STUDIES ON VARIOLA VIRUS

by

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ABSTRACT

The broad aspect of the growth of variola virus in human cells has been considered from a biological point of view.

A general discussion and review of the literature on the subject of smallpox and the forms in which the disease exists today summarises the background against which this present study was undertaken.

The experimental work embodies a comparison of the behaviour of variola major and variola minor strains in human cells, bearing in mind the possibility of finding new markers for the biological characterisation of variola virus strains.

The comparison was made by investigating the growth patterns of the International Reference Strains of variola major (Harvey) and minor (Butler).

The progress of cytopathic effect and production of infectious virus, complement-fixing antigen, diffusible antigen and haemagglutinin, and the development of virus DNA and antigen in situ in cells infected by the two variola strains were compared.

Particular attention was paid to the effect of incubation temperatures ranging from optimal to non-permissive for virus growth (35° - 41° C) upon the synthesis of virus products.

That the synthetic functions of Butler virus were characteristically more sensitive to supraoptimal incubation temperatures than were those of Harvey virus, agreed with the finding that 35° C was the optimal

temperature for Butler virus growth and 37° C for Harvey virus.

The production of haemagglutinin by variola virus was studied, and a differentiation of major and minor strains, based on haemagglutinin production in human cells at 37° - 39.5° C, attempted.

An enquiry into the role of several biological compounds in the growth of variola virus suggested that the metabolism of bicarbonate, arginine, and histone was linked here and played an important part in promoting variola infection in vitro.

The relevance of the results reported in this thesis to the diseases of smallpox and alastrim in man are discussed.

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ABBREVIATIONS

The following abbreviations have been used in the text:

CAM - chorioallantoic membrane of the chick embryo
CPE - cytopathic effect
HA - haemagglutinin
HA^n - haemagglutination
HAD - haemadsorption
QH - quantitative haemadsorption
RBC - red blood cells
RNA - ribonucleic acid
DNA - deoxyribonucleic acid
RNAase - ribonuclease
mRNA - messenger ribonucleic acid
HCO_3^- - bicarbonate
$[HCO_3^-]$ - bicarbonate concentration
CF - complement-fixing
FUdR - 5-fluorodeoxyuridine
BUdR - 5-bromodeoxyuridine
AO - acridine orange
FA - Fluorescent antibody
LD_{50} - Lethal $dose_{50}$, or the infectious dose necessary to cause death in 50 % of a group of test animals.
VC - variola-cowpox (hybrids) as in VC-hybrids
AR - alastrim-rabbitpox (hybrids) as in AR-hybrids

H37, H38.5, H39.5, B37, B38.5, B39.5 - Harvey or Butler infected human skin/muscle fibroblast cultures incubated at 37°, 38.5°, and 39.5° C and concentrated by drying from the frozen state.

CP - cotton-plugged cultures
SC - screw-capped cultures
CP/RB - cotton plugged cultures stoppered with rubber bungs (as controls)

Variola strains have been refered to by the strain name only (e.g., 'Harvey', 'Butler','Njotho'),and the group to which the strain belongs (major, minor, or African) were specified only when essential for purposes of reference.

CHAPTER I

Historical Introduction

The pan-eradication of smallpox has become a feasibility. The advent of modern techniques for handling vaccines has made it possible to render whole communities immune to the disease by the use, on a large scale, of the century-old method of vaccination. Freeze-drying has facilitated the preservation and storage of potent vaccine in tropical climates; the jet-gun has proved to be an efficient and acceptable method of dispensation.

With the means at their disposal, all that is required by the underdeveloped countries involved is the money and the organisation to make an anti-smallpox programme a reality.

The benevolence of the United States of America in granting foreign aid has made some states of West Africa fortunate in this respect.

Until all that is hoped for from vaccination campaigns has been achieved, clinical smallpox remains a problem.

In the overpopulated regions of Asia, endemic smallpox claims thousands of lives each year, maiming and disfiguring those who survive their infection; care and treatment of those afflicted is an overwhelming responsibility.

Jet aeroplane travel has made the accidental introduction of smallpox into non-endemic areas of the world a constant threat in spite of port-of-entry restrictions. Medical practioners must therefore be alert for the rare case which may occur. The difficulty lies, not in the diagnosis of the classical rash, but in recognising the significance of atypical symptoms such as dusky skin and collapse as seen in early fulminating cases of the disease, and in noticing the few small local lesions of mild and modified forms of smallpox easily missed in a less than thorough examination.

The epidemiology of smallpox has presented an interesting fact. Although the disease has now spread from its original foci to every part of the world, it has done so in two distinct forms, variola major and variola minor.

Variola major produces the more severe disease in man. Variolous patients are profoundly prostrated, while those with variola minor, or alastrim, are often ambulant and recover rapidly. The clinical picture is reflected in the corresponding mortality rates, 20-30 % and as much as 40-50 % in the unvaccinated in the case of smallpox, and less than 1 % in the case of alastrim.

The identity of both varieties has been retained. Extensive and rapid passage of variola minor through susceptible populations has not enhanced its virulence.

Ribas (1910) described the introduction of alastrim into Brazil from Africa via Bahia. Although the disease became very prevalent within a short time, it raised no alarm since it remained benign in character and the mortality rate, even among cases nursed under the most adverse conditions, was very low.

Ribas compared Brazilian alastrim with true smallpox and discussed the salient differences from the clinical aspect. Jorge (1924) summarised the subsequent spread of alastrim as it was imported from country to country throughout the world. Marsden (1948) reported an analysis of his clinical findings based on 13,686 confirmed cases occurring in London between 1928 and 1934.

Since many of the pressing difficulties concerning the efficient production of a useful anti-smallpox vaccine have been solved, one of the remaining scientific problems is to determine those differences between variola major and variola minor which are responsible for the diversity in the severity of the clinical disease in man.

Laboratory studies on smallpox and alastrim strains have revealed several important differences in the virus-host interactions. The work reported in this thesis was conducted in pursuit of others.

Torres and Teixeira (1935) first described the propagation of alastrim virus on the CAM of the fertile hen's egg, and Downie and Dumbell (1947) fully characterised the growth of variola major in

the chick embryo. The formation of pocks on the CAM has since proved to be an invaluable tool in the isolation and study of variola viruses and in the investigation of smallpox outbreaks.

Dinger (1956) compared virus strains isolated from an outbreak of 42 cases of alastrim in the Hague (de Jong, 1956) and from alastrim cases occurring outside this epidemic with strains of classical variola major and reported the first laboratory difference between smallpox and alastrim; variola major was more virulent for the chick embryo than variola minor. This fact was made apparent by the observation that, after inoculation into the 10 day old chick embryo, variola major persisted and could be recovered 6 days later by which time comparable inocula of variola minor had been eliminated.

The use of virulence for the chick embryo in the differentiation of variola major and alastrim was pursued further by Helbert (1957). He compared 5 strains of variola major with 5 strains of variola minor and showed that the approximate ID_{50} for minor strains was consistently at least 10 times greater than that for major strains. The 24-hour recovery of virus from livers of infected embryos was as much as several hundred-fold greater in the case of variola major than for variola minor. Helbert also remarked on histological differences seen in infected livers which suggested that variola major infections of the embryo also more seriously impaired the defence mechanisms of the host.

As a test, Helbert's method of determination of progressive mortality of chick embryos and recovery of virus from liver was laborious when used in the routine differentiation of variola strains. Nizamuddin and Dumbell (1961) devised a simpler but reliable technique based on the fact that the growth of variola major strains in the chick embryo is less susceptible to temperatures of incubation above 35° C than is variola minor [Figs. 1 and 2]. An inoculum of variola major expected to give 200 pocks at 35° C on the CAM produced about 100 smaller pocks in eggs at 38.5° C. An inoculum of variola minor giving 200 pocks at 35° C was completely suppressed in eggs at 38.5° C.

Furthermore, Dumbell, Bedson and Rossier (1961) showed that not only pock production, but also mortality rate and persistence of virus in the chick embryo were temperature dependent.

Bedson and Dumbell (1961) determined the temperature dependence of pock production for other viruses of the variola-vaccinia pox virus subgroup and found that their ranking order for ceiling temperature (that temperature above which the growth of the virus and development of virus-specific lesions is arrested) was the same as for chick mortality. Rabbitpox was established as the most virulent for the chick embryo and variola virus as the least virulent, variola minor being significantly less virulent than variola major.

This positive correlation between virulence and ceiling temperature in a single host was exciting evidence in support of a hypothesis propounded by Lwoff (1959) that the ability of a virus to

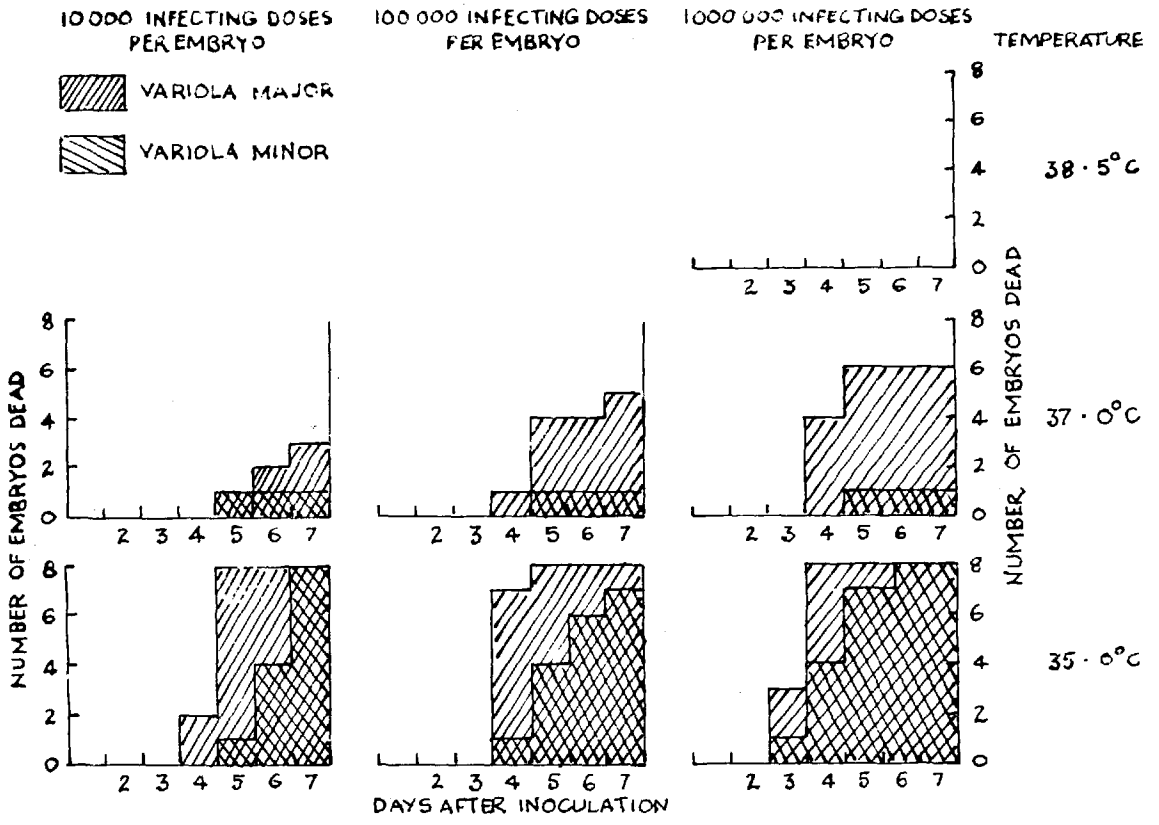


FIG. 1. CUMULATIVE DEATHS OF CHICK EMBRYOS AT DIFFERENT TEMPERATURES AFTER INOCULATION OF VARIOLA MAJOR AND VARIOLA MINOR VIRUSES ON THE CHORIO-ALLANTOIS

(DUMBELL, BEDSON, & ROSSIER, 1961.)

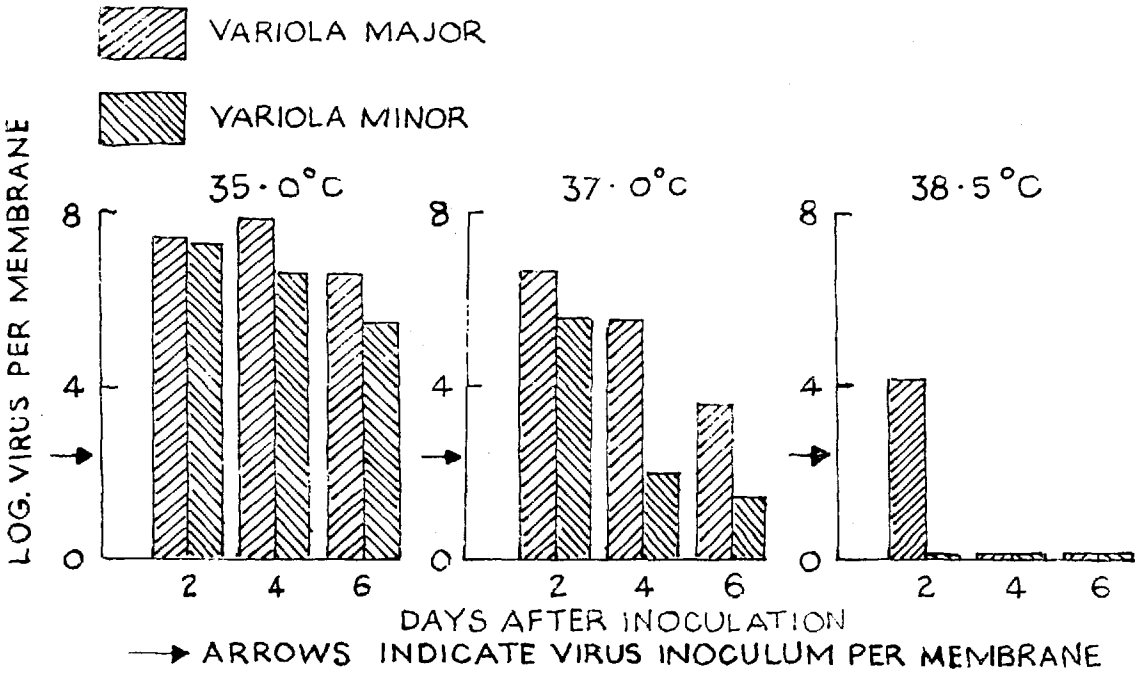


FIG. 2. VIRUS RECOVERED FROM CHICK CHORIO-ALLANTOIS OF EGGS INCUBATED AT DIFFERENT TEMPERATURES AFTER INOCULATION WITH VARIOLA MAJOR AND VARIOLA MINOR VIRUSES

grow at high physiological temperatures would be a favourable virulence factor.

However, although the chick embryo had been used as a common host for the study of the variola-vaccinia group of viruses, and in this host the order of ceiling temperature was correlated with the order of virulence, there was sufficient evidence to indicate that the order of ceiling temperature as determined in this host did not reflect the order of virulence of these viruses in other hosts such as man or the rabbit.

An attempt to evaluate other factors pertaining to virulence led Bedson and Dumbell (1964a,b) to prepare hybrids of variola major and cowpox (VC hybrids) and of alastrim and rabbitpox (AR hybrids). An analysis of 7 markers for AR hybrids and 8 markers for VC hybrids showed that all markers, which had been reasonably considered as positive virulence factors, segregated independently in pair-wise crosses. Virulence for the rabbit and for the mouse were found to be independent in AR hybrids. Neither were the hybrids correlated in their chick mortality to their ceiling temperature in this series. Bedson and Dumbell concluded that "... correlations between single characters and virulence are always likely to be fortuitous, and that one should not expect to find strict correlations of this kind in attempts to determine the characters which underlie virulence".

Since information concerning the comparative virulence and growth characteristics of viruses as determined in the chick embryo cannot be

validly extrapolated to predict the behaviour of strains in human tissues, a more complete understanding of the forces influencing the outcome of variola infection in man might be obtained from a detailed investigation of the behaviour of variola major and minor in cell types derived from the human host.

Gurvich and Marennikova (1964) compared the development of CPE of variola major and variola minor strains in human foetal skin/muscle cells, monkey kidney cells and chick embryo cells. Their results showed that temperatures above 37.5° C and 38° C prevented the onset of CPE in variola minor- and variola major-infected chick embryo fibroblast monolayers respectively. In cultures overlaid with agar, plaque-formation by variola major was inhibited above 37.5° C. However, egg preparations of both strains of variola virus caused a CPE in human embryo and monkey kidney cells at temperatures up to 39° C. The Butler strain of alastrim was an exception and failed to cause a CPE in either cell-type at this temperature. Adaption of strains by at least three passages in human embryo cells raised the ceiling temperature for CPE development 1° C.

Gurvich and Marennikova checked the specificity of CPE in their systems by haemadsorption and found "clear cut" haemadsorption accompanying all CPE. It was noted that both types of variola virus persisted in a potentially active state in cultures at increased temperatures (presumably above the determined ceiling temperatures of the virus-cell system in use) and was capable of inducing a

normal CPE on return to the "usual" temperature.

The results presented by Gurvich and Marennikova were qualitative and somewhat difficult to evaluate since no attempt was made to determine quantitatively the relative abilities of their variola major and minor strains to grow and produce a net increase in infectious virus at the temperatures tested in any of the three cell-types used. It would seem worthwhile to establish the pattern of growth of variola virus in human skin/muscle cultures.

Since variola major and variola minor are very closely related viruses (Helbert, 1957), it was considered in this case that the greater sensitivity to temperature of the growth cycle in the chick embryo might be more than fortuitously related to its lesser virulence in the human host. The ceiling temperature of alastrim in the chick embryo is 37.5° C, a temperature easily exceeded even in a mild fever, whereas that of variola major is 38.5° C.

Some evidence for an inter-relation between variola major and variola minor might be claimed from epidemiological observations. Variola minor was first reported in South Africa as 'Amaas' or 'Kaffir-pox' (de Korte, 1904). Ribas (1910) noted the arrival of alastrim in inland regions of Brazil by way of agricultural labourers travelling overland from the port of Bahia which was, at that time, receiving trading shipping directly from Africa.

It is possible that African conditions have been conducive to the evolution of many variants of variola virus, and the continued ambulance of members of nomadic tribes only slightly sick with the milder forms has encouraged the spread of variants of low, fixed virulence. One or more of these variants may have spread to the rest of the world as alastrim.

Scientific support for such an argument comes from the work of Bedson, Dumbell, and Thomas (1963). Using the temperature test (Nizamuddin and Dumbell, 1961), they were able to define a third and "intermediate" strain of variola virus; these strains had been isolated from cases of mild smallpox in Tanganyika and were "... either close to, or like, variola major in the temperature test, and yet like alastrim in the chick virulence test". Dumbell and Bedson (unpublished results) have also observed that some variola strains from other parts of Africa (including Transvaal, Congo, Bechuanaland, Nigeria, Sudan, Ethiopia, and Kenya) are detectably different from strains of true variola major and alastrim.

Annual reports of the mortality rates from smallpox indicate clearly that it is variola major which is prevalent in India and Pakistan and that variola minor is prevalent in Brazil. Mortality rates in African countries are lower than can be expected from variola major, but higher than can be expected from variola minor. It had been previously argued that variola major and minor were both present in these countries and that the figures represented the

overall death rate. It was also suspected that the figures were unreliably recorded and therefore statistically meaningless. However, in light of the results obtained from the laboratory studies on the African strains, it seems possible that the intermediate mortality rate of about 5 % is the result of the existence in these areas of strains of intermediate virulence.

Thus, the hitherto bifurcation of variola into two clinical categories, the major and the minor, is likely to be an oversimplification of a more complex taxonomical problem.

It was hoped that a further study of variola virus in human cells might reveal new characteristics which could be used as markers for the differentiation of variola strains. Information obtained in this way might also indicate the reasons for the lower virulence and stability of variola minor strains and reveal mechanisms whereby variola virus induces the clinical features seen in smallpox infections of man.

CHAPTER II

The Growth of Variola Virus in Human Skin/Muscle Fibroblast Cultures at Different Temperatures of Incubation

In order to create a system relating to the conditions for growth encountered by an invading virus in the human subject, the growth of the Harvey strain of variola major and of the Butler strain of variola minor in human skin/muscle foetal fibroblasts was studied at various temperatures of incubation. The temperatures chosen were similar to the temperatures of the dermal and subdermal areas of the body, of the deeper organs, and of the body undergoing a pyrexial episode.

Thus, the production of intracellular virus in, and the release of mature, infective virus from, infected cells was followed at the four incubation temperatures of 35°, 37°, 38°, and 39°-39.5° C during both single- and multiple-step growth cycles of infection.

Cellular and supernatant fractions from pairs of infected cultures were harvested at regular intervals for the duration of infection and were titrated for infective virus on the chorioallantoic membrane of fertile hens' eggs. Infectivity titres were expressed as pock forming units (pfu) per ml of undiluted harvest fluids, which is also an expression in terms of pfu per tube in this case.

Materials and Methods

(see appendix)

Results

Growth curves were constructed showing the formation of intracellular virus and release of extracellular virus from the infectivity assays of the samples harvested at intervals throughout single- and multiple-cycles of infection.

The logarithm of the pfu per ml of cellular or supernatant sample was plotted against the time of harvesting in hours (single-cycles of infection) or in days (multiple-cycles of infection). [Figs. 3 and 5]

In the following discussions, the Harvey strain of variola virus will be referred to simply as 'Harvey', and the Butler strain of variola minor as 'Butler'.

Virus detectable at four hours may be either adsorbed or free but not absorbed, and it could be assumed that this virus had played no part in the subsequent infectious cycle. Since this virus persists throughout the period of each experiment, the inactivation rate for variola virus being only 0.20 logs/day at 35° C (Bedson, 1964), the numerical value of the titre of the four-hour fractions was subtracted from the numerical value of all points for each growth cycle. The resulting growth curves, showing a zero baseline, are also given in order to further clarify the kinetics of each growth cycle. [Figs. 4 and 6]

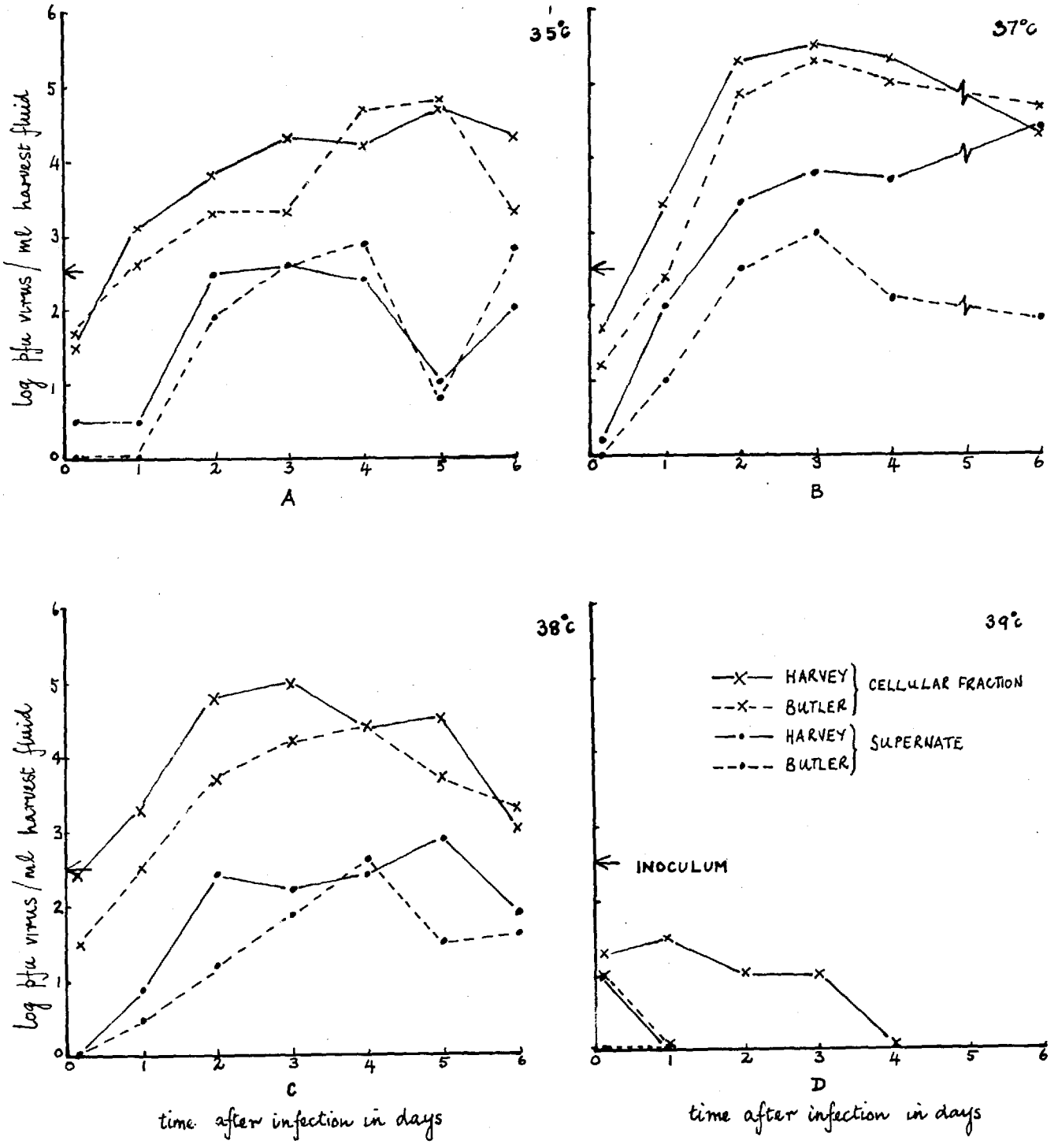


Fig. 3. Multiple-step growth curves of variola major (Harvey strain) and variola minor (Butler strain) in human skin/muscle fibroblasts incubated at 35°, 37°, 38°, and 39° C.

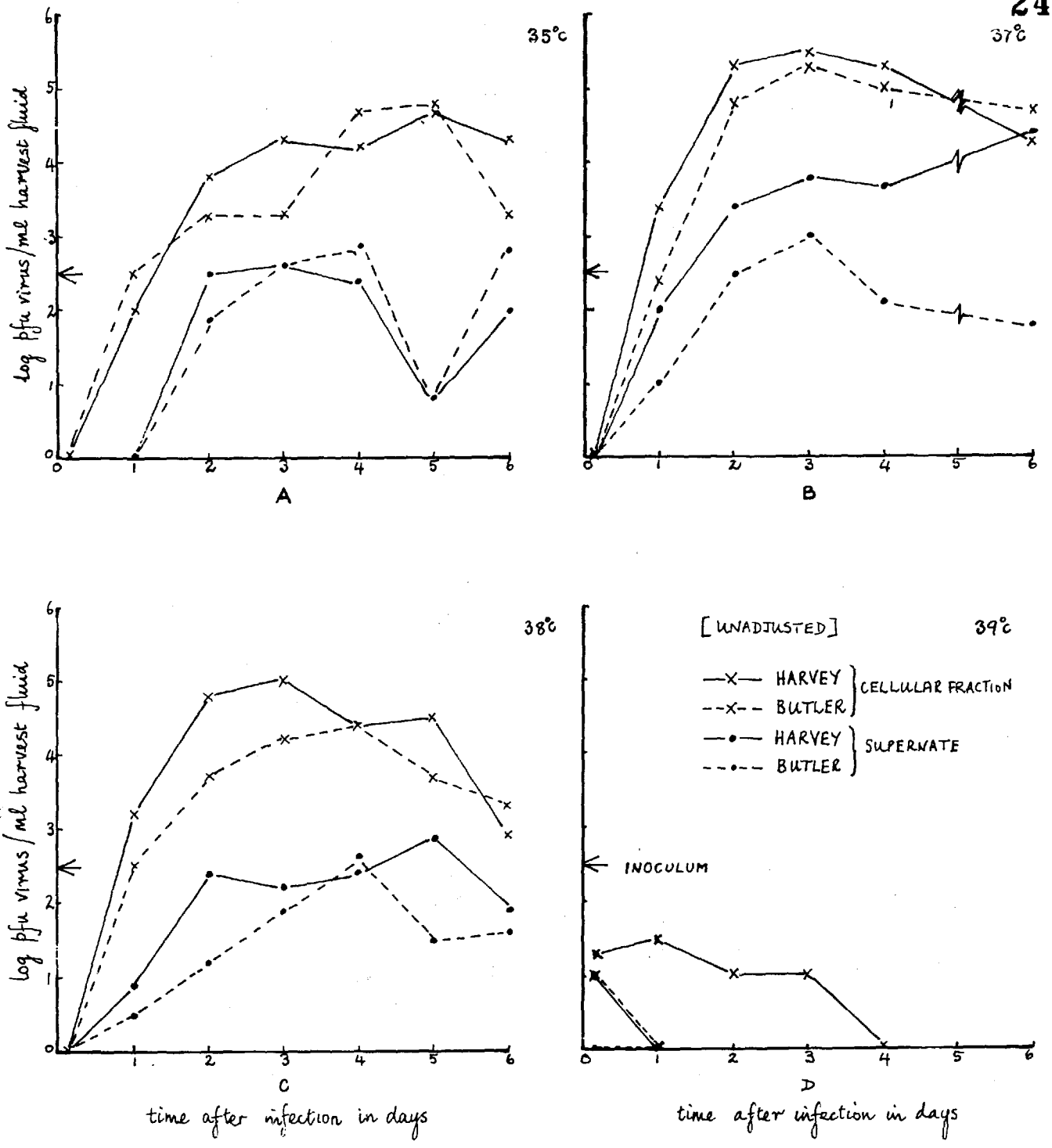


Fig. 4. Multiple-step growth curves of variola major (Harvey strain) and variola minor (Butler strain) in human skin/muscle fibroblasts incubated at 35°, 37°, 38°, and 39° adjusted to a zero baseline.

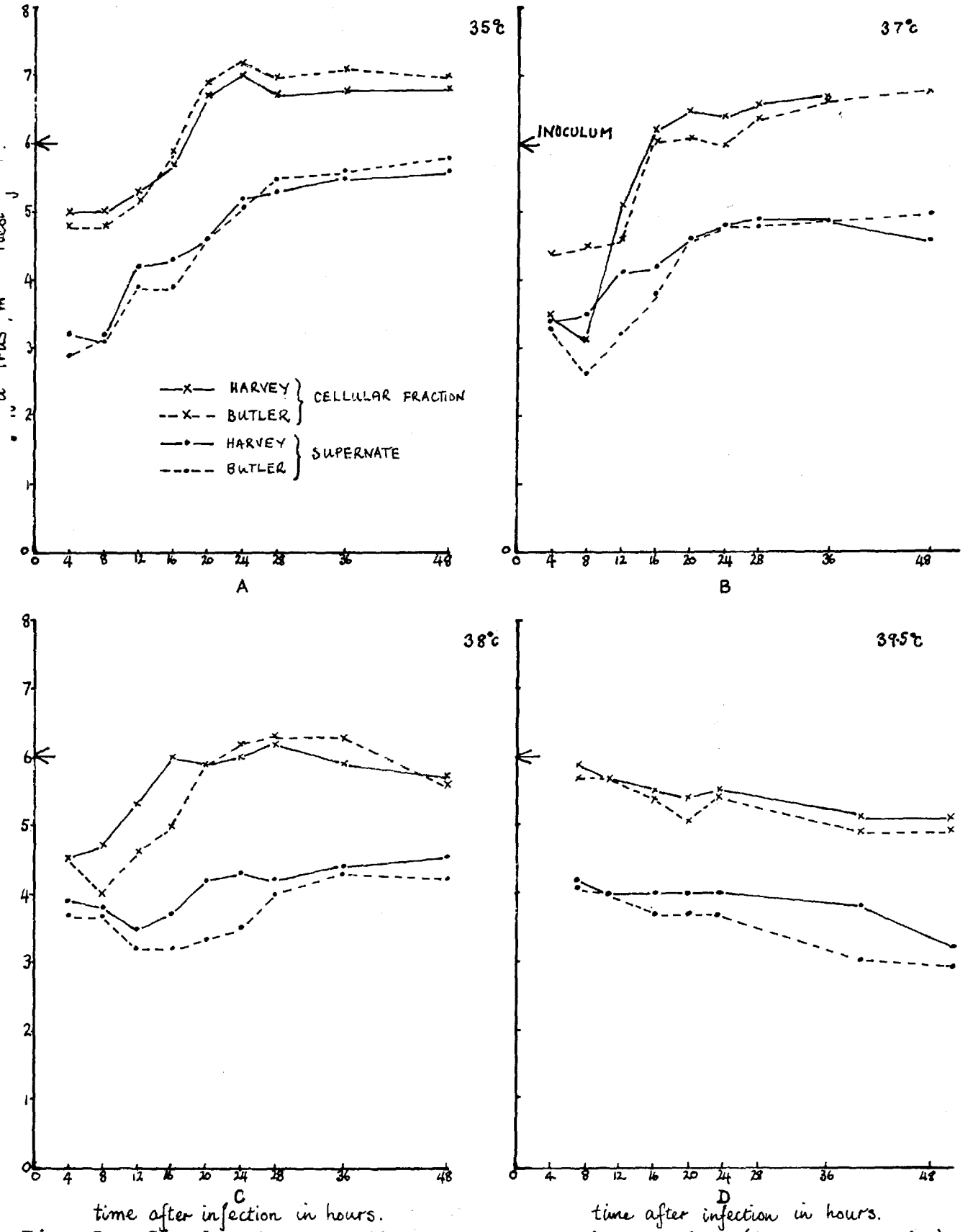


Fig. 5. Single-step growth curves of variola major (Harvey strain) and variola minor (Butler strain) in human skin/muscle fibroblasts incubated at 35°, 37°, 38°, and 39.5° C.

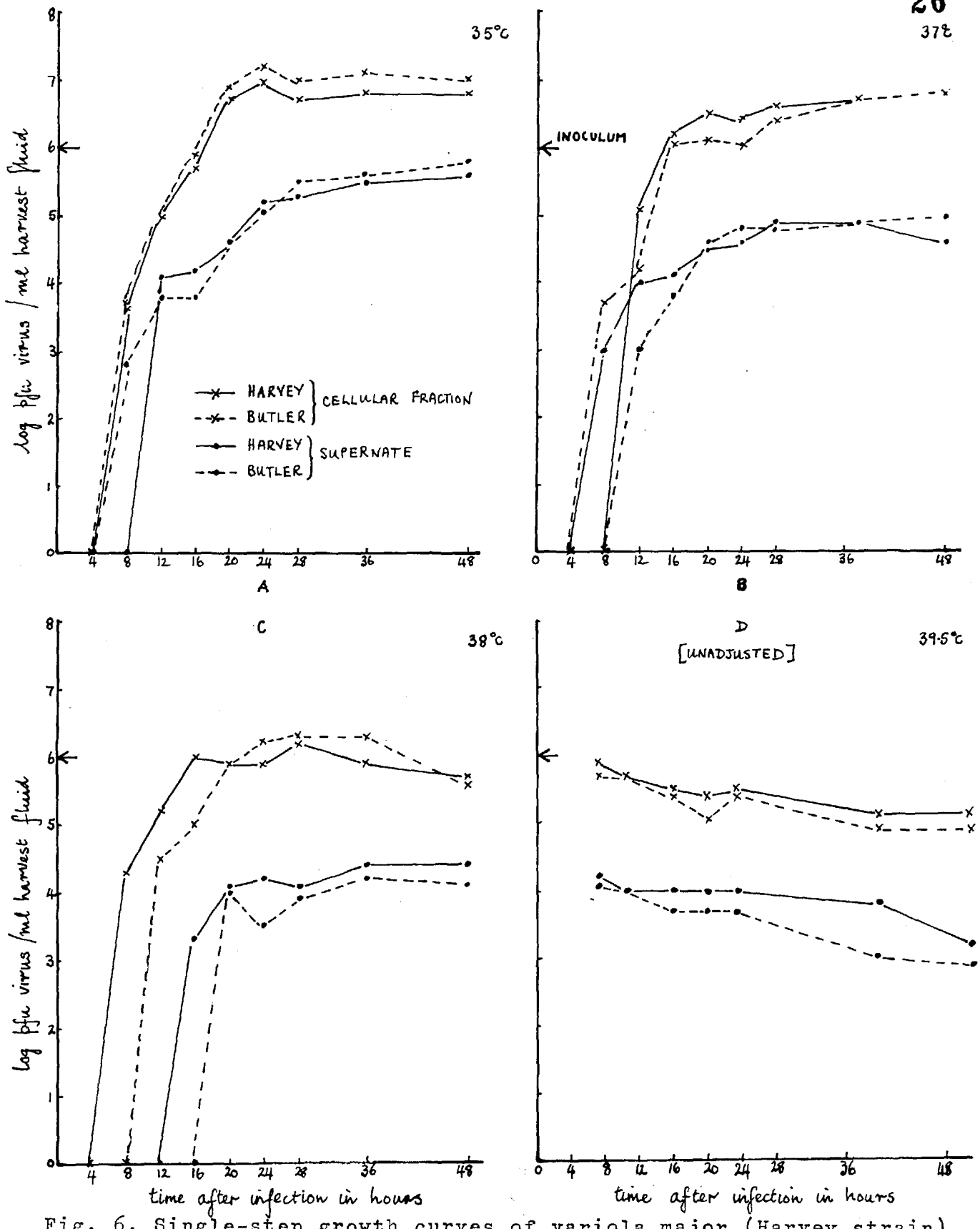


Fig. 6. Single-step growth curves of variola major (Harvey strain) and variola minor (Butler strain) in human skin/muscle fibroblasts at the stated temperatures adjusted to a zero baseline.

This procedure, however, created one anomaly. In the case of figure 6b, the infectivity titre of the extracellular virus of the Harvey strain appears to increase before an increase in the cell-associated virus was detectable. Reference to the corresponding unadjusted figure 5b shows that the lowest titre, at 8 hours, is two-fold less than the infectivity detected at 4 hours. No corresponding decrease was noted in the virus content of the supernatant fraction. Since this difference represented only a 4 % loss in infectivity in the cell-associated fraction and an 8 % increase in the infectivity of the supernatant fraction from 4 to 8 hours, the anomaly described is within experimental error and is therefore not considered to be significant.

In pointing out the salient features of a growth curve, the input dose of virus has been used as a baseline for describing and comparing increases and decreases in infectivity during the growth cycle, unless otherwise stated. Thus, an increase in infectivity of 100-fold would indicate that 10^8 pfu virus per ml had been recovered from cultures inoculated with 10^6 pfu virus per ml.

a) Multiple-step Growth Curves

At 35° C infectious virus was recoverable from the cellular fraction by the first day of infection and showed a steady increase until the third day. Since the rate of production of virus was greater for Harvey than for Butler, a net increase of 100-fold for

the Harvey strain and only 10-fold for the Butler strain was found at this time.

Virus progeny released into the culture medium was not detectable until the second day of infection. The titre of extracellular virus of both strains rose between one and two log units behind that of the corresponding cell-associated virus titre.

By the fifth day, a marked reduction in extracellular virus had occurred. This loss may have been due to readsorption and eclipse of progeny virus in a further cycle of infection; it was accompanied by a second increase in the level of intracellular virus. By the sixth day, the level of free virus in the supernatants had returned to approximately that recoverable at three days after infection.

A loss of cell-associated virus, particularly in the case of the Butler strain, was noted after the fifth day.

The maximum yield of infectious virus was recoverable on the fifth day and represented a total increase in infectivity of 100-fold for both strains of variola virus. The net increase in infectivity at the end of the multiple-step growth cycle (sixth day) was 60-fold for Harvey and 8-fold for Butler at 35° C. At this time, the infectivity recoverable from Harvey-infected cultures was almost totally cell-associated (99.5 %), whereas 25 % of the total virus formed in the Butler-infected cultures was found to be extracellular.

Both the Harvey and Butler strains show the maximum rate of virus production, maximum peak yield, and final net yield of infectious virus at 37° C. The maximum rates of release of virus progeny from cells infected with both strains were also attained at this temperature.

Both strains showed a sharp increase in intracellular virus by the first day of infection. The maximum level of intracellular virus occurred between the second and third day of infection and was 10-fold for Harvey and 100-fold for Butler greater than that attained in the same time at 35° C. Thus, the peak titre for both strains represented an increase of 1000-fold over the level of virus in the inoculum.

Extracellular virus was recoverable on the first day of infection at 37° C as compared with the second day at 35° C. Release of virus from infected cells was more efficient in the case of Harvey than that of the Butler strain at this temperature. On the first, second, and third day of infection, the free virus in the supernate of Harvey-infected cultures exceeded that of Butler-infected cultures by approximately one log unit. This difference was increased by an additional log unit by the fourth day due to a decline in the titre of extracellular virus of the Butler strain. This decline may have been due to a reabsorption of virus to infected cells.

By the sixth day, there was a very significant difference between the yield of free infectious virus in the two strains; 50 % of the total virus formed had been released from Harvey-infected cells, whereas 99.9 % of the total virus formed in the Butler-infected

cultures was still cell-associated. Thus, there was a 500-fold difference in the free virus yield of Harvey and Butler, although the net increase in total virus was the same (160-fold) for both strains.

At a temperature of incubation of 38° C there was a suppression of the rates of virus production and release, and in the infectivity yields of both viruses, but the variola minor strain was suppressed to a somewhat greater extent than was the variola major strain.

The titre of intracellular virus in Butler-infected cultures was 1/10 that in Harvey-infected cultures on the third day of infection when maximum titres had been obtained. The net increase of infectivity of Harvey cultures was 300-fold and that in Butler cultures 80-fold at this time. Thereafter, the virus detectable in the cellular fractions began to decline, and by the sixth day the remaining virus levels represented only a 5-fold increase in infectivity for both strains.

As at 37° C, extracellular virus was detectable by 24 hours after infection. It might have been expected that the difference in efficiency of the release of mature virus from cells noted at 37° C would be emphasized at a temperature 1° C higher. This was not the case. Although the initial rate of release of mature virus of the Harvey strain was somewhat greater than that of the Butler strain, the yield of free virus from Harvey-infected cells was suppressed at 38° C to much the same level as that for the Butler strain; 8 % and

2 % respectively of the total virus formed was released from the infected cells.

A temperature of incubation of 39° C was effective in inhibiting the growth and multiplication of both variola major and variola minor in human skin/muscle fibroblasts. No net increase in virus was detected at this temperature.

Infective virus of the Harvey strain persisted in association with the cellular fraction of infected cultures for three days after infection but was undetectable by the fourth day. All infective virus of the Butler strain was lost from infected cultures during the first 24 hours.

b) Single-step Growth Curves

The notable feature of the single-step growth curves of variola major and variola minor at 35° C was that there was no significant difference in the kinetics of growth of the two strains.

The eclipse phase lasted for approximately 8 hours, and by 12 hours the first increase in intracellular virus was detectable. After a 4 hour delay, there was a corresponding rise in the infectivity of the supernatant fraction. The virus content of the supernate was between 1/10 and 1/100 that of the corresponding cellular fraction throughout the growth cycle.

The maximum level of intracellular virus was detected at 24 hours and represented approximately a 10-fold increase over the virus content of the inoculum for both strains. This level of virus had been maintained at 48 hours, when 90 % of the total virus formed by Harvey and Butler remained cell-associated.

At 37° C the rate of virus production was slightly increased and the stationary phase began approximately 4 hours earlier than at 35° C for both Harvey and Butler. The yield of intracellular virus at this time was somewhat lower than at 35° C, but a small increase in intracellular virus during the second day of infection resulted in a total virus yield not different from that at 35° C.

After an initial lag in release of virus from Butler-infected cells, by 24 hours the yield of virus released into the supernatant was the same for both strains but was about 1/10 the yield attained during the stationary phase at 35° C. At 37° C approximately 99 % of the total virus produced during the growth cycle of both strains remained cell-associated.

Raising the temperature of incubation from 37° to 38° C resulted in some reduction of the virus yield from the infected cells.

The initial formation and release of mature progeny virus proceeded more slowly than at 37° C in Butler-infected cultures. The stationary phase of the intracellular virus was reached at 24 to 48 hours compared with 16 hours in Harvey-infected cultures; in the fluid

phase after 28 hours as compared with 20 hours with the Harvey strain. However, by 28 hours the virus content, both in the cellular fractions and the supernatant, of cultures infected with variola major or variola minor was not significantly different. Of the total virus recoverable at 48 hours, less than 5 % was recoverable as extracellular virus.

At 39°.5 C no increase in infectivity of either variola major or variola minor was detected. The level of virus of both strains persisting in association with the cell fraction and in the infectious form had decreased to a level representing only 10 % of the inoculum by 48 hours.

Discussion

Both the variola major and the variola minor strains were able to multiply from small and large inocula of virus at 35°, 37°, and 38° C but not at 39°.5 C in human skin/muscle fibroblast cultures.

The outcome, at each temperature of incubation, of the single growth cycles of the two strains was the same. By the time the stationary stage had been reached, the net yields of intracellular and extracellular virus of Harvey- and Butler-infected cultures were not different and had been similarly suppressed as the temperature of incubation was raised. However, the initial rate of virus formation, and particularly the rate of release of mature virus progeny, of the Butler strain was more sensitive to suppression by the higher

temperatures of 37° and 38° C than was that of the Harvey strain.

Since the net yield of virus produced was virtually the same for both strains at any one temperature, a difference in the ability of the strains to grow in human skin/muscle fibroblasts must stem from a difference in the ability of the two strains to initiate secondary cycles of infection.

Over the longer period of time taken for multiple-cycles of infection to occur, it was found that elevated temperatures of infection had a differentiating effect, not so much on the actual production of infective virus, but rather on the release of virus progeny from infected cells. Of the four temperatures tested, 35° C was found to be optimal for the liberation of virus from the Butler-infected cells (25 % of the total virus recovered on the sixth day was extracellular), whereas 37° C was found to be optimal for the release of virus from the Harvey-infected cells (50 % of the total virus recovered at six days was extracellular). At 38° C the rate of formation of virus of the Butler strain was also reduced to a lower value than that of the Harvey strain.

Given the limited scope for growth which exists for the duration of a single infective cycle in cells of a confined monolayer, a delay in virus formation and release conferred no overall disadvantage on the growth of the Butler strain as compared with that of the Harvey strain since the same total yield could be attained in time. But, where released progeny are the initiators of secondary and subsequent

cycles of infection, such a delay in the availability of new infectious Butler virus to reinfect more cells would result in a cumulative lag in the progress of infection as compared with a more rapid spread of infection by the Harvey virus strain which is capable of being promptly released from the host cell after maturation.

The multiple-step growth curves show that it would be possible for such a difference to accrue.

The efficiency with which virus is released and circulated in vivo could be a more important factor in determining the virulence of a virus strain than the actual growth in the cell.

If virus of the alastrim strain at normal body temperature (37° C) lacked the ability to spread from cell to cell as efficiently as virus of the variola major strains through slow release of progeny virus and at a 1° higher temperature incurred the added disadvantage of a slower rate of formation of virus, then it seems reasonable to suppose the following. In the intact living organism where many systemic factors may influence the progress of an infection, an alastrim infection would be more liable to be overcome by the defense mechanisms of the host and to cause less cell and tissue damage than a variola major infection spreading vigorously.

The growth and release of Butler virus at 35° C is as efficient as that of Harvey virus at the same temperature, and the release of Butler virus is greater at 35° than at 37° C. This fact could, in part, explain the observation that, although alastrim patients are

not as distressed by their virus infection as are patients with variola major, the skin eruptions (localised lesions resulting from virus growth) can be as extensive as those of cases of variola major. The surface temperature of the body being about 35° C compared with an internal body temperature of 37° C could thus be permissive of growth of variola major both externally and internally, while alastrim is predominately only able to form lesions in the skin.

CHAPTER III

The Cytology of Human Cells in Tissue Culture Infected with Variola Virus at Different Temperatures of Incubation

3.1 The Development of the Cytopathic Effect Induced by Large and Small Doses of Variola Virus

The growth of variola virus in human skin/muscle fibroblast cultures has been described in Chapter II. Mature, infectious virus was found to be released more slowly from cells infected with the Butler strain of variola minor than from cells infected with the Harvey strain of variola major, especially at incubation temperatures between 37° and 38.5° C. It was concluded that such a delay in the release of infective virus could result in a significant difference in the efficiency with which these two strains of variola virus are able to establish vigorously spreading infections in susceptible cell cultures.

To substantiate this supposition, the progress of the cytopathic effect (CPE) induced in human skin/muscle fibroblast cultures by variola virus at different temperatures of incubation was followed. Human skin/muscle fibroblast cultures were inoculated with large doses of variola virus in order to expose the entire monolayer to virus during the absorption period, and with small doses of variola virus in order to create discrete foci of infection from which virus can spread in secondary cycles of growth. Cultures inoculated with large doses of virus were incubated at 37°, 38°, 39° and 41° C, and those inoculated

with small doses of virus at 37°, 38.5°, and 39.5° C. Infected cultures were examined at regular intervals throughout infection by phase contrast microscopy.

(Materials and Methods - See Appendix)

Results

a) CPE from Large Doses of Variola Virus

Four hours after infection with Harvey at 37° C there were no visible signs of a beginning CPE [Fig. 7a]. The fibroblast cells retained the characteristic elongated spindle shape with a central oval nucleus containing usually a single nucleolus. The cytoplasm was granular and unrefractile.

By 8 hours obvious changes in the morphology of the majority of the cells in the infected monolayer had taken place. Retraction of cytoplasmic processes was beginning and the cells were assuming a more polygonal shape and an increasing granularity of the cytoplasm. In many of the cells the number of nucleoli/nucleus had increased. Small vacuoles were noted at the periphery of several cells. Cell boundaries were difficult to distinguish at this stage, although the nuclei were undistorted and quite discrete [Fig. 7b].

By 12 hours the cells were showing extreme pleomorphism, granularity, and fusion of the cytoplasms [Fig. 7c]. Contraction of the cytoplasm was resulting in 'microlesions' in the cell sheet which was

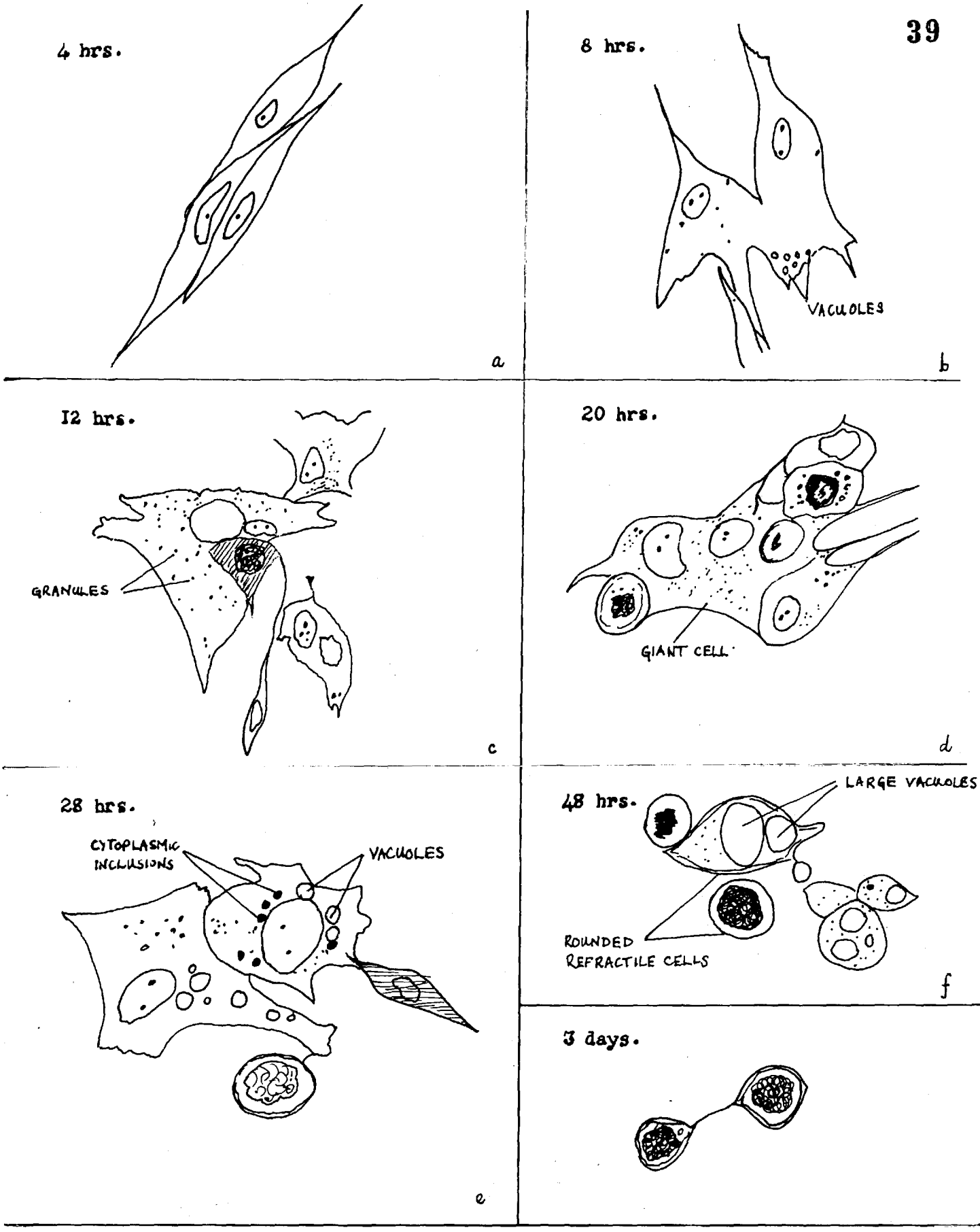


Fig. 7. Sequential stages of CPE induced by a large dose of Harvey virus in human fibroblast monolayers incubated at 37° C.

becoming increasingly irregular and ragged in appearance.

Progression of the CPE was marked by an increase in intensity of granularity and necrosis throughout the entire cell sheet [Fig. 7d]. Detail of the internal structure of the fused masses of cells was difficult to discern; but despite the extreme distortion of the cell cytoplasms, the nuclei remained relatively undamaged, although obscured by changes in the cytoplasm.

Large, dense cytoplasmic inclusions could be seen by 24 hours and by 28 hours vacuolation of the cytoplasm was often advanced [Fig. 7e].

The integrity of the cell sheets at 30 hours of infection had been lost. Many cells showed extreme vacuolation; but the majority of cells were rounded up and highly refractile, seeming to separate as cellular units from the fused mass into which they had previously merged. This was the last stage of CPE. At 48 hours most of the cells had fallen away from the glass surface, any remaining cells being completely necrotic.

Uninfected control tubes remained normal and unchanged throughout the experiment.

Butler at 37° C and Harvey at 38° C showed no obvious differences in the progress of CPE, but Butler at 38° C lagged quite considerably behind. At 28 hours the cell sheet was still intact, although the cells themselves were showing a degree of shortening and granularity. At 36 hours the cohesive forces maintaining the integrity of the cell sheet had been lost and the micolesions left by receeding cytoplasmic

processes were visible. The cells were markedly granular. However, by 48 hours little difference could be seen between Butler-infected cultures at 38° C and corresponding Harvey cultures. The cell sheet was entirely necrotic, but a significantly higher proportion of cells remained attached to the glass surface as compared with Harvey at 37° C and 38° C and with Butler at 37° C.

Subsequent observations have shown that large doses of Harvey and Butler virus are also able to induce a progressive CPE at 39° C and at 41° C. Infected cells at 41° C become rounded and refractile at an earlier time than at lower temperatures. Such a change was not seen in uninfected controls at 41° C.

b) CPE from Small Doses of Variola Virus

Where CPE was observed, infected cells in monolayers inoculated with a small dose of virus showed the same pattern of CPE-development as seen in cells of monolayers inoculated with large virus doses.

In comparison with the uniform distribution of CPE throughout the entire monolayer resulting from the simultaneous infection of all cells of a culture, small inocula set up discrete foci of infection, CPE spreading from the centre to the periphery of foci in a cell-to-cell manner. Thus, cells at the centre of foci had either fallen away from the glass (forming irregular plaques) or were rounded and necrotic. Those cells at the outer limits of foci, being at an earlier stage of infection, were losing the characteristic spindle shape to become polygonal and granular.

After 24 hours, cultures of both viruses at 37° C showed foci of shortened, granular cells and of necrosis gradually extending through the monolayer. By the eighth day, the entire cell sheet was necrotic, and many cells had left the glass surface.

At 38.5° C cultures of Harvey-infected cells showed large foci of necrosis from the first day. These extended, as at 37° C, to involve almost all the monolayer by the eighth day. However, in Butler-infected cultures, inoculated at 38.5° C, the foci of shortened, granular cells, which had developed by the first day, remained small and discrete throughout the course of the experiment and showed little degeneration into necrosis.

At 39.5° C, CPE did not develop in either Harvey-infected or Butler-infected cultures.

Discussion

It has been shown that both Harvey and Butler viruses are able to multiply and form infectious virus in cultures incubated at 35°, 37°, and 38.5° C, but not at 39°-39.5° C (Chapter II). It was interesting, therefore, to find that both viruses were able to induce a CPE at temperatures greater than that at which infectious progeny virus can be produced, and at least as high as 41° C.

Virus had been adsorbed at a constant temperature (25° C) before exposure to high temperatures of incubation, but infective virus was not the end product of the virus-cell interaction. Thus, either an

abortive or incomplete growth cycle had occurred causing a CPE, or cell damage was due to a non-specific cellular reaction to foreign material such as lysosomal activation (Allison and Mallucci, 1965).

Observations of the progress of CPE in variola-infected cultures were in support of conclusions drawn from the results of the growth study experiments described in Chapter II.

Single-step growth cycles had shown that initial delays in the release of mature virus from Butler-infected cells at higher temperatures of incubation behind that from Harvey-infected cells were overcome, and that the final virus yields of the two strains at each temperature were not significantly different. Similarly, the CPE in Butler-infected cultures at 38° C showed an initial lag but was no different from the CPE in Harvey-infected cultures at this temperature by 48 hours.

In cultures infected with small doses of virus, both Harvey and Butler were seen to have spread extensively at 37° C. At 38.5° C there was a very marked difference. Harvey-infected cultures showed necrosis which progressed at a similar rate as at 37° C, but the spread of infection of Butler virus was much retarded in comparison; foci of CPE remained confined even by the eighth day.

Thus, the slower rate at which Butler virus progeny matures and is released from the host cell does result in a much slower spread of virus from cell to cell in infected cultures.

That Harvey and Butler were unable to initiate foci of infection at 39.5° C was to be expected. As no virus production was possible at

this temperature, the CPE which would be induced in the small, but unnoticeable proportion of cells infected by the inoculum did not spread to adjacent cells.

3.2 The Application of Cytochemical Techniques in the Study of the Development of Variola Virus Antigens in Human Cells

a) Sequential Studies

Loh and Riggs (1961) followed the sequential development of virus antigens in HeLa cells multiply-infected with vaccinia virus.

Employing the technique of staining preparations of infected cells with the fluorochrome, acridine orange, they showed an early increase in cytoplasmic RNA and the appearance of centres of DNA synthesis at 4 hours after infection. A marginal fragmentation of these inclusion bodies at 8-10 hours was concommittant with the first increase in intracellular virus.

Using the fluorescent antibody technique and specific anti-'LS', anti-'NP', and anti-'HA' fluorochrome-coupled antisera, Loh and Riggs also demonstrated that 'LS' antigen (a virus surface component) was the first antigen to be detected which appeared at 4 hours followed by the 'NP' antigen (virus nucleoprotein) between 5-6 hours. Mixing of the 'NP' and 'LS' antigens occurred at 8-10 hours when fragmentation was seen by acridine orange staining and when maturation of progeny virus occurred. The 'HA' antigen (haemagglutinin) appeared at 10 hours.

Loh and Riggs concluded that the typical inclusion seen in vaccinia-infected cells was mainly 'NP' antigen.

In order to establish how far the growth cycle of variola virus proceeds at different temperatures of incubation, histochemical techniques were applied to coverslip preparations of variola-infected cells incubated at temperatures ranging from 37° C to 41° C.

A sequential study of the development of virus antigens in variola-infected cells was made employing the acridine orange (AO) staining technique for nucleic acids and the fluorescent antibody (FA) sandwich technique for the location of virus antigen. It was hoped that the cause of virus-induced CPE occurring above the permissive temperature for virus growth would also be revealed in this way.

Sites of virus antigen synthesis were identified by analogy with the work of Loh and Riggs (1961) on the vaccinia virus system.

(Materials and Methods - See Appendix)

Results: 1) Acridine Orange Staining

At 2 hours after variola infection at 37° C, the cytoplasm of cells in infected cultures showed a more intense staining than did the cytoplasm of cells in uninfected control cultures. Nuclear chromatin flecks and the nuclear membranes were more pronounced; the nucleoli were enlarged and often increased in number. Minute vacuoles were visible in the perinuclear area [Fig. 8a].

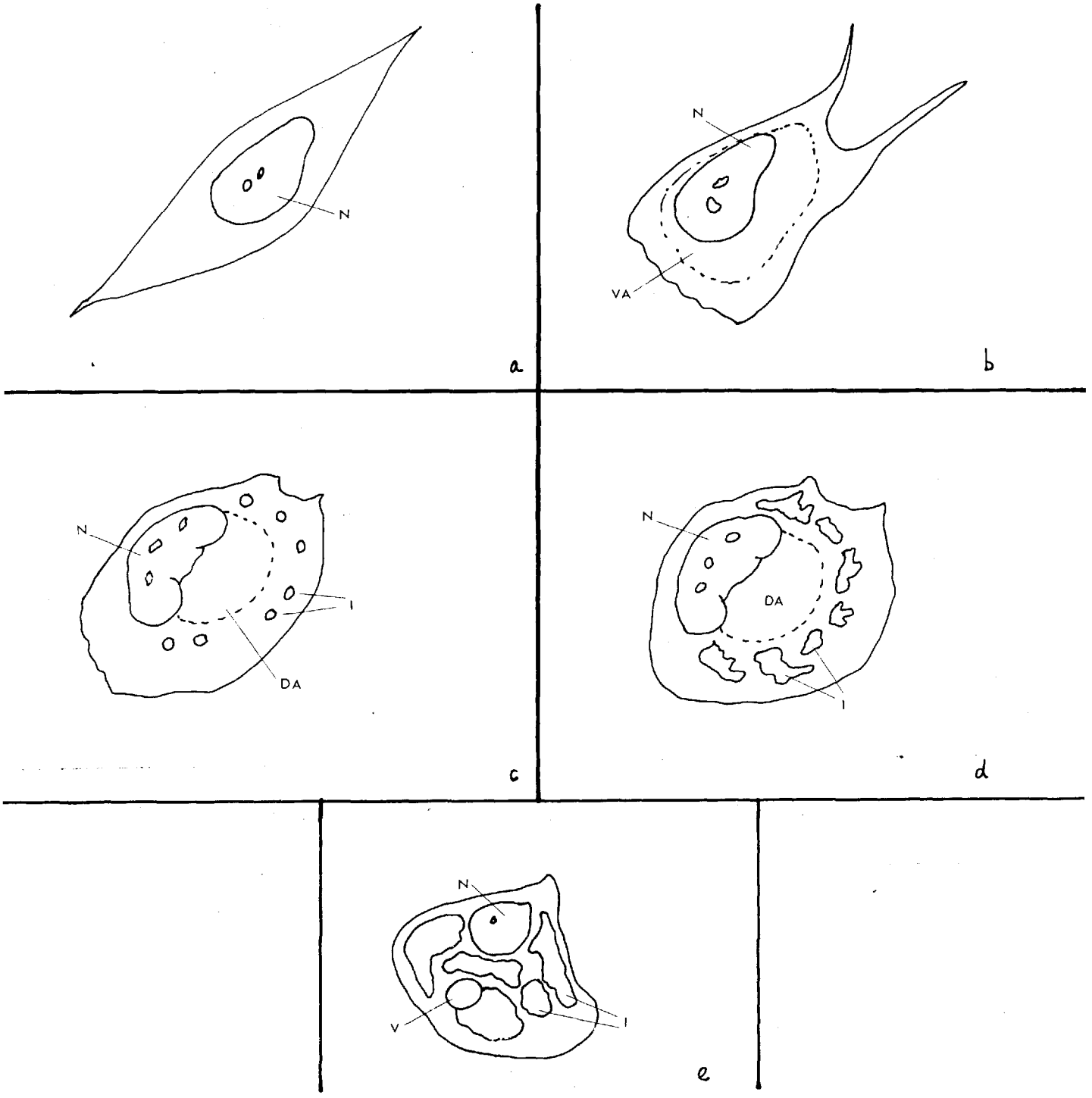


Fig. 8. Sequential development of virus DNA inclusions in a humaN fibroblast cell infected with variola virus incubated at 37° C and then stained with acridine orange. N = nucleus; I = inclusions; DA = dark area; V = vacuoles; and VA = vacuolated area.

By 6 hours perinuclear vacuolation was pronounced and cells were shorter and wider having lost the characteristic spindle shape [Fig. 8b].

DNA inclusions appeared at 7-8 hours as small spherical apple-green bodies often arranged in a hemisphere around an unstained area lateral to the nucleus [Plate 1]. This area displaced the nucleus to one side or to one end of the cell [Fig. 8c].

The inclusions increased in size as time progressed and after 10 hours a noticeable loss in the regularity of shape of the inclusions was seen. The irregularity was marked by 18 hours, when the majority of inclusions had assumed a very irregular shape [Fig. 8d]. Adjacent inclusions had merged by 24 hours.

Some intensity of staining was lost after this time and by 36 hours the cytoplasm of infected cells was riddled with diffuse yellowish-green inclusion material. Cells were rounded and had reached the final stage of necrosis [Fig. 8e].

Plate 2 illustrates the various stages of DNA inclusion development and the presence of the dark area in variola-infected human conjunctiva cells incubated at 37° C for 24 hours.

Haemadsorption, indicating the presence of the 'HA'-antigen, was first detectable at 10-12 hours after infection. It was interesting to find that haemadsorption was not directly correlated with inclusion formation since many cells showing all stages of inclusion development were seen which were negative for haemadsorption. Other cells at

Plate 1: Acridine orange-stained preparation. 800x Kodachrome II A

Cytoplasmic virus DNA inclusions in a variola-infected human skin/muscle fibroblast cell incubated at 37° C for 10 hours. The inclusions (I) are arranged around an unstained, dark area (DA) lateral to, and displacing the nucleus (N).

Plate 2: Acridine orange-stained preparation. 800x Kodachrome II A

Human conjunctiva cells infected with variola virus and incubated at 37° C for 24 hours. Five stages (I1-I5) of virus DNA inclusion development are to be seen; one cell contains an unstained, dark area (DA) displacing the nucleus (N).

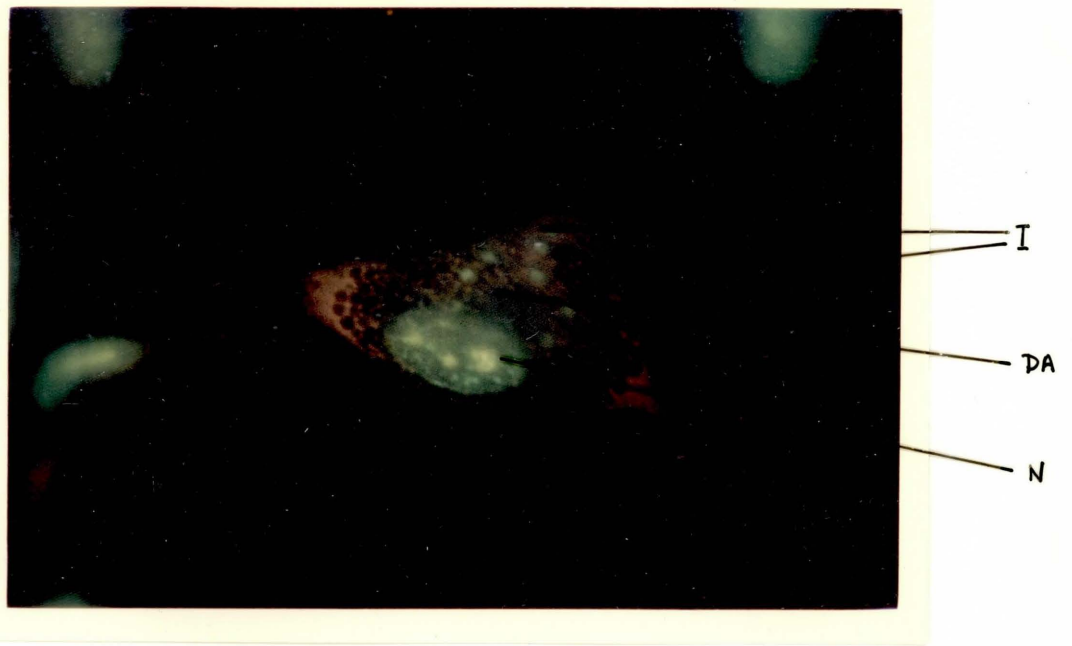


plate I

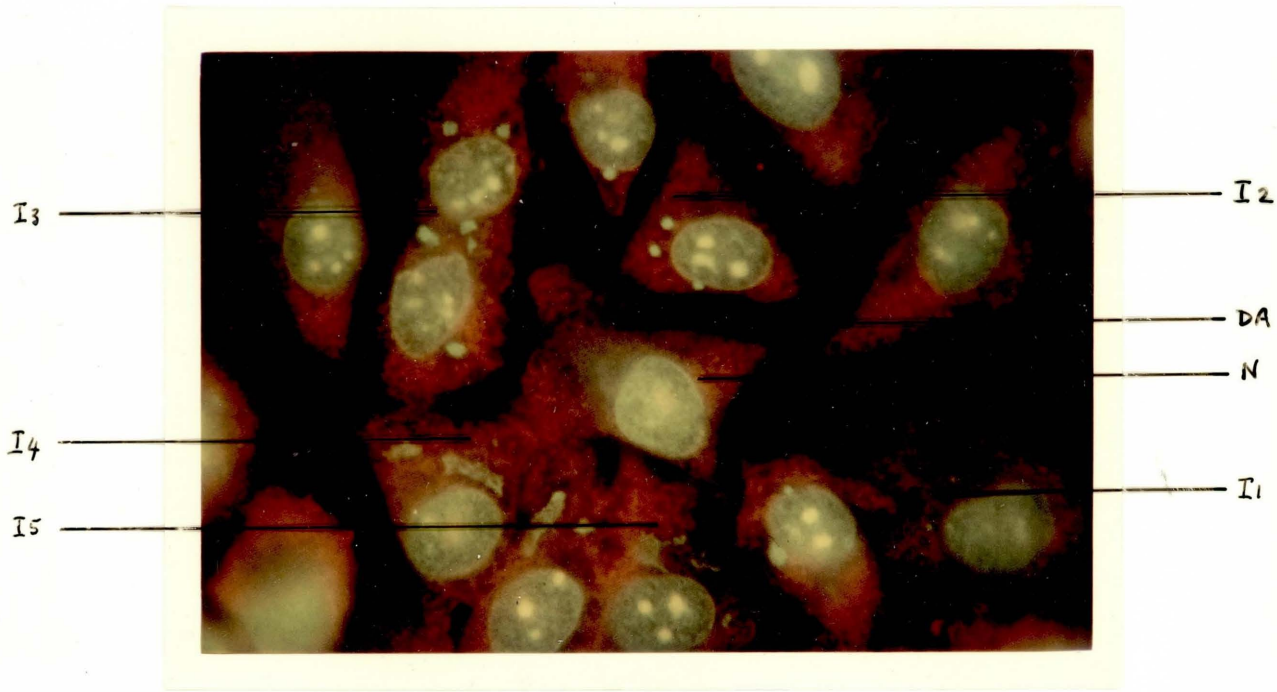


plate2

similar stages of inclusion formation were positive. The position of attachment of RBC showed no topographical relationship to the position of the DNA inclusions in the cells.

Incubation of infected cultures at 39.5° C did not completely prevent the formation of inclusions by either Harvey or Butler, although the production of infective virus did not proceed at 39° C (Chapter II).

At 8 hours when the centres of DNA synthesis had been established by both Harvey and Butler at 37° C, only about 1/10 the number of cells infected with Harvey virus contained inclusions at 39.5° C. A reduction in the multiplicity of inclusions in infected cells was noted. Cells of Butler-infected cultures at 39.5° C showed pronounced vacuolation, but less intensely stained cytoplasm than at 37° C. Only occassional cells contained DNA inclusions. When this was the case, there was usually only a single inclusion per cell.

By 24 hours about 70 % of infected cells at 37° C showed coalescing inclusions spreading through the cytoplasm. At 39.5° C in Harvey-infected cultures, less than 10 % of all infected cells contained diffuse inclusion material; most inclusions were still discrete, few in number per cell, and enlarged. The few inclusions which developed in Butler-infected cultures at 39.5° C also remained as enlarged, spherical bodies at a lower multiplicity per cell than for Harvey-infected cells at this temperature.

At temperatures ranging from 37° C to 39.5° C, the majority of cells of Harvey- and Butler-infected cultures developed a typical CPE irrespective of whether or not the cells contained DNA-synthesising foci.

Results: ii) Fluorescent Antibody Staining

AO stained preparations of variola-infected cells identified the site of virus DNA synthesis in infected cells.

It was noticed that in almost every cell one of the characteristic changes in morphology was the displacement and distortion of the nucleus by an area not stained by AO. At 37° C inclusions often formed around this area which was also present in cells at 39.5° C in which no inclusions had developed. For descriptive purposes this area will be termed the "dark area".

The dark area did not form in uninoculated controls at any of the temperatures tested and was therefore assumed to have been specifically induced at some stage in the virus growth cycle. It was of interest that this area should develop even when virus DNA synthesis appearen not to be taking place.

To determine whether the dark area was the site of virus antigen synthesis, infected coverslip monolayers were harvested and examined for the presence of virus antigen by using the fluorescent antibody sandwich technique.

Since the antivaccinial antiserum used in these experiments was prepared against whole virus, it was therefore polyspecific and reactive with all antigenic components present in infected cells. Thus, no differentiation could be made between the various virus antigens using this technique as in the work of Loh and Riggs where sera specific for 'LS', 'NP', and 'HA' were used.

Comparison of FA treated slides with duplicates stained with AO permitted some conclusions to be drawn as to the nature of various specifically fluorescent sites observed.

At 37° C the first appearance of fluorescence occurred at 6 hours as pinpoint round bodies lying around the already displaced nucleus and the site of the so-called dark area [Fig. 9a]. Comparison of this stage with a duplicate culture stained with AO identified the foci of fluorescence as being centres of DNA synthesis. The dark area was formed at this time as shown by the displacement of the nucleus and the arrangement of DNA inclusions, but was not fluorescing.

By 8 hours the inclusions had enlarged and some cells showed perinuclear fluorescence. The dark area showed slight fluorescence [Fig. 9b].

Preparations stained at 10-11 hours showed that coalescence of the DNA inclusions was beginning. Perinuclear fluorescence was more prominent in the infected cells of cultures and was spreading through the cytoplasm. The dark area was seen to contain a stellate-shaped body of increased fluorescence [Fig. 9c].

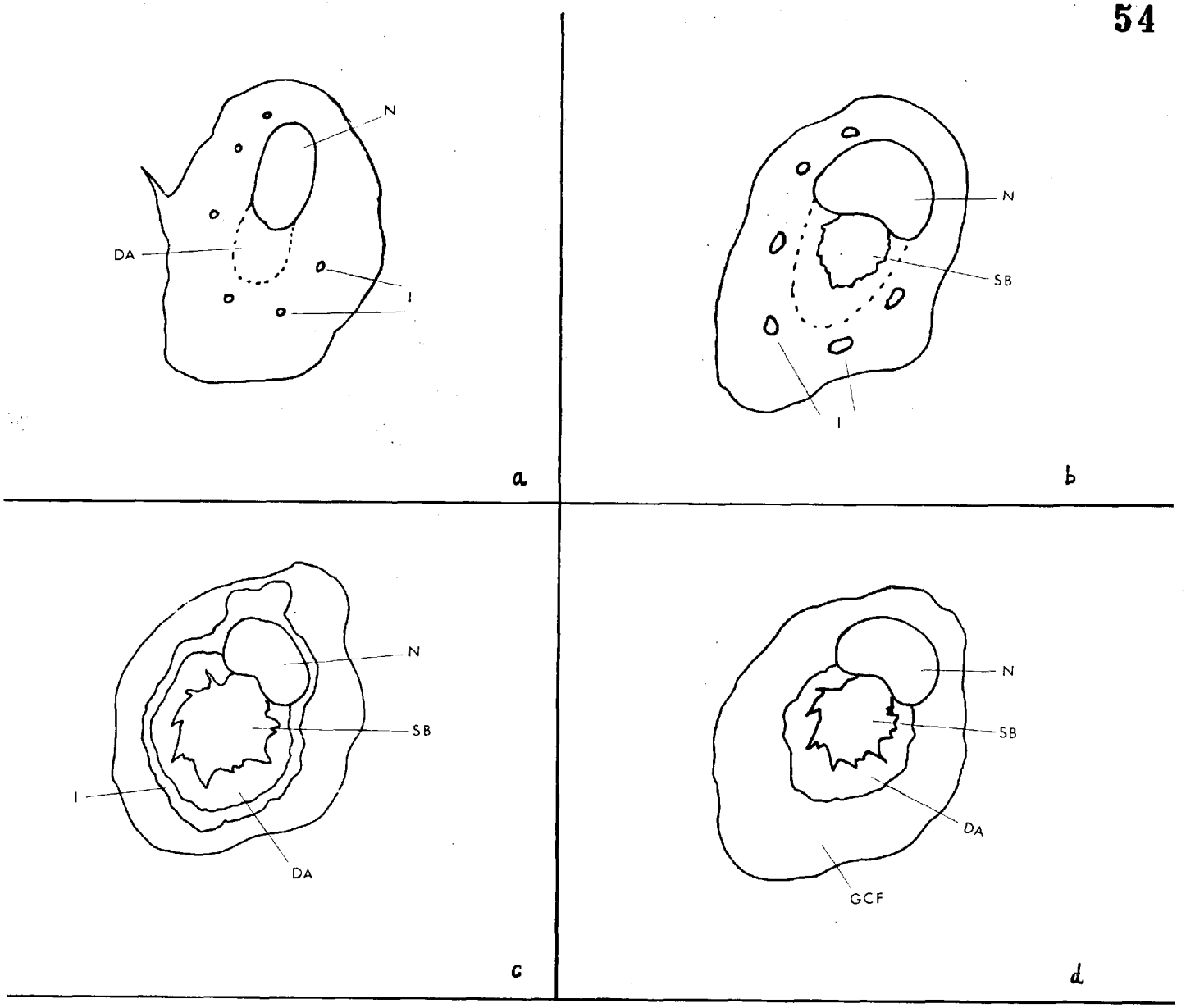


Fig. 9. Diagrammatic representation of the sequential development of sites of fluorescence in a human skin/muscle fibroblast cell infected with variola virus, incubated at 37° C, and then stained with the fluorescent antibody 'sandwich' technique.

N = nucleus; I = 'NP' inclusions; DA = dark area;
SB = stellate body; GCF = general cytoplasmic fluorescence.

Coalescence of inclusion bodies progressed and by 16 hours many cells showed bright fluorescence throughout the cytoplasm. The stellate body had enlarged and its internal structure had become finely reticular. At this and later stages, the former DNA inclusions were not distinguishable, having become dispersed throughout the cytoplasm.

As the time after infection increased, the number of cells showing total fluorescence increased. As cells became rounded and necrotic, the cytoplasm showed uniform fluorescence [Fig. 9d].

The typical fluorescence shown by variola-infected cells incubated at 37-39.5° C for 24 hours and stained by the FA sandwich technique is illustrated in Plates 3 and 4.

AO preparations of Butler-infected cultures incubated at 39.5° C showed that the majority of infected cells developed a single or very small number of DNA inclusions which remained as large, isolated round bodies in the cytoplasm and which failed to mature by coalescence.

FA preparations of such cultures showed a similar picture with respect to these inclusions. Infected cells stained at 24 hours showed that the cytoplasm had become generally fluorescent as in cultures incubated at 37° C. The 'stellate' body and the dark area also developed, but the former area appeared less conspicuous than at 37° C in the preparations examined. DNA inclusions remained as large discrete bodies rarely merging into the general fluorescence as at 37° C. Cells were also present which contained no inclusions, but the

Plates 3 and 4: Fluorescent Antibody Staining. 320x

Plate 3: Ektachrome high speed, Plate 4: Kodachrome II A

Human skin/muscle fibroblast cells infected with variola virus and incubated at 37° C for 15½ hours showing sites of virus antigen synthesis. The nucleus (N) of infected cells is displaced by an unstained area (DA) containing a 'stellate body' (SB) and is often surrounded by perinuclear fluorescence (PF). DNA inclusions have become diffuse (D),and general cytoplasmic fluorescence (GCF) is developing.

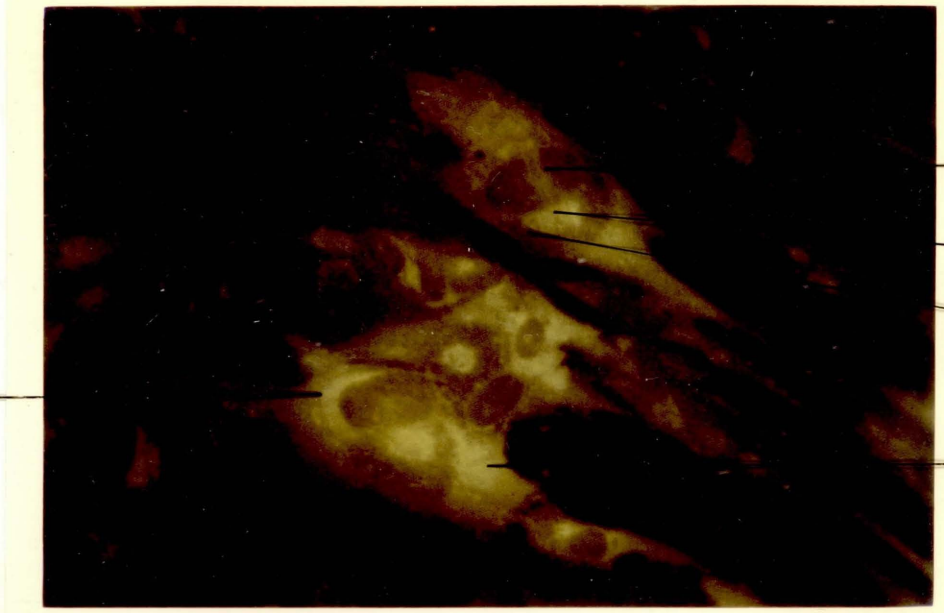


plate 3

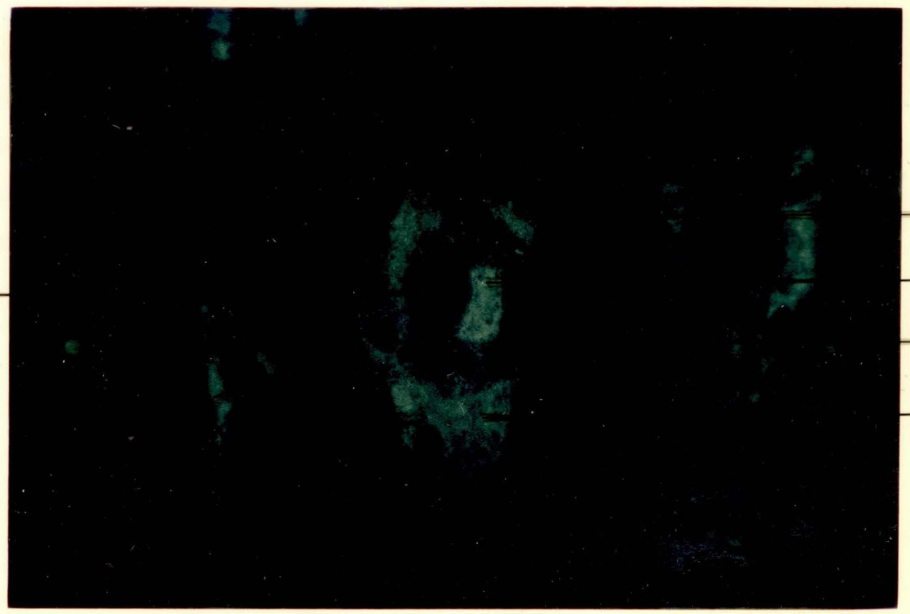


plate 4

dark area, stellate body, and general cytoplasmic fluorescence had developed. These observations are depicted in Plates 5 and 6.

At 41-42° C, as in cultures at all temperatures tested, a proportion of cells did not show fluorescence. In those cells which did, typical perinuclear, stellate body, and general cytoplasmic fluorescence was observed. It was noted at 39.5° C that the stellate body was less conspicuous than at 37° C; at 41-42° C this area was probably less developed than at 39.5° C.

48-hour Harvey-infected fibroblast cultures incubated at 41-42° C showed a marked rounding and refractility in a large proportion of the cells. This had not been apparent at 39.5° C and was not seen in uninoculated controls incubated at 41-42° C. FA staining showed that all such cells exhibited uniform fluorescence and therefore contained evenly distributed virus antigen [see Plate 7]. It is possible that infected cells were predisposed to rounding when exposed to such high incubation temperatures.

No inclusions identifiable as foci of 'NP' synthesis were noted.

Discussion

The work of Loh and Riggs showed that 'LS'-antigen was the first to be detected in the vaccinia-HeLa cell system. In the variola-human skin/muscle fibroblast system reported in this thesis, the

Plates 5 and 6: Fluorescent antibody staining. 320x Ektachrome

Human skin/muscle fibroblasts infected with the Butler strain of variola minor and incubated at 39.5° C for 28 hours showing sites of virus antigen synthesis. Virus inclusions (NP) have remained as discrete, but enlarged bodies in the cytoplasm of infected cells. The 'stellate body' (SB) within an unstained area (DA) has developed in some cells; nuclei (N) have become displaced, and general cytoplasmic fluorescence (GCF) has developed.

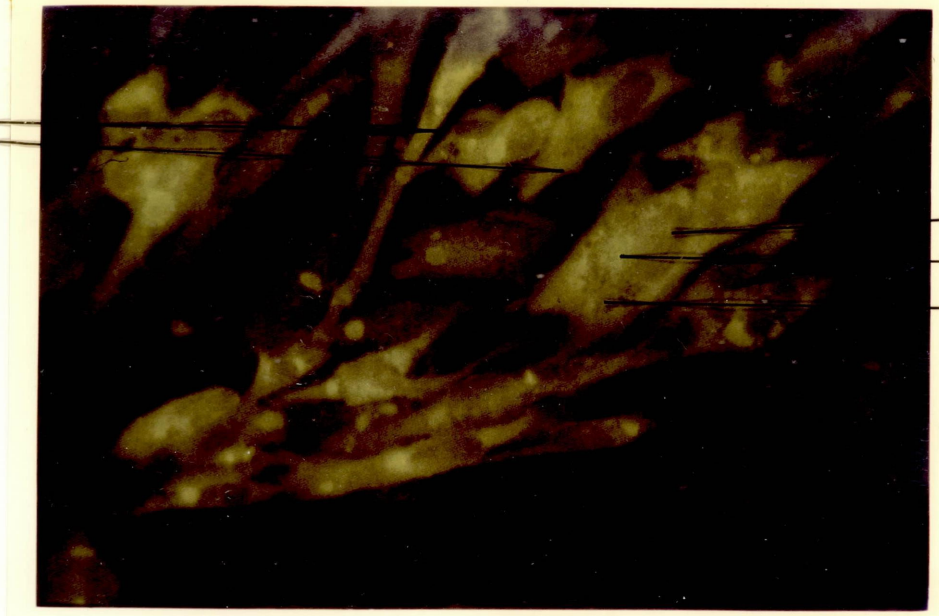


plate 5

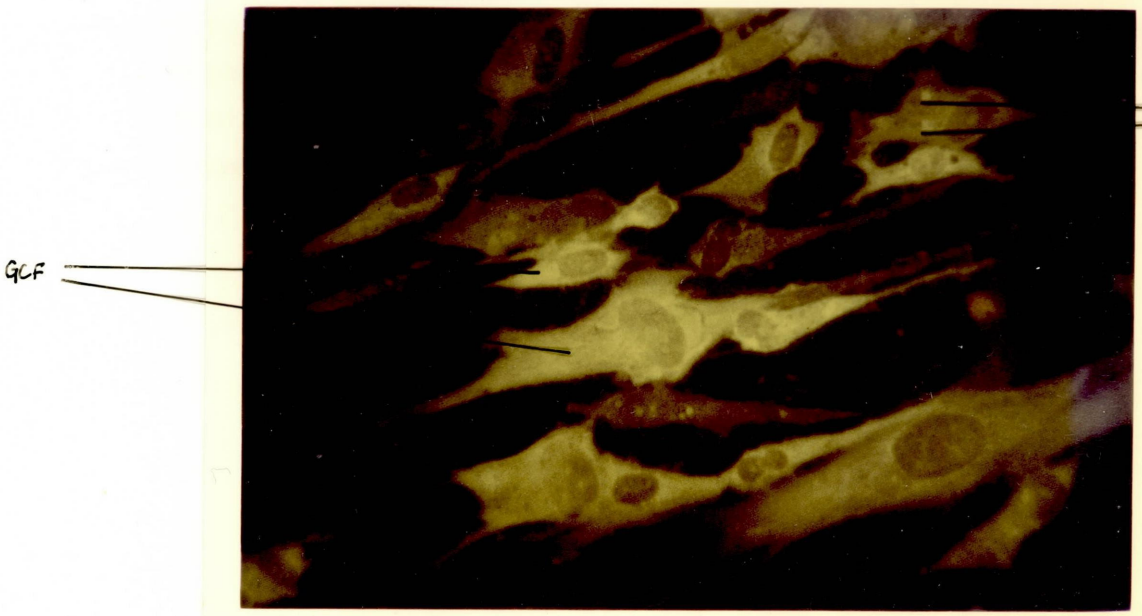


plate 6

Plate 7: Fluorescent antibody staining. 320x Ektachrome

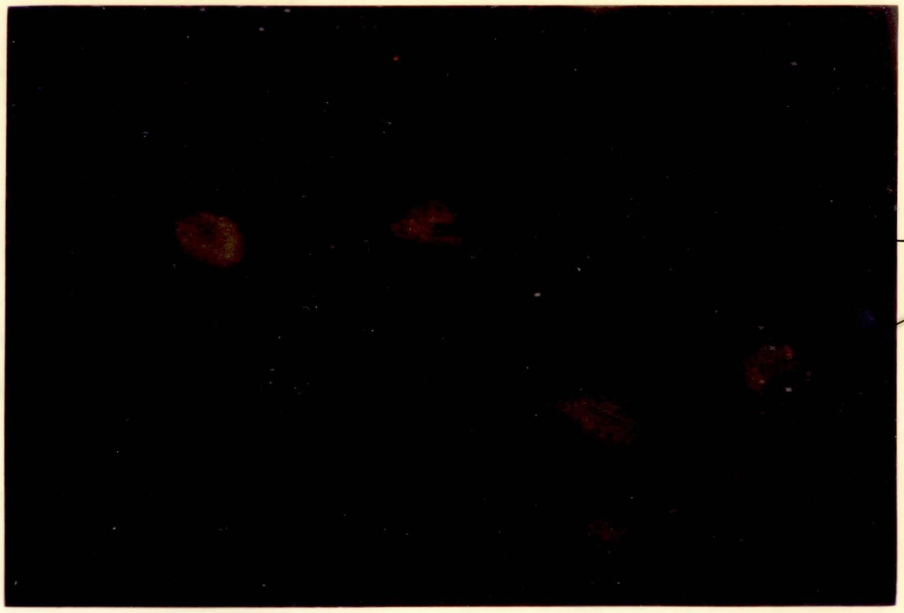
Human skin/muscle fibroblasts infected with the Harvey strain of variola major and incubated at 41°- 42° C for 48 hours. Rounded cells show uniform fluorescence (F).

(This is a poor quality photograph of the original preparation)

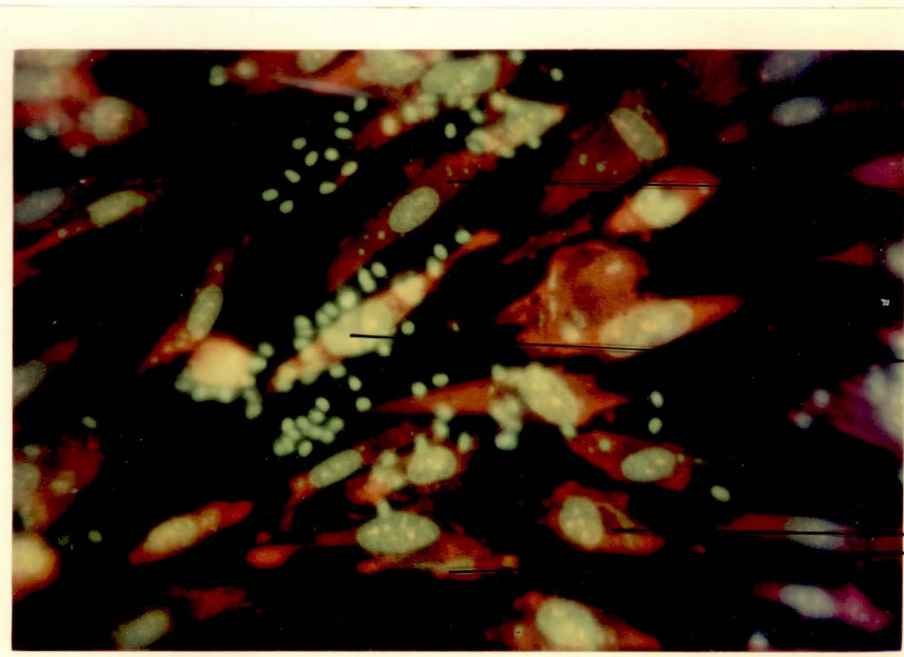
Plate 8: Acridine orange staining. 320x Kodachrome II A

A field of view of a variola-infected human skin/muscle fibroblast culture showing infected cells containing countable virus DNA inclusions (I) and cells positive for haemadsorption (HAD). Some cells contain diffuse inclusion material (D).

plates



7



8

'NP'-antigen associated with the DNA inclusions was the first to appear. A possible explanation for this discrepancy between two closely related systems is that the use of an antiserum made hyperimmune against whole virus precluded detection of earlier protein antigens through a lack of a high titre of antibodies to them in the serum. The use of a monospecific anti-'LS' antiserum might have rectified this anomoly.

The finding that the so-called dark area did indeed contain virus antigens was interesting, particularly since this area showed developmental changes during the progress of infection concurrently with the coalescence of foci of virus DNA synthesis. Maximum brightness and reticulation of the stellate body coincided with the merging of cytoplasmic DNA inclusions and with the spread of fluorescence throughout the general cytoplasm.

A second feature of interest to arise from these studies with FA was the fact that fluorescence of the stellate body and particularly of the cytoplasm in general developed at 39.5° C in Butler-infected cells and at 41-42° C in Harvey-infected cells. Since DNA inclusions remained identifiable as discrete, isolated bodies at 39.5° C and were completely absent at 41-42° C, the aforementioned fluorescence must have been attributable to the synthesis of antigen other than 'NP'. At 37° C the development of 'HA' at the cell surface (Oda, 1964) could not be eliminated as a cause of cytoplasmic fluorescence, but it has been shown that the 'HA' antigen, as detected by HAD, develops in less than 10 % of Butler-infected cells at 39.5° C and not at all in Harvey-infected cells at 41-42° C.

Thus, it is tempting to conclude,by a process of elimination, that fluorescence of the stellate body, of the perinuclear cytoplasm, and subsequently of the cytoplasm in general is due to the synthesis of antigens other than 'NP' and 'HA' in these locations.

'LS' antigen and other virus protein antigens synthesised de novo and possibly fulfilling structural functions may be implicated.

The results have shown that virus antigens can be synthesised in the infected cell without the participation of new virus DNA detectable in inclusion bodies in the cytoplasm.

Fluorescent antibody studies employing monospecific antisera would be essential to resolve the functional relationships of the various sites in which antigen synthesis has been demonstrated. Such antisera were not available at the time of these experiments.

b) Quantitative Studies

Cairns (1960) using autoradiographic and fluorescent antibody techniques established the following quantitative facts about the initiation of vaccinia virus infection in KB cells:

i) Each successfully infective virus particle creates its own centre of virus synthesis in the host cell.

ii) All centres of virus synthesis destined to develop in an infected cell begin simultaneously. One random virus particle, the initiator, overcomes or induces a specific mechanism controlling the initiation of all potential virus synthetic centres in the cell.

iii) Since there is a concommittance in the beginning of foci of virus synthesis in each cell, the number of foci in an individual cell remains constant until such time as the foci have enlarged to the extent of fusion.

iv) The number of cells in which initiation has taken place in an infected cell population increases with time. Those cells successfully infected with several particles have a greater probability of beginning synthesis at an earlier time after infection than those cells infected with a single virus particle.

Since vaccinia and variola are closely related members of the same poxvirus subgroup, it would be expected that the initiation of variola virus infection proceeds in the same manner as described by Cairns for vaccinia virus. If this assumption is correct, then the

frequency distribution of successfully established foci of DNA synthesis in cells of variola-infected cultures incubated at optimal, supraoptimal, and non-permissive temperatures could be examined.

In this manner, some quantitative information concerning the establishment and development of factories of DNA synthesis in variola-infected cells was collected.

The proportion of cells containing DNA inclusions, and the proportion of cells showing haemadsorption (HAD) of agglutinable fowl cells could be counted after the staining of infected cultures with acridine orange. The number of inclusions in each cell could also be counted [Plate 8]. Cells were recorded as containing diffuse inclusion material (D) when infection had proceeded to a stage where the inclusions were no longer discrete, but diffuse in the cytoplasm.

The following frequency distributions were investigated:

1) The proportion of cells in Harvey- and Butler-infected human skin/muscle fibroblast cultures incubated at 37°, 38.5°, and 39.5° C for 24 hours containing DNA inclusions and of cells showing HAD of agglutinable fowl cells.

2) The proportional distribution of discrete inclusions and diffused inclusion material (D) in Harvey- and Butler-infected human skin/muscle fibroblasts incubated at 35°, 37°, 38.5°, 39.5°, and 41-42° C for 15 hours.

3) The proportional distribution of discrete inclusions and diffused inclusion material (D) in Harvey- and Butler-infected human amnion

cells and human skin/muscle fibroblast cells incubated at 37°, 38.5°, and 39.5° C for 24 hours.

4) The proportional distribution of discrete inclusions and diffused inclusion material (D) in Harvey- , Butler- , and vaccinia-infected chick embryo fibroblast cells incubated at 37°, 38.5°, and 39.5° C for 24 hours.

5) The proportional distribution of discrete inclusions and diffused inclusion material (D) in Harvey-infected human skin/muscle fibroblasts incubated with and without 10^{-3} M hydroxyurea at 37° C for 24 hours.

Absorption was routinely carried out at 25° C for 2 hours and the inoculum was removed before placing cultures at the experimental incubation temperature. Every culture, therefore, had an equal chance of developing a Poissonian distribution of inclusions in the infected cell population during the absorption period. Any deviation in the distribution of inclusions in infected cells incubated above the optimum temperature from that distribution attained in baseline cultures incubated at the optimum temperature could therefore be assumed to be due to the temperature at which cultures had been incubated following absorption.

The frequency distributions to be discussed represent the proportional distribution of cells containing x number of inclusions or diffuse inclusion material at the time the counts were made and not the total proportion of cells in each culture which have developed one or more discrete inclusions and subsequently diffused inclusion

material during the course of infection.

The probability of development of inclusions and of HAD in a cell increases with time. Thus, it was essential in this instance to stop infection at a time when the maximum number of cells capable of developing inclusions and the property for HAD had done so, and when a minimum of the infected cells had proceeded to such an advanced stage of infection that internal cellular detail could not be resolved.

15-24 hours after variola-infection of human cells was chosen as a suitable time for the frequency distributions to be made. In Harvey-infected cultures at 35° or 37° C (baseline), most of the potentiality for inclusion formation and 'HA' production had been expressed, but at this time infection was not too advanced. After 24 hours, the loss of necrotic cells from the glass surface became increasingly significant and made frequency distributions performed after this time invalid.

Each count was qualified with the time after infection at which it was made.

(Materials and Method - See Appendix)

Results

1. See fig. 10

An analysis of the frequency of DNA-inclusion-containing cells in Harvey- and Butler- infected human fibroblasts showed that by 24 hours 8 %, 15 %, and 37 % fewer cells had been established as

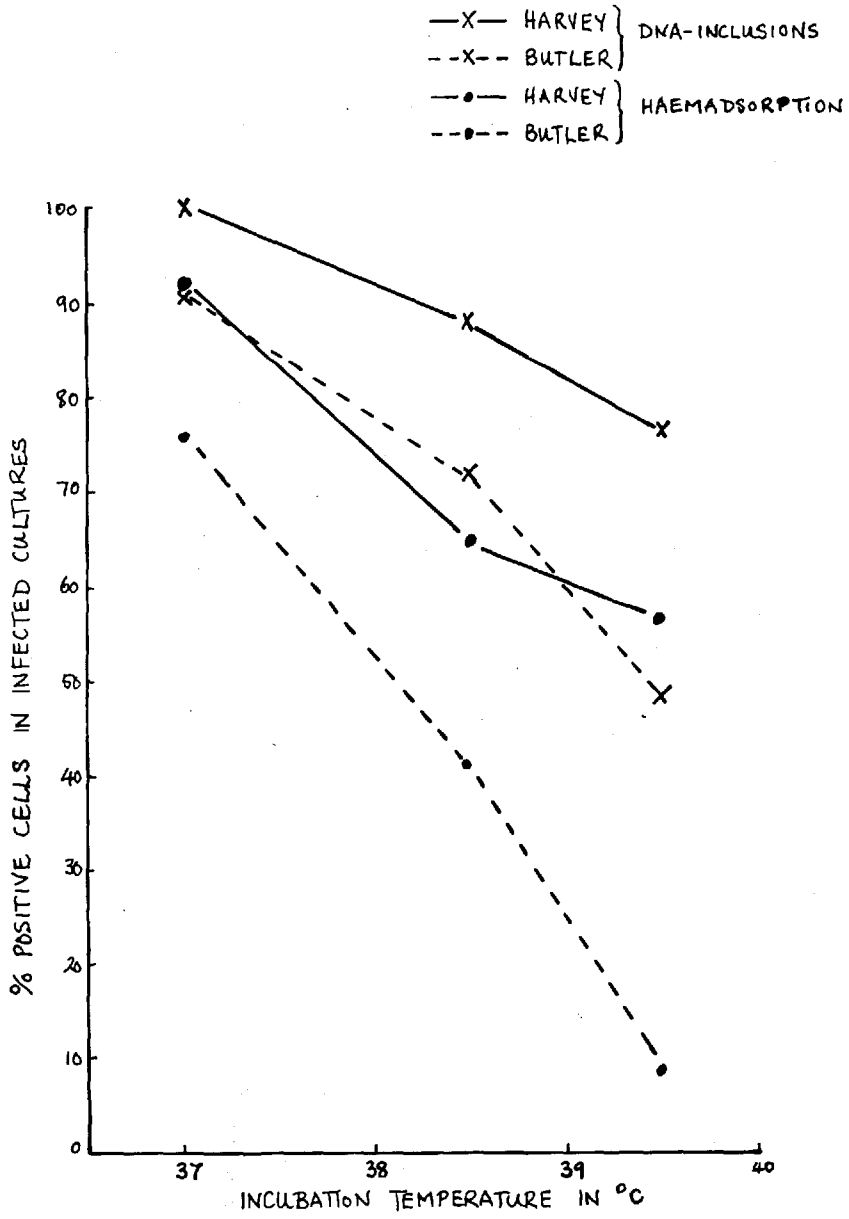


Fig. 10. The percentage of cells in Harvey- and Butler-infected human skin/muscle fibroblast cultures developing virus DNA inclusions and the property of haemadsorption by 24 hours at 37°, 38.5°, and 39.5° C.

infective centres at 37°, 38.5°, and 39.5° C, respectively, in Butler-infected than in Harvey-infected cultures.

At 37° C, 7 % of Harvey-infected DNA-inclusion-containing cells were negative for HA as detected by HAD. HA production is known to lag considerably behind the synthesis of other virus antigens (Loh and Riggs, 1961), but it was found that the proportion of cells containing DNA inclusions, but which were non-haemadsorbing, increased with the incubation temperature. This proportion was always greater in Butler-infected cultures than in Harvey-infected cultures at each temperature [Table 1]. The difference was particularly significant at 38.5° C and at 39.5° C.

TABLE 1

Virus Strain	37° C	38.5° C	39.5° C
Harvey	7 %	26 %	25 %
Butler	17 %	43 %	83 %

The percentage of cells at each temperature in Harvey- and Butler-infected human skin/muscle fibroblast cultures containing DNA inclusions, but which did not haemadsorb agglutinable fowl cells (24 hours after infection).

Thus, the establishment of successful infection as determined by the presence of virus DNA inclusions in cells was found to be more efficient for Harvey virus than for Butler virus at all three temperatures tested. Initiation of DNA synthesis in Butler-infected cells was particularly sensitive to a temperature of 39.5° C, and HA production

to temperatures of 38.5° and 39.5° C.

Plates 9, 10, and 11 illustrate the difference in the extent of HAD at 24 hours in Harvey-infected human skin/muscle fibroblast cultures at 37° and 39.5° C and in Butler-infected skin/muscle fibroblast cultures at 39.5° C.

2) See Fig. 11

An analysis of the distribution of inclusions among Harvey- and Butler-infected human skin/muscle fibroblast cells at 15 hours after infection showed that increased temperatures of incubation were effective in suppressing the stage of coalescence of inclusions.

Decreases in the proportion of cells with diffuse inclusion material were accompanied by increases in the proportion of cells containing lower multiplicities of inclusions.

'Maturation' of inclusions occurred as efficiently at 35° C as at 37° C in Harvey-infected cells, but was suppressed progressively at 38.5° and 39.5° C with a corresponding increase in the proportion of cells containing, in particular, 7 or fewer inclusions.

In Butler-infected cells 'maturation' of inclusions occurred most efficiently at 35° C, being increasingly suppressed at 37°, 38.5°, and 39.5° C. Fewer inclusions per cell were found at each temperature than in the corresponding Harvey-infected cultures; the number of cells containing single inclusions was greater and increased with temperature.

Little 'maturation' of inclusions was noted for either virus strain at 39.5° C, and no inclusions developed at 41-42° C.

Plates 9, 10, and 11: Acridine orange staining. 160x Kodachrome II A

A comparison of the extent of haemadsorption given by human skin/muscle fibroblast cultures infected with the Harvey strain of variola major and incubated at 37° C (Plate 9) and 39.5° C (Plate 10), and with the Butler strain of variola minor incubated at 39.5° C (Plate 11) for 24 hours.

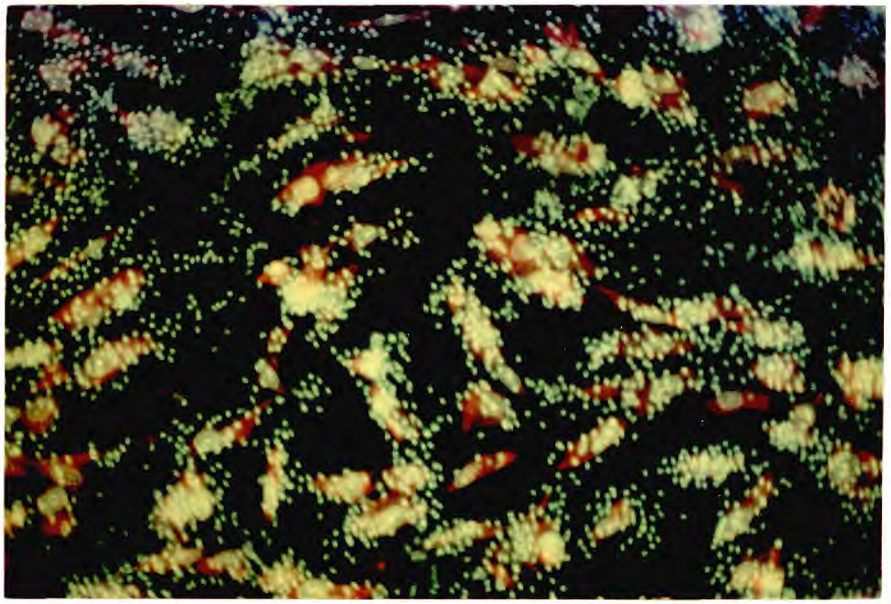


plate 9

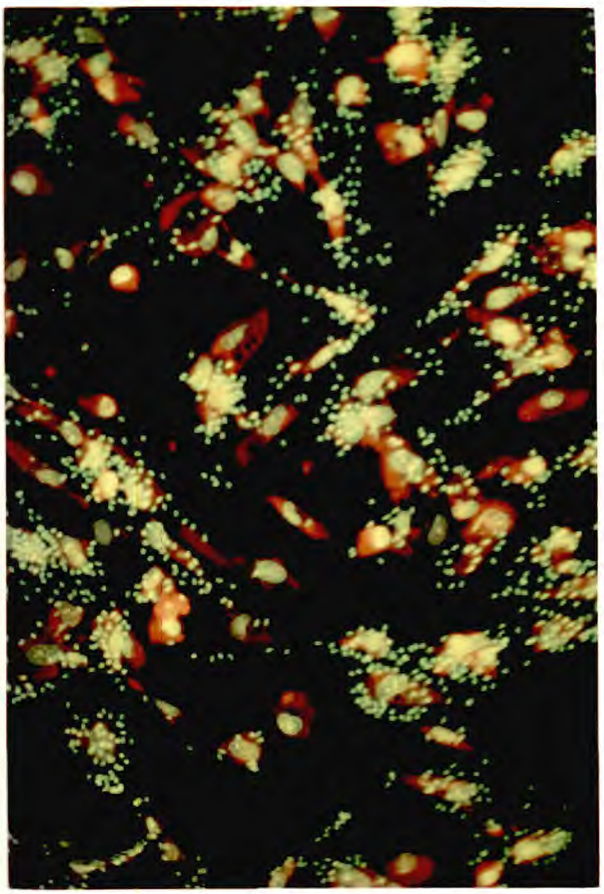


plate 10

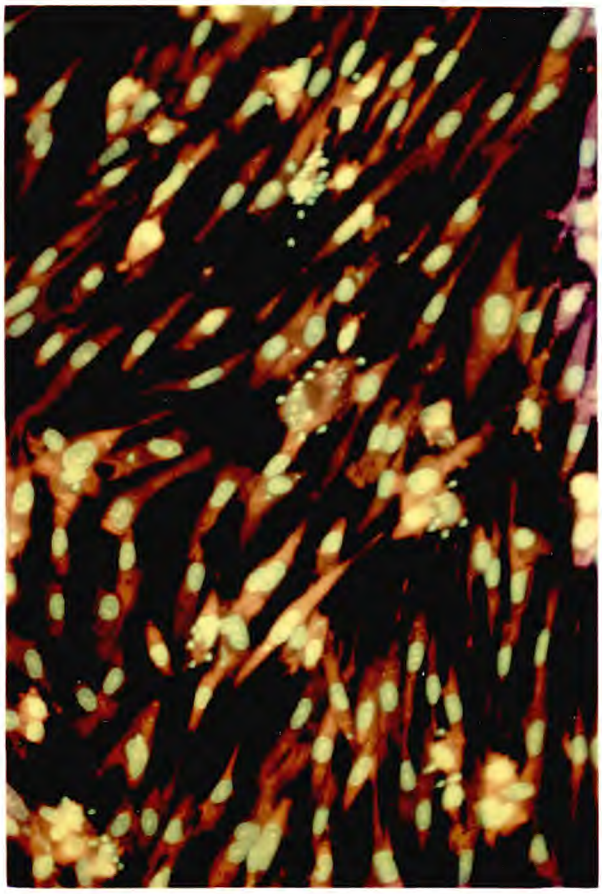
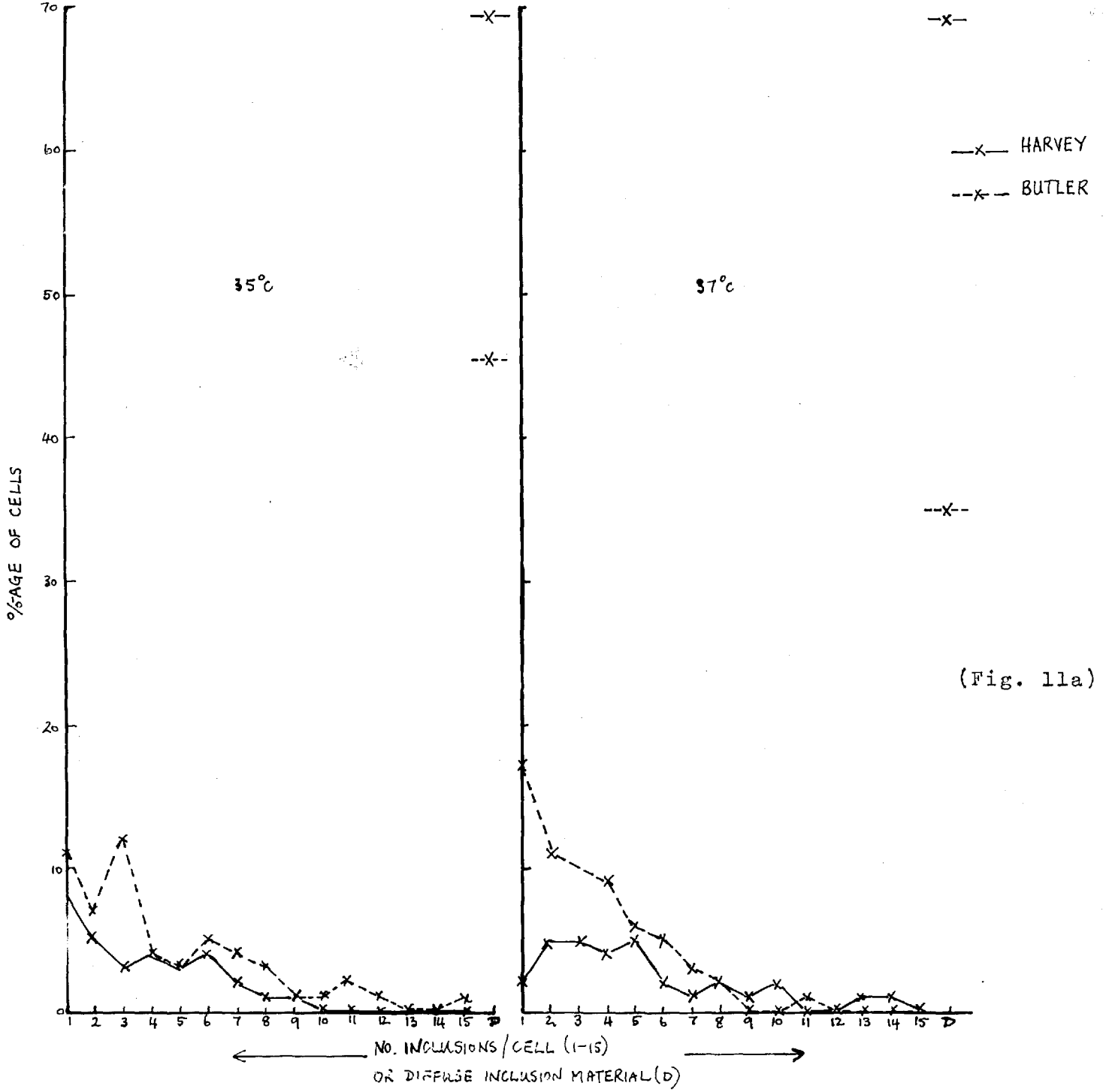
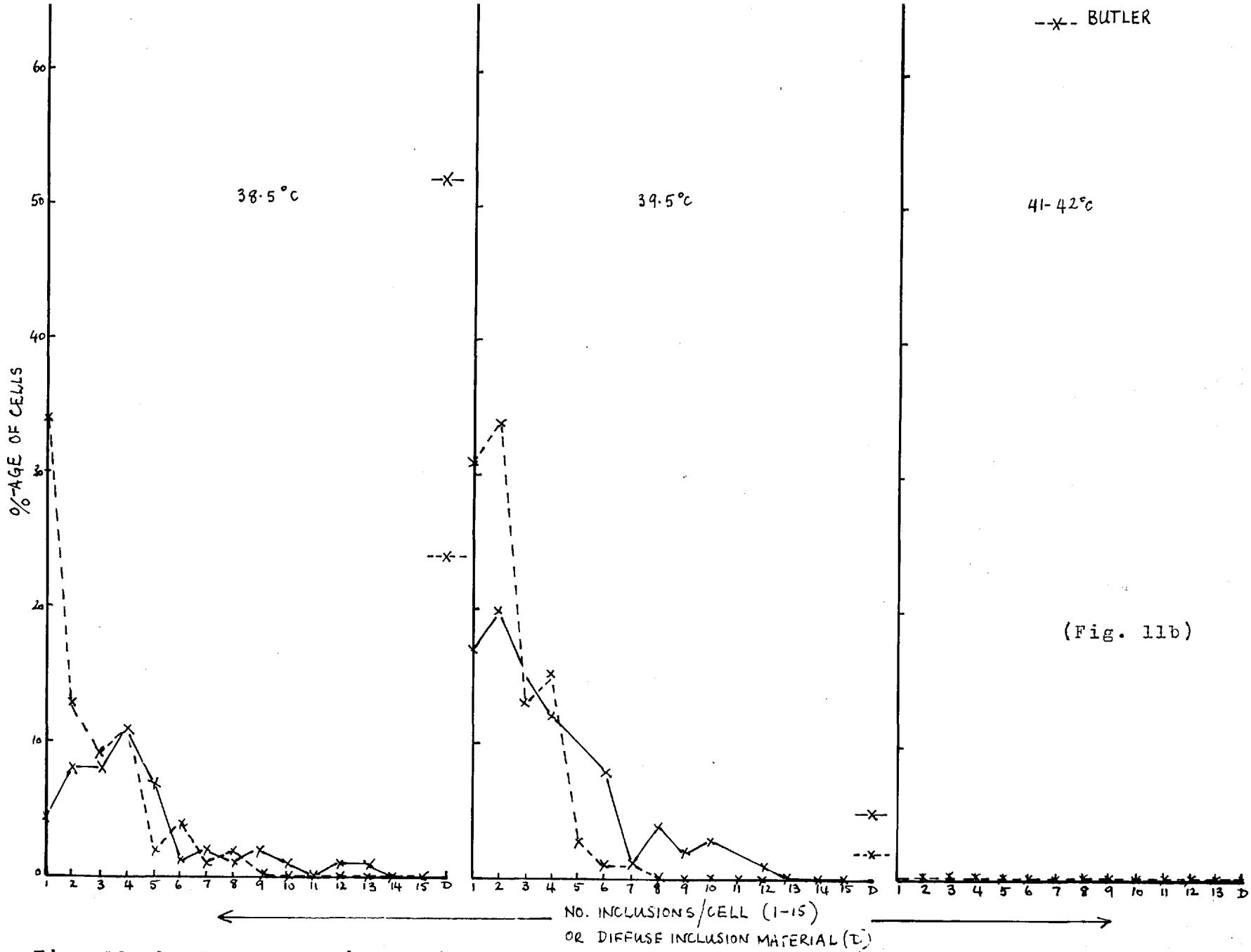


plate 11


HARVEY
BUTLER
35°C
37°C
%-AGE OF CELLS
0
10
20
30
40
50
60
70
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 D
NO. INCLUSIONS / CELL (1-15)
OR DIFFUSE INCLUSION MATERIAL (D)

(Fig. 11a)



(Fig. 11b)

Fig. 11a,b. The proportional distribution at 15 hrs of discrete DNA inclusions and diffused inclusion material in Harvey- and Butler-infected human fibroblast cells incubated at the stated temperatures.

These results correspond to the results obtained from the growth studies. The optimum temperature for both virus release and inclusion coalescence is 35° C in the case of the Butler strain and 37° C for the Harvey strain. The lag at 15 hours in the progress of development of foci of DNA synthesis in Butler-infected cells behind that in Harvey-infected cells corresponds to the situation at 15 hours as depicted by the single-step growth curves (Chapter II). That little fusion of DNA inclusions occurred at 39.5° C in cells infected with either Harvey or Butler virus is in accordance with the fact that no infective virus is produced at this temperature by either strain.

3) See Figs. 12 and 13

A comparison of the 24 hour frequency distribution of inclusions in populations of Harvey- and Butler-infected human skin/muscle fibroblasts with that in Harvey- and Butler-infected human amnion cells showed that DNA inclusion development was somewhat more efficient in infected skin/muscle fibroblast cells. No fusion of inclusions was detected in amnion cells at 39.5° C, whereas a small proportion of fibroblast cells showed this stage at this temperature.

By 24 hours the probability that cells containing the larger multiplicities of inclusions will have progressed to the stage of diffusion of inclusions is greater than at earlier times. Also, those cells containing single or small numbers of inclusions, being slower in initiation, are less likely to have reached this stage in maturation.

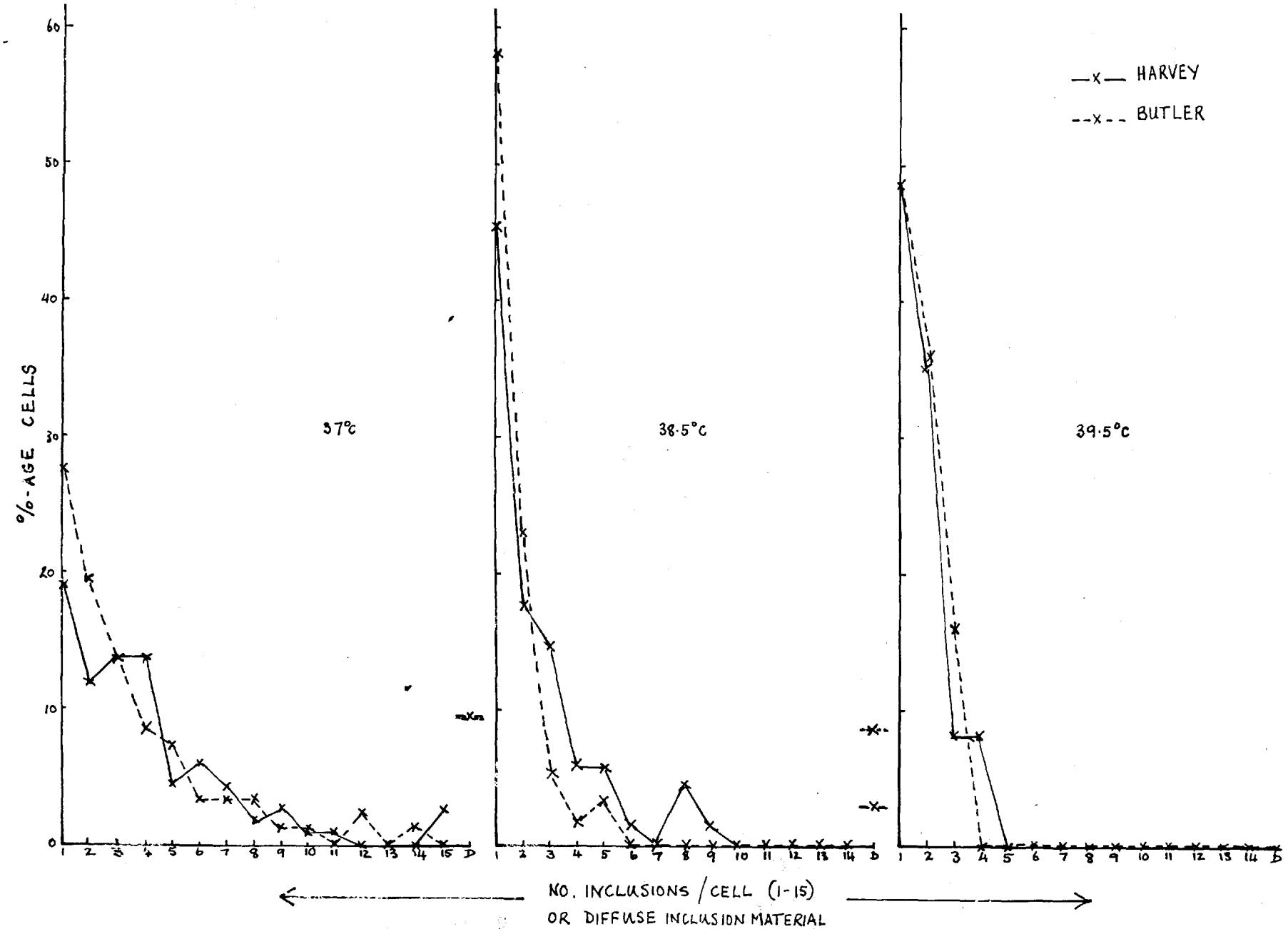


Fig. 12. The proportional distribution at 24 hours of discrete DNA inclusions and diffused inclusion material in Harvey- and Butler-infected human amnion cells incubated at the specified temperatures.

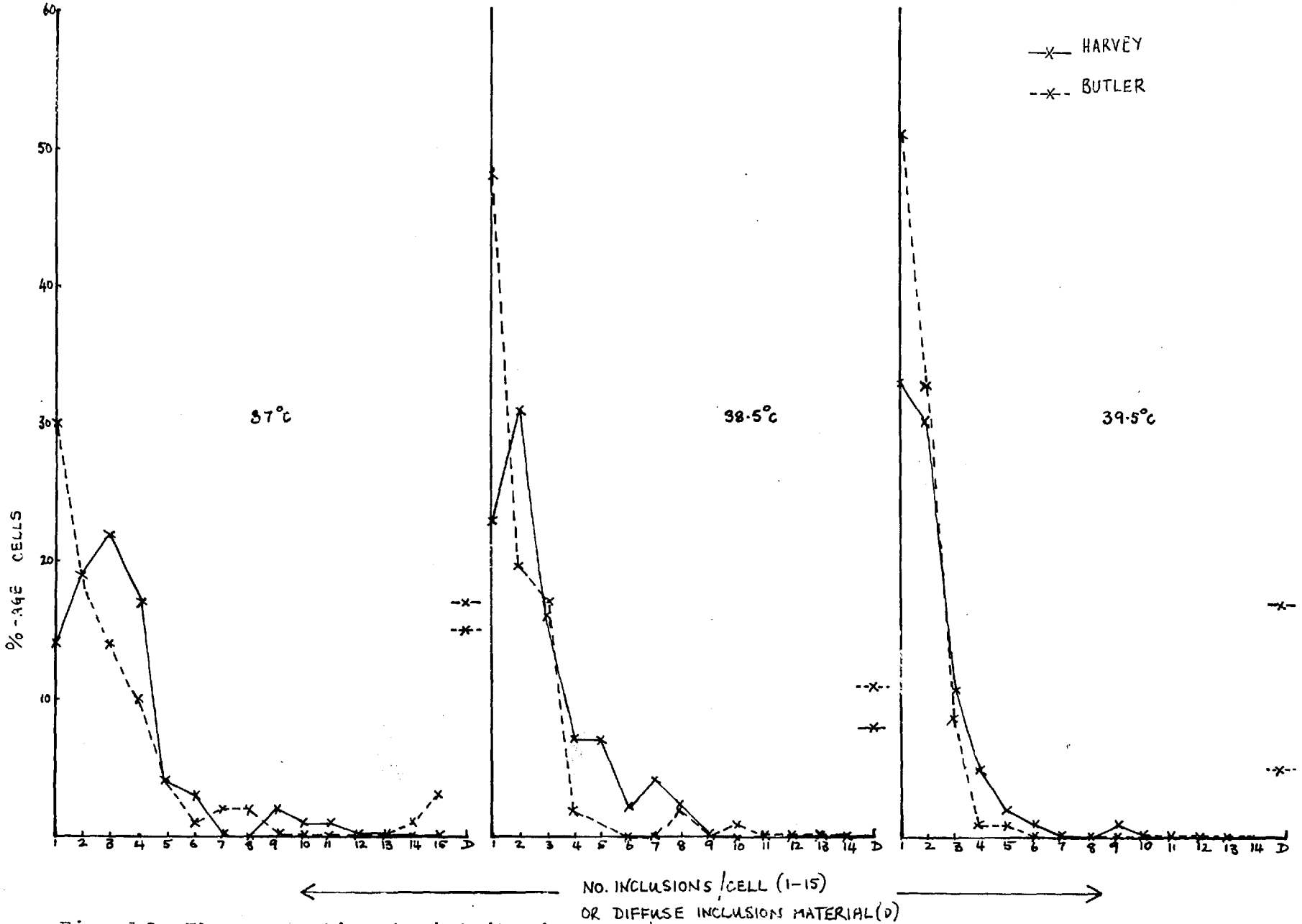


Fig. 13. The proportional distribution at 24 hrs of discrete DNA inclusions and diffused inclusion material in Harvey- and Butler-infected human fibroblast cells incubated at the specified temperatures.

Such cells, then, may be predicted to constitute the greater proportion of cells containing countable inclusions. This was found to be so. However, cultures incubated at 38.5° and 39.5° C could be seen to contain a significantly larger proportion of cells with one or two inclusions than did those incubated at 37° C; cells containing only one or two inclusions were particularly abundant in Butler-infected cultures.

4) See fig. 14

In human skin/muscle fibroblast cells the difference in ceiling temperature between the Harvey and Butler strains of variola major and minor, respectively, is not as marked as in chick embryo fibroblasts. It was of interest, then, to compare quantitatively the formation of DNA inclusions in these two cell systems. Vaccinia virus chick embryo fibroblasts were included in the study since this virus grows vigorously in chick embryo cells at all temperatures tested; the ceiling temperature of vaccinia virus as determined in the chick embryo is 41° C (Bedson and Dumbell, 1961).

The results of this experiment showed that, after 24 hours incubation, the proportion of Harvey-infected chick fibroblast cells containing diffuse inclusion material was 65 % and 60 % at 37° and 38.5° C, respectively. This proportion is more than 50 % greater than the proportion in Butler-infected chick embryo fibroblasts, being 32 % and 25 %, respectively. Correspondingly, Butler-infected cultures were richer in cells containing one or two inclusions only.

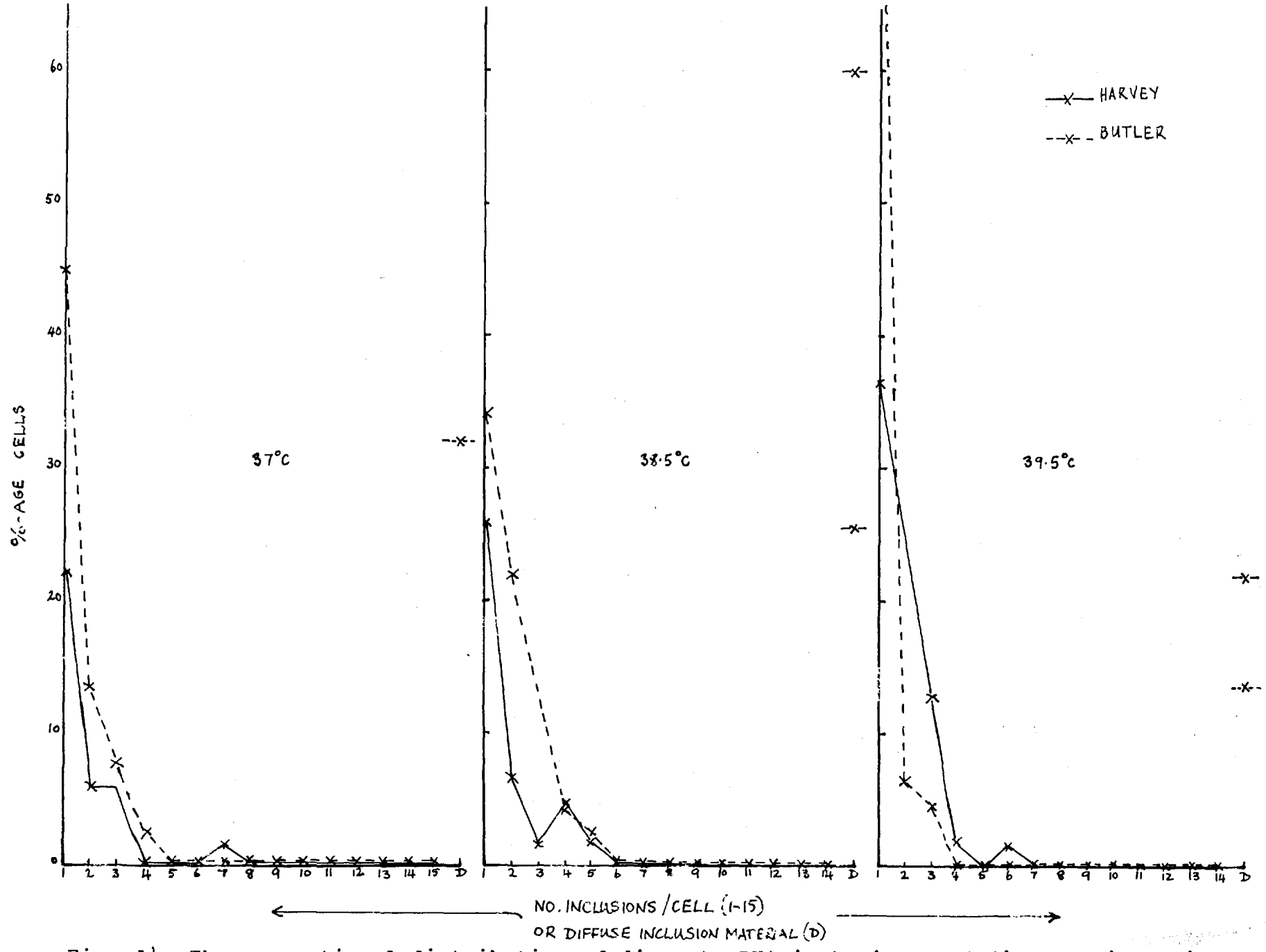


Fig. 14. The proportional distribution of discrete DNA inclusions and diffused inclusion material in Harvey- and Butler-infected chick embryo fibroblasts incubated at the specified temperatures for 24 hours.

This difference was more marked in chick embryo fibroblast cultures than in human skin/muscle fibroblast cultures as represented in fig. 13.

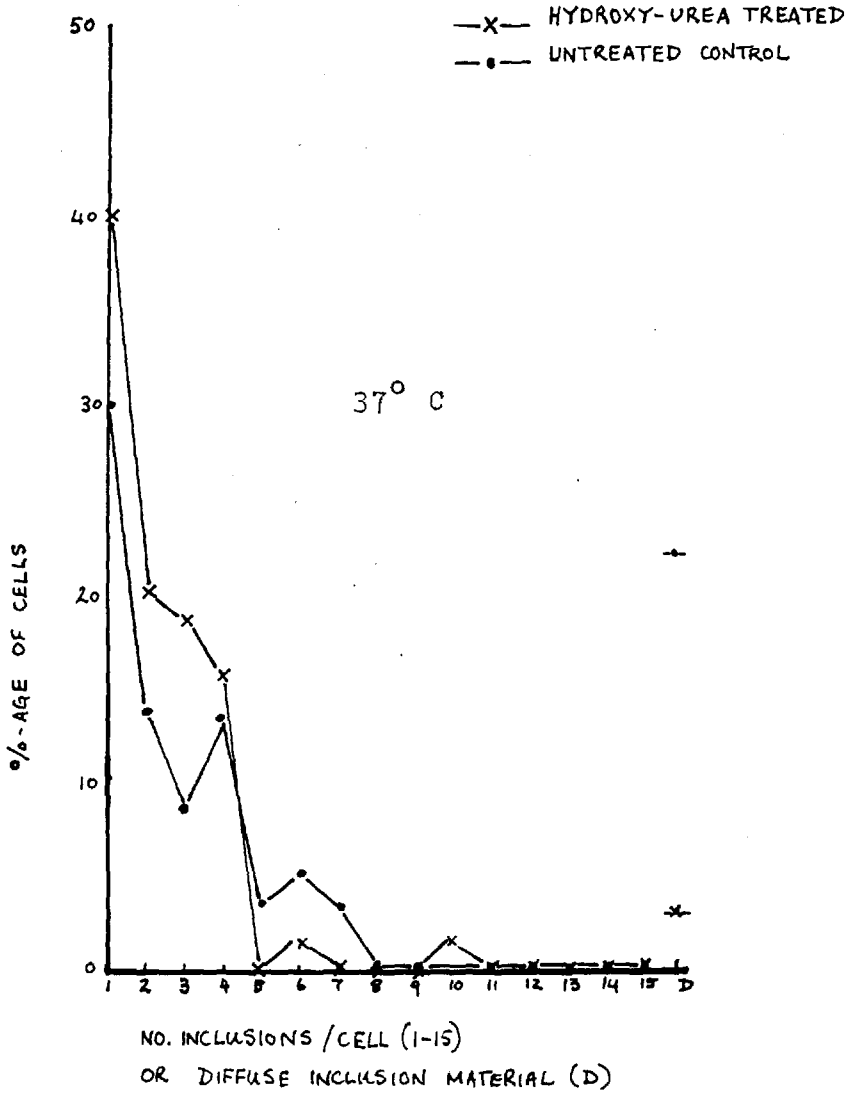
At 38.5° C Butler does not form pocks on the CAM, whereas Harvey does, but at a reduced number than at 37° C (Nizamuddin and Dumbell, 1961). At 38.5° C the frequency distribution revealed that 25 % of Butler-infected chick embryo fibroblast cells contained diffuse inclusion material as compared with 60 % of Harvey-infected cells. It is possible that this lack of development of inclusions in Butler-infected chick embryo cells indicates one reason for the difference in the behaviour of variola major and minor strains on the CAM at 38.5° C. Suppression of DNA synthesis would be reflected in a suppression of infective virus synthesis. A severe reduction in virus yield from the first infected cells would lead to a poor spread of infection to adjacent susceptible cells and to abortive pocks.

Vaccinia virus, which grows vigorously in chick embryo fibroblasts at the tested temperatures, showed coalescence of inclusions in 100 % of the infected cells at 37°, 38.5°, and 39.5° C by 24 hours.

5) See Fig. 15

10^{-3} M hydroxyurea has been shown to suppress the production of infective virus and HA in vaccinia-infected RK_{13} cells (Baxby, 1967). In Harvey-infected human fibroblasts the infectivity yield was reduced to 1/10, and HA production to 1/3 that in untreated controls. Hydroxyurea is known to suppress vaccinia virus production by virtue

Fig. 15. The proportional distribution of discrete DNA inclusions and diffused inclusion material in Harvey- and Butler-infected human fibroblasts incubated with and without 10^{-3} M hydroxyurea at 37° C for 24 hours.



of its effect on DNA replication (Rosenkrantz et al., 1966).

It was found that treatment of Harvey-infected human skin/muscle fibroblasts incubated at 37° C reduced the multiplicity of inclusions developing in any one cell and reduced the proportion of cells showing DNA inclusion coalescence. However, the total proportion of cells developing as centres of DNA synthesis in an infected population was not different to the proportion developing in untreated controls. Thus, a suppression of DNA synthesis by means other than 'high' temperature also resulted in a reduction in the establishment and development of DNA inclusions, although the inhibitory mechanisms are different and may well effect different stages in the pathway of virus DNA synthesis.

Discussion

Loh and Riggs (1961) showed that fragmentation and diffusion of inclusion material coincided with the onset of maturation of infective virus in cells. It has been assumed that this stage is equivalent to that scored 'D' in the quantitative studies described above.

The frequency distributions of the experiments have shown that the effect of supraoptimal temperatures of incubation is to suppress this stage of coalescence of DNA inclusions in infected cells. Furthermore, the proportion of cells containing high multiplicities of inclusions is reduced increasingly as the temperature of incubation approaches the non-permissive temperature for virus growth.

Thus, the delays noted in the rate of production and release of progeny virus and the reduction in the maximum yield of infectivity of Harvey and Butler virus at high temperatures of incubation can, at least in part, be accounted for by the suppression of the number of DNA inclusions developing in infected cells and in the proportion of inclusions able to reach the stage of coalescence.

Virus production and release in Butler-infected cells is more sensitive to supraoptimal temperatures of incubation than that in Harvey-infected cells. A corresponding sensitivity in inclusion production and development was noted during these studies.

Cairns (1960) showed that all factories of vaccinia virus synthesis began simultaneously in any one cell. It was noticed, however, during examination of AO-stained preparations of variola-infected cells, that once inclusions have been established in a cell their subsequent development was often asynchronous. Single cells were frequently seen to contain both small, discrete inclusions and large, irregular inclusions. Thus, once initiated, each inclusion would seem to develop independently of other inclusions in the same cell.

General Discussion of Cytological Studies

On the basis of results obtained from fluorescent antibody and acridine orange staining of variola-infected cultures, the failure of the infective process to yield mature infectious virus progeny at 39.5° C

may be due to the arrest in development of nucleoids.

Two temperature-induced blocks of the sequence of events controlling the establishment and subsequent development of DNA inclusions can be envisaged. The first block, which prevents the development of potential factories of virus synthesis so causing a reduction in the number of DNA inclusions established in cells incubated at supraoptimal temperatures, occurs early in the virus cycle. The second block, occurring much later in the cycle, prevents the coalescence of DNA inclusion bodies which normally preceeds, or is concommittant with, the assembly of mature virus.

The DNA which is formed in inclusions at 39.5° C would seem to be defective in some way since inclusions rarely become diffuse and infective virus does not form. If, however, this DNA is correctly assembled, then other steps vital to the maturation of progeny virus must also be suppressed at 39.5° C. Fluorescent antibody studies suggested that the synthesis of some virus proteins are probably relatively unaffected by high temperatures of incubation. However, "mistakes" may well be caused at such temperatures at one or several stages in structural protein synthesis also.

Evidence that a failure of the development of nucleic acid containing virus cores can explain an abortive virus infection at supraoptimal incubation temperatures is found in the reports of Stevens (1966) concerning Herpes virus and of Lebeurier and Hirth (1966) concerning Tobacco Mosaic virus. In both cases, temperatures

above the optimum for virus reproduction permitted the synthesis of virus capsids, but prevented the formation of the nucleoids. Electron micrographs of sections of infected cells showed that the capsids formed during infection were devoid of nucleoids and that no nucleoid-like elements were present in infected cells.

Electron microscopy of variola-infected cells incubated at high temperatures could serve to confirm or disprove the speculations made here concerning the production of virus components under such conditions.

3.3 Electron Microscopy of Variola-infected Human Skin/Muscle Fibroblasts

The production of virus-specific antigens, but not of mature infectious progeny virus occurs above the temperature permissive for variola virus growth. Some speculations have been made concerning the failure of the production of mature virus under these conditions.

In order to obtain direct information concerning the production of structural components of variola virus above the permissive temperature, Dr. J. G. Cruikshank kindly undertook to perform some preliminary examinations of variola-infected human skin/muscle fibroblasts prepared for electron microscopy.

Human skin/muscle fibroblasts infected with the Harvey strain of variola major at an input multiplicity of approximately 10 pfu per cell were incubated at 35° C and 40° C and examined by electron microscopy for the presence of virus structural components. Uninoculated controls incubated at the same temperatures were also examined.

(Materials and Methods - See Appendix)

Results: See electron micrographs with corresponding explanations

Plate 12

A section through a normal, uninfected human skin/muscle fibroblast cell incubated at 35° C. A rather vacuolated cytoplasm surrounds the nucleus (N) and its nuclear membrane (NM). The finely granular cytoplasm contains numerous fat bodies (FB) and mitochondria (M).

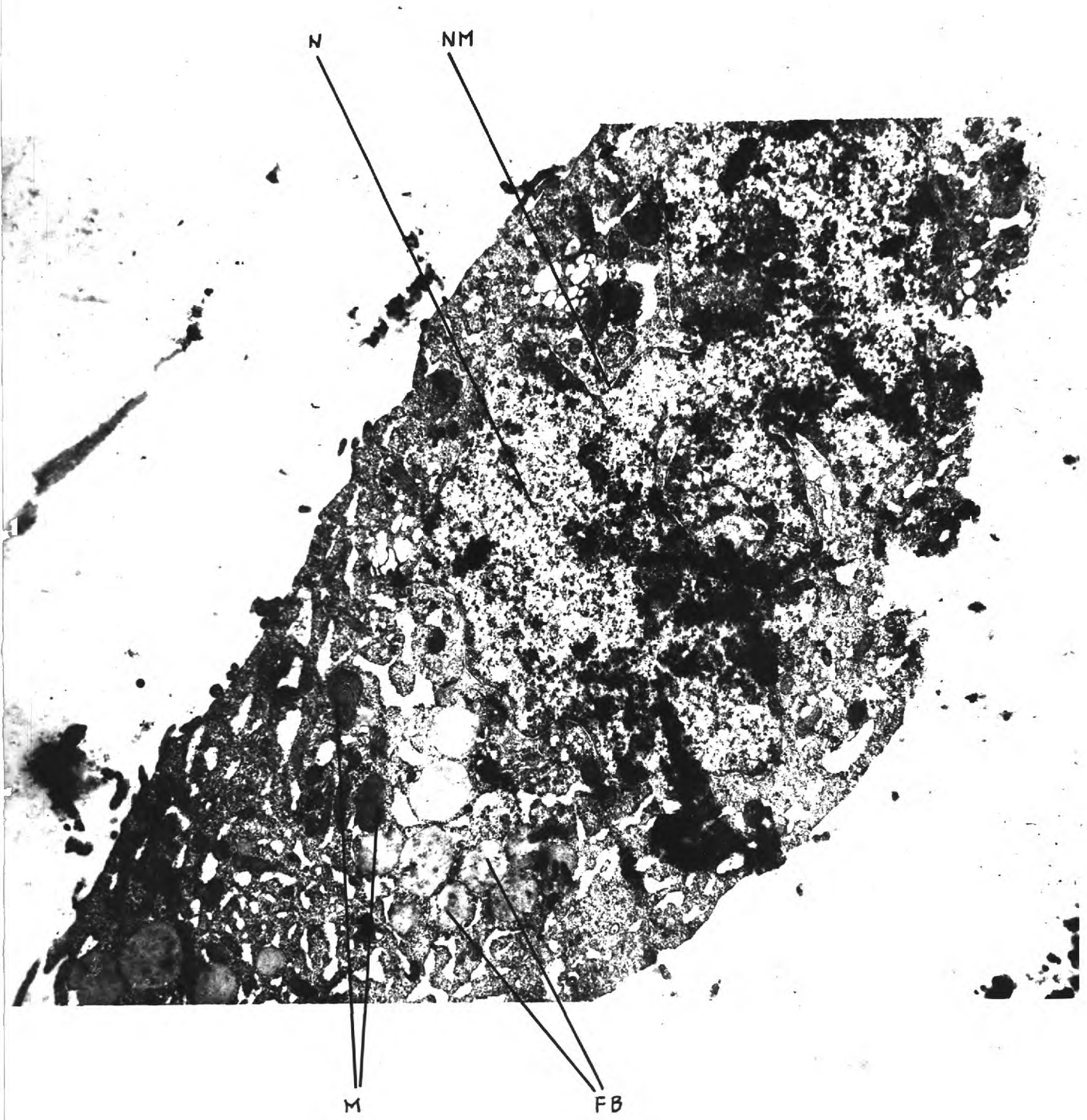

N
NM
M
FB

Plate 13

A section through a variola-infected human skin/muscle fibroblast cell 16 hours after infection and incubated at 35° C. Immature virus particles (VP) are developing in an area of viroplasm (V) which is devoid of mitochondria and other cellular structures. A dense core (C) is beginning to form within the area confined by the virus membranes of one particle (VMB).

30,000x

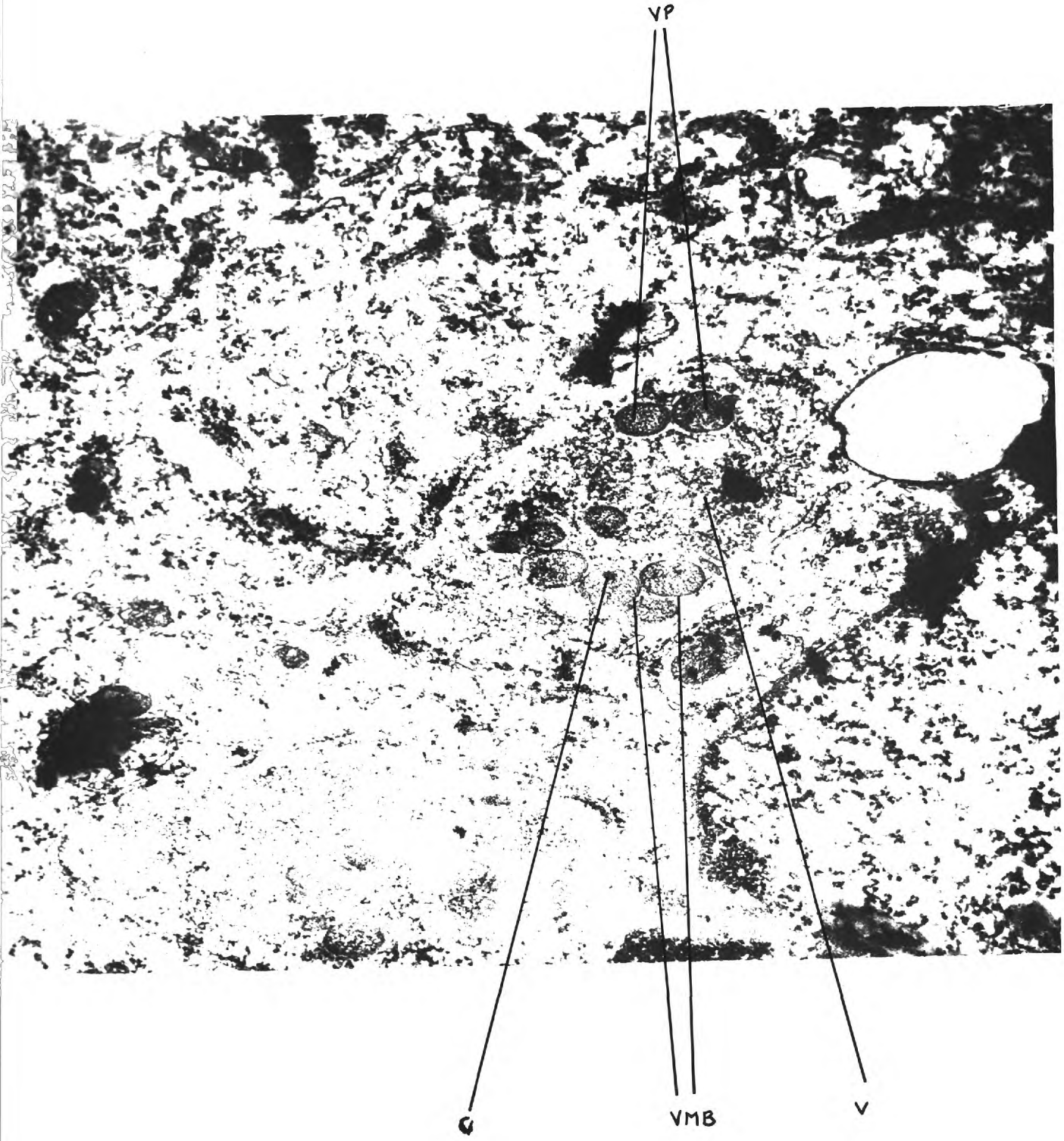

VP
VMB
V

Plate 14

A section through a variola-infected human skin/muscle fibroblast cell 16 hours after infection and incubated at 35° C. Immature virus particles (VP) are lying near the cell surface. A dense core (C) is forming in one particle.

20,000x

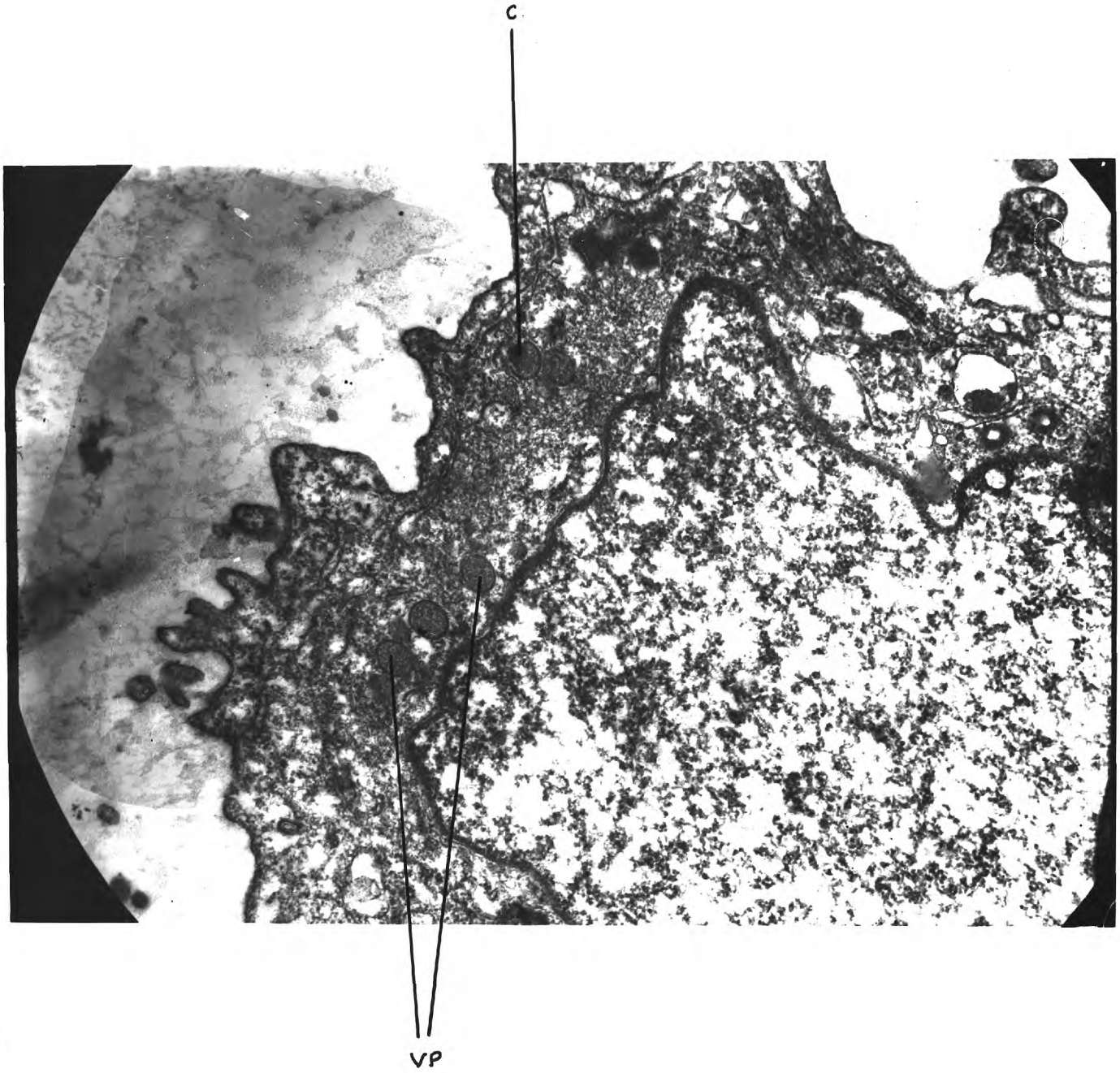

C
VP

Plate 15

A section through a variola-infected human skin/muscle fibroblast cell 16 hours after infection and incubated at 35° C. Nearly mature virus particles (VP) are lying at the cell surface prior to being released from the cell. Dense cores (C) completely fill the particles. A dumbell-shaped nucleoid (ND) can be discerned in the most mature of the virus particles.

36,666x

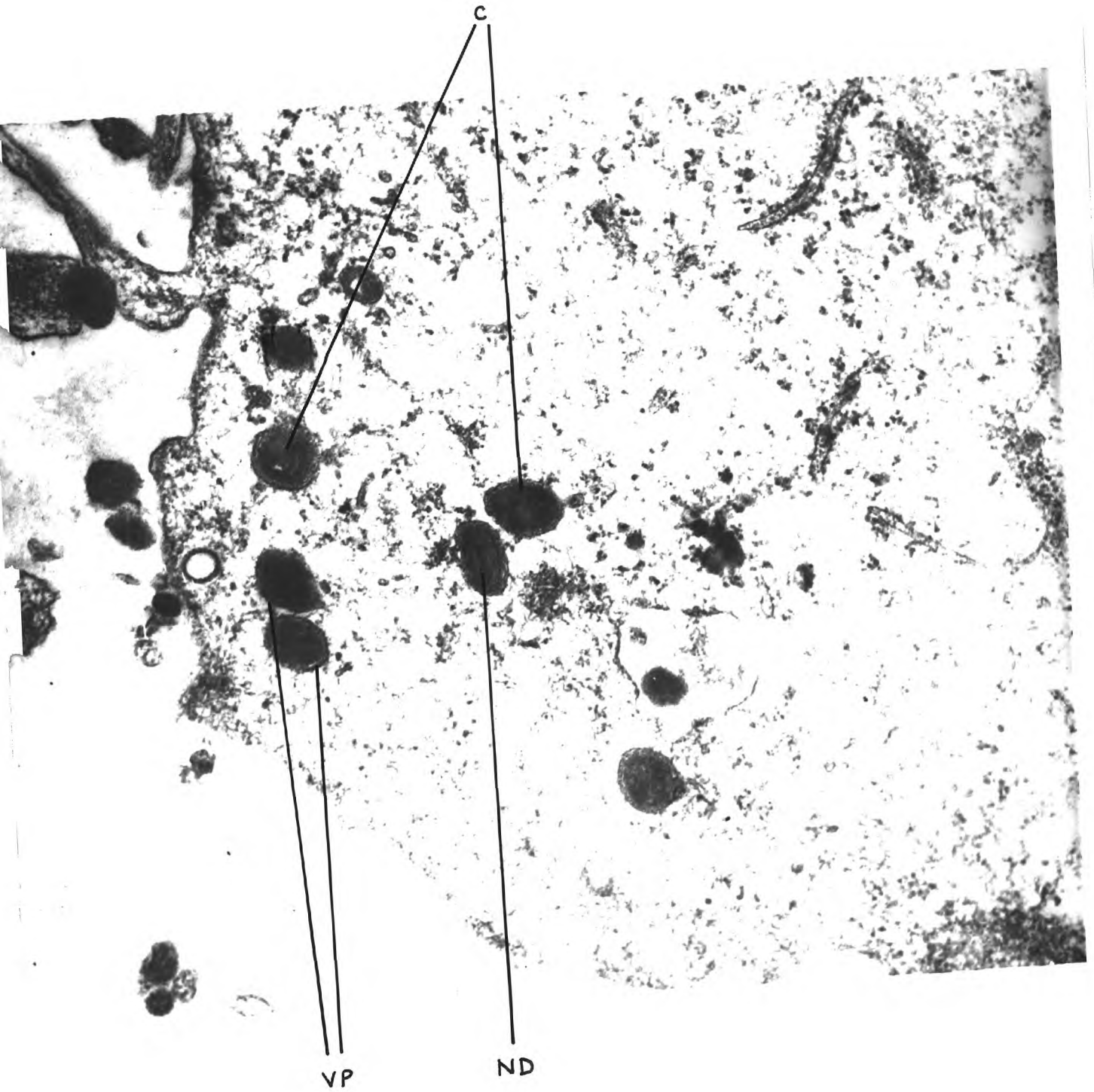
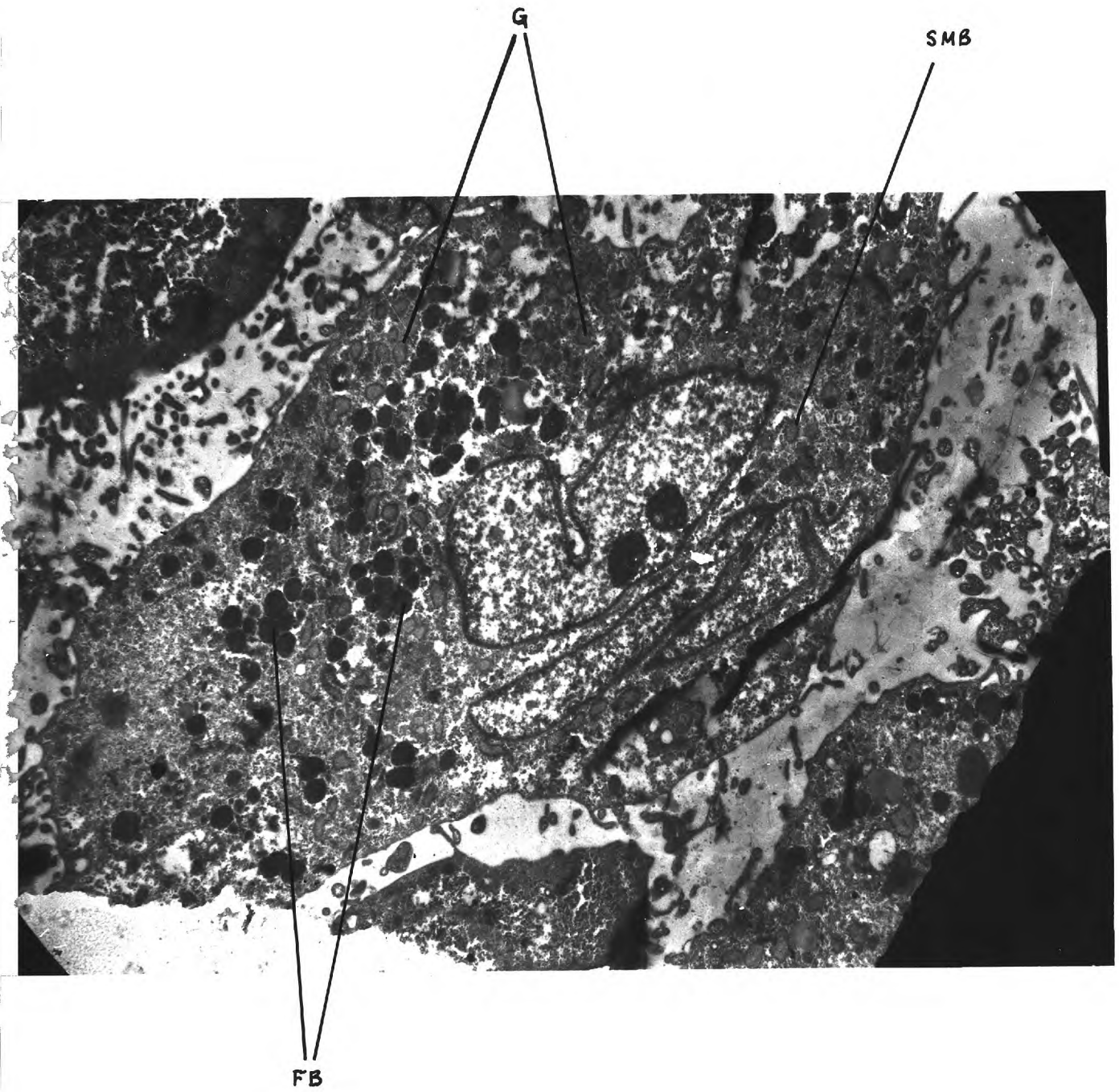

C
VP
ND

Plate 16

A section through a variola-infected human skin/muscle fibroblast cell 6 hours after infection and incubated at 40° C. Immature and mature virus particles are absent. Many electron-opaque fat bodies (FB) are apparent. The salient feature of this section is the presence of widely distributed smooth grey bodies (G) reminiscent of viroplasm, which are surrounded by a membrane. In many places, this membrane is seen to bear spikes (SMB).

35,000x


G
SMB
FB

Discussion

At 35° C variola virus particles form normally in infected human fibroblast cells. At 40° C no virus particles, either immature or mature, are seen in infected cells. Instead, an abnormal feature consisting of very many smooth grey masses surrounded by a membrane which often bears spikes is seen. Sequential studies have shown that this feature appears early after infection.

It is possible that this membrane, which sometimes appears smooth and sometimes bears spikes, could represent an abnormal assembly of virus membranes. Whereas the virus membranes are normally formed as small units suitable for individual virus 'coats', the membranes could be misassembled, at high temperatures, into much larger units forming bizarre shapes. It is interesting that this membrane encloses clear areas similar to the clear areas of viroplasm.

3.4 General Conclusions

Cytochemical studies of variola-infected cells incubated above the permissive temperature indicated that DNA synthesis was severely suppressed, that nucleoid development was arrested, but that the synthesis of some form of virus-structural protein was possible under these conditions.

Results obtained from the electron microscopical examination of cells infected and incubated in the same way are in general agreement with these conclusions. Both typical virus particles and nucleoid-like elements were absent in variola-infected cells incubated at 40° C, but a membrane, possibly representing an abnormal and misassembled virus membrane, was formed. However, the situation would appear to be more complex than was found to be the case with Herpes virus (Stevens, 1966) and with Tobacco Mosaic virus (Lebeurier and Hirth, 1966).

The significance of the findings from the electron microscopical studies of variola-infected cells at 40° C is not yet clearly understood, and the problem awaits a more exhaustive investigation.

CHAPTER IV

The Effect of the temperature of Incubation on the Formation of Variola Virus Soluble Antigens in Tissue Culture

The effect of the incubation temperature on the formation of three virus-specific products, DNA inclusions, virus antigen, and haemagglutinin, has been reported in Chapter III. The acridine orange staining technique, with and without accompanying haemadsorption, and the fluorescent antibody sandwich technique were used to detect the antigens in situ in infected cells.

Of these antigens, it is possible to assay total virus antigen by complement fixation tests and to analyse antigen diffusing as separate bands by means of immunodiffusion. It is also possible to assay haemagglutinin (HA) by haemagglutination tests.

The complement fixation and haemagglutination tests were applied to the cellular and supernatant fractions of infected cultures sampled during the course of single-step growth cycles of variola virus at various temperatures of incubation. The antigen content of Harvey- and Butler-infected human skin/muscle fibroblast cultures incubated at 37°, 38.5°, and 39.5° C for 3 days was investigated by immunodiffusion.

4.1 The Complement Fixing Antigen

The major complement fixing (CF) antigen is probably 'LS' antigen as described by Craigie and Wishart (1936) for vaccinia virus. This antigen is a protein antigen which is incorporated into the surface

structure of the virus particle (Marquardt, Holn, and Lycke, 1965), and which is produced during the infective cycle in excess of the amount required for the production of mature virus particles.

On standing, a suspension of elementary bodies undergoes dissociation of the LS antigen from its superficial position on the virus particle.

Both soluble LS and LS bound to the virion, and also virus NP antigen are able to fix complement.

Bearing in mind that other antigens may be responsible for a small proportion of fixation, the complement fixation test was used as an indicator system for the presence of LS antigen in variola-infected tissue culture harvests.

In order to establish a) the time of appearance, b) the rate of production, and c) the final yield of complement fixing antigen at various temperatures of incubation, four-hourly cellular and supernatant harvests taken during the course of single cycles of infection of Harvey and Butler virus in human skin/muscle fibroblasts were assayed for complement-fixing activity. The temperatures used were 35°, 37°, 38°, and 39.5° C for both virus strains. Harvey was also grown at the additional temperature of 40° C.

(Materials and Methods - See Appendix)

Results: See Figs. 16 and 17

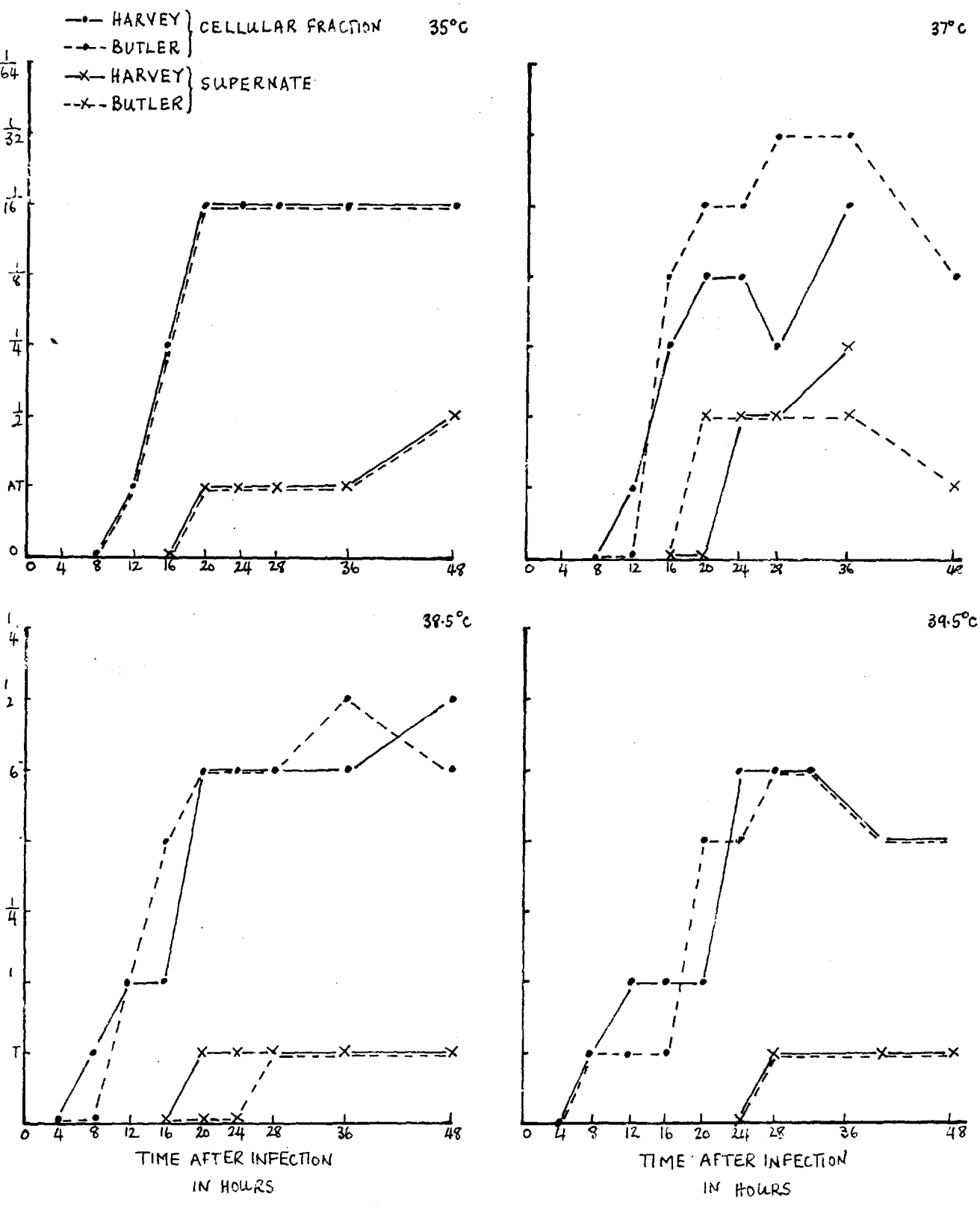
Almost all the complement fixing (CF) activity was cell-associated. The small amount of CF antigen present in the supernatant was probably attributable to loss of cellular material following cell necrosis. Maximum supernatant titres were found at 37° C.

The time of appearance, rate of production, and final concentration of CF antigen in Harvey- and Butler-infected cultures at any one temperature was not greatly different.

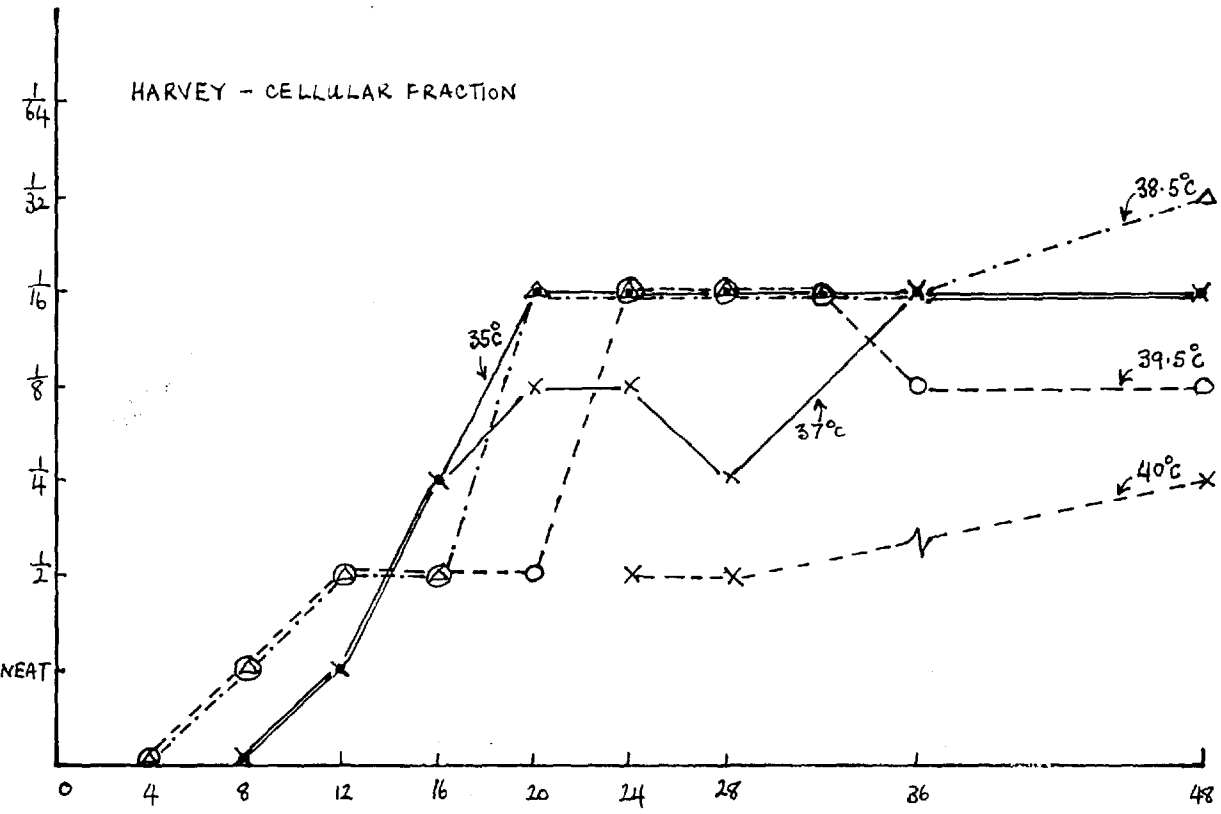
The first increase in CF antigen occurred between 8 and 12 hours at 35° and 37° C and slightly earlier, between 4 and 8 hours, at 38° and 39.5° C. The maximum yields of CF antigen were not altered by raising the incubation temperature from 35° to 38° C. At 39.5° C there was a lag in CF antigen production, greater in Butler-infected cultures than in Harvey-infected cultures, and the maximum yields were slightly reduced. A further delay in the appearance of antigen and a greater suppression of the maximum yield was noted in Harvey-infected cultures at 40° C.

Discussion

Since CF antigen was not significantly suppressed above temperatures at which NP and HA antigens are formed, this is evidence that these two antigens contribute very little to the CF activity of infected cell suspensions.



ig. 16. The production of complement-fixing antigen during single-step growth cycles of variola major (Harvey) and variola minor (Butler) in human skin/muscle fibroblasts incubated at the given temperatures



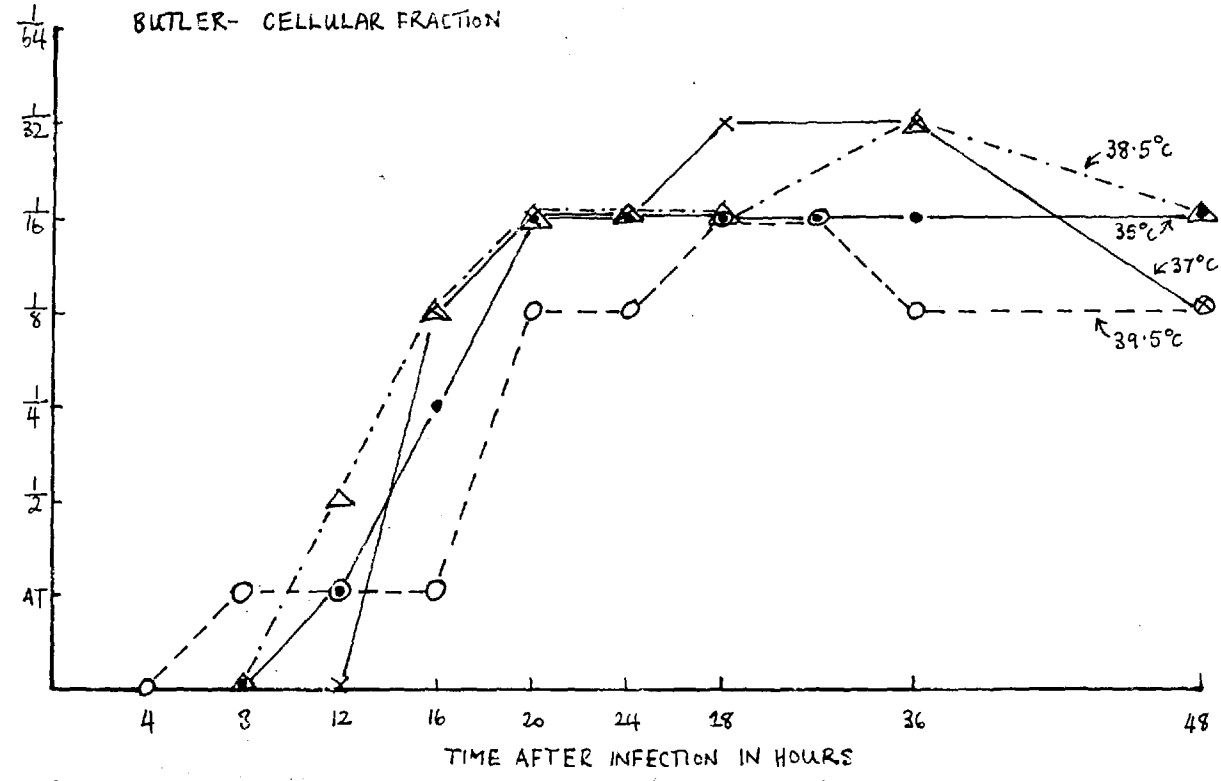


Fig. 17. Comparison of the complement fixing antigen content of cellular fractions of Harvey- and Butler-infected human skin/muscle cultures during single-step growth cycles at 35° to 39.5° C.

Assuming that the CF activity largely represents LS antigen, the results show that the production of at least one structural protein is thermostable at temperatures sufficient to suppress DNA synthesis and HA production.

The results of the complement fixation tests are in agreement with the results from fluorescent antibody studies. Both indicate that antigen(s), which cannot be accounted for by NP and HA, are produced at supraoptimal temperatures. This is further evidence that the synthesis of possibly a range of virus antigens takes place without the participation of new virus DNA. Above the permissive temperature for variola virus growth, virus structural proteins may be formed, but the synthesis of functional DNA or its incorporation into the elements of the nucleoid of virus particles is probably arrested.

Work done by Kajioka, Siminovitch, and Dales (1964), employing metabolic inhibitors to interrupt the cycle of replication of vaccinia (IHD strain) in Earle's L cells at known stages, has shown that vaccinia protein is synthesised in the absence of the synthesis of virus DNA in the cytoplasm. 5-Fluorodeoxyuridine (FUdR) treatment of cells permitted normal penetration and release of naked DNA from uncoated cores of vaccinia particles and was also without effect on the number of factories of viroplasm developing in the cytoplasm. However, these factories were smaller than those developing under normal conditions. Electron micrographs of treated, infected cells showed that those particles which had developed were 'immature' forms

and devoid of nucleoids. The particles were often misassembled, having several membranes. Similar results were obtained by treating cells with Mitomycin C (thought to depolymerise DNA and so prevent its net synthesis); "... protein synthesis can ensue under conditions of severely depressed DNA synthesis". Pseudorabies virus has been quoted as an example of assembly of structural protein into capsids in the absence of the formation of DNA-containing cores (Reissig and Kaplan, 1962).

Rosenkrantz, Rose, Morgan, and Hsu (1966) have more recently shown that appropriate concentrations of hydroxyurea prevented synthesis of vaccinia virus DNA, but not virus protein. The end product of replication under such conditions was an 'immature' virus particle lacking a nucleoid.

These observations and those of Becker and Joklik (1964) and Salzman, Shatkin, and Sebring (1964) have been taken to "... indicate that the replication of viral DNA is not required for its transcription into specific mRNA and consequently not required for the manufacture of proteins".

Supporting evidence has been tendered by Easterbrook (1963), Shatkin (1963), and Loh and Payne (1965) who have used FUdR to show that suppression or blockage of the production of infective virus does not prevent protein synthesis and the development of immature virus particles.

The electron microscopy of variola-infected human skin/muscle fibroblasts incubated above the permissive temperature has been discussed in chapter III. The results supported the speculation that virus DNA is not assembled into nucleoids under these conditions. However, 'immature' virus particles, as demonstrated in the metabolic inhibitor-treated cell systems reviewed above, were not seen in the variola-human fibroblast system at 40° C. An abnormal membrane occurring only in infected cells above the permissive temperature was demonstrated. This structure may possibly incorporate antigens detected by complement fixation tests.

4.2 Diffusible Antigens

Immunodiffusion is an essentially qualitative method for studying antigen and antibody systems. The basic principle lies in the mutual diffusion of antigens and antibodies from opposing wells in a layer of agar gel contained in a Petri dish (Ouchterlony, 1948).

Homologous antigens and antibodies diffusing towards each other form lines of precipitation (line components) where they meet at optimal concentrations. Where several homologous components are present in an antigen/antibody system, a series of line components will form between the antigen and antiserum wells. Each line of precipitation occurs at the point of optimal concentrations of the antigen/antibody pairs involved.

Two or more antigenic systems can be compared by allowing their components to diffuse through the agar gel from adjacent wells towards a common antiserum. Lines of precipitation will form where homologous antigens and antibodies in the systems under test meet.

If two or more adjacent antigenic systems contain a common component, then the lines of precipitation so formed will meet and fuse at the point of intersection. A "reaction of identity" common to these systems is thus produced. Where lines of precipitation are formed which do not have counterparts in adjacent systems, then the line components cross, without fusion at the intersection, all other lines of precipitation and pass on independently through the

agar gel. Such lines of precipitation are "reactions of non-identity".

A line of precipitation, however, may be formed by a complex of two or more antigenic components diffusing together. The 'LS' antigen of poxviruses, to be discussed later, is such a complex. If an adjacent antigen system contains one, but not the full complement of the components of the complex, then the line of precipitation formed by the antigenic complex and its homologous antibodies will be seen to divide. One part of the complex line component will fuse with the line of precipitation formed by the common antigen in the adjacent system, thereby giving a "reaction of partial identity"; the other part will pass on independently, without fusion, as a line of non-identity.

The immunodiffusion technique has been extensively used as a qualitative method for resolving antigenic components formed in poxvirus-infected tissues. Dumbell and Nizamuddin (1959) used gel-diffusion as a routine procedure for the laboratory diagnosis of smallpox. Rondle and Dumbell (1961) used immunodiffusion as a research tool for the investigation of the soluble antigens produced in the chick embryo chorioallantoic membrane and in the rabbit skin by infection with cowpox virus and its 'white variants'.

The immunodiffusion technique has been applied in this thesis to investigate possible qualitative differences in the diffusible antigens produced during the infection of human skin/muscle fibroblast cultures at 37°, 38.5°, and 39.5° C with the Harvey strain of variola major and the Butler strain of variola minor.

Cellular and supernatant material was harvested from variola-infected cultures and concentrated 70-fold by drying from the frozen state. The concentrated samples, denoted H37, H38.5, H39.5, B37, B38.5, and B39.5, were tested for diffusible antigen against a hyperimmune antivaccinia rabbit antiserum.

Lines of precipitation were identified by comparison with antigenic preparations giving known line components against the antivaccinia rabbit antiserum. Crude preparations of variola soluble antigen, extracted by centrifugation from infected chick embryo CAMs disintegrated by shaking with glass beads, give two lines of precipitation against an antivaccinia rabbit antiserum. The more prominent of these two lines is due to the soluble 'LS' antigen.

'LS' antigen is an antigenic complex containing a heat-stable antigen component 'S' and a heat-labile antigen component 'L'. In heated preparations of crude variola soluble antigen, the capacity for 'L' to combine with antibodies to 'L' antigen in an immune serum is lost. Thus, the single line of precipitation due to the LS complex in the unheated soluble antigen preparation gives a reaction of partial identity with the single line component given by heated soluble antigen when both are tested against an antivaccinia antiserum.

The line pattern given by concentrated preparations of variola soluble antigen derived from infected human skin/muscle fibroblasts was compared with the line patterns given by heated and unheated egg preparations of variola soluble antigen.

(Materials and Methods - See Appendix)

Results

i) See Plate 17

When tested against a hyperimmune antivaccinia antiserum three (H37, H38.5, and B37) of the six concentrated samples gave two lines of precipitation, (1) and (2). Each of the two line components was common to these three samples. Samples B38.5, H39.5, and B39.5 did not give lines of precipitation.

ii) See Plate 18

Line component (1) given by H37, B37, and H38.5 gave a reaction of identity with the single line component given by heated soluble antigen, and therefore a reaction of partial identity with the major line of precipitation given by unheated variola soluble antigen.

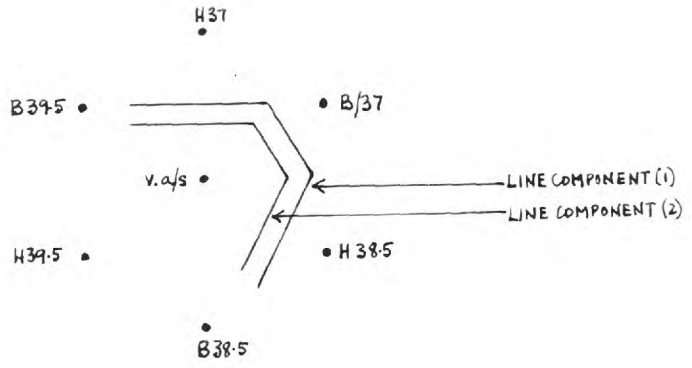
Discussion

Two antigenic components detectable by immunodiffusion against an antivaccinia rabbit antiserum are formed in variola-infected human skin/muscle fibroblast cultures at 37° C.

Line component (1) was identified with the line of precipitation given by the 'S' component of the 'LS' antigen of crude preparations of variola soluble antigen produced in egg and is therefore assumed to represent a structural protein formed by variola virus in human



PLATE 17.



KEY TO PLATE 17.

A comparison of the antigenic components produced in Harvey- and Butler-infected human skin/muscle cultures incubated at 37°, 38.5°, and 39.5° C. Preparations of Harvey- and Butler-infected cell cultures concentrated by drying from the frozen state, H37, H38.5, H39.5, B37, B38.5, B39.5; hyperimmune antivaccinial rabbit antiserum = v.a/s.

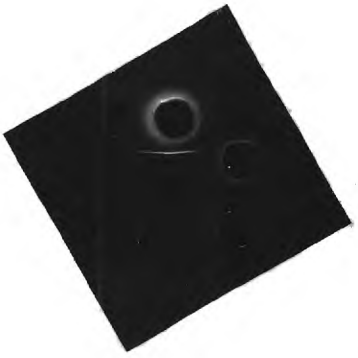
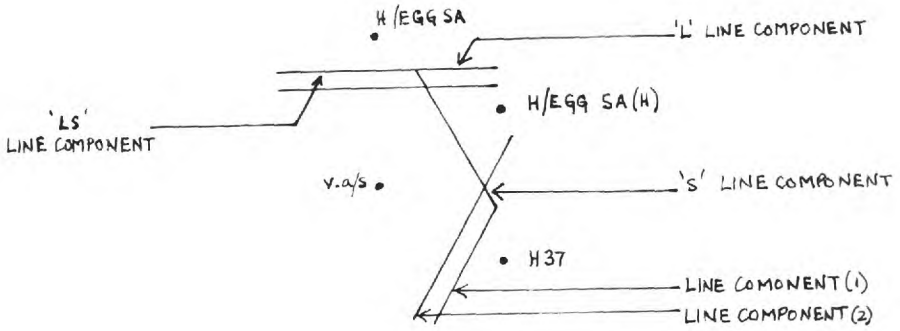


PLATE 18.



KEY TO PLATE 18.

A comparison of the antigenic components produced in Harvey-infected human skin/muscle cultures incubated at 37° C. with those contained in heated and unheated egg preparations of Harvey soluble antigen.

Preparation of Harvey-infected human cell culture concentrated by drying from the frozen state, H37; unheated and heated egg preparations of Harvey soluble antigen, H/EGG SA, and H/EGG SA(H) respectively; hyperimmune antivaccinial rabbit antiserum = v.a/s.

fibroblasts. A line identifiable with the 'L' component of 'LS' was not detected in these samples of variola-infected human fibroblasts. Line component (2) was not identified. It was not present in heated egg preparations of variola soluble antigen, but did not represent 'L' antigen.

Both the antigenic components represented by the lines of precipitation (1) and (2) were produced at 37° and 38.5° C in Harvey infected cultures, but only at 37° C in Butler-infected cultures.

In the preparation of infected tissue culture material for antigen analysis, fibroblast monolayers were infected under conditions allowing multiple cycles of infection. It has been shown (Chapter II and Chapter III-3.1) that the Harvey strain of variola major is able to grow and spread vigorously through susceptible cell cultures at both 37° and 38.5° C, whereas the Butler strain of variola minor is only able to grow and spread effectively at 37° C; at 38.5° C Butler virus spreads very slowly from original foci of infection. Neither Harvey nor Butler viruses are able to spread through cell cultures at 39.5° C. Hence, the detection of the two antigenic components (1) and (2) in samples H37, H38.5, and B37, but not in B38.5, H39.5, and B39.5 probably represented a quantitative difference in the amount of antigen formed, rather than a qualitative difference stemming from a difference in the ability of the two strains of variola virus to form antigens at the temperatures tested in cells which had been successfully infected.

Using the immunodiffusion technique on a microscale and employing monospecific antisera, Bedson and Cruickshank (unpublished results) have demonstrated up to seven antigenic components which are produced in variola-infected HeLa cells at a range of temperatures from optimal to supraoptimal. As the temperature of incubation is raised, there is a slight delay in the production of the antigenic components. At 40° C, which is above the permissive temperature for the Hinden strain of variola major used, the production of one of the prominent antigenic components (the 'LS' antigen) is considerably delayed and reduced in quantity.

The immunodiffusion tests reported here suggest that there is a quantitative, but not qualitative, difference in the diffusible antigens produced in variola major- and minor-infected human cells at a range of temperatures from optimal to non-permissive.

The investigation of the diffusible antigens produced during single-step growth cycles of the two strains of variola virus at various temperatures, using antisera specific for single antigenic components, would possibly confirm this conclusion.

4.3 The Haemagglutinin

Variola virus growing on the CAM of fertile eggs or in tissue culture causes the formation of a phospholipoprotein complex (haemagglutinin) which is not part of the virion and which will agglutinate the red blood cells of some hens (haemagglutination). Joklik (1966) has suggested that HA production is a virus-directed cellular function. HA is both virus-specific and cell-specific; it is neutralized by immune serum and yet its physical characteristics vary with the host cell source.

McCarthy and Helbert (1960) found no differences in the haemagglutinins of alastrim and smallpox virus in inducing anti-HA antibody in the human subject, or in the range of species of RBC agglutinated.

Marennikova, Gurvich, and Yumasheva (1964) found that HA was either present in very low titres or absent from tissue culture infected with variola virus. Experiments to be reported here will show that at optimal temperatures for infection the titre of HA in cultures infected with high doses of virus was such as would permit comparative studies under conditions of varied incubation temperatures.

It was briefly reported in chapter III that, under conditions of single-step growth cycles, HA production was increasingly suppressed as the temperatures of incubation were raised. Ha production by Butler virus was more temperature-sensitive than that by Harvey virus.

Thus, the thermostability of the process(es) resulting in the production of HA would seem to be a strain characteristic.

In order to study the kinetics of HA production at different temperatures of incubation, four-hourly cellular and supernatant samples were taken during the course of single-step growth cycles of Harvey and Butler. Growth cycles were conducted at 35°, 37°, 38°, and 39.5° C in human skin/muscle fibroblasts for both Harvey and Butler, and at the additional temperature of 40° C for Harvey.

(Materials and Methods - See Appendix)

Results: See Figs. 18 and 19

Discussion

Since very little HA activity was found in the supernates, the following discussion will be confined to that found in cellular fractions.

The effect of the temperature of incubation on the formation of the HA antigen as detected by HA assay confirmed the difference between the two strains mentioned above.

The highest titre of HA in Harvey- and Butler-infected cultures was attained at 35° C, being detectable after the 16th hour of infection. The initial rate of HA production was slightly slower

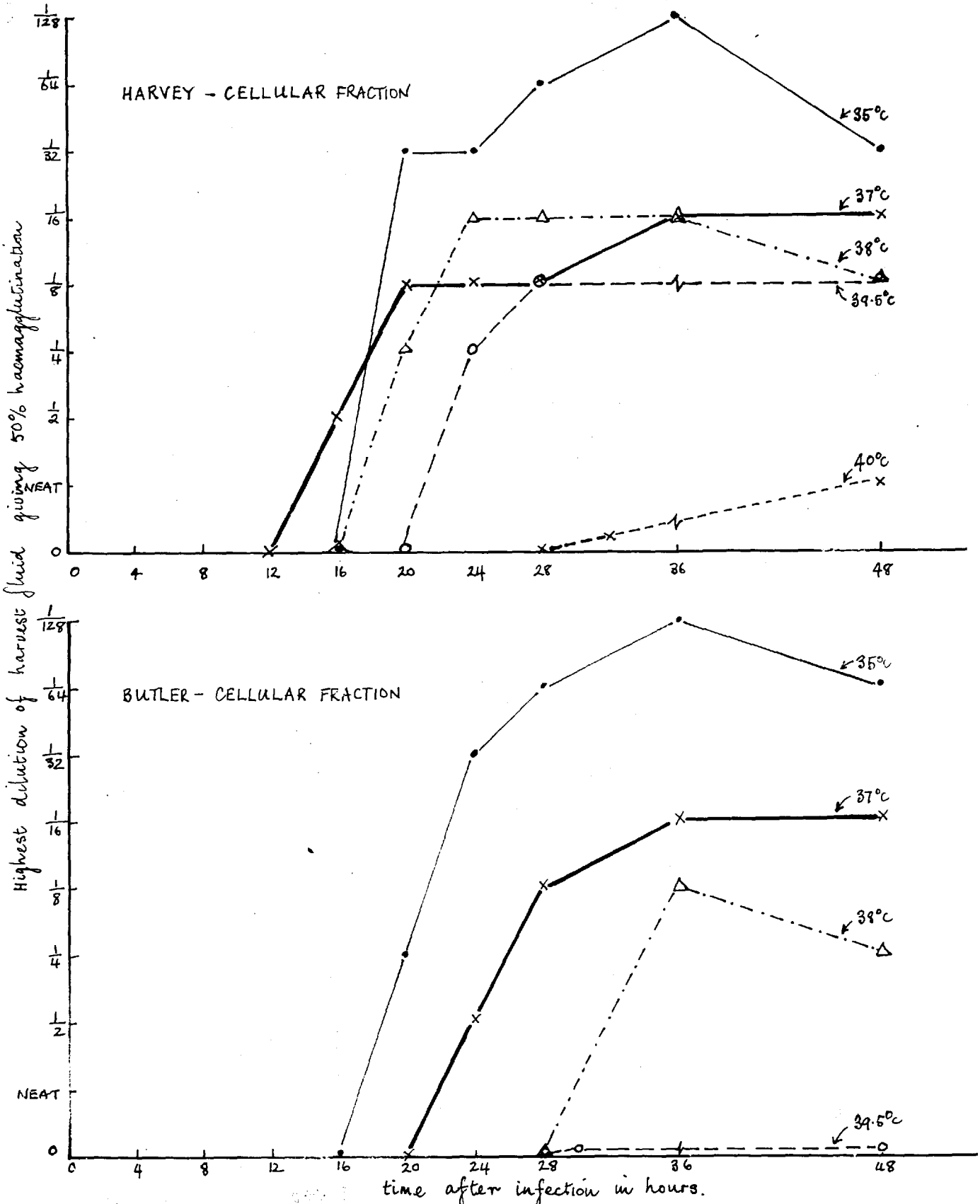


Fig. 18. Comparison of HA content of cellular fractions of Harvey- and Butler-infected human skin/muscle cultures during single-step growth cycles at 35° to 39.5° C.

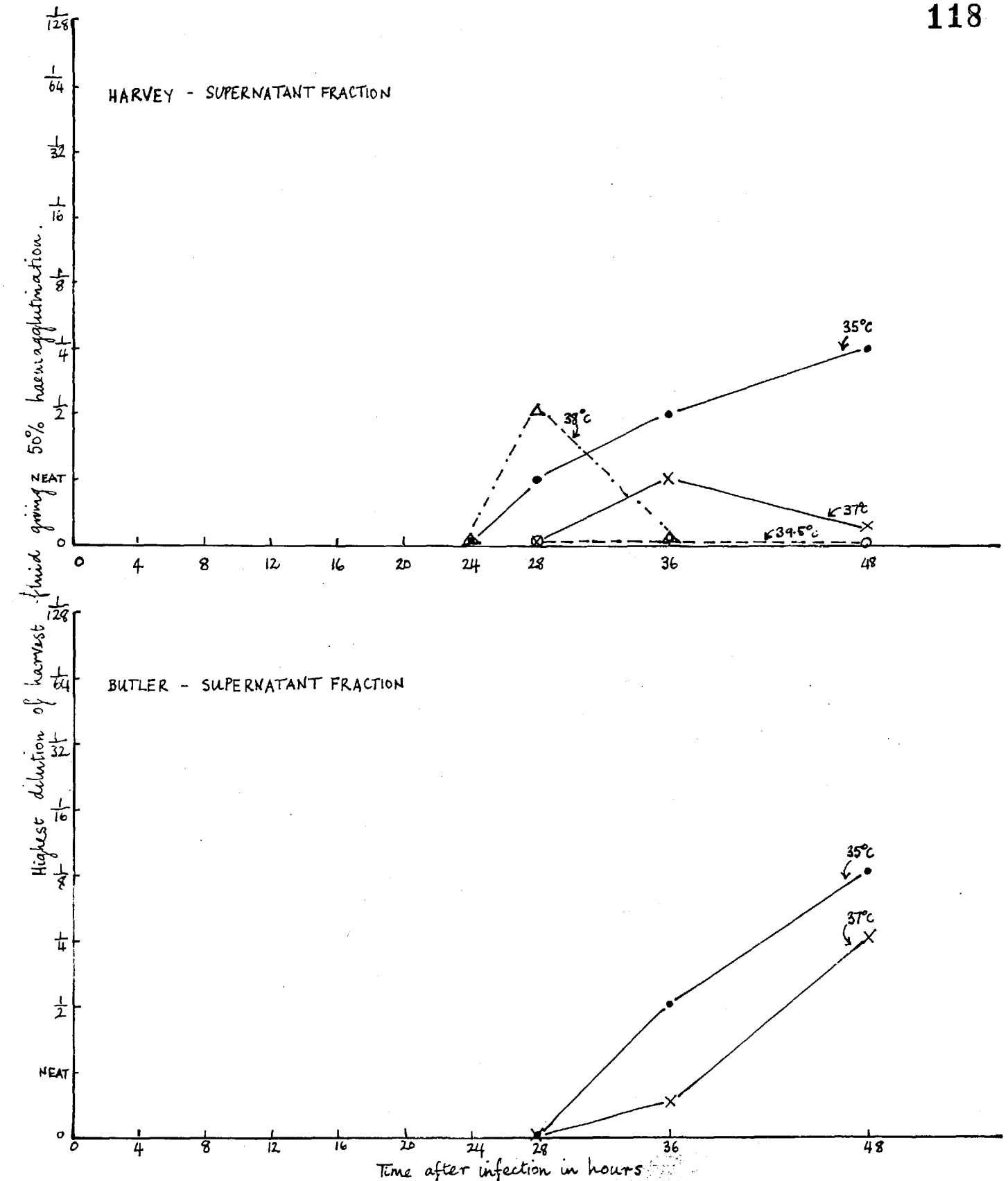


Fig. 19. Comparison of HA content of supernates of Harvey- and Butler-infected human skin/muscle cultures during single-step growth cycles at 35° to 39.5° C.

in the case of Butler-infected cultures, but the peak titre occurred at the same time as, and was not different from, that of Harvey HA.

At 37° C Harvey HA was detectable in infected cultures from the 12th hour, earlier than at 35° C. Butler HA did not appear until the 20th hour, later than Harvey HA at this temperature and later than Butler HA at 35° C. By 36 hours the maximum titres had been reached at 37° C and were 8-fold lower than the maximum titres at 35° C, being the same for both strains.

At 38° C the time of appearance of Harvey HA was delayed as compared with the time of appearance at 37° C, but the maximum titre remained the same as at 37° C. The appearance of Butler HA was further delayed until after 28 hours; the maximum titre at 36 hours was 2-fold less than the titre of Harvey HA at the same temperature. The HA content of the cell fractions of both strains of virus showed a decline after 36 hours.

The difference between Harvey and Butler HA production was even greater at 39° C. Harvey-infected cultures contained HA after the 20th hour, the final titre at 48 hours being the same as that at 38° C. Butler-infected cultures at this temperature contained no detectable HA at any time.

A temperature of 40° C considerably suppressed the formation of HA in Harvey-infected cultures as compared with the temperature of 39° C. A low titre of HA was detectable by 48 hours.

Thus, the time of onset of HA production, rate of production and final yield of HA in Butler-infected cultures was much more sensitive to suppression by raised temperatures of incubation than was that in Harvey-infected cultures. Between 35°- 38.5° C, the maximum titres of HA produced by the two strains was not greatly different at any one temperature. The significant differences between the production of HA by Harvey and Butler lay in (a) the yield of HA at 39.5° C, and (b) the time of onset of HA production at 37° and 38.5° C.

The pattern of HA production reflected the general pattern of virus growth as reported in Chapter II in that HA in Butler-infected cultures was more sensitive to temperatures of 37° and 38.5° C than was that in Harvey-infected cultures.

It was interesting to find that HA was formed in Harvey-infected cultures incubated at 39° C. At this temperature, discrete DNA inclusions were formed (Chapter III), but no increase in infectivity was detectable. Hence, HA was synthesised in the absence of mature virus production. It has been reported (Loh and Riggs, 1961; Oda, 1963) that poxvirus HA production is detectable only after production of mature virus has occurred. However, Baxby (1967) showed that virus production can be suppressed with or without an accompanying suppression in HA formation. 5-bromodeoxyuridine (BUdR) and mitomycin-C suppresses virus synthesis without affecting HA synthesis; FUdR and hydroxyurea suppresses both virus production and HA synthesis.

The property of HA production has been used as a genetic marker in analyses of the biological characters of poxviruses and in genetic studies (Fenner, 1958). Oda (1964) exploited the surface position

of HA in the genetic analysis of the HA marker by combining the techniques of plaque formation and HAD.

A comparison of the production of HA by Harvey and Butler viruses in human skin/muscle fibroblasts at a range of incubation temperatures suggests that HA production at a group of three temperatures, 37°, 38.5°, and 39.5° C, might prove to be a useful genetic marker in differentiating between smallpox and alastrim strains of variola virus in tissue culture. This possibility will be investigated in chapter V.

CHAPTER V

Haemagglutinin

Observations on the production of haemagglutinin (HA) in human skin/muscle fibroblast cultures infected with large doses of the Harvey and Butler strains of variola virus at 37°, 38°, and 39.5° C indicated that a differentiation between the two strains might be possible based on the relative capacity for HA production at these temperatures.

5.1 Some General Considerations

Before comparative experiments could be performed, it was necessary to investigate the following points:

a) The relationship between the HA particle and the phenomena of haemadsorption (HAD) and haemagglutination (HA^n).

Only a proportion of fowl have red blood cells (RBC) which are agglutinable by poxvirus HA. Because inagglutinable fowl cells have been said to haemadsorb to poxvirus-infected cell monolayers (Dekking, personal communication), it was decided to test the relationship between HAD, HA^n, and the HA particle.

Variola-infected cell monolayers, potentially capable of giving confluent HAD, were flooded with suspensions of known agglutinable and inagglutinable RBC in parallel and incubated at 37° C for 1 hour.

The infected monolayers flooded with agglutinable RBC were found to give confluent HAD, whereas those monolayers incubated with inagglutinable RBC showed that only a very small proportion of the RBC had been adsorbed.

These results indicated that each fowl has a certain proportion of 'agglutinable' and 'inagglutinable' cells in their population of RBC and that this proportion varies with individual birds. This idea is consistent with the fact that agglutinable RBC from different birds give varying titres of HA when the RBC are tested against a single antigen. It is not hard to visualize that when the proportion of agglutinable cells in a RBC population is low, any agglutination of RBC would be obscured by inagglutinated cells and thus pass undetected in a haemagglutination test.

Thus, on the basis of these results it would seem that a claim that inagglutinable RBC haemadsorb is unfounded.

Other results to be reported in this chapter show that no haemagglutinating activity was found in the absence of HAD. Accepting a threshold level factor for the detection of HA^n, the converse is also true.

It would seem that HAD and HA^n are '<u>in situ</u>' and '<u>in vitro</u>' expressions of a single factor HA.

b) The comparative merits of the available methods for HA assay.

Assuming that a superficial HA is the sole agent responsible both for <u>in vitro</u> haemagglutination and <u>in situ</u> haemadsorption, three

methods of assay were available:

i) direct assay of suitably disrupted cell suspensions by HA[a],

ii) direct HAD of infected monolayers, and

iii) assay of oxyhaemoglobin at 410 mμ released from haemadsorbed RBC. This is the quantitative haemadsorption (QH) method of Finter (1964).

Each method has its inherent advantages and disadvantages.

HAD was qualitatively the most sensitive since a small number of single-infected cells in a monolayer showing HAD were easily detected. Assay of the extent of HAD was limited to description of the number and size of foci of HAD in random fields of view or to the semiconfluence or confluence of HAD on the entire monolayer. No comparison of the quantity of HA produced was possible.

Parallel HA[a] assays and HAD assessments showed that where many small foci of HAD on a cell sheet were obvious, duplicate monolayers disrupted by sonication gave negative results even for the undiluted cell suspension in the HA assay.

A comparison of direct HAD with the QH method revealed that the difference in percentage transmission given by varying degrees of HAD was less than could be detected by inspection of HAD of monolayers by eye. Monolayers with many isolated foci of HAD gave percentage transmissions ranging from 75-77 %, whereas monolayers of semiconfluent HAD gave transmissions up to 72 %. The difference between no HAD and severe foci of HAD was only 1 %, less than the variation obtained from individual uninfected tubes.

Application of the QH method in this instance was therefore considered unnecessarily laborious and of no advantage.

The main advantage of the HA[n] assay was that all HA produced was harvested, whereas in methods involving HAD, no account can be made for haemadsorbing cells detached from the glass. The washing of the cell sheet to remove unadsorbed RBC necessary in both direct HAD and in the QH method only increased these losses.

HA[n] was quantitative in spite of a fairly high threshold level for detection of activity; HAD was very sensitive qualitatively.

It was therefore decided that direct HAD at 24 hours and 48 hours and HA[n] assay at 72 hours would be the most suitable ways of recording HA activity of infected cell sheets for comparative purposes.

The degree of haemadsorption shown by infected monolayers was scored in the following way:

confluent HAD	5
semiconfluent HAD	4
many merging foci of HAD	3
several isolated foci of HAD	2
several single HADing cells	1
no HAD	0

Haemagglutination was scored as follows:

100 %	HA[n]	+
75 %	"	75
50 %	"	50
25 %	"	25
trace	"	t
no	"	-

c) The dose-response relationship between virus input and the yield of HA.

Before direct comparisons of the HA yields of infected cultures could be made it was necessary to test the dependence of the final yield of HA upon the input multiplicity of virus.

Groups of confluent human skin/muscle fibroblast monolayers in tubes were inoculated with 5×10^7, 5×10^6, 5×10^5, 5×10^4, and 5×10^3 pfu Harvey virus/tube and incubated in triplicate at 37°, 38.5°, and 39.5° C for 72 hours. The HA yield in each group of cultures was assayed by the HAn test. The highest dilution of sonicated, harvested cell suspension giving 100 % HA was taken as the endpoint in the titration.

The results are expressed in fig. 20. At each temperature, those virus inocula (5×10^7, 5×10^6, and 5×10^5 pfu/tube) expected to give multiplicities greater than 1 resulted in a final yield of HA which did not vary in spite of a hundred-fold range in the virus dose. Below multiplicities of 1 (inocula of 5×10^4 and 5×10^3 pfu/tube), the final yield of HA varied with the virus dose; increasing proportions of susceptible cells failed to become infected during the absorption of virus as the virus dose was reduced.

Thus, as long as each cell of a culture is infected (and this can be established visually), accurate adjustment of the virus dose is not necessary for the comparison of HA production. A cell produces the same quantity of HA whether infected by 1 or by 100 virus particles.

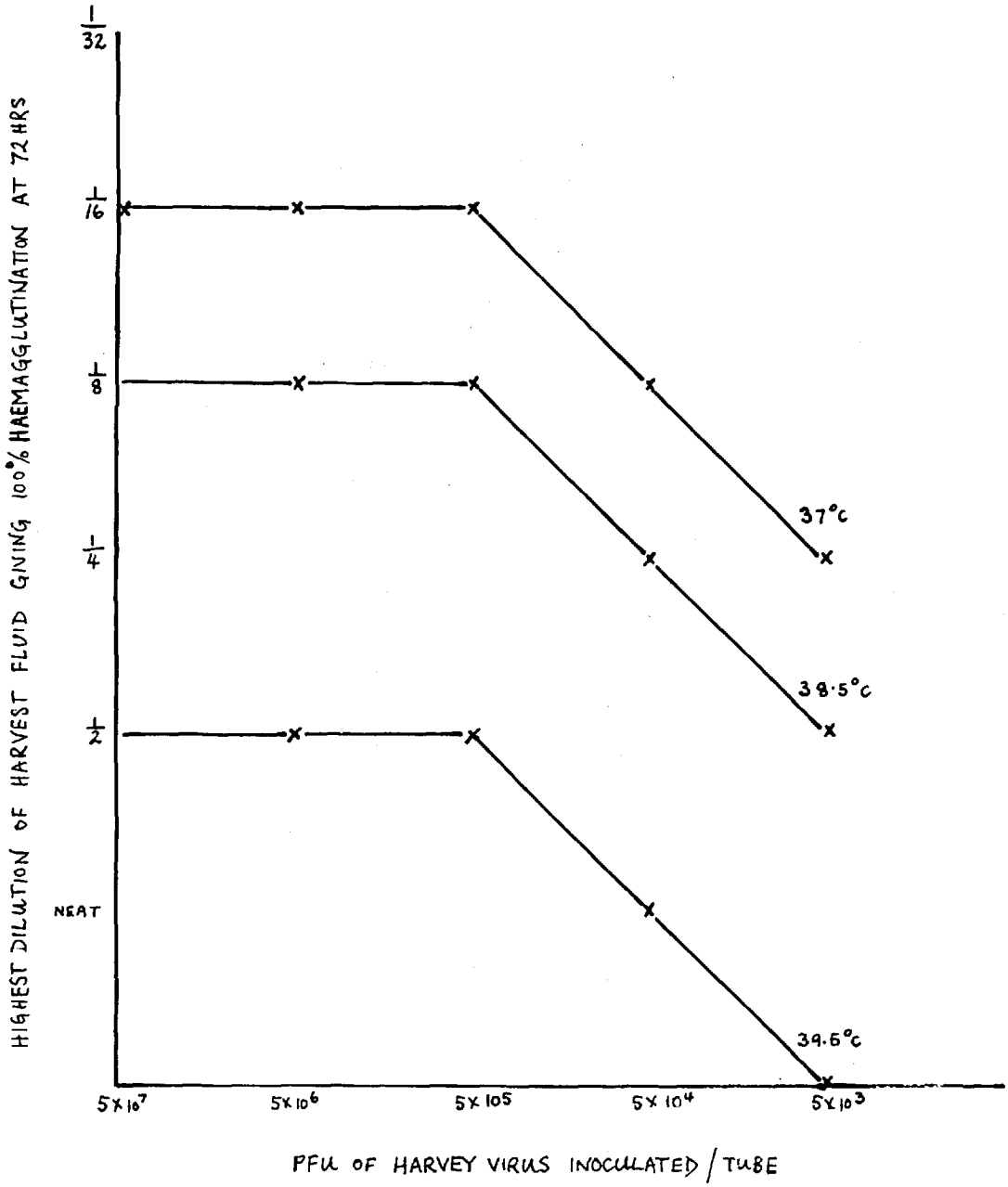


Fig. 20. The Dose-response relationship between virus input and HA yield as measured in Harvey-infected human skin/muscle fibroblast cultures incubated from 37° to 39.5° C for 72 hours.

d) Aggregation of the HA particle

The fall in titre shown by suspensions of HA on storage, particularly in the frozen state, has been shown to be due to an aggregation of HA particles into polymer complexes (Neff, Ackermann, and Preston, 1965).

In experiments to be described, samples thawed in readiness for HA assay were exposed to 15-30 seconds of ultrasonic vibration at 80 kc/s. The HA titres were restored to the original level after this treatment.

5.2 Production of HA in Various Cell Types

Table 2 shows the production of HA by Harvey and Butler in human skin/muscle fibroblasts at 37°, 38.5°, and 39.5° C as detected by HAD and HA^R tests.

TABLE 2

Strain	HAD (24 hrs)	HAD (48 hrs)	HA^R (72 hrs) N	1/2	1/4	1/8	1/16	1/32	1/64
Harvey 37°	5	5	+	+	+	+	+	+	+
38.5°	4	5	+	+	+	+	+	-	-
39.5°	2	4	+	+	+	-	-	-	-
Butler 37°	3	5	+	+	+	+	+	-	-
38.5°	1	4	+	+	-	-	-	-	-
39.5°	0	0	-	-	-	-	-	-	-

HA production by Harvey and Butler in human skin/muscle fibroblasts

The difference between the two strains lies in the extent of HAD at 37° C and 38.5° C at 24 hours, and in the extent of HAD at 38.5° and 39.5° C at 48 hours.

Although this difference is relative and not absolute, the difference in performance over the group of three temperatures is quite marked. Butler appears to show no HAD at 39.5° C, but it is known (chapter III) that a very small proportion of single cells do develop HA; the few positive cells were probably unnoticeable in a confluent cell sheet.

The possibility that other sources of tissue may support a greater difference in HA production at a range of temperatures than was found in human skin/muscle fibroblasts was next considered.

The following species of strains and lines of cells were screened:

1) Mammalian - human:

primary strain: amnion, lung
continuous line: Chang conjunctiva (Chang, 1954)

2) Avian - hen:

primary strain: chick embryo fibroblasts

3) Reptilian - turtle:

continuous line: $ThIB_2$-75 fibroblasts (Clark and Karzon, 1965)

Results - See Table 3

TABLE 3

Cell Strain	Virus/Temp. (°C)		HAD (24 hrs)	HAD (48 hrs)	HA^n (72 hrs) N	1/2	1/4	1/8	1/16	1/32
Human Amnion	Harvey	37	5	5	+	+	25	-	-	-
		38.5	3	5	+	+	t	-	-	-
		39.5	2	5	t	-	-	-	-	-
	Butler	37	2	5	+	+	75	t	-	-
		38.5	0	4	t	-	-	-	-	-
		39.5	0	3	-	-	-	-	-	-
Human lung	Harvey	37	4	5	+	+	+	+	+	+
		38.5	4	4	+	+	+	+	+	+
		39.5	4	4	+	+	+	75	t	-
	Butler	37	4	5	+	+	+	+	+	+
		38.5	2	2	t	-	-	-	-	-
		39.5	0	0	-	-	-	-	-	-

TABLE 3 cont'd

Cell strain	Virus/Temp (°C)		HAD (24 hrs)	HAD (48 hrs)	HA^{RE} (72 hrs) N	1/2	1/4	1/8	1/16	1/32
Human Chang Conjunctiva	Harvey	37	5	5	+	+	+	+	+	-
		38.5	4	5	+	+	+	+	-	-
		39.5	2	5	+	+	+	-	-	-
	Butler	37	3	5	+	+	+	+	-	-
		38.5	0	3	+	+	-	-	-	-
		39.5	0	0	-	-	-	-	-	-
Chick Embryo Fibroblasts	Harvey	25		4	t	-	-	-	-	-
		30		5	+	+	+	+	-	-
		37		5	+	25	t	-	-	-
		38.5		3	t	-	-	-	-	-
		39.5		2	t	-	-	-	-	-
	Butler	25		2	-	-	-	-	-	-
		30		4	t	-	-	-	-	-
		37		3	t	-	-	-	-	-
		38.5		2	-	-	-	-	-	-
		39.5		1	-	-	-	-	-	-
	(Vaccinia)	37	5		+	+	+	+	+	75
		41-42	3		-	-	-	-	-	-
Continuous Line Turtle Fibroblasts $TnIB_2$-75	Harvey	25		2	-	-	-	-	-	-
		37	5	5	+	+	+	+	+	+
		38.5	4	4	+	+	+	+	-	-
		39.5	2	2	-	-	-	-	-	-
	Butler	25		0	-	-	-	-	-	-
		37	4	4	+	+	+	+	+	-
		38.5	2	2	+	-	-	-	-	-
		39.5	0	0	-	-	-	-	-	-

The production of HA in Harvey- and Butler-infected cells of various species and types incubated at a range of temperatures from 25°-39.5° C.

Discussion

From Table 3 it can be seen that Harvey and Butler produced HA with varying degrees of efficiency in the several cell types at the range of temperatures tested.

Cells of rabbit origin, primary rabbit kidney and continuous live rabbit aorta, were also used. Since variola virus grew poorly in these tissues, experiments in HA production were not very successful. Primary rabbit kidney and rabbit aorta cells were not readily available in the laboratory and attempts to adapt Harvey and Butler to rabbit tissue were therefore abandoned.

It was found that the higher titres of HA were regularly obtained in human foetal lung cultures. Whereas Harvey produced good titres of HA at all temperatures in this cell strain even at 39.5° C, Butler produced little HA at 38.5° and 39.5° C.

As human foetal lung cells supported a greater overall difference in the ability of Harvey and Butler to produce HA at 37°, 38.5°, and 39.5° C, this strain of human cells was considered to be potentially useful in the differentiation between variola major and minor strains with respect to HA formation.

5.3 Smallpox, Alastrim, and East African Variola Strains Screened for HA production at Temperatures from 36°- 40° C in Human skin/muscle Fibroblast Cultures and in Human Lung Cultures

In order to establish whether the noted difference between the ability of the Harvey strain of variola major and the Butler strain of variola minor to produce HA at 37°, 38.5°, and 39.5° C was characteristic of major and minor strains in general, a number of strains isolated from cases of epidemic and endemic variola and known to be clinically major or minor were also tested for HA at a range of temperatures.

Several East African variola strains isolated from smallpox cases in Kenya and Tanganyika were also included in the tests. The East African strains which had been isolated from mild cases of smallpox had been found by Bedson, Dumbell, and Thomas (1963) to be "intermediate" between true variola major and minor in two laboratory tests. These strains, in general, behave more like variola major in the pock-reduction test, but more like alastrim in the chick-embryo virulence test.

(Materials and Methods - See Appendix)

Results

a) Screening in human skin/muscle fibroblast cultures (Figs. 21, 22, 23)

In spite of some internal variation, even among strains isolated from a single outbreak ('Campbell' and 'McGinty' from the Glasgow

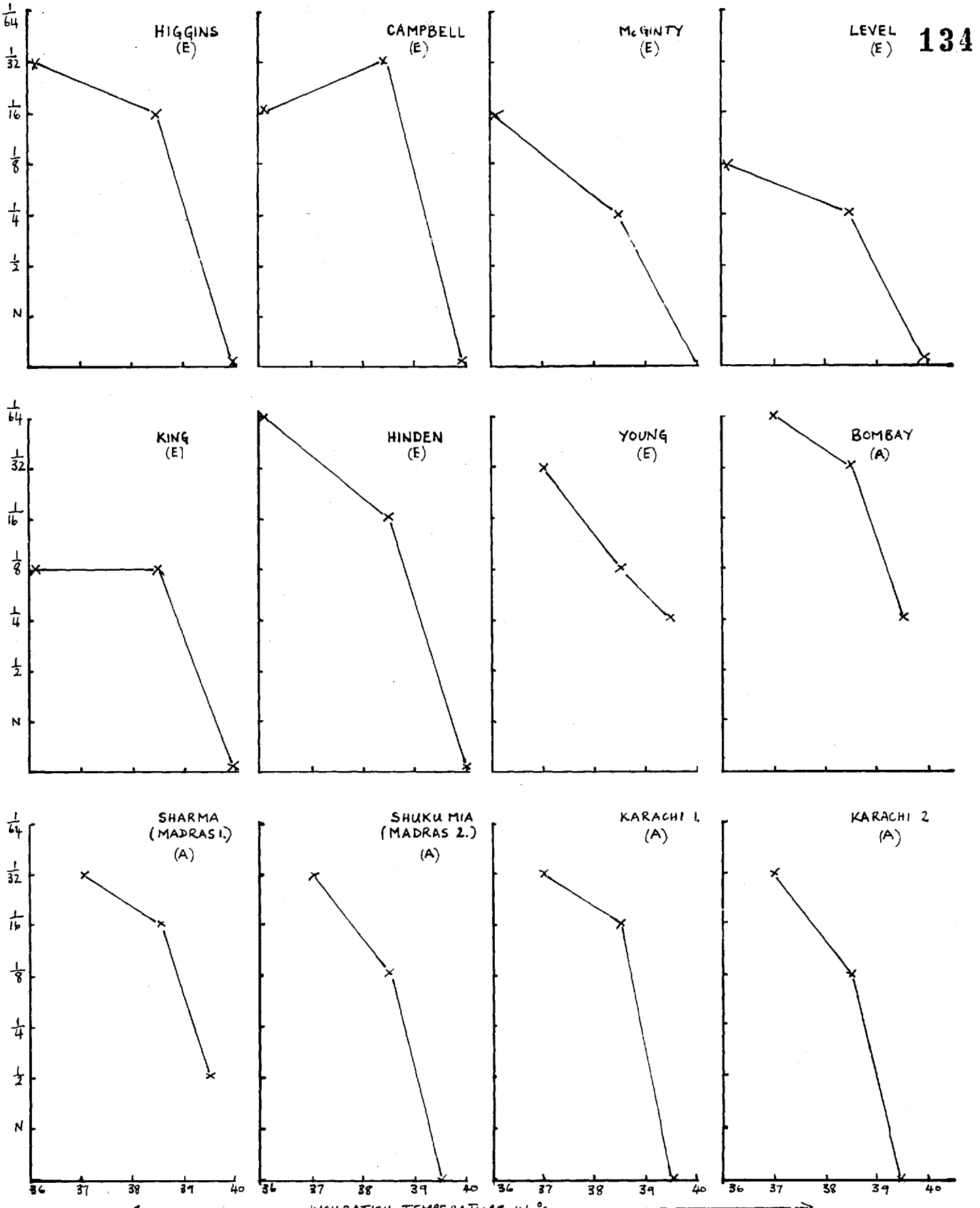


Fig. 21. The 72 hour yield of HA in human skin/muscle cultures infected with European (E) and Asian (A) strains of variola major and incubated from 36° to 40° C.

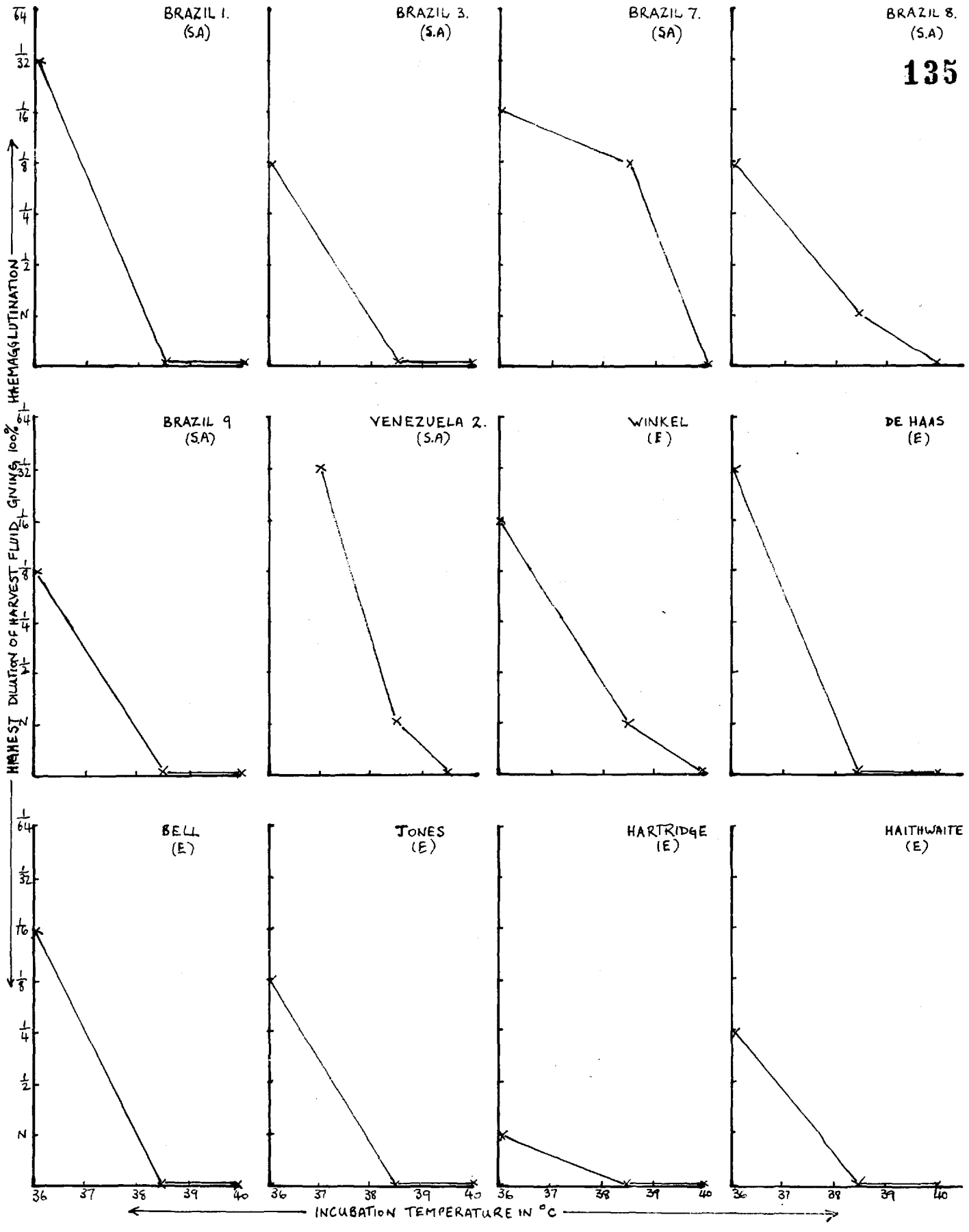
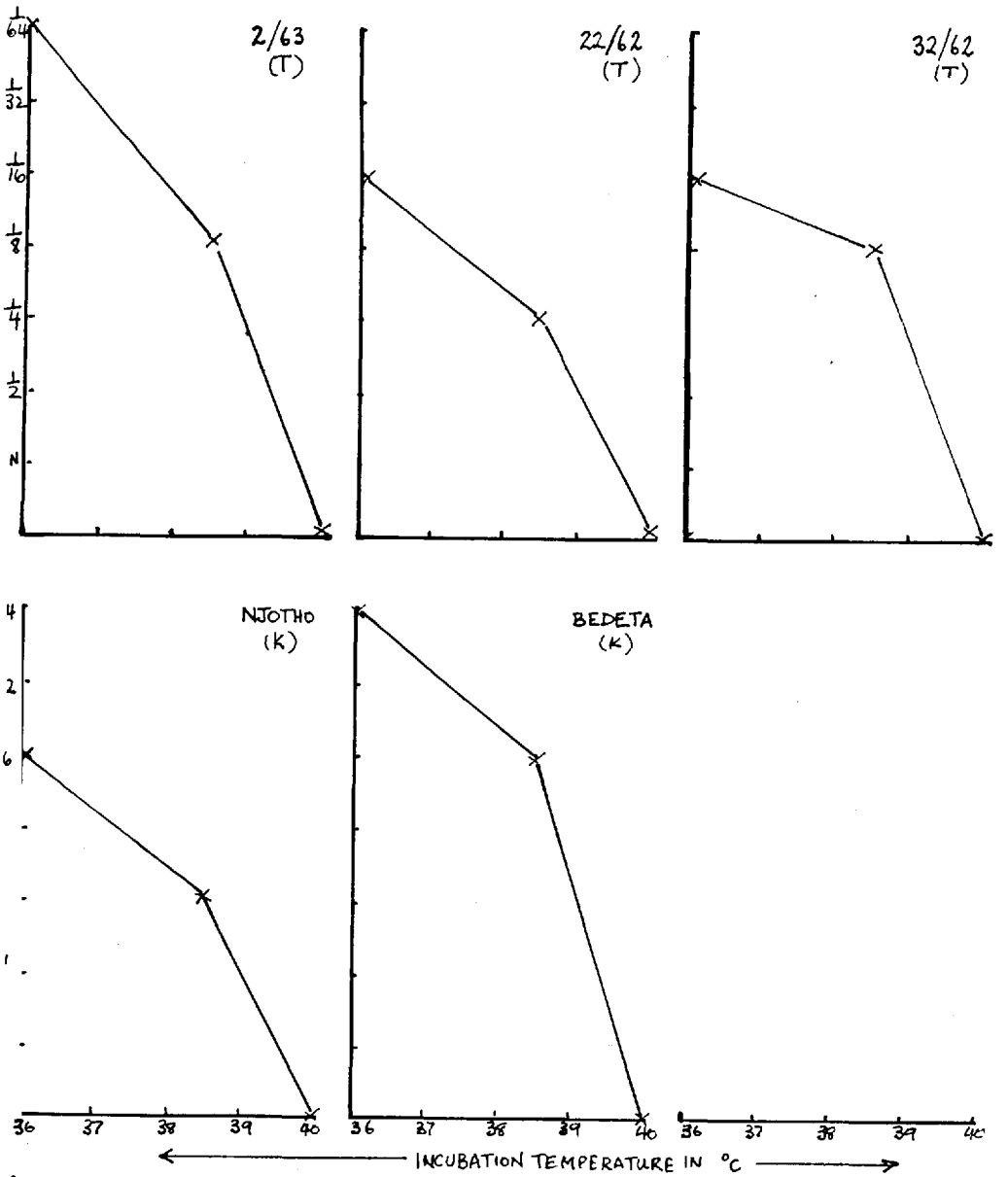


Fig. 22. The 72 hour yield of HA in human skin/muscle cultures infected with South American (SA) and European (E) strains of variola minor and incubated from 36° to 40° C.



ig. 23. The 72 hour yield of HA in human skin/muscle cultures infected with Tanganyikan (T) and Kenyan (K) strains of variola virus incubated from 36° to 40° C.

smallpox epidemic; Laidlaw and Horne, 1950), variola major strains from Europe and Asia, and variola minor strains from Europe and South America fell into two broad groups according to the yield of HA produced at a range of temperatures.

Variola major strains gave a good titre of HA at 38.5° C and often gave activity at 39.5°- 40° C, whereas variola minor strains produced very little or no detectable HA at 38.5° C and none at 39.5° C. Smallpox strains from Africa, both from Kenya and Tanganyika, resembled the variola major strains from Europe and Asia. Variola minor strains from South America resembled those from Europe with the exception of strain 'Brazil-7' which behaved more like a variola major strain in HA production.

An upper temperature of 40° C was used in testing many of the strains, but it proved to be slightly too high for detectable HA production even by variola major strains. The results obtained when strains were tested at 39.5° C as the highest temperature served to illustrate that more HA was detectable at this temperature. A temperature of 39.5° C was therefore more suitable than 40° C in this test and was routinely used when strains were screened in human lung cultures.

b) Screening in human lung cultures (Figs. 24; 25a,b; 26)

When variola strains were tested for HA production at a range of incubation temperatures in human lung cultures, the majority of

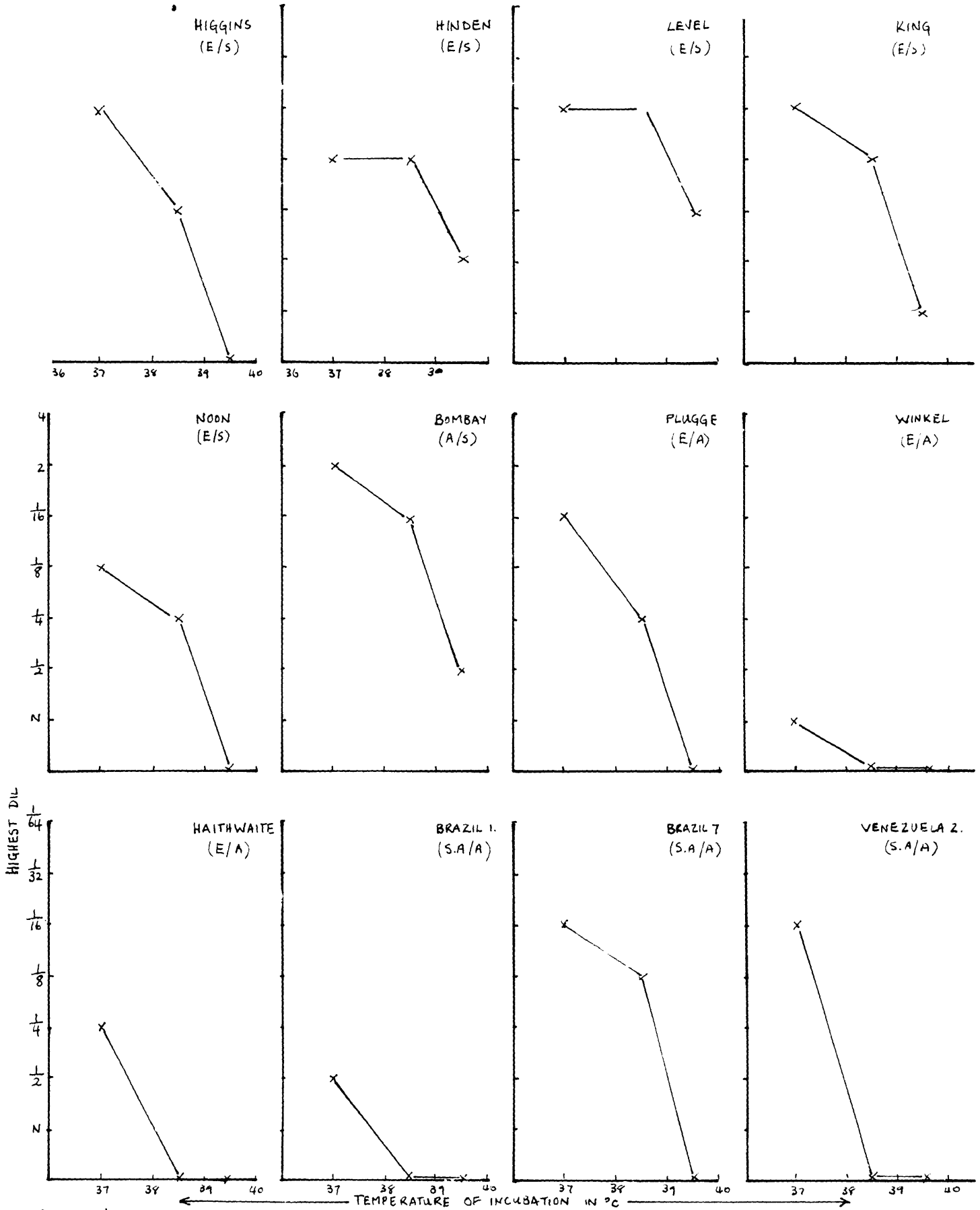


Fig. 24. The 72 hour yield of HA in human lung cultures infected with European (E/S) and Asian (A/S) strains of variola major and with European (E/A) and South American (SA/A) strains of variola minor

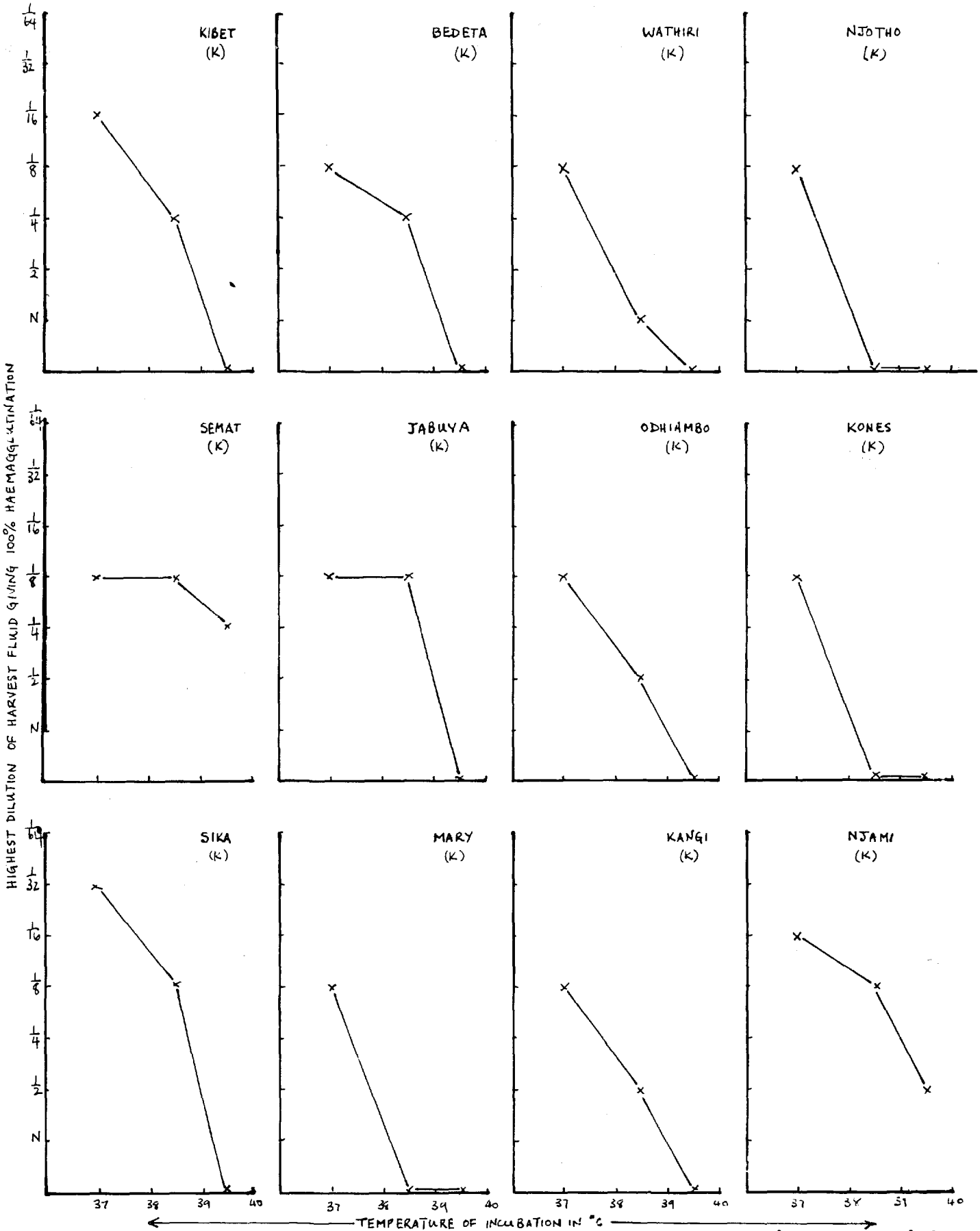


Fig 25a. The 72 hour yield of HA in human lung cultures infected with Kenyan strains (K) of variola virus incubated from 37° to 39.5°

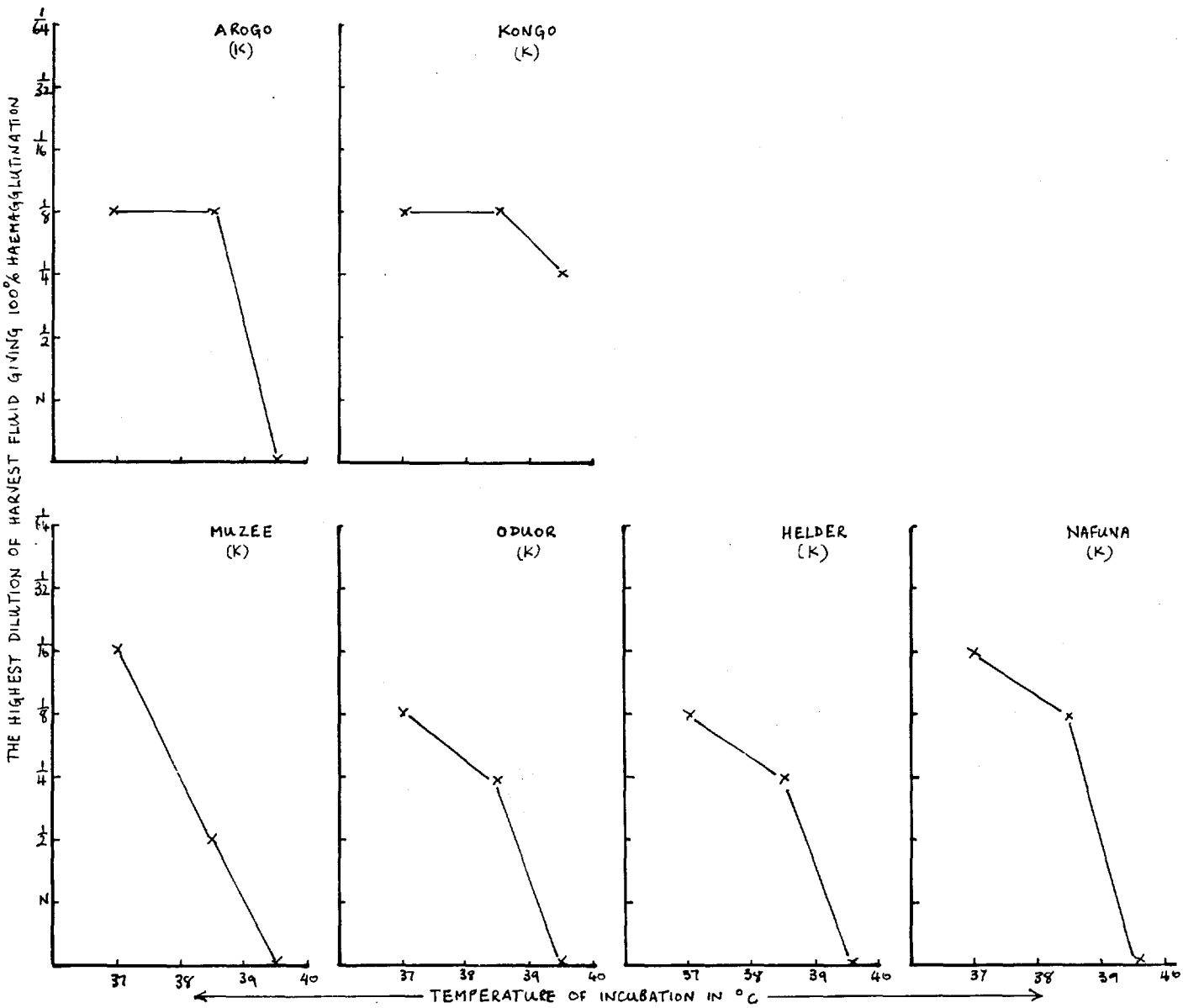


Fig. 25b. The 72 hour yield of HA in human lung cultures infected with Kenyan (K) strains of variola virus.

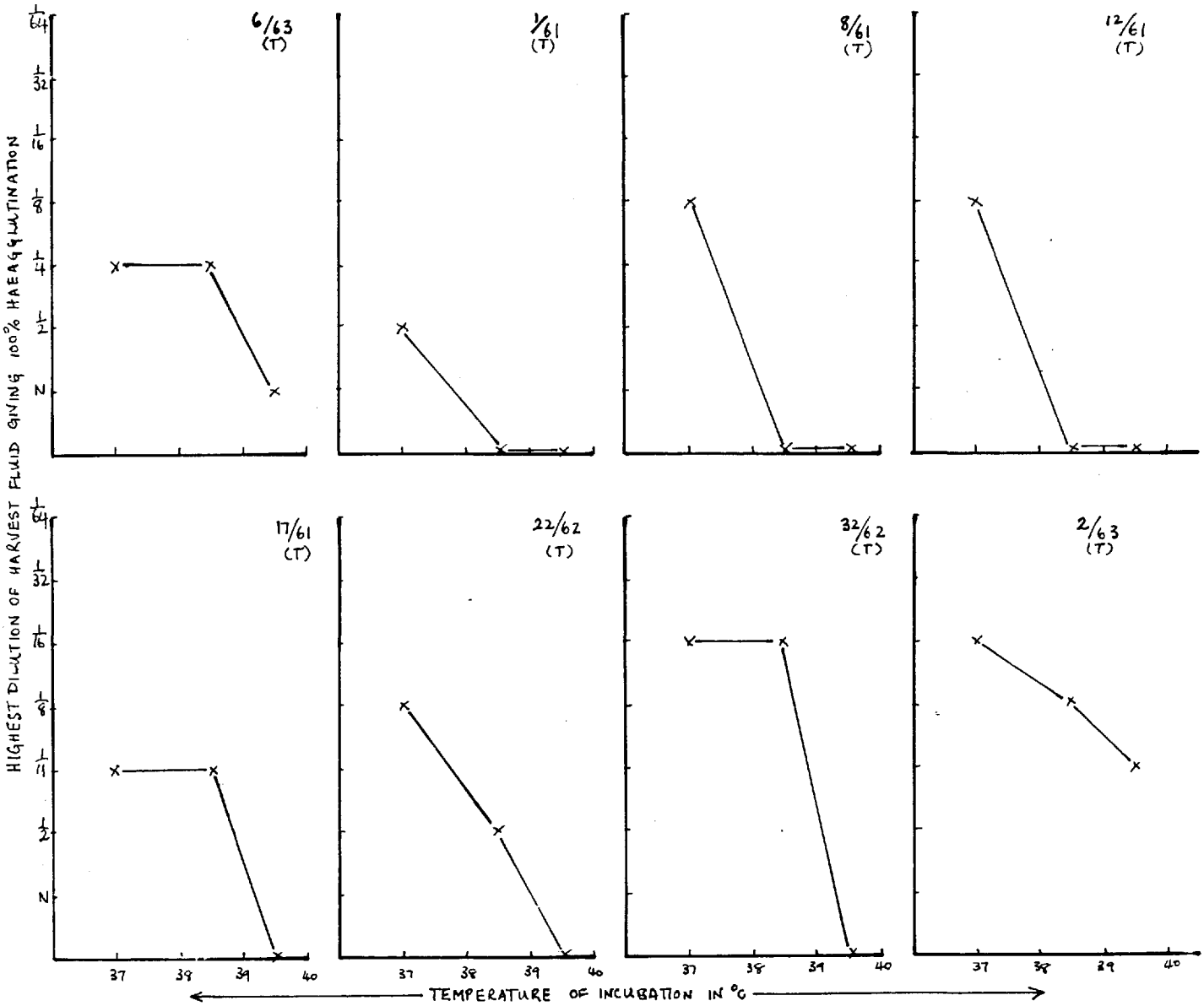


Fig. 26. The 72 hour yield of HA in human lung cultures infected with Tanganyikan (T) strains of variola virus incubated from 37° to 39.5° C.

major and minor strains again fell into two broad groups.

Most of the major strains produced as much, or almost as much, HA at 38.5° C as at 37° C, and also a fairly good titre at 39.5° C. Alastrim strains generally only produced detectable HA at 37° C. Exceptions were the 'Higgins' and 'Noon' strains of variola major and the 'Plugge' and 'Brazil-7' strains of alastrim between which there was no significant difference in this series of tests.

The results showed that human lung cultures were not significantly more sensitive to differences in HA production of variola major and minor strains at the three temperatures than were human skin/muscle fibroblast cultures.

The African strains, when tested in human lung cultures, showed a complete range of responses of HA production to temperatures of 37°, 38.5°, and 39.5° C from definitely variola major-like to definitely variola minor-like.

Both the Tanganyikan and Kenyan groups of variola strains were placed in ranking order.

Three subgroups were created. The first subgroup contained those strains behaving like variola major, showing HA production at all three temperatures. The second subgroup contained the intermediate strains which showed HA production at 37° and 38.5° C, but not at 39.5° C. The third subgroup contained those strains behaving like variola minor, showing HA production at 37° C only.

The members of each subgroup were then ranked weighting a high titre, where applicable, of HA at 39.5° C, then at 38.5° C, and then at 37° C as 1st, 2nd, and 3rd characters respectively.

The ranking orders with respect to the HA test of the two groups of African strains thus obtained are shown in Table 4.

TABLE 4

	HA test at 37°, 38.5°, 39.5° (in human lung)	Reduction in Pock titre at 38.3° C (log titre[35°]-log titre[38.3°])		Embryo Virulence Test D_4 Value	
	Strain	Strain	Value	Strain	Value
	Variola Major	(in general)	<0.5	(general)	4.5-5.5
	Kenyan Variola				
(1)	Semat, Kongo	Muzee	0.25	Sika	>5.6
(1)	Njemi	Sika	0.29	Njotho	>6.5
(2)	Sika	Odhiambo	0.34	Bedeta,Oduor	6.6
(2)	Nafuna	Wathiri	0.41	Kibet	8.4,7.0,>7.0
(2)	Jabuya,Arogo	Bedeta	0.45		
(2)	Kibet	Oduor	0.56		
(2)	Bedeta,Oduor,Helder	Kangi	0.57		
(2)	Muzee	Kibet	0.61		
(2)	Kangi,Odhiambo	Mary	0.62		
(2)	Wathiri	Njotho	0.66		
(3)	Mary	Helder	0.72		
(3)	Njotho,Konee	Konee	0.74		
		Nafuna	1.00		
	Variola Minor	(in general)	>2.00	(general)	>6.5

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TABLE 4 cont'd

HA test at 37°, 38.5°, 39.5° (in human lung) (°C)	Reduction in Pock titre at 38.3° C (log titre[35°]-log titre [38.3°])		Embryo Virulence Test D_4 Value	
Strain	Strain	Value	Strain	Value
Variola Major	(in general)	<0.5	(general)	4.5-5.5
Tanganyikan Variola				
(1) 2/63	1/61	0.3	2/63	5.2
(1) 6/63	2/63	0.4	12/61	5.9
(2) 32/62	22/62	0.7	6/63	6.0
(2) 17/61	6/63	0.7	22/62	6.4
(2) 22/62	12/61	0.8	8/61	6.7,7.0
(3) 8/61	8/61	1.0	1/61	7.1
(3) 12/61	32/62	1.3	32/62	8.3,8.6
(3) 1/61	17/61	1.8		
Variola Minor	(in general)	>2.0	(general)	>6.5

The ranking orders of East African variola strains (from variola major-like to variola minor-like) with respect to the HA test, the pock-reduction test, and the chick-embryo virulence test.

Of the Kenyan strains, 'Semat', 'Kongo', and 'Njami' behaved as variola major, and at the other end of the scale 'Mary', 'Njotho', and 'Kones' behaved as alastrim. The Tanganyikan strains '2/63' and '6/63' were like variola major, whereas strains '8/61', '12/61', and '1/61' behaved as variola minor. Every variation of reaction occurred

between the Tanganyikan and Kenyan strains at opposing ends of the ranking order of strains with respect to the HA test.

Dumbell, Bedson, and Thomas (1963) and Dumbell and Bedson (unpublished results) have tested many of these strains for their behaviour in the pock-reduction test and in the chick-embryo virulence test. Their results are also listed in Table 4. The African strains can be seen to be either intermediate or like variola major with respect to the pock-reduction test and either intermediate or like variola minor with respect to the chick-embryo virulence test.

Inspection of Table 4 shows that, from the results available, no regular correlation exists between the ranking orders of the African strains in the HA test and pock-reduction test nor between the ranking orders in the HA test and the chick-embryo virulence test. For example, strain '1/61' is definitely like variola minor in the HA and chick virulence tests, but is definitely like variola major in the pock-reduction test. On the other hand, 'Sika' and '2/63' resembles variola major in all three tests, and 'Bedeta' and 'Oduor' which are intermediate in the HA test are like variola major in the pock-reduction test and like alastrim in the chick virulence test.

Although Bedson and Dumbell (1961) demonstrated a positive correlation between the ranking order of poxviruses with respect to virulence and ceiling temperatures as determined in the chick

embryo, they later showed by hybridisation experiments (Bedson and Dumbell, 1964a,b) that virulence for the chick embryo and ceiling temperature in the chick embryo were independent properties.

The behaviour of variola strains, as illustrated by the African strains in human lung cultures, with respect to the HA test would also seem to be an independent property since no correlation was found with previously determined values for these strains for chick virulence and for sensitivity to temperature of growth on the chick CAM.

It is difficult to imagine that the production of HA is in any way a virulence factor, unless an ability to produce HA in human cells at high temperatures of incubation reflects a tenacity of virus-directed functions in the febrile human host.

Discussion

The possibility of a laboratory differentiation of strains of variola major and variola minor on the basis of HA production at 37°, 38.5°, and 39.5° C in human cells of foetal skin/muscle and lung origin has been investigated.

Variola major strains generally produced detectable HA at 37°, 38.5°, and 39.5° C, whereas variola minor strains generally produced HA detectable at 37° C but not at 38.5° and 39.5° C.

Although major and minor strains fall into two broad groups, group variation is sufficient to make the classification of a single variola strain tested alone unreliable, unless the strain in question gives a reaction which is very definitely characteristic of a major or minor strain.

Where individual African strains have been tested on two occassions in human lung cultures in the HA test, the results obtained each time were constant for each strain. The HA test would therefore seem to be another useful marker in the biological characterisation of the African strains in laboratory studies.

5.4 The Dependence of the Yield of HA on Growth Media Factors

During the course of experiments concerning the production of HA by variola virus, it became increasingly clear that the nutritional composition of the culture medium used during infection was important in determining the final yield of HA from infected cells.

An attempt to stabilize the rapid fall in pH of culture media during infection of cell monolayers had involved the use of a Zwitterion buffer known to be very effective in maintaining a pH of 7.4 under such conditions. The buffer, N-2 Hydroxyethylpiperazine-N'-2 Ethane Sulphonic Acid* (HEPES) substitutes for the normal $NaHCO_3$-5% CO_2-in-air buffering system used routinely in tissue culture media. HEPES is neither taken up by the cell nor metabolised. (Good et al., 1966)

When used as a culture medium during HA experiments, HEPES-buffered Eagles Basal Medium (EAG BM) (Eagles, 1955a,b) was found to cause an almost complete suppression of HA production as detected by HAD at 24 hours and HA^m at 72 hours.

In order to further investigate the production of HA under various types of media, parallel cultures of human skin/muscle cells were infected with a high dose of Harvey and Butler viruses and incubated at 37° C under culture media routinely used in tissue culture.

The effect on HA production of Parkers Medium 199 (Morgan et al., 1950)

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*pKa = 7.5; obtained from "Calbiochem", Los Angeles, California, U.S.A.

which had been previously used to maintain an uninfected monolayer of human fibroblasts and which was deficient in some of its constituent compounds was also tested (Table 5)

TABLE 5

Medium	g % Bicarb.	Virus	HAD (24 hrs)	HA* (72 hrs) N	1/2	1/4	1/8	1/16	1/32
199+10% liver digest ultrafiltrate	0.088	Harvey	5	+	+	+	50	t	-
		Butler	4 - 5	+	+	+	70	t	-
used 199+10% liver digest ultrafiltrate	0.088	Harvey	5	+	+	-	-	-	-
		Butler	3	+	+	-	-	-	-
199	0.088	Harvey	5	+	+	+	+	-	-
		Butler	4	+	+	-	-	-	-
HEPES EAG BM	0.110	Harvey	1*	t	-	-	-	-	-
		Butler	0	-	-	-	-	-	-
EAG MEM	0.088	Harvey	5	+	+	+	+	t	-
		Butler	5	+	+	+	+	+	t

*Band of HAD confined to the upper end of monolayer in tube

The effect of various culture media on HA production in Harvey- and Butler-infected human fibroblasts at 37o C.

It was found that HA production was most efficient under Eagles Minimal Essential Medium (EAG MEM) (Eagles, 1959); slightly more HA was produced in Butler-infected cultures than in Harvey-infected cultures. Previous use of 199 created a medium which was poor in supporting HA production.

As there are only minor differences in the concentrations of constituent compounds of EAG BM and EAG MEM, it was unlikely that the composition of EAG BM was responsible for the suppression of HA production rather than the presence of the compound HEPES.

Omitting liver digest ultrafiltrate from medium 199 reduced the yield of HA of Butler virus slightly.

Subsequent experiments showed that the suppression of HA production under HEPES-buffered EAG BM could be reversed by the addition of extra bicarbonate (HCO_3^-) to the medium. Extra HCO_3^- also increased the yield of HA under medium 199 and EAG MEM. (Table 6)

TABLE 6

Medium	HCO_3^- %	Virus	HAD (24 hrs)	HA[a] (72 hrs) N	1/2	1/4	1/8	1/16	1/32
199	0.176	Harvey	4-5	+	+	+	+	+	25
		Butler	3	+	+	+	+	+	50
	0.088*	Harvey	4-5	+	+	+	+	25	-
		Butler	3	+	+	+	+	+	50

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TABLE 6 cont'd

Medium	HCO_3^- (g %)	Virus	HAD (24 hrs)	HA^{RI} (72 hrs) Neat	1/2	1/4	1/8	1/16	1/32
EAG MEM	0.176	Harvey	5	+	+	+	+	25	-
		Butler	3	+	+	+	+	25	-
	0.088*	Harvey	5	+	+	+	75	-	-
		Butler	3	+	+	+	+	75	-
HEPES-EAG BM	0.198	Harvey	3	+	+	+	-	-	-
		Butler	3	+	+	+	-	-	-
	0.110*	Harvey	1	t	-	-	-	-	-
		Butler	0	-	-	-	-	-	-

*Standard Bicarbonate Concentration

The effect of the concentration of HCO_3^- in culture media on HA production in Harvey- and Butler-infected human fibroblasts at 37° C.

Since the concentration of HCO_3^- contained in HEPES EAG BM was slightly greater than that contained in both the EAG MEM and in 199 based media originally used in HA experiments (0.11 g % and 0.088 g %, respectively), it was not a deficiency of HCO_3^- in the medium which was causing suppression of HA production under HEPES EAG BM.

It is possible that, although HEPES cannot be taken up by the cell, it can exert an effect from without upon the metabolism of the cell. The fact that the addition of extra HCO_3^- to HEPES EAG BM reversed the suppression of HA may indicate that HEPES *per se* can block the availability to the cell of HCO_3^- in a utilizable form.

CHAPTER VI

A Survey of the Activity of Several Biological Compounds as Stimulators of Variola HA Production in Human Skin/Muscle Fibroblasts at 37° C

It was reported in Chapter V that the concentration of bicarbonate (HCO_3^-) available to infected cells was an important factor in haemagglutinin (HA) production. Addition of extra HCO_3^- to media reversed the suppressive effect of Eagles Basal Medium (EAG BM) buffered with the Zwitterion HEPES and improved HA yields under Eagles Minimal Essential Medium (EAG MEM) and 199-based medium. This fact was studied further.

(Materials and Methods - See Appendix)

6.1 The Effect of HEPES EAG BM, EAG MEM, and 199 media Containing Graded Concentrations of Bicarbonate on HA Production

Although the pH of media was unavoidably altered by addition of different bicarbonate concentrations ($[HCO_3^-]$), scrutiny of Table 7 shows that HA production was not directly related to the pH of the medium. Media of the same pH have supported different extents of HAD and HA titres.

An excess of 0.022 g % of HCO_3^- largely reversed the suppression of HA in HEPES EAG BM, but the yield of HA did not increase significantly

between the minimum and maximum concentrations of extra HCO_3^- added. This range, from 0.022 - 0.088 g % respectively, elicits a graded response in HA production in 199 medium and in EAG MEM.

It was found that in 199 medium the HA production by Butler was suppressed to a greater extent at low [HCO_3^-] than that by Harvey. This situation was reversed, however, in EAG MEM.

TABLE 7

Medium	HCO_3^- (g %)	HAD (24 hrs)	HA^R (72 hrs) N	1/2	1/4	1/8	1/16	1/32	Initial pH	24 hr pH	Final pH	Virus
199	0.176	4-5	+	+	+	+	+	25	7.4	>7.4	7.6	H
		3	+	+	+	+	+	50	7.4	7.6	—	B
	0.088*	4-5	+	+	+	+	25	-	7.1	7.3	6.8	H
		3	+	+	+	+	+	50	7.1	7.4	—	B
	0.044	4-5	+	+	+	50	-	-	6.8	7.1	6.6	H
		3	+	+	+	25	-	-	6.8	6.8	—	B
	0.033	4	+	+	50	-	-	-	6.6	7.0	6.4	H
		2	t	-	-	-	-	-	6.6	6.9	—	B
	0.022	1	75	t	-	-	-	-	6.4	6.9	6.4	H
		0	t	-	-	-	-	-	6.4	6.8	—	B
EAG-MEM	0.176	5	+	+	+	+	25	-	7.5	>7.4	7.7	H
		4	+	+	+	+	25	-	7.5	7.8	—	B
	0.088*	5	+	+	+	>50	-	-	7.3	7.4	7.1	H
		4	+	+	+	+	>50	-	7.3	7.3	—	B

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TABLE 7 cont'd

Medium	HCO_3^- (g %)	HAD (24 hrs)	HA^n (72 hrs) N	1/2	1/4	1/8	1/16	1/32	Initial pH	24 hr pH	Final pH	Virus
EAG-MEM	0.044	5	+	+	+	50	-	-	7.2	7.2	6.9	H
		4	+	+	+	+	>50	-	7.2	7.1	—	B
	0.033	4-5	+	+	>50	-	-	-	7.1	7.1	6.8	H
		4	+	+	+	25	-	-	7.1	7.0	—	B
	0.022	3	t	-	-	-	-	-	7.0	7.0	6.4	H
		4	+	+	t	-	-	-	7.0	7.0	—	B
HEPES EAG BM	0.198	4	+	+	+	-	-	-	7.2	7.3	7.4	H
		3	+	+	50	-	-	-	7.2	7.3	—	B
	0.154	4	+	+	+	-	-	-	7.1	7.2	7.1	H
		3	+	+	+	-	-	-	7.1	7.3	—	B
	0.143	4	+	+	+	-	-	-	7.1	7.2	7.3	H
		3	+	+	25	-	-	-	7.1	7.3-7.2	—	B
	0.132	4	+	+	+	-	-	-	7.1	7.2	7.2	H
		3	+	+	t	-	-	-	7.1	7.2	—	B
	0.110*	1**	(t)	-	-	-	-	-	7.1	7.2	7.1	H
		1**	(t)	-	-	-	-	-	7.1	7.1	—	B

*Standard bicarbonate concentration

**Band of HAD confined to upper end of monolayer in tube

The effect of varying bicarbonate concentrations on HA production by Harvey (H) and Butler (B) in human fibroblasts at 37° C.

Mosley and Enders (1962) investigating the dependence of the growth of virulent and attenuated strains of polio virus upon available HCO_3^- devised media in which polio virus could be grown in monkey kidney cell cultures in the presence or absence of HCO_3^-. (For media composition see appendix.)

Infected monolayers were incubated in tris-buffered 199 free of HCO_3^- in cotton-plugged tubes (CP) or in tris-buffered 199 containing HCO_3^- in screw-capped tubes (SC).

The method of Mosley and Enders was adopted in the investigation of an absolute requirement for HCO_3^- in variola HA production. Harvey- and Butler-infected human fibroblast monolayers were incubated in tris-buffered 199 with HCO_3^- (SC) and without HCO_3^- (CP). In the latter cultures, CO_2 produced during cell metabolism was free to diffuse away from the medium. In order to see what effect respiration-produced CO_2 fixed as HCO_3^- may have on HA production, CP cultures were also incubated with rubber bungs (CP/RB); escape of CO_2 was therefore prevented.

The results, tabulated in Table 8, indicated that HCO_3^- is essential for HA production. CO_2 formed during cell metabolism and fixed as HCO_3^- in the medium is sufficient for some restoration of HA formation.

TABLE 8

Medium	HCO_3^- (g %)	Virus	HAD (24 hrs)	Initial pH	24 hrs pH
SC	0.22	Harvey	4	7.0	7.4
		Butler	2	7.0	6.9
CP	none	Harvey	0	7.2	7.0
		Butler	0	7.2	7.0
CP/RB	estimated 0.03 from fixed CO_2	Harvey	3	7.2	7.1
		Butler	0	7.2	6.9

The effect of the presence and absence of HCO_3^- on the production of HA by Harvey and Butler in human fibroblasts incubated for 24 hours at 37° C.

6.2 The Influence of Culture Media Constituents on HA Production

Eagles MEM was regularly found to be somewhat better in supporting HA production in variola-infected cultures than 199-based media.

Although Parker's 199 medium contains a complete range of amino acids, vitamins, and growth factors, the concentration of certain of the essential compounds of the less comprehensive range present in EAG MEM is in excess of that in 199.

Thus it is not the absence of key compounds from 199 medium which makes it a poorer medium with respect to HA production, but

presumably the lower concentration of such compounds.

Table 9 lists those compounds common to 199, EAG MEM, and EAG BM with the appropriate concentrations in mg/litre of medium.

Both EAG MEM and BM are notably richer in growth factors than is 199. Eagles medium also contains six times the concentration of glutamine and approximately twice the concentration of the L-amino acids, arginine and histidine, than does 199.

TABLE 9

Compound	199 (mg/l)		EAG MEM (mg/l)	EAG BM (mg/l)	
L-arginine	70	FB	126.2	105	FB
L-cystine	20		24	24	
L-glutamine	50		292	292	
L-histidine	20	FB	38.3	24	FB
L-isoleucine	40	(DL)	52.4	52	
L-leucine	120	(DL)	52.4	52	
L-lysine	70	FB	73.1	58	
L-methionine	30		14.9	15	
L-phenylalanine	50	(DL)	33	32	
L-threonine	60		47.6	48	
L-tryptophan	20		10.2	10	
L-tyrosine	40		36.2	36	
L-valine	50	(DS)	46.8	46	
D-Ca-pantothenate	0.01		1	1	
Choline Chloride	0.50		1	1	
Folic acid	0.01		1	1	
i-inositol	0.05		2	2	

cont'd next page

TABLE 9 cont'd

Compound	199 (mg/l)	EAG MEM (mg/l)	EAG BM (mg/l)
Nicotinic acid	0.025	——	1
Nicotinamide	0.025	1	——
Pyridoxal·HCl	0.025	1	1
Riboflavin	0.01	0.1	0.1
Thiamin·HCl	0.01	1	1
Glucose	1000	1000	2000

Concentrations in mg/litre medium of constituents common to 199, EAG MEM and EAG BM media.

To establish which broad group of compounds was responsible for the 'enrichment' of Eagles medium, medium 199 containing a low concentration of HCO_3^- (0.022 g %) was supplemented with the following stock solutions:

stock solution (1): L-amino acids in mg/litre

Arginine (105) Leucine (52) Phenylalanine (32)
Histidine (24) Isoleucine (52) Threonine (48) Valine (46)
Lysine (58) Methionine (15) Tryptophan (10)

stock solution (2): L-amino acids in mg/litre

Tyrosine (36) Cystine (24)

stock solution (3): B vitamins in mg/litre

Choline Chloride (1) Pantothenic acid (1) Riboflavin (0.1)
Nicotinic acid (1) Pyridoxal (1) Thiamine (1)
Inositol (2)

stock solution (4): Folic acid (1 mg/litre)

stock solution (5): Glutamine (0.292 g/litre)

The basic medium 199 + 0.022 g % HCO_3^- could be expected to support a minimum amount of HA production. Addition of stock solution supplements at approximately the concentration present in EAG MEM should elicit an enhanced HA production in that group of infected cultures incubated with the medium containing the relevant supplement(s).

It was found that 199 supplemented with stock solution (1) was alone able to restore to some extent the production of HA in Harvey- and Butler-infected cultures. Harvey-infected cultures gave a greater response in HA production to the addition of extra amino acids than did Butler-infected cultures. Extra growth factors and glutamine were without effect.

Pyridoxal (vitamin B_6) has been implicated as a compound capable of forming a reversible complex with amino acids in order to assist the transfer of amino acids across the cell wall against a concentration gradient.

Although the B vitamin group as a whole had been found to be ineffective in enhancing HA production, it was possible that medium supplemented with both pyridoxal and amino acids might be more effective than medium supplemented with amino acids alone. This was not found to be the case and it was concluded that one or more amino acids *per se* account for the 'enrichment' factor in EAG MEM.

6.3 Replacement of Bicarbonate by Amino Acids

Since the L-amino acid concentration of culture media had some effect on the yield of HA in infected cultures, it was interesting to ascertain whether one or more amino acids could replace HCO_3^- necessary for HA production.

HEPES EAG BM was supplemented with the L-amino acids listed in Table 10 at their concentrations in EAG MEM. Infected cultures were incubated under these media for 24 hours at 37° C and tested for HAD (Table 10).

TABLE 10

Medium	Harvey: HAD (24 hrs)	Butler: HAD (24 hrs)
HEPES EAG BM	1*	0
HEPES EAG BM + 0.088 g % HCO_3^-	3	2
+ L-arginine	4	3
+ L-lysine	3	1
+ L-threonine	2-3	1
+ L-valine	1	0
+ L-histidine	1	0
+ L-methionine	0	0
+ L-phenylalanine	0	0

*Band of HAD confined to upper end of monolayer in tube

Effect of a/a supplemented HEPES EAG BM on HA production in Harvey- and Butler-infected human fibroblasts incubated at 37° C for 24 hours.

It was found that L-arginine was an efficient replacement for HCO_3^-. Lysine and threonine were less effective in that order, and histidine, valine, methionine, and phenylalanine were without effect. The extent of the reversal of HA suppression was always greater in Harvey-infected cultures than in Butler-infected cultures.

Thus, of the two amino acids present in a greater concentration in EAG MEM than in medium 199, only arginine was effective in replacing HCO_3^- in culture media in this instance.

Supplementation of HEPES EAG BM with 0.088 g % HCO_3^- or with the molar equivalent of arginine showed that arginine was only slightly less efficient in reversing the suppression of HA formation found in unsupplemented HEPES EAG BM (Table 11).

TABLE 11

Medium	Extra HCO_3^- added	Extra Arginine added	HA[a] (72 hrs)					
			N	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$
HEPES EAG BM	none	none	t	-	-	-	-	-
	0.088 g %	none	+	+	+	+	t	-
	none	10.5 mM	+	+	+	t	-	-

The relative efficiency of HCO_3^- and arginine in restoring HA production in Harvey-infected cells incubated with HEPES EAG BM at 37° C.

6.4 Replacement of Bicarbonate by Nucleic Acids and Related Compounds

Chang (1959) showed that HCO_3^- depletion of nutrient media suppressed the growth of Coxsackie B1 virus, polio virus, and vaccinia virus in human conjunctiva and HeLa cells. RNA (yeast-extracted), enzymatically-degraded RNA, and its nucleotides and nucleosides were all found to reverse the effects of this depletion . However, neither the purine and pyrimidine bases constituting RNA nor DNA and its derivatives were effective in this way.

It was proposed that the role of HCO_3^- in this instance was as a precursor of RNA. Depletion of HCO_3^- would result in a disturbance of cellular RNA metabolism which would cause an interruption of the synthesis of new protein in the cell. Hence, HCO_3^- depletion could lead to a suppression of virus protein synthesis in infected cells.

To test the possibility that HCO_3^- depletion was causing repercussions on nucleic acid metabolism connected with variola HA production, tris-buffered 199 containing 0.022 g % HCO_3^- was supplemented with calf thymus DNA and yeast-extracted RNA at a concentration of 1.23 μg/ml medium.

Since Chang found that purine and pyrimidine bases as such could not replace RNA in this case, it is possible that there is a pre-requisite for phosphorylated purine and pyrimidine bases or for the presence of D-ribose. Therefore, tris-buffered 199 was also supplemented with ATP (1 g/ml) as an example of a phosphorylated base, and with D-ribose (5 mg/ml).

It was found that DNA, RNA, ATP, and ribose were unable to reverse the suppression of HA production in the system tested here.

6.5 Replacement of Arginine by its Possible Biosynthetic Precursors

Since the L-amino acid arginine has so far been found to be the most effective compound in replacing HCO_3^- for the reversal of HA suppression under HEPES EAG BM, possible precursors of arginine (see fig. 27) were tested as replacements for arginine itself.

Supplementation of HEPES EAG BM with 3 mM arginine had been found to be nearly as effective as 0.088 g % HCO_3^- in supplementing HEPES EAG BM without significantly raising the pH.

For this reason, L-aspartic acid, citrulline, and carbamyl phosphate at 3 mM were used to supplement HEPES EAG BM and were compared with the activity of 3 mM arginine. The three precursor compounds were also tested in combinations (Table 12).

TABLE 12

Medium	Supplements	24 hr HAD in Harvey	Butler
HEPES EAG BM	+ arginine (3mM)	4	3-4
	+ L-aspartic acid (3mM) [A]	2	1
	+ citrulline (3mM) [C]	0	1
	+ carbamyl phosphate (3mM) [C/P]	0	0
	+ 3 mM A + C	1	0
	+ 3 mM A + C/P	0	0
	+ 3 mM A + C + C/P	0	0
	no additions	1*	0

*Band of HAD confined to upper end of monolayer in tube.

The effect of precursor compounds in restoring HA production in HEPES EAG BM in Harvey- and Butler-infected human fibroblasts incubated for 24 hours at 37° C.

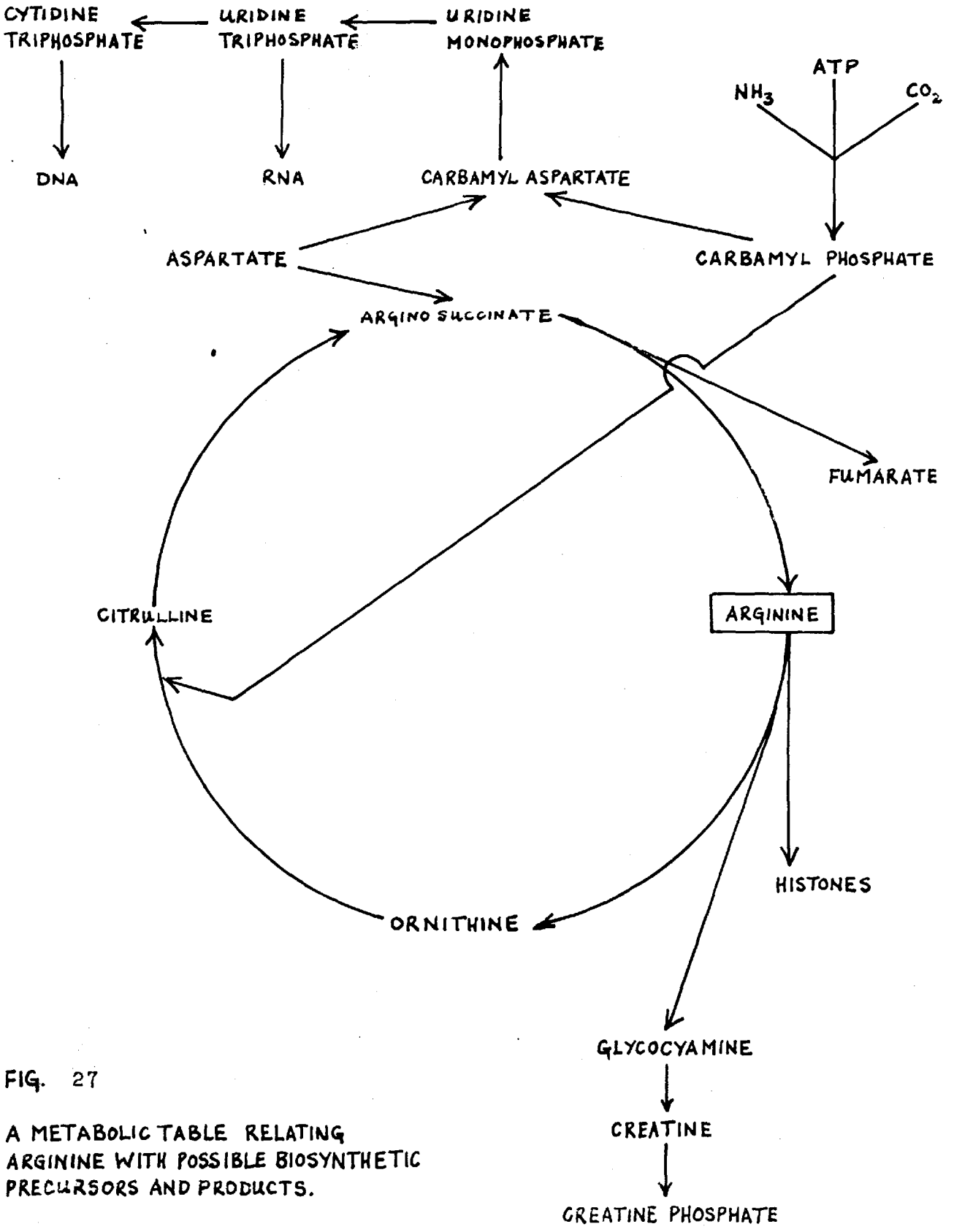


FIG. 27

A METABOLIC TABLE RELATING ARGININE WITH POSSIBLE BIOSYNTHETIC PRECURSORS AND PRODUCTS.

From the table it can be seen that aspartic acid (another L-amino acid) can replace arginine to some degree. Citrulline and carbamyl phosphate, alone or in combination with aspartic acid, had little effect in this series of experiments. Nothing was known, however, about the efficiency of uptake by the cells of these compounds from the medium. It may be that very little citrulline and/or carbamyl phosphate was taken up by the cells and that lack of reversal of HA suppression reflected this possibility rather than a lack of activity as arginine precursors in this system. An alternative possibility was that arginine in virus-synthetic pathways is synthesised via a different route.

6.6 Replacement of Arginine by Possible Biosynthetic Products

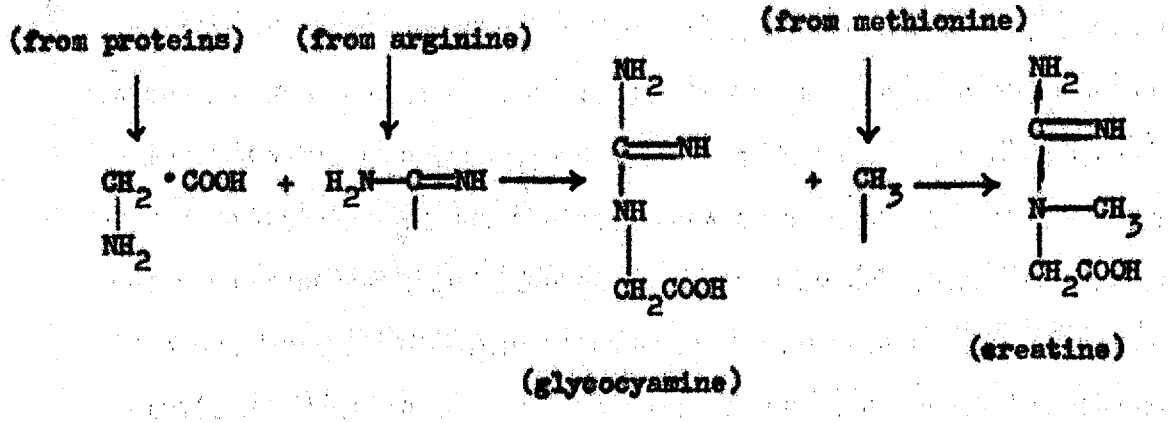
a) Creatine and Phosphocreatine

Creatine and its phosphorylated form phosphocreatine are found in high concentrations in muscle tissue. Creatine phosphate is the phosphogen or storage form of phosphate associated with high-energy bonding in vertebrates. During the active contraction of muscles the phosphocreatine level falls and the compound is resynthesised when the muscle is at rest. The specific function of phosphocreatine in muscle cells is to re-phosphorylate ADP to ATP in the reaction

Phosphocreatine + ADP ⟷ ATP + Creatine

which is mediated by the enzyme ATP-creatine transphosphorylase.

Arginine is involved in the cellular synthesis of creatine:



If the arginine requirement for phosphocreatine production in the skin/muscle fibroblast cell is increased during the variola virus infection, then in supplying the end product it should be possible to restore suppressed HA in the absence of additional arginine.

HEPES EAG BM was supplemented with creatine and phosphocreatine (Table 13) at a concentration of 1 mM.

TABLE 13

Medium	Supplement	24 hour HAD	
		Harvey	Butler
HEPES EAG BM	+ arginine	3-4	3
	+ creatine	1	2
	+ creatine-P	2	1
	no addition	1*	0

*Band of HAD confined to upper end of monolayer in tube.

Effect on HA production in Harvey- and Butler-infected human fibroblasts of arginine and biosynthetic products creatine and phosphocreatine.

The results show that neither creatine nor phosphocreatine were regularly efficient in restoring HA production, though as in the case of the precursors discussed in the previous section, nothing was known about the uptake of these compounds into the host cells.

b) Histones (CIBA Foundation Study Group, publ. no. 24, 1966)

Histones are proteins found in close association with nucleic acids in the cell and which contain an unusually high proportion of

the basic amino acids. Fractionation studies have suggested that "... histones are a group of a limited number of major proteins with possibly a number of minor fractions". A lysine-rich and arginine-rich histone fraction have been described in the above work where several fundamental functions for histones have also been suggested.

During the cellular synthesis of both arginine-rich and lysine-rich histones, the pool of arginine and lysine in the cell must be particularly heavily drawn upon. Indeed, Piez and Eagle (1958) found arginine and lysine to be incorporated into cellular proteins to a much higher proportion than were other amino acids and that only trace quantities of these two amino acids were detectable in the amino acid pool of several types of human cells. The reserve of the free arginine could be depleted in 24 hours if not replenished.

Bearing in mind that arginine and, to a slightly lesser extent, lysine have already been implicated as important factors at least for virus HA production in infected cells, the possibility that arginine- and lysine-rich histones were involved in the fate of these two amino acids was investigated. Arginine-rich, lysine-rich, and crude histones were tested as replacements for extra HCO_3^- and arginine in favouring an enhancement of HA production.

HEPES EAG BM was supplemented with crude histone (type II) and with purified arginine-rich (type IV) and lysine-rich (type III) histone* fractions at concentrations of 1, 0.1, and 0.01 mg/ml medium.

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*Obtained from Sigma

Harvey-infected cultures of human fibroblasts were incubated at 37° C under these media and tested for HAD at 24 hours (Table 14).

TABLE 14

Medium	Supplements	HAD (24 hrs)		
HEPES EAG BM	negative control: no supplement	2		
	positive controls: + 0.088 g% HCO_3^-	5		
	+ 10.5 mM arginine	4		
		histone concentrations		
		1 mg/ml	0.1 mg/ml	0.01
HEPES EAG BM	test + argine-rich histone	5	4	4
	+ lysine-rich histone	4	4-5	2
	+ crude histone	5	4-5	2

The effect of arginine-rich, lysine-rich, and crude histones on HA production in Harvey-infected human skin/muscle cells incubated at 37° C.

The results showed that arginine-rich histone was efficient in replacing HCO_3^- and arginine as requirements for HA production. Lysine-rich histone was less effective. Crude histone was slightly more effective than lysine histone at a concentration of 1 mg/ml, probably due to its content of the arginine-rich fraction.

The possibility that the histones were being used as sources of arginine and lysine rather than as substitutes for these compounds was not investigated.

However, the HA produced by histone stimulation was different to that stimulated by arginine; the HA very rapidly autoagglutinated when stored in suspension and caused a very coarse agglutination of chick red blood cells. These characteristics are like those shown by vaccinia HA produced in calf tissue (Rondle, unpublished results). Calf tissue HA in a high-titred suspension autoagglutinates in 6-7 hours on standing to give a suspension of little haemagglutinating activity, and also causes coarse agglutination. The histones used to stimulate HA production by variola virus in human cells had been prepared from calf thymus extracts (obtained from Sigma, London).

Since the physical properties of calf thymus histone-stimulated HA differed in at least two ways from arginine-stimulated HA, and since these two characteristics are also typical of HA produced in calf tissue, this is strong evidence that histone is used as such in enhancing HA production and not as a source of its component amino acids.

6.7 A General Discussion of Results described in Sections 6.1-6.6

The investigations reported in this chapter were not designed to fulfil a detailed study of the nutritional and biosynthetic requirements of variola-infected cells in the production of HA. But, having noted that the constituent status of nutrient culture media did seem to be important in determining the development of HA in infected cultures, the experiments conducted were more in the nature of a preliminary survey of the situation, and of the several possible approaches which might be made in investigating it for those avenues which promise worthwhile exploration.

In dealing with the response of the virus-cell interaction to variations in the constituents of culture media, HA production was used as an indication, rather than as an actual measurement in terms of virus growth, of the success of the virus replicatory cycle under such conditions.

Parallel HA assays and infectivity titres were not routinely carried out, but where they were there was evidence that a suppression of the infectivity yield accompanied suppression of HA production. Table 15 lists two examples.

It was therefore likely that HA suppression under media deficient in various respects reflected a suppression of the virus growth cycle as a whole.

TABLE 15

Medium	Supplement	Virus	Infectivity titre (72 hrs-pfu/ml)	HAD (24 hours)
199	0.176 g % HCO_3^-	Harvey	5×10^5	4-5
	0.088 " "	"	1.6×10^6	4-5
	0.022 " "	"	8×10^4	1
	0.176 g % HCO_3^-	Butler	9.9×10^5	3-4
	0.022 " "	"	1.3×10^4	0
HEPES EAG BM	1 mg/ml arg-hist.	Harvey	6×10^4	5
	0.001 mg/ml arg-hist.	"	$<5 \times 10^2$	1

The effect of various culture media on the production of HA and infectious virus in Harvey- and Butler-infected human fibroblasts at 37° C.

Experiments concerning the variation in $[HCO_3^-]$ of media were interpreted with some reservations . In varying the $[HCO_3^-]$ of culture media, unavoidable changes in pH occurred and it was difficult to dissociate entirely those effects due to variation of the $[HCO_3^-]$ and those due to pH differences. Adjustment of the pH of media only introduced the possibility of other inherent alterations, the extent of which would be unknown and which may even be on the $[HCO_3^-]$ itself.

The supplementing of media with amino acids and other biologically active compounds may also have effected a nutritional or metabolic balance through the action of feed-back regulatory mechanisms in the experimental cell cultures. Since nothing was known about the

efficiency with which compounds used as supplements were taken up into the cells, workable concentrations of these compounds were chosen on various arbitrary bases.

However, in spite of provisos, the indications are that HCO_3^- depletion itself is fundamental in its effects, and that arginine and subsequently the metabolism and cellular functions of histone are probably affected by this factor.

There is much documented evidence concerning the dependence of virus growth in host cells upon the presence of certain amino acids in the culture medium.

Rous, Bonifas, and Schlesinger (1963) showed that arginine was an absolute requirement for adenovirus type 2 growth in KB cells; in the presence of arginine, virus growth could take place in cells maintained in a balanced salt-solution with glucose and glutamine as the sole sources of carbon, nitrogen, and energy.

Tankersley (1963) tested the dependence of herpes simplex virus (HSV) growth in human cells upon amino acids. Eleven amino acids were required for optimal growth and the requirement for arginine was absolute; virus production and CPE did not occur under arginine-deficient media. Tankersley likened the suspension of HSV growth in arginine-deprived cells to the virus-cell relationship in latent infections.

Inglis (1966) investigated the amino acid requirements for the growth of HSV, vaccinia, and coxsackie B_5 in HK_{13} cells, paying

particular attention to the possible nutritional control of latency of infection. The HSV/RK_{13} system required both arginine and histidine for the production of infectious virus, but histidine-deficient media permitted the development of a CPE and presumably an incomplete growth cycle. Thus, arginine was again found to be an absolute requirement for both the initiation of CPE and virus production. A vaccinia/RK_{13} system showed no absolute requirement for arginine. In the absence of arginine and lysine, virus progeny was produced even though in a greatly reduced yield. The arginine and lysine requirements for the coxsackie B_3/RK_{13} system were intermediate between those of the HSV/RK_{13} system and the vaccinia/RK_{13} system.

In a completely unrelated system, Charles and Broadbent (1964) showed that CO_2 mutants of neurospora which were unable to utilize CO_2 in certain synthetic pathways were unable to do so because of disturbances on loci controlling carbamyl phosphate metabolism. The growth of mutants could be stimulated by the addition of citrulline, aspartate, arginine, uridine, or adenine depending upon the mutant concerned. This indicated that CO_2 was converted into carbamyl phosphate for use in two pathways, one for the synthesis of pyrimidines and one for arginine synthesis, two carbamyl phosphate-synthesis enzymes being involved.

An inter-reaction between HCO_3^- metabolism and arginine synthesis, and a dependence of virus synthetic pathways upon arginine and lysine have also been indicated in the study of the effects of supplementing

culture media with respect to the enhancement of HA production. The reverse and more basic approach would be to study the capacity for virus growth in terms of virus production under conditions of arginine and lysine depletion. Such a study is merited. It would be interesting in this connection to establish how far histone can substitute for arginine or lysine when these compounds are absent from, rather than their being used to enrich, the culture media. Another rewarding investigation would be to track the fate of HCO_3^- in infected cells by means of C^{14}-labeled bicarbonate in radioactive tracer experiments. This is the only way in which the sequence

$$HCO_3^- \longrightarrow \text{arginine} \longrightarrow \text{histone}$$

can be confirmed or disproved. The fate of lysine in infected cells could also be established in tracer experiments.

It would have been interesting to find that the energy-supplying process in muscle tissue was involved in the fate of arginine in the infected cell. One of the clinical features of smallpox in man is the muscle fatigue and collapse seen during the early stages of the disease. Alastrim produces this effect to a much lesser extent than does variola major. A quantitative difference in the arginine requirement for creatine synthesis in Harvey- and Butler-infected cells would have had great significance.

Even so, a lack of positive evidence for phosphocreatine involvement does not necessarily rule out this possibility; the system

may have been tested in the wrong way. If arginine is required in large amounts for a particular pathway in virus synthesis, and histone production may be just such a pathway, then in infected cells the arginine normally required for phosphocreatine production would be redirected for another purpose. According to the degree of avidity of the virus synthetic pathway for arginine, or to the greater demand for this precursor resulting from more extensive virus growth, more or less arginine would be made unavailable for conversion into phosphocreatine. A virus, weakly competitive with the normal cell requirement for arginine or which can only undergo limited growth in the host, would therefore have less effect on the normal functioning of the muscle than a virus strongly competitive for arginine or which is growing extensively in the host. Supplying the end product of phosphocreatine would relieve the need of phosphocreatine in muscle cells but would in no way satisfy the requirement for arginine in the deprived virus-specific reaction(s). Supplementing media with phosphocreatine as a replacement for arginine may not be expected to reverse HA suppression.

Variola major could then, by creating a greater metabolic imbalance in the host, cause more seriously detrimental effects than could alastrim.

Arginine-involvement and lysine-involvement in virus-specific histone synthesis would raise some interesting possibilities. One of the functions proposed for histones is to protect transcribed mRNA

from the action of cellular nucleases. (Busch, and Bonner, 1966)

Those RNA-ases coded for in the early stages of the virus cycle and which are not used in the expression of their coded function until later stages of the cycle would be particularly vulnerable to damage by RNA-ase but for the protective activity of associated histone.

In cells deprived of available bicarbonate, arginine in the intracellular pool and in the culture medium would be rapidly exhausted in biosyntheses. This could result in a deficiency of arginine for the production of new virus-coded histone; virus mRNAs may thus be lacking in the normal complement of protective histone. Professor K. R. Dumbell has suggested that mRNAs governing the expression of the property of HA production, being inadequately protected and being used late in the virus cycle, could be lost as a result of nuclease activity. No HA could be produced. Even if the mRNA were only slightly damaged, the coded message might still be unintelligible. Supplying a histone of extra-viral origin may serve to stabilize exposed mRNAs but may do so in such a way as to slightly alter the coded message. A HA of rather different physical properties could result.

Experimental results obtained do not conflict with such an argument.

Many speculations can be made concerning the effect upon the host cell and subsequently upon the intact organism of the requisitioning for virus synthetic pathways of precursor materials also essential to the normal functioning of the host.

Several promising aspects of this question have been reviewed here and are indicative of a wide scope for future research into the problem of how the symptoms of smallpox infections of man might be caused.

CHAPTER VII

General Conclusions

The following conclusions have been reached as a result of the experimental work reported in the text of this thesis:

1) Both the Harvey strain of variola major and the Butler strain of variola minor are able to grow in human skin/muscle cultures at 35°, 37°, and 38° C, but not at 39° C.

2) The optimal temperature for growth and release of mature virus in cultures of human skin/muscle fibroblasts is 35° C for the Butler strain of variola minor, and 37° C for the Harvey strain of variola major.

3) The release of mature virus from the host cell is more efficient in Harvey-infected cultures; in Butler-infected cultures the formation of intracellular virus and particularly the release of extracellular virus is sensitive to temperatures of 37° C and 38° C.

4) Between 35° and 38° C there is little difference in the net yields of infectious virus from single cycles of infection of Harvey and Butler virus in human skin/muscle cultures at any one temperature of incubation. Initial delays in the formation and release of Butler virus make a difference between the growth of Harvey and Butler in these cells apparent during multiple cycles of infection.

5) Both Harvey and Butler viruses cause a CPE in cells of human skin/muscle fibroblast cultures at 35° - 41° C successfully infected with virus during the absorption period at 25° C. Hence, virus causes CPE in

the absence of the production of mature infectious progeny.

6) Small doses of Harvey virus cause a spreading CPE in monolayers of human skin/muscle fibroblasts at 37° and 38.5° C, but not at 39.5° C. Small doses of Butler virus cause a spreading CPE at 37° C, but foci remain confined and non-necrotic at 38.5° C; no CPE is induced at 39.5° C. The difference in the vigorousness of growth can be attributed to differences in efficiency of release of virus by the two strains at these temperatures.

7) Increase in the temperature of incubation of variola-infected human skin/muscle fibroblasts reduces:
   a) the number of cells in a culture in which virus DNA inclusions develop, and in which HAD develops;
   b) the number of virus DNA inclusions per cell; and
   c) the number of cells in which DNA inclusions 'mature' and become diffused in the cell cytoplasm.

8) Variola-infected human skin/muscle fibroblasts showing the same advanced degree of inclusion formation may or may not have produced HA.

9) Once virus DNA inclusions are established within an infected cell, each inclusion develops independently; the inclusions in a single cell may be seen to be at various stages of development.

10) The development of DNA inclusions and HA in cells in Butler-infected human skin/muscle cultures is more temperature sensitive than that in cells in Harvey-infected cultures of the same cells.

11) The difference between the proportion of Harvey- and Butler-infected cells which contain few inclusions per cell and in which virus DNA

inclusion material has become diffuse is greater in chick embryo fibroblast cultures at 37° and 38.5° C than in human fibroblast cultures at the same temperatures. The difference in inclusion development seen in Harvey- and Butler-infected chick embryo cells suggests one reason for the difference in pock production of the CAM of the chick embryo at 38.5° C between the two strains.

12) Treatment of Harvey-infected human skin/muscle cells with 10^{-3} M hydroxyurea reduces the number of virus DNA inclusions developing in infective cells, reduces the number of cells in which DNA inclusion material has become diffuse, but does not reduce the number of cells capable of developing DNA inclusions in a culture. This concentration of hydroxyurea reduces variola virus production to 1/10 and HA production to ½-⅓ of that in untreated control cultures.

13) Two temperature-induced blocks in virus DNA synthesis are predicted. an 'early' block prevents the onset or a very early stage in DNA production, thus blocking the "initiation" of factories of virus synthesis; a 'late' block inhibits the organisation of DNA into nucleoids and thus blocks virus maturation.

14) Virus antigens are formed in variola-infected human skin/muscle fibroblast cells at temperatures ranging from 35° - 41° C. Antigen detected by complement fixation tests and by fluorescent antibody studies develops in the absence of detectable DNA synthesis. It is therefore possible that virus-specific proteins can be synthesised in the absence of new virus DNA synthesis.

15) An area, unstained by the AO stain, lying lateral to and displacing the nucleus in variola-infected human cells contains a site of variola virus synthesis as shown by FA studies. This area becomes

reticulate concommittantly with the merging of virus DNA inclusions; both processes precede the spreading of fluorescence from these sites and from a perinuclear area to involve the cytoplasm in general.

16) Fluorescent antibody studies have shown that virus antigen develops in variola-infected human skin/muscle fibroblast cells at temperatures at which the NP and HA antigens do not develop. Thus, the CPE seen at temperatures in which mature virus is not produced is caused by an incomplete, or abortive, virus growth cycle.

17) Infection with variola virus of skin/muscle fibroblasts at non-permissive temperatures for virus growth results in the absence of mature or immature virus particles. Electron microscopy has revealed a system of grey masses reminiscent of viroplasm surrounded by a membrane which sometimes shows a spiked surface. The membranes may possibly represent a grotesquely misassembled virus membrane.

18) There is little difference in the production of complement fixing antigen in Harvey- and Butler-infected human skin/muscle fibroblasts incubated at 35° - 39.5° C, although there is an initial delay in that produced at 39.5° C by both strains. The complement fixing antigen produced in Harvey virus at 40° C is suppressed compared with that at the lower temperatures of incubation.

19) Two antigenic components are formed in Harvey-infected human skin/muscle fibroblast cultures incubated at 37° and 38.5° C and in Butler-infected cultures incubated at 37° C which are detectable as lines of precipitation by gel-diffusion against a hyperimmune antivaccinia antiserum. Thes compononts were not detected in Harvey-infected cultures at 39.5° C and in Butler-infected cultures at 38.5° and 39.5° C in this series of experiments. One line compogent was identified with the 'S' component of variola LS antigen.

20) Harvey and Butler viruses differ significantly with respect to
    a) time of onset of HA production at 37° and 38.5° C, and
    b) the yield of HA at 39.5° C
in human skin/muscle fibroblast cultures. The onset of HA production during single-step growth cycles of Butler virus is increasingly delayed at 37° and 38° C, very little HA being produced at 39.5° C. The onset of HA production during single-step growth cycles of Harvey virus is not so markedly affected at these temperatures, and an appreciable yield of HA is attained at 39.5°C. Therefore, HA production at 37°, 38.5°, and 39.5° C is considered to be a possible marker for the biological characterisation of variola virus strains.

21) Haemagglutination and haemadsorption are *in vitro* and *in situ* manifestations of a single factor, haemagglutinin. The inagglutinable cells of a population of fowl red blood cells were not found to haemadsorb.

22) Where the infective multiplicity is greater than one, the yield of HA obtained in variola-infected human skin/muscle fibroblast cultures is independent of the multiplicity of infection.

23) Harvey and Butler virus, tested for production of HA in human skin/muscle fibroblasts, human amnion, human lung, human conjunctiva, turtle fibroblasts $ThIB_2$-75, and chick embryo fibroblasts, produced HA most efficiently in human lung cultures. The Harvey strain produced HA at 37°, 38.5° and usually at 39.5° C whereas the Butler strain produced HA at 37° C only.

24) Variola major and minor strains tested for HA production at 36°- 40° C in human skin/muscle fibroblast cultures and at 37°, 38.5°, and 39.5° C in human lung cultures formed two broad groups consistent

with the clinical classification of the strains. African variola strains tested for HA production at 37°, 38.5°, and 39.5° C in human lung cultures fell into three main groups, those resembling variola major, those like variola minor, and those which gave an intermediate response.

25) The ranking order of African variola strains with respect to HA production in human lung cultures at 37°, 38.5°, and 39.5° C was not correlated with their ranking orders with respect to the pock reduction test and the chick embryo virulence tests of Bedson, Dumbell, and Thomas (1963) and of Dumbell and Bedson (unpublished results).

26) At optimal temperatures of incubation, the yield of HA produced in variola virus-infected human skin/muscle cells depends on the availability of bicarbonate to the infected cell. Varied concentrations of bicarbonate in the culture medium used during infection elicit a graded response with respect to the yield of HA produced.

27) L-arginine and arginine-rich histone, and to a lesser extent aspartate, L-lysine, and lysine-rich histone can replace bicarbonate in reversing a medium-imposed suppression of HA production in variola-infected human skin/muscle fibroblast cultures.

28) A possible fundamental synthetic pathway in variola-infected cells, HCO_3^- ⟶ arginine ⟶ arginine-rich histones, has been suggested.

29) Calf thymus histone-stimulated HA resembles vaccinia HA produced in calf tissue in at least two ways: both types of HA rapidly autoagglutinate on standing, and both cause a coarse agglutination of sensitive fowl red blood cells.

30) The function of histone *per se* to bicarbonate-starved variola-infected cells is thought to be the replacement of that histone, not synthesised in such cells due to a lack of precursor material, which is essential for the protection of 'long-term' mRNAs from the deleterious effects of cellular nucleases. The translation of mRNA coded for HA may be prevented in bicarbonate-starved, and therefore histone-lacking, variola-infected cells. It is possible that the addition of histone from an external source stabilizes the mRNA but with a slight alteration, according to the characteristic of the histone used, to the coded message

The implications of these findings have been discussed fully in the relevant sections of the text.

In conclusion, the finding that the virus-directed functions of the Butler strain of variola minor are appreciably more thermosensitive than are those of the Harvey strain of variola major is consistent with the fact that the optimum temperature for virus growth and release in human skin/muscle fibroblasts was found to be 35° C for the Butler strain and 37° C for the Harvey strain of variola virus.

The ability of virus of the Harvey strain to initiate rampant infection in human cells *in vitro* through prompt release of mature virus, coupled with an ability to spread vigorously even at 38.5° C, is likely to favour an overwhelming and penetrating infection *in vivo* as is seen in classical smallpox in man. Bras (1952) reported the results of 177 post mortem examinations on patients who had died from smallpox in Djakarta in 1949. Gross lesions of the mucosa of the

respiratory and digestive tracts, liver, kidneys, testicles, and lymph nodes, in addition to those of the skin, were commonly found.

On the other hand, the slow release of mature progeny virus and a marked sensitivity to temperatures much in excess of 35° C of virus of the Butler strain growing *in vitro* in human cells would be likely to restrict virus growth *in vivo* to the cooler, dermal regions of the body, and to result in a more slowly spreading infection easily controlled or aborted by the natural defence mechanisms of the host. Such is seen in alastrim in man. Ribas (1911), Jorge (1924), and de Jong (1956) have agreed on the benign and superficial nature of alastrim. Patients are only moderately ill and rarely develop complications or evidence of internal disorder indicating that the internal organs are not seriously involved, if at all.

Studies on the role of biological compounds in implementing variola virus growth in human cells *in vitro* have suggested that precursor materials necessary for the normal functioning of the host cells are requisitioned for virus synthetic pathways. Since it seems likely that there is a requirement for cellular arginine for the synthesis of histone essential for the stabilisation of virus-coded mRNAs, then a virus, highly competitive for precursor material or which is growing vigorously, will be avid for the precursor arginine, and as a result, will synthesise adequate protection for mRNA from the action of cellular nucleases.

It is possible that mRNA so stabilised will confer on the virus a higher optimum temperature for growth than will the mRNAs of those viruses less avid for the precursor material necessary for histone synthesis.

If this is so, there is an interplay between the nutritional requirements of a virus during infection with the thermostability of the virus-directed functions and, consequently, with the efficiency of the growth of a virus in its host.

APPENDIX - MATERIALS & METHODS

Note 1: Viruses

Note 2: Tissue Culture

Note 3: M & M for Chapter II

Note 4: M & M for Chapter III

Note 5: M & M for Chapter IV

Note 6: M & M for Chapter V

Note 7: M & M for Chapter VI

Note 1: Viruses

1. Virus Strains

The following invaluable and comprehensive range of variola strains was kindly supplied by Professor K. R. Dumbell:

a) Variola Major:
- Harvey, Hinden (Middlesex, 1946)
- McGinty, Campbell (Glasgow, 1950)
- Level, King (Merseyside, 1958)
- Noon, Young (Yorkshire, 1952)
- Higgins (Staffordshire, 1948)
- Karachi 1 & 2 (Pakistan, 1961)
- Madras 1 (Sharma) (India, 1961)
- Madras 2 (Shuku Mia) (India, 1961)
- Bombay (India, 1961)

b) Variola Minor: (Alastrim)
- Winkel, Plugge, de Haas, de Jongh (The Hague, 1956)
- Brazil 1, 3, 7, 8, 9 (South America, 1959-1961)
- Venezuela 2 (South America, 1959-1961)
- Haithwaite (Lancashire)
- Hartridge (Italy)
- Butler (Rochdale, 1953)
- Jones, Pinson, and Bell (Birmingham, 1966)

c) East African Variola:

i) From Kenya:

Bedeta	Nafuma	Njotho	Helder
Arogo	Jabuya	Semat	Odhiambo
Kibet	Oduor	Wathiri	Kangi
Konea	Sika	Mary	
Musee	Njami	Kongo	

ii) From Tanganyika (1961-1963)

1/61	12/61
22/62	17/61
2/63	
8/61	
32/62	
6/63	

d) Vaccinia: Lister Institute Strain (egg passaged)

2. Diluents and Suspending Media

a) Glass doubly-distilled water buffered with phosphate - phosphate buffer (ionic strength 0.01) to pH 7.2 (PBDW) was used as a suspending medium and diluent for inoculation of virus onto the chorioallantoic membrane of chick embryos.

b) Normal saline buffered with phosphate-phosphate buffer (ionic strength 0.01) to pH 7.2 (PBSA) was used as a suspending medium and diluent for inoculation of virus into tissue culture.

c) 70 % glycerol (sterilised by autoclaving for 5 minutes at 10 lb/sq.in.) buffered with phosphate-phosphate buffer (ionic strength 0.01) to pH 7.2 (PBG) was used in the preparation of virus stock suspensions other than those of Harvey and Butler viruses.

3. Preparations of Virus Stock

Fertile hens' eggs were obtained commercially from Appleby Farms Ltd., Ashford, Kent and were incubated at 38.5° C in a "Westernette" incubator with hourly rocking for 12 days.

The eggs were candled 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).

Virus strains suitably diluted in PBDW were inoculated in 0.1 ml aliquots into groups of eggs in order to heavily infect the CAM. Those membranes of viable embryos showing confluent growth after three days incubation at 35.5° C were excised and placed aseptically into universal containers.

Sterile PBDW (1 ml) was added per membrane in the preparation of stock suspensions of the Harvey and Butler strains; 1 ml PBG was added per membrane in the preparation of stock suspensions of all other virus strains used.

The membranes were shaken vigorously in the suspending medium with sterile glass beads 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 supernate was carefully removed and constituted the stock suspension of virus.

Glycerol suspensions of virus were stored *in toto* at -20° C in bijou bottles.

Aliquots of virus suspension (0.1 ml) in PBDW were dispensed into bijou bottles and stored at -65° C until needed. Since the majority of experiments to be described involved the use of the Harvey and Butler strains, it was considered preferable to store this virus as aliquots rather than as a single suspension in glycerol at -20° C to be used repeatedly. The need to dilute out the glycerol before inoculation into tissue culture was eliminated, and also continual exposure to large temperature fluctuations resulting in subsequent and unknown losses in infectivity titres of the stocks was avoided. PBDW suspensions used in an undiluted form were brought to physiological isotonicity by the addition of 1 part x 10 concentrated saline to 9 parts PBDW suspension.

4. Assay of infective virus

Stock suspensions and harvested experimental tissue culture fluids were assayed for infective virus on the CAM of hens' eggs prepared for inoculation by the method of McCarthy and Dumbell (1961).

As a routine procedure, two suitable 10-fold dilutions in PBDW were made from the material to be titrated, and 0.1 ml aliquots of these dilutions inoculated into groups of 3-4 eggs per dilution. The eggs were sealed with a paraffin-wax/vaseline mixture, gently rolled to distribute the inoculum uniformly, and incubated at 35.5° C for 3 days. The CAMs were excised, and the pock count for each membrane made. Infectivity was expressed as pock-forming units (pfu) per ml

of undiluted virus suspension or harvest fluid.

The pfu:virus particle ratio was assumed to be equivalent for all virus strains used. Cruickshank and Bedson (unpublished results) have found the particle:infectivity ratio to be 6:1 - 10:1 for variola major and alastrim viruses tested on the chiek CAM.

5. Virus Inocula

In the experimental work described in this thesis, it was necessary to know the infectivity content of the inocula of Harvey and Butler virus used. Virus inocula of two infectivity titres were regularly used:

a) "large doses of virus" = 10^6 pfu virus/inoculum, and
b) "small doses of virus" = 300 pfu virus/inoculum.

Experiments with all other virus strains were not so critical. As long as inoculated cell monolayers were exposed to an infective multiplicity greater than one, accurate adjustment of input infectivities contained in inocula was not necessary. Dilutions of 1/10 (diluting out the PBG) of stock virus suspensions provided 0.1 ml aliquots of inocula of a sufficiently high titre for use.

Note 2: Tissue Culture

1. Culture Media

The following culture media were used for the routine growth and maintainance of tissue culture:

a) Growth medium: Eagles Minimal Essential Medium or Parker's 199 medium
+ 10% uninactivated foetal calf serum
+ 2 % of a 4.4 % solution of Sodium Bicarbonate "gased" with CO_2 to pH 7.2
+ 100 u/ml penicillin
+ 100 µg/ml streptomycin
+ 50 µg/ml nystatin
+ 1 µg/ml tylosin

b) Maintainance medium: Eagles Minimal Essential Medium or Parker's 199 medium
+ 10 % liver digest ultrafiltrate
+ 2 % of a 4.4 % Sodium Bicarbonate solution "gased" with CO_2 to pH 7.2
+ antibiotics as in growth medium (a)

The human skin/muscle fibroblast cultures used for the study of the growth of Harvey and Butler, during which samples were assayed for infectivity, haemagglutinin, and complement-fixing antigen, were grown and maintained on culture media based on EAG MEM. Chick embryo fibroblasts were grown in EAG MEM containing a 3 % bicarbonate solution.

All other routine tissue culture was grown and maintained in 199-based media.

2. Cell Strains

The following primary cell strains were initiated in the Laboratory:

Human Foetal Skin/Muscle Fibroblasts
Human Foetal Lung Cells
Chick Embryo Fibroblasts

The following cell strains were obtained as established tissue cultures:

Human Conjunctiva
Human Amnion - Flow Laboratories, Glasgow

$ThIB_2$-75 Turtle Fibroblasts - Dr. Clark, USA (Clark & Karzon, 1965)

Human conjunctiva and human amnion cultures were provided seeded in tubes and on coverslips ready for use. $ThIB_2$-75 cells were sub-cultured in the laboratory before use.

3. Culture Methods

a) Initiation of primary cultures

Skin/muscle tissue and the lungs were removed aseptically from normal human foetuses of 12-17 weeks gestation to provide tissue for the propagation of skin/muscle fibroblast cultures and lung cultures respectively.

Ten-day old chicken embryos were removed aseptically from fertile eggs, decapitated, and freed of viscera to provide tissue for the propagation of primary chick embryo fibroblasts.

Embryo tissue from these three sources was treated as follows:

Tissue fragments were washed in PBSA containing 100 u/ml penicillin and 100 µg/ml streptomycin, and then trypsinised in 100 ml aliquots of a 0.1 % (human tissue) or a 0.25 % (chick tissue) trypsin solution in saline in a waterbath maintained at 35° C. Sterile, teflon-coated magnets placed in the trypsinisation flasks and used in conjunction with external rotating magnets kept the trypsin solutions and tissue in constant agitation. After 30 minutes, the supernate was decanted and replaced by a fresh 100 ml aliquot of trypsin. Trypsinisation was repeated until the required amount of cell suspension had been obtained, or until the tissue supply was exhausted. The cells in the decanted supernates were sedimented by centrifugation at 1000 rpm for 10 minutes. Cell pellets were re-suspended in 10 ml growth medium minus calf serum.

Suspensions of chick embryo fibroblasts were subjected to a viable count, and diluted with complete growth medium to give concentrations of 2.5×10^6 cells/ml medium. Aliquots (1 ml) were dispensed into tubes. The tubes were gently shaken to ensure an even distribution of cells, and incubated at 37° C for 24 hours until confluent monolayers had formed. The monolayers were then ready for use in virus studies.

The volume of the suspensions of human skin/muscle fibroblasts and human lung cells in growth medium minus calf serum was made up to 80 ml with complete growth medium. The resulting suspensions were dispensed as 40 ml aliquots into 12 oz medical flat bottles. The bottles were gently shaken to ensure even distribution of the cells, and left to incubate undisturbed for 48 hours at 37° C. After 48 hours the bottles of cells were fed with fresh growth medium where needed, and subcultured for use in virus studies, and for the continued propagation of the cell strain, when the monolayers had become confluent.

b) Subculturing of cell strains

(human skin/muscle fibroblast cultures, human lung cultures, and $ThTB_2$-75 turtle fibroblast cultures)

To one confluent 12 oz bottle of cells, 2 ml of a 1 % trypsin solution was added and left in contact with the cell sheet for 30 seconds and then carefully decanted. The residual fluid was sufficient to complete cell stripping within 1-3 minutes at room temperature.

When the cell sheet began to break up, 2 ml of growth medium minus calf serum was added and the cells gently resuspended. The suspension was made up to 80 ml with complete growth medium and dispensed as 40 ml aliquots in 12 oz bottles for continued propagation of the cell strain. If monolayers in tubes for virus studies were

required, then the cell suspensions were made up to 10 ml with complete growth medium and dispensed as 1 ml aliquots in tubes.

Coverslip cultures were prepared by seeding 0.11 ml cell suspensions per coverslip.

Bottles and tubes were gently shaken and incubated at 35-37° C and refed when necessary until confluent monolayers had formed.

Since it seemed important to use cells in a similar metabolic condition for each experiment (Smith and Sharpe, 1960), those monolayers which had attained confluence overnight were used for virus studies.

Because of cell clumping, it was difficult to perform a meaningful viable cell count prior to each seeding of monolayers. Therefore, as a standard procedure, 10 tubes were seeded from the contents of a 12 oz confluent bottle of cells. The seeding rate was estimated to be approximately 3×10^5 cells/tube.

e) Cultures of cells (human amnion, human conjunctiva) received through the post ready for use were incubated overnight at 37° C after the transit medium had been replaced by fresh maintainance medium. This procedure allowed the cells to stabilize before use in virus studies.

4. Glassware

Soda-glass 12 oz bottles and bacteriological test tubes to be used for tissue culture were washed in "Pyroneg", rinsed in tap water and several changes of glass double-distilled water, dried and sterilized by dry heat. Bottles were screw-capped using silicone-rubber washers, and tubes were stoppered with non-toxic rubber bungs.

Soda-glass coverslips (1" x $\frac{1}{4}$") for use in cytological studies were rinsed in absolute alcohol and sterilized by dry heat.

5. Incubators

Extensive use was made of temperatures ranging from 25°- 41° C for the incubation of infected cultures and uninfected controls during the experimental work.

The following arrangements were made:

25° C - a constant temperature room maintained at 25° C by thermostatically-controlled infrared heat lamps.

30°- 35°C - a thermostatically controlled large-capacity incubator in which air circulation was maintained by a fan. The incubator temperature was adjusted to the required temperature and the constancy of temperature was checked by observation of thermometers placed near the incubated materials.

36°- 40°C - thermoelectronically-controlled 'Leec' incubators provided with fans for efficient air circulation. Incubator temperatures were monitored using an electrical temperature recorder with display-chart (Grant's Instruments, Cambridge). Incubators were kept in an ambient temperature of 25° C and were opened only when experimental work made this necessary. This step ensured that the temperature fluctuation was less than 0.5° C.

41° C - a small fan-operated incubator with a temperature fluctuation from 41° to 42° C. The bulbs of thermometers immersed in 10 oz bottles containing water and a liquid paraffin seal were placed in strategic positions in all incubators, and temperatures were checked regularly.

Note 3: (Chapter II)

1. Multiple-step Growth Cycle Experiments

Confluent monolayers in tubes were inoculated with virus at the rate of 300 pfu virus/tube. The virus was allowed to adsorb for two hours in the stationary state at the temperature of incubation for the experiment to follow. The inoculum was removed and the cell sheets washed with warm PBSA to remove unadsorbed virus. One ml of maintainance medium was added to each tube, and the tubes were placed at the required temperature for incubation. Duplicate tubes were sampled for each virus strain at 4 hours (baseline) and thereafter every 24 hours from the time of infection for six days. During sampling, the supernates from each pair of tubes were removed and pooled. One ml of fresh maintainance medium was added to the remaining cell sheet in each tube. The cellular and supernatant harvests were then stored at -20^{0} C until titrated for infected virus.

On the day of infectivity-assay, the specimens were thawed. Cellular fractions were subjected to two further cycles of freezing and thawing to liberate cellular virus, any monolayer still remaining attached to the glass being removed and resuspended in the medium by pipetting. Pairs of cellular fractions were pooled. Both the supernates and the cellular suspensions were then sonicated for 30 seconds (Electrosonic tank: Headland, London; operational λ = 80 kc/s) to break

down cell debris and to disrupt any viral aggregates. The samples were then titrated in eggs at suitable dilutions, and the titre of cell-associated and free, extracellular virus contained, respectively, in the cellular and supernatant fractions was calculated.

2. Single-step Growth Cycle ~~Supernatants~~ Experiments

Confluent monolayers in tubes were inoculated with virus at the rate of 10^6 pfu/tube in order to expose all the cells to virus during the absorption period. The virus was adsorbed for two hours in the stationary state at the temperature of incubation of the experiment. The inoculum was removed and the cell sheet washed three times with warm PBSA to remove unadsorbed virus, being of a significant quantity. One ml of maintainance medium was added to each tube and the tubes placed at the temperature of incubation.

Duplicate tubes were sampled for each virus strain at four-hourly intervals after infection until 28 hours, and at 36 and 48 hours. The cellular and supernatant fractions of each sampling were treated and assayed for infectious virus in the same way as described for the multiple-step growth cycle samples.

The cellular and supernatant samples harvested during the single-step growth cycles were also assayed for complement-fixing antigen and for haemagglutinin (see Note 5)

Note 4: (Chapter III)

1. Observation of CPE

Confluent monolayers of human skin/muscle fibroblasts in tubes were inoculated with 0.1 ml Harvey and Butler virus suspension containing (a) 10^6 pfu/inoculum and (b) 300 pfu virus/inoculum. The virus was absorbed for 2 hours at 25° C, the inoculum was removed, and 1 ml of fresh maintainance medium was added per tube. Tubes were then incubated in duplicate at the required temperature.

Those tubes inoculated with a large dose of virus were then observed four-hourly for 24 hours using a Vicker's microscope adapted for phase contrast microscopy by the addition of the McCarthy apparatus. Those tubes inoculated with a small dose of virus were observed by phase contrast microscopy twice daily.

2. Cytochemical Methods

Human conjunctiva and human amnion cells (obtained from FLOW Laboratories, Glasgow), human skin/muscle fibroblasts and chick embryo fibroblasts were grown to semiconfluence on sterile 1" x $\frac{1}{4}$" coverslips, and were infected with 10^6 pfu virus/coverslip. The inoculum was absorbed for 2 hours at 25° C, removed, and 1 ml medium added to each tube in which the coverslip culture was contained. Cultures were incubated at the required temperatures and harvested for cytochemical studies as required.

a) Acridine Orange Staining Method for Nucleic Acids

Coverslip cultures were washed briefly in PBSA pH 7.4 and fixed in absolute alcohol for 10 minutes. Staining was carried out in a 0.05 % solution of Acridine Orange-2G (AO) in McIlvaine's citrate-phosphate buffer pH 2.6 for 30 minutes. Coverslips were then washed rapidly in six changes of McIlvaine's buffer pH 2.6 and mounted in 20 % phosphate-buffered glycerol pH 7.4. Preparations were sealed with nail varnish and examined for the presence of cytoplasmic virus DNA inclusions using the ultraviolet microscope and oil-immersion lens.

After staining with AO, RNA-containing components of cells fluoresce flame-red, and DNA-containing components fluoresce apple-green.

Since fowl red blood cells (RBC) contain nuclei which stain green in AO treated preparations, haemadsorption by infected coverslip cultures of agglutinable fowl RBC can also be studied by AO staining. Coverslip cultures are flooded with a 1 % suspension of agglutinable RBC and incubated for 1 hour at 37° C with occassional agitation to resuspend unadsorbed RBC. The coverslips are then washed well in PBSA, fixed, and stained in the usual way.

b) Fluorescent Antibody Sandwich Technique for Staining Virus Antigen

Coverslip cultures were fixed in acetone for 10 minutes, air-dried, washed thoroughly in PBSA, and redried by blotting. The coverslips were placed in a moist chamber, flooded with two drops of a 1/10

dilution of a hyperimmune antivaccinia antiserum, and incubated for 30 minutes at 37° C.

Preparations were then washed in four changes of PBSA for a total time of 15 minutes and were replaced in the moist chamber.

Coverslips were flooded with two drops of fluorescein-conjugated anti-rabbit globulin (sheep origin - obtained from Progressive Laboratories Inc., Baltimore, U.S.A.: cat. no. 928) and re-incubated for 30 minutes at 37° C.

After a further washing in four changes of PBSA for 15 minutes, coverslips were mounted in 20 % phosphate-buffered glycerol pH 7.4 and examined using an ultraviolet light-illuminated microscope and oil-immersion lens.

Areas of virus antigen fluoresced brightly yellowish-green against a background of dim non-specific fluorescence given by cellular cytoplasm in the absence of virus antigen.

e) Preparation of Frequency Polygons

Quantitative studies were made by counting the number, and calculating the percentage, of haemadsorbing cells and cells containing DNA-inclusions in infected coverslip cultures incubated at a range of temperatures and stained with AO. The number and percentage of cells containing x number of DNA inclusions was also calculated by counting the number of DNA inclusions in infected cells. Frequency

polygons (Figs. 10-15) were constructed from these results expressed in percentages. Hydroxyurea obtained from Sigma, London was used in the quantitative studies as described in the text.

d) Colour Photography

Photographs were made as transparencies using Kodachrome II A (ASA 40) and Ektachrome (ASA 125) high speed film for artificial light produced by Kodak Ltd. using Gillet and Sibert (London) photographic equipment for use with microscopes. The UV-light illumination source was turned to maximum brightness giving 1/10 to 1 light unit reaching the film plane in the camera. Exposures ranged from 1-3 minutes using Ektachrome film and 2-5 minutes using Kodachrome II A.

Prints were processed from the resulting transparencies by Kodak Ltd., Hemel Hempstead.

3. Electron Microscopy

Cultures of human skin/muscle fibroblasts grown to confluence in 48 hours in Petri dishes were prepared using a growth medium consisting of :

Eagles Minimal Essential Medium
+ 10 % tryptose-phosphate broth
+ 10 % foetal calf serum
+ 2 % of a 4.4 % bicarbonate solution gassed with CO_2 to a pH of 7.4

The cultures were infected with variola virus as described in the text using 199 + 10 % liver digest + 2 % of a 4.4 % bicarbonate solution as maintainance medium and were prepared by Dr. J. G. Cruickshank for electron microscopy in the following way:

Infected monolayers and uninfected control monolayers were washed in Dulbecco Solution A and removed from the glass surface with a "rubber policeman". The pellets of suspended cells were fixed immediately in 5 % glutaraldehyde in 0.1 M phosphate buffer, washed overnight in cacodylate buffer, and post-fixed in buffered osmium tetroxide. The cell samples were passed through graded alcohols (20 %, 50 %, 70 %, 90 %, and absolute alcohol) and embedded in pre-polymerised methacrylate (1 part methyl:9 parts butyl) which was then polymerised overnight at 6° C.

Sections of embedded samples were cut on the model MT Porterbloum ultramicrotome and were examined in the Siemens Elmoscope 1 at magnifications of 2000 - 20,000 x. Photographs were made of interesting fields of view.

Note 5: (Chapter IV)

1. Complement Fixation Tests

The method used for the complement fixation tests was based on that laid down by Bradstreet and Taylor (1962).

(a) Materials:

standard antiserum - a hyperimmune antivaccinia rabbit antiserum used at a dilution of 1/320, the optimal titre of the antiserum as determined against a standard antigen prepared from Harvey-infected chick embryo CAMs.

test antigen - four-hourly cellular and supernatant samples harvested during the course of single-step growth cycles of Harvey and Butler viruses in human skin/muscle fibroblasts (see note 3).

complement - guinea pig complement stored at -55° C and used at $3HD_{50}$. (HD = haemolytic dose).

Red blood cells - sheep red blood cells stored in Alsevers anticoagulant and preservative solution, washed three times in diluent and made to a 4 % suspension.

Diluent - Ca-Mg buffered diluent designed for the complement fixation test.

(b) Method:

A unit volume of 0.1 ml was employed throughout the test. The method of long fixation was used. Each test antigen was dispensed in

duplicate in the undiluted form and in doubling dilutions from 1/2 to 1/64 in two adjacent rows of wells in perspex "agglutination trays". Unit volumes of standard antiserum at its optimal titre (the test series) were added to one row of antigen dilutions, and unit volumes of diluent (the control series) were added to the second row. Any anti-complementary activity contained in the test antigen material was thus detected and measured in the control series and could be taken into account when the final readings of the tests were made. Complement at a concentration of $3HD_{50}$ was then added to each well.

Control wells in which complement, or antigen, or antibody had been replaced by diluent were set up along with a cell control containing 3 unit volumes of diluent and to which only sensitised cells were to be added later in the test. A complement titration, involving wells containing $0.5HD_{50}$, $1HD_{50}$, and $3HD_{50}$ complement and two volumes of diluent in each well replacing test antigen and standard antibody, was made to ensure that the working concentration of complement of $3HD_{50}$ had been maintained and used in the test.

Plates were covered to prevent evaporation and were stored overnight at 4° C. A 4 % suspension of sheep RBC was sensitised with an equal volume of haemolytic serum at its optimal sensitising concentration and placed in a waterbath at 37° C for 10 minutes to complete the sensitisation of the cells. The sensitised cells were then stored overnight at 4° C.

On the day of the test, plates were warmed in a 37° C incubator for 30 minutes. Sensitised cells were returned to the waterbath at 37° C for 30 minutes.

One volume of sensitised cells was delivered to each well. Plates were incubated at 37° C for 30 minutes with shaking at the beginning, after 15 minutes, and at the end of incubation. The cells were allowed to settle at 4° C before reading the results of the test.

The results were graded according to the degree of fixation of complement. The endpoint was taken as being that dilution of test antigen which gave 50 % fixation (50 % lysis) or a reading of 2 as scored by the method of Bradstreet and Taylor.

2. Gel-Diffusion Tests

a) Preparation of Antigens

(i) Confluent monolayers of human skin/muscle fibroblasts grown in 12 oz medical flats were infected with 10^6 pfu per bottle of virus of the Harvey and Butler strains. Absorption was carried out at 25° C for 2 hours and the inoculum removed. 35 ml of maintainance medium was added per bottle, and one bottle of infected cells for each virus strain incubated at 37°, 38.5°, and 39.5° C for three days. The bottles and contents were then stored at -20° C until the day on which the antigens were to be prepared.

The bottles were thawed at room temperature and the cell monolayers resuspended into the supernates using "rubber policemen". The antigen-containing materials were then dialysed in 8/32" Viskin tubing against distilled water at 4° C with daily changes of distilled water for seven days.

The contents of the dialysis sacs were transfered to 250 ml centrifuge pots capped with viskin tubing into which had been cut a slit. The antigen-containing solutions were then shell-frozen in a mixture of acetone and solid CO_2 and dried overnight from the frozen state in an "Exal-Virtis" lyophilisation apparatus. When the "Pirani" gauge for total gases and the "Genevac" gauge for dry gases gave the same reading indicating that all moisture had left the frozen samples, the drying process was complete.

The lyophilised antigen material was harvested and the separate samples H37, H38.5, H39.5, B37, B38.5, and B39.5 were made up to a concentration of 10mg/ml in saline. A concentration of the antigenic material of approximately seventy-fold had been effected.

ii) Concentrated suspensions of Harvey virus in PBDW prepared from the infected CAM of hens' eggs and separated from cellular debris as described in Note 1.3 were used as concentrated preparations of variola soluble antigens from egg after adjusting to isotonicity with 10x concentrated saline.

(iii) Egg preparations of Harvey soluble antigen in bijou bottles were incubated at 56° C for 2 hours in a waterbath to provide preparations of heated variola soluble antigen.

b) Preparation of the Ouchterlony Plates

5 ml aliquots of a 1 % solution of New Zealand 'Davis' agar in saline containing 1 part in 10,000 of sodium azide and stored in the fluid phase at 56° C were dispensed into 2" petri dishes. The agar was allowed to gel in the petri dishes at room temperature, and when cold and firmly set an isometric pattern of wells was cut into the agar 4 mm in diameter and 4 mm apart using a perspex template and a stainless steel cutter sharpened from the inside.

Antigen or antiserum was added to the wells as required until the meniscus just disappeared. Plates were left to develop for 24 to 48 hours at room temperature and then examined for lines of precipitation.

3. Haemagglutination Tests

Fresh fowl cells, collected in an equal volume of Alsever's citrate-dextrose anticoagulant from birds with known poxvirus haemagglutinin-sensitive RBC, were used. Before use, the RBC were washed three times in PBSA and made up as a 1 % suspension containing 1 % normal heat-inactivated rabbit serum to stabilise the red cells and to prevent non-specific auto-agglutination.

A unit volume of 0.25 ml was used throughout the test. The diluent was PBSA.

Cellular and supernatant samples collected from single-step growth cycles of Harvey and Butler virus in human skin/muscle fibroblast cultures (see Note 4) and stored at -20° C were thawed and ultrasonicated for 30 seconds to disperse aggregates of HA which are known to occur on storing in the frozen state (Neff, Ackermann, and Preston, 1965).

Samples were dispensed in the undiluted form and as doubling dilutions from 1/2 to 1/64 in rows of wells in perspex "agglutination trays".

A sample of known haemagglutinin titre was also diluted as a positive control, uninfected cell material was diluted as a negative control, and wells containing diluent only (to which RBC were to be added) acted as cell controls.

One volume of 1% RBC suspension was added to each well and plates incubated at 37° C for 1 hour with shaking before incubation, after 15, 30, and 45 minutes, and at the end of incubation in order to resuspend the RBC.

Plates were left at room temperature for 1 hour, and the results were recorded when the cells had settled. 100 % haemagglutination was recorded as '+', and an estimate of lesser degrees of haemagglutination (75 %, 50 %, 25 %) and trace (t) haemagglutination was noted.

Spot haemagglutination-inhibition tests were performed on positive samples on samples giving doubtful results in order to confirm that

haemagglutination was specific. Duplicate dilutions, from undiluted to the highest dilution known to have given haemagglutination, were made for each sample to be tested. One series of dilutions was incubated for one hour at room temperature with 1 unit volume per well of a 1/64 dilution of a hyperimmune antivaccinia rabbit antiserum, the other series of dilutions with one volume per well of diluent as a positve control. One volume of 1 % suspension of RBC was then added to each well, and the plates were incubated as described for the straightforward haemagglutination test.

If positve haemagglutination reactions are specific for virus haemagglutinin, then antigen incubated with antiserum shows no haemagglutination as compared with the expected titre given by the positive control. Immune sera fail to inhibit haemagglutination if this is non-specific.

Although it was impossible to conduct all haemagglutinin assays on the same day with RBC from the same fowl, conditions for the titration within each group of growth samples were constant, and positive controls of known titre were included in each group of samples so that valid comparisons of results obtained on different occassions could be made.

Note 6: (Chapter V)

1. Assay of Haemagglutinin

a) Haemagglutination test - (see method described in Note 5.3)

The cell monolayer of each sampled culture was resuspended into its supernatant, subjected to 30 seconds sonication, and like pairs of culture material pooled. These constituted the test antigens and were diluted for HA assay.

b) Haemadsorption Test

0.2 ml of a 1 % suspension of agglutinable fowl RBC in saline were added to the culture medium of the monolayers in tubes to be tested for haemadsorption (HAD). The cultures were incubated with the RBC at 37° C for 1 hour with occassional shaking to resuspend unadsorbed RBC. Monolayers were then carefully washed twice with warm PBSA, and 1 ml of fresh maintainance medium was added to each tube. Cultures were examined by phase contrast microscopy, and an estimate of the extent of HAD, if any, was made.

c) Quantitative Haemadsorption Method (Finter, 1964)

0.2 ml of a 1 % suspension in saline of agglutinable fowl RBC was added to the culture medium of monolayers in tubes to be tested. The cultures were incubated at 37° C for 1 hour with occassional shaking to resuspend unadsorbed RBC. Supernates of tubes were decanted and monolayers were washed twice with PBSA. 2 ml of distilled water

was added to each tube to lyse the adsorbed RBC. After 10 minutes, when haemolysis was complete, tubes were placed in a waterbath at 37° C for the mixing of contents by convection to ensure a uniform concentration of haemoglobin without unduly disturbing the cell monolayers.

The absorption and percentage transmission given by the resulting haemoglobin solutions was determined in a Beckman spectrophotometer at a wavelength of 410 mμ at which point oxyhaemoglobin shows a maximum peak. The results of the different samples were compared.

2. Production of Haemagglutinin in Various Cell Types

Confluent tube monolayer cultures of the various cell types described in the text were inoculated with 10^6 pfu virus/tube of the Harvey and Butler strains of variola. Absorption was carried out at 25° C for 2 hours, the inoculum was removed, and 1 ml fresh maintainance medium was added to each tube. Tubes were then placed at the appropriate temperature of incubation in groups representing each virus and each cell type.

The haemadsorption test was applied to cultures from each group sampled at 24 and 48 hours, and the haemagglutination test to cultures sampled at 72 hours.

3. Screening Variola Strains in Human Skin/Muscle Fibroblast Cultures and in Human Lung Cultures

Confluent tube monolayer cultures of human skin/muscle fibroblast cells and human lung cells were infected with 0.1 ml aliquots of 1/10 dilutions of high-titred glycerol stock suspensions of variola virus strains stored at -20° C. Such inocula were sufficient to give an infective multiplicity of greater than one in each monolayer. Absorption was carried out at -25° C for 2 hours, the inoculum removed, and 1 ml of fresh maintainance medium (Note 2.1) added. Tubes were placed in duplicate at the appropriate temperatures for incubation. After 3 days, the cultures were harvested and prepared for assay of the haemagglutinin content (Notes 6.1 and 5.3).

The following virus strains were used:

a) in human skin/muscle fibroblasts:

major { Higgins, Campbell, McGinty, Level, King, Hinden, Young, Bombay
Sharma (Madras 1), Shukla Mia (Madras 2), Karachi 1, Karachi 2

minor { Brazil 1, 3, 7, 8, 9, Venezuela 2, Winkel, de Haas, Bell,
Jones, Hartridge, Haithwaite

Tanganyika { 2/63, 22/62, 32/62

Kenya { Njotho, Bedeta

b) in human lung cells:

major { Higgins, Hinden, Level, King, Noon, Bombay

minor {Plugge, Winkel, Haithwaite, Brazil 1,7, Venezuela 2

Tanganyika {6/63, 1/61, 8/61, 12/61, 17/61, 22/62, 32/62, 2/63

Kenya {Kibet, Bedeta, Wathiri, Njotho, Semat, Jabuya, Odhiambo, Kones, Sika, Mary, Kangi, Njami, Arogo, Kongo, Muzee, Oduor, Helder, Nafuna

4. Experiments with three basic culture media

a) materials

Eagles Minimal Essential Medium
4.4 % Sodium bicarbonate solution
liver digest ultrafiltrate
} obtained from Burroughs Wellcome Co., London

Parker's 199 } obtained from Glaxo, Ltd.

Eagles Basal Medium, buffered with HEPES (See note 7.1aii), was made up from the constituent ingredients according to the formula for Eagles Basal Medium, and contained 0.11 g % bicarbonate.

"Used" 199 + 10 % liver digest ultrafiltrate was prepared by incubating 199 + 10 % liver digest ultrafiltrate with a monolayer of metabolising uninfected human skin/muscle cells for 4 days.

b) Method

Confluent monolayers in tubes of human skin/muscle fibroblasts were infected with 10^6 pfu per tube of virus of the Harvey and Butler strains. Absorption was carried out for 2 hours, the inoculum removed, and 1 ml of the appropriate culture medium as described in the text added to each tube before incubating at 37° C.

Infected cultures were sampled at 24 hours for the haemadsorption test and at 72 hours for the haemagglutination test.

Note: 7 (Chapter VI)

1. Materials for Nutritional Studies

a) Basic Media

Eagles Minimal Essential Medium, Eagles Basal Medium, and Parker's 199 medium obtained as described in Note 6.4 were used as basic media. The following buffering systems have been used to buffer the basic media:

i) 2 % of a 4.4 % sodium bicarbonate solution with a gaseous phase of 5% CO_2 in air (the bicarbonate concentration was reduced as required).

ii) 0.646 g/100 ml medium of HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid) (Good et al., 1966). HEPES-buffered Eagles Basal Medium contained 0.11 g % bicarbonate.

iii) Tris (hydroxymethyl) aminomethane buffer at a final concentration of 0.01 M.

The basic media were supplemented as described in the text with the following biological compounds: (obtained from Sigma, London)

L-amino acids (listed in text) Grade A
B-vitamins (listed in text) Grade A
Calf thymus DNA
Yeast extract RNA
Adenosine triphosphate
D-ribose
DL-citrulline (Grade 1)
Carbamyl phosphate,
Dilithium salt
Creatine·H_2O (practical grade)
Phosphocreatine hydrate, sodium salt

Calf Thymus histone:
(type II crude histone)
(type III lysine-rich histone)
(type IV arginine-rich histone)

b) Experimental media of Mosley and Enders (1962)

	Medium for:	
	(i) screwcapped cultures	(ii) cotton-plugged
10x concentrated 199	1 ml	1 ml
13.3% solution $Na_2HPO_4 \cdot 12H_2O$	0.1 ml	0.1 ml
1 M tris buffer	0.1 ml	0.1 ml
4.4% $NaHCO_3$ solution	0.5 ml	——
Distilled Water	8.3 ml	8.8 ml
1 N HCl dropwise to:	pH 7.0	pH 7.2

cotton-plugged cultures were incubated in a humidified atmosphere.

2. Method

Confluent monolayers in tubes of human skin/muscle fibroblasts were inoculated with 10^6 pfu per tube of virus of the Harvey and Butler strains. Absorption was carried out at 25° C for 2 hours, the inoculum removed, and 1 ml of the appropriate experimental medium added to each tube as described in the text.

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