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THE VIRUSES OF VERVET MONKEYS AND OF BABOONS IN SOUTH AFRICA

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ABSTRACT

In this thesis are presented briefly the results of studies extending over the period 1955 to 1974.

The use of vervet monkeys in South Africa for the production and testing of poliomyelitis vaccine made acquaintance with their viruses inevitable; and the subsequent introduction of the baboon as a laboratory animal of major importance also necessitates a knowledge of its viral flora.

Since 1934 when Sabin and Wright described the B Virus which was recovered from a fatal human infection contracted as the result of a macaque monkey bite, numerous viral agents have been isolated from monkeys and baboons. In the United States of America, Dr. Robert N. Hull initiated the classification of simian viruses in an SV (for Simian Virus) series according to cytopathic effects as seen in unstained infected tissue cultures. In South Africa, viruses recovered from monkeys and baboons were designated numerically in an SA (for Simian Agent) series on the basis of cytopathic changes seen in stained preparations of infected cells. Integration of these two series is in progress.

Simian viruses in South Africa have been recovered mainly through the inoculation of tissue cultures with material obtained by means of throat and rectal swabs, and also through the unmasking of latent agents present in kidney cells prepared as tissue cultures. Some evidence concerning viral activity has been derived from serological tests.

In this thesis the classification of simian viruses later proposed by Dr. Hull in 1968 has been adopted. This

comprises five categories based on nucleic acid type and strandedness, and on ether sensitivity. It has not been possible to provide conclusive proof for the inclusion of a number of South African viruses in the categories to which they have been assigned under this scheme, but the available evidence suffices to indicate that representatives of the five main categories occur in South Africa. Some viruses found in this country are recognized as prototype strains in the internationally recognized classification of simian viruses, and one vervet adenovirus has been widely distributed and studied for its oncogenic properties. The herpesvirus SA8 is sufficiently closely related to the dangerous B Virus of macaques to warrant extreme care in the handling of non-human primates. This virus has been recovered from baboons as well as vervet monkeys. It is becoming evident that viruses recovered from one species may eventually be found to be more common in another species.

In South Africa much remains to be done in the field of simian virology, and in this thesis are indicated techniques which could be further exploited and expanded to increase our knowledge of this group of potentially dangerous viruses.

Dedicated to

KARL HABEL

Victim of B Virus infection which has brought to an end the work of one of the most eminent virologists of our time.

ACKNOWLEDGEMENTS

The studies summarized in this thesis extended over the period between 1955 and 1974 while the candidate was Head of the Enteric Virus Research Unit and Head of the Poliomyelitis Vaccine Safety Testing Section at the Poliomyelitis Research Foundation in Johannesburg. It would be invidious to single out for special thanks any of the numerous assistants who have collaborated in these investigations, but grateful acknowledgement is made for the help received from all of them. Throughout the text, the term 'we' is used in recognition of this teamwork

Individual acknowledgements are made where required, when particular studies were carried out by others not under the direct supervision of the candidate. Thanks are due to the Board of Trustees and the Director of the Poliomyelitis Research Foundation for providing laboratory facilities and for bearing the cost of the photography. A special word of thanks is due to Mrs. A. Strong for typing this thesis.

ABBREVIATIONS USED

C	Degrees Celsius
CNS	Central Nervous System
CPE	Cytopathic Effect
H & E	Haematoxylin and Eosin
r.p.m.	Revolutions Per Minute
TCID50	Viral Dose Infective for Fifty per Cent of the Tissue Cultures Used
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
Log	Logarithmic Value

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1. INTRODUCTION

Virology expanded rapidly after the report by Enders, Weller and Robbins (1949) that poliovirus could be cultivated in vitro in non-neural human tissues. Extension of their techniques led to the identification of many viruses whose existence was previously unknown or only suspected; their detection being chiefly through observation of the changes produced in infected cells, before electron microscopy made possible visualization of the virus particles themselves.

The way was thus opened for the development of a vaccine against poliomyelitis. Use of the monkey, both as a source of tissue for cultivation of the virus and as a test animal for the efficacy and safety of the vaccine, made acquaintance with the viral flora of monkeys essential. It was chiefly in response to this need that identification of the viruses of South African vervet monkeys was pursued. Later, when the baboon gained prominence as a laboratory animal of major importance, it was felt appropriate that a start should be made in the study of its viruses. A number of other workers are now contributing to knowledge of the viruses of these primates, but much still remains to be done. This thesis describes mainly only the beginnings that were made as a pioneering effort, but it attempts to indicate also areas which need to be further developed.

In our own studies the exigencies of vaccine safety testing limited the extent to which detailed investigations could be carried out; and in many instances recognition of the presence of a virus through characteristic cellular changes had to suffice for practical purposes. Nevertheless, a number of South African simian viruses have been sent on request to laboratories in other countries to aid their research or to serve as prototype strains in the classification of viruses.

The term cytopathic effect (CPE) is usually applied to cellular changes perceptible through the light microscope. Chemical fixation followed by staining

increases the amount of detail to be seen, and frequently the observable changes are sufficiently characteristic to identify the type of virus responsible. On the other hand, some agents such as rubella virus may multiply in certain cells without causing visible changes, and other means must be adopted to detect their presence. In the search for viruses of simians in South Africa we have placed considerable reliance on observation of CPE in stained tissue cultures. The limitations of this technique are well understood, but the routine use of staining in the examination of infected tissue cultures has revealed much which would otherwise have passed undetected. In the majority of instances, the production of inclusions has indicated changes due to viral infection.

In his perspicacious article on intranuclear inclusions, Cowdry (1934) drew attention to the fact that cytologists and virologists employ the word 'inclusion' differently; the former applying it to bodies that have nothing to do with viruses, while virologists 'have simply appropriated the word' to indicate visible abnormal cellular material produced as a result of viral infection. The nature of viral inclusions varies considerably. They may mark the site of viral replication, as do the nuclear bodies of herpes-infected cells; or they may reflect abnormal metabolism at a distance from the place of viral assembly, as is possibly the case with the nuclear inclusions of picornavirus-infected cells. In the latter instance, the pale-staining zone in the cytoplasm where virus is being replicated is not usually referred to as an inclusion, although it has sufficient substance to displace and distort the nucleus.

The taxonomy of African monkeys does not appear to have reached finality. In the words of Hilleman (1960) 'Probably it is only the monkeys that understand the classification of the green monkey. This is a very confused situation. All the green monkeys belong to the species Cercopithecus aethiops, but there are twenty subspecies or races. By common parlance the South African green monkeys are usually called vervets, the Northeast

African greens are grivets, and the East African greens are called African green monkeys.' The term 'guenon' is commonly used to encompass most of the African cercopithecus monkeys.

In South Africa there are two kinds of vervet monkeys: the common green vervet, Cercopithecus aethiops pygerythrus, named for its peri-anal rufous hair; and the less common samango monkey, Cercopithecus mitis labiatus. Because of its relative rareness the samango is not generally used as a laboratory animal. On one occasion a troop of sixteen samangos was trapped in the Eastern Cape and sent to the Poliomyelitis Research Foundation where throat and rectal swabs were taken by us. From the throat of one samango the paramyxovirus SA10 was recovered. Cercopithecus aethiops pygerythrus is used in large numbers for the provision of kidneys for tissue cultures in poliomyelitis vaccine production as well as in vaccine safety testing and in routine diagnostic virology. In the text of this thesis, 'vervet' will be applied to C. aethiops pygerythrus, and 'samango' to C. mitis labiatus.

The chacma baboon, Papio ursinus, is widely distributed throughout South Africa, and it is now used extensively as a laboratory animal in surgical and immunological research. A major stimulus towards the study of baboon viruses was the possible hazard to animal handlers, particularly when baboons were given immunosuppressive drugs. Within the limits of our experience, serious viral infections in baboons have not followed immunosuppression; but the potential danger should not be disregarded. The viral flora of the baboon deserves intensive study, particularly in view of the herpesviruses already recovered from it.

In the United States of America, Dr. Robert N. Hull and his associates at the Lilly Research Laboratories in Indianapolis reported (Hull et al., 1956) the recovery of eight viruses from tissue cultures of monkey kidney cells. Thereafter, Hull's laboratory became the main reference centre for simian viruses. During the next

two years he published two more papers (Hull and Minner, 1957; Hull et al., 1958); bringing the number of viruses in his series, which was designated SV for Simian Virus, to twenty-six. These were divided into four groups based on CPE noted in unstained cultures. The SV sequence included viruses numbered up to fifty-nine, but certain numbers fell away when homology was established.

In South Africa, extensive use of the vervet monkey for poliovirus vaccine production and testing from 1955 onwards yielded a number of viruses. Initially we described (Malherbe and Harwin, 1957) characteristic cytopathic changes forming seven categories in a series designated SA for Simian Agent. Six of the agents responsible for these changes were recovered from uninoculated monkey kidney cells. In the following year we noted (Malherbe and Harwin, 1958) the isolation from vervets of a herpesvirus related to the B Virus of macaque monkeys, which we numbered SA8. By 1962 a further four categories were added to the SA series (Malherbe, 1962); and in 1963 we extended it to SA15, publishing coloured photomicrographs of the changes seen in stained cultures (Malherbe, Harwin, and Ulrich, 1963).

Interchange of viruses between Hull's laboratory and our own was established for comparative studies; but it was agreed that for practical reasons the arrangement in SV and SA series should be retained for the time being. It is probable that the classification of viruses proposed by the International Committee for the Nomenclature of Viruses (ICNV), recently summarized by Wildy (1971) will be generally adopted; and within the WHO/FAO Programme on Comparative Virology, the Working Team for Simian Viruses is currently using the ICNV classification as the basis for the nomenclature of prototype simian virus strains.

In an important monograph, Hull (1968) reviewed the existing information about simian viruses. He proposed that these agents be classified under five headings based on nucleic acid type and strandedness, and on ether sensitivity. These five headings have been adopted for

the discussion of South African simian viruses in this thesis.

The SA grouping was based mainly on changes observed in the infected cell, and initially took little cognizance of the physical and chemical properties of the viruses or of their serological differences. Under the broad SA headings will undoubtedly be found serologically distinct strains among the numerous isolates stored for further study. The application of techniques such as organ culture of respiratory epithelium should greatly extend our knowledge of simian respiratory viruses; while the prolonged cultivation of explants of various tissues would probably reveal a wide range of viruses latent throughout the bodies of monkeys and baboons: the number of agents already recovered from the kidney is indicative of the potential yield from other sites.

Monkeys and baboons to some extent share environments with each other and with man, both before and after capture ; and it is not yet possible to state with certainty that a particular virus is peculiar to the primate species from which it was isolated. There is evidence that certain human viruses are readily conveyed to monkeys following capture; and from our own limited studies there emerges the possibility that some viruses first recovered from monkeys may prove to occur more commonly in baboons.

South Africa is specially favoured in having virus laboratories within reasonable distance of a number of trapping sites, and on several occasions we took specimens from monkeys and baboons shortly after capture. This approach deserves further attention, particularly since the acquisition of fresh virus infections proceeds rapidly with increased contact and stress following capture and transport.

2. MATERIALS AND METHODS

The procedures outlined here represent basic methods

which were followed with occasional minor modifications.

Laboratory Records

The laboratory records giving details of relevant tests and experiments carried out in the Enteric Virus Research Unit and the Poliomyelitis Vaccine Safety Testing Section of the Poliomyelitis Research Foundation from 1955 to 1974 are intact at the time of submission of this thesis. Many thousands of stained coverslip cultures showing positive findings were kept from 1957 onwards, and some of the illustrations record the appearance of infected tissue cultures involved in early passages of viruses.

Tissue Cultures

Primary vervet kidney cells comprised the main testing system, since they are susceptible to a wide range of simian and human viruses. The limitations in using relatively few testing systems are acknowledged, but one versatile system adequately applied outweighs multiple systems yielding little additional information for much extra trouble and expense. Some viruses have only single cycles in certain cell types: inclusions may be produced, but incomplete and therefore non-infectious virus particles are released. Such a system can nevertheless be useful for detecting the presence of an agent when virus yield in the harvested fluid is of no importance.

(a) Primary Cell Cultures

Trypsin-dispersed vervet kidney cells were grown as monolayer tissue cultures in stationary bottles or in roller tubes. The tubes usually contained free coverslips forming part of the cultures. A fluid nutrient medium based on Hanks' balanced salt solution, with 0,5 per cent lactalbumen hydrolysate and 0,5 to 5,0 per cent horse or calf serum, was used.

Primary rabbit kidney or human amnion cells were grown in M150 medium containing calf serum.

(b) Continuous Cell Line Cultures

Various animal and human cell lines were used to test the spectrum of susceptible cells. These included the W138 line of foetal human lung fibroblasts developed at the Wistar Institute in Philadelphia, and the LLC-MK2 line of rhesus kidney cells obtained from the Lilly Research Laboratories in Indianapolis. Adult horse serum, in later years replaced by foetal or unsuckled calf serum, was used to supplement the tissue culture media. Serum was heated at 56C for thirty minutes to inactivate non-specific inhibiting substances and to reduce the possibility of introducing equine or bovine viruses. Antibiotics in suitable concentrations were included in the media to inhibit bacterial contaminants.

Throat and Rectal Swabs

These were agitated in two millilitres of gelatin-buffered-saline solution containing 750 units of penicillin, 750 micrograms of streptomycin, 500 micrograms of neomycin, and 200 units of mycostatin per millilitre. The suspensions were then centrifuged at 2500 r.p.m. for thirty minutes, and the supernatant fluids used.

Blood

Blood for virus isolation was collected in a plain tube and allowed to clot. The serum, together with some free red blood cells, was used as inoculum for tissue cultures.

Inoculation of Tissue Cultures

When large volumes of virus fluids were needed, tissue culture bottles inoculated with 0,5 to 2,0 millilitres of infective fluid were used. It was found that roller tubes provided a more sensitive system than stationary cultures, and in the majority of investigations roller tubes were used with or without free coverslips. Cultures were incubated at approximately 37C for varying periods up to thirty days, with fluid changes at five to seven day intervals. At the termination of an experiment,

cells were scraped from the sides of the vessel and harvested with the fluid medium. Harvests were usually stored at -20C or -70C without preservatives.

Staining of Coverslip Cultures

Immediately after virus fluid was harvested, five to seven millilitre amounts of Bouin's fixative were introduced into the tubes containing the coverslip cultures. The fixative was allowed to act for at least one hour, and was then replaced by seventy per cent ethyl alcohol in water for at least twelve hours. This procedure was adopted in view of the recommendation of Reissig and Melnick (1955) concerning the measures required to ensure shrinkage of inclusion material to render it more distinct. In the staining technique, Ehrlich's haematoxylin was followed by very rapid differentiation in acid-alcohol solution, the rinsed culture then being stained with one per cent alcohol-soluble eosin. The coverslip was mounted in polystyrene dissolved in dibutyl phthalate and xylene.

Virus Titration

Titration of virus was carried out in two to four roller tube cultures for each tenfold dilution. Tests were terminated at periods appropriate for each virus, usually after seven to ten days. In most instances the unstained CPE determined the endpoint; but when extreme accuracy was required, or when agents otherwise undetectable such as the Marburg virus were titrated, coverslip cultures were stained to ascertain the endpoint. The method of Reed and Muench (1938) was employed to calculate the viral dose infective for fifty per cent of the tissue cultures used (TCID50). Titres were expressed as numbers of TCID50 per unit volume, the standard inoculum for a roller tube culture being 0,1 millilitre.

Neutralization Tests

Antisera were routinely inactivated at 56C for thirty minutes. Virus suspensions were usually centrifuged at

2500 r.p.m. for thirty minutes, the supernatant fluid being used. Serum-virus mixtures were held for periods varying from one hour at 37C to two hours at room temperature with overnight storage at 4C, depending on the lability of the virus tested. A virus challenge dose of 100 TCID50 for each tissue culture tube was regarded as optimal; but as is well-known, this is often not possible to achieve precisely. The challenge dose was checked by means of back-titration of non-neutralized virus at the time of performance of the test.

Biochemical Tests

These conformed to internationally accepted procedures. The standard book of reference was that edited by Lennette and Schmidt (1964).

Small Animals

Mice, guineapigs and rabbits were obtained from colonies maintained by the S.A. Institute for Medical Research. Syrian golden hamsters were reared by us from stock obtained from the Sloan-Kettering Institute for Cancer Research, New York.

Anaesthetics for Monkeys and Baboons

Initially, hexobarbitone sodium administered intravenously was used; but later phencyclidine hydrochloride became available and was given intramuscularly.

Histological Sections

Most of the animal tissues taken at autopsy for histological study were processed and stained by Mrs. Z. Kirsch and her assistants in the Histology Unit of the Poliomyelitis Research Foundation. Examination of the tissue sections was done by the candidate.

Photomicrographs

Stained tissue cultures were photographed under the light microscope by the candidate, using a Wild M20

microscope fitted with a Nikon Microflex Model EFM attachment for Polaroid camera, with built-in light meter. Polacolor Type 108 Land Film was exposed for one second at an ASA setting of seventy five. Clear blue filters were inserted at the light source which was a twenty-four volt 150-watt quartz-iodine projector lamp in a metal housing. After removal from the camera, each print was allowed to develop for one minute before it was peeled off to dry. Marked variations in colour occurred with minor adjustments of light transmission as well as with different batches of Polacolor film. Polaroid photography involves sacrifice of definition of detail for the convenience of instant results.

Other colour photographs were printed by Kodak Limited from transparencies prepared by Mr. M. Ulrich of the Photographic Department of the S.A. Institute for Medical Research. Mr. Ulrich also made the black-and-white reproductions of electron micrographs.

Through the courtesy of Professor K.E. Weiss, Director of the Veterinary Research Institute, and Professor D. Verwoerd, Head of the Molecular Biology Section, at Onderstepoort, electron micrography of tissue culture materials provided by us was carried out by Dr. G. Lecatsas using a Siemens 1A electron microscope.

The electron micrograph of SA7 adenovirus was taken with an Hitachi Model Hu 1113 microscope at Baylor College of Medicine in Houston, Texas, and it has not been published elsewhere. This micrograph was sent to us by Professor W.E. Rawls who gave permission for its inclusio- in this thesis.

3. DNA ETHER RESISTANT VIRUSES

Under this heading Hull placed adenoviruses and papovaviruses.

Adenoviruses

The category designated SA7 by us in 1957 comprised

adenovirus strains. By 1970 we had made over two hundred isolations of adenoviruses from vervet monkeys and baboons; recovery of virus from the gut being six times more frequent than from the throat, suggesting that multiplication in the pharynx is of shorter duration than in the gut.

Simian adenoviruses produce characteristic nuclear inclusions in vervet kidney cells. Fig. 1 illustrates the appearance of an uninfected vervet kidney monolayer tissue culture stained with haematoxylin and eosin; and the subsequent plates of virus-infected tissue culture cells should be compared with it. Fig. 2 shows a vervet kidney culture infected with the simian adenovirus SA7 strain C8, with nuclear inclusions at different stages of development. The final form of the inclusion is that of a dense basophilic body surrounded by a retraction space or halo within the nuclear membrane, together with eosinophilic inclusion material lying in this space next to the nuclear membrane. Two such late-stage inclusions are seen in this plate. Some simian adenovirus strains also produce small deeply-staining eosinophilic cytoplasmic inclusions in vervet kidney cells.

The most important vervet adenovirus isolated is that designated SA7 strain C8, commonly referred to as SA7 in the numerous studies carried out on it in this country and overseas. No attempt will be made here to review the extensive work done on this virus. Strain C8 was recovered from a rectal swab taken from vervet monkey No. 865 in Cage 8 during the course of an experiment in November 1955 to test whether any of ten monkeys given a measured dose of Mahoney strain virulent Type 1 poliovirus by gastric catheter would excrete the same virus. Rectal swabs and blood samples for viraemia were taken daily for the ten-day duration of the test. Prior to the start of the poliovirus feeding, rectal and blood specimens were negative, but shortly after commencement of the experiment, adenovirus infection showed itself in the animals, finally involving eight of them. All the blood samples were negative. One monkey excreted adenovirus daily for the duration of the experiment, while seven others excreted

adenovirus intermittently. Poliovirus was not recovered. The virus fluid administered was checked to ensure that only poliovirus was present in it and that SV40 was absent.

Adenovirus recovered from the gut of the monkey in cage 8 was sent to Hull who included it among the eighteen simian adenoviruses tested by him and his colleagues (Hull et al., 1965) for oncogenicity in newborn hamsters. They found that five of seven adenoviruses from macaque (rhesus or cynomolgus) monkeys produced clinically detectable tumours in hamsters over periods ranging from fifty-three to 280 days, whereas the vervet C8 strain caused tumours which were visible within thirty-one to forty-one days. This virus was also oncogenic in suckling rats. We subsequently confirmed the oncogenicity of SA7C8, and Fig. 3 shows a hamster which had been inoculated by us subcutaneously at twenty-four hours of age less than thirty days previously.

The ability of the virus to transform hamster cells in vitro was reported by Whitcutt and Gear in 1968. Fig. 4 shows a focus of transformed cells in a tissue culture made from the skin of a forty-eight-hour-old hamster prepared and inoculated with SA7C8 six days previously by Dr. J.M. Whitcutt and photographed by us. We have observed that during the period in which clones of morphologically altered cells are forming the transformation foci, adenovirus nuclear inclusions can be seen in cells at a distance from the foci, but no inclusions are found in the transformed cells. Further studies on in vivo tumour-producing properties of the cells which have been transformed in vitro, and on the chromosomal abnormalities of the transformed cells, have been published by Bey and Whitcutt (1972).

SA7C8 virus has been widely distributed to workers in other countries. Careful studies have confirmed that there is no admixture of other agents, and Fig. 5 shows details of the virion in an unpublished electron micrograph taken at Baylor College of Medicine in Houston, Texas. It is of interest to note that Rapoza (1967) reported that of eighteen simian adenovirus prototype strains studied in

order to classify them by haemagglutinating properties, SA7C8 alone comprised his Group 4 in which no haemagglutination whatsoever of rat, rhesus or guineapig red cells was observed.

Antiserum to SA7C8 was prepared in rabbits by us, and in neutralization tests a number of vervet isolates were found to be serologically of the same type. Among the vervet adenoviruses not neutralized by C8 antiserum two strains, B105 and C626, were found to differ serologically also from each other. Since the adenoviruses in Hull's SV series include sixteen serotypes, it is highly probable that more serologically distinct types will be found in due course among the vervet adenoviruses.

Papovaviruses

The name papovavirus was coined by Melnick (1962). This group of agents includes the papilloma viruses of rabbits and other animals, the polyoma virus of mice, and the vacuolating virus of monkeys designated SV40. No papovavirus has been indentified with certainty as originating from South African primates. It may, however, be appropriate to mention here our experience with SV40 and the agent we have called SA12.

Sweet and Hilleman (1960) first described the virus placed in Hull's series as SV40. It originated from macaque monkeys and contaminated live poliovirus and adenovirus vaccines prepared in the United States. There is no convincing evidence that the virus occurs naturally in African primates, but Meyer et al. (1962a) experimentally infected three vervet monkeys by the nasal route, and SV40 was still present in the kidneys of all three monkeys when they were sacrificed four months after infection. The potential peril of introducing SV40 accidentally into the cercopithecus population of Africa is obvious.

We observed the virus as a contaminant in tissue culture fluids received from the United States in 1957 and the succeeding years; but being then in our salad days, green in judgement, we did not publish the finding. Fig. 6 illustrates the characteristic nuclear changes seen in

October 1957, in the second passage on vervet kidney of SV40 virus isolated from fluid sent to us from the United States as a harvest of human enterovirus. While cytoplasmic vacuolation is characteristic of SV40 infection of vervet kidney cells, the basophilic and usually ill-defined nuclear inclusions are a more constant sign of infection and can readily be seen in stained cultures.

We were thus well-acquainted with the virus when the first batches of seed virus received for oral poliomyelitis vaccine production were found to be contaminated with it. Until SV40 had been eliminated from the seed virus stocks in 1962 by means of neutralization with specific anti-SV40 serum, this papovavirus was present in our vaccine production and testing areas. In April 1960 a set of uninoculated control vervet kidney tissue culture bottles, comprising a total of eighty-one control bottles from eleven individual monkey kidney batches, was submitted for safety testing, and from one of these bottles there was isolated an agent which we designated SA12 (Malherbe, 1962). The nuclear changes it produced resembled somewhat those of SV40, but they tended to be less hazy in outline, as shown in Fig. 7. We subjected this virus to a number of tests and found that it was not neutralized by our anti-SV40 serum and that it did not produce tumours in hamsters. Hull (1968) with marked reservations included SA12 as a possible candidate in the simian papovavirus group on the basis of the similarity between the nuclear inclusions of SV40 and SA12.

In 1972 we again infected vervet kidney cells with SA12 for examination under the electron microscope by Dr. G. Lecatsas of Onderstepoort. The culture fluid contained numerous small virions resembling SV40 when negatively stained with phosphotungstic acid (Fig. 8), but thin sections of infected cells revealed two types of virus particles. Those situated in the nucleus and inside the endoplasmic reticulum measured approximately forty-five nanometers in diameter; while those lying free in the cytoplasm were larger, having a diameter of about fifty-three nanometers, and they appeared to possess ar

outer envelope. Fig. 9 shows the smaller virions in the nucleus and the larger particles in the cytoplasm. Fig. 10 further illustrates the larger particles with their envelopes. In Fig. 11 are seen the smaller virions within the endoplasmic reticulum. Examination of uninoculated cells of the same tissue batch did not show any latent contaminating virus. It was then considered probable that SA12 may be a mixture of SV40 and a second agent derived from the kidney tissue of the original bottle culture of April 1960. This consideration led us to re-examine the stained coverslip culture in which SA12 had first been noted. It was now seen that about seventy per cent of the cells had nuclear inclusions, and about five per cent had small basophilic cytoplasmic inclusions which had not been observed when the coverslip was examined in 1960. Fig. 12 shows a cell with both nuclear and cytoplasmic inclusions in the first passage form the original bottle culture. We briefly reported these findings in 1973 (Malherbe and Strickland-Cholmley, 1973).

Skin papillomas of vervet monkeys have been observed infrequently by us, but the limited studies carried out on them have not indicated a virus aetiology.

4. DNA ETHER SENSITIVE VIRUSES

Under this heading Hull placed simian herpesviruses and poxviruses. He subdivided the herpesviruses into Groups A and B; and there are representatives of both these groups among the South African viruses. We have not encountered simian poxviruses in this country.

Herpesvirus Group A

In 1958 we reported (Malherbe and Harwin, 1958) the isolation of SA8, now recognized as a prototype within the group which includes the B Virus of macaque monkeys and other herpesviruses more recently recovered from South American primates.

Sabin and Wright (1934) described the simian

herpesvirus which they called the B Virus, presumably from the initial of the patient W.B. from whom the virus was recovered after his death following the bite of a rhesus monkey in 1932. The virus has subsequently been recognized as occurring in nature in rhesus (Macaca mulatta) and cynomolgus (Macaca irus) monkeys, producing in these animals a mild self-limiting clinical disease manifested by oro-pharyngeal ulceration; but the agent can remain latent in the kidney for prolonged periods. The B virus is highly dangerous to man, affecting especially the central nervous system. The result is death or complete incapacitation through prolonged coma or extensive paralysis. Some human infections have followed monkey bites; but in some instances there was no known preceding injury, so presumably the virus can enter by the conjunctival or oropharyngeal routes. In a recent case, the first manifestation was that of herpes zoster, B virus being isolated from the skin lesions. Paralysis and complete permanent disability followed.

The danger of B virus infection from macaque monkeys has considerably complicated the production of poliovirus vaccine prepared from the kidneys of these monkeys, as well as the vaccine neurovirulence test carried out in them. Africa is singularly fortunate in thus far having no natural infection with B virus in its simians; but there still exists the danger that it may one day be spread from imported macaques. SA8 virus is sufficiently closely related to B virus to warrant very careful precautions when handling African monkeys and baboons.

SA8 virus was first isolated by us in July 1957 from a vervet monkey No. B264 which had been inoculated with formalin-inactivated poliovirus vaccine by the combined intramuscular, intracerebral, and intraspinal routes, according to the prescribed safety testing regulations. Paralysis of the left leg due to trauma at the time of inoculation was noted over the first ten days; but the right leg became paralysed on the eleventh day, remaining so until the animal was sacrificed on the eighteenth day. Pooled tissues from cerebral cortex, lumbar cord, and

cervical cord were made into suspensions for the inoculation of vervet kidney cell cultures, and a virus producing the CPE characteristic of B virus was isolated. Histological examination of the central nervous system of this monkey revealed marked destruction of both grey and white matter in the lumbar cord (Fig. 13) with inflammatory infiltration by lymphocytes and monocytes. The thoracic cord was also severely affected, but the cervical cord and medulla showed only a moderate degree of inflammation. A few lesions were present in the pons, but none occurred above it. Pre-inoculation serum from the monkey contained no detectable SA8 antibody and was negative for viraemia. Terminal blood taken on the eighteenth day contained antibody against SA8. Exhaustive tests confirmed that the vaccine inoculated was not contaminated with SA8, and it is probable that the trauma of the intraspinal injection of inactivated vaccine precipitated viral multiplication in an area of the cord where SA8 was latent.

One year later, in August 1958, our second isolation of SA8 was made under similar circumstances. In the course of a safety test on another batch of formalin-inactivated poliovirus vaccine, monkey No. B710 was inoculated by the prescribed triple route. Weakness of both legs due to the trauma of intraspinal injection was seen during the first seven days, and on the eighth day the left leg became paralysed. The animal was immediately sacrificed, and SA8 virus was isolated from pooled central nervous tissues. Extensive damage to both grey and white matter was found in the lumbar cord, but at higher levels up to the pons only mild inflammatory changes were present. As in the first case, the infiltrating cells were chiefly lymphocytes and monocytes.

Two more isolates of SA8 virus have been made by us: strain B188 from a vervet throat swab taken in 1966; and strain W16 from a baboon rectal swab taken in 1969 (Malherbe and Strickland-Cholmley, 1969a). Becker and Baker (1971) reported the recovery of a herpesvirus serologically related to B virus from the saliva of a baboon in Cape Town. Since their virus was not invariably fatal for rabbits, it

was probably a strain of SA8. Kalter (1970) stated that his team in the United States had recovered SA8 on five occasions from baboon rectal swabs, as well as once from a baboon vaginal swab,and once from oral lesions in a baboon. Kalter and Heberling (1971) found SA8 antibody in the sera of forty-two baboons sent from Kenya to Texas; but since eleven of forty macaques tested also had SA8 antibody, the source of infection in the baboons is not absolutely certain. However, this evidence does suggest that SA8 infection may occur far north of South Africa.

Vervet kidney tissue cultures infected with SA8 show nuclear inclusions in most of the cells after the first day, and by the second day virtually all cells are taken up in syncytia which range in size from small to large. Fig. 14 illustrates the advanced changes seen on the second day after infection in a vervet kidney cell culture; and Fig. 15 shows, at a higher magnification, another culture similarly harvested on the second day following infection. The inclusions are typically herpetic, with chromatin distributed in small clumps throughout the inclusion material before the inclusion has contracted. Nuclear bubbles, somewhat resembling those produced by adenovirus, may also be seen. Other cell types can be infected with SA8, but syncytia develop most rapidly in vervet kidney cell cultures.

In his monograph, Hull (1968) stated that no information was then available on the susceptibility to SA8 of human cells or of the continuous rhesus kidney cell line LLC-MK2. We have infected human foetal diploid lung cells with SA8: by the fourth day the culture sheet was largely intact but virtually every cell nucleus contained an inclusion with the characteristic stippled chromatin in it, and syncytia were scanty and small. The KB line of human carcinoma cells yielded SA8 titres of $10^{5,5}$ and $10^{6,5}$ TCID50 per 0,1 millilitre. We also infected primary human amnion cell cultures with SA8: by the thirteenth day there had appeared foci of infected cells and small syncytia, cells normal in appearance remaining between the foci. In a continuous line of human amnion cells the viral change was more rapid, being extensive by the second

day. In LLC-MK2 rhesus cells the changes were slower than in vervet kidney, complete involvement in syncytia being reached only on the fourth day; but a titre of $10^{5,5}$ per 0,1 millilitre was obtained. In primary chick embryo cells infected by us with SA8 widespread changes with nuclear inclusions were present by the second day, but no syncytia were seen.

Hull noted that in rabbit kidney cells B virus produced a CPE quite unlike that seen in monkey kidney cells: the rabbit cells rounded up without enlargement into giant cells. We have infected primary rabbit kidney cells with SA8, and after the first day destruction was far advanced. Virtually every nucleus had an inclusion, and in the stained preparation very small syncytia could be seen which might not have been noticed in the unstained culture. Thus the CPE produced by SA8 in rabbit kidney cells corresponds relatively closely to that seen by Hull in unstained B-virus-infected rabbit kidney cultures.

SA8 virus is completely inactivated by heating at 50C for one hour, or 56C for fifteen minutes. Molar magnesium chloride at 4C for one hour did not reduce the virus titre, but it did not protect against viral inactivation at 50C for one hour. SA8 virus was not completely inactivated by exposure to 1:4000 formaldehyde concentration at room temperature for nine days. Our SA8 harvests remained viable at -20C for at least eight and a half years, without any preservative. No significant drop in titre was noted in harvests held at 4C for seven weeks; but at 37C the titre fell by one log after twenty-four hours, and after four days at 37C no virus could be detected.

Not wishing to handle B Virus in South Africa, we did not use it as a challenge virus, but we did set SA8 against anti-B-virus sera obtained from several sources. Hull carried out cross-neutralization tests using B Virus, SA8, and other herpesviruses as challenge viruses. In his 1968 monograph, Hull shows in his Table 14 the results of such tests. These indicate that while B virus and SA8 antisera will neutralize B virus, SA8 and Herpes simplex viruses,

there is no neutralization of the two simian viruses by Herpes simplex antiserum. Thus the presence of Herpes simplex antibody in man is unlikely to afford protection against B virus or SA8. Neutralization tests on the simian herpesviruses require complement in order to demonstrate satisfactory antibody levels.

In rabbits, B virus inoculated intradermally on the thorax or abdomen invariably produces skin lesions which are followed by hind-leg paralysis and death. In Hull's tests, SA8 gave only skin lesions in rabbits; but our inoculation of thirty-one rabbits with SA8 by either the intradermal or subcutaneous routes resulted in skin ulceration at the site of inoculation during the second week in most of the animals, and in fifty per cent of the rabbits there was hind-limb weakness which in some cases progressed to paralysis. Several paralysed rabbits were sacrificed by us for viral investigation of the central nervous system, but two paralysed rabbits left unsacrificed died. Thus although death is not inevitable in rabbits inoculated with SA8 as it is with B virus, it can nevertheless occur.

In passing, it may be of interest to note that Sabin and Wright (1934) found that the histological examination of B-virus-infected rabbits was complicated by the presence in the central nervous system of inflammatory lesions caused by Encephalitozoon cuniculi which was endemic in their rabbits. We encountered the same problem when we were investigating SA8 in rabbits, which led us to publish the first description of E. cuniculi in rabbits and mice in South Africa (Malherbe and Munday, 1958). Acquaintance with this parasite, now called Nosema cuniculi, is essential for the correct interpretation of results when virus experiments are carried out on rabbits or mice derived from colonies in which the parasite is endemic.

Both Sabin (1934) and Hull (1968) noted that B virus causes encephalitis in only occasional mice inoculated with it, and that passage of brain material from such mice is negative. We inoculated SA8 intracerebrally into 108 four-to-six-week-old mice and found no signs of illness during the observation periods of twenty-three to

twenty-eight days.

No information is available from other workers concerning the behaviour of SA8 in newborn mice. We inoculated two litters each comprising six twenty-four-hour-old white mice, each mouse receiving 0,01 millilitre SA8 virus intracerebrally. Weakness, paralysis or death occurred in ten of the twelve mice between the third and eighth days after inoculation. Four of the weak or paralysed mice were sacrificed for histological examination: all showed extensive areas of necrosis in the brain with numerous herpetic retracted nuclear inclusions, chiefly in neurones but also occurring in glial cells in the vicinity of the necrotic areas. The virtually total absence of inflammatory cellular reaction was a striking feature. Cerebellar involvement also was found, but in those instances where spinal cord sections were examined no lesions were seen. The two remaining mice became sick after the twenty-first day: one was discarded, and the other was sacrificed for histological examination. A calcifying area of necrosis was present in the cerebrum; but inflammatory reaction was absent, and there were no lesions in the five spinal cord levels examined. We also inoculated six twenty-four-hour-old white mice by the combined intraperitoneal and subcutaneous routes, but no evidence of infection was found.

It is well-known that in the natural infection of macaque monkeys with B virus there is a self-limiting occurrence of oral ulcers, but the virus remains latent in the kidney. Information concerning transmission of B virus to cercopithecus monkeys appears to be confined to the incident cited by Hull (1968) concerning which Dr. Ruth Kirschstein, of the United States Food and Drug Administration, kindly supplied us with details in a personal communication. During the course of an experimen carried out for another purpose, B virus was accidentally transmitted from rhesus to vervet and patas monkeys by th oral route. The only evidence of infection was recovery o the virus from throat swabs taken from these monkeys, and the period of excretion did not extend beyond two weeks.

The circumstances of the first two isolations of SA8 from vervet monkeys have been described above. We experimentally infected four monkeys which had been pre-tested to ensure absence of antibody against SA8. One vervet and one samango were given high-titre SA8 orally under anaesthesia to be certain of access to the pharynx; another vervet was inoculated subcutaneously with 0,5 millilitre and intramuscularly with 0,5 millilitre of SA8 in one leg; while a second samango was given 0,5 millilitre intradermally in divided doses. The four monkeys were observed for twenty-one days and then sacrificed. There was no histological evidence of SA8 infection in the CNS or viscera in any of these animals. Of nine monkeys tested for SA8 antibody prior to the above experiment, five vervets were positive and were thus excluded from the experiment. SA8 has never been isolated from the many thousands of vervet kidney batches prepared for tissue cultures at the Poliomyelitis Research Foundation. Thus the virus appears to differ significantly from B virus in that while infection may be relatively common in vervets, latency in the kidney is virtually absent and oral ulceration due to SA8 has not been observed. It seems probable that natural infections with SA8 in monkeys and baboons in South Africa occur with little or no clinical illness.

In a personal communication, Hull informed us of an African green monkey which was found to have an anti-SA8 serum titre of 1:4 but was negative for antibodies against other herpesviruses. The animal was inoculated with SA8 intradermally and by scarification of the lip. Small lesions developed at the sites of inoculation, and three weeks later the antibody titres for SA8, B virus, and Herpes simplex had all risen to 1:2048. In another personal communication Hull stated to us that rabbits hyper-immunized with Herpes simplex virus can develop antibody to B virus which protects them against challenge with B virus. There is no doubt that B virus, SA8 and the human Herpes simplex virus are interrelated; but interpretation of the relationships depends upon the testing systems used.

Herpesvirus Group B

The name cytomegalovirus was introduced by Weller et al., (1960), the agent up to that time being known as salivary gland virus. Strains of guineapig and murine origin had been cultivated by various workers in homologous tissue cultures, and Smith (1956) propagated the human virus in fibroblast cultures.

We recovered similar agents from uninoculated vervet kidney tissue cultures twice in 1956 and twice in 1957. Our report (Malherbe and Harwin, 1957) constituted the first description of the cultivation of simian viruses belonging to this group: we placed them in the category SA6, and illustrated the characteristic nuclear inclusions and the formation of giant cells by cytophagocytosis. Although the virus sent to Hull did not survive the journey (Hull, 1968), other workers successfully cultivated SA6 sent to them; and our strain ST 383, which had been isolated from an uninoculated vervet kidney tissue culture in 1960, is accepted as the prototype of simian cytomegalovirus. Black et al., (1963) also reported recovery of cytomegalovirus from salivary gland and kidney tissues of African cercopithecus monkeys.

We have frequently isolated latent cytomegalovirus from uninoculated vervet kidney tissue cultures; and from 411 vervet throat swabs tested between 1958 and 1969, thirteen isolations of cytomegalovirus were made. Fig. 16 illustrates the characteristic CPE seen in vervet kidney cells. The nuclear changes typically begin as deeply-staining inclusions appearing on one side or both sides of the nucleoli; the inclusions then enlarge or fuse to form an elongated mass. The infected cell tends to round up, and the cytoplasm stains purple-red. There occurs ingestion of one infected cell by another, leading to the formation of giant cells containing nuclei with inclusions. This process of cytophagocytosis is entirely different from that involved in the formation of a syncytium in which cytoplasmic membranes of contiguous cells are lysed by enzymatic action and the nuclei drift together. Such syncytium formation can be caused by a number of viruses

including other types of herpesvirus.

The presence of cytomegalovirus in the kidney illustrates the fact that the infection is systemic and blood-borne. Various viscera are infected, including the salivary glands which comprise a useful index of the incidence of cytomegalovirus infection. Fig. 17 shows a histological section of a vervet parotid gland in which can be seen a cell with the typical purple-red-staining nuclear inclusion surrounded by a retraction halo within the nuclear membrane. Around the nucleus is the deeply-staining cytoplasm which contrasts clearly with the paler uninfected cells. Such evidence of cytomegalovirus infection has been found by us in both pygerythrus and samango vervets, as well as in baboons. In one series, the salivary glands of twenty-six of forty vervets examined histologically showed cytomegalovirus inclusions, and the virus was recovered in tissue cultures inoculated with suspensions of three pools of parotid tissue taken from a total of fifteen of the monkeys in this series (Malherbe, 1962).

In 1970 we sent fifty-five vervet sera to Dr. P.D. Parkman, then Head of the Section on General Virology at the Division of Biologics Standards, United States National Institutes of Health, at his request for cytomegalovirus antibody studies. In a personal communication, Dr. Parkman stated that by complement fixation tests the sera showed an unexpectedly high incidence of cytomegalovirus antibody, fifty-three of the fifty-five sera being positive for one or both of two cytomegalovirus antigens used. The fact that sera reacted differently to different strains of cytomegalovirus antigen suggests that more than one type of cytomegalovirus is active in South Africa.

We examined histologically salivary glands taken from twenty-seven baboons, and of these four showed cytomegalovirus inclusions. Further evidence of the presence of the virus in baboons was obtained through isolation of cytomegalovirus by us from the rectal swab of a baboon in 1962.

Unclassified Herpesviruses

The virus designated SA15 (Malherbe et al., 1963) was isolated in 1962 in the course of a poliomyelitis vaccine safety test No. 2SN27, originating from the vervet kidney cells used. It occurred in one roller tube in a subinoculation from a culture bottle, and recovery from the fluid harvested from this tube was repeated successfully twice. Since horse serum had been introduced into the cultures, it was considered possible that the virus may have been of equine origin. Through the courtesy of Dr. B.J. Erasmus, of the Veterinary Research Institute at Onderstepoort, we obtained equine herpesviruses Type 1 and 2 for cultivation in vervet kidney cells; but the cytopathic effects of these viruses were quite different from that of SA15. Using antisera against equine herpesviruses supplied by Dr. Erasmus, we showed that SA15 was not neutralized by Type 1 or Type 2 equine herpes antiserum.

On original isolation SA15 produced only occasional syncytia. In these giant cells were nuclei filled with basophilic inclusion material, as well as nuclei with eosinophilic inclusions somewhat resembling those caused by adenovirus. Some of the nuclei were markedly enlarged. Fig. 18 illustrates the variation in size of nuclei, and also shows inclusions with bubbles or vacuoles. It was shown conclusively by us that SA15 is not a combination of foamy virus and another agent such as herpesvirus or adenovirus. The syncytia and both types of inclusion are integral features of the cytopathic effect.

A virus producing identical changes was isolated in 1972 from an uninoculated vervet kidney culture in vaccine safety test No. SPD 598 in which horse serum was not used.

The first isolate was taken as the prototype strain for study. It has the general properties of a herpesvirus, but electron microscopy has not been carried out. In vervet kidney cells the most characteristic feature is the syncytium containing herpetic nuclear inclusions which vary much in appearance. Cytoplasmic vacuolation resembling foamy virus effect may be found in the syncytia.

We were unable to detect changes in HeLa or KB human carcinoma cells infected with SA15; and while human foetal diploid fibroblasts showed some nuclear changes, viral multiplication did not occur in them.

One hundred and twenty-three infant white mice twenty-four or forty-eight hours old were inoculated with SA15 by various routes including intracerebral, intraperitoneal and subcutaneous injection. The litters were observed for at least twenty-one days, and usually one mouse from each litter was sacrificed on the tenth day for histological examination. No clinical or histological evidence of SA15 infection was found. Fifty-seven weanling mice averaging twenty-four grams in weight were inoculated by similar routes and were observed for periods between twenty-one and fifty-two days without clinical illness. Eight guineapigs inoculated intraperitoneally remained afebrile for twenty-three days; and three guineapigs inoculated intracerebrally remained healthy until discarded after fifty-two days.

Two vervet monkeys were each inoculated with 1,0 millilitre of SA15 intrathalamically, while two other vervets received 0,1 millilitre intraspinally in the lumbar cord. The animals were sacrificed after thirty days, and only glial traumatic lesions with minimal lymphocytic cuffing of vessels at the sites of inoculation were found, there being no extension of inflammation above or below.

SA15 remained viable in tissue culture harvests stored at -70C for eighteen months but not for four years. Infectivity was retained by harvests held at 4C for thirty-six days.

During 1968 and 1969 we recovered from the throats of baboons ten isolates of an agent which produced in vervet kidney tissue cultures changes (Fig. 19) which were similar to those caused by the vervet agent SA15. The baboons involved comprised two groups: those tested by us at a catching point in the North-Western Transvaal; and those held by the Department of Surgery at the University of the Witwatersrand, of which the probable origin was the

same catching area we visited. Five isolates came from baboons held in single cages at the catching point, and one isolate was from a baboon anaesthetized and swabbed by us in the trap in the mountains. We reported the recovery of this agent (Malherbe and Strickland-Cholmley, 1969 b); and when the herpesvirus-like appearance of the virions (Fig. 20) had been demonstrated in electron micrographs taken by Dr. G. Lecatsas and Miss J. Hoogenhout at Onderstepoort, using osmium-fixed vervet kidney cells infected by us, we briefly reported that the agent had the structure of a herpesvirus (Malherbe and Strickland-Cholmley, 1970).

5. RNA SINGLE STRANDED ETHER RESISTANT VIRUSES

Under this heading Hull placed picornaviruses which he subdivided into Enteroviruses and Not Enteroviruses. Our SA4 virus was put into the latter category together with Hull's SV4 and SV28 because these had been isolated only from monkey kidney tissue cultures; but we have subsequently recovered strains of SA4 from the gut of monkeys, and we consider that this virus should now be classified as a simian enterovirus. We do not have evidence on which to divide our vervet and baboon enteroviruses into further subgroups, and we have not identified clearly any rhinoviruses. Our simian picornaviruses will thus, for present purposes, be regarded as forming a single group containing serological variants. The category SA4 was made for a picornavirus recovered from vervet kidney cells, and the category SA5 for a serologically different picornavirus isolated from a vervet rectal swab. Both of these agents produce similar cytopathic changes.

SA4 was isolated from an uninoculated control vervet kidney tissue culture bottle in the safety test L79 in November 1956, and isolation from the bottle was successfully repeated. The virus produced in vervet kidney cells the CPE typical of picornaviruses (Fig. 21). A titre of $10^{7,5}$ TCID50 per millilitre was obtained in vervet cells.

Using a challenge dose of 100 TCID50, SA4 was neutralized by antisera against the three poliovirus types which had been prepared in monkeys in 1956, and this suggests that SA4 was prevalent in monkeys here during that year. No CPE was produced in rabbit kidney cells; there was no inhibition of replication in the presence of 5-iododeoxyuridine; and resistance to ether and chloroform was demonstrated. The virus was stable at pH 3,5 for three hours at room temperature, while Echovirus 28 used as a control under identical conditions was labile; thus proving that SA4 was not a rhinovirus. Molar magnesium chloride protected more than four logs of SA4 at 50C for one hour. The virus survived storage at -70C for over ten years.

We prepared an antiserum against SA4 in rabbits, and in tests using Hull's SV4 and SV28 as challenge viruses in parallel with SA4 we showed that SA4 was serologically distinct although some minor antigenic relationship may exist between these viruses.

Two further strains of SA4 have been isolated by us from uninoculated vervet kidney tissue cultures, in 1958 and 1963; while during 1963 and 1964 we recovered eight serologically similar viruses from vervet rectal swabs. SA4 would therefore appear to be an enterovirus which has a viraemic phase resulting in occasional latency in the kidney.

SA5 prototype strain B165 was isolated from the rectal swab of a sick vervet monkey in May 1958. Before being tested the swab suspension was stored frozen at -20C for one year. After isolation of the virus in tissue culture, re-isolation from the rectal swab suspension was successful. The first harvest had a titre of $10^{4,25}$ TCID50 per millilitre. A challenge dose of 100 TCID50 was tested against antisera to SA4, the three types of poliovirus, and Echoviruses Types one to fourteen. No neutralization was obtained with any of these antisera. Antiserum against SA5 was prepared in rabbits, and a cross-neutralization test using SA4 and SA5 with their antisera confirmed that these two viruses are serologically distinct.

SA5 produced in vervet kidney cells the CPE characteris

tic of picornaviruses (Fig. 22) with the nucleus displaced and crushed by the paranuclear cytoplasmic zone which is the main site of virus assembly. The agent was resistant to ether and chloroform, and had the general properties of an enterovirus. Second passage harvest fluid was stored at -20C for more than two years with retention of viability. Third passage fluid was sent to Hull who placed the virus as a prototype strain in the category of simian enteroviruses.

Of the ninety virus isolates made by us from 273 baboons, only one was a picornavirus; but from vervet throat and rectal swabs taken between 1958 and 1967 we recovered ten strains of picornavirus which were not neutralized by either SA4 or SA5 antisera. Thus multiple serotypes exist within the group of South African picornaviruses.

6. RNA SINGLE STRANDED ETHER SENSITIVE VIRUSES

This category comprises myxoviruses and paramyxoviruses arranged in two groups: Group A agents which haemadsorb erythrocytes, and Group B agents which do not haemadsorb red cells.

The word 'haemadsorption' was introduced by Shelokov et al., (1958) to describe the reaction by which erythrocytes of various kinds, including guineapig, chick, sheep, human O, and rhesus red cells, adhered to monolayer tissue culture cells infected with viruses capable of haemagglutinating, before haemagglutinins were released into the culture medium. The test had the advantage of simplicity: erythrocytes were introduced into infected roller tube cultures and readings were taken after three to five minutes. Negative tubes were re-incubated at 37C for up to eight days, the red cells being replenished every two days. While haemadsorption correctly indicated the presence of haemagglutinating viruses, it was implied that lack of haemadsorption would indicate absence of haemagglutinating viruses. It is now well-known that

viral haemagglutination may depend upon pH, as in the case of arboviruses, or upon temperature and the species of red cells used, as Rapoza (1967) has shown for simian adenoviruses. Thus while Hull may be quite correct in selecting haemadsorption to differentiate between the particular Group A and Group B agents he has cited, it would be desirable to know more precisely the conditions under which the test should be performed; or else to substitute, as the distinctive criterion, haemagglutination by clearly defined techniques.

The subject of simian myxo- and paramyxoviruses is fraught with difficulties. Ruckle (1957) first described cultivation of the MINIA (monkey intra-nuclear inclusion agent) virus which was latent in monkey kidney cells used for tissue cultures. She found the agent to be identical to human measles virus, and Meyer et al., (1962 b) subsequently demonstrated the ease with which rhesus monkeys caught in India contracted measles infection within six weeks from their human handlers. The virus SV5 which Hull included in Group A is commonly latent in the kidneys of monkeys used in other countries. It is possibly of human origin, but differences of opinion exist about its place in the classification of human viruses if this is so. Hull (1968) presented results indicating that SV5, SV41, and the human Parainfluenza Type 2 virus are closely related, but that differences between them may be demonstrated by complement fixation and haemagglutination-inhibition tests. Hsiung (1972) proposed that SV5 should be classified as Parainfluenza Type 5 to form an additional member in the group of four human parainfluenza viruses already recognized.

These problems arise partly from the ease of exchange of viruses between animals and man, and partly from variations in the results of tests done in different laboratories as a consequence of slight modifications in technique or of mutant selection in the virus strains used. Much therefore remains to be done by way of standardizing reagents and techniques before the vexed question of the classification of human and simian myxo-

and paramyxoviruses can be settled.

Group A Viruses

This group presents the greatest difficulties. In South Africa the haemadsorption test is carried out at 37C and 4C on the uninoculated control vervet kidney tissue culture bottles which comprise twenty-five per cent of the total amount of tissue cultivated in each individual monkey kidney batch used for poliovirus vaccine production; and guineapig erythrocytes are used in this test which appears to be designed chiefly to detect SV5. Our safety testing techniques include the staining of coverslips in subinoculations, and any paramyxovirus would also be detected by this means. Very few agents in this group have been recovered from uninoculated cultures of monkey kidney by us. We have encountered false positive haemadsorption, but this can be checked by staining cultures. Dowdle and Robinson (1966) first drew attention to nonspecific haemadsorption resulting from the use of erythrocytes stored for varying periods at different temperatures; and we have confirmed that guineapig red cells kept at 4C for five or more days can give non-viral haemadsorption. Haemagglutination of guineapig erythrocytes in the culture medium may also be observed if bovine serum is present, without haemadsorption occurring.

The human paramyxoviruses characteristically produce in vervet kidney cells one or more of the following: cytoplasmic inclusions, nuclear inclusions, and syncytia. We have cultured in vervet kidney tissues known strains of human paramyxoviruses including Parainfluenza Types 1 to 4; mumps virus from a clinical case; respiratory syncytial virus; and measles strains both from avirulent vaccine and from clinical cases. In addition we have cultured the prototype strain of SV5 received from Hull. The following points summarize our observations in stained preparations:

(a) Cytoplasmic inclusions only: Parainfluenza Type 1.

(b) Cytoplasmic inclusions and Syncytia: SV5; Parainfluenza Type 2;

	Respiratory Syncytial Virus; Mumps (if not first passaged through human cells).
(c) Cytoplasmic inclusions, small nuclear inclusions, and syncytia:	Parainfluenza Type 3; Parainfluenza Type 4 (this type shows only scanty nuclear inclusions).
(d) Cytoplasmic inclusions, large nuclear inclusions, and syncytia:	Measles; Mumps if first passaged through human cells.

Using these criteria to assess the probable identity of simian agents isolated by us, we have found ourselves entering a field more extensive than we had anticipated.

From uninoculated vervet kidney cultures we have recovered two agents, VK625 in May 1965 and VK710 in October 1965, which produced cytopathic changes similar to those of measles virus. These are the only measles-like viruses recovered from uninoculated monkey kidney cells during more than a decade of testing twenty-five per cent control cultures in vaccine safety testing. This low incidence of measles-like agents contrasts strikingly with that experienced by workers in other countries. On one occasion we recovered from uninoculated vervet kidney cells another agent, P72/16/2, which produced very scanty small nuclear inclusions but numerous cytoplasmic inclusions which resembled those of a paramyxovirus except that in each infected cell there were both brightly-staining small eosinophilic cytoplasmic inclusions as well as larger more dull-staining cytoplasmic inclusions. The photographic technique used does not discriminate between these two types of inclusion, but Fig. 23 shows the general form of the cytoplasmic inclusions produced by this agent.

Another strain of paramyxovirus-like agent, VK798, was recovered from uninoculated vervet kidney cells in 1973. This strain was characterized by small nuclear inclusions and irregular cytoplasmic inclusions, and the destruction of tissue by it was unusually rapid. Thus there would appear to be at least three kinds of agents producing paramyxovirus-like CPE which may be latent in vervet kidney cells.

When cercopithecus throat and rectal swab suspensions were tested, still more paramyxovirus-like agents appeared. We obtained eighty-seven such isolates, seventy-two coming from monkey throat swabs and fifteen from rectal swabs. The majority of these viruses produced small nuclear inclusions and relatively large dull-staining irregular cytoplasmic inclusions resembling those caused by the agent we categorized as SA9 in 1963 (Fig. 24). Syncytia were also usually present, and the combination of CPE features resembled most nearly those of the human parainfluenza Type 3 except that the cytoplasmic inclusions of the simian agents were less firm in texture than those of the human virus. Some of the monkey agents not only produced small nuclear inclusions, firm cytoplasmic inclusions, and occasional syncytia, but they also caused the tissue to assume a shredded appearance with the cells becoming elongated and stringy. Such agents included the prototype strain SA10 (Fig. 25) which was obtained from the throat of a samango monkey, and which Harwin (1966) found to be related serologically to Parainfluenza Type 3. From the throat of a vervet was isolated the virus B47 which caused a measles-like CPE and which was the prototype of our category SA13. Harwin (1966) considered that this agent also belonged in the Parainfluenza Type 3 group; but the cytopathic changes of SA13 strain B47 (Fig. 26) are quite distinct from those of SA10. No serological relationship with measles could be demonstrated. A further variation of paramyxovirus-like CPE was noted in the case of strain D490 which was recovered in 1963 from the throat of a vervet. This agent caused syncytia with firm cytoplasmic inclusions but no nuclear inclusions.

In brief, we have recovered from uninoculated vervet

kidney cells and from vervet throat and rectal swabs agents producing cytopathic changes which suggest five different kinds of paramyxovirus. It must be emphasized that the exigencies of routine work precluded detailed studies on most of these agents, and the above considerations serve only to indicate the wide range of viruses awaiting investigation which should expand considerably Hull's Group A.

Group B Viruses

Under this heading Hull placed five types of foamy virus, together with our SA1 which is serologically identical to Type 1 foamy virus but which he included because it is frequently referred to in the literature. These agents produce syncytia but no nuclear or cytoplasmic inclusions. They have been called foamy viruses because of the large cytoplasmic vacuoles visible in unstained syncytia; but vacuolation is not always present, and syncytium formation is the constant feature. Fig. 28 shows a syncytium produced by a strain of vervet foamy virus, in which there is no vacuolation; while Fig. 29 illustrates vacuolation in another syncytium in the same culture.

The first report of the agent was made by Rustigian, Johnston and Reihart (1955) who isolated four strains from rhesus monkey kidney cells. Since that time the virus has been recognized as the most common latent agent in the kidneys of macaque and cercopithecus monkeys; and Johnston (1971) has recorded that seven serotypes of simian and ape foamy virus exist. Our first isolation of foamy virus was made in 1955; and with the extensive use of vervet kidney tissue for poliovirus vaccine production and testing, our acquaintance with the virus has increased correspondingly. On the average, about forty per cent of vervet kidney monolayer cultures show well-developed syncytia four weeks after initial cultivation of the cells. Development of the changes is slow and focal as the infection spreads to contiguous cells. Ragged gaps occur in the tissue sheet as syncytia degenerate and fall off the surface of the culture vessel. A number of cell types, including human, simian,

rabbit and guineapig cells, are susceptible to foamy virus, but probably primary rabbit kidney is the most sensitive. In some instances we have observed that subinoculation of SA1 foamy virus from primary vervet kidney cells into primary rabbit and vervet kidney cultures in parallel resulted in more extensive syncytial changes in the rabbit cells by the seventh day, which may reflect a difference in behaviour between types of SA1. We have not proceeded with type identification beyond identifying one strain as Type 1 with antiserum supplied by courtesy of Dr. Robert Rustigian; and then subsequently identifying ten other strains of SA1 as belonging to the same serotype, using an antiserum prepared by us in rabbits. It is probable that more than one serological type of foamy virus will be found in South African vervets and baboons. Stiles et al., (1964), who first reported foamy virus Type 3, speculated that certain serotypes may be found to be associated with different species of monkeys. They recovered only Type 1 from forty-five of 100 rhesus monkeys imported from Northern India, whereas thirty-six of 100 kidney batches from East African cercopithecus monkeys yielded Types 1, 2 and 3; the last-named type being found in twenty-one of these batches. These authors considered that foamy virus is probably a respiratory virus, because Johnston had only been able to recover it from throat swabs and not from urine or stools. In 1968 and 1969 when we were testing baboons under optimal conditions, we recovered foamy virus from the throat on thirty-two occasions, from rectal swabs on four occasions, and from the blood on four occasions. The fact that the virus is encountered with such frequency from the kidney indicates that viraemia must be common and the spread through the body extensive. Viraemia may, however, be of short duration, as we have isolated foamy virus from the blood a few times only; yet many bloods of monkeys and baboons have been screened by us for the presence of virus. The foamy agent is sensitive to lipid solvents and to low pH, and it is unlikely that it would survive passage through the stomach and the upper intestine Presence of the virus in the rectum may have followed

infection there as a result of viraemia. The high incidence in simian kidney is demonstrated by means of prolonged cultivation of kidney cells, which permits very small amounts of latent virus to increase. Extensive changes in kidney cultures held for relatively short periods would suggest a recent infection of the kidney. It is possible that prolonged cultivation of other simian tissues may unmask foamy virus, as well as other viruses, in them.

As cited by Hull (1968), other workers have shown that the foamy virus particle has an RNA core with a helical structure. We have confirmed that the agent is sensitive to ether and chloroform; and in our studies the virus was destroyed when held at room temperature for three hours at pH 3,5. SA1 kept at 4C showed a fall in titre over the first six days, but viability at a low titre was maintained at 4C for up to eighty days. Exposure to 50C for one hour resulted in a fall in titre without complete elimination of infectivity, but the presence of molar magnesium chloride abolished infectivity after one hour at 50C. Storage at -70C was possible for over two months, but storage at -20C was unsuccessful. The presence of foamy virus in cultures of vervet kidney did not interfere with the propagation of various other viruses in the same cultures. Some implications of this will be considered in Section 11 under Multiple Infections.

7. RNA DOUBLE STRANDED ETHER RESISTANT VIRUSES

Under this heading Hull placed three reoviruses SV12, SV59 and SA3.

Verwoerd (1970) proposed the name ' diplornavirus' for double-stranded RNA viruses, the size range being about forty to eighty nanometers in diameter. He recognized two sub-groups: one, with reovirus as its prototype, has a double-layered capsid with ninety-two capsomeres in the outer layer; and the other, represented by bluetongue and horsesickness viruses, has a single-layered capsid consisting of thirty-two capsomeres. In

the latter sub-group Verwoerd placed as a potential member our simian agent SA11, on the grounds of nucleic acid indentity and particle size. We will therefore consider SA11 under this main heading.

(a) Reovirus

The term 'reovirus' was proposed by Sabin (1959). Up to that time the prototype strain of human Echovirus Type 10 had been noted to produce cytopathic changes which, as seen in unstained preparations, were different from those caused by the other Echoviruses. In 1957 we reported (Malherbe and Harwin, 1957) that in stained cultures we had observed eosinophilic cytoplasmic inclusions in vervet kidney cells infected both with Echovirus Type 10 and with the simian agent SA3. Sabin (1959) acknowledged that this was the first description of reovirus inclusions. With antiserum kindly supplied by Dr. Sabin we established that there was homology between SA3 and Echovirus Type 10 which then became known as Reovirus Type1. Cross-neutralization tests carried out in both Hull's and our laboratories showing that serological relationships existed between Echovirus Type 10, SV12, SV59 and SA3 were reported by us (Malherbe, 1959). Rosen (1960) by means of the haemagglutination-inhibition test demonstrated that SV59 belongs in the reovirus Type 2 group, whereas the other strains mentioned belong to Type 1. Rosen's technique, as Hull (1968) has pointed out, appears to give a clearer differentiation between reovirus serotypes than does the neutralization test. Primates, cattle and mice (Rosen, 1962), and probably other animals as well, harbour reoviruses, but thus far only three types have been recognized. It is not yet known whether serotypes other than Type 1 occur in South African primates.

In stained cultures the eosinophilic cytoplasmic inclusions are distinctive. They begin as small bodies which coalesce to form large inclusions which characteristically in the later stages lie partly across the nucleus. The texture of the reovirus inclusion appears denser than that of most of the paramyxovirus

inclusions discussed in the previous section. Advanced reovirus infection in vervet kidney cells leads to nuclear degeneration, and occasionally small eosinophilic nuclear bodies may be seen; but in general, nuclear inclusion formation is not a typical feature of reovirus CPE. In Fig. 30 can be seen the usual form of reovirus inclusions in vervet kidney cells. The very distinctive appearance of these inclusions was of considerable assistance to us when we were assessing quantitatively the viruses passing through sewage purification systems (Malherbe and Strickland-Cholmley, 1967 a) and when we identified the viruses present in effluents from the Johannesburg abattoir (Malherbe, Strickland-Cholmley and Geyer, 1967). Even single cells infected with reovirus could be identified with certainty in tissue cultures infected at the same time with other viruses. Vervet kidney cells are probably the most sensitive cells for the detection of vervet reovirus. The LLC-MK2 continuous line of rhesus cells can be used for propagation of the virus, and a number of human cells, including primary amnion cultures, will show the typical inclusions. Inclusions were not observed with certainty in primary rabbit kidney cultures infected with SA3.

The prototype strain of SA3 had titres of about $10^{5,5}$ TCID50 per 0,1 millilitre in vervet kidney culture harvests, and culture fluids remained infective after storage for eleven years at -70C. The virus was stable at pH 3,5 and 7,4 for three hours at room temperature, and it was not inactivated by molar magnesium chloride after one hour at either 4C or 50C. The fact that reovirus is resistant to ether and chloroform greatly facilitated studies on bacterially contaminated fluids such as sewage effluents; and SA3, propagated in large amounts in tissue cultures, was successfully used by us to seed water purification systems in order to test their efficiency.

Our prototype strain L66AA was isolated from an uninoculated vervet kidney tissue culture in 1956, and numerous other isolations have been made by us from uninoculated vervet kidney cells. In addition, between

and 1963 we obtained twenty-two reovirus strains from vervet throat swabs; eighty-nine strains from vervet rectal swabs; and one isolation from a vervet salivary gland. In 1969 we recovered reovirus from a baboon rectal swab. We could not relate reovirus infection to any recognizable disease in monkeys, and this is in keeping with our observation (Malherbe and Strickland-Cholmley, 1967 a) of a very high rate of excretion of reovirus by human communities showing no evidence of disease attributable to the virus. On the other hand, the reovirus which is endemic in our mouse colony appears to produce a runting syndrome with thymic necrosis; while massive eosinophilic necrosis of hepatocytes, and large cytoplasmic inclusions in cerebral neurones, may be found in the mice. This endemicity of reovirus in the mice complicated our studies, but it appeared that we could not successfully infect adult or suckling mice with the prototype strain of SA3 by any of a number of routes.

SA 11 Virus

The prototype strain, H96, of this virus was recovered from a vervet rectal swab taken in 1958. The swab suspension was stored at -20C for one year before it was inoculated into vervet kidney cells. Re-isolation of the virus from the swab suspension was successful. Only a slight degree of change was seen in the unstained culture at the twelfth day after inoculation, but the coverslip fixed and stained on that day showed that while the tissue sheet was mainly intact, there were occasional degenerated cells. Approximately five per cent of the total number of cells showed multiple eosinophilic cytoplasmic inclusions of a kind not previously encountered. Fig. 31 illustrates the characteristic inclusions which may be loculated and somewhat resemble the negri bodies of rabies. The cytoplasm around the nucleus stains more deeply in infected cells. Small light-red-staining bodies may be seen in the nuclei of some cells, but these are not commonly present. The cells finally shrink and stain a deeper red, and in such degenerated cells may still be seen the cytoplasmic

inclusions. One such shrunken cell is present in Fig. 31.

Two more strains of SA11 were recovered from monkeys: one from a vervet throat swab in 1958, and the other from a vervet rectal swab in 1963. In both these cases adenovirus was also present in the same specimen, but the stained coverslips clearly showed individual cells infected with each of the two viruses, although some cells with a double infection were noted. Certain strains of simian adenovirus produce small eosinophilic cytoplasmic inclusions in vervet kidney cells, but these can readily be distinguished from those caused by SA11.

During a study of viruses in sewage in Johannesburg in 1965, we isolated five strains of a virus producing cytopathic changes identical to those of SA11. The agent was recovered from the mixed washings of the intestines of cattle and sheep. Another similar agent was isolated from the Klip River which received agricultural run-off surface water. We designated the abattoir virus O (for Offal) Agent (Malherbe, Strickland-Cholmley and Geyer, 1967). A detailed comparison of SA11 and the O Agent was completed for presentation at the John F. Enders Symposium held in Boston in 1967 (Malherbe and Strickland-Cholmley, 1967 b). The two viruses both contain RNA, and the naked virions have a diameter of approximately 57 nanometers. The agents resemble each other in being resistant to sodium deoxycholate and to ether and chloroform, and by their failure to be stabilized by molar magnesium chloride at 50C for one hour. SA11 was stable at pH 3,5 at room temperature for three hours, but the O Agent showed some lability at pH 3,5. The two viruses were not neutralized by standard antisera against the three reovirus types, but some of our uninoculated rabbits had neutralizing substance in their serum, which suggests that the host range may extend beyond monkeys and cattle or sheep.

Els and Lecatsas (1972) studied the morphology of the virions of SA11 and the O Agent by means of negative staining with phosphotungstic acid under the electron microscope, and they demonstrated the similarity in structure of the two agents. Dr. G. Lecatsas kindly

supplied two unpublished electron micrographs of the virus particles propagated in LLC-MK2 cells. Fig. 32 shows SA11 naked virions with a probable number of thirty-two capsomeres; and Fig. 33 illustrates the envelopes around SA11 particles, which increase the diameter to about seventy-two nanometers. These authors found envelopes commonly around SA11 particles, but less than one per cent of O Agent virions had envelopes.

SA11 inoculated by us into newborn mice by various routes did not produce lesions, and no tumours were produced in hamsters over the forty-four-day observation period. The O Agent also appeared to be non-pathogenic for small animals. Six vervet monkeys were tested for antibody against SA11 and the O Agent, and the sera of five of these monkeys neutralized both viruses. The sixth monkey, whose serum at a dilution of 1:8 was negative in the neutralization test, was used for intracerebral inoculation of O Agent. 0,25 millilitre amounts of O Agent fluid containing $10^{5,9}$ TCID50 were inoculated into each thalamus. The monkey remained clinically well until it was sacrificed on the thirty-second day. Histological sections showed a marked mononuclear inflammatory reaction in the thalami with minimal neuronal damage, and there was no extension to other parts of the CNS. One of the five monkeys showing antibody to the agents at a serum dilution of 1:8 was inoculated in the lumbar cord with $10^{5,8}$ TCID50 of O agent in 0,2 millilitre of virus fluid. Paralysis of the right leg developed on the fifth day, and the animal was sacrificed two days later. In the lumbar cord there was a traumatic lesion and a marked mononuclear inflammatory reaction, but there was no extension up the cord. The O Agent was recovered from the lumbar cord.

Thus SA11 and the O Agent represent previously unknown animal viruses. We could not establish, within the time available to us, whether the O Agent came from cattle or sheep, but the virus was enteric in origin, being found in gut washing effluent. The significance of this agent in veterinary medicine has not yet been established.

8. SURVEYS OF VIRUS INCIDENCE

A limited number of investigations into virus incidence were carried out, not with the object of exhaustively delineating the viral flora of South African monkeys and babcons, but in order to answer certain practical questions. A continuing surveillance of latent viruses in vervet kidneys was effected through extensive use of kidney tissue cultures for vaccine production and control as well as for research, and some of the findings will be considered in Section 10.

Predictably, viral incidence varied greatly from time to time, being subject to a number of factors which included closeness of contact between individuals, pre-existing immunity, and the effects of stress. Visits were made to two monkey catching areas and one baboon catching site; partly in an attempt to find out which viruses were present in the animals before direct human contact had occurred, and partly to study the effects of transportation on the spread of viral infection. The former purpose was largely frustrated because of the shortness of the period between trapping and the caging at close quarters which takes place in such catching stations. It was, however, possible to examine a number of vervets and baboons at the traps. Monkeys were run singly from the trap into a portable cage with retractable back to immobilize them for anaesthesia; while for baboons a pistol firing darts loaded with anaesthetic was used. At least two throat and two rectal swabs were taken and immediately agitated in gelatin-buffered-saline; and blood was drawn from the femoral vein. All specimens were held in cooled containers during transit to the laboratory.

Throat and rectal swabs taken from monkeys and baboons held at laboratories also provided useful information. Often it was not possible to examine these specimens at once, and occasionally suspensions stored at -20C for as long as a year yielded viruses. Many labile viruses must have been lost through storage, and the results given should be interpreted in the light of the limitations

imposed at the time by the exigencies of routine work. In view of all these variables, and in the interests of brevity, only a summary will be given of the isolations made from 468 cercopithecus monkeys and 273 baboons during the period between 1958 and 1969.

Vervet Throat Swabs Virus	No. of Isolates
Adenovirus	30
Cytomegalovirus	13
SA8 herpesvirus	1
Enterovirus	4
Foamy Virus	30
Other Paramyxovirus-like	72
Reovirus	22
SA 11	1

Vervet Rectal Swabs Virus	No. of Isolates
Adenovirus	180
Enterovirus	21
Foamy Virus	2
Other Paramyxovirus-like	15
Reovirus	89
SA 11	2

Baboon Throat Swabs Virus	No. of Isolates
Adenovirus	4
SA15-like herpesvirus	10
Foamy Virus	32

Baboon Rectal Swabs Virus	No. of Isolates
Adenovirus	32
Cytomegalovirus	1
SA8 herpesvirus	1
Enterovirus	1
Foamy Virus	4
Reovirus	1

Baboon Bloods Virus	No. of Isolates
Foamy Virus	4

In a number of instances multiple infections were present in individual animals, or both throat and gut were infected with the same kind of virus. In view of the high rate of viral latency in the kidney, which implies a preceding viraemia, it is surprising that only four isolations of foamy virus were made from baboon bloods. Very numerous vervet and baboon blood and serum samples have been tested for viraemia, with negative results. In the years before 1960 when monkeys inoculated with inactivated poliovirus vaccine were checked for viraemia, many hundreds of blood samples were found to be negative for all detectable viruses; and in our study on the Marburg virus, when particularly careful examination was made of tissue cultures inoculated with monkey and baboon sera, the findings were again negative.

Marburg Virus

It is still not known where the Marburg virus originated, but since it was conveyed to man by cercopithecus monkeys exported from Africa, the question of whether it may be found in South Africa justifies mention of the subject here.

In 1967 there occurred a haemorrhagic disease among laboratory workers in Marburg and Frankfurt in Germany, and in Belgrade in Yugoslavia. Thirty-one persons became ill, and of these seven died. The infection was conveyed by contact with the tissues of cercopithecus monkeys imported a short while previously from Uganda. Owing to war in the Middle East, the monkeys had been flown to Europe by way of England instead of Cairo, and they had been taken from the plane at London to spend the night in a large shed in the company of forty-eight other species of animals including birds and reptiles. On arrival in Europe the monkeys were not quarantined for a sufficiently long period, but were used for laboratory purposes during the incubation period of the disease. The nature of the illness in man, the properties of the causal agent, and studies attempting to trace the origin of the infection have been reviewed by Siegert (1972), and were the subject of a symposium held in Marburg in 1969 to which we contributed two papers.

At the time of the outbreak, sera from several patients were sent from Germany to the Poliomyelitis Research Foundation in Johannesburg for arbovirus antibody tests. One of these sera had been taken on the sixth day of illness, and it was given to us to attempt virus recovery from it. The serum was inoculated into vervet kidney tissue cultures, but no significant changes were seen in the living cells. Coverslip cultures fixed and stained after the twenty-third day of observation demonstrated the presence of numerous eosinophilic cytoplasmic inclusions (Fig. 34) in cells forming a tissue sheet showing no other cytopathic change. We immediately warned workers in other countries that tissue cultures infected with Marburg virus might seem to be negative unless they were stained to show inclusions. Study of the virus here was confined to two persons working with it out of normal hours to reduce the risk of accidental spread. During 1967 and 1968 there continued an exchange of information and materials between ourselves and the very few teams studying the agent in other countries. Further isolations of the virus were made

by us both from sera taken from patients in Germany during the acute phase of the illness, and from animals inoculated in Britain. Our initial findings were briefly reported in 1968 (Malherbe and Strickland-Cholmley, 1968).

We continued to concentrate on aspects of the behaviour of the virus in tissue cultures, which appeared not to be receiving full attention elsewhere, until Kalter et al., (1969) reported that antibody to Marburg virus had been detected in the sera of a number of primates, including African vervets and baboons, by means of the complement fixation test using beta-propiolactone-inactivated antigen prepared from the spleens of guineapigs infected with Marburg virus. It then became a matter of urgency to us to find out whether antibody to Marburg virus could be found in South African primates, and we decided to adopt methods which would yield conclusive results. Steps were taken to ensure that the microtiter complement fixation test was performed by us in precisely the same manner as that used by the United States workers. Two vervet monkeys were infected intramuscularly by us. One of these, No. D788, died on the ninth day and its liver tissue was made into a suspension which was then treated with fluorocarbon for use as our positive antigen in the complement fixation test. The virus was not inactivated, and the liver antigen had an infective titre of $10^{3,0}$ TCID50 per millilitre. The second monkey, No. D787, became very ill and was undoubtedly about to die from massive liver failure when, acting on the possibility that antibody might be present after the tenth day of the disease, we exsanguinated it on the sixteenth day following virus infection. The serum did indeed contain sufficient antibody for it to be used at a working dilution of 1:20 in the complement fixation test. This is the only known positive Marburg antiserum derived from a monkey with the active infection. Uninfected monkey liver and serum were used as negative controls. Our positive antigen was checked against convalescent sera from five German patients in parallel with the U.S. positive antigen, and it was found that both antigens reacted with Marburg antibody, but our antigen had

a higher titre than that of the U.S. antigen. Through the courtesy of Dr. Robert Kissling of the Communicable Disease Center in Atlanta we obtained sufficient supplies of the U.S. positive and negative antigens to enable us to set each serum tested by us simultaneously against the two positive and the two negative antigens, with other appropriate controls included in each test.

The complement fixation test was carried out on 268 baboon and 211 vervet sera collected since 1967 from animals derived from a number of areas in South Africa (Fig. 35). Many of the baboon sera from the Cape were obtained through the invaluable co-operation of Professor H.D. Brede and his assistants at Stellenbosch University. Of all these sera, only forty-three proved to be anticomplementary. Strong reactions with the U.S. positive antigen were given by 133 baboon sera, but of these none reacted significantly with our positive antigen. Of the vervet sera 159 gave strong reactions with the U.S. positive antigen, and seven reacted with our positive antigen. These seven positive sera also reacted in varying degrees with negative monkey liver antigen, and we considered that they did not provide convincing evidence of Marburg virus infection. Thus we confirmed that the U.S. antigen appeared to show the presence of Marburg antibody in baboon and vervet sera; but we felt that these were false positive reactions for Marburg virus antibody, although they might be detecting another antibody whose identity we did not attempt to establish. Other workers also failed to find valid evidence of Marburg virus infection in African primates other than those involved in the original outbreak (Simpson et al., 1968; Slenczka et al., 1971). Our studies on the Marburg virus were presented at the international symposium held in Marburg in 1969 (Strickland-Cholmley and Malherbe, 1971; Malherbe and Strickland-Cholmley, 1971).

The lack of serological evidence of Marburg virus activity in South Africa was supported by the fact that the virus had not been found in over 3000 vervet kidney tissue batches used by us since 1955; nor had it been

detected in any of the numerous specimens taken for virus isolation, including all the sera collected in 1969 for the Marburg virus tests.

It was our original impression that the eosinophilic cytoplasmic inclusions which we observed in Marburg virus-infected cells differed in appearance from other inclusions we had noted previously. Recent re-examination of coverslip cultures showing the various inclusions we have seen since 1957 has lessened the confidence we felt earlier that Marburg inclusions could be readily distinguished from all the others; but it has served to strengthen our conviction that primates and their tissues should be handled as if potentially infected with a dangerous virus, and that the staining technique should be mandatory in any laboratory handling primate tissue cultures. The origin of the Marburg virus remains unknown, and there is no assurance that it will not one day appear unexpectedly in South Africa.

In 1967 we did not possess special security facilities for the handling of dangerous viruses when we isolated the agent. It was necessary then to have made a hood under negative pressure in which infected tissues could be manipulated and complement fixation tests carried out, the exhaust air being sterilized by a combination of fine filters and ultraviolet lamps. Animal work was done in a closed system constructed largely by ourselves, consisting of cages surrounded by a framework of slotted angle iron bars and covered with plastic sheeting secured with adhesive tape. The exhaust air from this system was sterilized by exposure to heated wire coils and to a complicated system of ultraviolet lamps. Thus it was possible with improvised equipment to carry out hazardous work, but South Africa should have at least one permanent security laboratory with adequate facilities for the handling of exotic and dangerous viruses.

9. ARBOVIRUSES

Little information has been published about the

incidence of arboviruses in primates in South Africa, and we did not carry out any studies in this field.

Weinbren (1955) tested monkey sera for the presence of West Nile antibody by means of the neutralization test. He found that of 246 monkeys from the Eastern Cape, sixty were positive; and of 159 monkeys from the Northern Transvaal, eighteen were positive. Only eight monkeys from Natal were tested, and all were negative.

McIntosh (1970) reported results of a survey of primate sera for antibodies to five Group A, four Group B, and four Bunyamwera Group arboviruses; using mainly the haemagglutination-inhibition test and occasionally the neutralization test. One hundred monkeys trapped at Ndumu in Natal from January 1964 to December 1967 showed a higher incidence of antibodies than monkeys trapped during 1968 and 1969. From these one hundred vervets was obtained evidence of infection with Sindbis virus (Group A) and West Nile virus (Group B), but the highest infection rate was for Chikungunya virus (Group A) which had affected fifty-two of the hundred monkeys. Positive findings for Semliki Forest virus antibody in thirty-eight monkeys were interpreted as serological group reactions arising from Chikungunya virus infection. Sera from nine of seventy-six baboons trapped in 1963 and 1966 in the Eastern Transvaal lowveld adjoining the Kruger National Park were positive for Chikungunya virus antibody; but sera from fifty-nine baboons caught in the Transvaal and the Cape in 1967 and 1968 were all negative for this virus.

10. THE KIDNEY AND LATENCY

Experience with monkey kidney tissue cultures leads to the conclusion that latency in the kidney follows viraemia, and that prolonged cultivation of the kidney cells themselves may be necessary to reveal the latent viruses which are there in small amounts after viruria has ceased.

Cultivation of primary human kidney cells on a similarly extensive scale is not possible, and the evidence from

human material must needs be limited. Benyesh-Melnick et al., (1964) recovered three strains of cytomegalovirus, three adenovirus serotypes, and one strain each of measles, varicella and coxsackie B1 from cultures of kidneys taken from sixty-six children under one year of age. Hsiung (1968 a) reported that during a three-year period in which 124 lots of human kidney cultures were prepared, seven virus isolations were made which included measles, foamy, reo-, adeno-, and myxo-viruses. The finding of foamy virus in human kidney is not entirely surprising, in view of the report of recovery of a syncytium-producing virus from human nasopharyngeal carcinoma cells which is stated to resemble foamy virus in both morphology and morphogenesis (Achong et al., 1971). It is probable that as experience widens, more latent viruses will be found in the kidney and other organs of man and animals.

Hsiung (1969) studied the effects of prolonged quarantine and the separation of monkey species on the recovery of latent viruses from the kidney. Irrespective of species or season, rhesus and African green monkeys yielded virus in fifty per cent of kidney tissue batches both when the species were kept separate and when they were housed together, and when they were used with or without prolonged quarantine periods of thirty days or more. When the two kinds of monkeys were kept in close contact with each other without prolonged quarantine, the viruses from both species included SV5, measles, foamy virus and SV 40. When the two species were separately housed and quarantined for at least thirty days, SV5 and measles viruses were not recovered. Hsiung concluded that quarantine appeared to eliminate SV5 and measles from the kidney, whereas SV40 and foamy virus remained latent for a longer period.

The time required for latent viruses to become apparent in monkey kidney cultures was also noted by Hsiung (1968 b). When she maintained cultures for fourteen to twenty-one days after planting, the percentage showing SV40 was 2,6 and that showing foamy virus was 3,5. When cultures were held for twenty-nine to fifty-five days, the percentages for SV40 and foamy virus rose to 28,0 and 24,0

respectively.

If Hsiung's conclusion is correct that prolonged quarantine eliminates certain viruses such as measles from the kidney but leaves others latent for a long time, the relative absence of measles and the common occurrence of foamy virus in our own monkeys which are as a rule quarantined for one or more months may be accounted for. Conclusive proof on this point would be difficult to obtain. Quarantining of our monkeys has not reduced the rate of foamy virus latency significantly irrespective of season. We do not know if foamy virus infection can be conveyed congenitally with resultant tolerance. From year to year the recovery of foamy virus remains relatively constant, as the following examples show:

July - December 1970	91 kidney batches 52 (57 per cent) foamy virus
January - July 1971	127 kidney batches 70 (55 per cent) foamy virus
July - December 1972	81 kidney batches 34 (42 per cent) foamy virus
January - June 1973	71 kidney batches 30 (42 per cent) foamy virus

There is no doubt that close contact between monkeys shortly after capture is responsible for much of the rapid spread of virus with resultant latency in the kidney, whether this be for short or long periods. Our own field studies confirm this explosive spread of virus infection, and the experience of many workers, as summarized by Hsiung (1968 a) also supports it. Some of the viruses, such as SV5 and measles, may originate from the animal handlers; but in other instances the spread is of naturally

occurring agents, such as B virus to which antibody was found in ten to twenty per cent of newly caught rhesus monkeys, the incidence rising to 100 per cent in laboratory monkeys.

Viruses other than foamy virus recovered by us from vervet kidneys usually comprise less than about one per cent of the total isolations, although the figure may occasionally be higher. We reported (Malherbe, 1962) that during a period of ten and a half months, from 615 individual monkey kidney batches held for periods of four weeks or longer, with subinoculations into roller tubes from the original bottles, 196 virus isolations were made, comprising 144 strains of foamy virus, thirty-four strains of the agent designated SA2, seven strains of reovirus, ten strains of cytomegalovirus, and one strain of SA12. The effect of a short cultivation period was also noted: during the three-year period 1957 through 1959, kidney cultures prepared from single or at most two monkeys totalling 1033 animals yielded only forty-two strains of virus when the cultures were incubated for not more than three weeks after preparation. A strain represented collectively all the isolates of a particular virus from a single tissue batch.

11. MULTIPLE INFECTIONS

The practical question of whether latent simian viruses in kidney cultures used for the safety testing of poliomyelitis vaccine would interfere with the detection of other viruses led us to combine various agents in tissue cultures. When vaccine testing was confined mainly to checking for the presence of residual poliovirus in formalin-inactivated vaccine, we showed that the detection of small amounts of poliovirus was feasible in tissues infected with foamy virus. Later, when live virus vaccine was introduced, it became important to know whether any latent simian virus would interfere with other simian viruses which might contaminate the vaccine, and further

viral combinations were made. Then, since vervet kidney cultures are also used in research and diagnostic virology, the same practical consideration prompted us to combine human viruses with foamy virus which is the most common latent virus in vervet kidney. The syncytia of foamy virus infection are formed by the enzymatic dissolution of cytoplasmic outer membranes, after which the nuclei and the fused cytoplasms appear to retain their normal functions to the extent of supporting the development of viruses either in the nucleus or the cytoplasm. As a rule, the second agent used to super-infect foamy-virus-infected cells would also produce cytopathic changes in cells lying between the syncytia, and cells containing either cytoplasmic or nuclear inclusions could be observed in the process of being drawn into the syncytia.

Examples of foamy virus in combination with other viruses in vervet kidney cells are illustrated in a number of the photographs in this thesis. Fig. 36 shows nuclei infected with human herpes simplex virus in a foamy virus syncytium. Syncytia can be formed by some strains of human herpesvirus, but in this instance the strain of herpes did not form syncytia. In Fig. 37 is seen the combination of foamy virus with a strain of human adenovirus, the inclusions retaining some of the typical adenovirus characteristics. SV40 inclusions, on the other hand, showed a somewhat altered appearance in the syncytia, as illustrated in Fig. 38. The typically purple-red staining of cells infected with simian cytomegalovirus could be recognized with ease, and in Fig. 39 is seen the nucleus of a cytomegalovirus-infected cell from which the cytoplasm appears to have become detached in the syncytium; while other SA6-infected cells are being drawn in at the edge of the syncytium. The cytoplasmic inclusions of simian reovirus SA3, as shown in Fig. 40, were smaller than in single cells but could still be readily recognized in foamy virus syncytia. Marburg virus inclusions sometimes assumed very unusual elongated shapes, as can be seen in Fig. 41. Hsiung (1969) has also commented on the value of the staining technique for detecting multiple infections

in tissue cultures infected with simian and other viruses.

When specimens of throat and rectal swabs were taken from monkeys and baboons, multiple viral infections were occasionally encountered. The combination of SA11 and adenovirus in the same culture or the same cell has already been mentioned, as well as the fact that reovirus-infected cells can be distinguished among cells infected with other agents. In Fig. 42 is illustrated a cell containing the nuclear inclusion of an adenovirus and the cytoplasmic inclusion of a reovirus, noted in a culture inoculated with a vervet rectal swab suspension. In this culture there was also evidence of the presence of both viruses in other cells individually infected.

A further stimulus to mixing viruses deliberately was the concept that the combination of a syncytium with nuclear and cytoplasmic inclusions, which is observed in cytopathic changes due to a number of viruses, might originally have resulted from the fortuitous conjunction of different viruses which then formed a stable genetic entity, and that such a process might be repeated experimentally. We were unable, within the limits of our investigations, to create such a combination which would remain stable on serial passage. Most of the viruses combined with foamy virus destroyed the tissue before syncytia could form when the mixtures were passaged; except in the special case of SA2 which will be considered below. The experiments served, however, to show that cytopathic changes due to different viruses could be detected in tissue cultures having multiple infections; and that the presence of latent viruses need not interfere with the use of vervet kidney cells as a useful system in virology, provided that an adequate amount of uninoculated control tissue is examined to account for the presence of any viruses detected.

The category we designated SA2 still poses a problem. On numerous occasions we have observed that in uninoculated cultures showing the presence of foamy virus SA1 there may be occasional syncytia with well-retracted nuclear inclusions. Such inclusions are typically viral in appearance,

as is shown in Fig. 43 which demonstrates a syncytium in an uninoculated culture. These inclusions disappeared after one or two serial passages. It was possible to produce syncytia with similar inclusions by mixing foamy virus with other agents such as herpesvirus or adenovirus, but such mixtures inevitably led to rapid destruction of the tissue by the second agent. Thus while we suspected that naturally-occuring SA2 effect might be due to a second agent in a foamy-virus-infected culture of vervet kidney cells, we were unable to unmask such a second agent. In the course of safety testing oral poliomyelitis vaccine we noticed an unusually high incidence of SA2-like syncytia in stained subcultures from tissues used in the neutralization test which is intended to reveal non-poliovirus contaminants. It was an easy matter to trace the syncytial component to latent foamy virus in the vervet cells used for the tests, but the inclusions required explanation. In such tests, anti-poliovirus serum produced in horses was used to neutralize the vaccine virus, and this antiserum was also added to the culture medium when it was replenished in the bottle cultures and when subinoculations were done, in order to prevent breakthrough by the poliovirus. By performing serial subcultures of the syncytial agent for a number of passages in the presence of poliovirus antiserum and then omitting the antiserum at the next subculture, it was possible to recover and identify the poliovirus which inevitably broke through when the antiserum was omitted. Once it was established that poliovirus was causing the inclusions, we could construct experiments in which the syncytia with inclusions could be produced intentionally. Other picornaviruses, including Echovirus Type 1 and the simian enterovirus SA4 were also used successfully, and it is probable that a number of other picornaviruses would produce the same effect. These findings were briefly reported by us (Malherbe et al., 1963; Malherbe, 1973).

It is generally accepted that the main site of poliovirus replication is in the cytoplasm. Beale et al., (1956) described the large cytoplasmic paranuclear zone

which develops in the infected monkey kidney cell and which displaces and distorts the nucleus. These authors also observed small eosinophilic nuclear inclusions in a few of the poliovirus-infected cells. Reissig et al., (1956) also observed the cytoplasmic mass, and they published very clear coloured photographs of the small nuclear inclusions which they found as early as four hours after virus inoculation. Our routine use of haematoxylin and eosin staining of infected cultures has also shown the nuclear bodies in picornavirus-infected monkey kidney cells; and we have observed that echoviruses and the attenuated strains of poliovirus may in the early stages of infection produce a large, but only faint-staining, central eosinophilic body in the nucleus, with a faint halo around it within the nuclear membrane. The smaller deeper-staining inclusions illustrated by Reissig and her colleagues can be identified in the early stages of infection, but they may also be present in nuclei that have undergone more advanced changes. Fig. 44 shows two such eosinophilic bodies lying near to the nucleolus in a vervet cell in the early stage of infection with an attenuated vaccine strain of poliovirus. As the cytopathic changes progress, distortion of the nucleus tends to obscure such inclusions, but these may still be seen at the late stage of infection shown in Fig. 45.

If a vervet kidney cell roller tube culture is infected with vervet foamy virus and is maintained until syncytium formation can be seen in the living cells, the addition of a small dose of approximately 100 to 300 TCID50 of picornavirus will result in the formation of large nuclear inclusions in a proportion of the syncytia at the same time as the earliest visible CPE of picornavirus can be seen in the unstained culture. If the culture is at that time fixed and stained with haematoxylin and eosin, syncytia with large nuclear inclusions can be clearly seen, while a proportion of the cells lying between the syncytia will show the characteristic picornavirus changes illustrated in Fig. 45. A syncytium with poliovirus inclusions produced in this way is shown in Fig. 46. This

culture was infected deliberately with foamy virus whic formed obvious syncytia by the eighth day when it was superinfected with 100 TCID50 of attenuated poliovirus Type 1. Four days later the early changes due to poliovirus were noted and the coverslip culture was fixed and stained. In addition to syncytia without inclusions, seventy-four syncytia with inclusions were counted in this coverslip culture. Counts of up to 179 syncytia with inclusions in coverslip cultures measuring approximately seven by twenty-two millimetres have been made in such experiments. If anti-poliovirus serum is added at that early stage, it is possible to carry out serial subinoculations of the poliovirus-foamy-virus combination which will continue to produce syncytia with inclusions as long as the antiserum is present. In vaccine neutralization tests in which the mixture of poliovirus and its antiserum is added to kidney cells in culture, similar syncytia with inclusions are produced if latent foamy virus is present in the cells (Fig. 47). Similar changes were induced by superinfection with Echovirus Type 1 (Fig. 48), and by simian enterovirus SA4 (Fig. 49).

12. ENIGMAS

'There are more things in heaven and earth, Horatio,
Than are dreamt of in your philosophy.'

Hamlet

Cytoplasmic Inclusions in Vervet Neurones

In 1966 we reported (Malherbe, 1966) that eosinophilic cytoplasmic inclusions could frequently be seen in neurones of the brain, cord, and spinal ganglia of the vervet monkey. It was felt that the observation should be recorded, because misinterpretation of the significance of these inclusions can occur when histological studies are carried out on monkeys inoculated with material suspected to contain virus.

In the cerebrum the inclusions are found most commonly

in the thalami. They are usually round or oval in outline, and lie clearly within the cytoplasm. Their texture is smooth, although loculations or blue-staining areas in them have occasionally been seen. There is normally only one inclusion in a cell, but infrequently they may be multiple. There is a tendency for groups of cells to contain them. Fig. 50 shows a thalamic neurone in which there is an inclusion measuring 3,0 by 4,5 micrometers.

Inclusions in the spinal ganglia occur at any level, and are found in nearly every vervet monkey. Their shape is more irregular, and they are less smooth in texture than the inclusions in cerebral neurones. When sectioned transversely they seem to consist of plates or lamellae which present a needle-like appearance. Inclusions somewhat similar in shape and texture are infrequently seen in anterior horn neurones.

These inclusions of the brain, cord and ganglia in no way resemble Nissl bodies. Those in the cerebrum resemble viral inclusions closely, but on a number of occasions we have attempted to cultivate virus from histologically positive thalami and ganglia by various means without success. Electron microscopy has not been tried.

SA 14 Agent

The category designated SA14 was descriptive of cytopathic changes produced by an agent recovered from vervet kidney. The cytoplasm of the cells tended to be drawn into long processes, and the nuclear chromatin was stippled or coarsely clumped. Attempts to recover mycoplasma by culture on artificial media failed, but electron microscopy by Dr. G. Lecatsas of Onderstepoort showed bodies similar to mycoplasmas; and it is possible that the SA14 agent is a mycoplasma with special requirements for artificial cultivation.

The 'Unreo' Agent

On a number of occasions an agent producing cytoplasmic inclusions, and sometimes nuclear inclusions of a

similar character, has been recovered from uninoculated vervet kidney cells. Infected cells tend to occur in groups, suggesting that spread of the agent may be from cell to adjacent cell. The inclusions are smooth in texture, and they somewhat resemble those of a reovirus except that they do not coalesce to form large bodies, and they do not stain evenly with eosin, some parts of the inclusions being bright orange while other parts are grey, leading to our use of the term 'two-tone' for the staining of these bodies. Fig. 51 illustrates the shape and texture of the cytoplasmic and nuclear inclusions, but it does not demonstrate the 'two-tone' tinctorial quality. Serial passages of infective material have been continued for protracted periods of months at a time, with no progressive increase in the cytopathic changes. At some passage levels the inclusions were numerous, yet scanty in succeeding passages. On occasions there have been runs of infected kidney batches, with long intervals between such occurrences. The agent was noted in ten batches of vervet kidney cells between May and September 1965, and it was encountered again in 1967, 1972 and 1973. Unstable at low temperature storage, the agent has to be passaged without delay. It is relatively cell-bound, and passage of some cellular material is necessary, but the bodies seen are quite clearly not merely cell debris. Due to the bright staining of the inclusions with eosin, affected cells can be distinguished readily at a magnification of 125x. The cytopathic changes are different from those produced by any of the probable paramyxoviruses already considered above; and in our view the inclusions are quite distinctive and unlike those of other viruses. The erratic behaviour in serial passages suggests that our culture system is marginal for some component necessary for replication of the agent.

The Bronn Agent

In 1967 we screened thirty-seven vervet monkeys held in traps on a farm in a catching area on the bank of the Limpopo River in the North-western Transvaal, and from

rectal swab suspensions taken from four of these monkeys we recovered an agent which produced small basophilic cytoplasmic inclusions. Fig. 52 illustrates the inclusions produced by the strain recovered from the monkey Bronn No. 68. This rectal swab suspension was inoculated into vervet kidney cells, and by the third day advanced degeneration was noted, small basophilic inclusions being present. The harvested fluid was stored for over two years at -20C, and on passage in October 1969 changes were again seen by the third day, when the culture shown in Fig. 52 was fixed and stained. About forty per cent of the cells contained these inclusions. In February 1970 the fluid stored at -20C since July 1967 was again passaged, and by the third day about ten per cent of the cells contained inclusions. No further studies have been carried out.

Similar inclusions were observed in 1963 in kidney cultures inoculated with the rectal swab suspension of a monkey held at the Poliomyelitis Research Foundation, and from both throat and rectal swab suspensions taken from another monkey there.

Less dense in texture but somewhat similar in appearance were the inclusions seen in vervet kidney batch No. VK 292 prepared in December 1970, and illustrated in Fig. 53. This agent did not produce degeneration of the cell sheet.

The problem of the double infection in SA12 has been discussed. It seems probable that there is a group of agents producing small basophilic cytoplasmic inclusions in vervet kidney cells, but not all of these agents cause marked degeneration of the cultures.

13. POSSIBLE SOURCES OF ERROR

The fact that some viruses are replicated without producing visible cytopathic changes has already been mentioned, and such agents will pass unrecognized unless other techniques are employed to detect them. Thus it is probable that some specimens regarded as negative in

tissue culture may in reality be positive; yet the use of multiple techniques for routine purposes is, as a rule, not feasible. Furthermore, the host range for a virus may be restricted because enzymes needed for viral replication may be inactive in the cell system used; or evidence of infection in the detector system may be very slowly produced and difficult to observe. These factors will lead to errors due to omission of appropriate techniques. Delay, inadequate conditions of storage, or incorrect preparation of a specimen can lead to much waste of labour and materials when specimens which contained virus at the time they were taken prove unsuitable for use when inoculated. These false negative results can be avoided to some extent by careful planning. All in all, we probably reveal in practice only a fraction of the viruses for which we search.

Errors on the positive side can also be made. It is very seldom that a tissue culture does not show a few areas of cellular change probably due to the action of bacteria held down by the antibiotics used in the culture medium. L-forms may occasionally be seen, but more often the changes are only assumed to be due to bacterial toxins. These changes may include alterations in the appearance of nucleoli, resulting in their simulating nuclear inclusions.

Fungal toxins can produce similar changes even when the fungus is small and not visible in that part of the culture accessible for examination by staining methods. Engelbrecht and Purchase (1969) and Engelbrecht (1970) exposed primary vervet kidney cells to the actions of various mycotoxins, and through the courtesy of these authors we were permitted to examine the stained cultures they had prepared at the National Nutrition Research Institute of the South African Council for Scientific and Industrial Research. Both aflatoxin and sterigmatocystin produce in vervet kidney cells small eosinophilic nuclear bodies which could be mistaken for viral inclusions. In our studies of the non-viral causes of inclusions, we observed that Actinomycin D can induce in vervet kidney cells both small multiple eosinophilic bodies (Fig. 54)

as well as large retracted basophilic bodies (Fig. 55) in the nucleus, after exposure to 0,6 micrograms of Actinomycin D per millilitre of culture medium for twenty-four hours. Both Figs. 54 and 55 illustrate cells in the same coverslip culture.

Probably more important is the presence of amoebae in tissue cultures. These usually belong to the genus Acanthamoeba (Hartmannella). Hull's SV24 was originally believed to be a virus, but later it was proved to be an amoeba (Hull, 1968). Dr. C.G. Culbertson, of the Lilly Research Laboratories in Indianapolis, became the recognized authority on these amoebae, particularly when Hartmannella and Naegleria amoebae were incriminated in human cases of encephalitis; and our long-standing association with Dr. Culbertson enabled us to be well aware of the pitfalls due to the presence of amoebae in tissue cultures. Although we were watching for amoebic contamination of our cultures, it was not until recent years that we encountered amoebae in both primary vervet and primary rabbit kidney cultures.

Chang (1961) recorded the isolation of a transmissible cytopathic agent in tissue cultures of epithelial-like human liver cells which he had inoculated with blood from a case of infectious hepatitis. Chang and Humes (1962) proposed the name 'lipovirus' for this agent, on the hypothesis that it was a virus consisting of DNA within a surface coat rich in lipid. During the succeeding years a considerable literature had developed on the subject of the lipovirus, but in 1966 the agent was found to be a Hartmannella amoeba (Chang et al., 1966).

Pereira et al., (1966) reported the recovery of a cytopathic agent isolated in HeLa cells and rhesus monkey kidney cells from materials taken from six patients with respiratory or more generalized infections. The authors noted that the most characteristic effect was the appearance of discrete shrivelled crenated cells adhering in large numbers to the glass after the cytopathic process had become complete. They also noted that stained coverslip cultures taken within seventy-two hours of infection showed

that there were small eosinophilic nuclear inclusions, either single or multiple, surrounded by clear haloes to be seen in some of the HeLa and kidney cells. They concluded that the properties of the agent studied thus far were compatible with it being a virus which they named the Ryan Virus. Subsequently Armstrong and Pereira (1967) described the results of electron microscopy of cultures infected with this agent. The tissue culture cells showed no virus particles, but there were present also cells of a non-mammalian type possessing tubular cristae in their mitochondria, which resembled the cristae of protozoal mitochondria. This led to re-assessment of the Ryan Virus, and it was identified as an amoeba of the genus Hartmannella. Through the courtesy of these authors we obtained infected fluids, and we studied the methods of cultivation of the amoeba and its effects on tissue culture cells.

Long after we had ceased to handle the Ryan agent, we began to encounter amoebae in our own tissue cultures, and they have appeared from time to time during the past few years. Fig. 56 shows two amoebic trophozoites stained with haematoxylin and eosin, originating from an uninoculated vervet kidney bottle culture in October 1970. The culture showed a moderate degree of non-specific degeneration at the twenty-first day of holding, and on subinoculation amoebae were present in all three roller tubes used. The coverslip showing these two trophozoites was harvested on the fifth day when virtually all the tissue culture cells had degenerated and fallen off the glass. The pointed cytoplasmic processes which have given the name Acanthamoeba to the Hartmannellid amoebae are visible with dark-field illumination, but not with the staining method used in this Figure. The small round nucleus with the characteristic central karyosome can be seen in each trophozoite. In Fig. 56 the elongated amoeba is approximately eighteen micrometers in length. In Fig. 57 is seen the rounded pre-cystic form of an amoeba lying in the cytoplasm of a vervet kidney cell, indenting the nucleus. The diameter of the rounded amoeba is about five micrometers,

and in the living cells such forms resemble ingested erythrocytes. The fully-developed cysts of Hartmannellid amoebae have a wrinkled shell in which there are several pores.

The 'Ryan Virus' amoeba was noted by Dr. Pereira and her colleagues to produce nuclear bodies in some of the tissue culture cells. We have seen similar changes in some cells only, when vervet kidney cultures have contained amoebae. Fig. 58 shows the pre-cystic form of an amoeba in the cytoplasm of a vervet kidney cell of which the nucleus contains several eosinophilic bodies very closely resembling viral inclusions. The fact that such inclusion-like bodies are usually noted in only a few cells suggests that individual amoebae may vary in their ability to affect the enzymatic systems of cells, resulting in the production of abnormal products seen as inclusions; or that there may be present in certain amoebae a virus which can either make the amoeba toxigenic or directly infect the mammalian cell. Since bacteria and algae have their own viruses, it is not outside the bounds of possibility that amoebae can have viruses too. Whatever the mechanism, however, the nuclear changes result from interaction between amoebae and kidney cells.

14. CONCLUSION

The observations presented in this thesis may serve to indicate the very extensive field of simian virology in which new and unsuspected agents appear from time to time. The isolation rate for viruses is directly proportional to time spent and care taken; and while much may come by way of routine work as a by-product of it, scientifically sound investigations of the properties of the viruses recovered require undivided attention and the use of facilities whose adequacy must be reviewed periodically in the light of experience gained.

The danger resulting from simian virus infection of man provides a cogent reason for thorough understanding of

subhuman primate viruses; but the very variety of the agents in this field invites deeper insights and the revision of laboratory procedures required to obtain them.

THE VIRUSES OF VERVET MONKEYS AND OF BABOONS IN SOUTH AFRICA

VOL. II

Hubert Henri Malherbe

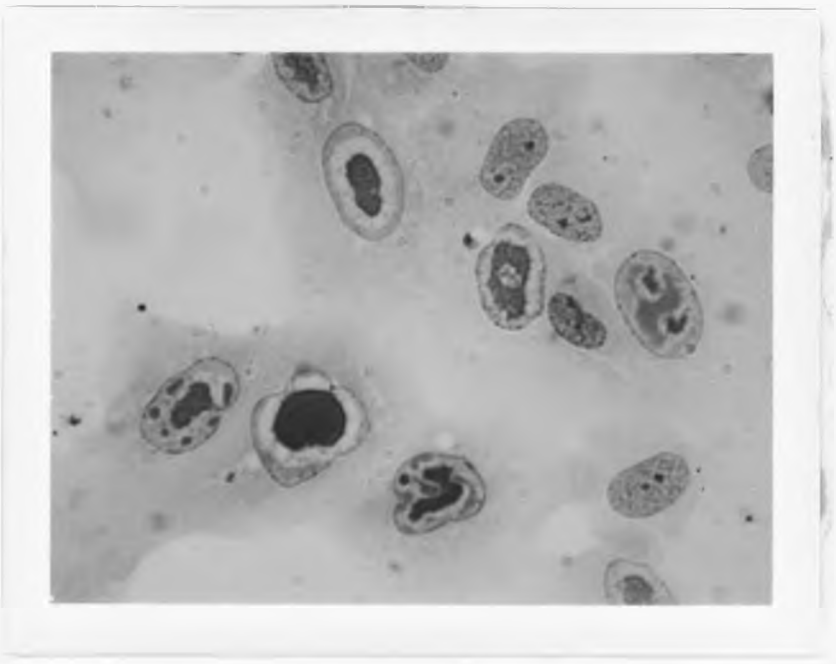

Fig. 1. Normal Vervet Kidney Culture
Haematoxylin and Eosin Stain
Coverslip 5609E 650x

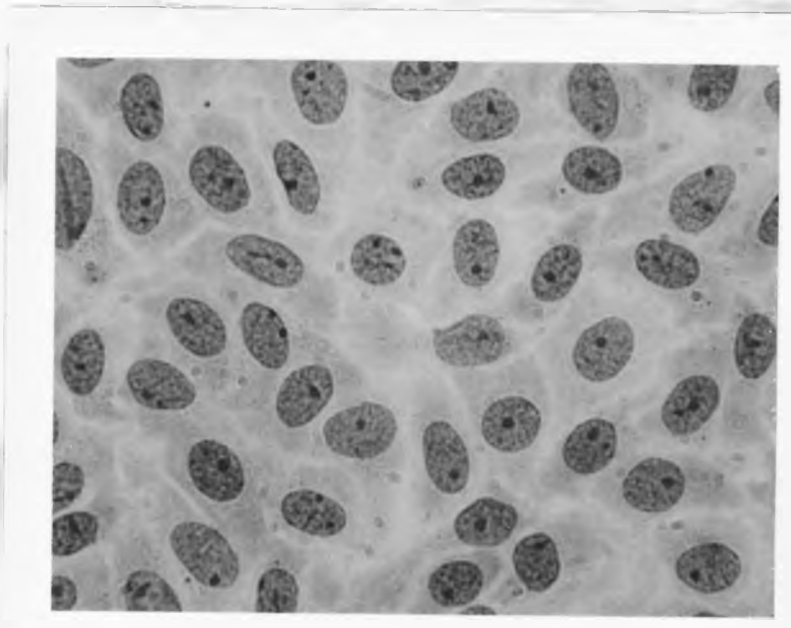

Fig. 2. Vervet Adenovirus Inclusions
Coverslip 5379D 650x H & E



Fig. 3. Tumours Produced in Syrian Golden Hamster by Vervet Adenovirus SA7C8

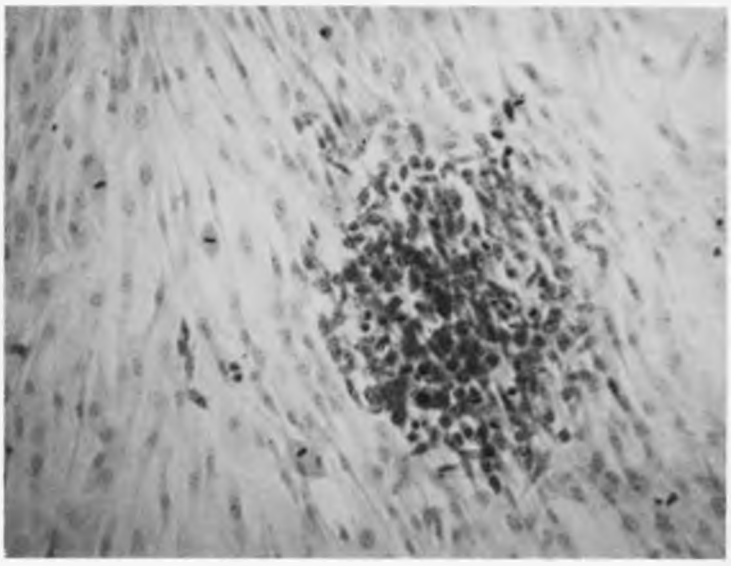

Fig. 4. SA7C8 Adenovirus Transformation Focus in Hamster Skin Culture, Day 6. 162x H & E

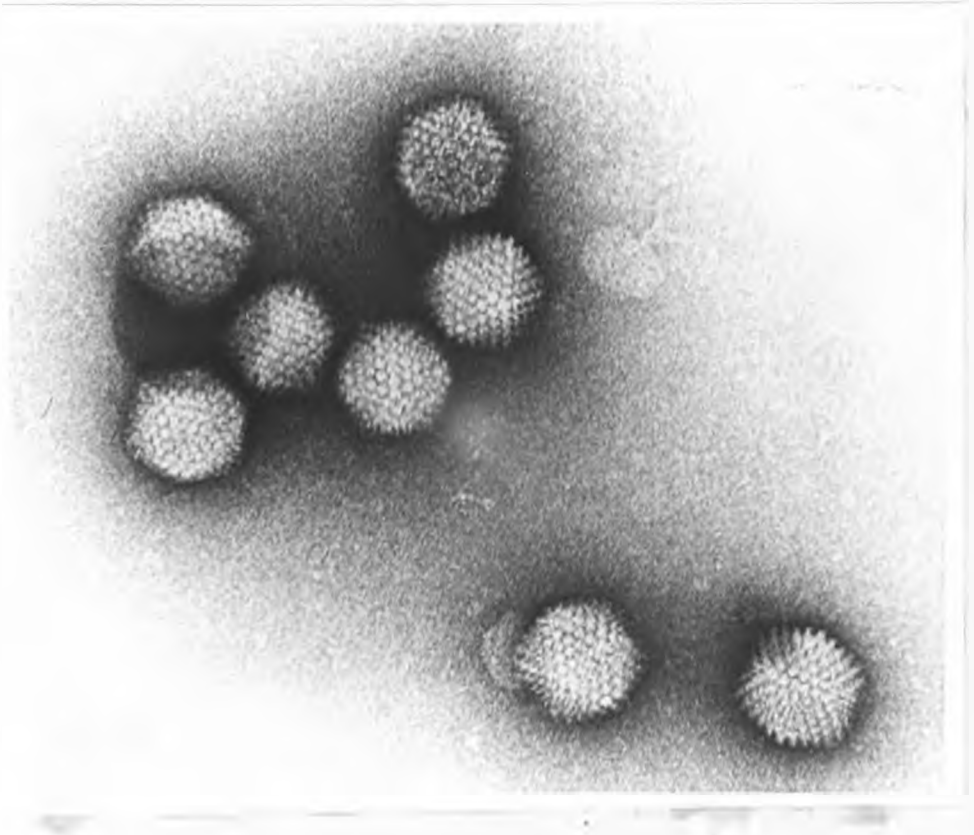

Fig. 5. Electron Micrograph of SA7C8 Adenovirus
Baylor College of Medicine BR-135 R-1
Uranyl Acetate 215 000x

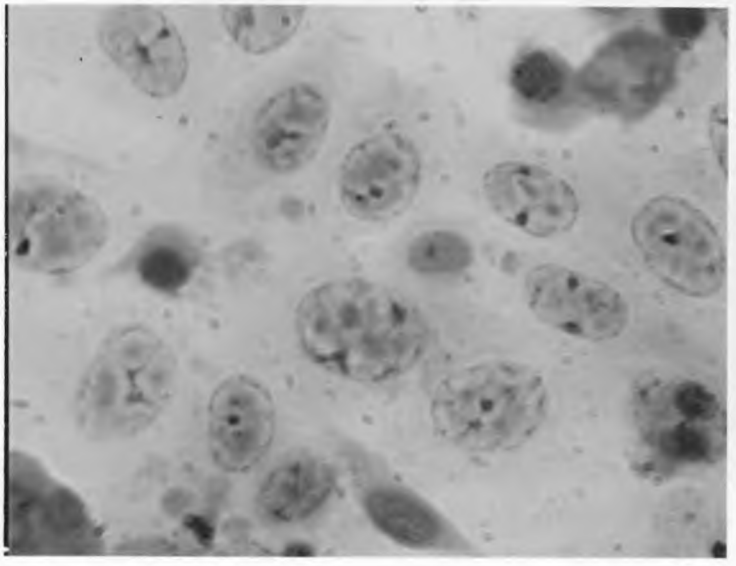

Fig. 6. SV40 in Vervet Kidney Cells
Second Passage, October 1957
Coverslip 244B 650x H & E

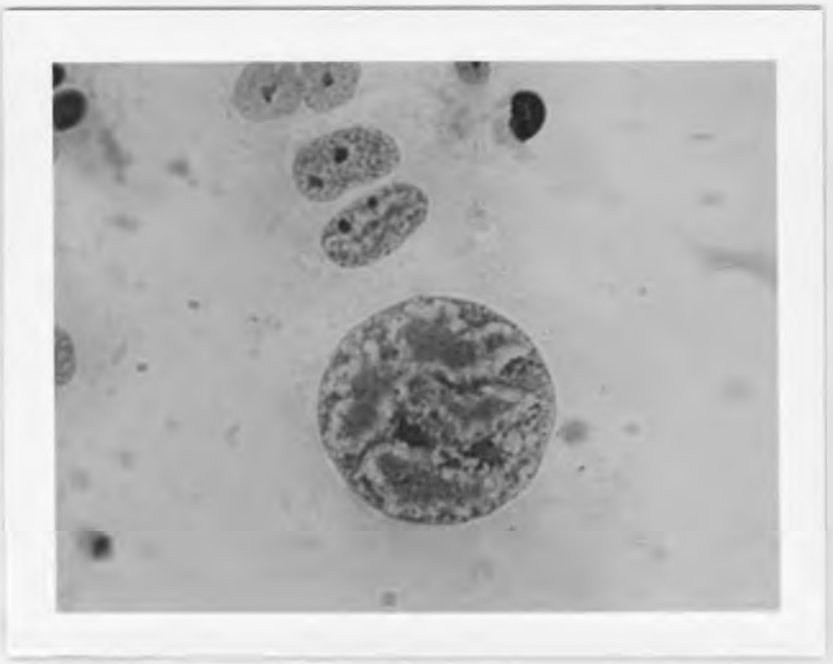

Fig. 7. SA12 in Vervet Kidney Cells
Coverslip 3526B 650x
H & E

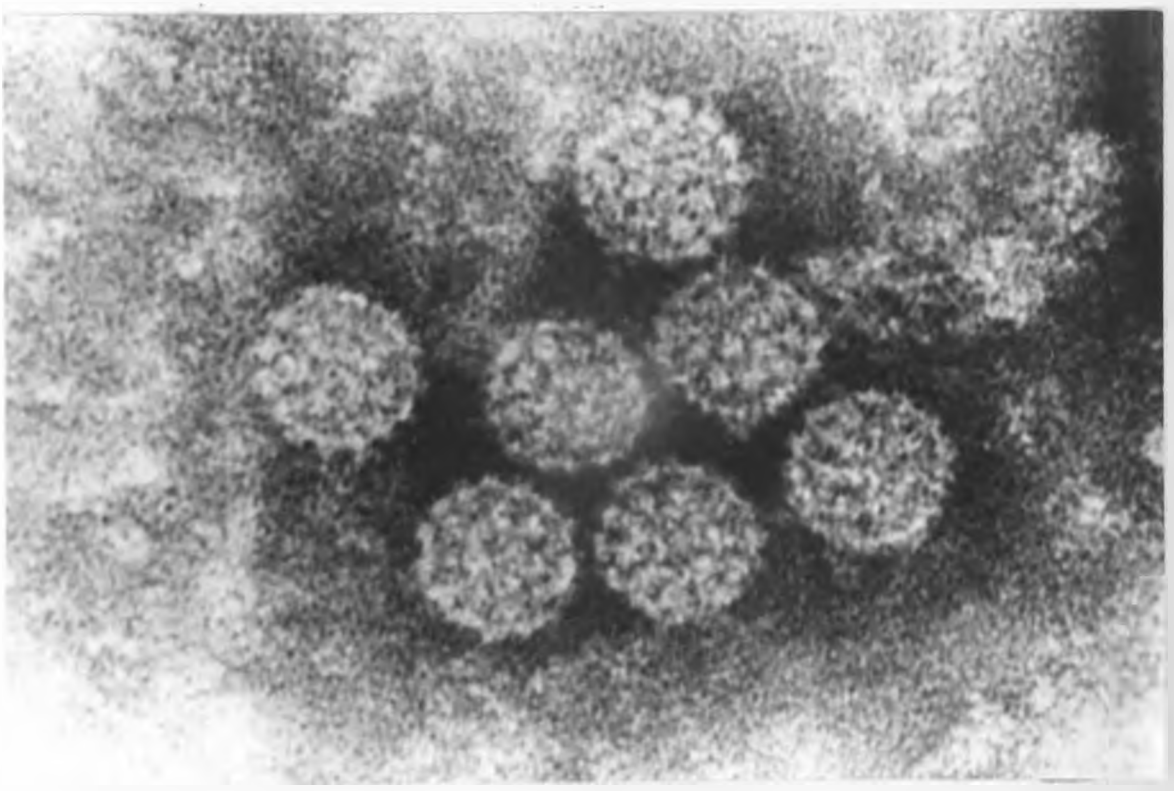

Fig. 8. Electron Micrograph of SA12 Small Virions
Dr.G.Lecatsas October 30, 1972
Phosphotungstic Acid 500 000x

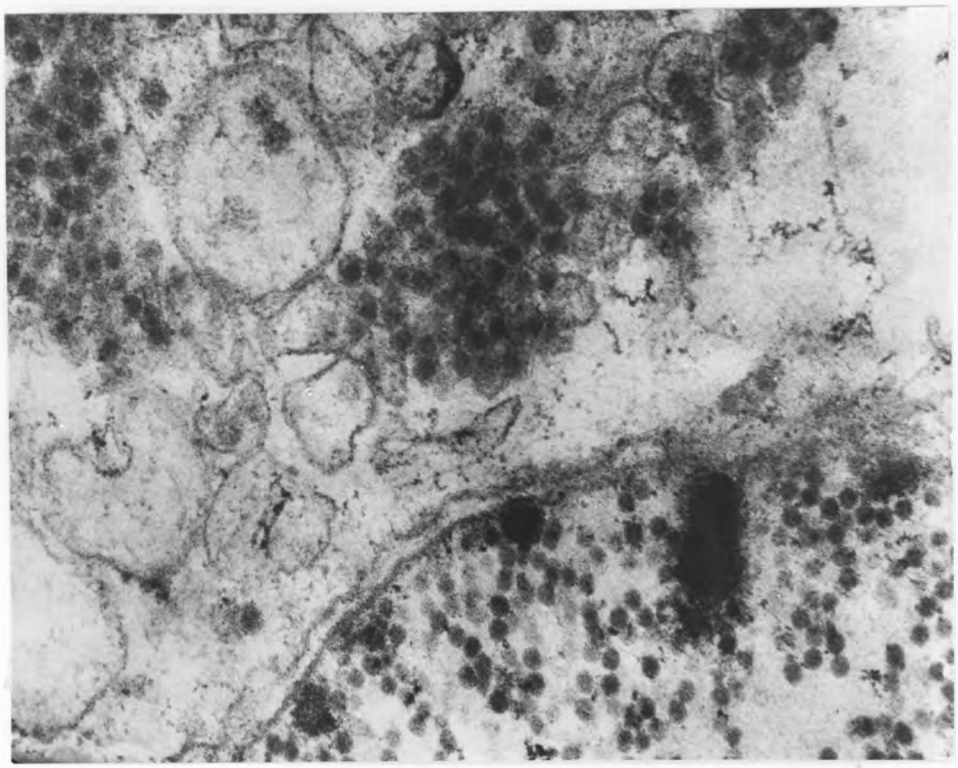

Fig. 9. Electron Micrograph of SA12 Small and Large Virions Dr.G.Lecatsas 8/12/72 Osmic Acid 80 000x

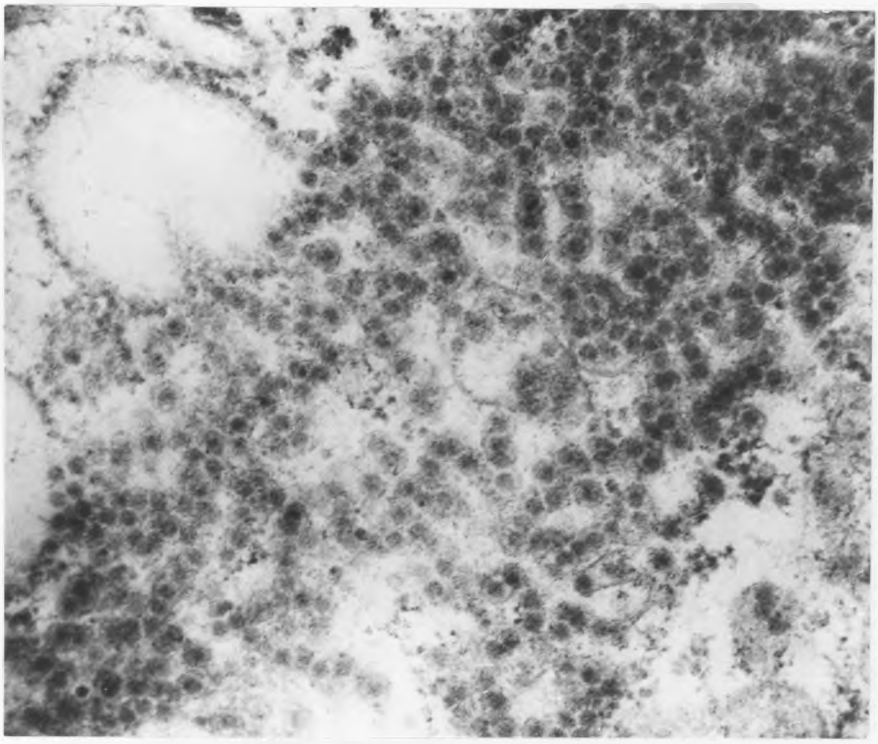

Fig. 10. Electron Micrograph of SA12 Large Virions Dr.G.Lecatsas 4/12/72 Osmic Acid 80 000x

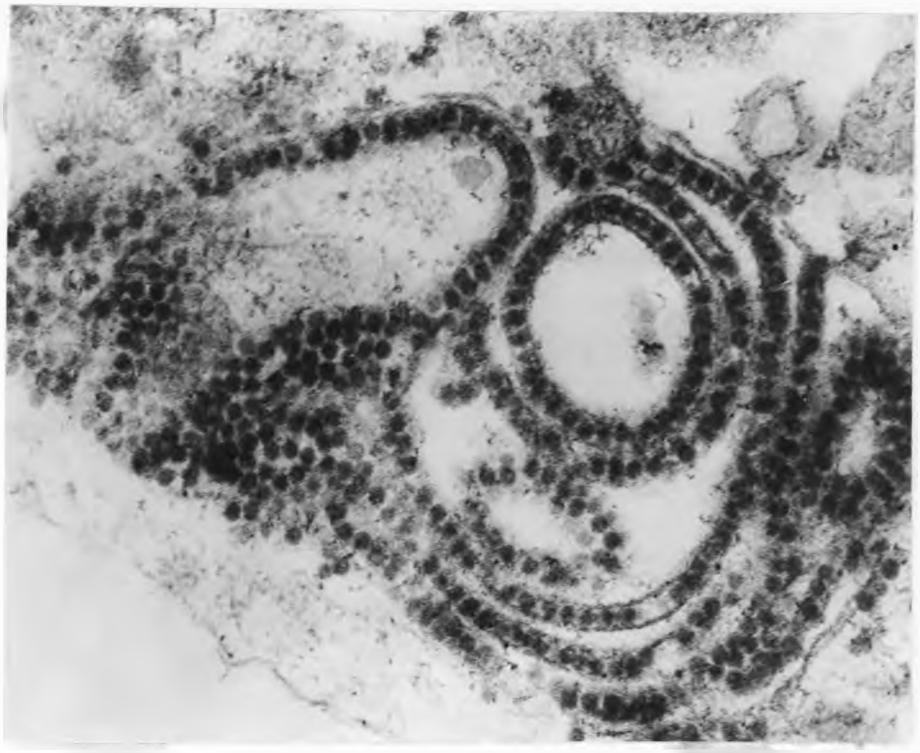

Fig. 11. Electron Micrograph of SA12 Small Virions in Endoplasmic Reticulum
Dr.G.Lecatsas 8/12/72
Osmic Acid 80 000x

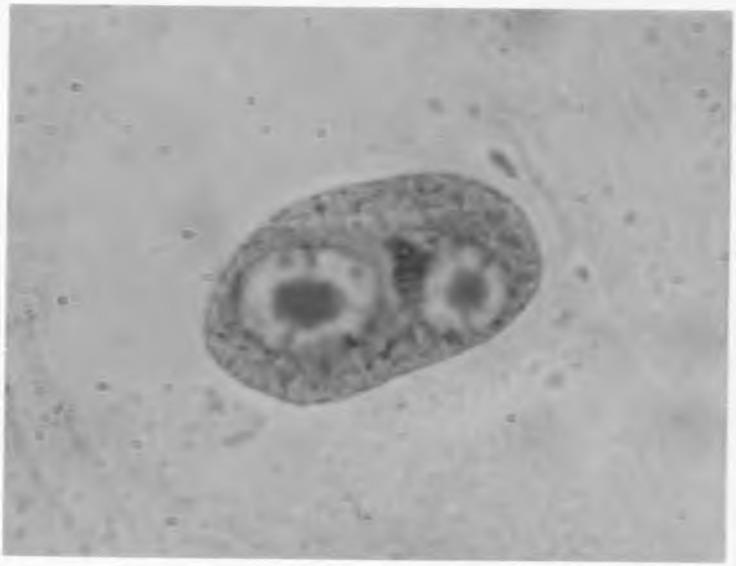

Fig. 12. SA12 Small Basophilic Cytoplasmic Inclusions in Vervet Kidney Cell
Coverslip 1718C 3/6/60 1625x
H & E

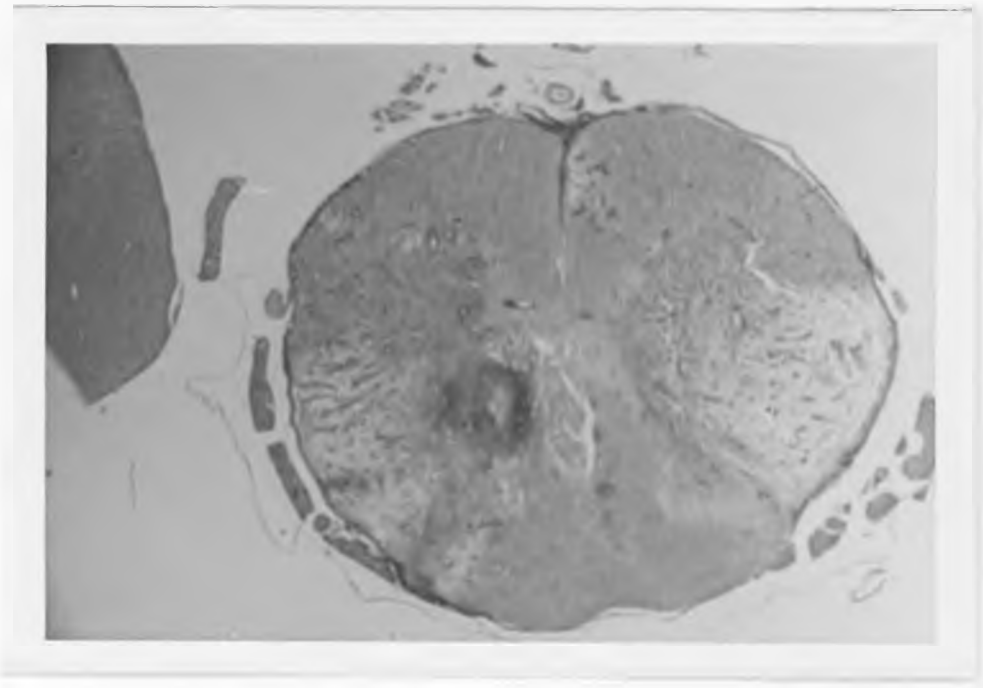

Fig. 13. SA8 in Lumbar Cord of Vervet B 264
Histology Section 6927A 12x

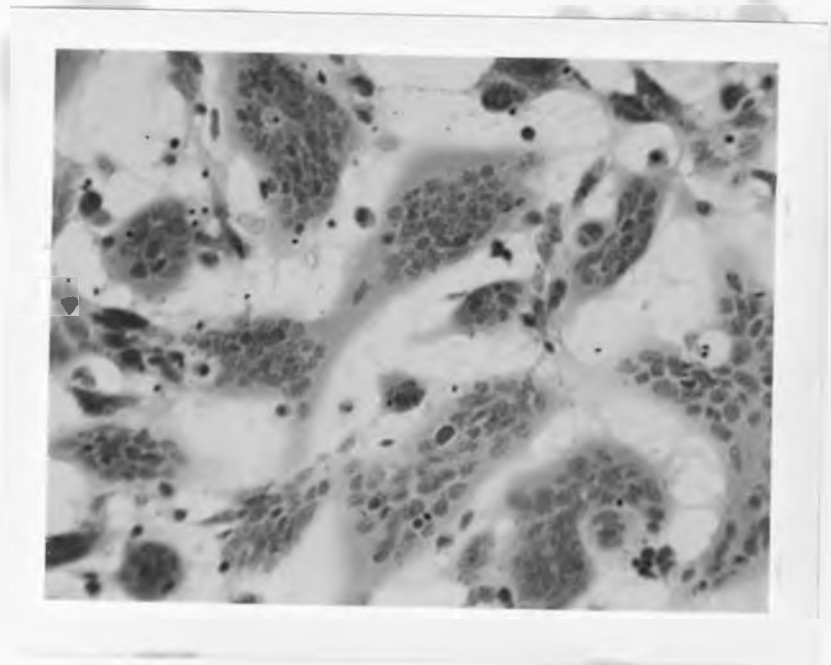

Fig. 14. SA8 in Vervet Kidney Cells
Coverslip 3829D 162x H & E

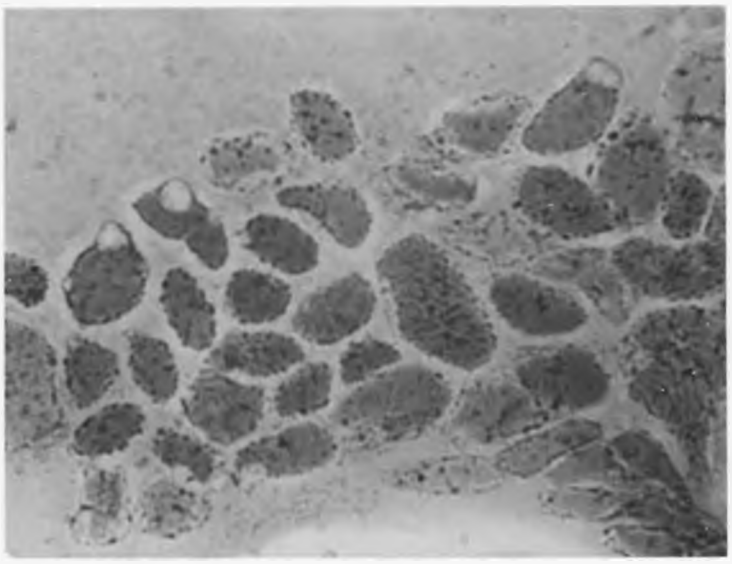

Fig. 15. SA8 Strain B264 in Vervet Kidney Cells Coverslip 4161A 650x H & E

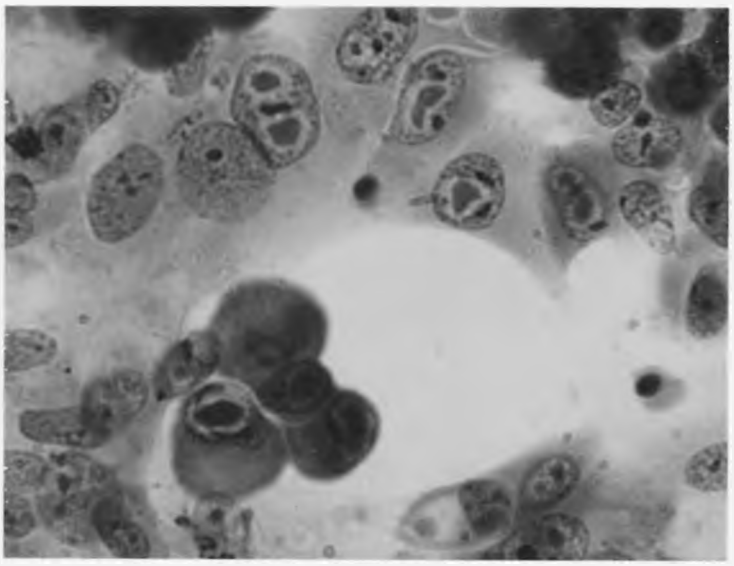

Fig. 16. SA6 in Vervet Kidney Cells Coverslip 5157A 650x H & E

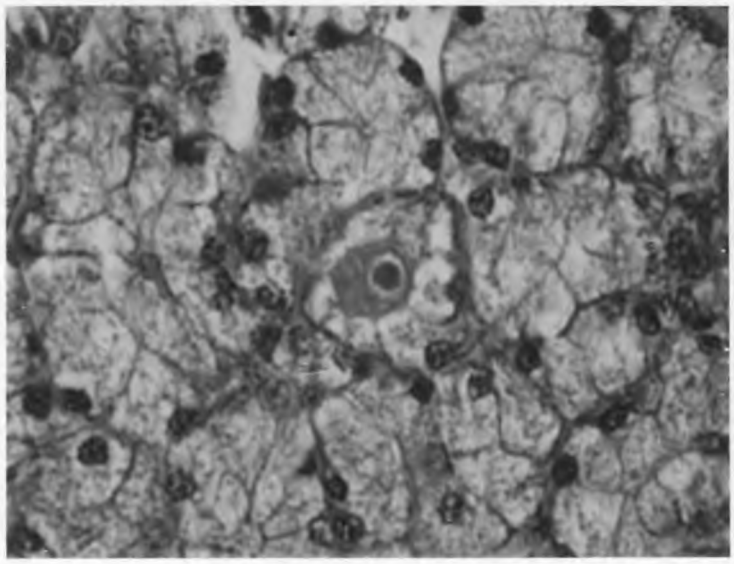

Fig. 17. Cytomegalovirus Inclusion in Vervet Parotid Gland Histology Section 10160A 650x H & E

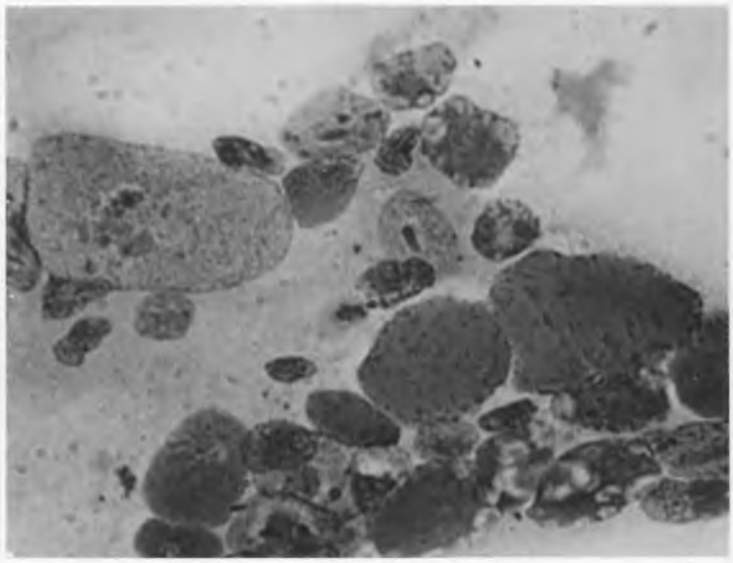

Fig. 18. SA15 in Vervet Kidney Cells Coverslip 5316B 650x H & E

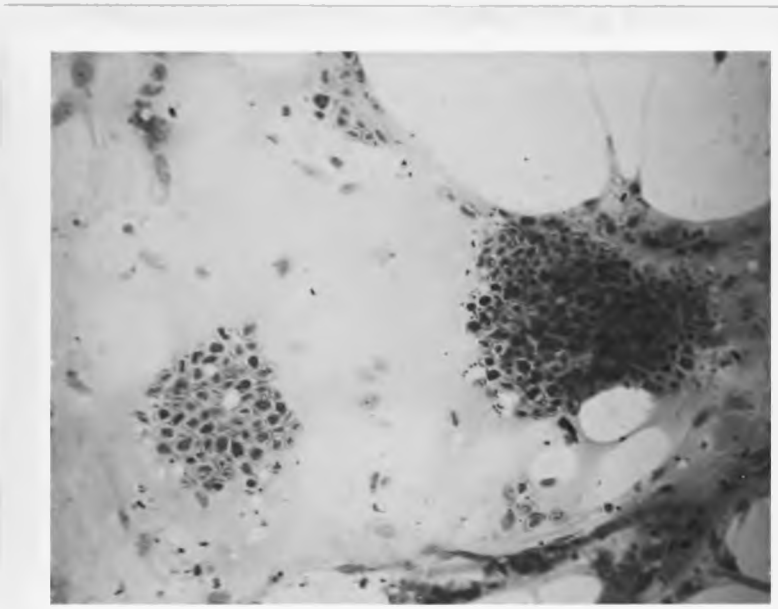

Fig. 19. Baboon Herpes in Vervet Kidney Cells
Coverslip A57A 162x
H & E

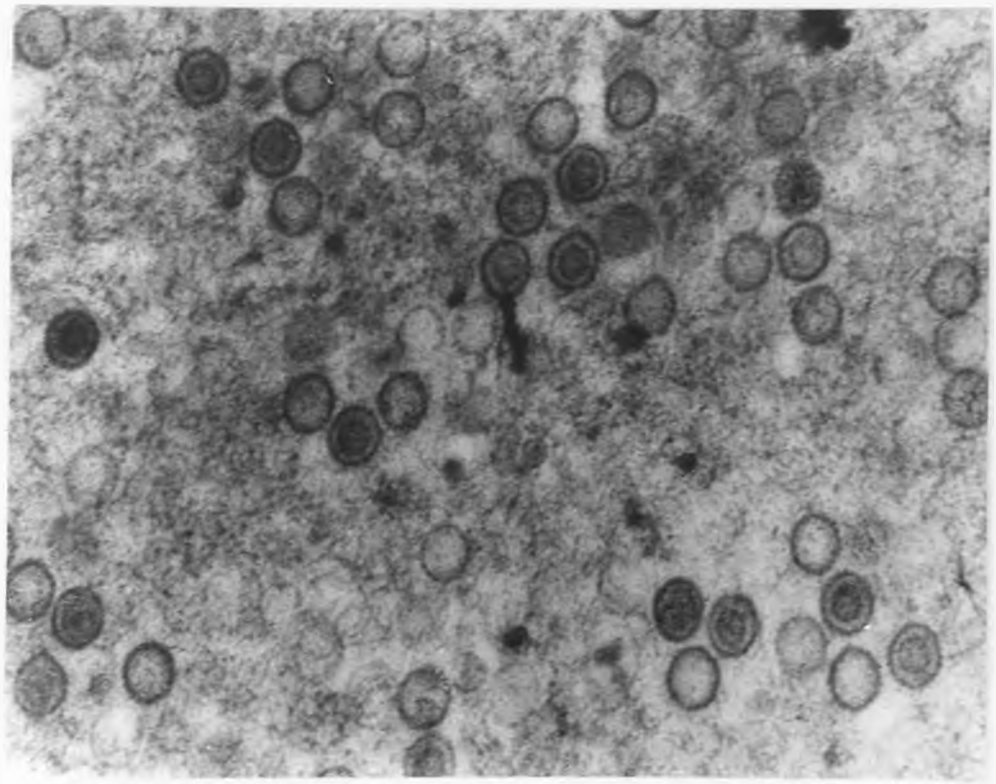

Fig. 20. Electron Micrograph of Baboon Herpes
Strain 28T2 Dr.G.Lecatsas 16/2/70
80 000x Osmic Acid

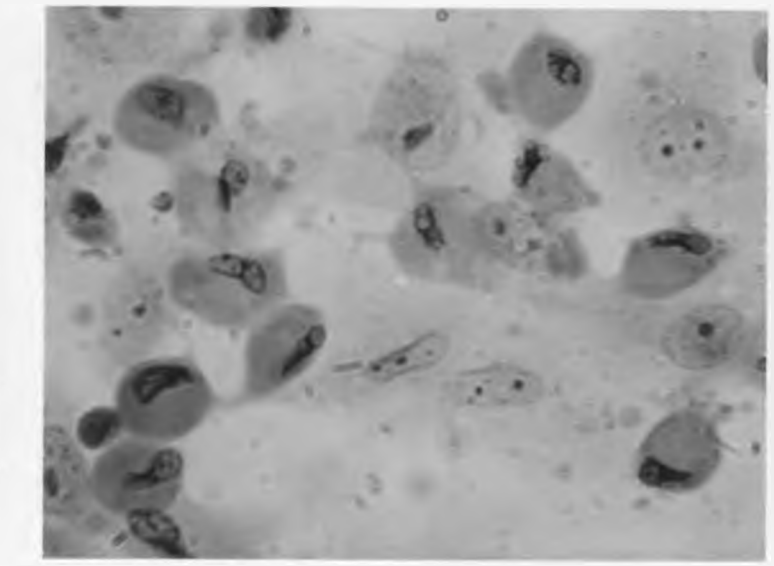

Fig. 21. SA4 in Vervet Kidney Cells
Coverslip 1190B 650x H & E

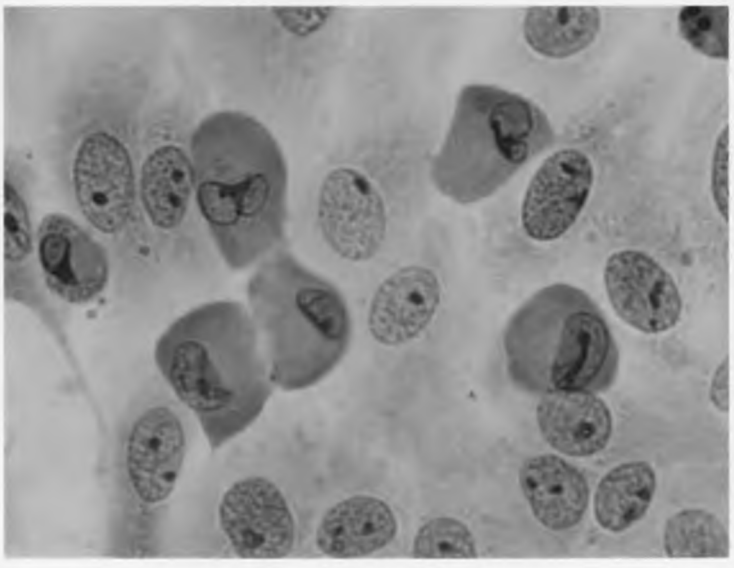

Fig. 22. SA5 in Vervet Kidney Cells
Coverslip 5179D 650x H & E

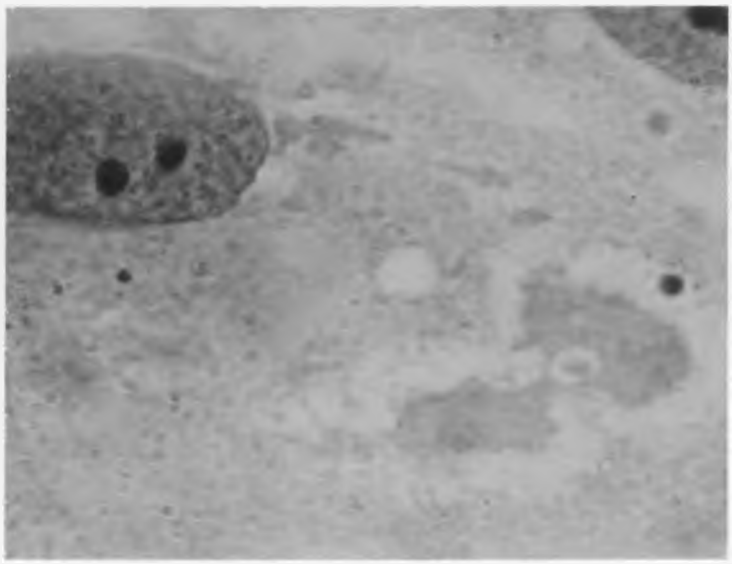

Fig. 23. P72/16/2 Agent in Vervet Kidney Cell
Coverslip C276F 1625x H & E

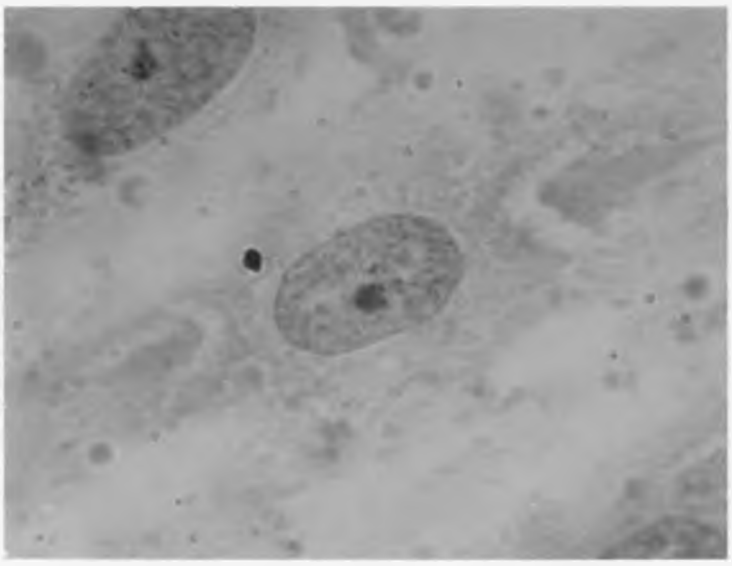

Fig. 24. SA9 in Vervet Kidney Cells
Coverslip 379B 1625x H & E

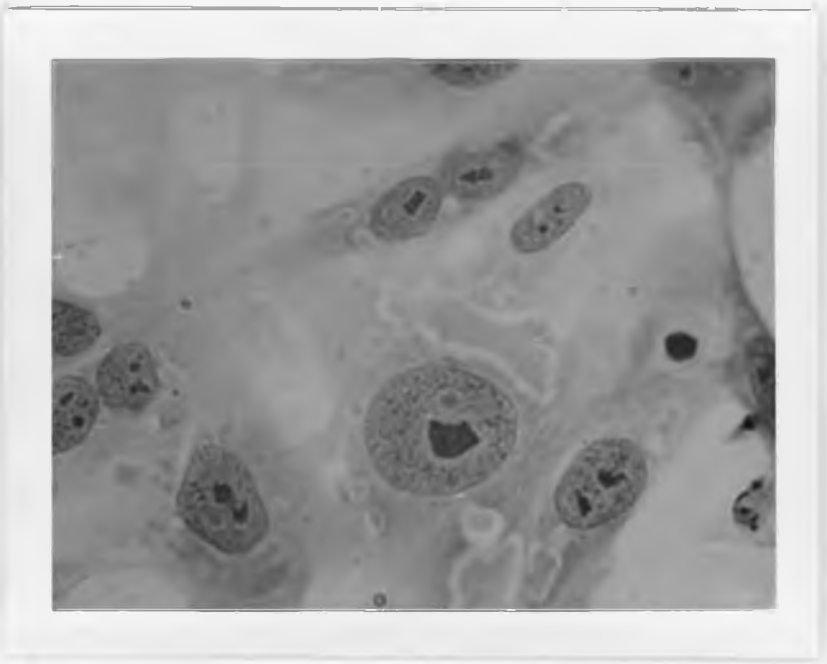

Fig. 25. SA 10 in Vervet Kidney Cells
Coverslip 3841B 650x H & E

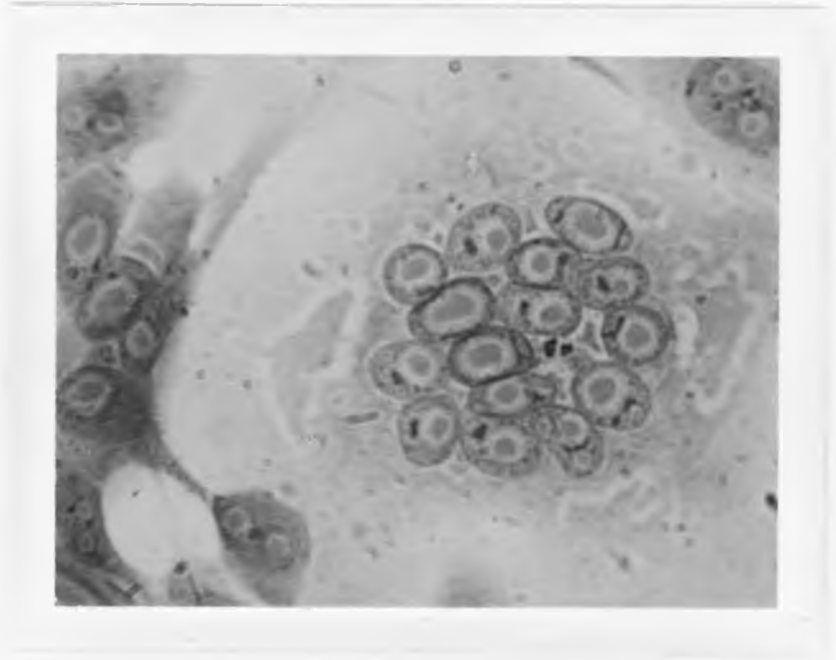

Fig. 26. SA 13 Strain B47 in Vervet Kidney
Cells Coverslip 5271C 650x H & E

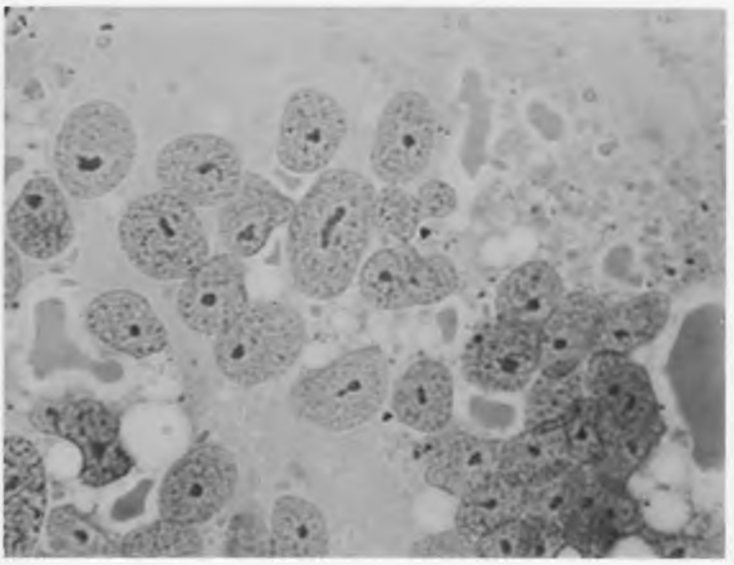

Fig. 27. Agent D490 in Vervet Kidney Cells
Coverslip 7306G 650x H & E

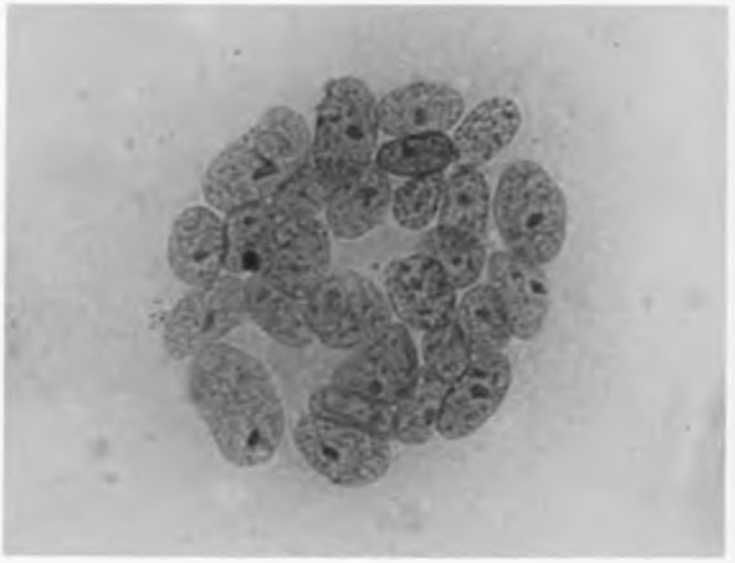

Fig. 28. SA 1 Without Foam
Coverslip 5195B 650x H & E

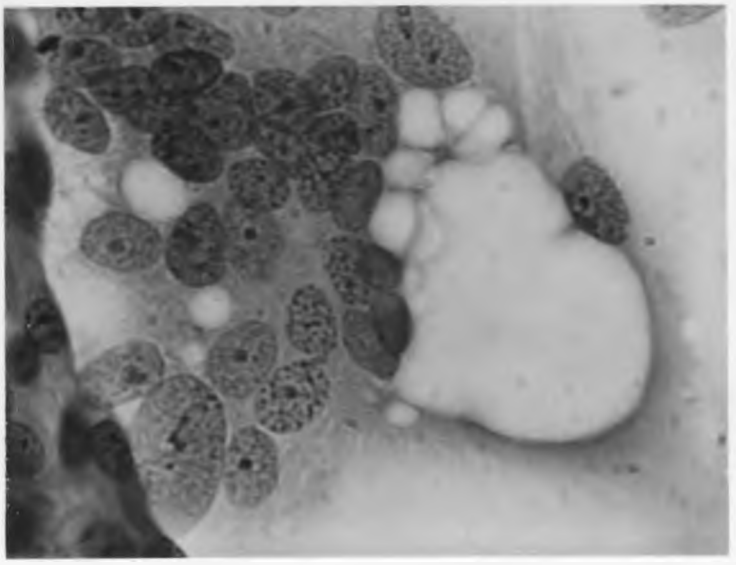

Fig. 29. SA 1 With Foam
Coverslip 5195B 650x H & E

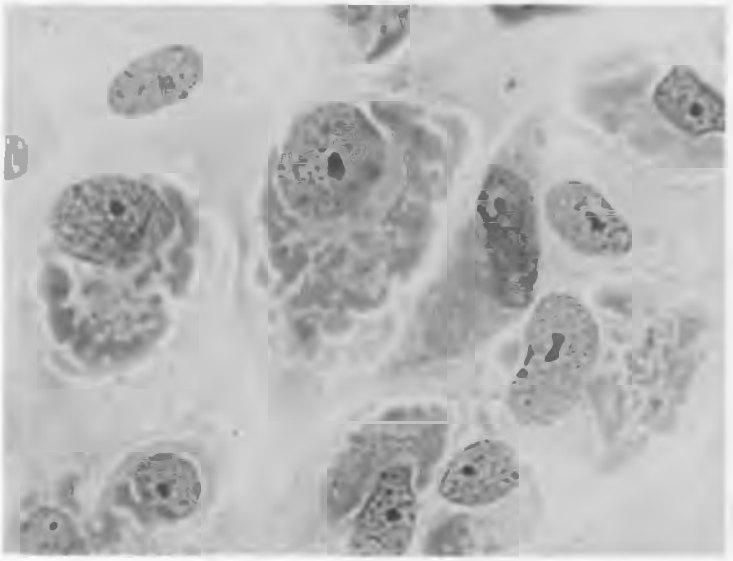

Fig. 30. SA 3 Reovirus in Vervet Kidney Cells
Coverslip 5977A 650x H & E

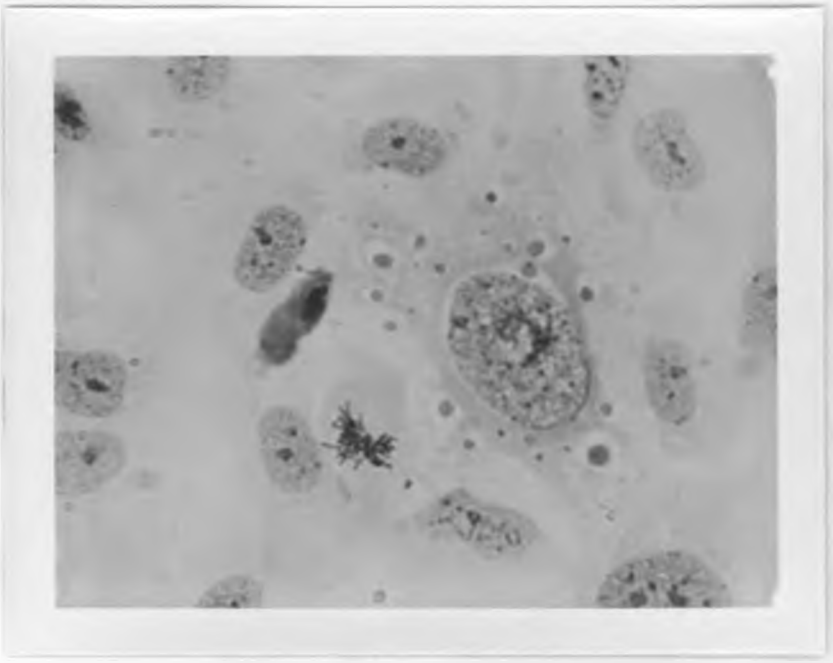

Fig. 31. SA 11 in Vervet Kidney Cells
Coverslip 7614A 650x H & E

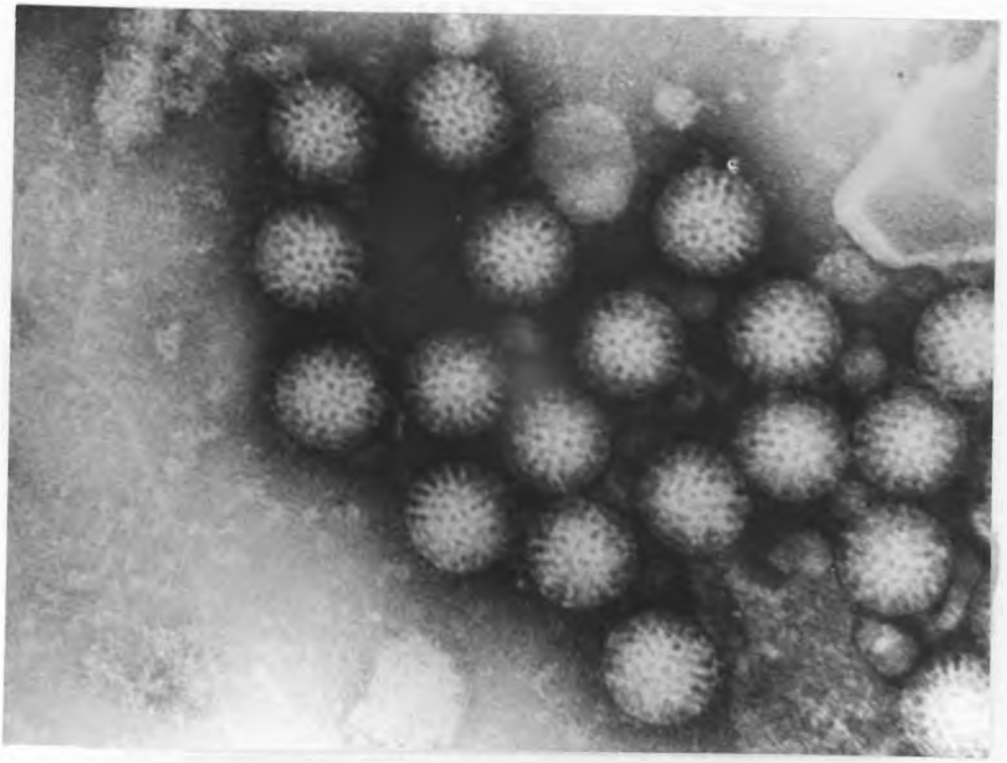

Fig. 32. SA 11 Naked Virions. Electron Micrograph
Dr.G.Lecatsas Phosphotungstic Acid
200 000x

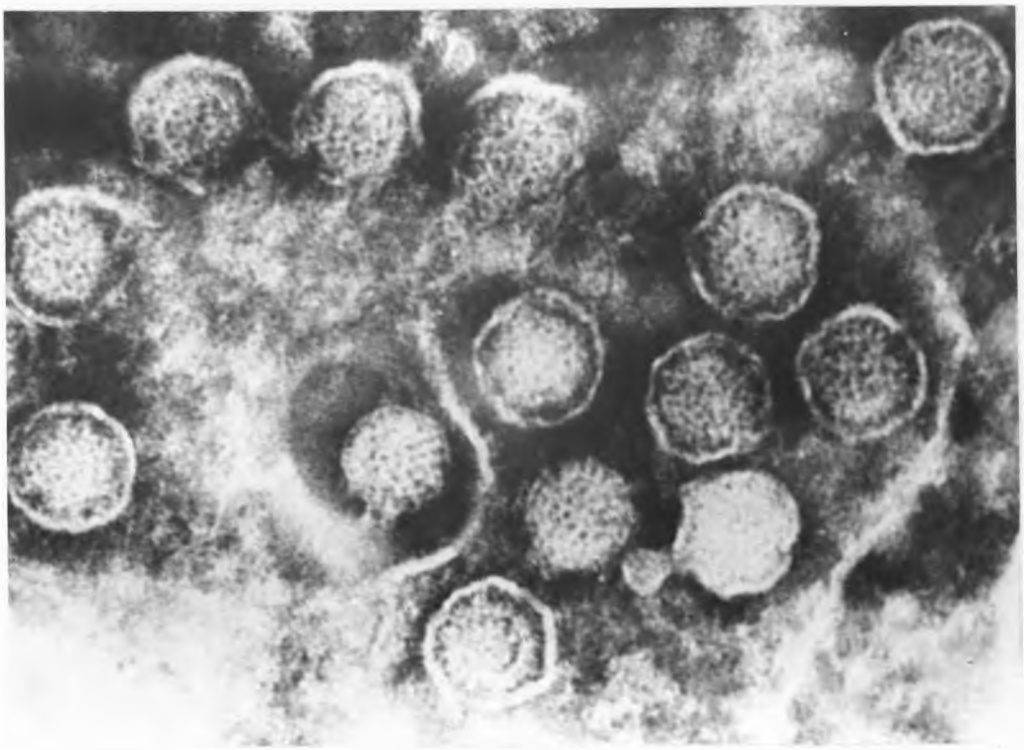

Fig. 33. SA 11 Virions with Envelopes Electron Micrograph Dr.G.Lecatsas Phosphotungstic Acid 270 000x

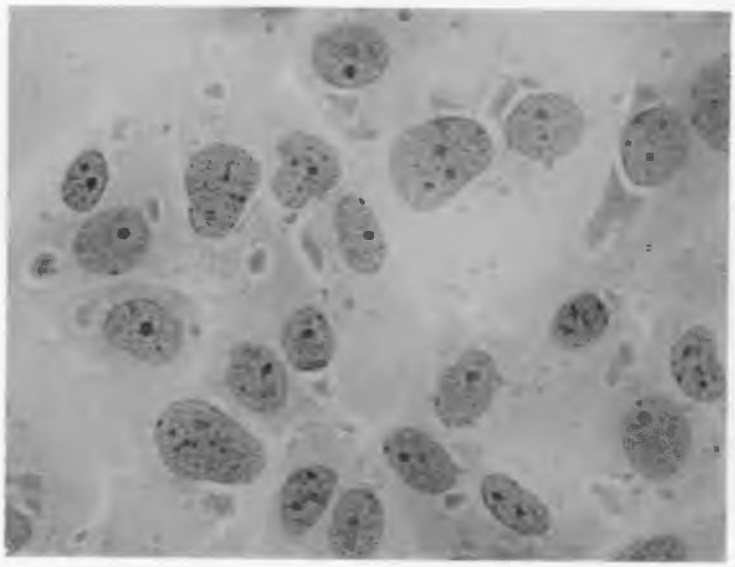

Fig. 34. Marburg Virus in Vervet Kidney Cells Coverslip A36U 650x H & E

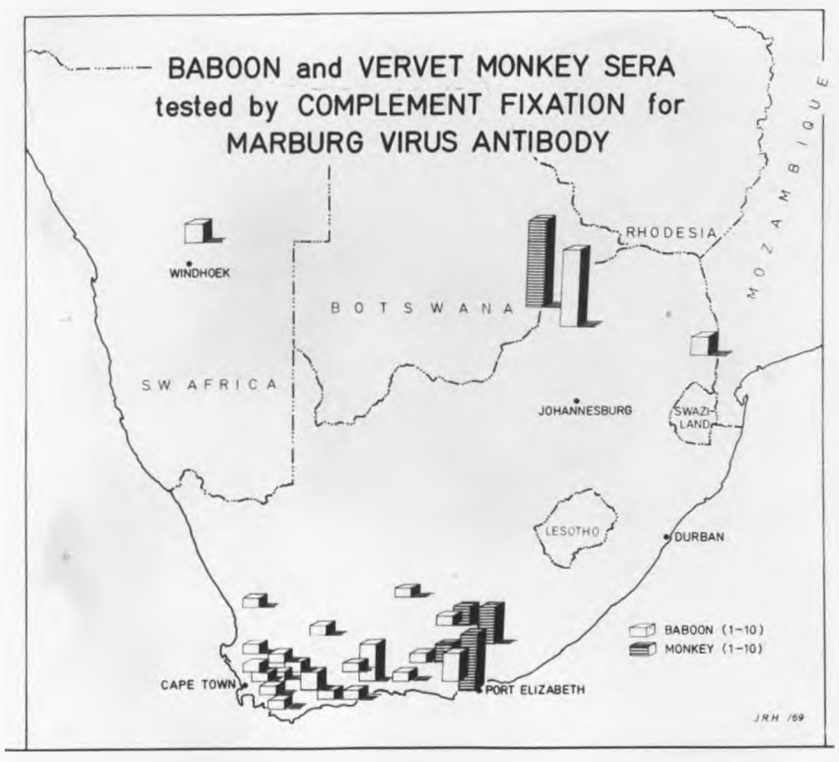

Fig. 35. Marburg Virus Antibody Survey Map

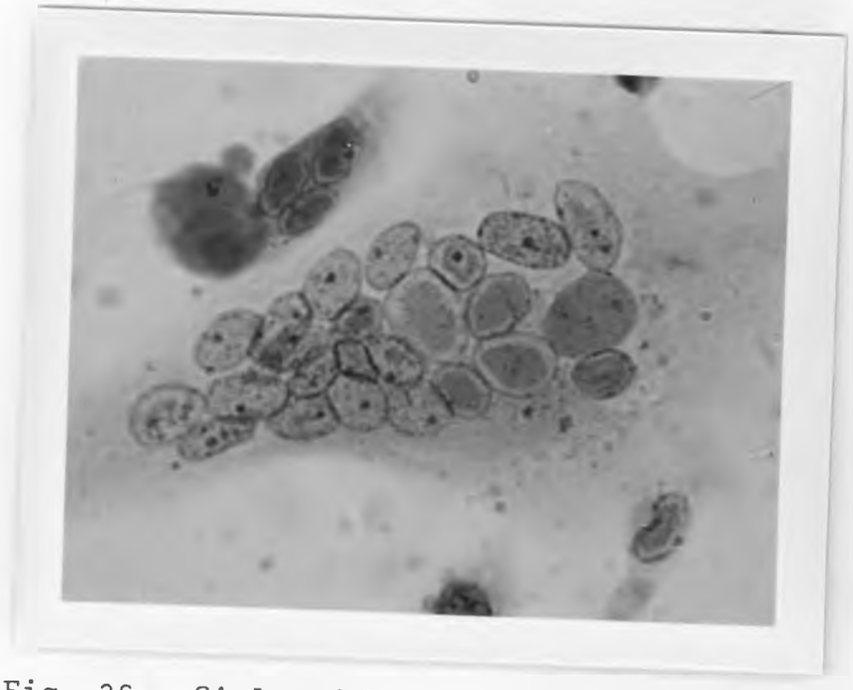

Fig. 36. SA 1 and Herpes Simplex Viruses in Vervet Kidney Cells
Coverslip 3828D 650x H & E

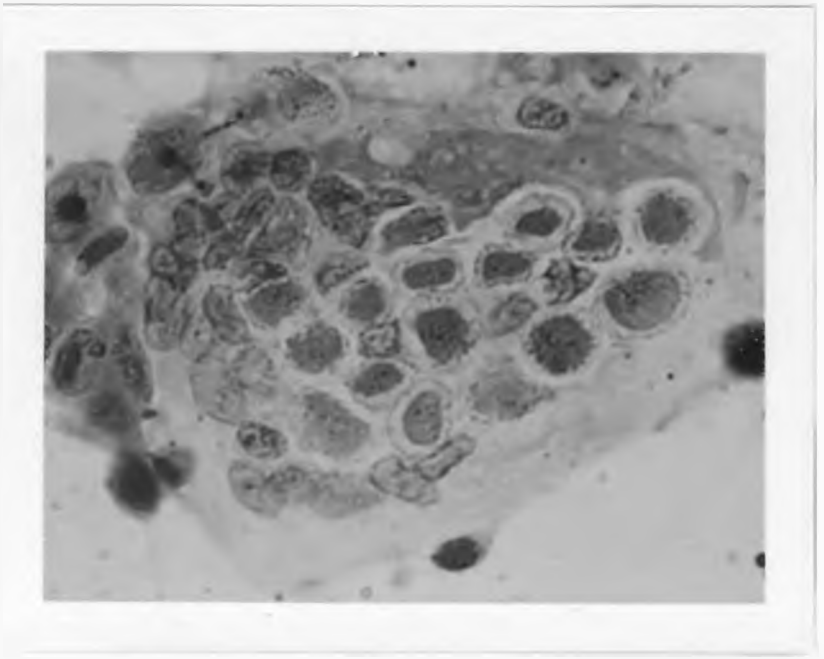

Fig. 37. SA 1 and Human Adenovirus in Vervet Kidney Cells
Coverslip 6325D 650x H & E

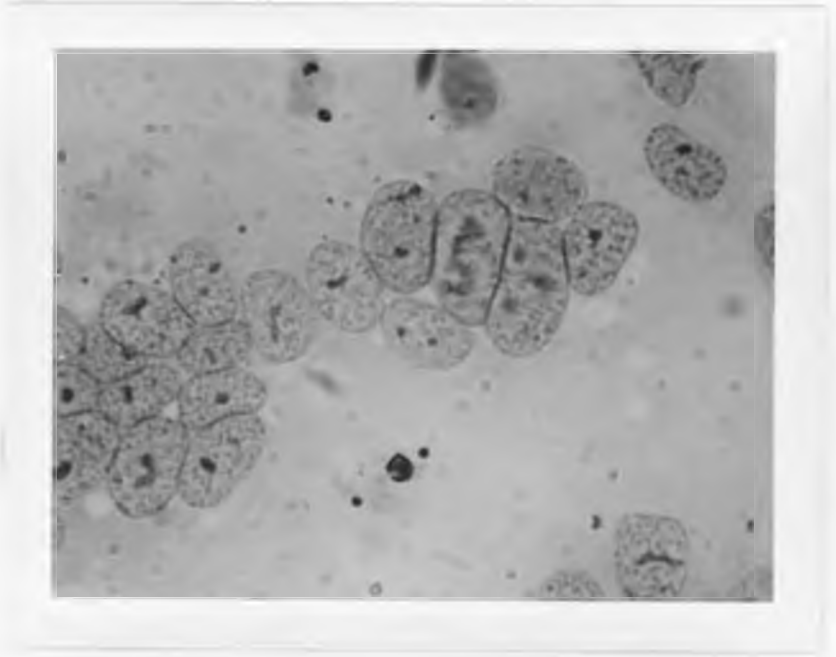

Fig. 38. SA 1 and SV 40 in Vervet Kidney Cells
Coverslip 3337D 650x H & E

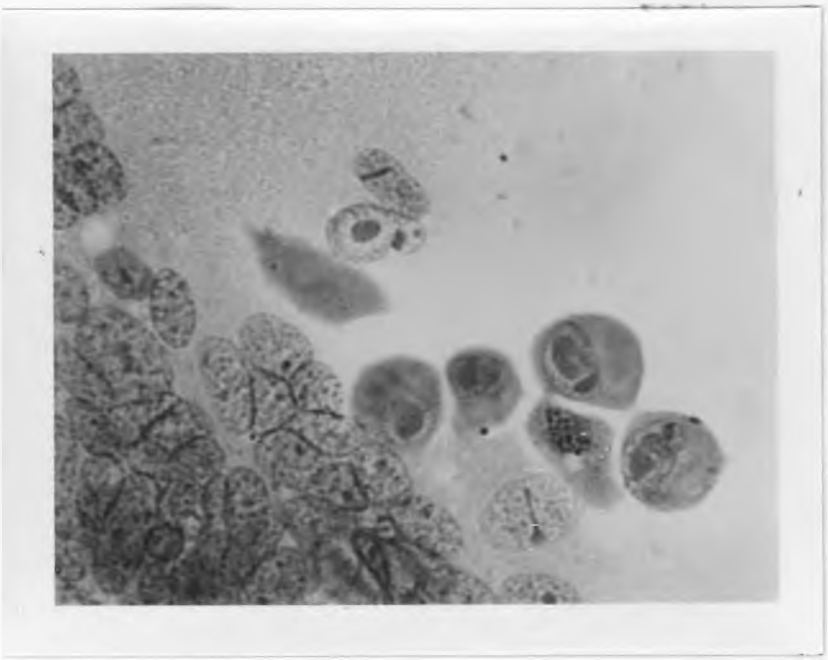

Fig. 39. SA 1 and SA 6 in Vervet Kidney Cells
Coverslip 6169A 650x H & E

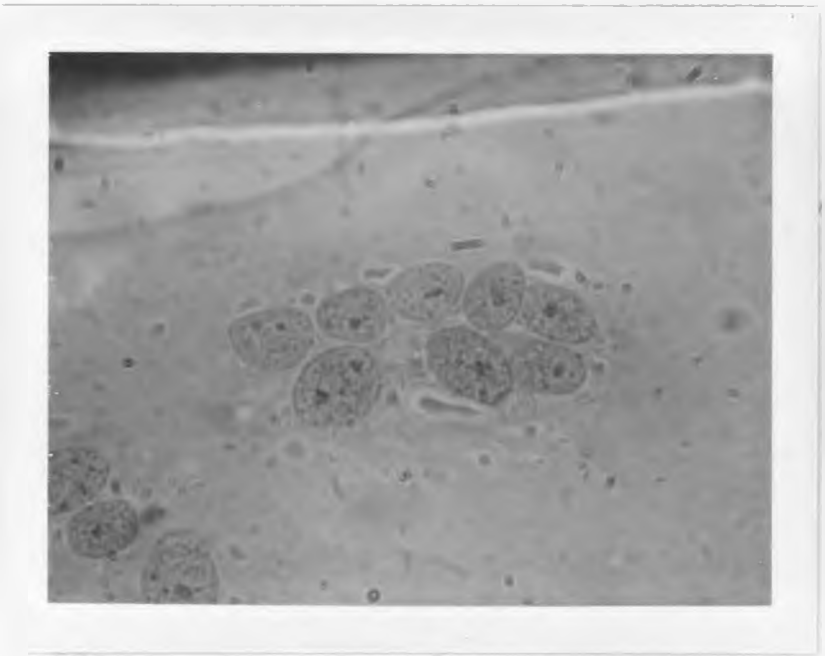

Fig. 40. SA 1 and SA 3 in Vervet Kidney Cells
Coverslip 5754B 650x H & E

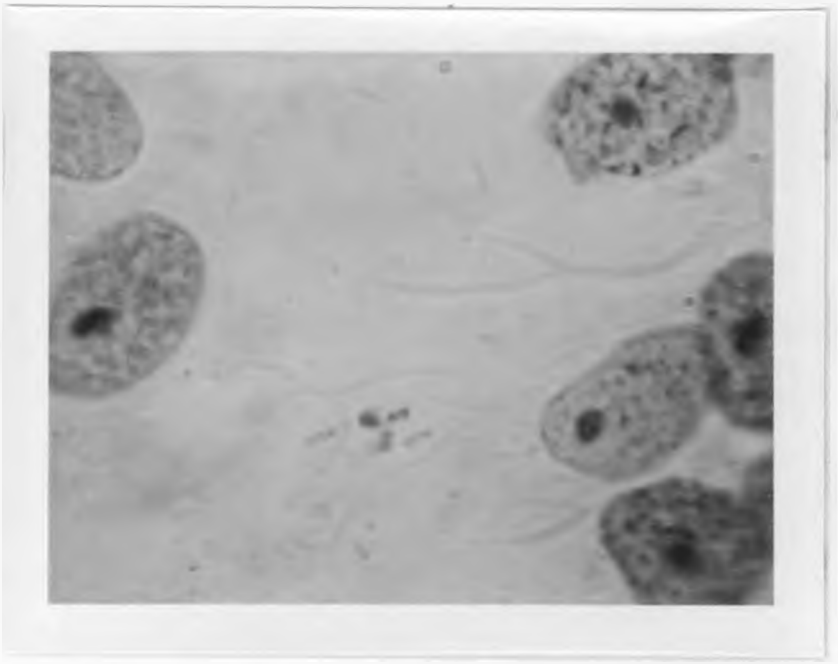

Fig. 41. SA 1 and Marburg Virus in Vervet Kidney Cells
Coverslip A140E 1625x H & E

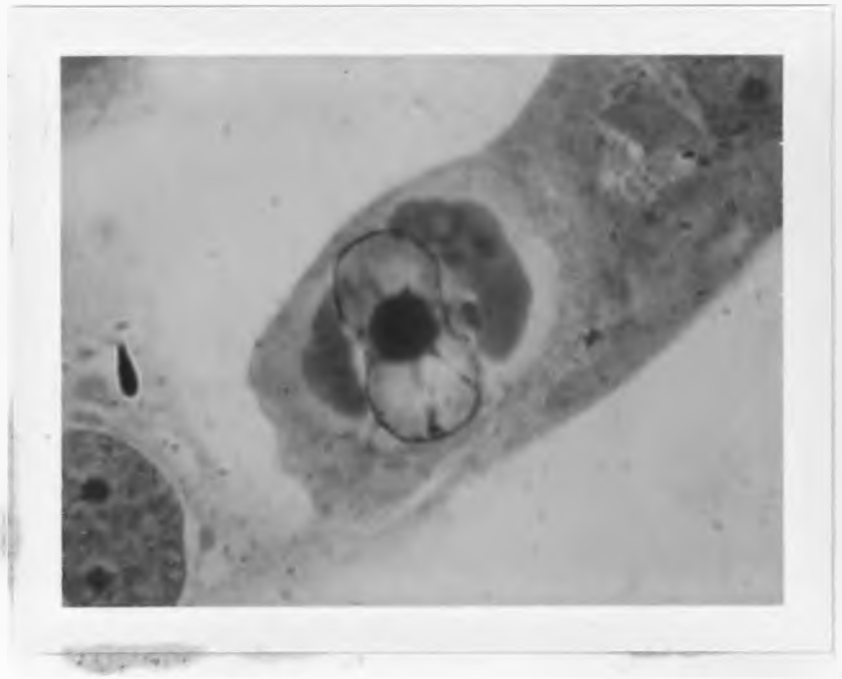

Fig. 42. Reovirus and Adenovirus in Vervet Kidney Cell
Coverslip 8995B 1625x H & E

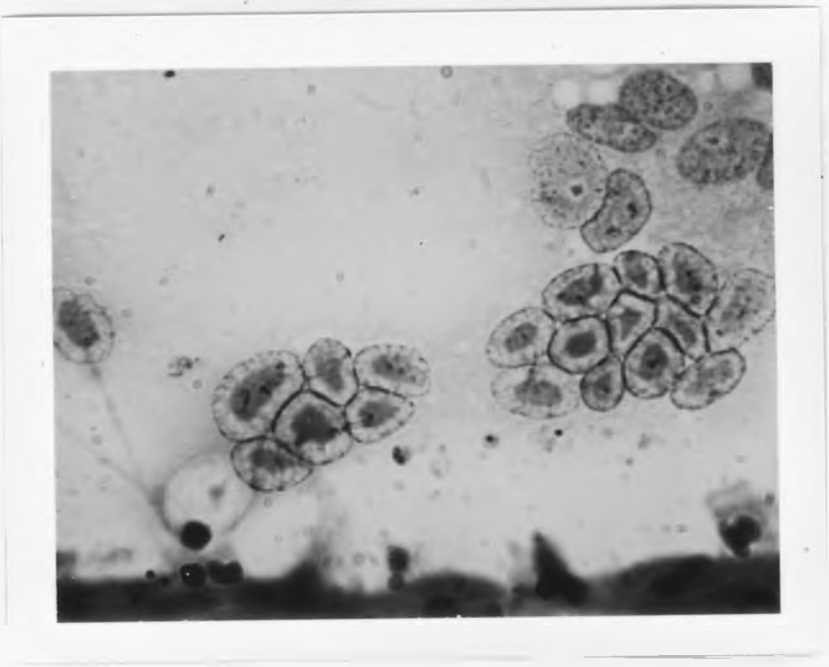

Fig. 43. SA 2 in Uninoculated Vervet Kidney Cells
Coverslip 5011D 650x H & E

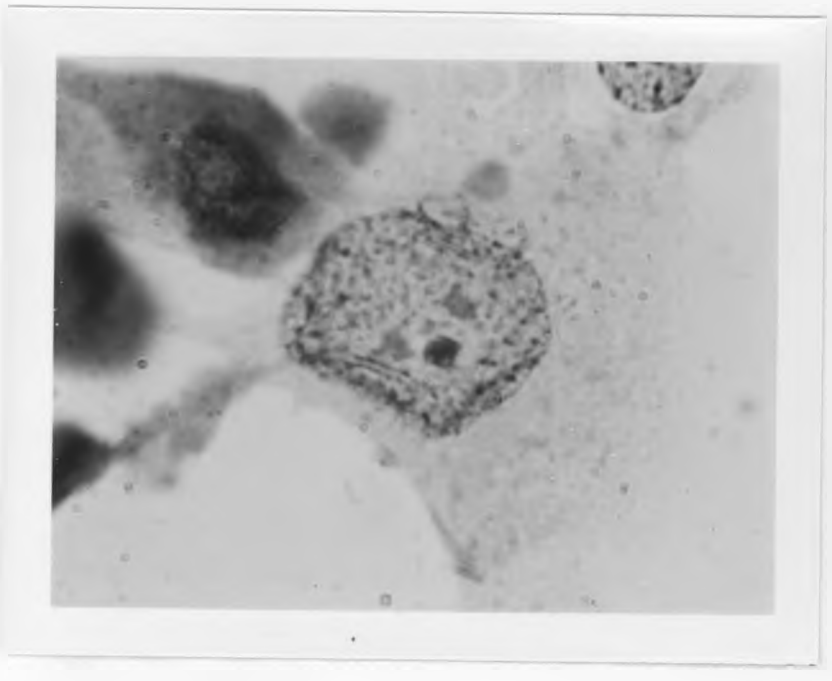

Fig. 44. Poliovirus Nuclear Inclusions in
Vervet Kidney Cell
Coverslip 5920G 1625x H & E

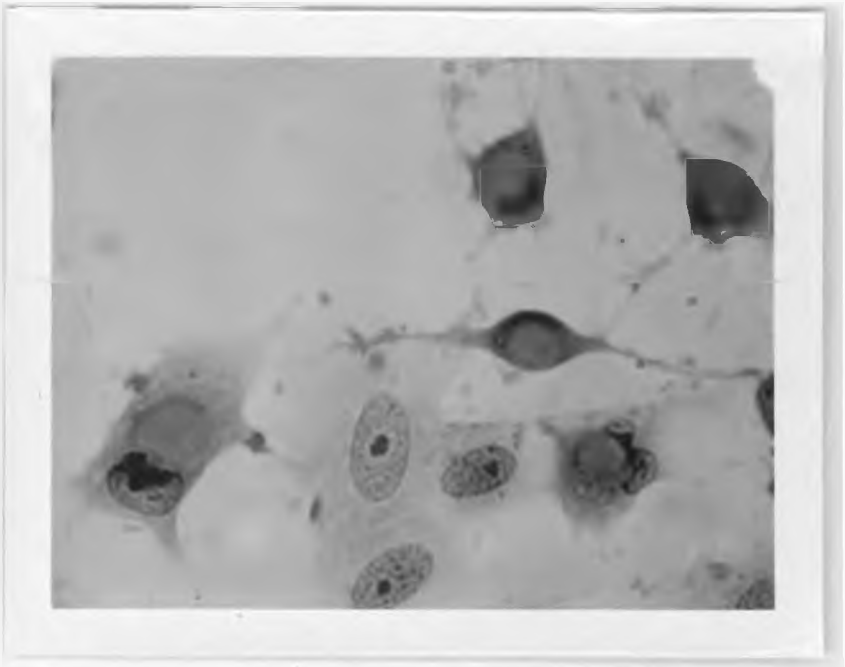

Fig. 45. Late Stage of Poliovirus Infection in Vervet Kidney Cells
Coverslip 5920H 650x H & E

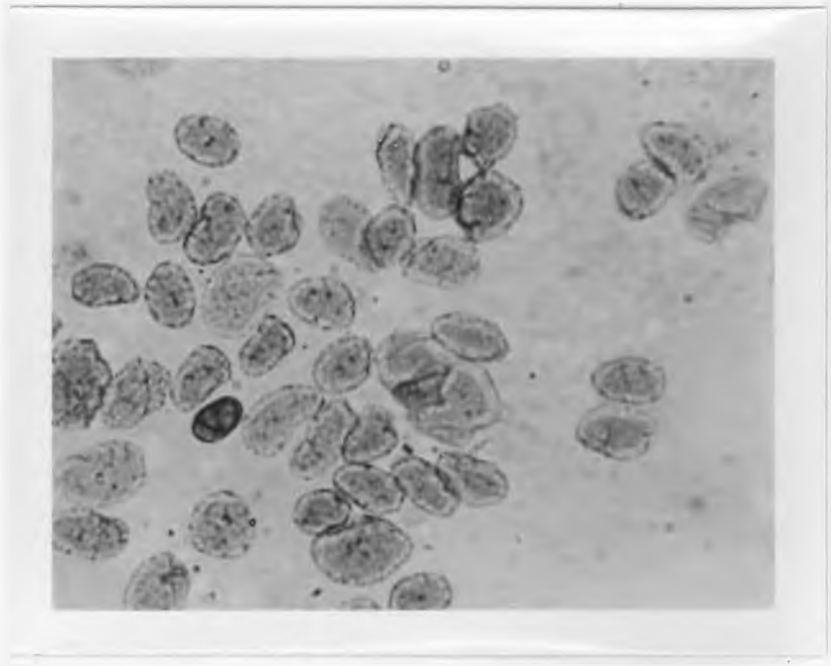

Fig. 46. SA 1 and Poliovirus in Vervet Kidney Cells
Coverslip 5920P 650x H & E

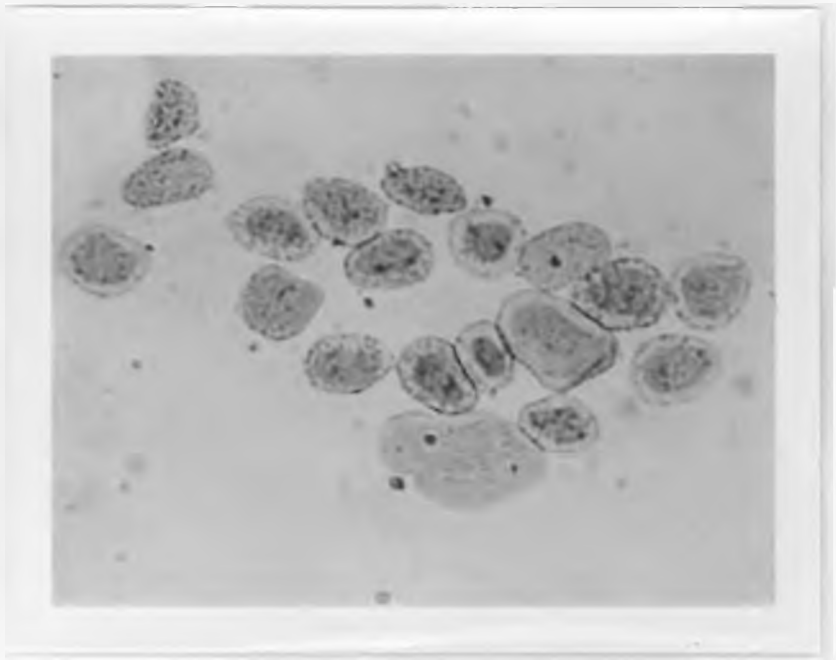

Fig. 47. SA 1 and Poliovirus from Neutralization Test
Coverslip 5710E 650x H & E

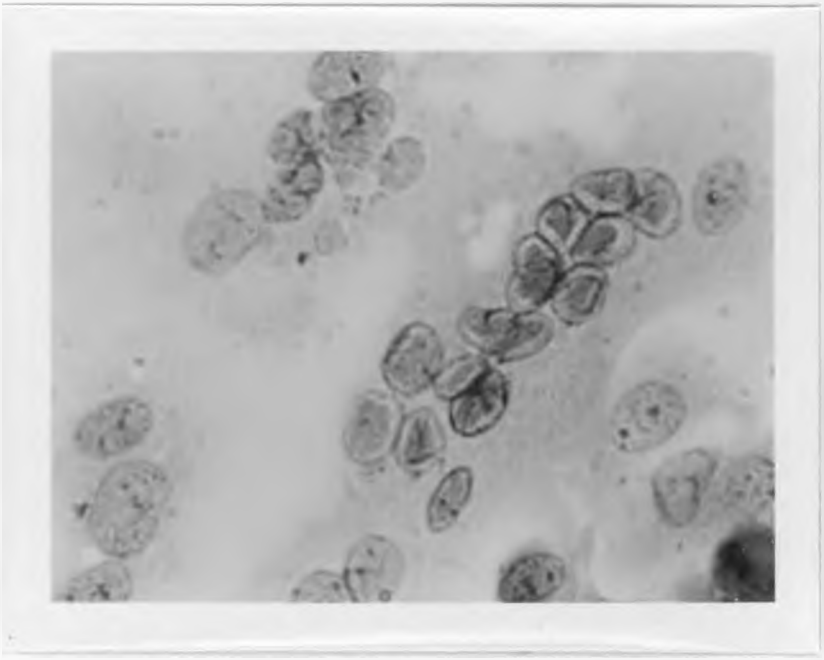

Fig. 48. SA 1 and Echovirus Type 1 in Vervet Kidney Cells.
Coverslip 5947J 650x H & E

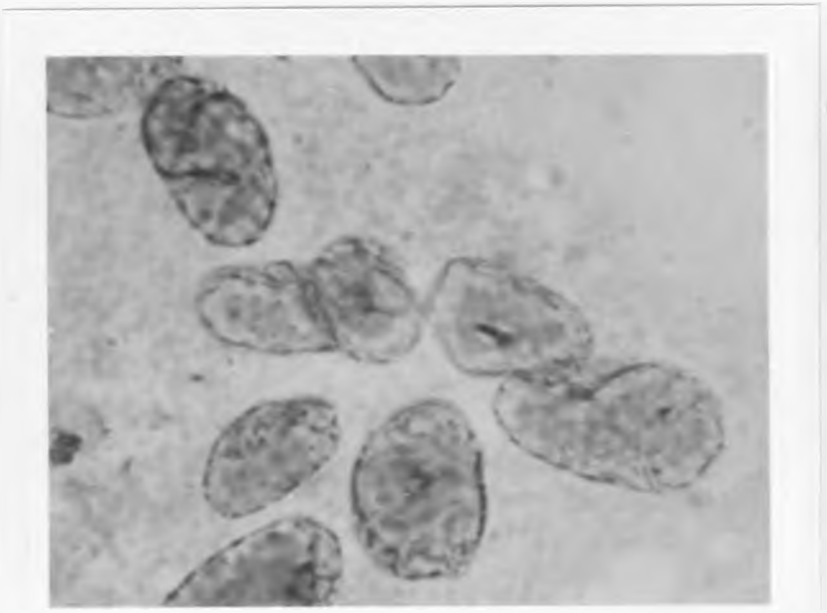

Fig. 49. SA 1 and SA 4 Enterovirus in Vervet Kidney Cells
Coverslip D610C 1625x H & E

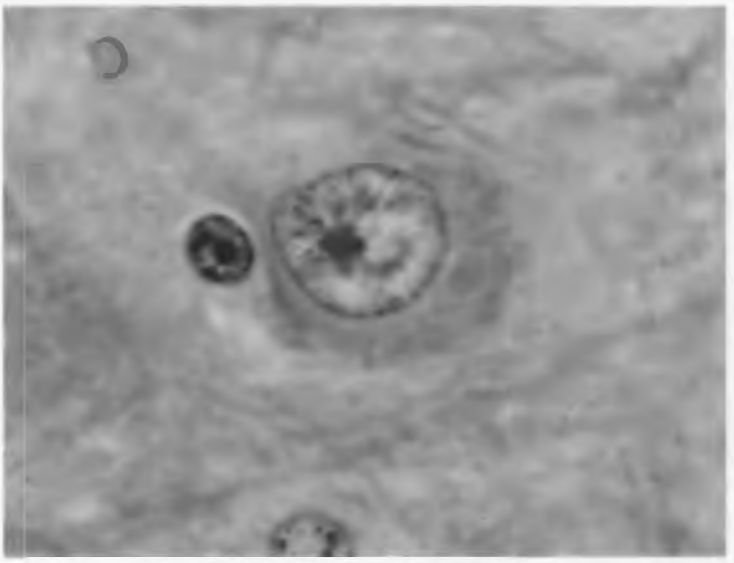

Fig. 50. Cytoplasmic Inclusion in Vervet Thalamus Neurone. Histological Section 23001G 1625x H & E

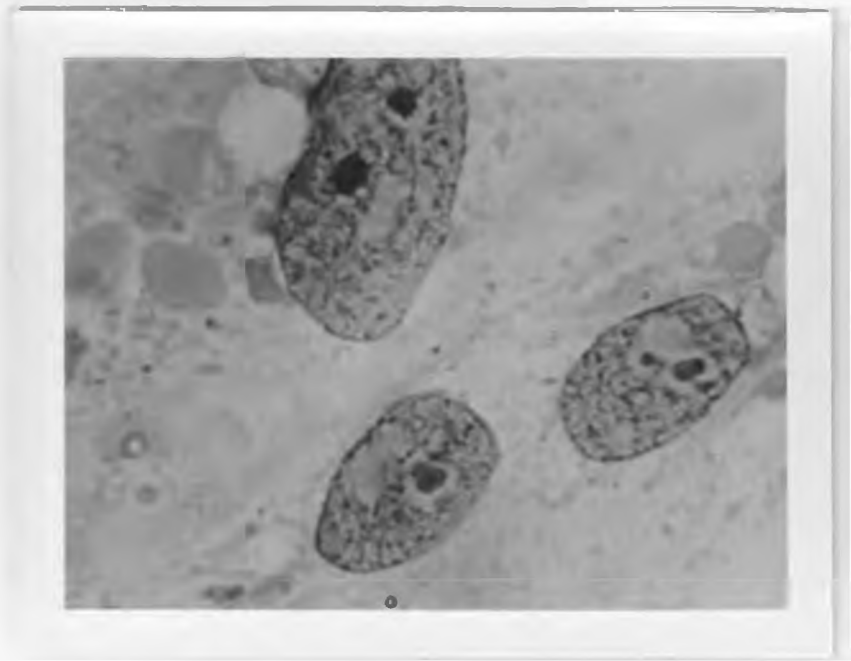

Fig. 51. Unreo Agent in Vervet Kidney Cell
Coverslip 6999R 1625x H & E

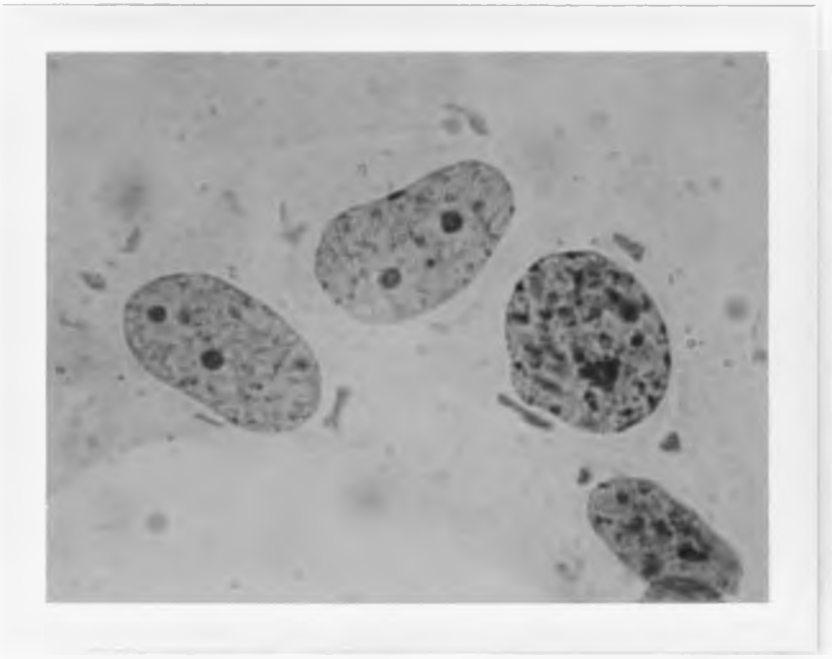

Fig. 52. Bronn Agent in Vervet Kidney Cell
Coverslip A268A 1625x H & E

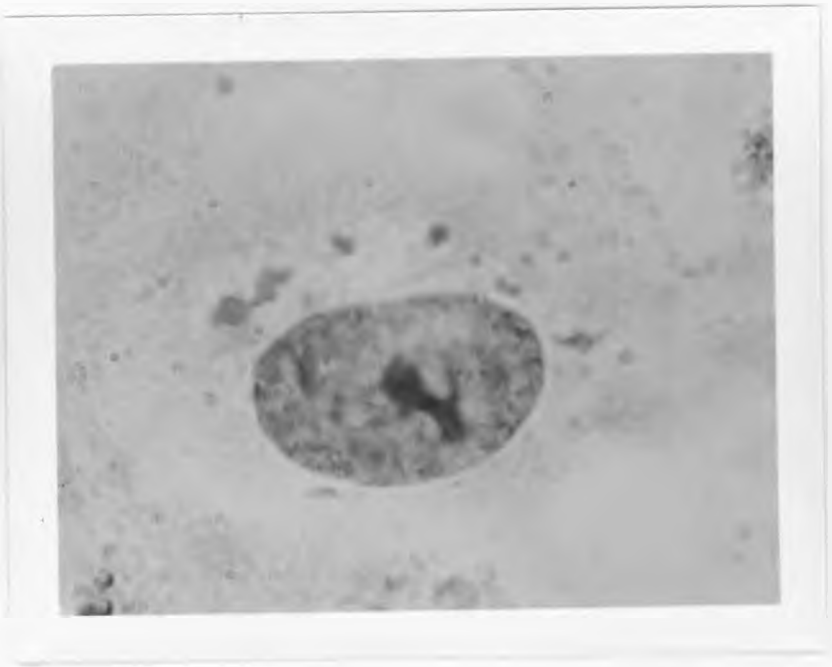

Fig. 53. VK292 Agent in Vervet Kidney Cell
Coverslip B69L 1625 x H & E

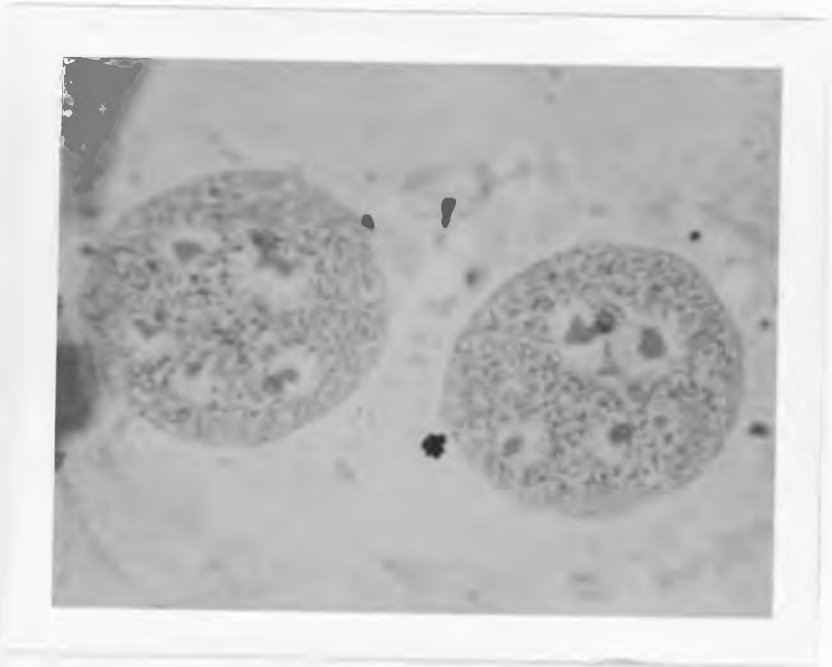

Fig. 54. Actinomycin D Inclusions in Vervet
Kidney Cell
Coverslip 8236D 1625x H & E

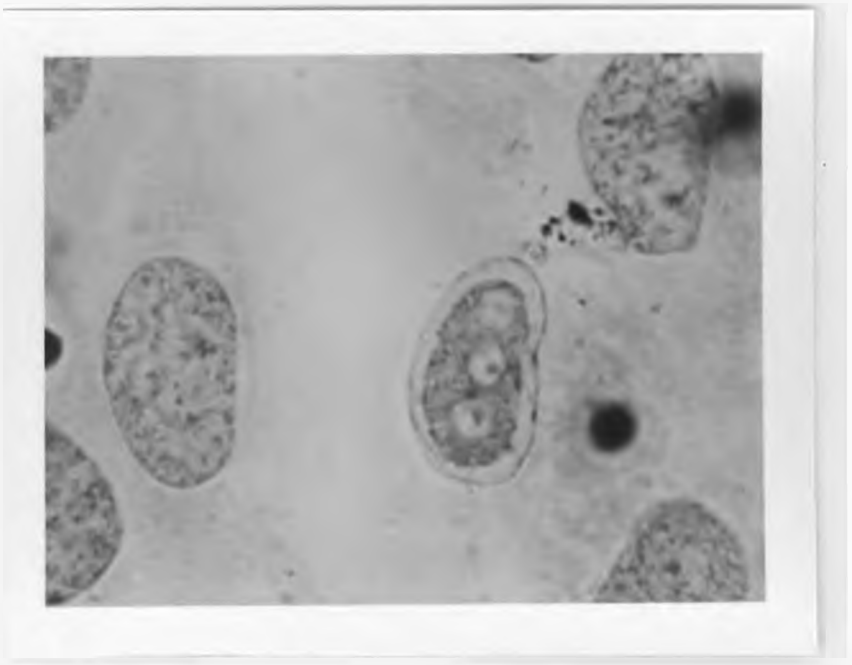

Fig. 55. Actinomycin D Basophilic Inclusion in Vervet Kidney Cell
Coverslip 8236D 1625x H & E



Fig. 56. Acanthamoeba Trophozoites in Vervet Kidney Tissue Culture
Coverslip A881K 1625x H & E

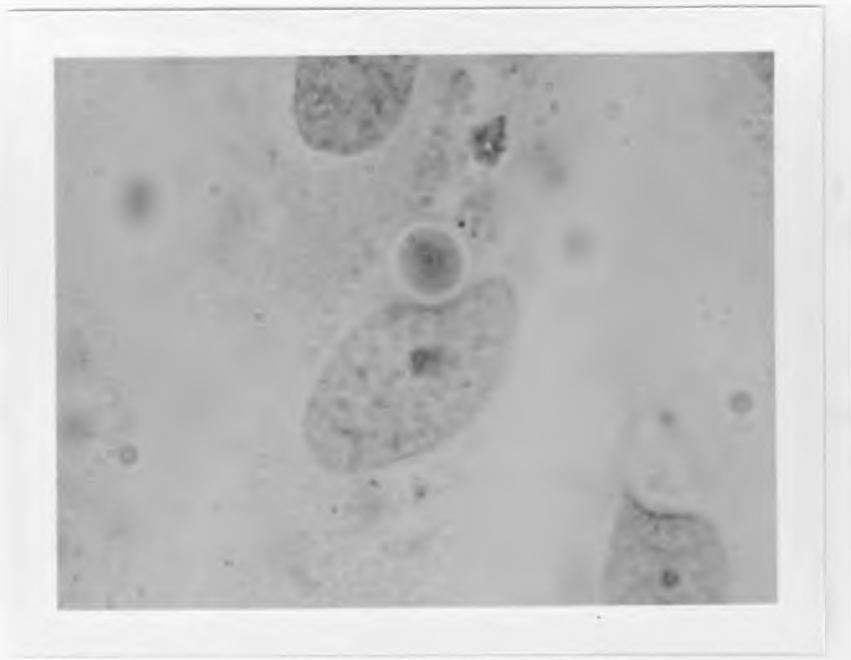

Fig. 57. Pre-cystic Form of Amoeba in Vervet Kidney Cell
Coverslip B55D 1625x H & E



Fig. 58. Nuclear Bodies Induced by Amoeba in Vervet Kidney Cell
Coverslip B55E 1625x H & E

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