J. Hyg., Camb. (1982), **89**, 389–397
Printed in Great Britain

The pathogenicity of variola virus

A comparison of the growth of standard strains of variola major and variola minor viruses in cell cultures from human embryos

By K. R. DUMBELL and D. G. T. WELLS

St. Mary's Hospital Medical School, London W2

(*Received as part of an academic tribute to A. W. Downie*)

SUMMARY

The international reference strains of variola major (Harvey) and of variola minor (Butler) were grown in cultures of skin and muscle cells from human embryos. The development of infective virus, complement-fixing antigen, haemagglutinin and cytological changes were followed at four temperatures between 35 and 40 °C. No significant difference was found in the amount of virus produced by Harvey or Butler viruses at any of the experimental temperatures, but Harvey attained the plateau titre at 16 h, some 4 h ahead of Butler in the cultures incubated at 38 °C. Harvey also produced a higher and more prolonged yield of virus in the extracellular medium of cultures, inoculated at low multiplicity and incubated at 37 °C. At 38 °C small inocula of Harvey produced foci which developed and spread till the whole culture was necrotic; Butler foci did not spread and remained relatively undeveloped at this temperature.

Staining with acridine orange showed the development of cytoplasmic inclusions at all temperatures up to 39·5 °C, at which temperature most inclusions remained round and discrete and susceptible to digestion with DNase. The yield of virus at all temperatures correlated well with the number of cells in which the DNA cytoplasmic inclusions became irregular in outline, diffuse and insusceptible to digestion with DNase. It was concluded that elevated temperatures, up to 39·5 °C affected principally a maturation phase in the development of the virus.

Equal amounts of complement-fixing antigen were produced at all temperatures and by either virus, but the late product, haemagglutinin was depressed at elevated temperatures much more in cultures infected with Butler than in cultures infected with Harvey. This was clearly shown by haemadsorption; in cultures, infected at high multiplicity and incubated at 39·5 °C Harvey gave semi-confluent haemadsorption, while only an occasional haemadsorbing cell could be found in the cultures infected with Butler virus.

It is concluded that there were two ways in which temperature affected the growth of variola viruses in cultures of human embryo skin and muscle cells. The total yield of virus was reduced by inhibition of a stage in the maturation of the virus with a cut off point betweeen 39·5 and 40 °C at which no cells produced infective virus, and with little observable differences between Harvey and Butler viruses. Changes at the surface of virus-infected cell, involving virus release and haemagglutinin, were affected independently of virus maturation; in these changes Butler was much more sensitive than Harvey to elevated temperatures. The relevance of these observations to the development of variola major and variola minor in man is discussed.

https://doi.org/10.1017/S0022172400070960 Published online by Cambridge University Press

INTRODUCTION

The eradication of human smallpox has been followed by a virtual cessation of work on variola virus; a few studies only are in progress and these have been sponsored by the World Health Organization. It is appropriate to record any observations that have been made on variola viruses to complete the historical account of a remarkable event in the history of medicine; some of these studies may also be relevant to future understanding of viral pathogenicity.

Fears have been expressed that a world population in which routine smallpox vaccination is no longer practised may become liable to increased incidence and severity of infections due to other orthopoxviruses. The reasons why variola, alone among the orthopoxviruses, was a virulent human pathogen have so far proved elusive. This series of papers will approach the question by comparing variola strains, known to have different levels of virulence, in the hope that a laboratory correlate of human pathogenicity may be found that could refer also to other orthopoxviruses isolated from non-human sources.

The international reference strains of variola major (Harvey) and of variola minor (Butler) have been compared for their growth and virulence in the chick embryo (Dumbell, Bedson & Rossier, 1961). This paper presents the results of a comparison of these two viruses in human diploid cell cultures.

MATERIALS AND METHODS

Virus strains. Variola major, strain Harvey and variola minor, strain Butler were used at comparable low-passage stocks prepared from heavily infected chick chorioallantois (CAM). Clarified extracts of confluent membranes were made up to 50% with glycerol and stored at −20 °C. For some experiments virus was deposited by high speed centrifugation, the pellet resuspended in 0·01 M citrate phosphate buffer pH 7·2 and stored in 0·2 ml aliquots at −65 °C.

Cell cultures. Limbs and lungs of human embryos of 12–17 weeks gestation were made available through the courtesy of Dr Kay and the Royal Marsden Hospital. Cell suspensions were prepared by trypsinization and grown in Eagles MEM medium supplemented with 10% fetal calf serum. Cultures were maintained in MEM without serum but with 10% liver digest ultrafiltrate. Cell cultures were used from the 4th to the 30th passage. No significant differences were found over this range or from one fetus to another.

Incubators (LEEC) fitted with circulating fans were used and the temperature was continuously monitored throughout each experiment. The incubators were kept in a constant ambient temperature of 25 °C in order to minimize temperature fluctuations and were opened as infrequently as possible. By these means temperatures were kept within 0·25 °C of the desired value.

Assays. Infective virus was assayed by pock counts on CAM using four eggs per dilution and counting those dilutions which averaged between 10 and 200 pocks per membrane. Complement-fixing antigen was assayed by a standard technique, using as antiserum an hyperimmune antivaccinia rabbit serum. The optimal dilution of antiserum was determined against a standard antigen prepared from CAM inoculated with strain Harvey. Fixation was 18 h at 4 °C and tests were set

https://doi.org/10.1017/S0022172400070960 Published online by Cambridge University Press

up using an unit volume of 0·1 ml. Haemagglutinin was assayed by the method of McCarthy & Helbert (1960) using vaccinia-sensitive erythrocytes drawn from two selected birds bled alternately at weekly intervals.

Cytological observations. The general development of cytopathic changes in inoculated tubes was followed by phase-contrast microscopy. For more detailed studies, cells were grown on floating coverslips (1 × ¼ in.) which were inoculated at the stage of semi-confluence with 10^6 pock forming units (p.f.u.) of virus per coverslip. Coverslips were fixed in absolute alcohol, stained 30 min in 0·05% acridine orange G in citrate phosphate buffer at pH 2·6 washed thoroughly in buffer at pH 2·6 and mounted in 20% glycerol, buffered at pH 7·4 for fluorescence microscopy.

RESULTS

Single cycle experiments

Cultures of human embryo skin and muscle cells were inoculated at a multiplicity of approximately 10 p.f.u. per cell and the inoculum was removed after 2 h adsorption at 37 °C; the cultures were then washed with three changes of warm phosphate buffer saline (PBSA) to remove unadsorbed virus. Parallel series of inoculated cultures were incubated at 35, 37, 38 and 39·5 °C. Duplicate samples were taken at four-hourly intervals from 0–28 h and stored frozen. On the day of assay the cultures were thawed and subjected to two further cycles of freezing and thawing and to 30 s treatment in an ultrasonic bath (Headland, London). Specimens were assayed for infective virus, for complement-fixing antigen and for haemagglutinin.

No significant difference was found between Harvey and Butler in the yield of infective virus or complement fixing antigen at any temperature. The yield of virus at 8 h was 5–10% of the inoculum and after this the yield increased at all temperatures except 39·5 °C. At 35 °C the plateau titre of 10^7 p.f.u. was reached at 20 h. At 37 °C the plateau titre was reached at 16 h but was at the lower level of $10^{6\cdot4}$ p.f.u. At 38 °C the plateau titres were only slightly above the level of the inoculum and were reached at 16 h (Harvey) and at 20 h (Butler). There was no evidence of viral replication at 39·5 °C.

Equal amounts of complement-fixing antigen, with an ultimate titre of 16, were produced by both viruses at all temperatures, even at 39·5 °C at which temperature no increase in infective virus was noted.

Haemagglutinin (HA)

There was a notable difference in the production of haemagglutinin by Harvey and Butler viruses, particularly at the higher temperatures of incubation. At 35 °C little difference was observed; each virus produced a titre of 32 by 24 h, though Butler lagged a few hours behind Harvey in reaching this level. This lag was more pronounced at 37 °C. Harvey gave a titre of 8 by 20 h which was maintained to 28 h, whereas Butler only attained a titre of 8 in the 28 h sample. At 38 °C Harvey produced a titre of 16 at 38 °C and of 8 at 39·5 °C by 28 h, but at this time no HA was demonstrable at either temperature in the preparations of Butler. When incubation was continued until 36 h some HA was produced by Butler at 38 °C but none at 39·5 °C. Incubation till 36 h also resulted in some further increase in

https://doi.org/10.1017/S0022172400070960 Published online by Cambridge University Press

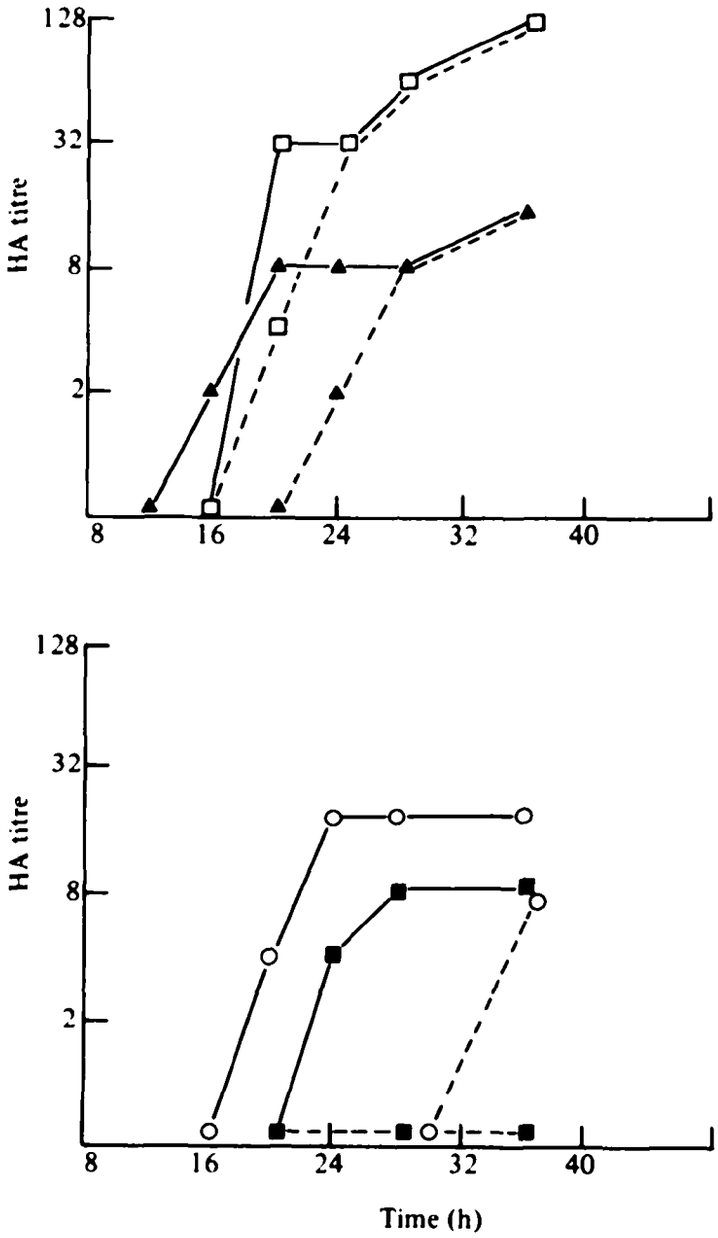

Fig. 1. Production of haemagglutinin by Harvey (——) and by Butler (– – –) viruses in cultures of human embryo skin and muscle cells incubated at □, 35 °C; ▲, 37 °C; ○, 38·5 °C; and ■ 39·5 °C. Haemagglutinin titres are the reciprocals of the end point dilution. Time is shown in hours after inoculation with 10^6 p.f.u. per tube of $2 \cdot 10^5$ cells.

HA titre by both viruses at 35 and 37 °C. These results are shown graphically in Fig. 1.

The production of haemagglutinin was also assessed by means of haemadsorption tests. It was shown in preliminary tests that maximum yield of HA was obtained with an inoculum of 5×10^5 p.f.u. per tube; higher inocula, up to 5×10^7 p.f.u. gave no increase in yield of HA. For the haemadsorption (HAd) tests inocula of 10^6 p.f.u. or greater were used in all tests. Haemadsorption was read and scored at 24 and again at 48 h. Tubes infected in parallel were harvested at 72 h and titrated for HA as described above. The results are shown in Table 1. Although there was some increase in the number of haemadsorbing cells between 24 and 48 h, the high multiplicity of the inoculum and the results of the HA tests at 72 h combined to suggest that this was due to a lag in development of HA rather than to an extension of the infection to additional cells. It would seem that a simple HAd test at 39·5 °C

https://doi.org/10.1017/S0022172400070960 Published online by Cambridge University Press

Table 1. *Production of haemagglutinin and demonstration of haemadsorption in human embryo cell cultures, inoculated with Harvey or Butler viruses and incubated at* 37, 38·5 *and* 39·5 °C

Virus	Temperature	Haemadsorption* 24 h	Haemadsorption* 48 h	Haemagglutinin† 72 h
Harvey	37	4	5	32
	38·5	4	4	32
	39·5	4	4	8
Butler	37	4	5	32
	38·5	2	2	Trace
	39·5	0	0	None

* Haemadsorption coded as: confluent-5; semi-confluent-4 many foci in each microscopic field, but majority of cells negative-3; one or a few foci in each field-2; foci found in occasional fields-1; no haemadsorption-0.

† Haemagglutinin titres given as reciprocals of highest dilution to give 50% or more haemagglutination.

is sufficient to distinguish between Harvey and Butler viruses and possibly between variola viruses in general. Results of a follow-up study of this have already been published (Dumbell & Huq, 1975).

Cytological changes

Large doses of either Harvey or Butler were capable of producing a progressive cytopathic effect (CPE) at 39·5 °C and this happened somewhat more rapidly than at 35 °C. The sequence of events at 35 °C was the same for either virus. At 4 h post inoculation the cells had retained the characteristic spindle shape of the controls but by 8 h retraction of cytoplasmic processes was evident and the cells were assuming a more polygonal shape with increasing granularity of the cytoplasm. Cell boundaries were difficult to distinguish but the nuclei were undistorted. By 12 h the cells were pleomorphic and showing fusion of cytoplasms. Contraction had resulted in microplaques in the cell sheet which had become irregular and ragged in appearance. The CPE progressed to produce fused masses of cells with large cytoplasmic inclusions and vacuolation by 24 h. Later the fused masses of cells shrank, became fully necrotic and detached from the glass. This description would also serve for both viruses at 37 °C and for Harvey at 38 °C. Butler lagged behind at 38 °C, taking from 36 to 48 h to achieve a fully necrotic cell sheet.

Cytoplasmic inclusions stained by acridine orange. At 35 °C cytoplasmic inclusions appeared by 7–8 h after inoculation as small, regular apple-green bodies varying in number from one up to more than ten per cell. These inclusions increased in size with time and after 10 h began to become irregular in outline. By 18 h the majority of the inclusions were irregular in shape and diffusing through the cytoplasm. At 39·5 °C the inclusions were less numerous and retained the discrete, rounded shape even up to 48 h. Treatment of the coverslips with DNase prior to acridine orange staining removed the small round inclusions but did not affect the diffuse inclusions. This suggested that a maturation phase of the virus was inhibited at 39·5 °C and also was confirmed that the acridine orange staining was picking out the DNA factories. The proportion of cells showing DNA inclusions

https://doi.org/10.1017/S0022172400070960 Published online by Cambridge University Press

Table 2. *DNA inclusions at* 16 h *in cells inoculated with Harvey or Butler virus and maintained at* 37, 38·5 *or* 39·5 °C.

Virus...	Harvey			Butler		
Temperature...	37	38·5	39·5	37	38·5	39·5
% Cells with DNA inclusions	100	88	77	91	72	49
% inclusion-bearing cells with diffuse inclusions	69	52	5	35	24	2
Successful* cells per tube ($\log_{10}$)	5·1	4·9	3·9	4·8	4·5	3·3
Virus yield† per tube ($\log_{10}$)	6·4	5·9	5·3	6·1	5·9	5·0
Virus per successful cell ($\log_{10}$)	1·3	1·0	1·4	1·3	1·4	1·7

* The number of cells showing diffusion of DNA inclusions in the cytoplasm, for a tube estimated to contain $2{\cdot}10^5$ cells.

† Virus yield estimated 4 h later than inclusions to approximate completion of virus maturation.

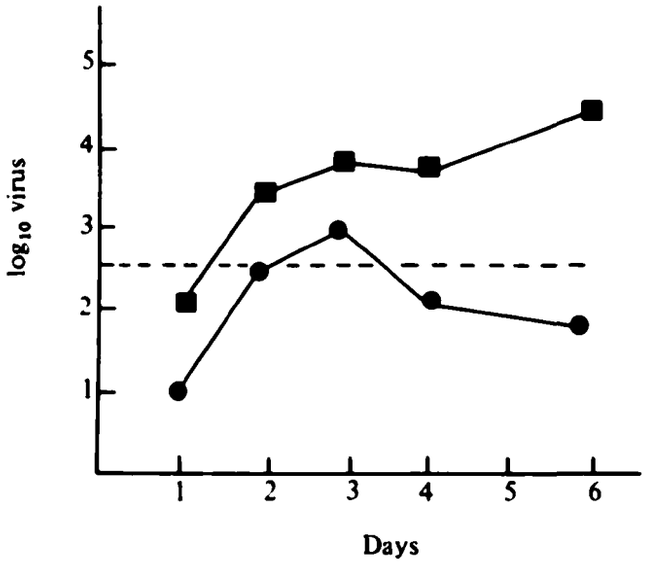

Fig. 2. Amount of virus recovered from the medium of tubes of human embryo skin and muscle cells inoculated with Harvey (■) or Butler (●) virus and incubated at 37 °C. (– – –) shows amount of virus inoculated.

and the proportion in which these inclusions became diffuse is shown in Table 2 for the different temperatures of incubation. It is to be noted that even at 39·5 °C a small number of cells supported diffusion of the DNA inclusions. Table 2 also shows that the total yield of virus bore an approximately constant relation to the number of cells in which maturation of virus could be presumed to have occurred.

Multiple cycle experiments

These were set up in the same way as the single cycle cultures except that an inoculum of 300 p.f.u. per tube was used. Timed samples were taken at 4 h post inoculation and thereafter at daily intervals for 6 days. The medium was removed

https://doi.org/10.1017/S0022172400070960 Published online by Cambridge University Press

from each tube and frozen separately, so that estimates could be made of the amount of virus released from the infected cells.

The only significant difference between Harvey and Butler was in the amount of extra-cellular virus in those cultures incubated at 37 °C. These results are illustrated in Fig. 2. It will be seen that extracellular virus, equivalent in amount to the inoculum was first observed in Harvey-infected cultures during the second day and that the amount continued to increase slowly until the end of the experiment. Butler, in contrast showed only a small amount of extracellular virus between the second and third day and production did not continue. At 38 °C a sustained level of extracellular virus was produced only by Harvey, but at no time did this exceed the level of the inoculum. At 39·5 °C no extracellular virus was detected with either virus.

Cytopathic effect

At 35 °C and at 37 °C cultures of either virus showed foci of shortened, granular cells by 24 h. These foci became necrotic and gradually extended throughout the cell sheet which was completely necrotic by the 8th day. At 38 °C foci were visible by 24 h. Those due to Harvey continued to extend to involve almost all of the cell sheet by the 8th day. Those due to Butler remained small and discrete and neither extended in area nor developed into the full necrotic picture. At 39·5 °C CPE was not detected in cultures inoculated with either virus and the cells retained their normal appearance, as did control uninoculated cultures at this temperature.

DISCUSSION

Hahon, Ratner & Kozikowski (1958) were the first to describe the effect of temperature on the growth of variola virus on the CAM. In 1961 Nizamuddin and Dumbell showed that representative strains from variola major had a higher ceiling temperature for growth on the CAM than had representative strains from variola minor. The stage in the growth cycle at which temperature inhibited growth was studied for variola major virus in HeLa cells, (Cruickshank & Bedson, 1968; Bedson & Cruickshank, 1969) and for variola minor virus in chick embryo cells (Cooper & Bedson, 1973). Because of the different hosts, the results are not strictly comparable, but with each virus a block in maturation was found at elevated temperature. The effect of temperature on the growth of variola major and variola minor viruses in cell cultures from chick and human embryos and from monkey kidney was studied by Gurvich & Marennikova (1964), but only for the production of plaques and CPE. They noted that the results depended to some extent on the passage history of the virus; a few passes in the cells to be used for the experiment were sufficient to raise the maximum temperature at which CPE developed. The present report is the first detailed comparative study of the growth of variola major and variola minor viruses in cell cultures. In confirms that elevated temperature blocks a maturation stage in the development of the virus but showed that there was insignificant difference between the two viruses in this respect. Harvey virus differed from Butler virus in events which relate to the surface of virus-infected cells, and are independent of intracellular maturation. Thus, Harvey virus was released into the medium to a higher level and over a longer period than Butler

https://doi.org/10.1017/S0022172400070960 Published online by Cambridge University Press

virus from cultures maintained at 37 °C. Also haemadsorption of vaccinia-sensitive fowl erythrocytes was shown by Harvey-infected cells even at 40 °C while haemadsorption to Butler infected cells was completely inhibited at 39·5 °C. The development and extension of discrete foci due to Butler was inhibited at 38 °C, though Harvey foci at this temperature extended till the whole sheet became necrotic.

These results are not necessarily at variance with the difference in ceiling temperature for Harvey and Butler on the CAM. Ceiling temperature is based on the development of pock lesions on the CAM of eggs inoculated with 10^2–10^4 p.f.u. of virus (Nizamuddin & Dumbell, 1961). At 38·25 °C the pocks produced by Harvey and other strains of variola major were considerably smaller in size and somewhat fewer in number than at 35 °C, but retained their general pock characteristics. Butler and other strains of variola minor did not produce visible pocks at all at 38·25 °C. These changes with temperature do not necessarily imply a decreased amount of virus as the basic cause, but could as well be a secondary result of increasing difficulty of the viruses in spreading to adjoining cells and so building up a pock lesion. Helbert (1957) had found that the amount of variola major and variola minor viruses recovered from the CAM did not differ by more than twofold but that in the livers of chick embryos inoculated on the CAM variola major virus was recoverable in amounts up to several hundred times the amounts of variola minor virus, and that there was a difference in the cumulative mortality of the embryos. From the results of Dumbell *et al.* (1961) it can be seen that the mortality of chick embryos inoculated with Harvey or Butler was 100% at 35 °C. At 37 °C the mortality due to Butler had been all but abolished, but Harvey was still capable of killing most of the embryos.

It is tempting to see in these results an explanation for the difference between variola major and variola minor in man. A reduction in the extension of local lesions and a delay or reduction in the dissemination of virus from them could be expected to result in a less severe disease, and could act in concert with a developing immunity. The febrile stage in variola begins at or about the time that virus is disseminated throughout the body, and is of the magnitude to produce the changes described in this paper. It would be unwise to extrapolate too widely from the results of a single virus of each type and the matter might be reconsidered when more extensive laboratory results are available.

REFERENCES

BEDSON, H. S. & CRUICKSHANK, J. G. (1969). The relation of the DNA polymerase to an early temperature-sensitive event in the replication of variola virus. *Journal of General Virology* **4**, 413–422.

COOPER, R. J. & BEDSON, H. S. (1973). Temperature-sensitive events in the growth of alastrim virus in chick embryo cells. *Journal of General Virology* **21**, 239–251.

CRUICKSHANK, J. G. & BEDSON, H. S. (1968). Temperature-sensitive events in the growth of variola virus in HeLa cells. *Journal of General Virology* **3**, 255–266.

DUMBELL, K. R., BEDSON, H. S. & ROSSIER, E. (1961). The laboratory differentiation between variola major and variola minor. *Bulletin of the World Health Organization* **25**, 73–78.

DUMBELL, K. R. & HUQ, F. (1975). Epidemiological implications of the typing of variola isolates. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **69**, 303–306.

https://doi.org/10.1017/S0022172400070960 Published online by Cambridge University Press

GURVICH, E. B. & MARENNIKOVA, S. S. (1964). Laboratory diagnosis of smallpox and similar viral diseases by means of tissue culture methods. III. Additional modes of differentiating viruses of the pox group in tissue culture. *Acta Virologica* **8**, 435–442.

HAHON, N., RATNER, M. & KOZIKOWSI, E. (1958). Factors influencing variola virus growth on the chorioallantoic membrane of embryonated eggs. *Journal of Bacteriology* **75**, 707–712.

HELBERT, D. (1957). Smallpox and Alastrim. Use of the chick embryo to distinguish between the viruses of variola major and variola minor. *Lancet* **i**, 1012–1014.

MCCARTHY, K. & HELBERT, D. (1960). A comparison of the haemagglutinins of variola, alastrim, vaccinia, cowpox and ectromelia viruses. *Journal of Pathology and Bacteriology* **79**, 416–420.

NIZAMMUDIN, MD. & DUMBELL, K. R. (1961). A simple laboratory test to distinguish the virus of smallpox from that of alastrim. *Lancet* **i**, 68–69.

https://doi.org/10.1017/S0022172400070960 Published online by Cambridge University Press