimposed on the pH-R_{ϕ} plot shown in Fig. 36 for native virus, both components survived this treatment and no difference in the relative mobilities of the two components was observed.

In further experiments, virus was incubated at 37° with trypsin (Difco 1/250 or Seravac Laboratories, Cape Town) before it was centrifuged and submitted to zone electrophoresis. The results of several such experiments are presented in Fig. 43, where, once more, the R_{ϕ} values obtained by this treatment are superimposed on the spectrum obtained for native virus preparations.

Figure 42. The $R_{\emptyset}$ values of Coxsackievirus B1, after preincubation of the virus with 2 mg/ml lecithinase-C at 37° for 30 min, superimposed on the curves obtained on submitting native Coxsackievirus B1 preparations to zone electrophoresis (Fig. 36), after submitting to density gradient zone electrophoresis at pH 5.0.

▣ virus preincubated with lecithinase-C.

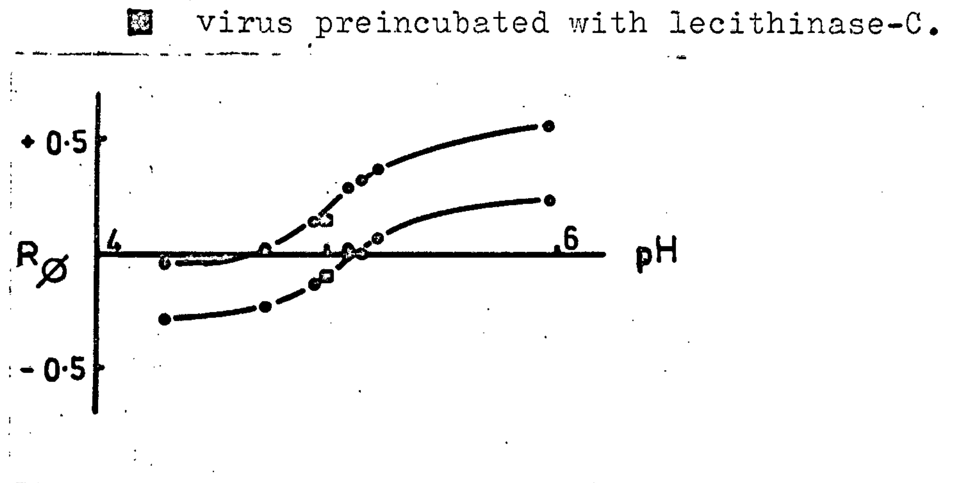
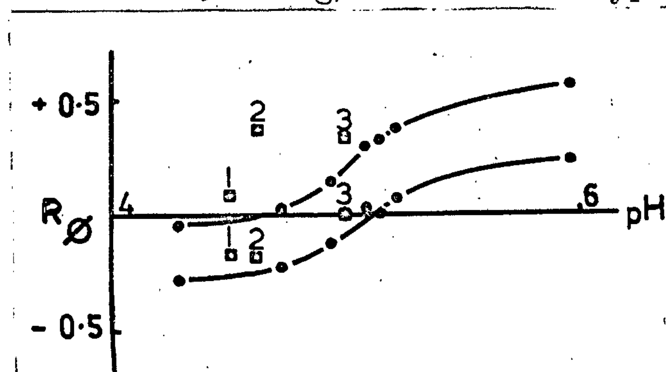


Figure 43. Coxsackievirus B1 was incubated with 2 batches of trypsin for varying periods of time before it was subjected to zone electrophoresis. $R_{\emptyset}$ values were calculated and superimposed on the curves obtained on submitting native preparations of Coxsackievirus B1 to electrophoresis at different pHs (Fig. 36).

▣ virus preincubated with trypsin.

1. 2 mg/ml Difco 1/250 trypsin, 60 min.
2 & 3. 1 mg/ml Seravac trypsin, 30 min.



While such treatment did not affect the existence of either of the two components, the relative electrophoretic mobility of the "fast" component of the virus was increased considerably, the increase in relative mobility depending on the trypsin preparation and time of incubation of virus with the enzyme. The mobility of the "slow" component of the virus was also altered, the proportion of negative charges on the surface increasing.

6.4.3.1.2. Coxsackievirus B 3.

Coxsackievirus B 3 preparations were similarly treated with enzymes. Pre-treatment of the virus with 2 mg/ml trypsin (Seravac Laboratories, Cape Town) resulted in the observation presented in Fig. 44 where the $R_{\emptyset}$ values obtained on submitting such a preparation to zone electrophoresis are superimposed on the spectrum obtained for untreated virus preparations (Fig. 37). While such treatment once more did not affect the titre of the two components, the relative electrophoretic mobility of the "fast" component of this virus was increased considerably, while that of the "slow" component remained unaltered.

Virus was incubated with 30 units/ml neuraminidase (Calbiochem, U.S.A.) at 37° for 30 min. before it was sedimented by ultracentrifugation and the pellet submitted to zone electrophoresis. When the $R_{\emptyset}$ values obtained by such treatment were superimposed on the spectrum obtained for native virus, it was observed that both components were present and that their relative electrophoretic mobilities had not been altered (Fig. 45).

When Coxsackievirus B 3 was submitted to two cycles of fluorocarbon (Arcton 113; Imperial Chemical Industries) extraction (Cramer, 1964) before submitting to zone electrophoresis the resulting $R_{\emptyset}$ values obtained (Fig. 46) showed that such treatment had influenced neither the existence nor the relative electrophoretic mobilities of

Figure 44. Coxsackievirus B3 was incubated with 1 mg/ml trypsin (Seravac Laboratories) at 37° for 30 min before it was subjected to sucrose density gradient zone electrophoresis at pH 4.88. The $R_{\emptyset}$ values of the two components were calculated and superimposed on the curves obtained on electrophoresis of native Coxsackievirus B3 (Fig. 37). Note that while the mobility of the "slow" component remained unaltered, the electrophoretic mobility of the "fast" component was increased considerably.

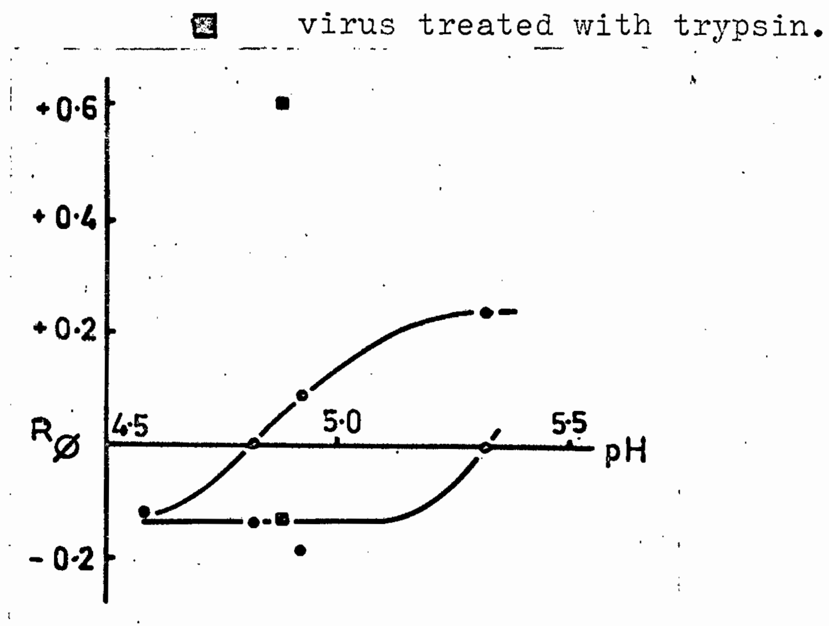
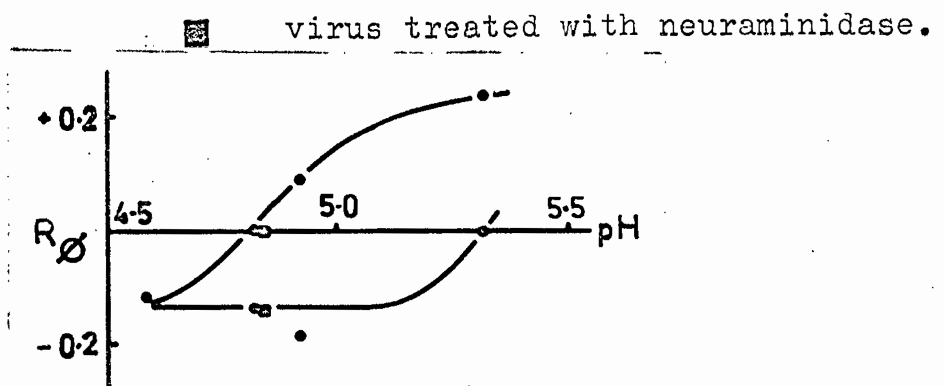


Figure 45. Coxsackievirus B3 was incubated with 30 units/ml neuraminidase (Calbiochem) for 30 min before it was submitted to zone electrophoresis at pH 4.90. When the $R_{\emptyset}$ values of the two components were superimposed on the curves for native virus (Fig. 37) it is seen that the enzyme had no effect on the mobility of the 2 components.



the two components.

In a further experiment, the two components of Coxsackievirus B 3 were separated by zone electrophoresis at pH 4.82. The two components were diluted with PBS, centrifuged at 30 000 rpm for 90 min, and the pellets resuspended in a CaCl solution for buoyant density determinations by the method of Polson & Levitt (1963). When the infectivities of samples collected after centrifugation from CsCl density gradients were plotted against their densities, the curves presented in Fig. 47 were obtained. The buoyant density in CsCl of the "slow" component was approximately 1.31 g/cc while that of the "fast" component approximately 1.34 g/cc.

In another experiment, the two components of Coxsackievirus B 3 were separated by zone electrophoresis and the column fractions containing the majority of the infectious units of the two components were diluted to 10 ml with distilled water. The virus in these samples was sedimented by centrifugation at 30,000 rpm for 120 min. The pellets were resuspended in distilled water and recovered from this washing by centrifugation at 35 000 rpm for 105 min. in the No.40 rotor of a Spinco model L centrifuge. The resulting pellets were resuspended in distilled water, stained by mixing with an equal volume of 2% phosphotungstic acid pH 7.2 and examined in the elætron microscope. Examination revealed the presence of "complete" particles in both preparations. In preparations of the "slow" component, however, "coreless" particles were observed. These empty particles were absent in preparations of the "fast" component. There was approximately one "coreless" particle for every 2 or 3 "complete" particles, in preparations of the "slow" component.

Figure 46. Coxsackievirus B3 was submitted to 2 cycles of fluorocarbon (Arcton 113) extraction before being examined by zone electrophoresis at pH 4.92. The $R_{\emptyset}$ values obtained were superimposed on the curves obtained for native virus (Fig. 37). This treatment had no effect on the mobilities of the 2 electrophoretic components of the virus.

▪ virus extracted with fluorocarbon.

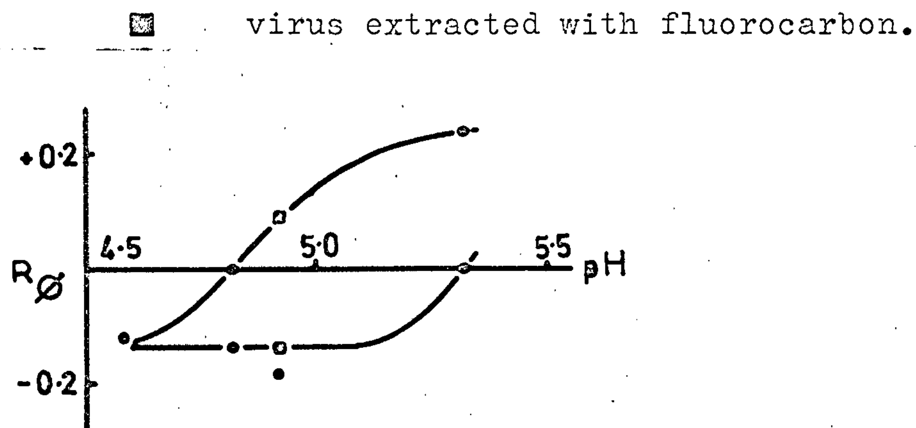
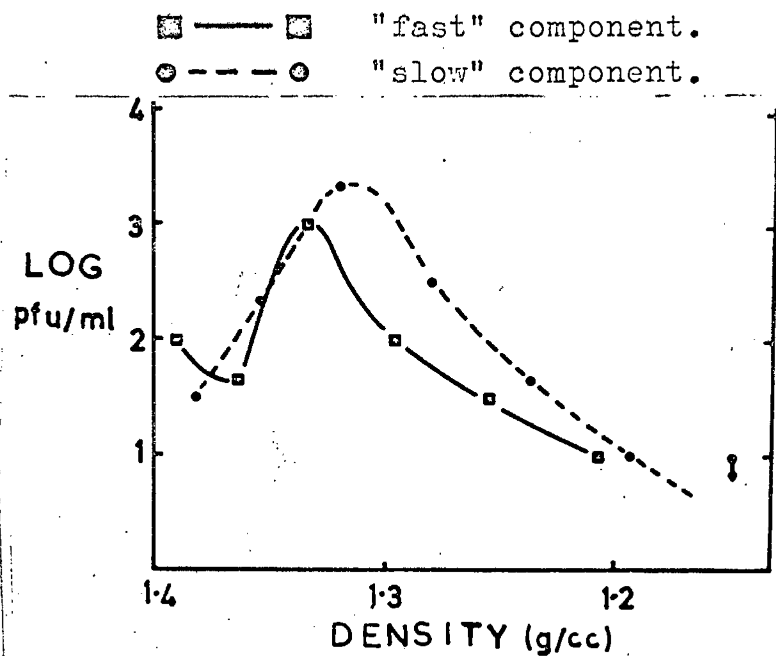


Figure 47. The 2 components of Coxsackievirus B3 were separated by sucrose density gradient zone electrophoresis at pH 4.82. Virus was concentrated from fractions collected and centrifuged in a CsCl density gradient. The figure shows the distribution of infectivity in the gradients after centrifugation.

▪——▪ "fast" component.

○- - -○ "slow" component.



6.4.4. DISCUSSION.

The results of the experiments recorded in this chapter on a limited number of human enteroviruses do not allow definite conclusions to be made as to relationships between the isoelectric points and the electrophoretic mobilities at pH 8.6. It is probable that these two physical properties cannot be correlated. For instance, Polson & Deeks (1962) found that all strains of poliovirus, most strains of Coxsackie group A viruses (including Coxsackievirus A9) and Coxsackievirus B3 could be included in a single group, as they had the slowest electrophoretic mobilities at pH 8.6 of the entire human enterovirus group. In the present study it was found that these viruses had widely differing isoelectric points, from pH 4.39 for the mouse-adapted strain of MEF_1 poliovirus to pH 5.33 for Coxsackievirus A9.

Despite the limited evidence presented here, three groups may be presumed to exist for these enteroviruses studied, on the basis of their isoelectric points (Table 28).

Table 28. The grouping of a number of enteroviruses on the basis of their isoelectric points.

Group 1 pH ± 4.4	Group 2 pH ± 4.7	Group 3 pH > 5
echovirus SA 1	original MEF_1 poliovirus	Coxsackievirus A 9
CBO virus	Coxsackievirus B 4	Coxsackievirus B 1 ("slow" component)
mouse-adapted MEF_1 poliovirus	Coxsackievirus B 1 ("fast" component)	Coxsackievirus B 3 ("slow" component)
	Coxsackievirus B 3 ("fast" component)	
	reovirus type 1	

The first of these consists of viruses having an isoelectric point at or near pH 4.4, namely ecbovirus SA 1 (IEP at pH 4.40), CBO virus (IEP at pH 4.45) and the mouse-adapted strain of MEF_1 poliovirus (IEP at pH 4.39). The second group consists of viruses having an isoelectric point near pH 4.7, namely the original strain of MEF_1 poliovirus (IEP at pH 4.79), Coxsackievirus B 4 (IEP at pH 4.65) and the "fast" components of Coxsackieviruses B 1 and B 3 (IEPs at pH 4.65 and pH 4.82, respectively. Reovirus type 1 (IEP at pH 4.65) may also be included in this group. The third group contains Coxsackievirus A 9 (IEP at pH 5.33) and the "slow" components of Coxsackievirus B 1 and B 3 (IEPs at pH 5.14 and pH 5.42, respectively).

The results of experiments performed on the two infectious components of Coxsackieviruses B 1 and B 3 revealed that the existence of the two components was not affected by incubation of crude virus preparations with trypsin, lecithinase-C and neuraminidase, although the mobilities of both components of Coxsackievirus B 1 components, and the "fast" component of Coxsackievirus B 3 were altered after incubation with trypsin. Two cycles of fluorocarbon extraction did not affect the mobility of the two components of Coxsackievirus B 3. These studies strongly suggested that the "slow" component of both viruses did not consist of virus particles associated with cellular debris, as was originally suspected, as these treatments would have removed from such suspected associations lipids, proteins, carbohydrates, and their complexes, resulting in a single electrophoretic component. Crude preparations of Coxsackieviruses B 1 and B 3 must therefore consist of two types of component which are separable on the basis of their surface properties.

That the separated components of Coxsackievirus B 1 were neutralised to the same extent by immune serum prepared to crude antigen could mean that the two components, while differing in electrophoretic mobility and hence in net surface charge, were equally neutralized by a single antibody. On the other hand, it is possible that immune serum to the crude antigen, prepared in mice, contained antibodies to the two components in equivalent amounts.

While the results of a single experiment in which the buoyant densities in CsCl of the two components of Coxsackievirus B 3 were determined (Fig. 47) were not entirely convincing due to the points on the curves being widely separated from one another, indications were obtained that the "fast" component had a buoyant density of approximately 1.34 g/cc, which is the value accepted as the buoyant density of enterovirus infectious particles (Melnick & McCombs, 1966), while the "slow" component had a buoyant density of approximately 1.31 g/cc. This observation suggested a similarity between these components and the "complete" and "coreless" particles of ecbovirus SA 1 (section 4.3.2.), although these particles were inseparable by zone electrophoresis in a sucrose density gradient. While electron microscopic examination of the two components of Coxsackievirus B 3 revealed the presence of "coreless" particles in preparations of the "slow" component, the ratio of these particles to "complete" particles being approximately 1 to 3, it is possible that a large number of these "coreless" particles was initially present in preparations, but that they remained behind in the supernatant fluid when the sample from the zone electrophoresis sucrose gradient column was centrifuged to recover the virus for electron microscopic examination.

The viscosity of the solution may have been sufficiently high to have prevented the recovery of most of the "coreless" particles in this centrifugation, as these particles would have a sedimentation coefficient much lower than that of "complete" particles.

It is suggested that the "slow" component consists of clusters of large numbers of "coreless" particles, these particles trapping a large number of "complete" particles within their matrices, the opalescent band in the electrophoresis column which was always associated with this component thus being a result of light scattering by these aggregates. This would explain why enzymic treatments did not affect the existence of the two components, why the two components of Coxsackievirus B 1 were neutralised to the same extent by immune serum, and why the two components of Coxsackievirus B 1, when separated by zone electrophoresis and propagated separately in secondary cultures of monkey kidney cells, both gave rise to the same two electrophoretic components.
The difference in the electrophoretic mobilities of the two components may be due to there being less net surface charge available on the clusters as compared with individual free particles, or may be due to the "coreless" particles, unlike those of echovirus SA 1, having different surface properties from the "complete" particles. This aspect will be discussed in chapter 7. The observation that at and below pH 4.58 the two components of Coxsackievirus B 3 were inseparable when they were subjected to density gradient zone electrophoresis suggests that the latter explanation (i.e. that the two types of particle have different surface properties) is most likely, the differences in net surface charges at this hydrogen ion concentration being too small to allow for effective separation of the components.

Viewed in this light, the "slow" components of the two group B Coxsackieviruses (i.e. Coxsackieviruses B 1 and B 3) should not be included in Table 28. Of the entroviruses examined, only Coxsackievirus A 9 should thus be included in group 3 of Table 28, i.e. viruses having an isoelectric point above pH 5.

7. CONCLUSIONS.

7.1. Haemagglutination.

Ecbovirus SA 1 was shown to haemagglutinate red blood cells at 4° but not at $20-24^{\circ}$ or 37° (3.3.). All bovine enteroviruses which have been shown to haemagglutinate red cells do so, with one exception, at 4° but not at room temperature or 37°. The exception is one of Falk's (1964) bovine enterovirus strains, which was capable of agglutinating red cells to the same titre at 4° and at 20°. Rosen (1964) pointed out that viruses may cause haemagglutination at 4° or 37°. While haemagglutination tests were often performed at room temperature for the sake of convenience, no viruses were ever shown to haemagglutinate red cells better at room temperature than at either 4° or 37°. Human enteroviruses have optimum haemagglutination temperatures of either 4° or 37°. Coxsackieviruses B1 and B5 haemagglutinated optimally at 37° (Rosen & Kern, 1961), echovirus 6 at 37° (Lahelle, 1958), while Coxsackievirus A21 haemagglutinated optimally at 4° (Johnson & Lang, 1962). In a survey on a number of human enteroviruses Kern & Rosen (1966) found that approximately 40% of 906 strains agglutinated red cells at 4° <u>or</u> 37°. The ability to haemagglutinate red cells only at temperatures near 4° thus seems to be a property common to all bovine enteroviruses, and a reason for the inclusion of ecbovirus SA 1 within this group of animal viruses.

A possible explanation for this phenomenon exists in terms of the kinetic theory, i.e. that the electrostatic bond between enteroviruses and susceptible red cells may be disrupted by dispersive forces such as Brownian motion at higher temperatures. The observation by Gibson (1955) that the lowering of temperature slows ionic exchange across the red cell membrane may also have some bearing on this phenomenon.

The enhancement of haemagglutination of ecbovirus SA 1 by the divalent cations of calcium (3.3.) may have a simple explanation. The isoelectric points of all red cells appear to be below pH 5, although exact figures are difficult to produce owing to lysis of red cells in media of low pH (Prankerd, 1961). The isoelectric point of ecbovirus SA 1 is at pH 4.40. Thus, under conditions for optimal agglutination of red cells by this virus (pH approximately 6) both participants in the reaction carry a net negative charge on their surfaces, which would tend to favour mutual repulsion. An excess of divalent cations such as Ca^{++} would tend to neutralize such charges and allow the specific groups on the surfaces of the two participants to interact.

Evidence exists in the literature, however, that the enhancement of haemagglutination of ecbovirus SA 1 by calcium ions may not be explained in such simple terms. Calcium ions are firmly bound to the human red cell membrane (Streef, 1939) and addition of more Ca^{++} decreases the permeability of human red cells (Bolingbroke & Maizels, 1959). Thus, two of the features necessary for the optimal agglutination of red cells by ecbovirus SA 1, namely the presence of the Ca^{++} and a temperature of 4°, are both features which decrease the exchange of ions across human red cell membranes.

The red cell surface is complex, and what applies to human red cells may not apply to those of other species. For instance, the red cells of ruminants (sheep, ox and goats) lack lecithin which is present in the cells of man, pigs, rabbits, ducks, chicks, cats, dogs and guinea pigs (Turner, 1957).

Divalent cations have long been known to be of importance in many biological systems. For example,

Ca^{++} and Mg^{++} stabilize trypsin and other proteases, reduce denaturation of bovine serum albumin on heating at 52° for 2 hr., and stabilize this protein against attack by trypsin (Gurd & Wilcox, 1956). In the field of virology, Norman & Veomett (1960) found that high NaCl concentrations resulted in a decrease in the rate of thermal inactivation of poliovirus RNA. Holland, Hoyer, McLaren & Syverton (1960) showed that the infectivity of poliovirus RNA for Hela cells was enhanced by 2M $MgSO_4$. The effect appeared to be partly due to hypertonicity on the cells and partly due to the action of high concentration of divalent cations on the viral RNA itself, without causing an enhancement of adsorption. Pollard (1960) suggested that one of the results of heating a virus was a basic structural alteration of the particle due to the differential expansion of the component parts of the virus (protein and nucleic acid) under the action of heat. Wallis & Melnick (1961) thus suggested that the protection afforded by cations, particularly divalent cations, in high molar concentrations to enteroviruses when heated, was due to the cations penetrating the particle and reinforcing the structure during heat stress.

Wallis & Melnick (1962b) further reported that the addition of 25 mM $MgCl_2$ to maintenance media enhanced the susceptibility of cultured monkey kidney cells to infection by poliovirus. While the mechanism was not understood, they claimed it was not dependent upon increasing the rate of adsorption of virus to cells, even though Bachtold, Bubel & Gebhardt (1957) had previously found that 1 mM Mg^{++} increased the rate of adsorption of poliovirus to cells.

It thus appears that the main effect of cations in stabilizing enteroviruses and their nucleic acids to heat is, as suggested by Holland <u>et al.</u>, (1960), due to

the facilitation of hydrogen bonds by the cations which stabilize the structural entities, and not due to an increase in virus adsorption to cells. This evidence suggests that the effect of Ca^{++} in enhancing the haemagglutination of ecbovirus SA 1 was not primarily due to these ions neutralizing negative charges on the surfaces of the red cells and virus particles and thus improving the conditions for the two participants in the reaction to come together. The enhancement of haemagglutination by calcium ions may, as has been stated above, be due to the cations decreasing the exchange of ions across the red cell membrane.

7.2. The typing of bovine enteroviruses.

La Placa and his coworkers (1963, 1964, 1965) found that bovine enteroviruses could be divided into two main serotypes and a small miscellaneous group of viruses serologically unrelated to these two serotypes or to each other. They found that all the strains in serotype 1 agglutinated rhesus monkey red cells while the other strains did not. They therefore proposed that this feature be a criterion for the inclusion of a bovine enterovirus in serotype 1. Huck & Cartwright (1964) found that their serotype M63, though clearly belonging to La Placa's serotype 1, failed to haemagglutinate rhesus monkey red cells. Verwoerd _et al._ (1967) compared ecbovirus SA 1 serologically with a number of other enteroviruses and found that this virus belonged to the Weybridge serotype 134 of Huck & Cartwright (1964), a serotype which is serologically unrelated to either of La Placa's two main serotypes. In the results of experiments reported here on ecbovirus SA 1 (3.3.), the virus was found to be capable of causing the agglutination of rhesus monkey red cells, even in the absence of 140 mM $CaCl_2$.

As a result of this observation, and the observation of Huck & Cartwright (1964) for their serotype M63, the ability of a particular bovine enterovirus to haemagglutinate rhesus monkey red cells might well be used as a preliminary screening test for newly isolated strains for inclusion in La Placa's serotype 1, but not as an absolute criterion.

7.3. Determination of isoelectric points.

The method employed in this study to determine the isoelectric points of enteroviruses (5.5.2.), namely sucrose density gradient zone electrophoresis in succinic acid-NaOH buffers of ionic strength 0.1 and measuring the distance of virus migration in relation to the distance migrated by the reference substance, phenol red, proved to be reliable. When the isoelectric point of human serum albumin was determined by this method a figure of pH 4.72 was obtained, a value which is in close agreement with values obtained by using other methods, e.g. pH 4.64 (Stenhagen, 1938), pH 4.9 (Moyer & Moyer, 1940), pH 4.71 (Baldwin, Laughton & Alberty, 1951) and pH 4.7 (quoted by Fruton & Simmonds, 1958).

Most determinations of isoelectric points of proteins have been made in buffers, usually of acetic acid and sodium acetate, of ionic strength 0.1. The valency of the buffers is critical, the change from a univalent buffer salt mixture to one containing divalent ions having a decided effect on mobilities (Longsworth, 1941). The effect of increasing the salt concentration is to shift the isoelectric point over to the acid side, salts with divalent anions causing the greatest shift, those with divalent cations the least (Lloyd, 1926). While to date no reports of the determination of isoelectric points of viruses by means of isoelectric focussing in "natural" pH gradients (Vesterberg & Svensson, 1966) have appeared

in the literature, it might be expected that the effect of this method would be to shift the isoelectric points of viruses to the alkaline side, as such determinations are carried out in the absence of salts. It is thus more practical, when determining the isoelectric points of viruses as a physical property, to make such determinations under standard conditions of ionic strength, e.g. 0.1 as in the experiments reported here, as these conditions are easily reproducible.

Owing to the result obtained for the isoelectric point determination of human serum albumin by submitting the protein to zone electrophoresis in sucrose density gradients (5.5.3.), it was thought that this method would be a precise method for the determination of isoelectric points of viruses.

The possible effect of the host cell on the isoelectric points of enteroviruses has been discussed (5.6.) Although Polson & Deeks (1962) found that the electrophoretic mobilities of certain human enteroviruses propagated either in monkey kidney cells or the brains of suckling mice were identical at pH 8.6, the possibility of the host cell having some effect on the surface composition of these viruses and consequently their charge densities should not be ignored. Podoplekin (1964), for instance, found that echoviruses propagated in primary cell cultures, but not those grown in cultures of stable cells, had haemagglutinating properties. Johnson & Lang (1962) found that Coxsackievirus A 21 after passage in KB or Hela cell cultures lost the capacity to haemagglutinate human red cells. In these instances, at least, the host cells appear to have had some effect on the production of haemagglutinating or non-haemagglutinating viruses, possibly being accounted for by the stable cells favouring the multiplication of non-haemagglutinating variants in the enterovirus populations. This phenomenon

may account for the failure to detect a haemagglutinin in preparations of echovirus SA 1 by Verwoerd et al. (1967) who used the stable cell line, BHK-21, for the bulk cultivation of the virus. In the present report this virus was found to have a haemagglutinin after it had been propagated in primary cultures of calf kidney cells.

It is thus possible that enteroviruses propagated in primary cell cultures will have constant isoelectric points, irrespective of the animal species from which the cells were obtained.

The variation in surface properties of tobacco mosaic virus strains propagated in the same host plant is considerable. A number of investigators, using several techniques, have found that different strains of the virus may have isoelectric points ranging from pH 3.67 to pH 4.90 (Oster, 1951; Gordon & Price, 1953; Ginoza & Atkinson, 1955). On the other hand MacDonald, Price & Lauffer (1949) found that two strains of southern bean mosaic virus, "yellow" and "regular", had identical isoelectric points, and the electrophoretic mobilities of the two strains were identical when tested between pH 3.9 and pH 7.2.

Among the adenoviruses, the variation in surface properties of different strains appear to be minimal. Six adenovirus strains all had isoelectric points at pH 3.15 ± 0.15 (Brandt et al., 1963).

The finding that different enteroviruses had different isoelectric points (6.4.3.) was not unexpected. Different human enteroviruses attach to different sites on Hela cells. Pretreatment of cells with certain enzyme solutions, e.g. trypsin and chymotrypsin resulted in the different treatments selectively preventing the attachment of polioviruses or group B Coxsackieviruses,

indicating that the cellular receptors for polioviruses differed from those for group B Coxsackieviruses (Zajac & Crowell, 1965a). Crowell (1966) found that exposure of Hela cells to high concentrations of Coxsackievirus B3 produced cells which were refractory to the attachment of Coxsackievirus B1, whereas type 2 poliovirus was able to attach normally to such cells. Similarly, type 2 poliovirus interfered with the attachment of type 1 poliovirus without affecting the rate of attachment of Coxsackievirus B3. This would contribute towards the different isoelectric points observed here for Coxsackie-and-polio-viruses.

7.4. Applications of the isoelectric point.

Experimental results presented in this investigation (6.2.3.) reveal that ecbovirus SA 1, a "spherical" animal virus normally stable at its isoelectric point, was precipitated from solution at its isoelectric point by low concentrations of polyethylene glycol. Apart from the use of this property as a preliminary stage in virus concentration and purification, it may also be of use in the estimation of the isoelectric points of viruses which normally remain in suspension at their isoelectric points. The technique of isoelectric precipitation may be used to determine the isoelectric points of such viruses in suspension in solutions containing 3% (w/v) polyethylene glycol.

Yeast nucleic acid was used to aid in the precipitation of virus in infected culture fluid of pH 3.5 at temperatures of 0° to 5° as a first step in virus concentration for the preparation of Salk type inactivated poliovirus vaccines (Bachrach & Breese, 1968). Isoelectric precipitation was used to concentrate and purify

adenoviruses for vaccine production (Brandt *et al.*, 1963). For attenuated poliovirus vaccines the World Health Organisation advises that the virus be grown in primary cultures of monkey kidney cells in the absence of serum, and that the virus pools after harvesting be passed through appropriate filters to ensure bacterial sterility. No further purification of vaccines is recommended provided a virus concentration in cultures of monkey kidney cells of 1 x 10^7 $TCID_{50}$/ml for the infectivity of the vaccines be achieved (Potash, 1968). The results obtained on submitting volumes of culture fluid containing ecbovirus SA 1 in suspension to multi-membrane electrodecantation suggest that this technique might be used with advantage in the concentration of virus before inactivation in the preparation of Salk type vaccines, and in the concentration and purification of attenuated live poliovirus vaccines, from large volumes of culture fluid.

It was proposed from this investigation that the enteroviruses may be arranged in at least three groups on the basis of their isoelectric points. The observation that the isoelectric point of the mouse-adapted MEF_1 strain of poliovirus, which has lost its neurovirulence for monkeys, is markedly lower than that of the original MEF_1 poliovirus strain, which is neurovirulent for monkeys, may be of considerable importance. Whether the observation of this association is coincidental is not known, and experiments to determine whether this is a genetic marker for polioviruses are not yet complete. On the other hand, two other enteroviruses of IEP group 1 which have as yet not proved pathogenic for man or monkeys, namely ecbovirus SA 1 and CBO virus, have isoelectric points very similar to that of the mouse-adapted strain of MEF_1 poliovirus.

It has been shown that the surface properties of attenuated poliovirus strains differ from those of neurovirulent poliovirus strains. This is evident in the example of the E marker (elution marker) as attenuated poliovirus is adsorbed to a DEAE-cellulose column at pH 7.1 while neurovirulent poliovirus is eluted from such a column (Hodes, Zepp & Ainbender, 1960). On the other hand, attenuation of polioviruses does not result in a change in antigenicity of the viruses (Diwan, Ozaki, Fulton & Benyesh-Melnick, 1963).

As the RNA extracted from attenuated polioviruses had the same thermolability characteristics as intact virus (T marker), Gerber & Kirschstein (1960) suggested that the infectious RNA carried the genetic information which determines the degree of neurovirulence. That an alteration in surface properties of polioviruses coincides with attenuation appears to be merely incidental as is illustrated by the results of Harter & Choppin (1965). They found that attenuated and neurovirulent strains of all three types of poliovirus adsorbed to human and rhesus monkey brain and spinal cord matter at similar rates, which did not support an hypothesis that attenuation of poliovirus reflects a failure of the virus to adsorb to cells of the primate nervous system. This suggested that the adsorption of polioviruses to cells was not involved in an explanation of attenuation.

While the alteration in the surface properties of attenuated poliovirus strains is thus not sufficient to explain the mechanism of attenuation, the isoelectric point marker, if indeed it be proved to be a genetic marker for neurovirulence, would be of considerable use in the identification of attenuated or unattenuated poliovirus strains.

7.5. The electrophoretic components of Coxsackieviruses.

The two electrophoretic components identified for Coxsackieviruses B1 and B3 (6.4.3.) do not appear to be identical with the complete and coreless particles observed for ecbovirus SA 1 (4.3.2.2.) in CsCl density gradients as the latter components had identical electrophoretic mobilities.

Human enterovirus preparations are known to contain components of different antigenicity. Le Bouvier (1955) first recognized this phenomenon and demonstrated that two antigens were present in preparations of all three types of polioviruses. The presence of these antigens was confirmed, and their physical characteristics determined, by Mayer et al. (1957), Roizman, Mayer & Rapp (1958), Hummeler & Hamparian (1958), Hummeler & Tumilowicz (1960) and Hummeler, Anderson & Brown (1962). The antigens were recognized by complement fixation and gel precipitin tests and were termed C and D antigens. The D antigens were faster sedimenting infectious particles, while the C antigens were slower sedimenting non-infectious particles containing no RNA. Kipps et al. (1957), by means of complement fixation tests, recognized the presence of two sedimenting components in preparations of MEF_1 poliovirus which they interpreted in terms of size. It is likely, however, that these two components were the D and C antigens of this virus and differed not in size but in density.

Schmidt & Lennette (1962) demonstrated the presence in preparations of group B and group A9 Coxsackieviruses of two antigens for each strain by means of the Ouchterlony gel double diffusion technique. While they were unable to obtain a satisfactory separation of the two antigens of Coxsackieviruses B4 and B6, they were able to show that one of the antigens was a group antigen, the other being

specific for the particular virus. Schmidt, Dennis, Frommhagen & Lennette (1963) separated the two antigens by centrifugation in a CsCl density gradient, demonstrating that they were comparable to the C and D antigens of the polioviruses. Frommhagen (1965) characterized the two antigens of Coxsackievirus B5 chemically and physically. Electron microscope studies with material prepared by shadow casting with uranium revealed that the C antigen showed a flattening, although the antigens were of the same size. Nucleic acid analyses revealed that the D antigen contained 22% RNA, while the C antigen contained less than 2% RNA.

In the light of these findings, it is likely that the "slow" and "fast" components found on submitting preparations of Coxsackieviruses B1 and B3 to density gradient zone electrophoresis (6.4.3.) correspond, respectively, to the C and D antigens of these viruses. As the isoelectric points of the two components in each instance differed considerably, it is likely that the D and C particles of Coxsackieviruses B1 and B3 have widely differing surface properties. The finding that both components were infectious for monkey kidney cells would tend to confirm the hypothesis (6.4.4.) that "complete" particles were trapped in aggregates of "coreless" particles, as the C antigens are not infectious.

Poliovirus particles of C antigenicity may or may not contain RNA (Roizmann _et al._, 1958). Such particles which contain RNA are not infectious, presumably due to a change in the basic structure of the RNA. While the C and D antigenic particles of the Coxsackieviruses have not been studied to the same extent as those of the polioviruses, it is probable that some Coxsackievirus particles which contain RNA are of the C antigenic type, and are thus noninfectious, as are the "empty" C antigenic particles.

The presence within an enterovirus particle of intact, infectious RNA must exert a stabilizing influence on the protein subunits which make up the capsid of the particle, due to ionic or hydrogen bonding, which confers on the protein subunits their quaternary, and perhaps even their tertiary, structure. This hypothesis ensures that particles of one virus type containing intact, infectious RNA would have identical antigenicity. If such particles contained no RNA, or contained RNA which had lost its infectivity and hence its basic structure, the net effect would be a change in at least the quaternary structure of the protein subunits, altering the antigenicity, and the net surface charge, of the particles. This hypothesis offers an explanation for the presumed aggregation of the "coreless" or C particles of Coxsackieviruses B1 and B3. While the net effect is one of decreasing the negative charge on the surface of these particles (which make up the "slow" components of these viruses), the alterations which may occur in the quaternary structure of the protein subunits may not all be identical, and there may be sufficient differences in the chemical and physical nature of the protein subunits on two adjacent particles for these two particles to be linked together. Furthermore, there may be similar differences in the chemical and physical nature of the protein subunits of adjacent C and D particles which would allow a linkage between the two particles. In this manner, aggregates of noninfectious C and infectious D antigenic particles could be formed.

Such an hypothesis would help to explain the observations that the C antigens of the polioviruses (Hummeler & Hamparian, 1958) and the Coxsackieviruses (Schmidt & Lennette, 1962) are group-specific. If the quaternary structure of the protein subunits making up the capsids of the particles were all altered, it is likely that some of these altered protein subunits on the surfaces of different

virus types would share a group identity. This is not an unreasonable suggestion when one considers that the RNA of the enteroviruses is the genetic determinant of these viruses, and alone carries the information for the replication of complete, infectious particles. The specific viral RNA contained in the infectious particles would thus confer a specific stability on the quaternary structure of the protein subunits of infectious particles, which would explain the type-specificity of these particles.

This hypothesis, i.e. that the nature of the surface of an enterovirus particle is determined by the quaternary structure of the protein subunits comprising the capsid, and that the quaternary structure of the protein subunits is determined by the viral RNA, furthermore indicates the relationship between the viral RNA and the protein capsid. The "slow" electrophoretic components of Coxsackieviruses B1 and B3, which are presumed to be aggregates of infectious D and noninfectious C antigenic particles, have higher isoelectric points than the "fast" components, which are presumed to consist solely of infectious D antigenic virions. This means that the C antigenic particles bear less net negative surface charge than the D antigenic particles. This in turn implies that more protons are present on the "inner" surfaces of the capsids of infectious D antigenic particles than are present on such surfaces of noninfectious C antigenic particles, indicating that the stability conferred on the quaternary structure of the protein subunits by the viral RNA is mediated by hydrogen bonds.

A single infectious electrophoretic component was detected in preparations of Coxsackievirus B4 (6.4.3.). It has been mentioned above that Schmidt & Lennette (1962) were unable to obtain satisfactory separation of the two antigens of Coxsackievirus B4. It is therefore likely that the two antigens associated with this virus have very

similar surface properties, or that the relative number of C particles present is very low.

The single infectious electrophoretic component of high isoelectric point determined for preparations of Coxsackievirus A9 (6.4.3.) may be explained in the following way. The C antigens, which are the group antigens for all Coxsackieviruses, should have more or less identical surface properties irrespective of the virus preparation. Thus the "slow" electrophoretic components of the Coxsackieviruses might be expected to have approximately similar isoelectric points, a result which has been found to be true as is seen for those components included in isoelectric point group 3 of Table 28 (6.4.4.). The inclusion of Coxsackievirus A9 in this group must mean that all the infectious D particles in preparations of this virus are trapped within aggregates of the noninfectious C particles which confer the high isoelectric point on this virus. It is thus possible that "uncontaminated" infectious D particles of Coxsackievirus A9, if this hypothesis is correct, have a lower isoelectric point which will allow them to be included in group 2 of Table 28. This further implies that only groups 1 and 2 of Table 28 are valid.

7.6. The effect of trypsin on the surface properties of enteroviruses.

The observation that treatment of virus with trypsin at a concentration of 1 mg/ml markedly increased the electrophoretic mobilities of both components of Coxsackievirus B1 (6.4.3.1.1.) and the "D" component of Coxsackievirus B3 (6.4.3.1.2.) may be of considerable significance if the initial reaction between these viruses and susceptible cells is electrostatic in nature.

Gard (1943) found that the infectivity of a preparation of murine encephalomyelitis virus (GDVII) was enhanced by treatment of the preparation with trypsin. Morris (1952) found that this virus, which before treatment agglutinated

human type 'O' red cells at 4° and not at higher temperatures, was able to haemagglutinate these red cells also at 20° after incubation with trypsin (0.1% solutions). Haemagglutinin recovered by elution at 37° after adsorption at 4° was able to haemagglutinate red cells at 20° after treatment of the haemagglutinin with trypsin, indicating that at least part of the tryptic action was directed against the haemagglutinin itself, i.e. the surface of this enterovirus.

Matheka & Mayr (1963) found that the infectivity of Teschen virus, a porcine enterovirus, which had been reduced by cold storage was "reactivated" by treatment with trypsin. The reactivation process appeared to be due to the digestion of a protein which had caused the virus to become aggregated, and did not appear to be due to a direct action on the surface of the particles. Usmankhodzhayev & Zakstelskaya (1964) found that treatment of reoviruses with trypsin unmasked haemagglutinins. This was presumed to be due to the destruction of non-specific mucoprotein inhibitors, as Lerner, Cherry & Finland (1963) found that the receptors on red cells forreoviruses were mucoproteins.

Whether the action of trypsin on the electrophoretic mobilities of Coxsackieviruses B1 and B3 (6.4.3.1.) was due to the removal of nonspecific protein inhibitors on the surfaces of particles, or was due to the direct action of trypsin on the surfaces of the particles, is not known. Trypsin hydrolyses only peptide bonds whose carbonyl group is contributed by an amino acid that has a positively charged side group, i.e. arginine or lysine (Neurath, 1964). The enzyme may hydrolyse the chain at every peptide bond adjacent to an arginine or lysine residue, giving rise to polypeptide fragments with arginine or lysine residues at the end . Arginine and lysine residues are present in hydrolysates of poliovirus protein capsid (Luria & Darnell,

1968). Arginine is present at a concentration of 6.6 g/100 g amino acid, and lysine is present at a concentration of 5.0 g/100 g amino acid.

When considering the route of infection in man of the enteroviruses, the observations that the net negative charge on the surfaces of Coxsackieviruses B 1 and B 3 was increased considerably by treatment of the viruses with trypsin may be of importance in natural infections with enteroviruses, especially those of the ileum which are presumed to occur through infection of the lymphoid cells of the Peyer's patches (Rhodes and Van Rooyen, 1968). In man, the concentration of trypsin in the intestinal tract is highest in the duodenum and proximal jejunum (Borgström, Dahlqvist, Lundh & Sjövall, 1957), being as high as 780 µg/ml (which compares with the concentration of 1 000 µg/ml used here). An enterovirus infecting man through the Payer's patches is thus subject to relatively high concentrations of trypsin in its passage through the duodenum and jejunum, which would have the effect of increasing the net negative surface charges on these particles (at least, in the case of Coxsackieviruses B1 and B3, and very likely applicable to all enteroviruses as they have so many properties in common), possibly resulting in an enhanced affinity for susceptible cells.

Finally, the results of these physico-chemical studies have confirmed that ecbovirus SA 1 is a typical member of the enterovirus group and indicate further relationships within this group of animal viruses. The echoviruses of human origin and ecboviruses of bovine origin appear to have a feature in common apart from the name "orphan". The full and empty particles found in preparations of ecbovirus SA 1 are electrophoretically inseparable and probably antigenically identical, similar results being found with echovirus 12 by Halperen _et al._ (1964). On the other hand

empty and full virus particles found in preparations of polio- and Coxsackieviruses have different antigenicities (Le Bouvier, 1955; Schmidt and Lennette, 1962).

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