~~STUDIES ON THE~~ VARIANTS OF VARIOLA VIRUS

BY

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ABSTRACT

Two laboratory tests are known which distinguish variola major and variola minor strains. They are the mortality rate and ceiling temperature in chicken embryos. There has been some indication from these two tests that intermediate strains of variola exist in Africa but further marker characters were needed to categorize variola strains more fully. Three possible new marker characters are described in this thesis. They are : the development of haemadsorption at 40° in cultures of human embryonic skin and muscle, the induction of cytopathic effects in the same cells and the production of haemagglutinin in cultures of HEp2 cells. Cross comparisons between these five tests showed that they did not all measure the same virus properties. In particular the haemadsorption test gave results which supplemented those obtained by ceiling temperature tests in chick embryo chorioallantois. Two hundred and eighteen strains of variola have been subjected to these two tests and a proportion of these to the other three tests as well.

The variola strains used were well established variola major and minor and a number of strains from various parts of Africa as well as a smaller number from Pakistan and Indonesia. Comparison of the results established the existence of at least one variola 'family' other than major and minor and also suggested the existence of further variants. The laboratory tests were done without knowledge of the origins of the strains and this lends greater interest to the observation that strains from certain areas were similar in the profile of their characters. While this work was in progress viruses were isolated from sporadic cases of smallpox-like illness which proved to be indistinguishable from monkey pox. Five such strains were obtained which were compared with standard strains of monkey pox and with variola. The question of the possible interconversion of variola and monkey pox viruses was examined by investigating the properties of four viruses isolated from animals which shared more properties in common with variola than did monkey pox. Some attempts were made to induce variola variants in the laboratory but it proved to be very stable and no significant success was obtained. The significance of these observations is discussed and relevant literature is cited.

CHAPTER I

INTRODUCTION

Smallpox has been known as an acute exanthematous and highly infectious disease since very ancient times. From time to time the disease would break out in epidemic form and cause much suffering and panic in the population claiming thousands of lives. Even its complication and scarring cause thousands of children, men and women to be crippled and handicapped both mentally and physically. The disease was a great terror up to 17th century when Jenner introduced vaccination which came as a boon to human sufferings and miseries. Still in this 20th century we are depending on vaccination which has been improved in various ways since Jenner's time. Up till now there is not any effective specific therapy for this disease. It has been found that a compound called N-Methylisatin β - thiosemicarbazone

gives a promising result against intracerebral infection of mice with variola virus (Bauer et al 1962). Further Bauer et al (1963) carried out some preliminary studies in Madras which suggests that this compound (Methisazone) may be effective in preventing smallpox in exposed family contacts. But the drug which will cure the smallpox is yet to be discovered.

Starting from Jenner's time and gradually afterwards by the proper isolation of the disease and by effective vaccination, the smallpox epidemic seems to be almost controlled in most parts of the world, and countries which have become non-endemic maintain their status by strict quarantine regulations. But in the darker side of the world, in the poor underdeveloped, densely-populated countries the disease continues endemic and hundreds of human lives are still being claimed each year. These areas are endemic foci and a source of constant threat to the rest of the world. Modern fast travel and fast trading between the countries, make it very difficult to maintain the endemic and non-endemic areas of smallpox apart. This problem can only be solved if the whole world could be made free from smallpox. The World Health Organization has undertaken a world wide programme of smallpox eradication. The plans are carried out by sending experts to different areas, who by proper planning of mass vaccination and by rapid isolation and identification of the disease, are trying to get rid of the disease country by country. This programme is going on very effectively and it is confidently assumed

that after a short period no smallpox will exist on earth.

The causative organisms of the disease are v. major and v. minor viruses of the variola-vaccinia subgroup of Poxviruses. Clinically the disease is highly infectious and is characterised by fever followed in 2 - 3 days by a macular rash which transforms into papules, vesicles, pustules and lastly scabs. Healing is accompanied by scarring. The disease caused by both types of viruses runs the same pattern and differs by the severity, mortality rate and liability to complications. The most amazing difference is in the mortality rate. Smallpox caused by v. major has the mortality rate 20 - 30%, sometimes as much as 40 - 50% in the unvaccinated. V. minor causes disease with a mortality rate less than 1% irrespective of vaccinated or non-vaccinated populations.

Dixon (1962) classified the disease caused by v. major into nine types based on principal clinical characteristics. Type I does not give any picture typical of smallpox. After 12 days incubation period the patient is suddenly ill with severe prostration. The patient usually dies within 36 - 48 hours. The diagnosis both clinical and at autopsy is impossible unless smallpox is thought of and laboratory facilities are used for the detection of virus. If the patient is not dead within 48 hours, erythema appears on face and body and the patient's condition deteriorates. The patient dies with massive haematemesis or intestinal haemorrhage. There is no vesicular eruption. Downie et al (1969a) noted that pregnant mothers

are more likely to develop haemorrhagic (Type I) smallpox than non-pregnant women of the same age. They suggested that the circulating steroids in the blood during pregnancy might play a part in the haemorrhagic smallpox. Type II and III are called malignant confluent and semi-confluent respectively. The mortality rate is 70 - 80% and 15 - 20% respectively. In both cases the clinical appearance is different from 'classical smallpox'. There are not any vesicular or pustular stages in these types. Erythema and haemorrhage is common. Types I, II and III occurs mostly in unvaccinated group. Type IV and V are called benign confluent and semi-confluent respectively. These two types are called classical smallpox clinically. The mortality rate is 20 - 30% and 12 - 15% respectively. Incubation period is 12 days. Pre-eruptive illness is great with high temperature. The temperature drops in the next 48 hours with the appearance of the macular rash. The rash is of centrifugal origin (Ricket 1908 quoted by Dixon). The macules are gradually replaced by papules and then by vesicles. The vesicular lesions start to grow in size so that by 8 or 9 days the body is covered with pearly vesicles. The vesicle changes into a pustule which dries up and develops a scab. When this occurs, secondary fever and mild toxemia develops. Dixon (1948) found by treating cases with antibiotic that secondary fever is an integral part of some cases of smallpox. By the fourteenth day of the disease the scabs usually begin to fall out leaving scar marks.

Type VI and VII are called discrete and mild as mortality is about 2% and 1% respectively. Vesicles are very few in number and it occurs both in vaccinated and unvaccinated cases.

Type VIII is called abortive. Mortality is nil here. It occurs mostly in vaccinated persons. This is very important because diagnosis is often missed. Papules if present are very few and dry up quickly. This also is very important as the patient is completely unaware of the disease and can spread it among the population. Type IX also occurs in well vaccinated persons who only show an episode of temperature. The diagnosis of this type is often missed if smallpox is not being thought of and the specimens are not properly examined for smallpox.

When the disease occurs in the vaccinated, the normal picture of smallpox is deviated and 'cropping' occurs. The whole process undergoes quick maturation and lesions dry up without pustulation.

Type VII, VIII and IX are very important from the epidemiological point of view as the true diagnosis is often missed. The patient might pass unnoticed and risk of spreading the disease among the population is great.

Subclinical infection of smallpox in the form of conjunctivitis was reported by Kempe et al (1969). They showed that smallpox virus can be isolated from the conjunctival fluid and swab from patients who showed signs of conjunctivitis only without the clinical picture

of smallpox. Dekking (1967) also found smallpox virus in seven mothers who had conjunctivitis after their children died of smallpox. So diagnosis of conjunctivitis in the area of endemic foci of smallpox must always include smallpox as a possibility.

The smallpox caused by v. minor clinically is the same as smallpox caused by v. major (Type IV - VII). The disease tends to be very mild in comparison to that of v. major. Marsden (1948) found only a very few deaths, less than 1% out of his 13,686 patients. The toxic side of the disease is almost nil. Pre-eruptive illness is comparatively rare. After the usual incubation period the rash occurs. The rash undergoes the stages of macule, papule, vesicle and pustule. Jorge (1924) said that the cycle of maturation is more rapid than that of the v. major. The lesions are much smaller and occupy a more superficial position in the skin. Marsden and Jorge both confirmed that loculation and umbilication are almost entirely absent in v. minor. Secondary fever is uncommon. The disease can occur in both the vaccinated and the unvaccinated.

The serological picture is the same in both the diseases. Neutralising antibody and haemagglutinin-inhibiting antibody appears after first 6 days of the illness. Complement fixation antibody can be detected usually on the 8th day. The neutralising antibody reaches high concentration during convalescence and remains for many years (Downie and McCarthy 1958, Downie et al 1969b). McCarthy and Downie (1953) showed that in cases of v. minor the serological test

could not differentiate it from v. major. The serology of recently vaccinated persons could not be differentiated from that of smallpox and alastrim. McCarthy et al (1958) showed that the antibody response in vaccinia occurs from 8th day of vaccination. Neutralising antibody remains for a long time. Complement fixation antibody can be found for 6 months and antihaemagglutinin also goes down very quickly.

Complications of infection with v. major are widespread throughout the body. These include massive suppuration of skin or keratitis and corneal ulceration. Effusion of both serous and purulent types might occur into joints. Encephalomyelitis is also another complication. In v. minor no such complications are seen.

The history of smallpox caused by v. major can be traced many centuries back and it is considered to be one of the most ancient diseases which prevailed in human history. The origin of smallpox is unknown. Dixon (1962) described in his book seeing the picture of the skull of Ramases V (1100 B.C.) - "Allowing for changes due to the process of mumification the lesions bear a considerable resemblance to smallpox". Variola major is also known to be in China and India from earliest times. Its spread across Europe, Africa and America can be traced. It appeared to travel from India towards the west in Syria and then Arabia and finally to Egypt and Spain. From Spain it spread all across Europe. Jorge (1924) described its pathway from Portugal to America crossing Atlantic in 16th century. It

was also introduced to South Africa from India in 1713 (Mitchell 1922).

Observing the mortality rates of different epidemics at different places it has been found that v. minor is a permanent variant from v. major. V. minor can be traced back to central Africa which existed in Kaffirs from very long time (Fehrsen 1922). Also it can be traced back to South America in Brazil in 1910 (Ribas 1910). The disease appears to occur in labourers of coffee plantations and was apparently very prevalent in the interior of the country. In Cuba the same disease can be traced and it was found that spread of the disease in North America can be traced back to Cuba. In Brazil this disease was called "Alastrim", and this name of the disease has become widely used. From the central part of Africa the disease spread to the northern part along the Mediterranian. In Britain the first case of v. minor, in 1919, seems to have been transported by ship which called at Alexandria and Gibraltar (Copeman 1920). Since then up to 1935 there were several outbreaks and incidence of v. minor. In Europe, Denmark, Sweden and Switzerland all have had outbreaks of v. minor.

In India, Pakistan and Bangla Desh the incidence of v. minor is apparently little or nil. Strains from and around Calcutta are always found to be v. major (Sarkar & Mitra 1967, Sarkar et al 1970).

There is not any evidence where it seems to be that v. major is converted to v. minor or vice versa. Both the diseases might occur

as epidemics which remained true to type. Marsden's large series of cases also shows that in variola minor there is no tendency to a change of type.

At present the main reservoir of v. major in the world is found in Asia and Africa. The places are India, Pakistan, Bangla Desh, Burma and to some extent Vietnam, Cambodia and Indonesia. In Africa some foci exist in Congo, Nigeria and in Ethiopia. But by the modern techniques and methods used in the laboratories for the detection and characterisation of the variola major virus, the virus from some parts of Africa has been found out to differ somewhat from v. major strain (Bedson et al 1963). Epidemiologically also they are found to be different as they have lower mortality rates than v. major. Epidemiological and laboratory studies (Downie et al 1963) show that only v. minor has been present in Brazil for several years.

The source of smallpox virus is only man. It is transmitted from man to man by close contact. There seems to be no known animal reservoir (Arita and Henderson 1968). Subclinical type of infection is also seen to be very rare. For this reason it is very easy to locate the disease whenever it breaks out and also it is possible to locate the possible contacts. Another favourable factor is that the patient is only capable of spreading disease for two weeks and as the incubation period is about two weeks also, it is possible to isolate the contacts before they have spread the infection any further.

In the endemic area when smallpox is introduced in a community, control of the disease depends on early diagnosis, reporting, prompt isolation of the disease and vaccination. Diagnosis can be done as soon as typical rash appears. The patient then must be isolated quickly to the hospital. The intimate contacts of the house should be watched carefully for two weeks. The people who visited the house from the two weeks before should be observed. In case of children going to school his or her classmates also should be observed. Chance of infection by contact in public places like markets, meetings and trains are very small (WHO Surveillance - Containment Operations Principles and Operational Procedures). Contacts traced out can be promptly vaccinated and watched for further outbreak of the disease. If this procedure can be carried out very promptly no second generation of the disease may occur.

The proper disposal of the scabs of the patient should be carried in mind. In the humid, temperate condition virus seems to remain alive for a considerable period of time. McCallum and McDonald (1957) studied the survival of smallpox scab at a temperature of 30° - 40° and found virus did not survive longer than 6 months but at 20° - 25°C the virus could be recovered after 17 months. The clothing and bedlinen of the patient must be sterilised first before giving it to the laundry. There is a report of an outbreak in England occurring in a laundry from infected bedlinen (Cramb 1951).

In non-endemic areas there is no chance of sporadic infection

in the community but there is always a chance of imported cases from endemic areas. In Britain 1962 all cases were found to be imported from India and West Pakistan. Here the special check-up should be done at airports and seaports. The cases of suspected persons must be kept in quarantine hospital. A valid vaccination certificate must be required for persons arriving from endemic areas.

Sometimes the smallpox is confused with other diseases like varicella. Also vaccinomodified smallpox and Type I, II, III cannot be diagnosed clinically. So a laboratory with modern techniques and efficient technicians must be available in both the endemic and non-endemic areas to identify smallpox as soon as possible.

Vaccination itself cannot prevent the disease fully but it can prevent the spread whenever there is an epidemic in the community. History of vaccination can be traced back from Jenner's time when he first introduced vaccination from cowpox by hand to hand method. As the time passed the quality of vaccine and the mode and method of vaccination were improved gradually. The cowpox virus was replaced by vaccinia virus which is the same as variola virus having the same identical antigenic properties but differs in only biological marker characters. The origin of this virus is obscure. Downie (1965) believes that properties and characters of most strains now in use suggests that they have been derived from cowpox.

Effective vaccination in the endemic areas nowadays is the main

work to be carried out to control and eradicate the disease. The child under the age of 6 months must be vaccinated successfully with good, potent vaccine. The whole population should be successfully vaccinated every three years or even at shorter intervals when there is a threat of epidemic.

In the endemic-free areas the child should be vaccinated primarily at 6 months and after 5 - 10 years (WHO technical report 1964). The interval can be adjusted to suit convenient landmarks such as school entry. Persons who work at the ports, hospitals or laundries must be vaccinated at the interval of 5 - 10 years. The person who intends to visit an endemic area must be vaccinated before travelling.

The immunity conferred by primary vaccination develops at about 8 days and it wanes gradually. Observations in the endemic areas show that 95% were protected for 5 years against v. major and 100% against v. minor. After successful revaccination protection certainly extends up to 20 years in respect of v. major and probably life long in regard to v. minor (Henderson 1971).

It is shown that sometimes vaccination in smallpox contacts is too late to ensure protection from the disease. In these cases passive protection might be achieved by administration of immune gamma-globulin early in the incubation period as in measles. It was tried in India by Kempe et al (1961). They found in over 400 close family contacts who were given vaccinial immune gamma-globulin and vaccinated,

there were 8 cases of smallpox as against 29 cases of similar control group without gamma-globulin. Later Marennikova (1962) prepared gamma-globulin from serum of immunised animal which also showed effective results in the prevention of smallpox.

As the virus introduced in the body during the vaccination is a live virus, there is always a chance of complication of vaccination. Normally the vaccinia virus has a very low but definite pathogenicity for man. When primarily introduced, it leads to regional lymphadenopathy, systemic malaise and fever. In eczematous skin it turns to be eczema vaccinatum. Sometimes generalised vaccinia results. The post-vaccinial encephalitis also is not a very uncommon feature. The pregnant mother vaccinated during pregnancy might give birth to a child with generalised vaccinia. It has been found out that there is less chance of complication if the child is vaccinated under one year of age.

In the non-endemic areas where there has been practically no smallpox for several years yet the vaccination programme has continued, it is found that the cost of vaccination has been substantial both from human health and from money. Complications following from vaccination resulted in 6 - 12 deaths each year, more than 150 serious complication and over 2,000 days of hospitalisation (Neff et al 1967, Lane et al 1969). This risk of vaccination has now raised a serious problem to the non-endemic countries.

Nanning (1962) found that post-vaccinial complications are lessened

if vaccination is carried out with hyper-immune gamma-globulin. This was also confirmed by Marennikova (1962).

Recently many experiments have been carried out to produce bacteria-free vaccine. Kaplan and Micklem (1961) tried production of vaccine in chick embryo tissue culture and Jackson et al (1956) tried in chorioallantoic membrane of chick embryo. The vaccines they produced are bacteria-free but the immunity conferred with these types of vaccine are yet to be determined.

The vaccine which was produced before was not very stable as it was in the lymph form. The titre of virus used to fall very rapidly so the problem of production and preservation of stable vaccine was thought of. The standard amount of live vaccinia virus should be at least 5×10^7 pk.f.u. in CAM of chick embryo in order to have a successful primary vaccination. By the development of freeze-dried vaccine (Collier 1955) it was possible to keep the vaccine stable for a large period of time at room temperature (Cockburn et al 1957, Gross et al 1957). This vaccine can be kept at $4^{\circ}C$ for an indefinite period of time. By the introduction of this freeze-dried vaccine the eradication problem of smallpox in hot climates and under-developed countries has been made much easier.

The possibility of smallpox eradication from the world is feasible within a short period of time. The World Health Organization has taken the project very seriously supplying proper help both by giving aid and

potent vaccine. As there is no known animal reservoir of the disease at present it will not be very difficult to check the spread of the disease by second generation with potent vaccine. Rapid isolation and identification of the disease by expert laboratories and clinicians will help to solve the problems.

Once upon a time smallpox was a problem throughout the world. With the help of extensive vaccination and proper control of the disease most civilised countries have been able to check and banish the disease. By 1950 considerable areas of the world including most of Europe, North America and Australia did not have the disease except as occasional importations. By 1967 many additional countries in central and South America, Middle East Asia no longer had smallpox problem. A recent review noticed that not more than 27 countries in the world experience endemic smallpox (Henderson 1968). If proper care is taken to control the disease by prompt vaccination and isolation in those places then these 27 countries may also enjoy freedom from smallpox in the near future.

Laboratory Identification of Variola Infection

The variola infection can be easily diagnosed clinically when there is an epidemic. But in the case of vaccino-modified smallpox or type VII, VIII and IX, laboratory aid is required to diagnose smallpox. In non-endemic countries smallpox might be mistaken for chickenpox and so prompt laboratory aid is required for diagnosis. The laboratory identification of smallpox can be done by direct or indirect method.

Direct identification is to demonstrate the virus particle or the antigen from the lesion or from body. Indirect identification is to demonstrate antibody in the blood serum which suggests infection. Recently vaccinated persons cannot be distinguished from the smallpox-infected person by indirect method. Direct identification can be done in various ways :

i) Light microscopy - The smear taken from maculo-papular rash or from vesicle or pustule. Gutsteins method of staining (Kempe 1964) shows number of typical elementary bodies. The result must be confirmed by other methods.

ii) Virus particles also can be demonstrated by fluorescent antibody technique (Murray 1963). Sometimes the impurities might give false fluorescence giving a false positive result.

iii) Most modern and quick method of identifying virus is electron microscopy by negative contrast technique. It was first tried by Van Rooyen in 1948 but later, by improving the staining method and other techniques, Naggington and Macrea (1965) made it very useful for quick diagnosis in half an hour. Cruickshank et al (1966) examined material from 30 patients suffering from smallpox obtaining positive results with 28 patients. In the other cases the virus was not in sufficient quantity in the material which was later proved positive by other tests. However, the negative result should be confirmed by other tests. The electron microscope cannot differentiate between the

variola and vaccinia viruses.

iv) The isolation and identification of variola viruses can be done on 12 days old embryonated eggs. The chorioallantoic membrane is the specific site of production of lesion and shows definite and specific pocks. Another advantage is besides isolation and identification of virus, culture of the virus can be done in membrane. This method is very sensitive and if there is any viable virus particle in the material, definitely it will give rise to pock. This method was standardized and improved by McCarthy and Dumbell (1961). It requires 3 days to have a definite result. This method does not only isolate the virus but can also differentiate between the pox group viruses such as vaccinia, variola, monkey pox, cowpox, etc.

v) Demonstration of smallpox antigen by precipitation

Precipitation in agar gel was first used to diagnose smallpox by Dumbell and Nizamuddin (1959). In this test positive results appear within two hours, and is considered to be the most efficient and quickest method of diagnosis. But a negative result does not rule out smallpox. It is said that considerable amount of viral antigen must be present to make the test positive. This test has been widely adopted for routine use (WHO expert Committee on smallpox 1964).

vi) Demonstration of antigen by CFT - soluble antigen of smallpox might be found in early smallpox cases. Downie and McDonald (1953) using CF test by antivaccinial rabbit serum found positive

antigen in patients blood in early stage. But this occurs mostly in fulminating and haemorrhagic cases of smallpox (Downie et al 1969a). Other CF tests found to be useful by using vesicular fluid or crust extracts.

vii) Various tissue cultures are used for the identification of smallpox. Tissues like chick embryonic fibroblast, human embryonic fibroblasts and tumour cell cultures have been used. Marennikova (1963) showed that variola virus causes definite cytopathic effects in tissue culture and that this can be successfully used for purpose of early diagnosis. But the sensitivity of the tissues to the virus seems to be very poor unless it is given in high dose.

Antibody to smallpox virus usually appears by the 7th day of the disease. So after 7 days antibody can be demonstrated by CF test, neutralisation test or by haemagglutination-inhibition tests. Very often anti-haemagglutinins are the first demonstrable antibodies and Marennikova reported that 80% of smallpox cases had significant HA titres by the 7th day. Vaccination, on the other hand, only rarely gives a rapid response with anti-haemagglutinin, and this difference may be of use when only a single specimen of serum is available. According to Downie et al (1969) post-vaccinial sera very rarely gave a positive precipitation-in-gel test with smallpox antigens and this may also be a helpful point. It must be realised, though, that none of these serological tests is conclusive in people who have been vaccinated.

Difference between V. Major and V. Minor

The major differences between the two viruses is in their virulence for human host. V. major has a case mortality of 20%, although a mortality of 40 - 50% may be reached in the unvaccinated. On the other hand v. minor has got a case mortality of less than 1%. Epidemiologically it has been easy to say which type prevails in a community but difficulty arises when there are only few cases of v. major and if they are all vaccinated. In these cases clinically or epidemiologically it is almost impossible to decide which type exists.

Serologically many experiments have been carried out to differentiate between the two viruses for a long time (McCarthy and Downie 1953, Downie and Dumbell 1956) but without much success. Only recent laboratory studies have shown that the two viruses can be differentiated by their biological marker characters. These are as follows :

1) Virulence in chick embryo

Dinger (1956) first demonstrated in the laboratory that the amount of v. minor recovered in CAM of chick embryo is very small amount in comparison to that of v. major. He used 8 days old chick embryos to carry out his experiments and he did not mention at what temperature he incubated the eggs after inoculating with virus. Later Helbert (1956, 1957) carried out the chick mortality test in 12 days old chick embryos at 35^{o}C with both type of viruses and found that v. major virus is more virulent to chick embryos than v. minor with the same dose of virus. He also found that the amount of v. major virus

extracted from the liver of the infected embryo was at least 10 times higher than v. minor using the same dose of virus.

2) Ceiling temperature test

Nizamuddin and Dumbell (1961) found another very simple and effective method to differentiate between v. major and v. minor virus. They observed that v. minor virus failed to produce any pocks on the CAM of 12 days incubated chick embryo at 38.3°C, whereas v. major can produce pocks though the pock size is reduced and fewer in number. Dumbell et al (1961) also confirmed these above two findings.

3) Haemadsorbtion and haemagglutination test

Gurvitch et al (1964) showed by chick embryo cell culture that v. major produces cytopathic effect and haemadsorption up to 38°C whereas v. minor does up to only 37°C. Wells (1967) carried out extensive experiments to show the cytopathic effect and production of haemagglutinin on human embryonic fibroblast cell by v. major and v. minor virus. She found that v. major generally produces detectable haemagglutinin up to 39.5°C whereas v. minor strain produces detectable haemagglutinin up to 37°C. She also found that a very poor haemadsorption occurs with v. major at 39.5°C and no haemadsorption occurs at all with v. minor at that temperature.

Test numbers 1 and 2 are confirmed standard tests for differentiating between v. major and v. minor. The disadvantage of test number 1 is that it requires a large number of eggs to carry out the experiment

and the result is only obtained after 7 days. Test number 2 has the advantage that it requires only a few eggs to carry out the experiment and the result can be obtained within 72 hours. The disadvantage is that the test is very temperature sensitive. A fluctuation of 0.5°C may affect the result considerably, so a very accurate incubator with minimum fluctuation is needed for this test.

Test number 3 requires more observations and experiments to confirm the findings, and to access their usefulness in distinguishing variola strains. This will be reported in the present thesis.

Intermediate Strains

Bedson et al (1963), while investigating 23 strains from Tanganyika by chick embryo mortality and ceiling temperature test, found that the strains were difficult to fit in either of the v. major or v. minor groups. They observed that the strains have a low virulence for chick embryo like v. minor but they do produce pock at 38.3°C like v. major. The pocks were more reduced in size and in number than those of v. major at this temperature. This suggested the possibility that variola strains might not fall necessarily into either the major or minor category, but that intermediate strains might exist. Certainly the case mortality reports from Tanzania, and East Africa generally would bear this out. They range throughout the year mostly between 3 and 10% with an average of about 5%. This is much less than the mortality of variola major in the unvaccinated and significantly more than that of variola minor. Of course similar figures might come from a country where both strains were

endemic and the total figures represented a summation of outbreaks of both types. Another difficulty is our ignorance of how accurately the reported figures represent the true state of affairs.

It seemed that laboratory studies could help in two different ways. Firstly, by examining many strains from a particular area it could be seen whether they were uniform in behaviour or whether some behaved like variola major and others had lower virulence. This investigation involved the extension of established tests to many more specimens.

Another valuable laboratory study would be to discover other properties of variola virus which could be used as marker characters. The more such properties there are, the more precisely could variola viruses be compared and described. Some attempts are described in this thesis and it is felt that it is now possible to delineate various 'families' of variola and that their geographical distribution does not run counter to the laboratory findings. The plan of the work has been affected by the available sources of smallpox specimens. Collections were available from Kenya, Tanzania and West African states. These have formed the main body of the work and the results have been compared with group of variola viruses from Brazil, Indonesia and Pakistan. From other areas in the world only a few strains were available, insufficient to establish any general patterns.

Human cases of monkey pox

While these studies were in progress, great excitement was raised by the occurrence in various parts of West Africa of sporadic cases of smallpox-like illness without traceable contacts. From each of these cases a virus was isolated which could be differentiated from variola but not from monkey pox. The epidemiological investigation, as yet unpublished, indicated the possibility of contact with local monkeys. This raises some small doubt about the non-existence of any animal reservoir of variola. The question to the laboratories would relate to the possibility of interconversion between variola and monkey pox. Some viruses have been isolated with properties intermediate between these two and in Chapter VIII some preliminary investigation of this topic will be reported.

CHAPTER II

CHICK EMBRYO MORTALITY

Dinger (1956) was the first to describe a laboratory method which distinguished between variola major and variola minor. His method was based on the longer period over which variola major virus could be recovered from the membrane compared with minor. Dinger's work was soon superseded by Helbert (1957) who measured the dose mortality relations for inoculation of the chick embryo with strains of variola major and variola minor. Helbert found that the minor strains were consistently less virulent than the major strains. The drawback of Helbert's method is the large number of eggs required for each strain of virus examined. In this type of test, the only information gained from each egg is whether the embryo survives or dies within the period of the experiment. Clearly an embryo which

dies three days after inoculation will in general have suffered a more virulent infection than one which survives until the 7th day after inoculation. Some technique in which survival time is measured should therefore yield equivalent information about the virus from fewer eggs. A difficulty about survival time in a finite experiment is how to incorporate the surviving embryos into the mathematical assessment. Gard (1940) had proposed plotting the reciprocal survival time against a measure of the dose inoculated. Thus, the longer an individual survives the smaller its contribution and this after, say seven days, can be regarded as vanishingly small. This method was used by Bauer (1960) for his chemotherapeutic experiments on mice and was adopted by Bedson & Dumbell (1961) to measure virulence of pox viruses for the chick embryo. A plot of reciprocal survival time against the log dose of inoculum gave results sufficiently close to linear for use in characterizing virus strains. A linear plot should be described by two measures, either two points on the line, or one point and its slope. Bedson and Dumbell found that all the viruses they used gave plots with more or less the same slope and they described the virulence by the logarithm of that inoculum which would give a mean survival time of four days. They were interested to compare the virulence of several different pox viruses and were able to find doses of each one appropriate to include a mean survival time of 4 days, though the equivalent doses ranged from less than 10 pk.f.u. to over 10^6 pk.f.u. In the present work comparing strains of variola virus, many were found with very low pathogenicity for the chick

embryo and their D_4 values involved extensive extrapolation from the data. The factor with these viruses which could be kept within defined limits was the inoculum and a better statistic for comparison of variola strains is the harmonic mean survival time for a standard inoculum. The particular level of 10^5 pk.f.u. was chosen as it could always be obtained by interpolation, and the result from at least two and usually three levels of inoculum was used. In a mortality experiment some embryos die early from non-specific causes and it is customary to disregard any deaths which occur in the first forty-eight hours after inoculation. Nevertheless it is probable that some embryos may die specifically from variola infection during the second day and these will be missed from the count. When recriprocal survival time is the measure it makes a relatively large difference to the statistic is one embryo is changed from day 2 or 3 or day 3 to 4 and this difference diminishes as the time interval increases. The reciprocal survival time with large doses of virus is therefore likely to be underestimated. It is not necessary for present purposes to have the 'correct' formula. All that is required is some way of smoothing and summarizing the mortality data so that they may be simply presented and so that repeated estimations will give the same answer.

Some workers have described single dose methods. Sarkar & Mitra (1967) inoculated embryos on the CAM with 10^6 pk.f.u. and obtained mortalities varying from 0 to 100% with undoubted strains of variola major. All this work was done with virus in the first egg passage.

This was not possible in London. By the 2nd or 3rd passage in eggs we have consistently had 100% mortality with doses of 10^6 pk.f.u. of variola major and substantial mortalities even from undoubted strains of variola minor. Marennikova (1969) also reported results from a single inoculum of 100 - 1000 pk.f.u. Attempts to repeat her work were unsatisfactory and it was found that the survival of embryos was markedly affected by small changes in the inoculum when this was of the order of 100 - 1000 pk.f.u. It is concluded that the results presented by Marennikova cannot be taken as reliable.

More recently Baxby (1971) has returned to the older more wasteful technique of estimating LD50. It is to be hoped that his example will not be followed.

One critical variable in these mortality experiments is the temperature - a factor that is not mentioned by some authors. Bedson, Dumbell and Rossier (1961) showed that temperatures of 35°, 37° and 38.5° markedly affected the mortality of embryos, the higher temperatures permitting greater survival of embryos. While investigating the mortality rate in embryos inoculated with low doses of virus the present author investigated even lower temperatures down to 33°. This increased the proportion of embryos which died following a given inoculum, but did not increase the discriminatory power of the technique between variola major and variola minor.

Materials and method:

i) Eggs and incubation of eggs

ii) Candling and dropping of CAM — See materials and methods

iii) Virus strains — of Chapter III

iv) Preparation of viral stock

v) The mortality test:

Determinations were made of the mortality rates of 12 day-old chick embryos after inoculation on chorioallantois membrane (CAM). For each virus three separate doses of inoculum were used, a group of 6 embryos being inoculated with each dose. The eggs were incubated at 35°C, and candled daily to determine the time of death in each. Confirmation of death was made by opening the egg and observing the CAM and embryo directly. Deaths occurring in the first two days after inoculation were regarded as non-specific. The observations were terminated on the 7th day. Control titrations were made to determine the actual doses of viruses employed in each test.

Result:

The results of the chick embryo mortality test with v. major and v. minor and strains from Kenya, Tanzania and West Africa are shown in Tables II-1 to II-5. Five strains of v. major were tested which showed mean survival time (ST) with 10^5 pk.f.u. inoculum of between 3.9 to 4.2 days (Table II-1). Three strains of v. minor showed ST at 10^5 pk.f.u. of between 6.2 to 7.7 days (Table II-2). The ST at 10^5

TABLE II - 1

Chick Embryo Mortality test with V. Major

Strain	Log_{10} Inoculum	RST	"D4"	RST (10^5)	ST (10^5)
Cameron	5.5 4.5 3.5	0.26 0.25 0.22	4.8	0.265	3.9
Harvey	5.5 4.5 3.5	0.29 0.24 0.20	4.6	0.27	3.7
Level	5.3 4.3 3.3	0.32 0.25 0.19	4.3	0.30	3.3
McGregor	5.9 4.9 3.9	0.29 0.23 0.17	5.2	0.24	4.2
Ismat	5.7 4.7 3.7	0.25 0.24 0.19	5.1	0.25	4.0

Note in this and subsequent Tables

RST	mean reciprocal survival time
RST(10^5)	reciprocal survival time calculated for inoculum of 10^5 pk.f.u.
ST(10^5)	Survival time (days) for inoculum of 10^5 pk.f.u.
D4	Inoculum to give mean survival of 4 days

TABLE II - 2

Chick Embryo Mortality test with V.Minor

Strain	Log_{10} Inoculum	RST	RST (10^5)	ST (10^5)
Butler	6.9	0.25	0.16	6.25
	5.9	0.22		
	4.9	0.15		
Plugge	5.3	0.17	0.13	7.7
	4.3	0.06		
Brazil-8	5.4	0.16	0.15	6.7
	4.4	0.14		
	3.4	0.05		

TABLE II - 3

Chick Embryo Mortality test with Kenyan Strains

Strain	Log_{10} Inoculum			RST			RST (10^5)	ST (10^5)
Njotho	6.3	5.3	4.3	0.28	0.25	0.17	0.22	4.5
Muzee	5.7	4.7	-	0.24	0.21	-	0.22	4.5
Oduor	6.7	5.7	4.7	0.25	0.24	0.22	0.22	4.5
Bedeta	6.2	5.2	-	0.24	0.22	-	0.21	4.8
Wathiri	6.2	5.2	4.2	0.24	0.22	0.20	0.21	4.8
Khadeja	5.5	4.5	3.5	0.23	0.20	0.17	0.21	4.8
Joseph	5.5	4.5	3.5	0.21	0.19	0.19	0.20	5.0
Lefer	5.7	4.7	3.7	0.23	0.20	0.00	0.20	5.0
Kibet	7.2	6.2	5.2	0.20	0.16	0.14	0.12	8.3

TABLE II - 4

Chick Embryo Mortality test with Tanzanian Strains

Strain	Log_{10} Inoculum			RST			RST (10^5)	ST (10^5)
EA2/63	5.9	4.9	3.9	0.27	0.24	0.21	0.24	4.2
EA1/61	5.8	4.8	3.8	0.22	0.20	0.17	0.20	5.0
EA8/61	6.7	5.7	4.7	0.25	0.24	0.23	0.23	4.3
EA6/63	5.6	4.6	3.6	0.24	0.22	0.19	0.23	4.3
EA22/62	6.6	5.6	4.6	0.26	0.23	0.19	0.20	5.0
EA12/61	7.0	6.0	5.0	0.23	-	0.18	0.18	5.6
EA32/62	6.2	5.2	4.2	0.20	0.18	0.18	0.18	5.6
Ilembula	5.6	4.6	3.6	0.19	0.15	-	0.16	6.2
EA17/61	5.3	4.3	3.3	0.14	0.15	-	0.15	6.7
EA5/61	6.7	5.7	4.7	0.16	0.15	0.07	0.12	8.3

TABLE II - 5

Chick Embryo Mortality test with West African Strains

Strain	Log_{10} Inoculum			RST			RST (10^5)	ST (10^5)
Mangumba	5.3	4.3	3.3	0.30	0.27	0.16	0.29	3.5
U. Volta	5.0	4.0	3.0	0.25	0.19	0.08	0.26	3.8
Congo 2	5.3	4.3	3.3	0.26	0.09	0.07	0.225	4.5
S-Leone 1	5.1	4.1	3.1	0.21	0.05	-	0.20	5.0
Congo 4	5.1	4.1	3.1	0.20	0.15	-	0.19	5.3
Congo 1	5.2	4.2	3.2	0.19	0.11	0.13	0.18	5.6
Banza	5.4	4.4	3.4	0.18	0.14	0.05	0.17	5.9
Niger 1	5.4	4.4	3.4	0.19	0.15	0.09	0.17	5.9
Niger 3	5.6	4.6	3.6	0.22	0.11	0.03	0.15	6.7
Togo 1	5.5	4.5	3.5	0.15	0.10	-	0.125	8.0
B-Maria	6.4	5.4	4.4	0.13	0.11	0.11	0.11	9.1
S-Leone 4	5.6	4.6	3.6	0.08	0.06	0.04	0.07	14

pk.f.u. of Kenya, Tanzania and West Africa varied between 3.5 to 14 days (Table II-3, II-4 and II-5). Therefore it can be seen from the results that the mean survival time at 10^5 pk.f.u. of v. major and v. minor groups of viruses are well separated from each other. The strains from Africa showed a wide variation overlapping both v. major and v. minor.

CHAPTER III

CEILING TEMPERATURE TEST

Nizamuddin and Dumbell (1961) found a simple way to distinguishing between v. major and v. minor by the inability of v. minor to produce pocks on eggs inoculated at 38.3°. At this temperature v. major gives rise to pocks on CAM of 12 days-incubated chick embryo though they are reduced in size and number. By raising the temperature by another 0.3°, v. major also stops producing pocks. So 38.3° is said to be the ceiling temperature of v. major. This has become a standard test in differentiating between v. major and v. minor virus.

The advantage of this test over the chicken embryo mortality test is that this test requires fewer eggs to perform the experiment. Also, the result can be obtained within 72 hours.

Materials and methods:

i) Eggs and incubation of eggs :-

Fertile hen's eggs used throughout the experiments described were obtained from Appleby Farm, Kent. These were incubated in a Westernette egg incubator at a temperature of 38.5°C with hourly rocking for 12 days.

ii) Candling and dropping of CAM :-

The eggs were candles for viability on the day of use and were prepared for inoculation of the chorioallantoic membrane (CAM) by the method of McCarthy and Dumbell (1961).

iii) Virus strains :-

Standard v. major from England and v. minor from Europe and S. America were used. The other strains used were strains from Kenya, Tanzania and West Africa.

iv) Preparation of viral stock :-

Virus strains suitably diluted in distilled water were inoculated in 0.1 ml amounts into groups of eggs in order to heavily infect the CAM. Those membranes of viable embryos showing confluent growth after 3 days incubation at 35° were excised and placed aseptically into Universal bottles containing beads. Sterile 70% glycerol (1 ml to each membrane) was added and the membranes were shaken vigorously to release the virus from the membrane tissue. The resulting suspension was centrifuged at low speed (1000 rpm for 10 minutes in an MSE bench

centrifuge) to sediment cell debris. The supernatant was carefully removed and constituted the viral stock suspension. The stocks were stored at -20°C in bijoux bottles throughout the experiment.

v) The ceiling temperature test :-

Chicken embryos 12 days old were inoculated on the CAM with approximately 50 and 500 pk.f.u. per 0.1 ml in order to have countable pocks. Two sets of eggs were inoculated. One set was kept at 35° for control and another set was kept in 38.3° incubators. Known v. major and v. minor strains were always placed along with the test to have a positive and a negative control. The incubator was thermo-electronically controlled and provided with a fan inside for efficient and uniform air circulation. The temperature fluctuation was less than 0.2° with this system. Thermometers kept on each shelf and at each corner showed uniform temperature throughout. The incubator was opened for a minimum period of time when the eggs were kept inside.

After 3 days the membranes of the eggs were excised, washed in 2% formalin in water and spread out in a black tray. Pocks were counted in a good light. The pocks at 38.3° were difficult to see on an ordinary black dish, so the membranes were examined over a white transparent petri dish. The pocks could be seen as small white, opaque dots. The test was repeated with some strains more than once and the result was found to vary very little.

Results:

The results of the ceiling temperature test of v. major and v. minor and strains from Kenya, Tanzania and West Africa are shown in Tables III-1 to III-5.

The log reduction of titre was calculated by subtracting the log pk.f.u. at 38.3° from log pk.f.u. at 35°. This enabled comparison of the results of different strains with known v. major and known v. minor viruses from the same base line.

Five strains of v. major were observed for ceiling temperature and the log reduction of titre was found to vary between 0.20 to 0.35 (Table III-1) average being 0.26. Six strains of v. minor (Table III-2) were observed and the log reduction of titre was found to be more than 2.5 in each case. This is a clear distinction but Bedson, Dumbell & Thomas (1963) have reported that strains from Tanzania were as a group different from v. major in that the average reduction in titre at 38.3 was greater than for known strains of v. major. Strains from various parts of Africa have been tested and the results are shown below.

Ten strains from Kenya (Table III-5) were tested of which Joseph, Khadeja, Lefer, Teresia and Kongo were tested by present author and the results of the rest were taken from Dumbell. It was found that Joseph, Muzee and Teresia gave log reductions of the titre of 0.25, 0.25 and 0.28 respectively. Lefer gave 0.37, Bedeta and Khadeja gave 0.45 and 0.47 respectively. Kongo gave 0.50, whereas Njotho and Kibet gave over 0.60. In average, they gave a log reduction of titre of 0.43.

TABLE III - 1

Result of ceiling temperature tests with V. major

Strain	Dilution of virus used in eggs 0.1 ml	Number of pk.f.u. at 35°	mean	Number of pk.f.u. at 38.3°	mean	log pk.f.u. at 35° -log pk.f.u. at 38.3°
Cameron	$10^{-6.0}$	34,31,40	35	20,22,14	18.7	0.27
Harvey	$10^{-6.0}$	54,48,36	46	22,18,22	20.6	0.35
Level	$10^{-6.0}$	41,46, D	43.5	21,27,32	26.6	0.20
McGregor	$10^{-6.0}$	44,56,63	54.3	23,29	26	0.32
Ismat	$10^{-5.0}$			159,93	126	0.28
	$10^{-6.0}$	21,27, D	24	14,17	16.5	

TABLE III - 2

Result of ceiling temperature tests with V. minor

Strain	Dilution of virus used in eggs 0.1 ml	Number of pk.f.u. at 35°	mean	Number of pk.f.u. at 38.3°	mean	log pk.f.u. at 35° -log pk.f.u. at 38.3°
Butler	$10^{-5.0}$	22,21,18	20.3	0.0	0	>2.5
	$10^{-6.0}$			0.0		
Plugge	$10^{-5.0}$	13,14,28	18.3	0.0	0	>2.5
	$10^{-6.0}$			0.0		
Kershaw	$10^{-5.0}$	18,11, D	14.5	0.0	0	>2.5
	$10^{-6.0}$			0.0		
Br-8	$10^{-5.0}$	28,33,20	27	0.0	0	>2.5
	$10^{-6.0}$			0.0		
Br-10	$10^{-5.0}$	19,13, D	16	0.0	0	>2.5
	$10^{-6.0}$			0.0		
Br-25	$10^{-5.0}$	39,26,20	28.3	0.0	0	>2.5
	$10^{-6.0}$			0.0		

TABLE III - 3

Result of ceiling temperature tests with strains from Tanzania

Strain	Dilution of virus used in eggs 0.1 ml	Number of pk.f.u. at 35°	mean	Number of pk.f.u. at 38.3°	mean	log pk.f.u. at 35° -log pk.f.u. at 38.3°
EA1/61	$10^{-6.0}$	78,46 62,66	63	18,21,29	23	0.44
	$10^{-5.0}$			80,63		
EA8/61	$10^{-6.0}$	36,25,49	36.3	4, 6	6	0.78
EA12/61	$10^{-5.3}$	62,82 101,116	90	10,19 23,26	19.5	0.66
EA17/61	$10^{-3.7}$	72,89 95,142	100	11,14 21,21	17	0.77
	$10^{-5.0}$			0,1,1,3,		
EA25/62	$10^{-5.7}$	9,13,18 19,22	16	3	1.6	1.7
	$10^{-4.3}$			54,63,85		
EA32/62	$10^{-5.3}$	41,45,55 67	52		67	0.89
EA2/63	$10^{-6.0}$	57,79,91 110	84	23,38,39	33	0.40
	$10^{-4.0}$			90,140,		
EA6/63	$10^{-5.0}$	46,46,56	49	200	140	0.54
	$10^{-5.0}$			153, D		
Ilembula	10^{-600}	29,31,36	32	8,11	9.5	0.53

TABLE III - 4

Result of ceiling temperature tests with strains from West Africa

Strain	Dilution of virus used in eggs 0.1 ml	Number of pk.f.u. at 35°	mean	Number of pk.f.u. at 38.3°	mean	log pk.f.u. at 35° -log pk.f.u. at 38.3°
Banja	$10^{-5.0}$ $10^{-6.0}$	21,17,15	17.6	22, D 2, 8	4.25	0.64
Mangumba	$10^{-5.0}$ $10^{-6.0}$	14,18, D	16	84, D 5, 3	5.5	0.46
Congo-2	$10^{-5.0}$ $10^{-6.0}$	22,19, D	20.5	42,73 13,18	10.6	0.29
Congo-4	$10^{-5.0}$ $10^{-6.0}$	11, 8, D	10	15,21 2, 2	1.9	0.72
Niger 1	$10^{-5.0}$ $10^{-6.0}$	13,16,22	17	15, D 3, 3	2.5	0.83
Niger 3	$10^{-5.0}$ $10^{-6.0}$	6,17,22	15	11,24 3, 2	2.12	0.84
Togo 1	$10^{-5.0}$ $10^{-6.0}$	29,21,23	24.3	139,97 6, 8	9.4	0.41
U. Volta	$10^{-5.0}$ $10^{-6.0}$	12,15, D	13.5	7, 7 0, 0	7	1.29
S. Leone 4	$10^{-5.0}$ $10^{-6.0}$	18,13, 5	12	21, 9 0, 0	15	0.90
Guinea-2	$10^{-5.0}$ $10^{-6.0}$	23,28, D	25.5	22, D 4, 6	4.7	0.73

TABLE III - 5

Result of ceiling temperature tests with strains from Kenya

Strains	Dilution of virus used in eggs 0.1 ml	Number of pk.f.u. at 35°	mean	Number of pk.f.u. at 38.3°	mean	log pk.f.u. at 35° -log pk.f.u. at 38.3°
Joseph	$10^{-5.0}$ $10^{-6.0}$	33,47,59	46.3	>200, >200 25,28	2.65	0.25
Lefer	$10^{-5.0}$ $10^{-6.0}$	13,15,18	15.3	79, D 7, 6	7.0	0.34
Khadeja	$10^{-5.0}$	108,110, 120	113	21,43,43 45	38	0.47
Kibet	$10^{-6.0}$	69,76,76	74	12,19,24	18	0.61
Njotho	$10^{-4.0}$	20,27,39 41	32	3, 5, 6 13	7	0.66
Muzee	$10^{-6.0}$	18,20,25	21	4,13,18	11.7	0.25
Bedeta	$10^{-6.0}$	67,79,81 87	79	21,29,34	28	0.45
Wathiri	$10^{-6.0}$	229,232	231	49,91, 126	89	0.41
Teresia	$10^{-5.0}$ $10^{-6.0}$	34,42,47	40.7	>200,D 19,23	21	0.28
Kongo	$10^{-5.0}$ $10^{-6.0}$	27,30,19	25.3	97,62 8, 2	6.5	0.59

Joseph, Muzee, Lefer and Teresia closely resembles v. major with respect to ceiling temperature.

Nine strains from Tanzania were observed of which seven strains were previously tested by Bedson, Dumbell & Thomas (1963) (Table III-3). The log reductions of titre in this group ranged from EA2/63 at 0.40 to EA25/62 at 1.70. The average reduction was 0.75.

Ten strains from West Africa were observed (Table III-4) and the result of log reduction of titre found to vary between 0.29 and 1.29. The average log reduction of this group found to be 0.71. The average for strains from Tanzania and West Africa is significantly higher than the average for Kenyan strains or known variola major.

Discussion:

The difference between v. major and v. minor on the basis of their ceiling temperatures was described by Nizamuddin and Dumbell (1961). Their findings were confirmed by Dumbell et al (1961) and Bedson and Dumbell (1961). This test was found to be very convenient with respect to the time factor and number of eggs required when compared with Helbert's chick mortality test.

Bedson et al (1963), while performing ceiling temperature tests on some strains of variola viruses from Tanganyika, found that the strains behaved like v. major yet they were far more temperature sensitive at 38.3^{o} than v. major. Sample strains from West Africa when tested for ceiling temperature have given results like those from Tanzania. This

is not true of Kenyan strains. The clinical picture of the disease in all three areas is reported to be milder than smallpox caused by v. major, the mortality rate being about 5%.

It is doubtful how valid the reported mortality figures are from these areas but there seems to be no difference between Kenya and Tanzania in this respect. Whether the intermediate pock reduction figures reflect an intermediate human pathogenicity is uncertain. However, a more extensive survey of strain from Kenya to be reported in a later chapter shows a bimodal distribution with one mode in the v. major area and the other close to that of Tanzanian strains. It suffices for the moment to show that intermediate values can be obtained from this test, which may be respresentative of geographical groups of variola virus. There still remains an obvious and greater difference between these African viruses and true v. minor as judged in this test.

The pock-reduction test is delicately poised and repeated tests on the same strain may vary. This is illustrated in Table III-6. Although the results of a single test are not fully reliable, a definite trend is noticed in repetitions, which confirms the validity of group comparisons.

TABLE III - 6

Repetition of ceiling temperature test with some strains

Virus	log reduction in titre at 38.3° on repeated tests	Average Reduction
Harvey	0.23, 0.23, 0.28, 0.29, 0.33, 0.33, 0.34, 0.35, 0.36, 0.37, 0.38, 0.39, 0.42, 0.45, 0.52, 0.54, 0.57, 0.67, 0.69, 0.70.	0.42
EA12/61	0.48, 0.59, 0.66, 0.67, 0.70, 0.82, 0.93	0.69
EA8/61	0.78, 0.90, 0.94, 0.95, 0.96, 1.0	0.92

CHAPTER IV

HAEMADSORPTION (HAD) IN HUMAN EMBRYONIC FIBROBLAST (HEF)

Marennikova (1963) first observed the cytopathic effect of variola virus on skin muscle fibroblast cultured from human embryo. Gurvitch and Marennikova (1964) and Marennikova, Gurvitch and Yumashova (1964) reported the growth and cytopathic effect of variola virus on tissue cultures at different temperatures. They also reported the use of HAD to reveal the effects of variola even before the appearance of cytopathic effect. They showed that the cytopathic effect of some strains of variola could be observed at temperatures up to 40^{0} if the virus had been previously passed in human cell cultures.

These results were confirmed and extended by Wells (1967). She found a considerable difference in the ability to produce

haemagglutinin at 38° by v. major and v. minor virus. No increase in titre of virus was found at a temperature of 39° or over, but the Harvey strain of v. major was found to produce a small amount of haemagglutin even at 40°. HAD was found to be more sensitive than the haemagglutination test.

Materials and methods:

i) Culture media for HEF :

a) Growth medium -

Eagle's minimum essential medium (MEM)

+ 10% uninactivated calf serum

+ 2% of a 4.4% solution of sodium bicarbonate with indicator

+ 1% glutamine stock solution

+ 100 units/ml penicillin

+ 0.1 mgm/ml streptomycin

b) Maintenance Medium -

Eagle's MEM

+ 2% uninactivated calf serum

+ 2% of a 4.4 solution of sodium bicarbonate with indicator

+ 1% glutamine stock solution

+ 100 units/ml penicillin

+ 0.1 mgm/ml streptomycin

ii) Culture methods :

a) Primary cultures:

Skin/muscle tissues were removed aseptically from normal human foetus of 12 - 17 weeks gestation. The tissues were washed in phosphate buffered saline (PBSA) containing 100 units/ml penicillin and 0.1 mgm/ml of streptomycin, and then chopped into fine fragments. The fragments were trypsinised in 100 ml of 1% trypsin solution in PBSA at a temperature of 35°. Sterile teflon-coated magnets were placed in the trypsinisation flask and rotated by an external rotating magnet to keep the trypsin solution and tissue in constant movement. After half an hour the supernatant was decanted and replaced by a fresh 100 ml of trypsin. Trypsinisation was repeated about 4 times or until the required cell suspension was obtained. The cells in the decanted supernatant were sedimented by centrifugation in a MSE minor centrifuge at 1000 rpm for 10 minutes. Cell pellets were resuspended in 10 ml growth medium and finally 150 ml growth medium was added. The resulting suspension was dispensed in aliquots of 25 ml into 12 oz. flat prescription bottles. The bottles were gently shaken to ensure even distribution of the cells. The bottles were left flat at 37° to incubate undisturbed for 48 hours. After 48 hours the cells of the bottle were fed with fresh growth medium if needed. Confluent monolayers were formed in 4 - 5 days and were then ready for

subculturing.

b) Subculturing of HEF:

The medium was removed from a confluent 12 oz bottle of cells, 2 ml of 0.25% trypsin solution was added and left in contact with the cell sheet for one minute and then carefully removed. The residual fluid was sufficient to complete cell stripping within 1 - 3 minutes at 25°. When the cell sheet began to break up, 2 ml of growth medium was added and the cells gently resuspended by pipetting up and down. The suspension was distributed to another two 12 oz bottles and 25 ml of growth medium was added to each and the bottles returned to the incubator.

If viral studies required monolayers in tubes, then the cells from a bottle were resuspended in 20 ml of growth medium and distributed in 20 tubes (6" x ⅝" soda glass tube). The mouth of the tubes were closed by rubber bungs and incubated in stationary racks at an angle of 5° to the horizontal at 37° for 2 - 3 days. After 2 days, when confluent monolayers were attained, the tubes were ready for experiments.

iii) Red Blood cells:

Fresh vaccinia-sensitive fowl red blood cells in Alsever's solution was obtained from a commercial source. The cells were washed

thrice with PBSA containing 0.5% sterile uninactivated calf serum. A dilution of 0.5% of red blood cell was made for use in the test.

iv) Viral preparation :

See Materials and methods of Chapter III

v) Viruses used :

vi) The HAD test :

a) Tubes which had confluent growth of HEF were ready for inoculation. The growth medium was removed from the tube and 0.2 ml of virus dilution in maintenance medium containing not less than 2×10^5 pk.f.u. per 0.1 ml was added to each tube. The virus was adsorbed at 25° for one hour after which the inoculum was removed and replaced by one ml of maintenance medium. Four tubes were inoculated with each virus of which two were incubated at 37° and two at 40°. Control uninoculated tubes and tubes inoculated with standard v. major and v. minor viruses were included in each batch of tests.

Tubes were examined at the end of 24 hours for cytopathic effect and 0.1 ml of RBC was added to one tube from each temperature. Tubes were returned to the incubator for 30 minutes and HAD read under phase microscope at a magnification of 80x.

The incubation was continued for a further period of

24 hours when red blood cells were added to the second batch of tubes at each temperature. The significant measurement was the degree of HAD at 40° after 48 hours incubation. When the inoculum exceeded 10^5 pk.f.u. per 0.1 ml per tube, confluent HAD occurred after 24 hours at 37°. This was taken as an indication that all cells had been infected and that the inoculation titre had been adequate. Some viruses were very slow to produce HAD at 37° even with 10^5 pk.f.u. at 24 hours. In those cases if confluent HAD did not occur at 37°, the titre of that virus suspension was checked by titrating on CAM. If the virus titre was found to be below 10^5, then a new suspension was prepared and the experiment was repeated. All viral strains were used between 1 - 6 egg passage and care was taken to use virus stock within a period of 3 months from the date of preparation.

The degree of HAD was recorded according to the following arbitrarily chosen score.

Description of HAD	Score
Confluent HAD, no cells seen free of attached RBC	5
Confluent HAD, not more than approximately 15% of the cells free of RBC	4

Numerous foci of HAD; Approximately 50% cells free of RBC	3
Many small foci of HAD; At least one per microscopic field	2
Occasional small foci of HAD but most microscopic field shows no HAD	1
NO HAD	0

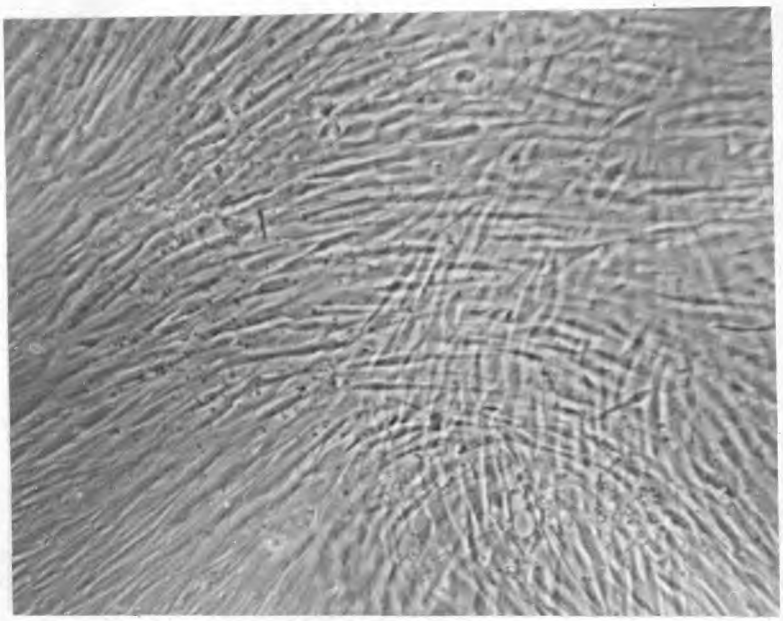
Quantitative assessment of HAD is illustrated in Plate IV - 1

Finally, for the interpretation of results it was found convenient to classify the variola virus into three groups. The grouping was done according to the score as above. Those are as follows :-

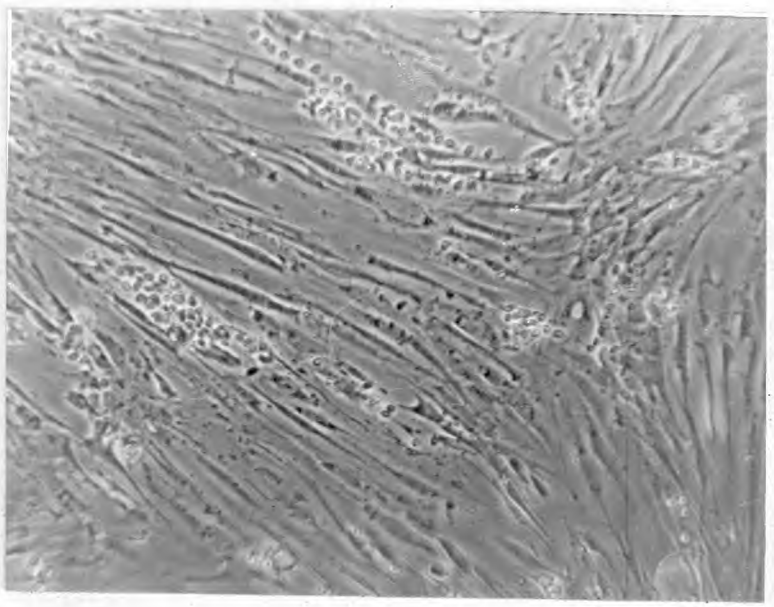
Group	Description	Score
A	Confluent HAD or nearly confluent	4 or 5
B	Many discrete foci of HAD, appearing in each field	2 or 3
C	Little or no HAD many fields clear	0 or 1

Sometimes repeated HAD tests with the same virus were done to confirm that results were repeatable.

b) Experiments were also carried out with different doses

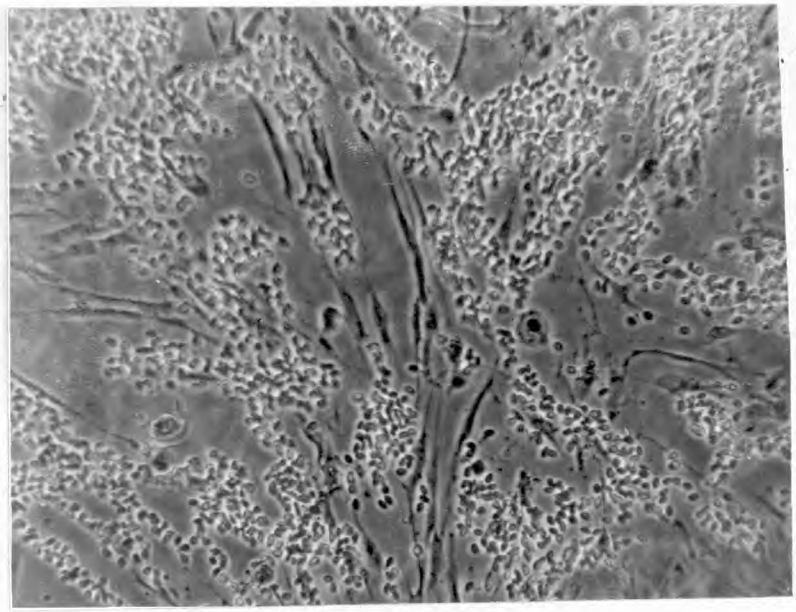


(a) Uninfected human fibroblast

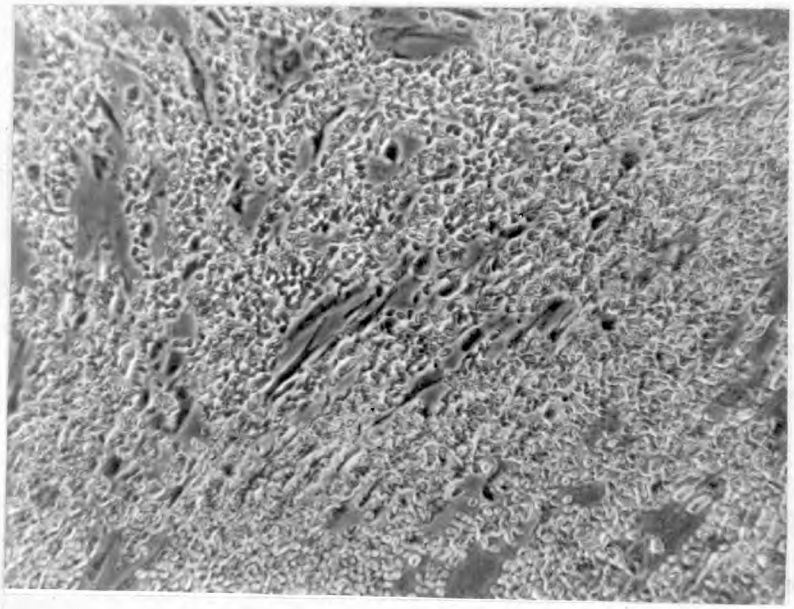


(b) Infected human fibroblast : Haemadsorption Score, 2

Haemadsorption of variola strains on human fibroblasts



(c) Infected fibroblast : Haemadsorption Score 4



(d) Infected fibroblast : Haemadsorption Score, 5

Haemadsorption of variola strains on human fibroblast

of v. major and v. minor viruses. The tubes were inoculated with 10^5, 10^4, and 10^3 pk.f.u. per 0.1 ml of both viruses separately.

c) Experiments were also carried out with strains of v. major and v. minor viruses at 35° and 39.5°.

d) Experiments were performed with heat-inactivated and ultraviolet light-irradiated viruses of v. major and v. minor strains. The strains of viruses named Cameron and Plugge were used. These were heated at 56° in a water bath for 45 and 90 minutes at a dose of 10^5 pk.f.u. unit. Then the heated strains were inoculated to the tubes and kept at 37°. The same two viruses were irradiated in a petri dish containing 10^5 pk.f.u. per 0.1 ml in maintenance medium. The times used were 5, 7 and 10 minutes. The test was then carried out as usual, and tests were also put up to show that all virus had been inactivated.

Results:

a) The result of HAD at 37° and 40° with v. major and v. minor and strains from Tanganyika, West Africa and Kenya are shown in Tables IV-1 to IV-4.

All the strains of v. major (Table IV-1) can be grouped in A as they produced confluent HAD at 40°. Only one strain called Ismat from the outbreak of England 1962 shows less HAD and falls in Group B.

TABLE IV - 1

Result of Haemadsorbtion with V.major and V.minor Strains at 37° and 40°C

Group	Strain	HAD				Group
		24 hrs		48 hrs		
		37°	40°	37°	40°	
V.major	Cameron	5	4	5	5	A
	Harvey	5	4	5	5	A
	Level	5	4	5	5	A
	McGregor	5	4	5	5	A
	Ismat	5	2	5	3-4	B
V.minor	Butler	5	0	5	1	C
	Plugge	4	0	5	0	C
	Kershaw	5	0	5	0	C
	Br-10	4	0	5	0	C
	Br-25	3	0	5	0	C
	Br-8	3	0	5	2	B

TABLE IV - 2

Result of HAD of Strains from Kenya

Strain	HAD				Group
	24 hrs		48 hrs		
	37°	40°	37°	40°	
Kibet	5	2	5	4	A
Njotho	5	4	5	5	A
Muzee	5	3	5	5	A
Bedeta	5	3	5	4	A
Wathiri	5	3	5	5	A
Teresia	5	2	5	3	B
Kongo	4	1	5	2	B
Joseph	5	2	5	2	B
Lefer	5	2	5	3	B
Khadeja	5	1	5	2	B

TABLE IV - 3

Result of HAD with Strains from West Africa

Strain	HAD				Group
	24 hrs		48 hrs		
	37°	40°	37°	40°	
Banja	4	2	5	3	B
Nigeria 7	5	1	5	2	B
Congo 2	2	2	5	2	B
Congo 4	5	1	5	3	B
Niger 1	5	2	5	3	B
Niger 3	5	2	5	3	B
Togo 1	4	2	5	3	B
Upper Volta	3	2	5	2	B
Sierra Leone 4	4	2	5	2	B
Guinea 2	5	2	5	3	B

TABLE IV - 4

Result of HAD with strains from Tanganyika

Strain	HAD				Group
	24 hrs		48 hrs		
	37°	40°	37°	40°	
EA1/61	3	0	5	3	B
EA8/61	3	2	5	3	B
EA12/61	4	2	5	2	B
EA17/61	3	1	5	3	B
EA25/62	4	2	5	2	B
EA28/62	4	2	5	2	B
EA32/62	5	2	5	3	B
EA2/63	5	2	5	3	B
EA6/63	4	2	5	2	B
Ilembula	5	2	5	3	B

All the strains of v. minor (Table IV-1) produced very little or no HAD at 40° and they can be classified in Group C. Here also an exception can be found in the case of Brazil-8, a strain from Brazil in 1961. This shows discrete HAD even at 40°. So it falls in Group B.

The strains from Kenya (Table IV-2) show a mixed result. Strains Joseph, Lefer, Khadeja, Teresia and Kongo can be placed in Group B, whereas Kibet, Njotho, Muzee, Bedeta and Wathiri can be placed in Group A for their confluent HAD.

The strains from West Africa and Tanzania (Tables IV-3 and IV-4) are found in Group B as they show discrete HAD at 40°.

b) Table IV-5 shows the effect of different levels of inoculum on the production of HAD. Even with v. major an inoculum of 10^5 pk.f.u. was required to produce confluent HAD within 24 hours. The number of cells per tube was normally about 200,000 so this corresponds approximately to a multiplicity of infection of 0.5 to 1.0. Lesser inocula produced focal patches of HAD which spread at 37° to become confluent in 48 hours. Variola minor normally showed confluent HAD within 24 hours if the inoculum contained at least 10^5 pk.f.u. Some strains, however, were "slow producers" and even high inocula of the order of 10^5 - 10^6 did not produce confluent HAD till 48 hours at 37°. In the HAD tests virus was used at a dilution expected to contain at least 10^5 pk.f.u. per 0.2 ml inoculum, and the appearance of confluent HAD

TABLE IV - 5

Result of HAD with different dose

Group	Strain	Dose of Pk.f.u. per 0.1 ml	HAD			
			24 hrs		48 hrs	
			37°	40°	37°	40°
V.major	Harvey	10^5	5	5	5	5
		10^4	3	2	5	2
	Cameron	10^5	5	4	5	5
		10^4	4	2	5	3
		10^3	2	1	4	2
V.minor	Butler	10^5	5	0	5	1
		10^4	3	0	3	0
	Plugge	10^5	4	0	5	0
		10^4	3	0	4	0
		10^3	1	0	3	0

after 24 hours at 37° was taken as confirmation of this. Any virus stock which did not do this was titrated on CAM immediately to check that the inoculum had contained the expected dose. If this was so, the virus was then classed a "slow producer". Sometimes, particularly with v. minor strains, it was found that the titre had fallen. A new stock of this strain was then prepared and the HAD test repeated.

c) The results of HAD at different temperatures with v. major and v. minor strain are shown in Table IV-6.

Harvey strain of v. major shows a confluent HAD at a dose of 10^5 pk.f.u. in 35°, 37°, 39.5° and 40° respectively. It suggests that the HAD production in v. major does not depend on temperature between 35° to 40°.

But in the case of v. minor it shows that a few single cells throughout the field haemadsorb at 39.5° but none do so at 40°. Brazil-25, a v. minor strain, shows a slow production of haemagglutinin at 24 hours but it was confluent after 48 hours. The titre of virus when checked on CAM showed the dose administered was greater than 10^5 pk.f.u.

d) Virus inactivated with ultraviolet light or by heating at 55° shows no HAD at all even up to 3 days of incubation at 37°.

Discussion:

The haemagglutinin production at 40° of v. major and v. minor

TABLE IV - 6

Result of HAD at different temperatures

Group	Strain	Dose of Pk.f.u. per 0.1 ml	HAD							
			24 hrs				48 hrs			
			35°	37°	39.5°	40°	35°	37°	39.5°	40°
V.major	Harvey	10^5	5	5	5	5	5	5	5	5
V.minor	Butler	10^5	5	5	2	0	5	5	2	0
	Brazil 25	10^5	3	3	1	0	5	5	1	0

strain can be differentiated when observed by HAD test. According to the degree of HAD they may be classified into Group A and Group C respectively where Group A denotes confluent HAD and Group C denotes little or none.

Most strains from West Africa and Tanzania can be classified into Group B because of their ability to produce discrete HAD at 40°. The mortality rate of smallpox cases of Tanzania is reported to be lower than that of v. major and higher than that of v. minor. The strains from Kenya show a mixed result. Out of 10 strains 5 strains were found to fall in Group A and 5 strains in Group B. The implication of this will be discussed in a subsequent chapter.

V. major shows confluent HAD with 35°, 37°, 39.5° and even at 40°. V. minor shows confluent HAD at 35° and 37° but at 39.5° the HAD production is very little and nil at 40°. This agrees with the results of Wells (1967) and Gurvitch (1964).

The HAD test is very easy to carry out and for this test very accurate temperature control is not necessary. It has been found that a deviation of 0.5° on either side of 40° does not invalidate the test. This is in contrast to the ceiling temperature test where a variation of 0.25° may seriously affect the result. For this reason the haemadsorption test recommends itself as a distinguishing criterion between variola major and variola minor. However, even in the small series presented in this chapter, strains of well documented v. major

and v. minor have each turned up in the B category. This seems to indicate that this test is measuring a different viral property from that assessed in the ceiling temperature test, which successfully distinguishes between all strains of v. major and v. minor so far encountered. Further discussion of this point will be taken later in the thesis.

CHAPTER V

HAEMAGGLUTINATION PRODUCTION IN HEp2 CELLS

After the demonstration of HAD production of variola virus in human embryonic fibroblasts, another cell line of human origin was tried for HAD production. The cell line used was HEp2, originally derived from human larynx carcinoma (Fjelde 1955, Toolan 1954). When infected with variola virus the cells round up and become highly refractile. When HAD test was performed it was very difficult to read the score of HAD because of the round and refractile cells. So it was decided to perform haemagglutination (HA) tests with the infected cell cultures.

Materials and method:

1) Medium for the cell culture

a) Growth medium

Minimum essential medium

+ 10% calf serum

+ 3% of a 4.4 solution of sodium bicarbonate

+ 1% glutamine

+ penicillin and streptomycin at the usual concentrations

b) Maintenance Medium -

Same as growth medium but 2% calf serum added instead of 10% calf serum

2) Cell line and subculture of cell line :-

The cell line was obtained from St. Thomas' Hospital, London. Subculture dose as same as described in fibroblast subculture with growth medium. From a 12 oz bottle cell culture 50 tubes of cells usually obtained.

3) Virus and preparation of viral stock :-

See materials and methods of Chapter III

4) Red blood cell :-

Vaccinia-sensitive fowl red blood cells were washed and prepared as described for the HAD test in Chapter IV. The final concentration of red blood cells used in this case was one per cent.

5) HA test :-

After removal of growth medium virus was inoculated at a dose

of 10^5 pk.f.u. per 0.1 ml into cell cultures in test tubes. The inoculated tubes were kept for one hour at room temperature for adsorption after which time the inoculum was removed and 1 ml of maintenance medium added to each tube. Two tubes were inoculated at each temperature with each virus. A pair of uninoculated tubes were included as a control. A known v. major virus and a known v. minor virus were also inoculated with each batch of tests. The tubes were incubated at the required temperature for 72 hours. After 72 hours, the tubes were examined for CPE and then frozen at -20°C. The tubes were then allowed to thaw and the cells scraped off. The suspension in maintenance medium was transferred to a bijoux bottle and then treated in an ultrasonic bath for 1 minute. This was done to disperse the aggregates of haemagglutinin which result from freezing (Neff et al 1965). After sonication the stock was ready for use in the haemagglutination test.

6) Haemagglutination (HA) test :-

The virus samples prepared as above were dispensed in 0.25 ml volumes as doubling dilutions from 1/2 to 1/256 in rows of wells in perspex agglutination trays. The diluent used was phosphate buffered saline. When dilution was done, 0.25 ml of 1% washed vaccinia-sensitive red blood cells were added to each well. A sample of known haemagglutinating activity was also diluted as a positive control. Uninfected cell material was diluted as a negative control and wells containing diluent only served as red blood cell controls. After addition of

red blood cells, the plate was shaken to complete mixing and was incubated at 37° for one hour with shaking at 15 minute intervals and at the end of the incubation period to resuspend the red blood cells. Plates were then left at room temperature for one hour and the results recorded when the cells had settled. End points were taken as those dilutions giving 50% agglutination of red blood cells.

Results:

The HA tests with the Cameron strain of v. major at different temperatures (Table V-1) showed that at 35° and 37° Cameron had a titre of 1/64 and 1/128 respectively. At 38° the titre fell to 1/16 and at 40° no HA could be demonstrated. The Plugge strain of v. minor (Table V-1) showed no HA at all at any temperature. Consequently more v. major and v. minor strains were tested at 37° and 38° (Table V-2) and were found to give the same results as Cameron and Plugge. The HA titre produced by strains from Kenya, West Africa and Tanzania are shown in Tables V-3, V-4 and V-5.

Ten strains from Kenya (Table V-5) were examined at 37° and 38° and all of them were found to produce HA varying from a titre of 1/8 to 1/64 at 37°, and from nil to 1/8 at 38°. Strains from Tanzania (Table V-3) showed some variability. At 37° strains 12/61, 17/61, 25/62 and 2/63 produced a very low titre (1/8). The rest showed no HA. At 38° none of them produced any HA. Ten strains from West Africa (Table V-4) also were tested and only two of them Togo 1 and Upper Volta produced HA of a titre 1/8 and 1/4 respectively: the rest were negative

TABLE V - 1

Haemagglutinin Production at different temperatures

Group	Strain	Temp. of Incubation	HA titre after 72 hrs
V.major	Cameron	35°	1/64
		37°	1/128
		38°	1/16
		40°	-
V.minor	Plugge	35°	-
		37°	-
		38°	-
		40°	-

TABLE V - 2

Haemagglutinin Production by V.major and V.minor

Group	Strain	HA titre after 72 hrs at	
		37°	38°
V.major	Cameron	1/128	1/16
	Harvey	1/16	-
	Level	1/128	1/16
	McGregor	1/8	-
	Ismat	-	-
V.minor	Butler	-	N.D.
	Plugge	-	-
	Kershaw	-	N.D
	Br-8	-	-
	Br-10	-	N.D.
	Br-25	-	N.D.

N.D. = Not done

TABLE V - 3

Haemagglutinin Production by Strains from Tanzania

Strain	HA titre after 72 hrs at	
	37°	38°
EA1/61	-	-
EA8/61	-	-
EA12/61	1/4	-
EA17/61	1/8	-
EA25/62	1/4	-
EA28/62	-	-
EA32/62	-	-
EA2/63	1/4	-
EA6/63	-	-
Ilembula	-	-

TABLE V - 4

Haemagglutinin Production by Strains from West Africa

Strain	HA titre after 72 hrs at	
	37°	38°
Banja	-	-
Nigeria 7	tr	-
Congo 2	-	-
Congo 4	tr	-
Niger 1	tr	-
Niger 3	tr	-
Togo 1	1/8	tr
Upper Volta	1/4	tr
Sierra Leone 4	tr	-
Guinea 2	-	-

TABLE V - 5

Haemagglutinin Production by Strains from Kenya

Strain	HA titre after 72 hrs at	
	37°	38°
Joseph	1/16	tr
Lefer	1/16	tr
Khadeja	1/16	-
Kibet	1/8	-
Njotho	1/32	1/4
Muzee	1/64	1/8
Bedeta	1/16	tr
Wathiri	1/64	1/4
Teresia	1/32	1/2
Kongo	1/32	tr

even at 37°. At 38° with the exception of the above two strains, none showed any HA.

Discussion:

These results appeared to show a regular difference between v. major and v. minor strains, but it seemed strange that v. minor would not produce HA in HEp2 cells even at 37° in spite of the appearance of an extensive cytopathic effect. It was decided to pass one strain (Plugge) three times in HEp2 cell cultures and test it again. When this was done HA was detected (after 3 days at 37°) to a titre of 1/32. However, three further strains, Butler and Dunbar from the England outbreak of 1953 and Brazil-25 were passed four times in HEp2 and no HA was demonstrated even from cultures showing confluent CPE. There was no lack of virus production. The yield from the third pass was titrated on the CAM and showed a titre of more than 10^6 pk.f.u. per tube. In a further experiment the third pass of Plugge in HEp2 was passed once on the CAM and the harvest from this did not give any HA on the first inoculation into HEp2 cell cultures. No time was available to pursue these interesting results further. The rapidity with which the virus behaviour was modified in a new host cell system would seem to rule out the selection of a mutant line. Also the results indicate the possibility that host materials might be included in the virion which were effective in homologous systems but much less so in heterologous cells. Variola major would then seem to have the capacity to over ride or bypass the necessity for this particular control mechanism. The possible implications of these results are so important

that much further study of this topic should be undertaken.

In the meantime this test provides a simple method which has distinguished between all strains of v. major and v. minor so far tested, without involving much material or the very accurate control of temperature. A further advantage is that the majority of the presumed intermediate strains behave like variola minor in this test.

CHAPTER VI

CYTOPATHIC EFFECT (CPE) OF VIRUSES ON HUMAN FIBROBLASTS

While performing the HAD test on the cells, the cytopathic effect (CPE) on the cells by the virus at 40° was noticed and found to be of some interest. Wells (1967) described the CPE of v. major and v. minor viruses on human embryonic fibroblasts. She showed that both v. major and v. minor viruses produce CPE up to 41° with large doses of virus. With small doses of virus CPE did not develop at 39.5° in cultures inoculated with either virus. CPE was also observed by Brown et al (1959) with vaccinia virus and Appleyard et al (1962) with rabbit pox virus.

Materials and methods:

i) Growth medium for cell - See Materials and methods of Chapter IV

ii) Primary cell culture and subculture of human embryonic fibroblast - See Materials and methods of Chapter IV.

iii) Virus and preparation of viral stock - See Materials and methods of Chapter III.

iv) Test for Cytopathic Effect (CPE) :-

a) Tubes were inoculated with a dose of at least 10^5 pk.f.u. (0.2 ml diluted in maintenance medium) and were adsorbed at 25° for 1 hour. Then the viral inocula were replaced by 1 ml of maintenance medium and the tubes were kept at 37° and 40° in pairs. Known v. major and v. minor were always kept as control and normal, uninoculated tubes were also incubated as normal controls. After 24 hours and again after 48 hours the tubes were examined for CPE under phase microscope at a magnification of 80x.

b) The test was also carried out with different doses of virus. The tubes were inoculated with 10^5, 10^4, 10^3 pk.f.u. and CPE observed at 37° and 40° after 24 and 48 hours.

c) The test was also carried out with heat-inactivated and ultraviolet light-inactivated virus - See Materials and methods of Chapter IV.

Results:

a) At 37° after 24 hours, v. major and v. minor infected tubes

produced considerable amount of CPE. The cells appeared to be thickened, shortened and irregular in size. They started to separate from each other. After 48 hours the whole cell sheet became necrotic and cells after rounding up started falling off from the glass surface. Uninfected, control tubes remained normal and unchanged throughout the experiment. At 40°, after 24 hours v. major shows the same type of CPE as at 37° but it had not progressed so far. After 48 hours, the cells started to separate from each other and the cell sheet started to disintegrate. V. minor at 40° does not produce any CPE up to 48 hours or even up to 5 days.

In order to check whether the virus was infecting the whole cell sheet after 24 hours, 0.5% RBC (see HAD test) was added to the tube at 37°. If confluent HAD resulted then it was considered to be standard infection. Some strains did not show confluent HAD after incubation at 37°; in those cases their titres were checked on CAM and if found not satisfactory then fresh preparations were made and the experiments were repeated.

The amount of CPE was scored as follows (see also Plate VI - 1).

-	No CPE
+(1+)	CPE only observed in places, and consisting of shortening and thickening cells.
++(2+)	Shortening and thickening of cells now seen all over cell sheet.
+++(3+)	Shortened, thickened cells now separating from each other and revealing irregular outlines.

PLATE VI - 1

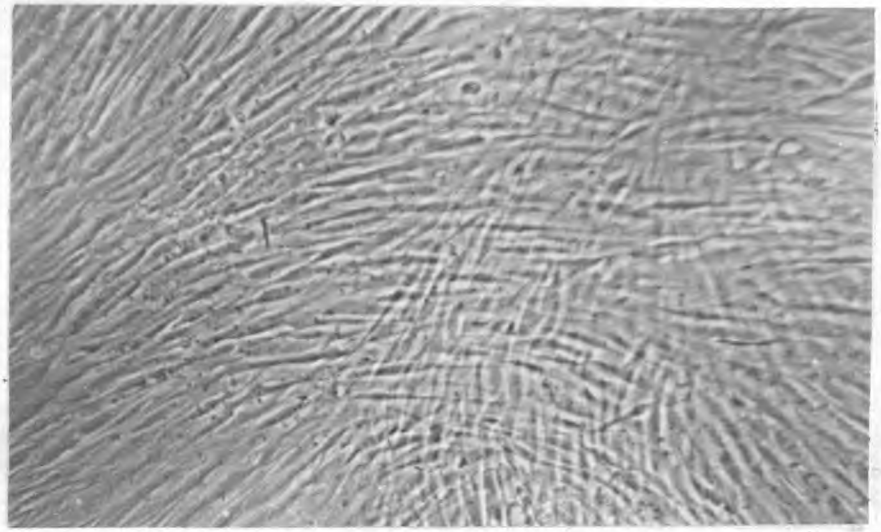
Cytopathic effect of variola strains on human fibroblasts

(a) Uninfected human fibroblast

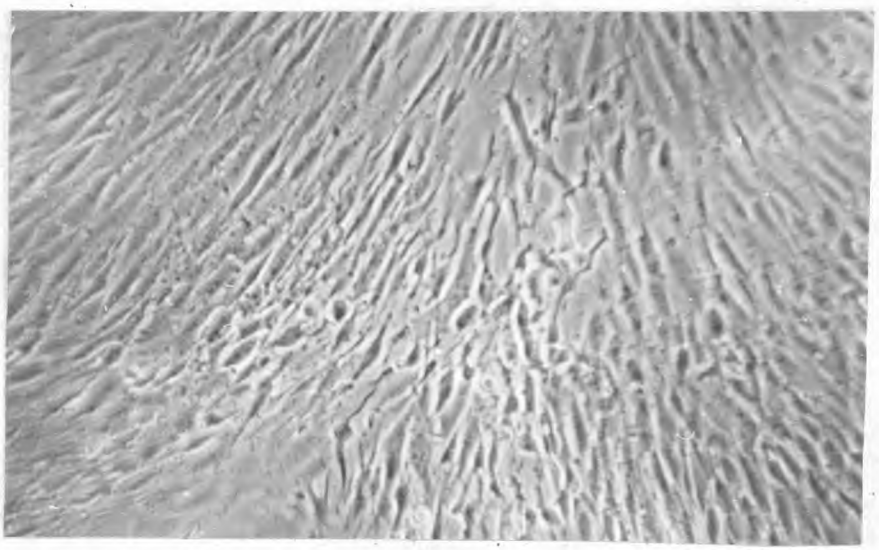
(b) Infected human fibroblast showing CPE 2+

(c) Infected human fibroblast showing CPE 4+

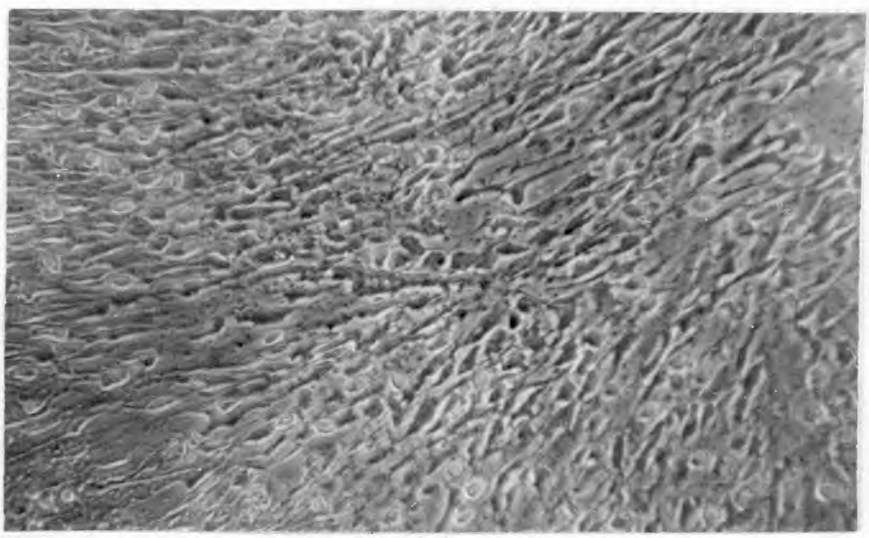
PLATE VI - 1



(a)



(b)



(c)

++++(4+) Cell sheet partly or wholly disintegrated, with scattered surviving shrunken cells or groups of cells.

On the basis of this scoring the results of CPE of v. major, v. minor and strains from Kenya, Tanzania and West Africa are shown in Tables VI-1 to VI-4.

V. major (Table VI-1) shows CPE confluent and necrotic after 48 hours at 37°. At 40° after 48 hours the CPE was 4+ but the cell sheets were not as severely disintegrated as at 37°. One strain 'Ismat' showed only 2+ at 40°. V. minor (Table VI-1) showed no CPE at all at 40° after 48 hours with the exception of 'Br-8' which showed CPE of 3+. However, at 37° after 48 hours the CPE from variola minor was 4+ and indistinguishable from that of v. major.

Eight strains from Kenya (Table VI-2) behaved like v. major. The CPE at 37° and 40° after 48 hours are 4+. Kibet and Kongo were found to produce only 1+ or 2+ CPE at 40°.

Four strains from Tanzania (Table VI-4) showed 3+ CPE at 40° after 48 hours and 2+ CPE was produced by EA17/61, EA32/62, EA6/63 and Ilembula. EA8/61 and EA2/63 produced scattered CPE. However, at 37° after 48 hours they all produced confluent CPE.

Of ten strains from West Africa (Table VI-3), none showed 3+ or 4+ CPE at 40° after 48 hours though they produced confluent CPE at 37° after the same period of time. Banja, Niger 3, Congo 4, Togo 1 and

TABLE VI - 1

Result of CPE of HEF with V. Major and V. Minor Virus

Group	Strain	CPE			
		24 hrs		48 hrs	
		37°	40°	37°	40°
V.major	Cameron	++++	+++	++++	++++
	Harvey	++	++	++++	+++
	Level	++++	++++	++++	++++
	McGregor	++++	+++	++++	++++
	Ismat	+++	++	++++	++
V.minor	Butler	++	-	++++	-
	Plugge	++++	-	++++	-
	Kershaw	++	-	+++	-
	Br-8	+++	+	++++	+++
	Br-10	+	-	+++	-
	Br-25	+	-	++++	-

TABLE VI - 2

Result of CPE of HEF with strain from Kenya

Strain	CPE			
	24 hrs		48 hrs	
	37°	40°	37°	40°
Joseph	++++	++	++++	++++
Lefer	++++	+++	++++	++++
Khadeja	++++	+++	++++	+++
Kibet	+++	+	++++	++
Njotho	+++	++	++++	+++
Muzee	++++	+++	++++	++++
Bedeta	++++	+++	++++	++++
Wathiri	++++	+++	++++	++++
Teresia	++++	+++	++++	+++
Kongo	+++	+	++++	+

TABLE VI - 3

Result of CPE of HEF with Strains from West Africa

Strain	CPE			
	24 hrs		48 hrs	
	37°	40°	37°	40°
Banja	+++	+	++++	++
Nigeria 7	++++	-	++++	+
Congo 2	++	++	+++	+
Congo 4	++	-	+++	++
Niger 1	++	-	+++	-
Niger 3	+++	++	+++	++
Togo 1	+++	-	++++	++
Upper Volta	++	+	+++	-
Sierra Leone 4	++	-	++++	-
Guinea 2	+++	++	++++	++

TABLE VI - 4

Result of CPE of HEF with strains from Tanganayika

Strain	CPE			
	24 hrs		48 hrs	
	37°	40°	37°	40°
EA1/61	+++	++	++++	+++
EA8/61	++	+	++++	+
EA12/61	+++	-	++++	+++
EA17/61	++	-	++++	++
EA25/62	+++	+	+++	+++
EA28/62	+++	++	++++	+++
EA32/62	++++	++	++++	++
EA2/63	++	-	+++	+
EA6/63	+++	+	++++	++
Ilembula	+++	+	++++	++

Guinea 2 produced only 2+, Nigeria 7 and Congo 2 produced only 1+ CPE and Niger 1, Upper Volta and Sierra Leone 4 were found to produce no CPE at all at 40° after 48 hours.

b) The CPE with different doses of v. major and v. minor are shown in Table VI-5, Cameron, the v. major strain shows a 4+ CPE at 37° after 48 hours with 10^5, 10^4 and 10^3 pk.f.u. At 40° after 48 hours the tube containing 10^5 and 10^4 pk.f.u. show confluent CPE but with 10^3 pk.f.u. on the other hand shows only scattered CPE. Plugge, the v. minor strain at 37° after 48 hours 10^5 and 10^4 pk.f.u. shows 4+ and 3+ CPE respectively but with 10^3 pk.f.u. shows 2+. At 40° at 48 hours Plugge does not show any CPE with any doses.

It suggests that at lower doses the rate of development of CPE of Plugge at 37° is less than that of Cameron but at higher doses the rate of development at the same temperature is same with both the viruses.

c) Heat-inactivated and ultraviolet light-inactivated preparations of Cameron and Plugge did not produce any CPE when observed up to 72 hours.

Discussion:

V. major was found to produce definite 4+ CPE at 40° with a dose of 10^5 pk.f.u. V. minor, on the other hand, did not produce any CPE at 40°. Wells (1967) described how v. major and v. minor both produce CPE up to 41° with large doses of virus and with small doses

TABLE VI - 5

Result of CPE with different doses of V.major and V.minor

Group	Strain	Dose of Inoculum per 0.1 ml	CPE			
			24 hrs		48 hrs	
			37°	40°	37°	40°
V.major	Cameron	10^5	++++	+++	++++	++++
		10^4	++	+	++++	+++
		10^3	+	-	+++	+
V.minor	Plugge	10^5	+++	-	++++	-
		10^4	++	-	+++	-
		10^3	+	-	++	-

CPE did not develop at all at 39.5° with either virus. Her findings do not correlate entirely with the findings in this thesis but her work was done with just one strain each of v. major and v. minor. Gurvitch and Marennikova (1964) observed the CPE of v. major and v. minor on chick embryo fibroblast at different temperatures ranging from 35° to 40°. They observed that a dose of 1 x 10^3 pk.f.u. of v. major does not produce CPE at 40° and v. minor also stops producing CPE at 37°. However, in human embryonic fibroblasts v. major produces scattered CPE at 40° and v. minor does produce CPE at 37°. So it might be suggested that CPE production of v. major and v. minor at different temperatures is related to their host cells.

On human embryonic fibroblast, v. major in general with a dose of 10^5 pk.f.u. show 4+ CPE at 40° with the exception of one strain. V. minor strains with the same dose of virus on the other hand show no CPE at all at 40° with the exception of one. On the basis of production of CPE at 40° v. major and v. minor can be differentiated into two broad groups. Strains from Kenya, with the exception of two strains, can be broadly placed into v. major group. Strains from Tanzania are more variable. Out of 10 strains, 4 show 4+ CPE and the other 6 show 1+ or 2+ CPE. These strains, therefore, are very difficult to fit in either of the groups. West African strains produce 2+ CPE or no CPE at all. Among 10 strains, 3 do not produce any CPE and the rest are 1+ or 2+. Why this variation occurs among the strains in CPE production is not known. The relation of HAD production and CPE

are found to be of some interest in these strains. They will be dealt with in a subsequent chapter. It is just noted here that the results of this test are different from those of the HAD tests in these cells and may very likely represent a different property of the virus.

Heat-inactivated or ultraviolet light-irradiated v. major or v. minor virus does not produce any CPE on human fibroblasts. Kitamura (1971) showed that v. major produces hyperplastic foci in HeLa cell cultures in which iododeoxyuridine in the culture medium inhibited virus growth by more than 99%. However, he used a different cell culture system and a different mode of inactivation. Appleyard et al (1962) found that rabbit pox virus, when inactivated by ultraviolet light or sodium azide, produced CPE in ERK cells. Brown et al (1959) found inactivated vaccinia produced CPE in mouse foetal lung cells.

CHAPTER VII

GEOGRAPHICAL DISTRIBUTION OF VARIOLA STRAINS

The tests described and illustrated in previous chapters have been extended as far as time would permit to cover as many variola strains as possible. Most attention has been paid to variola from Africa, the original source of v. minor and the area where strains of intermediate virulence are perhaps most likely to be found. Most of the African strains have come from Tanzania and Kenya between 1961 and 1965 or from various countries in Western Africa between 1968 and 1971. In all, 102 African strains have been examined by some of the tests described. A number of strains had also been sent from Indonesia and from the area round Lahore in Pakistan. The controls were provided by recognised strains of v. major and v. minor. The major strains were taken from well-described outbreaks in Britain since 1945. Most of the v. minor strain derived from

Brazil where the preponderance of v. minor in recent years has been amply established on clinical grounds. Strains were also tested from the outbreaks in England and Holland in 1953 and 1954.

The results obtained are set out in Tables VII-1 to VII-8. The rest of this chapter will be devoted to an analysis and commentary on these results. It will be seen that most attention has been paid to the pock reduction test and the haemadsorption at 40° in HEF cells. These two tests have been applied to the majority of the strains examined, but the others have so far only been done for a sample of the strains available.

It can be seen immediately by reference to Tables VII-1, VII-2 and VII-3 that there is a clear distinction between the group of v. major strains and the groups of v. minor. Not all the tests however, give the clear cut difference between the two groups that is afforded by the pock-reduction test or the mortality rate in chick embryos. This point will be taken up later.

CORRELATION BETWEEN VARIOUS PAIRS OF TESTS

It is important to know for the somewhat arbitrary choice of tests available, whether any two are measuring the same character of viruses or different properties. Most of these questions can be resolved satisfactorily by inspection of the appropriate data without resort to statistical analysis.

TABLE VII - 1

V. Major strains from England 1946 - 1962

Source	Name	Egg pass	ST(10^5)	log pk.f.u. $35^0/38^0$	HAD on HEF	CPE on HEF	HA on HEp2
Middlesex 1946	Harvey	E_5	3.6,3.7 3.8	0.44	A	+++	1/16
Merseyside 1946	Tinkler	*		0.15	A	+	
" 1948	King	E_4		0.36	A	++++	
" 1948	Level	E_2	3.3,3.3 4.0	0.20	A	++++	1/128
Stafford 1948	Higgins	*		0.46	A		1/8
Glasgow 1950	Cameron	E_5	3.1,3.6 3.8,3.9	0.27	A	++++	1/128
"	Robertson	E_5		0.29	A	++++	1/16
"	McLeod	E_5		0.18	A	+	
"	Campbell	E_6		0.28	A	++++	
Pennines 1953	Young	*		0.36	A	++++	1/64
"	Noon	*		0.22	A	++++	1/64
"	Douglas	E_5		0.33	A	++	
Liverpool 1959	McGregor	E_2	4.2	0.24	A	++++	1/8
London 1962	Ismat	E_2	4.0	0.28	B	+++	-
" 1962	O'Hara	E_2		0.40	B		
Wolverhampton 1962	McLenon	*	4.5	0.18	B	+	-
Bradford 1962	Bradford	E_2		0.27	B	++	
Cardiff 1962	Shuker	*	4.8		A	++	-

ST(10^5) = mean survival time after 10^5 pk.f.u. HAD = Haemadsorption
HEF = Human embryonic fibroblast HA = Haemagglutination
* Egg pass history not known CPE = Cytopathic effect

TABLE VII - 2

V. Minor strains from Europe

Source	Name	Egg pass	ST_5 (10^5)	log $pk_\delta f.u._\delta$ $35^\delta/38^\delta$	HAD on HEF	CPE on HEF	HA on HEp2
England 1953	Butler	E_6	6.25	>2.5	C	-	-
"	Haithwait	E_7		>2.5	C	-	-
"	Kershaw	E_6		>2.5	C	-	-
"	Schoffield	E_9		>2.5	C	-	-
"	Dunbar	E_7		>2.5	C	-	-
England 1966	Bell	E_2		>2.5	C	-	-
"	Law	E_2		>2.5	C	-	-
"	Jones	E_3		>2.5	C	-	-
"	Pinson	E_2			C	+	-
Holland 1954	Plugge	E_9	7.7	>2.5	C	-	-
"	Dehaas	E_6		>2.5	C	-	-
"	Dejong	E_5		>2.5	C	-	-
"	Winkel	E_7			C	-	-
Italy 1945	Hartridge	E_3		>2.5	C	-	-

TABLE VII - 3

V. Minor strains from South America

Source	Name	Egg pass	ST_5 (10^5)	log pk.f.u. $35^o/38^o$	HAD on HEF	CPE on HEF	HA on HEp2
Sao Paulo 1960	Br 2	*		>2.5	C		
"	Br 3	*		>2.5	C	-	
"	Br 5	*			C	+	-
"	Br 10	*	16.6	>2.5	C	-	-
"	Br 17	*		>2.5	C	-	
"	Br 18	*		>2.5	C	-	
"	Br 20	*		>2.5	C		
"	Br 21	*			C	-	
"	Br 22	*			C	-	-
Sao Paulo 1968	1C1	*			C	-	
"	6C1	*		>2.5	C	-	
Pernambuco 1960	Br 1	E_4		>2.5	B	+	-
"	Br 9	$*^4$		>2.5	B	+++	-
"	Br 14	*			C	-	
Bahia 1960	Br 4	*		>2.5	C	-	
"	Br 7	*		>2.5	B	++	1/16
"	Br 11	*		>2.5	C	-	
"	Br 19	*		>2.5	C	-	
"	Br 24	*			C	-	
"	Br 26	*		>2.5	B	-	
Paraiba 1960	Br 8	*	6.7	>2.5	B	+++	1/4
Minas Gerais 1960	Br 6	*		>2.5	C		
"	Br 13	*		>2.5	C		
"	Br 15	*		>2.5	C	-	
Rio Grande de Norte 1970	Br 23	*		>2.5	C		

Table VII - 3 contd.

Venezuela 1970 " "	Ven 1 Ven 2 Ven 4	* * *		>2.5 >2.5 >2.5	C C C		-
Place not known	Br 25	*		>2.5	C	-	-

* egg pass not known

TABLE VII - 4

Virus strains from Tanzania

Source	Name	Egg pass	ST_5 (10^5)	log pk.f.u. $35^0/38^0$	HAD on HEF	CPE on HEF	HA on HEp2
Northern, Babuti 1962	EA32/62	E_4	5.6	1.31	B	++	-
Northern, Moshi 1961	EA5/61	*	8.3	0.97	B	+++	
"	EA6/61	*			B	+++	1/64
"	EA8/61	E_4	4.4	1.0	B	+	-
Dar-es-Salam 1961	EA9/61	*		0.73	B	++	
"	EA10/61	*		0.93	B	-	
"	EA12/61	*	5.6	0.70	B	+++	1/4
"	EA14/61	*		0.80	B	±	
"	EA15/61	E_4		0.56	B	±	
"	EA16/61	*			B	-	
Dar-es-Salam 1963	EA6/63	E_3	4.4	0.7	B	++	-
Central, Dodoma	EA12/62	*		1.3	B	+	
" 1962	EA13/62	*		0.9	B	+++	
"	EA25/62	E_4	12.5	1.5	B	+++	1/4
"	EA27/62	*		0.7			
"	EA28/62	E_4		1.5	B	+++	-
E. Lake Maswa 1963	EA2/63	E_6	4.2	0.44	B	-	1/4
"	EA3/63	E_5		0.6	B	+++	
E. Lake, Butinbura 1962	EA22/62	*	5.0	0.7			1/2
Tanga 1961	EA17/61	E_5	6.7	1.4	B	++	1/8
Iringa Njombe 1962	EA30/62	E_4		0.63	A	+++	
Iringa Mlole 1962	EA44/62	E_5		0.48	A	+++	
Iringa Ilembula 1965	EA2/65	E_3	6.2	0.47	B	++	
"	Ilembula	E_3	6.2	0.47	B	++	-

Table VII - 4 contd.

Place unknown	EA1/61	E_3	5.0	0.42	B	+++	-

TABLE VII - 5

Virus strains from West Africa

Source	Name	Egg pass	ST_5 (10^5)	log pk.f.u. $35^0/38^0$	HAD on HEF	CPE on HEF	HA on HEp2
Nigeria 1963	Nigeria 3	E_3		0.66	B	+++	
"	Kano 2	E_4		1.8	B		
"	Kano 4	E_3		0.6	B		
Nigeria 1963	Kudano 1	E_3		0.72	B		
Nigeria 1969	N.Nigeria	E_2		0.91	B	±	
Nigeria 1970	Nigeria 4	E_3		0.81	B	++	
"	Nigeria 5	E_3		0.67	B	+	
"	Nigeria 6	E_2		0.90	B	++	
"	Nigeria 7	E_3		0.75	B	++	tr
"	Nigeria 8	E_2		0.47	B	++	
Niger 1969	Niger 1	E_2	5.9	0.75	B	±	tr
"	Niger 2	E_3		0.68	B	++	
"	Niger 3	E_3	6.7	0.93	B	++	tr
U. Volta 1968	U. Volta	E_3	3.9	1.29	B	±	1/4
Togo 1969	Togo 1	E_3	8.0	0.35	B	++	1/8
"	Togo 2	E_3		0.27	B	++	1/32
S. Leone 1968	S. Leone 1	E_2	5.0	0.6	B	+	tr
"	S. Leone 2	E_3		1.1	B	++	
"	S. Leone 3	E_4		0.54	B	++	
S. Leone 1969	S. Leone 4	E_4	14	1.07	B	±	tr
Guinea 1969	Guinea 1	E_2		0.65	B	-	
"	Guinea 2	E_3		0.71	B	++	-

Table VII - 5 contd.

Congo 1963	Luk	E_5		1.0	B		
"	Kombe	E_4		0.9	B		
Congo 1968	Banja	E_3		0.55	B	++	-
"	Kegali	E_2		0.62	A	+++	
Congo 1969	Lusambula	E_2		0.73	B	+	
"	Mangumba	E_3	3.4	0.60			
Congo 1970	Congo 1	E_1	5.6	0.7	B	+++	
"	Congo 2	E_3	4.5	0.3	B	++	-
"	Congo 3	E_2		0.62	B	+++	
"	Congo 4	E_3	5.3	0.72	B	+	tr
"	Congo 5	E_3		0.51	B	-	
"	Congo 6	E_3		0.83	B	+	
"	Congo 7	E_3		1.2	B	++	tr
Gabon 1963	B-Maria	E_2	9.1	0.3	B		

TABLE VII - 6

Virus strains from Kenya and East Africa

Source	Name	Egg pass	ST_5 (10^5)	log pk.f.u. $35^o/38^o$	HAD on HEF	CPE on HEF	HA on HEp2
Nakuru 1964	Odour	E_2	4.5	0.6	B		
"	Wathiri	E_2	4.8	0.4	A	++++	1/64
"	Ndungu	E_1		0.5	A		
Kitale 1964	Nafuna	E_2		1.0	B	++++	
"	Ekiru	E_2		0.6	A		
Kisii 1964	Joseph	E_2	5.0	0.25	B	++++	1/16
"	Teresia	E_2		0.28	B	+++	1/32
"	Arogo	E_2		0.87	B	+	
"	Kongo	E_2		0.50	B	+	1/32
Eldoret 1964	Charles	E_3		0.2	A	+++	
"	Muchaii	E_2		0.1	A		
Kakamega 1964	Mwenje	E_2		0.4	A		
"	Wiyalira	E_2		0.3	A		
"	Agneto	E_2		0.5	A		
Molo 1964	Mwoniki	*		0.41	A	+++	
Nairobi 1964	Bedeta	E_2	4.8	0.4	A	++++	1/16
Fort Londian 1967	Osombo	E_2		0.3	A		
Fort Hall 1967	Wachuti	E2		0.2	A		
Machakos 1967	Syokau	E_2		0.7	B		

Table VII - 6 contd.

French Somalia 1970	Djibouti	E_3		1.38	B	++	
Place not known 1964	Kibet	E_2	8.3	0.6	A	++	1/8
	Njotho	E_2	4.5	0.7	A	+++	1/32
	Dishon	E_2		0.4	A		
	Kones	E_2		0.7	B	-	
	Kanji	E_2		0.6	A		
	Njeri	E_2		0.6	B		
	Khadeja	E_2	4.8	0.6	B	+++	1/16
	Sika	E_1		0.3	A		
	Muzee	E_1	4.5	0.3	A	++++	1/64
	Kiboji	E_1		0.6	B		
	Mary	E_2		0.6	A		
	Helder	E_3		0.7	B		
	Odhiambo	E_3		0.3	B	++	
	Semat	E_2		0.2	A		
	Mburu	E_2		0.2	A		
	Ngani	E_2		0.31	A	++++	1/32
	Jabuya	E_2		0.53	B	+++	
	Leunda	$*^2$		0.4	A		1/16
	Taputany	E_5		1.0	B		
	Lefer	E_5	5.0	0.37	B	++++	1/16
	Aheno	E_3		0.7	B		
	Mukanyo	E_2		0.97	B	++	

TABLE VII - 7

Virus strains from Indonesia

Source	Name	Egg pass	ST_5 (10^5)	log pk.f.u. $35^0/38^0$	HAD on HEF	CPE on HEF	HA on HEp2
Java - Surbaja 1961	Maridjah	E_5		0.24	B		
Djakarta 1968	Djakarta I/1a	*		0.78	A		
"	Djakarta II/2a	*		1.23	A		
Surakarta 1968	Sur-623	*		0.65	A		
"	Sur-626	*		0.73	A		
Bandung 1968	Jojoh	E_3		0.64	A		
"	Tjuatjua	E_2		0.46	B		
Bandung 1970	W.J. 1	E_3			A		
"	W.J. 2	E_2			A		
"	W.J. 3	E_1		0.9	B		
"	W.J. 4	E_2		0.4	A		
"	W.J. 5	E_3			A		
"	W.J. 6	E_2		0.2	B		
"	Ban 2	E_3		0.54	A		
"	Ban 3	E_1		1.59	B		
Tjiandjur 1970	Tjiandjur	E_3			A		
"	Tjiandjur 1	E_2			A	+++	
"	Tjiandjur 2	E_3		0.22	A	++++	
"	Tjiandjur 3	E_3		0.39	B	-	
Brebes 1969	Dima	E_2		0.2	A		
"	Taswin	E_1		0.5	A		
"	Kasiral	E_2	3.5	0.2	A	++++	1/16
"	Tamin	E_3		0.8	A		

Table VII - 7 contd.

Bogor 1970	Mimin	E_1		0.9	B	±	
"	Ereh	E_1		1.0	B	-	
"	Rubiah	E_2	4.0	0.2	B	+	1/4
"	Sugandi	E_1	4.2	1.0	B	-	tr
"	Maesarah	E_3		1.25	B	++	
Tjimahi 1970	Tjiamahi	E_3		0.33	A		
N. Sumatra Tapanuli 1969	Imron	E_5		1.4	B	++	1/64
"	Raahme	E_5	4.4	1.5	B	++	1/32
N.Sumatra 1971	Nurbaiti	E_2	3.9	0.8	A	+++	1/64
	Nurjahati	E_2		0.5	A	++++	
	Nurlys	E_2		0.8	A	+++	

TABLE VII - 8

Virus strains from Pakistan

Source	Name	Egg pass	ST_5 (10^5)	log pk.f.u. $35^o/38^o$	HAD on HEF	CPE on HEF	HA on HEp2
Sind							
Karachi 1961	Kar 1	E_4		0.2	A	++++	
	Kar 2	E_4		0.7	A	+++	
	Kar 5	E_3		0.3	A	+++	
	Kar 8	E_3			A	++++	
	Kar 9	E_3		0.3	A	+++	
Punjab							
Lahore 1969	Rafik	E_1	3.5	0.3	A	++++	tr
	Nasreen	E_1		0.1	A		
	Misba	E_1		0.5	A	++++	1/64
	Ashiq	E_2	3.6	0.51	A	++++	tr
	Asad	E_2		0.72	A		
	Nasim	E_2			A		
	Nasar	E_2		0.62	A		
	Fawzia	E_2		0.57	A	+++	
	Ramjan	E_3	3.9	0.68	B	++	
Punjab							
Bahawal Nagar 1969	Sudan	E_1		0.2	B		
	Jamil	E_1		0.6	B		
	Sajeda	E_1		0.3	B	+++	1/16
	Badar	E_3		0.84	B	++	
	Kalsum	E_3		0.27	B		
	Talia	E_1		0.73	B	+++	
	Zubeida	E_2		0.3	A		
	Asgar	E_3		0.15	A		
Place unknown	Razak	E_2		0.5	A		
	Farid	*	5.0	0.66	B	+++	

Comparison of pock-reduction at 38.3° with chick embryo mortality

None of the v. major strains tested has a reduction in pock titre exceeding 0.5 log units, and the estimated survival time from an inoculum of 10^5 pk.f.u. was regularly less than 4.5 days. These were taken as the limits for v. major. All the v. minor strains had pock titre reductions of more than 2.5 log units and survival times in excess of 6 days with an inoculum of 10^5 pk.f.u. They thus formed a group well separated from variola major. The question of intermediates was dealt with in the following way. Survival times between 4.5 and 6.0 days were taken as an intermediate group. Pock reduction values tend to be somewhat variable but values in excess of 0.7 can safely be taken as being different from variola major and repeatable. Values in this group ranged from 0.7 - 1.5, which is still well short of the figure established for variola minor. The intermediate group with pock reduction between 0.5 and 0.69 was admittedly uncertain but very few strains, in fact, fell into this region. Thirty strains from Africa have been tested for chick embryo mortality and the classified results are set out in Table VII-9, with the numerical analysis following in Table VII-10. It is immediately apparent from the latter table that there is no tendency for strains of high or low virulence in the one test to be associated with a similar result in the other test. This is further emphasized in Table VII-9 where also it can be seen that there is no obvious grouping according to country of origin. It has to be concluded that these two tests do not measure the same character, even though either serves to

TABLE VII - 9

Comparison of pock reduction at 38.3° and chick embryo mortality of African strains

ST(10^5)	Pock reduction at 38.3°C			
	0 - 0.49	0.5 - 0.69	0.7 - 1.5	>2.5
0 - 4.5	All v. major 2/63, Congo 2	Mangumba	8/61, 6/63 Njotho, U.Volta	-
4.6 - 6.0	Joseph, Bedeta Muzee, Lefer, 1/61	Banja S. Leone 1	32/62, 5/61, 12/61, 22/62, Congo 1, Niger 1 Congo 4	-
> 6.0	Ilembula B-Maria Togo 1	Kibet Khadeja	25/62, 17/61, S. Leone 4, Niger 3	All v. minor

TABLE VII - 10

Numerical analysis of data in Table VII - 9

ST(10^5)	Pock reduction at 38.3°C			Total
	0 - 0.49	0.5 - 0.69	>0.7	
0 - 4.5	2	1	4	7
4.6 - 6.0	5	2	7	14
> 6.0	3	2	4	9
Total	10	5	15	30

differentiate variola minor from variola major. It seems probable that the factors affecting pock production at 38.3° are not the only factors determining the survival of the chick embryo. No attempt was made to estimate the amount of virus in the livers of the embryos, as in the extensive data of Sarkar and Mitra (1967) this correlated almost completely with mortality rates.

Comparison of pock-reduction test at 38.3° and haemadsorption at 40° in HEF

The results of these two tests are summarized in Table VII-11. It can be seen that, despite considerable overlap, the three groups: variola major, African strains in haemadsorption Group A and Group B respectively all have distributions with different modal values. There is also a suggestion of a second mode in the African 'B' group in the pock-reduction range from 1.1 units to 1.5 log units. There is nevertheless a considerable overlap between African 'B' and v. major. Reference to Table VII-3, will also show that several strains of v. minor from Brazil were classed as 'B' in the haemadsorption test. The two inferences from these results are that haemadsorption and pock-reduction measure different viral properties and also that haemadsorption would not serve as a test to distinguish v. minor from v. major with accuracy.

As both chick mortality and haemadsorption differ from the pock-reduction test, it remains to compare these two. This is not very profitable with the African strains as the great majority of these

TABLE VII - 11

Comparison of pock reduction test at 38.3° and HAD at 40° in HEF

HAD Group	Decline in titre on CAM at 38.3°C						
	0.1	0.3	0.5	0.7	0.9	1.1	1.1-1.5
V.major (A)	0	12	5	-	-	-	-
Africa "A"	1	9	8	6	0	-	-
Africa "B"	0	6	11	28	12	8	8

are in Group B. The group taken for this comparison was the 18 strains from Kenya, Indonesia and Pakistan which had been tested for mortality. All of these three areas has more or less equal distribution of A and B strains. The results are summarized in Table VII-12, in which the two groups of A and B are put in ranking order of their survival times. Although these two groups have different median values the overlap is so great that it is hard to see any correlation between mortality rate and haemadsorption.

Comparison of HA titre in HEp2 cells with haemadsorption in HEF

There seemed no reasonable point of division in the HA titres so these have been classified into two groups only, positive with a titre of 1/4 or more and negative with a titre of less than 1/4. The overall picture of the 78 strains examined for HA is shown in Table VII-13. From this it will be seen that the majority of 'A' strains are positive for HA, and all 'C' strains are negative. 'B' strains are equally divided between positive and negative. In Table VII-14 the results are shown according to country. It is interesting to note that all Kenya strains were positive for HA irrespective of their HAD group. The HA results therefore differentiate clearly between haemadsorption groups A and C, but might add slight additional clarification when applied to B group strains.

Comparison of CPE with haemadsorption in human fibroblast cultures

78 strains have been tested for CPE in HEF cells at 40°. The results were graded from 0 to 4+ and are now classed into three groups.

TABLE VII - 12

Comparison of chick embryo mortality and HAD group with strains from Kenya, Indonesia and Pakistan

HAD Group	Survival times after inocula of 10^5 pk.f.u.							
A	3.5	3.5	3.6	3.9	4.5	4.5	4.8	8.3
B	3.9	4.0	4.2	4.4	4.8	5.0	5.0	5.0

TABLE VII - 13

Comparison of the HA titre of HEp2 with HAD in HEF

HA titre	HAD Group			Total
	A	B	C	
Positive	18	19	0	37
Negative	2	21	18	41
Total	20	40	18	78

TABLE VII - 14

Geographical distribution of data in Table VII - 13

HA titre	Kenya	Tanzania	W.Africa	Asia	V. major	V. minor
Positive	AAAAAA BBBB	BBBBB	BBB	AAA BBBB	AAAA AAAA	BB
Negative	-	BBBB B	BBBB BBBB BB	AA B	BB	BB 18 x C

1)	4+	with marked disintegration of cell sheet and shrinking of cells
2)	3+ & 2+	with cells shortened and thickened and possibly beginning to separate from each other
3)	1+ & 0	With CPE only observable in small scattered areas or not at all

It is clear from Table VII-15 that these two properties are closely related, and that each reveals an intermediate group. There is some scatter distribution, but some at least of this might be reduced if the tests were several times repeated. A breakdown of this comparison by country is shown in Table VII-16.

It can be concluded that the CPE results do not add significantly to the haemadsorption results obtained in the same cells.

Some haemadsorption and CPE tests were done in culture of human embryonic lung. The results are set out in Tables VII-17 and VII-18 and show essentially similar results. These results can therefore be taken as confirmatory tests of those given earlier for the HEF cells.

GEOGRAPHICAL DISTRIBUTION OF VARIOLA STRAINS

1. Variola major in Britain

Most variola major strains were haemadsorption Group A. It is interesting to find four strains in the B group and further that all of them were isolated from the outbreaks in England in 1962. These tests were done 'blind' in the sense that the present author did not

TABLE VII - 15

Comparison of CPE with haemadsorption in human fibroblast cultures

CPE Group	Haemadsorption Group			Total
	A	B	C	
++++	14	2	0	16
+++) ++)	3	21	0	24
+ 0	0	12	26	38
Total	17	35	26	78

TABLE VII - 16

A breakdown of the comparison by country

CPE Group	Pakistan	Indonesia	Kenya	Tanzania	W.Africa	V. major	V. minor
++++	AAA	A	AAAA BB	-	-	AAA AAA	-
+++) ++)	BB	A BB	AA BB	BBBB	BBB BBB	BB	BBB
+) 0)		BB	B	B	BBB BB	B	BB Cx26

TABLE VII - 17

HAD and CPE on human embryonic lung and human embryonic fibroblast after 48 hrs at 40°

Strain	Name	HAD		CPE	
		H.E.L.	H.E.F.	H.E.L.	H.E.F.
V. major	Young	A	A	++++	++++
	Cameron	A	A	++	++++
	Mrs. King	A	A	++++	++++
	Campbell	A	A	++++	++++
	Harvey	A	A	++++	+++
	Ismat	A	B	++	+++
	Shuker	A	A	++	++
	McLenon	B	B	+	+
V. minor	Winkel	C	C	-	-
	Haithwait	C	C	-	-
	Butler	C	C	-	-
	Ven. 4	C	C	-	-
Indonesia Strains	Tjiandjur 1	A	A	++	+++
	" 2	A	A	++++	++++
	" 3	B	B	-	±
	Ereh	B	B	-	-
	Maesarah	B	B	±	++
	Rubia	B	B	-	+
	Sugandi	B	B	-	-
Tanzania	EA8/61	B	B	±	+
Pakistan	Kar-2	A	A	+++	+++
	Kar-5	B	A	-	+++

H.E.L. = Human embryonic lung

H.E.F. = Human embryonic fibroblast

TABLE VII - 18

HAD and CPE on human embryonic lung and human embryonic fibroblast after 48 hrs at 40°

Strain	Name	HAD		CPE	
		H.E.L.	H.E.F.	H.E.L.	H.E.F.
Congo 1968	Banja	B	B	++	++
"	Kigali	A	A	++	+++
Congo 1970	Congo 1	B	B	+++	+++
	Congo 2	B	B	++	++
	Congo 3	B	B	++	+++
	Congo 4	B	B	-	+
	Congo 5	B	B	-	-
	Congo 6	B	B	-	+
	Congo 7	B	B	+++	++
Nigeria 1970	Nigeria 4	B	B	±	++
	Nigeria 5	B	B	+	+
	Nigeria 6	B	B	-	++
	Nigeria 8	B	B	-	++
S. Leone 1968	S.L. 1	B	B	±	+
	S.L. 2	B	B	±	++
	S.L. 3	B	B	-	++
	S.L. 4	C	B	-	±
Upper Volta	U.Volta	B	B	±	+
N. Nigeria	N. Nig	B	B	-	±
Niger	Niger 1	B	B	-	±
Guinea	Guinea 1	B	B	-	-

H.E.L. = Human embryonic lung

H.E.F. = Human embryonic fibroblast.

know the origin of the strains at the time the tests were done. It is even more fascinating to find the strain for the Cardiff outbreak of 1962 to be an 'A', and the epidemiological evidence (DHSS 'Smallpox 1962') indicated that this group of cases results from a separate importation into the country. The original source was apparently West Pakistan where both A and B strains have been found (Table VII-8).

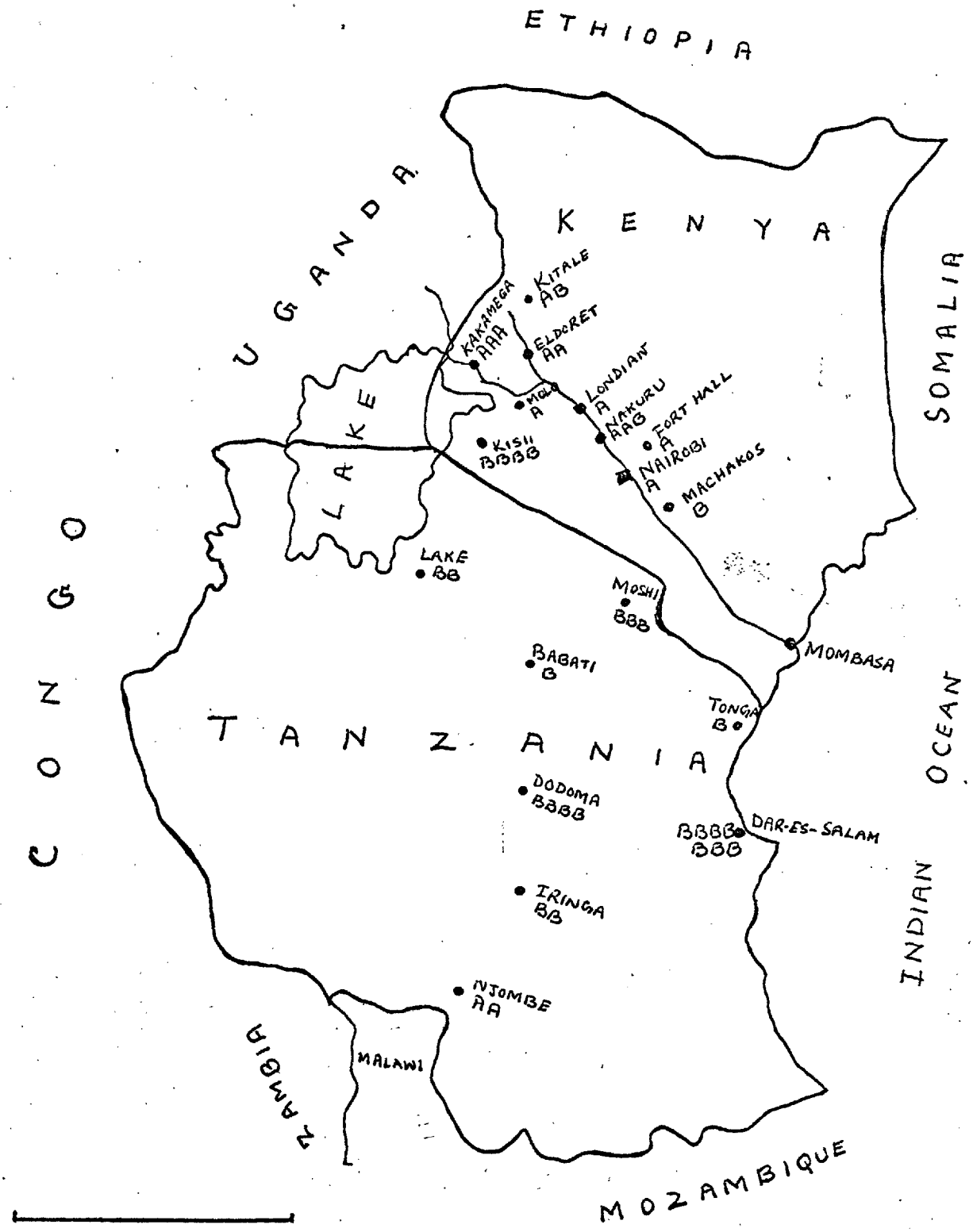
2. Variola minor

Most strains of variola minor were haemadsorption group C. There were five exceptions to this all from Brazil. It will be seen from Table VII-3 that these five strains come from the adjoining provinces Pernambuco, Bahia and Paraiba in Northern Brazil and account for 50% of the strains examined from these three provinces.

3. Tanzania

None of the 25 Tanzanian strains behaved like variola minor. Only two of them were in Group A and 21 were 'B'. The median value for pock reduction was between 0.70 and 0.73 compared to 0.28 for the v. major. Of the smaller number of mortality tests done the median survival time was 5.6 days for Tanzania compared to 4.0 days for v. major. It seems clear the variola endemic in Tanzania in 1961 - 63 was a different variola family from the Asian variola major, and was also distinct from v. minor. The two 'A' strains in the Tanzania isolates both came from near Njombe (Figure VII-1), in the South West of Iringa district and well removed from the origins of the other

FIGURE VII - 1



Scale 1 : 10,000,000

Tanzanian strains.

4. Kenya

Forty-two strains isolated from cases in Kenya in the years 1962 - 1965 were fairly evenly divided between Group A and Group B. The place of origin is known for 19 of them as listed in Table VII-6 and reference to the location map in Figure VII-1, reveals some interesting possibilities. The main arterial road comes up from the coast at Mombassa, through Nairobi and then divides to join again at the border into Uganda. All the 'A' strains were from places along this highway, and the more remote districts yielded a higher proportion of 'B's, particularly from Kisii which is getting near the border with Tanzania.

5. West Africa

All but one of the West African strains were HA in Group B. The exception came from the Congo in 1968, but its history is rather uncertain. Further differentiation of Group B's might come from consideration of pock-reduction, where there was evidence of a bimodal distribution (Table VII-11) or from HA production in HEp2 cells where group B strains were evenly divided between positive and negative (Table VII-13). Taking first the pock reductions, it seems reasonable to begin by dividing the region into three areas, (a) the Congo and Gaboon in the South, (b) the adjoining countries of Nigeria, Niger, Upper Volta and Togo and (c) Sierra Leone and Guinea. The last group only contains six strains, but the other two are shown in ranking

order in Table VII-19, and it can be seen that their distributions are not significantly different. There are some interesting geographical points about the 'Niger' group, which may be relevant. The two strains with the lowest figure for pock-reduction (0.27 and 0.35) are the only strains representing Togoland. The four strains from the other end of this group came from the inland areas Niger, Northern Nigeria and Upper Volta. The two strains from Togo are also noteworthy in that they both produce haemagglutinin in HEp2 cells. This is summarised in Table VII - 20.

Bedson & Dumbell (1961) showed that the thermostability of the variola major virion at 55° was much greater than that of variola minor. They used the same technique as that employed by Fenner (1958) and examined two strains of each virus. This test has been used to study the monkey pox-like viruses described in the next chapter and the two West African strains (Togo 1 and Upper Volta) were included among the controls. Both of these suffered reductions in titre significantly greater than that of v. major or monkey pox though not as great as that reported for v. minor. The significance of this observation cannot be assessed until more West African strains have been tested in this way.

Indonesia

The Indonesian strains are divided between Group A and B without any regular geographic association. It will be noted, however, in Table VII-7 that small groups of strains collected from particular

TABLE VII - 19

Results of pock reduction tests with 'Congo' group and 'Niger' group virus strains

Province	Pock reduction at 38.3°							
Congo Group	.30	.30	.51	.55	.60	.62	.62	
	.70	.72	.73	.83	.90	1.0	1.20	
Niger Group	.27	.35	.47	.60	.66	.67	.68	.72
	.75	.75	.81	.90	.92	.93	1.3	1.8

TABLE VII - 20

Geographical grouping of 17 African 'B' strains, examined also for HA in HEp2 cells and pock reduction, at 38.3°
Figures show proportion of strains in each category

	HEp2 - HA positive	HEp2 - HA negative
Reduction in pock titre like v. major	Kenya 4/4 Togo 2/2	Congo 2/4
Reduction in pock titre: intermediate	Upper Volta 1/1	Congo 2/4 Sierra Leone 2/2 Niger 2/2 Nigeria 1/1 Guinea 1/1

localities all belong to the same group. Thus, from Brebes in Java in 1969 and from North Sumatra in 1971, all strains were Group A. In Bogor, Java in 1970 and North Sumatra in 1969 all were in Group B.

Pakistan

A few strains were tested which had been sent from Karachi in 1961 but most strains had been collected by Dr. Gordon Heiner in 1969 in Lahore and the surrounding district. It is noteworthy that 8 of 9 strains from Lahore City were Group A while 6 of 8 strains from the local village of Bahawal-Nagar were Group B.

CHAPTER VIII

THE RELATION BETWEEN VARIOLA AND MONKEY POX VIRUSES

Smallpox-like illness in Africa

In three countries of Africa, where smallpox had not been observed for at least 18 months, 6 suspected cases of smallpox in children were reported between August and December, 1970. No source of infection could be traced or identified and none of them transmitted infection to other susceptible persons (WHO 1971). Another case was demonstrated early in 1971. The viruses from some of those patients were isolated and extensive studies have been made. It was found that all cases were not vaccinated. Virus strains obtained were :-

Year	Country	Name
1970	Liberia	Liberia I and II
"	Congo	Congo-8-Lombe

1970	S. Leone	V70-266
1971	Nigeria	V71-82

Studies were undertaken with these strains and comparison was made with standard v. major virus. These were as follows :

1) Pock morphology :-

The viruses were inoculated in egg at a dilution to obtain discrete pocks. The pocks were found to be like monkey pox pocks. At 35° they were small, flat and pinkish in appearance, often haemorrhagic in the centre. But sometimes at higher temperatures they looked like v. major pocks - white, raised and shiny, and it was difficult to distinguish from v. major virus. Baxby (1969) reported the change in pock morphology with change of temperature. He observed that the white variant of cowpox virus showed white pocks at 35°, but when they were incubated at 33° the CAM produced all red pocks similar to the ordinary haemorrhagic standard cowpox strain.

2) Chicken embryo mortality test :-

These isolated strains were found to be as lethal as monkey pox to chick embryo. They were definitely more lethal than v. major. Their survival time in days with 10^{5} pk.f.u. inocula was found to correlate with monkey pox (Table VIII-1).

3) Ceiling temperature test on CAM :-

V. major does not grow on the CAM at a temperature of 39°

TABLE VIII - 1

Results of various tests with strains of smallpox-like disease in humans and smallpox-like virus isolated from animals

Group	Name	Egg Pass	Log reduction of titre at 38.3°	Log reduction of titre at 39°	Log reduction of titre after heating for 20 mins at 55°C	ST (10^5)
Monkey pox	Denmark	E_4	0.05	1.22	2.46	2.94
Strains from smallpox -like illness in man	Liberia I	E_4	0.18	1.31	2.74	3.0
	Liberia II	E_3	0.17			
	Congo-8-L	E_3	0.20			2.70
	V70-266	E_3	0.20	1.33		
	V71-82	E_4	0.07			3.12
V. major	Cameron	E_6	0.27	>2.5		3.57
	Harvey	E_6	0.40	>2.5	2.1 (1)	3.57
Variola -like strain isolated from animal	64-7275	E_4	0.10	>2.5	1.47	4.0
	64-7255	E_4	0.23	>2.5	1.59	3.57
	Chimp 9	*	0.11	>2.5	2.32	4.0
	V71-16	E_2	0.18	>2.5	2.89	

(1) Result taken from Bedson (1964)

* No history obtained

but monkey pox produces pocks at that temperature. These strains also show pocks at 39° (Table VIII-1).

4) Heat inactivation at 55° :-

Fenner (1958) carried out heat inactivation tests on vaccinia, cowpox and rabbit pox at 55° for 40 minutes and found that the drop in their titre varied from 10-fold to 40-fold. Bedson (1969) did the inactivation test more elaborately on some more groups of pox virus and carried out the test for 20 minutes. He found that the ceiling temperature and thermal stability of pox viruses are distinct and have completely unrelated characters. He also observed that the thermal stability of pox viruses varies between the different groups. V. major and monkey pox virus have got different log reduction titres of heat stability. On the basis of his findings the heat inactivation test was carried out with some of these strains.

Method: The eggs were inoculated with respective virus at a suitable dose to give confluent membranes after 72 hours. The membranes were aseptically excised in a universal bottle containing glass beads and 1 ml of phosphate buffered saline solution was added per membrane. The membranes were shaken vigorously to remove the virus from the tissue. The extract was centrifuged at 1000 rpm for 15 - 20 minutes to sediment the crude debris. The supernatant was taken and preserved. The heat experiment was done on the same day as the extracts were prepared. Volumes of 0.5 ml virus suspension were sealed in ampoules and were then completely immersed in a 55°C water-bath for

20 minutes. After 20 minutes, the ampoules were quickly removed from the bath and cooled by placing in cold water. The infectivity titration of the heated materials from the ampoules were carried out, along with unheated controls, on egg membranes.

Result: V. major is found to be slightly more stable when heated for 20 minutes at a temperature of 55° than monkey pox. Only one of these new strains has been tested and this was found to resemble monkey pox (Table VIII-1).

5) CPE on human fibroblasts :-

The CPE of monkey pox on fibroblast is different from that of v. major. The rate of CPE production is quicker than that of v. major. The cells become elongated, thin and the whole cell sheet is trabeculated giving a net-like appearance. In case of v. major the cells are shorter, thicker, irregular and separated from each other. The monkey pox also shows CPE at 40°. The strains isolated show the same CPE as monkey pox (Table VIII-2A).

6) HAD on fibroblasts :-

The strains show confluent HAD like both monkey pox and v. major at 40° (Table VIII-2A).

7) HA on HEp2 cells :-

Monkey pox produces a considerable amount of HA on HEp2 cells at 37°, 38° and even at 39°. V. major also produces HA to a high titre at 37° but it falls rapidly at 38° and no HA could be recorded

TABLE VIII - 2 (A)

Results of various tests with strains of smallpox-like disease in humans and smallpox-like virus isolated from animals

Group	Name	Egg Pass	HEF after 48 hrs			Titre of HA on HEp2 after 72 hrs at		
			CPE at 40°	HAD at 40°	Group	37°	38°	39°
Monkey pox	Denmark	E_4	++++	4	A	1/256	1/256	1/32
Strains from smallpox -like illness in man	Liberia I	E_4	++++	5	A	1/256	1/256	1/64
	Liberia II	E_3	++++	5	A	1/128	1/128	1/64
	Congo-8-L	E_4	++++	5	A	<1/256	1/256	1/64
	V70-266	E_3	++++	5	A	1/256	1/128	1/32
	V71-82	E_4	++++	5	A	1/256	1/256	1/64
V. major	Cameron	E_6	++++	5	A	1/128	1/32	-
V. minor	Plugge	E_9	-	0	C	-	-	-
Variola -like strains isolated from animals	64-7255	E_4	++++	2	B	1/8	-	-
	64-7275	E_4	+++	2	B	1/64	-	-
	Chimp 9	*	+++	5	A	1/32	1/8	1/4
	V71-16	E_2	++	3	B	1/128	1/16	-

* No history obtained

at 39°. These strains follow the pattern of monkey pox regarding HA (Table VIII-2A).

8) CPE on rabbit kidney cells (RK_{13}) :-

Egg-passed strains of monkey pox produced degenerative, cytolytic and necrotic lesions in RK_{13} cell lines at a very rapid rate, but egg-passed strains of v. major produced proliferative hyperplastic foci by clumping up of cells one upon another followed by a surrounding hollow space. After 96 hours these hyperplastic foci might break up and be followed by a spreading degenerative lesion but at a much slower speed. These new strains produce lesions just like monkey pox on RK_{13} cells (Table VIII-2B).

Gispen et al (1967) found that v. major could not be passed regularly on RK_{13} cells whereas monkey pox and vaccinia could. The new strains Liberia I, II and Congo-8-Lombe were successively passed 8 times on RK_{13} cells without difficulty. On the other hand major strains have also been passed serially in RK_{13} without difficulty in this laboratory though the time intervals were longer than for monkey pox strains. This is of some interest. The line of RK_{13} cells maintained in this laboratory is grown in medium containing rabbit serum. This serum is obtained in large batches after preliminary tests of suitability. A few experiments have been set up with RK_{13} cells in medium with different batches of rabbit serum or with calf serum. It can be said that variola grew with much greater difficulty in these different sera but the matter has not been fully investigated. It is

TABLE VIII - 2 (B)

Results of various tests with strains of smallpox-like disease in humans and smallpox-like virus isolated from animals

Group	Name	Egg Pass	RK_{13} at 37°C after 48 hrs	
			HA titre	CPE
Monkey pox	Denmark	E_4	<1/256	Cytolytic and degenerative ++++
Strains from smallpox -like illness in man	Liberia I	E_4	1/256	"
	Liberia II	E_3	1/256	"
	Congo-8-L	E_4	<1/256	"
	V70-266	E_3	1/256	"
	V71-82	E_4	1/256	"
V. major	Cameron	E_6	-	Hyperplastic foci with clumping up of cells +
V. minor	Plugge	E_9	-	Hyperplastic foci +
Variola -like strains isolated from animals	64-7255	E_4	1/32	Huperplastic and degenerative ++
	64-7275	E_4	1/16	"
	Chimp 9	*	-	"
	V71-16	E_2	1/256	Cytolytic and degenerative ++++

* No history obtained

assumed, for the present, that some factor in the available batch of rabbit serum allows variola virus to develop in RK_{13} cells.

9) HA on RK_{13} cells :-

The egg-passed strain of monkey pox and these newly isolated strains produce HA in RK_{13} cells to a high titre at 37° (Table VIII-2B). The egg-passed strains of v. major usually do not produce HA in RK_{13} cells or sometimes they produce a very low titre. However, the RK_{13}-passed strains of v. major show a moderate amount of HA when compared to that of egg-passed strains. No HA was obtained when an egg-passed strain from West Africa (Congo-1) was inoculated in RK_{13} cells but after one passage in RK_{13}, Congo-1 was found to produce HA to a titre of 1/64 on the second passage. Variola minor strains did not produce HA in RK_{13} cells when egg-passed strains were inoculated. The Plugge strain of v. minor was tested in the second passage in RK_{13} and was found to be negative. Time has not been available to continue this line of investigation.

10) Inoculation of rabbit skin :-

Professor McCarthy (personal communication) inoculated Liberia I, Liberia II and Congo-8-Lombe intradermally into rabbits and found that they produced large papules more than 1.5 cm in diameter with central necrosis and haemorrhage and a surrounding area of induration. A standard monkey pox strain also showed similar lesions. The inocula used by McCarthy were from 10 - 1000 pk.f.u. per insertion. Variola on the other hand gives a transient small, non-necrotic papule when

inoculated in much larger doses. (McKinnon & Defries 1928, Bedson & Dumbell 1964).

Smallpox-like viruses isolated from animals

In 1964, 3 strains of pox virus were isolated from healthy rhesus monkey kidneys being processed in Holland for poliomyelitis work. Of these three strains 64-9411 behaved like monkey pox in all respects but the other two, 64-7255 and 64-7275 behaved more like variola than monkey pox in laboratory tests. Recently another strain (Chimp 9) was isolated from a healthy chimpanzee kidney obtained from Equatorial province of the Congo. This was also found to vary from monkey pox virus. Furthermore, another virus strain like variola was isolated from a gerbil (V71-16) which had been examined as part of an arbovirus survey.

The following tests were performed on these strains and results were as follows :

1) Pock morphology :-

64-7255, 647275, Chimp 9 and V71-16 gave the same pock morphology as v. major on CAM of chick embryo at 35°.

2) Chick embryo mortality test :-

They behaved in the same way as v. major virus regarding chick embryo mortality (Table VIII-1). Their survival time was found to be like v. major. The mortality test with V71-16 was not performed.

3) Ceiling temperature test :-

64-7255, 64-7275, V71-16 and Chimp 9 behaved the same as v. major. They do not grow at 39° (Table VIII-1).

4) Heat inactivation test at 55° :-

64-7255 and 64-7275 gave a result very similar to v. major. Chimp 9 and V71-16 gave 2.32 and 2.89 respectively which is somewhat different from v. major and close to monkey pox (Table VIII-1).

5) CPE on human fibroblast :-

At 40° after 48 hours the CPE with 64-7255 is 4+, with 64-7275 and Chimp 9 are 3+ and with V71-16 is 2+. However, at 37° after 48 hours all give 4+ (Table VIII-2A).

6) HAD on human fibroblast :-

At 40° after 48 hours the HAD is 'A' with Chimp 9 and 'B' with 64-7275, 64-7255 and V71-16 (Table VIII-2A).

7) HA on Hep2 cells :-

At 37° V71-16, 64-7275, Chimp 9 and 64-7255 produce HA at a titre of 1/128, 1/64, 1/32 and 1/8 respectively.

At 38° the HA titre of V71-16 reduces to 1/16 and Chimp 9 to 1/8 and the remaining two strains do not produce any HA. At 39° Chimp 9 only produces a titre of 1/8 and V71-16 produces nothing. So in respect of HA at 37°, 38° and 39° these strains behave much like v. major (Table VIII-2A).

8) CPE on RK_{13} cells :-

At 37° after 48 hours 64-7255, 64-7275 and Chimp 9 produced hyperplastic foci followed by degenerative lesions much like v. major. However, V71-16 forms cytolytic and degenerative lesions from the very beginning like monkey pox (Table VIII-2B). V71-16 produced cytolytic and degenerative lesions from the beginning. Strain 64-7255 could not be passed serially in RK_{13} cells under the same conditions that permitted passage of variola major strains.

9) HA in RK_{13} cells :-

An egg-passed strain of Chimp 9 did not produce any HA on RK_{13} cells. 64-7255 and 64-7275 produced a very low titre of HA on RK_{13} but V71-16 produced HA up to 1/256 (Table VIII-2B). However, the RK_{13}-passed strain of Chimp 9 also produced HA to a considerable titre. V71-16 is clearly different from variola major in this test.

Gispen has reported that 64-7255 and 64-7275 both produce haemorrhagic lesions in rabbit skin, but that this does not occur in every rabbit inoculated, as with monkey pox, but only in a proportion of rabbits. So far as is known, no variola virus tested in rabbit skin has produced a haemorrhagic lesion.

Discussion:

Chimp 9, 64-7255, 64-7275 behaved like v. major in most tests. V71-16 also behaved similarly but it produces CPE and HA like monkey pox on RK_{13} cells and also behaves like monkey pox in heat-inactivation tests.

As 64-7275, 64-7255 and Chimp 9 are very similar to v. major, the question arises whether there is a natural animal reservoir of smallpox virus. Arita and Henderson (1968) reported that smallpox epidemics happened in monkey populations in several places along with human epidemic. So it is possible that monkeys or apes might carry the smallpox virus. On the other hand no evidence has been produced suggesting that monkeys played any part in the epidemiology of smallpox. It is also doubted whether the smallpox observed in monkeys was truly variola. It is possible that it was caused by a virus in the herpes group which is known to produce profuse exanthemata in monkeys. (Noble, personal communication). Experimental inoculation of monkeys with variola has produced only mild, non-fatal disease.

The established facts are firstly, that a mild smallpox-like illness in man has yielded on several occasions a virus which in laboratory tests is not distinguishable from monkey pox, secondly that viruses have been isolated from monkeys not clinically ill which are at least closely similar to variola.

Two problems arise. The first is how to identify precisely a virus in the pox group. Our failure to distinguish two separate isolates may reflect more ignorance than knowledge. This will be taken up in the general discussion. The other problem, in which the laboratory may be of some help is the likelihood, or otherwise, of natural inter-transformations between variola and monkey pox. This also will be considered in the general discussion.

CHAPTER IX

GENERAL DISCUSSION AND CONCLUSIONS

Most virus groups can be subdivided and individual viruses typed by serological reactions. Complement fixation tests often detect all viruses within a group and individual types are separated by neutralisation tests. It may be said that this is the standard method for identifying viruses. Difficulties are encountered in the vaccinia-variola subgroup of the pox viruses. Cross reactions by complement fixation tests (Downie and McDonald 1953) and by neutralisation tests (Downie and McCarthy 1950) are so extensive that these methods do not suffice for the precise identification of a virus isolate. Consequently, recourse has to be taken to a pattern of results over a series of tests for biological marker characters. Differences in results of such tests with two viruses can show that they are not identical but it is not easy to establish absolute

identify between separate isolates. Some biological markers are fairly unequivocal, and give a clearcut result, but many depend on quantitative measurements or fine grading of responses which vary throughout a range and merge with the possible response of another virus. It can be said that the more biological marker characters are revealed in a virus group, the more the chance of differentiating them or identifying them accordingly.

Up to 1956 it was impossible to differentiate between v. major and v. minor in the laboratory. Dinger (1956) first showed the difference between the two viruses according to the severity of virulence to chick embryo. Helbert (1957) modified the test. Nizamuddin and Dumbell (1959) described the temperature sensitivity system by which the two groups of the viruses can be differentiated successfully. Bedson et al (1963) while performing these two tests with some of the strains from Tanzania in Africa, found difficulties in placing these strains in either of the two groups described above. They seem to share the properties of both the viruses on the basis of one test or the other. In chick embryo virulence tests these strains behaved like v. minor and in ceiling temperature tests they behaved like v. major though the reduction of pock titre was greater than v. major. The case mortality figures also were intermediate between those of v. major and v. minor. Bedson et al (1963) mentioned the possibility of the existence of an intermediate group of variola viruses but more studies were required to test this hypothesis. Also,

more marker characters were needed to confirm the existence of such a new group.

Marennikova (1963) mentioned the cytopathic effect of pox virus on tissue culture. Wells (1967) showed the HA production of v. major, v. minor and strains from Africa in relation to temperature in human embryonic fibroblasts. She could not establish a definite group but indicated the possibility of differentiating between v. major and v. minor in respect of temperature and HA production. More extensive work has been done based on her findings. Three new possible methods of differentiating between v. major and v. minor by their biological characters have been described in this thesis. All three based on tissue culture systems. In general it can be said that all three tests can differentiate between v. major and v. minor though with a few exceptions. These exceptions are also of some interest because when the geographical distribution of these strains is studied, they seem to originate from the same source and same time.

The strains from Africa when subjected to these tests do not fall completely in either of the v. major or v. minor group. Either some of them behave differently or some of them share properties with either of the two groups. It has been mentioned earlier that they share the properties of both v. major and v. minor in respect of ceiling temperature test and chick embryo mortality test. When HAD and CPE tests on fibroblasts are included, the strains from various countries in West

Africa and from Tanzania fall into the same group which seems to be different both from v. major and v. minor. However some strains from Kenya behave like v. major and some behave like those from Tanzania. In respect of HA production in HEp2 cells the Kenyan strains are positive like v. major and West African strains are negative like v. minor : Tanzanian strains again lie in between. The chick embryo mortality rate is similar for strains from all of these countries. The minor differences are often constant for all strains from a particular area, and this suggests the possibility of further subgroups. Further tests for marker characters are required and more strains would be required to establish this point.

The tests do not always correlate with each other indicating that they measure different viral properties. However, when HAD and CPE on fibroblasts are compared it seems that the African strains in general fall into a broad group which is different from both v. major and v. minor. This indicates an intermediate group. The comparison of HAD on fibroblasts and the reduction of pock titre in CAM also broadly suggests that African strains belong to an intermediate group. This hypothesis can be strengthened further when their geographical location is studied. It has been found that strains showing similar marker characteristics usually come from the same source. The Kenyan strains which behave like v. major originate from townships along the main highway from Mombassa to the Ugandan border, which suggests an influence of importations from Asia. Those Kenyan strains which behave

like other African strains are mostly from areas on either side of this highway, suggesting a more completely indigenous origin.

Whether this intermediate African variola is a permanent variant from v. major or v. minor or if this strain originated in Africa the same time as the two other groups is not known. Again, whether it has originated by mutagenic change from v. major is not known. Attempts have been made to produce variants of v. major in the laboratory. The Cameron strain of v. major was subjected to increasing doses of ultraviolet irradiation and at each time several single pocks were picked up and these were tested for possible changes in their biological characters. Everytime they retained characters identical with their parent v. major. Another attempt was made by passaging single pocks from infected chick embryos which died early or late in mortality tests. After eight such consecutive passages a small but reproducible difference was shown in the marker characters of the clonal line which showed a high mortality rate and the pock line showing a low mortality rate. The results obtained were not significant enough to establish any variation. So by testing the virus by the above two methods for laboratory variation it can be said that v. major virus is a very stable virus which does not change under the conditions used.

Another interesting feature of the work described is the study of the strains from smallpox-like illness from Africa. These strains behave in every way like monkey pox when subjected to the available

tests for the biological characters. But there is no history of any monkey pox epidemic in the areas from which these strains were isolated and neither was the disease transmitted to potentially susceptible persons. How they get into the human population or smallpox virus is transformed to monkey pox-like virus is not known for certain. Further complications are introduced by the isolation of smallpox-like strains from normal monkey kidney, chimpanzee and gerbil. These strains when subjected to the available tests, behave like v. major in most respects. These observations raise the question whether this is a true smallpox virus and whether there is after all an animal reservoir of smallpox. Again, the lack of definite criteria prevent an unequivocal answer at the present moment. This stresses the need to develop more marker characters. Regarding the question of an animal reservoir, it seems from the past literature and history that there is no evidence of an animal carrier. It might be said that proper identification of smallpox virus in normal animals could be missed. If the existence of animal reservoirs can be proved then this will alter the smallpox eradication campaign programme throughout the world.

Another possibility that might be considered is that monkey pox is transformed to a new strain having v. major like properties in a normal monkey. Changes in the properties of a virus can occur when the virus is passed in animals. Similarly, variants of monkey pox with virulence in man may arise by this mechanism. No conclusions can be drawn with regard to the validity of these suggestions at

present. Resolution of these problems will depend on finding more biological marker characters which give different results for variola and monkey pox viruses.

To conclude, the following points are observed :

(1) Three new marker characters in tissue culture are described by which v. major and v. minor can be distinguished.

(2) The African strains of variola studied by the tests available show a possibility of at least one intermediate group, perhaps more.

(3) Some strains from humans with smallpox-like illness behave like monkey pox and some strains isolated from normal monkeys behave like v. major. This suggestion of possible transformation between these two viruses is in conflict with both epidemiological evidence and with the results of laboratory experiments designed to produce such transformation. It does, however, demand vigilance until these questions can be more adequately resolved.

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