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Hybrids derived from the viruses of variola major and cowpox

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INTRODUCTION

The isolation of presumptive hybrids from the viruses of variola major and cowpox has already been described (Dumbell & Bedson, 1964). We report here the detailed characterization of the 16 clones of virus obtained. As with the alastrim–rabbit pox hybrids considered in the previous paper (Bedson & Dumbell, 1964), attention has been paid to the behaviour of the ceiling temperature character in recombination and to the possible relationship it and other markers might have to the virulence of these viruses. Both sets of hybrids may also be regarded as making a minor contribution to pox virus genetics, in that variola viruses have now been shown to form hybrids with other members of the variola–vaccinia group.

TESTS FOR MARKER CHARACTERS

Three of the seven marker characters used in the study of the alastrim–rabbit pox hybrids have not been used for the variola major–cowpox hybrids. The markers excluded were haemagglutinin production, thermal stability and mouse virulence. The first two were not considered to offer worthwhile differences in the present system, while mouse virulence was omitted for reasons of personal convenience.

Tests for pock morphology, type of plaque in chick embryo tissue culture and rabbit virulence have been made as described for the alastrim–rabbit pox hybrids. Details of the tests for ceiling temperature and for three additional markers are given below.

Ceiling temperature

In initial tests the ceiling temperatures were determined as described by Bedson & Dumbell (1961). The presence or absence of pocks was noted at the temperatures 38·5, 39, 39·5 and 40° C. using an inoculum giving an average of 50–100 pocks per membrane at 35° C.

A second series of tests was performed to determine more precisely the reduction in pock-forming efficiency at the temperature concerned. In these tests, counts were made of the pocks present in groups of three to four eggs incubated at each of the relevant temperatures and in a control group at 35° C. Inocula larger than 100 pock-forming units of virus were used if the temperature was such that very few or no pocks were to be expected. The extent to which pocks were reduced at any temperature was taken as: $\log_{10}$ pock titre at 35° C. $-\log_{10}$ pock titre at the temperature concerned. For each virus the values obtained were then plotted

against temperature. In the experiments 35° C. was used as the control temperature as a matter of convenience.

Cytoplasmic inclusions

Portions of infected C.A.M. were fixed in 10% formal-zenker for 1 hr., washed and embedded in paraffin. Sections were stained with haematoxylin and eosin.

Egg virulence

Virulence for the chick embryo was determined from the mortality rates of 12-day embryos inoculated on the C.A.M. and incubated at 35° C. For each virus three separate doses of inoculum were used, a group of six eggs being inoculated with each dose. The D4 value—i.e. the log dose of virus giving a mean survival time of 4 days—has been calculated from the results as previously described (Bedson & Dumbell, 1961).

Diffusible LS antigen

Extracts of heavily infected C.A.M.s were examined for the presence of a diffusible LS antigen in agar-gel precipitation tests using an anti-serum supplied by Dr C. J. M. Rondle. This had been prepared in a rabbit by the intravenous injection of a preparation of LS antigen made from vaccinia-infected rabbit material by the method of Shedlovsky & Smadel (1942). Reservoirs 4 mm. in diameter with centres 5 mm. apart were made in a layer of agar 1 mm. deep on a microscope slide. The agar was at a 1% concentration in isotonic phosphate-buffered saline pH 7·3 containing 0·01% merthiolate. The antigen extracts were made in isotonic phosphate-buffered saline pH 7·3, 1 ml. per membrane.

PROPERTIES OF THE VARIOLA MAJOR–COWPOX CLONES

In the following account it will be convenient to refer to the 16 clones of virus prepared from the variola major–cowpox system as VC clones 1–16. Not all 16 were independent, for VC2, 3 and 4 were taken deliberately from a single clone in order to provide an internal check on the methods used and on the stability of the cloned viruses. Similarly VC10 and 15 came from the same clone, but in this case there was at first a suggestion from the pock appearance that the clone might not be pure.

The results of applying tests for the seven marker characters to the VC viruses and to the parent viruses are presented in Table 1. These results are discussed in greater detail in the following sections which deal with each marker character separately.

Pock morphology

The pocks of cowpox are large, ulcerated and conspicuously red from haemorrhage while those of variola are smaller, not ulcerated and white. Despite these well-marked differences pock appearance has not been a particularly useful marker in classifying the VC clones. This may have been in part due to its use at the onset to exclude viruses with pocks of the cowpox type, but the chief difficulty was that

Table 1. *Characters of cowpox, variola major and hybrids developed from them (VC 1–16)*

Virus	Pock type	Cyto-plasmic inclusions	Egg virulence (D4 value)*	Diffusible LS antigen	Ceiling temperature (° C.)	Plaque type	Plaques appear (days)	Rabbit virulence†
Cowpox	Red, ulcerated	+	3·5	0	40	Trabeculated	2	+
Variola major	White, non-ulcerated	0	4·5–5·5	+	38·5	Heavy-rimmed	4	0
VC 1	Red, ulcerated	+	.	0	40	Trabeculated	2	+
VC 2	Intermediate, ulcerated	+	5·6	0	40	Trabeculated	2	+
VC 3	Intermediate, ulcerated	+	.	0	40	Trabeculated	2	+
VC 4	Intermediate, ulcerated	+	.	0	40	Trabeculated	2	+
VC 5	Intermediate, ulcerated	0	2·3	0	38·5	Trabeculated	3	0
VC 6	White, non-ulcerated	+	.	0	40	Trabeculated	3	0
VC 7	Intermediate, ulcerated	+	2·5	0	40	Trabeculated	3	+
VC 8	White, ulcerated	0	7·0	+	38·5	Heavy-rimmed	4	±
VC 9	Red, ulcerated	+	3·4	0	40	Trabeculated	2	+
VC 10	White, ulcerated	+	.	0	39·5	Trabeculated	2	±
VC 11	Red and white, ulcerated	+	.	0	39·5	Trabeculated	2	±
VC 12	White, ulcerated	+	3·9	0	40	Heavy-rimmed	3	±
VC 13	Intermediate, ulcerated	+	4·2	0	39	Trabeculated	2	+
VC 14	Intermediate, ulcerated	+	3·2	0	40	Trabeculated	3	+
VC 15	White, ulcerated	+	.	0	39·5	Trabeculated	2	±
VC 16	White, ulcerated	0	.	0	40	Trabeculated	2	±

* Log dose of virus giving harmonic mean survival time of 4 days.

† +, Papule with haemorrhage and necrosis; ±, papule without haemorrhage or necrosis; 0, insignificant lesion.

of analysing the very wide variety of appearance in terms of the parental viruses. Two viruses (VC1 and 9) had pocks indistinguishable from those of cowpox. One (VC 6) had pocks like those of smallpox, but perhaps rather smaller than is typical of this virus. The rest were all either white or grey and ulcerated to a varying extent. A number had some degree of central haemorrhage and these have been recorded in Table 1 as being of intermediate colour.

After 3 days' incubation at 35° C. the pocks of many of the VC clones resembled those produced by different strains of vaccinia after 2 days. Thus VC8, 10, 12, 15 and 16 had pocks very similar to those of dermo-vaccinia, VC2, 3, 4 and 14 had pocks like neurovaccinia and VC7 and 13 gave pocks more like those of the Utrecht strain of rabbit pox. Such descriptions are, of course, only approximations and take little account of minor distinguishing features.

Cytoplasmic inclusions

The histology of cowpox-infected C.A.M. is characterized by the presence of very numerous compact or 'solid' eosinophilic cytoplasmic inclusion bodies (Downie, 1939), whereas C.A.M. infected with variola virus shows only granular cytoplasmic inclusion material. Sections were examined from both confluent infections and discrete pocks for each VC virus and there were only three (VC5, 8 and 16) which did not show the solid inclusions characteristic of cowpox.

Egg virulence

In previous work (Bedson & Dumbell, 1961) it was established that cowpox differs from variola major in its virulence for the chick embryo and that this difference could be expressed quantitatively in terms of the log dose giving a mean survival time of 4 days (the D4 value). Cowpox is the more virulent and has therefore a lower D4 value (3·5) than that of variola major (4·5–5·5). D4 values for eight of the VC clones were determined. Among these there were two like cowpox (VC9 and 14), two of intermediate virulence (VC12 and 13) and one like variola major (VC2). Perhaps the most interesting finding was that there were also clones with values outside the parental range. Both VC5 and 7 appeared significantly more virulent than cowpox, while VC8 was definitely less virulent than variola major.

Diffusible LS antigen

It has been shown by Rondle & Dumbell (1962) that antigen component *f* cannot normally be demonstrated in agar gel-diffusion analysis of extracts of cowpox-infected tissue. They suggest that this component, which appears to be a part of the LS antigen complex, is present in such extracts in a non-diffusible state. In extracts of variola-infected tissue this component is readily demonstrable. Extracts of C.A.M. infected with cowpox, variola major and VC clones 1–16 have been examined for the presence of antigen *f* using an antiserum prepared by Dr C. J. M Rondle which reacts specifically with this antigen in gel-diffusion tests. Only variola major and VC8 gave a line of precipitation with this serum used both unconcentrated and after fivefold concentration.

Ceiling temperature

The values shown in Table 1 were obtained from the initial tests in which small doses of virus were used and in which only the presence or absence of pocks was noted. Despite their limitations these tests revealed a considerable variety amongst the VC clones. There were 10 clones which behaved like cowpox, giving pocks at 40 but not at 40·5° C. The latter point established their difference from strains of vaccinia which gave clear-cut lesions in parallel tests at 40·5° C. Three of the clones (VC10, 11 and 15) formed pocks at 39·5 but not at 40° C., while the remaining three did not appear to form pocks at 39·5° C. One (VC13) gave pocks at 39 but not at 39·5° C. and the other two (VC5 and 8), like variola major, formed pocks at 38·5 but not at 39° C.

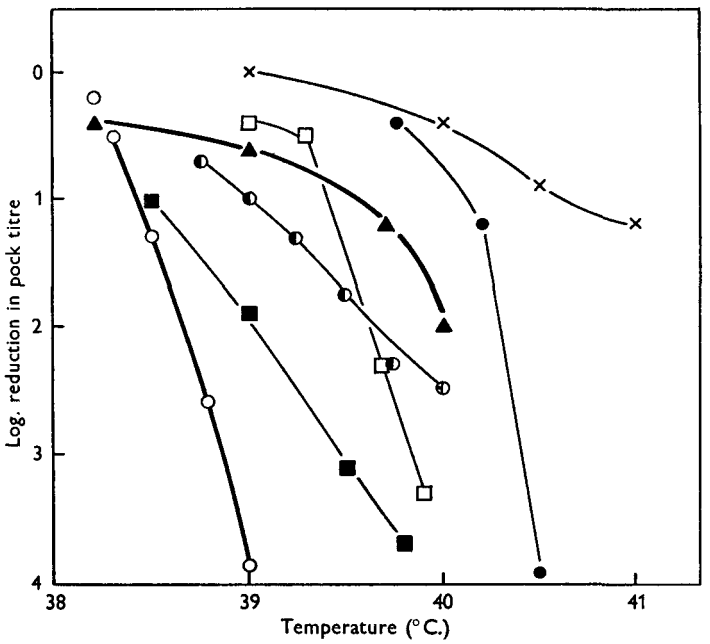

Fig. 1. Plots of log. reduction in pock titre, based on titre at 35° C., against temperature for variola major (○—○), VC8 (■—■), VC13 (◐—◐), cowpox (▲—▲), VC10 (□—□), VC16 (●—●) and vaccinia (×—×).

These last results were surprising, for it appeared that three of the VC clones would not grow at 39·5° C. although the original reactivation had taken place at this temperature. A second series of tests was therefore made in order to determine more precisely the decline in pock-forming efficiency with rising temperature. Each of the VC clones was examined and log values for the reduction in pock titre at the relevant temperatures were obtained. These values were then plotted against temperature. Some examples of the plots are shown in Fig. 1. The parent viruses, cowpox and variola major, and also vaccinia, are included for comparison. Among the VC clones five different types of plot were recognized. The first corresponds closely to that of cowpox and was seen with seven of the clones (VC1, 2, 3, 4, 6, 7 and 9). The remaining four types are shown by the four examples given in Fig. 1.

Of the viruses not shown in the figure, VC12 and 14 were like VC16, VC11 and 15 were like VC10, and VC5 was like VC8.

Although these results do not conflict with those of the initial tests, they affect their interpretation. In the first place they showed that none of the VC clones was exactly like variola major and that none of them was completely incapable of growth at 39·5° C. They also showed that three clones (VC12, 14 and 16) had slightly higher ceiling temperatures than cowpox, even though none of them reached the range of vaccinia. But perhaps the most interesting point to emerge was that not all the viruses reacted to rising temperature in the same way. With some viruses, especially variola major, VC10 and 16, there came a point at which pock-forming efficiency declined abruptly. At this stage there was a fall of about 2 log units within a range of 0·5° C. With these viruses the value obtained for the ceiling temperature is practically independent of the dose of virus used in the test. However, other viruses showed a much more gradual decline in pock-forming efficiency. This type of response was shown by VC5, 8 and 13 and also to some extent by cowpox. For these viruses, the dose of virus used in the test becomes of critical importance. Indeed, for them, the ceiling temperature of pock formation can only be defined in terms of some arbitrarily chosen level of reduction in pock numbers. It will be seen from Fig. 1 that the ceiling temperatures recorded in Table 1 correspond in most cases to a temperature at which there is a reduction in pock-forming efficiency of 1 log unit.

Plaques in chick embryo monolayers

The major difference between the plaques of cowpox and variola major was in the speed of their formation but there was also a difference in the type of plaque produced. Those of cowpox were present at 2 days and were trabecular in appearance (Pl. 1A). After 3 days they had become much larger and secondary plaques were usually visible (Pl. 1B). With variola major the monolayers appeared normal at 2 days and definite plaques with a characteristic heavy rim were not seen until 4 days (Pl. 1C). Specific lesions were in fact visible at 3 days but at this stage they were present only as densely staining dots (Pl. 1D). It was thought that the plaques developed from these foci by central necrosis and detachment of cells. In some dishes in which development was uneven, lesions in various stages of transition were observed. An attempt was made to gain a better understanding of the changes involved using both phase contrast microscopy of lesions in tube cultures and conventional microscopy of monolayers fixed and stained with haematoxylin and eosin. Although there were often small giant cells in the bases of plaques there was no massive syncytial formation and it appeared that both the densely staining dots and the heavy rims of plaques were attributable to the more intense staining of cells undergoing degeneration.

Among the VC clones there were 10 that gave plaques of the cowpox type present at 2 days. Only one (VC8) behaved like variola, giving heavy-rimmed plaques at 4 days. The other five were intermediate in that plaques were first seen at 3 days. In four cases (VC5, 6, 7 and 14) the plaques were of the trabeculated type. Two examples—VC14 at 3 days and VC7 at 4 days—are shown in Pl. 1E and F,

respectively. The fifth (VC12) gave heavy-rimmed plaques. The lesions of this virus at 3, 4 and 5 days are shown in Pl. 1G, H and J. The target-like appearance at 5 days is reminiscent of the 'ring-zone' phenomenon observed with fowl pox virus (Mayr & Kalcher, 1961).

Rabbit virulence

In tests of virulence in the rabbit skin there were three main kinds of response. First, there were the papules which proceeded to extensive central haemorrhage and necrosis, usually accompanied by considerable oedema. This type of result was given by cowpox and eight of the VC clones. In Table 1 this has been recorded as rabbit virulence +. VC7 appeared to differ slightly from the others in that the lesion was always slower to evolve and did not develop haemorrhage or necrosis until after the 5th day. At the other extreme was the response given by variola major and by VC 5 and 6. With these viruses there were either no lesions at all or at best small soft papules which began to regress after 3 days and had practically disappeared by 5 days. This type of response is shown in Table 1 as rabbit virulence 0. Intermediate between these were the six VC clones, shown in Table 1 as having rabbit virulence ±, which gave well-developed firm papules which did not progress to haemorrhage or necrosis.

ANALYSIS OF RESULTS

From the results presented in Table 1, it is clear that there are many different kinds of virus amongst the VC clones. Two (VC1 and 9) were indistinguishable from cowpox but none had all the characters of variola major. Of the others, VC11 should be excluded from consideration because its mixed pocks suggested that it may not have been a pure clone. VC3 and 4 can also be excluded for they were indistinguishable from VC2, and VC15 was exactly like VC10. This leaves 10 separate new kinds of virus, each of which differed from the parent viruses and from the other nine.

The stability of these new viruses was next considered. Some evidence on this point is contained in the results already presented, e.g. the lack of any difference between VC2, 3 and 4 and between VC10 and 15. Nevertheless the point was thought of sufficient importance to warrant further experiments. Each of the 10 new types of virus was passed twice on the C.A.M. at high concentration. Fresh stocks were prepared from the membranes of the second pass. These were tested for pock morphology, type of plaque, diffusible LS antigen, rabbit virulence and for ceiling temperature. In no instance was a change found in the characters of the new stock. Additional evidence of stability was obtained for VC12. A heat-inactivated preparation of this virus was reactivated on the C.A.M. at 39·5° C. with variola major. Several clones of virus were derived from the resulting pocks and each proved similar to VC12 in respect of pock and plaque type, ceiling temperature and the absence of diffusible LS antigen.

It must also be considered whether these new viruses are to be accepted as hybrids or whether they are white variants of cowpox (Downie & Haddock, 1952). In answer to this question it may be said that all the arguments advanced in the

case of the alastrim–rabbit pox (AR) hybrids (Bedson & Dumbell, 1964) apply with at least equal force to the VC viruses. Thus it is to be noted that 6 of the 10 new types of virus were developed from a single reactivation pock. There is also the point that, whereas rabbit pox has been shown to produce a very wide variety of white variants (Gemmell & Fenner, 1960), a corresponding variety has not been seen amongst the white variants derived from the nine strains of cowpox virus extensively studied in our department. The difference between rabbit pox and cowpox in this respect is confirmed by the difficulty we have experienced in obtaining wild cowpox by crossing its white variants (unpublished observations) although this can readily be achieved with rabbit pox (Gemmell & Cairns, 1959; Gemmell & Fenner, 1960).

As with the AR hybrids, it is possible to study the segregation of individual marker characters by examining the pairwise crosses that have been encountered. These are shown in Table 2. From the table it will be seen that some of the markers have been arbitrarily redefined in such a way as to class intermediate results with one or other of the parental types. The number of markers has also been increased to eight by considering the speed of plaque formation and type of plaque as separate characters. In the scheme shown, there are 56 possible pairwise crosses and 44 of them were present among the 10 new VC viruses. For every pair of characters at least one of the two possible crosses was found and in 16 instances both the pairwise cross and its reciprocal were present. There is, therefore, good evidence that each of the markers chosen is capable of segregating independently. Although a high proportion of the pairwise crosses has been found among the VC viruses, it must be noted that we have studied only a very small number of clones in relation to the large number of hybrids which potentially exist. Even neglecting intermediate results, as in Table 2, there are 254 possible new types of virus.

GENERAL DISCUSSION OF VC AND AR HYBRIDS

In this discussion reference will be made not only to the results of the present work but also to the alastrim–rabbit pox (AR) hybrids described in the preceding paper (Bedson & Dumbell, 1964).

Four of the markers which have been used in these studies have not previously been used in genetic work with the pox viruses. That involving the LS antigen would seem to be ideal for this kind of work because of the ease of testing large numbers of clones. It is a pity that the prospect of developing further antigenic markers of this type does not at present appear more hopeful. Both virulence for the chick embryo and the presence or absence of solid inclusions are too laborious for large-scale use, but the fourth new marker—ceiling temperature—does not suffer from this defect. The recovery of hybrids with intermediate ceiling temperatures has shown that it is a graded character and the more detailed study of the VC hybrids has revealed some of its complexities. Nevertheless, simple tests are all that have been required for its use as a marker with both sets of hybrids.

The behaviour of the viruses in chick tissue culture is also of interest. Variola major and alastrim have not previously been shown to produce plaques in chick

Table 2. *Analysis of pairwise crosses of individual marker characters amongst the VC clones*

	Cowpox markers							
Variola major markers	Pocks on c.a.m. ulcerated	Cyto-plasmic inclusions	Egg virulent (D4 < 4·0)	Diffusible LS antigen absent	Ceiling temperature ⩾ 39·5° C.	Plaques Trabecular	Plaques 2 days	Rabbit virulent (+ in Table 1)
Pocks non-ulcerated	—	+	0	+	+	+	0	0
Cytoplasmic inclusions absent	+	—	+	(+)	+	+	+	0
Not (D4 > 4·0) egg virulent	(+)	+	—	(+)	+	+	+	+
Diffusible LS present	+	0	0	—	0	0	0	0
Ceiling temperature < 39·5° C.	+	+	+	(+)	—	+	+	+
Plaques rimmed	+	+	+	(+)	+	—	0	0
No plaques at 2 days	(+)	+	+	(+)	+	(+)	—	+
Not rabbit virulent (0 or ± in Table 1)	(+)	(+)	+	(+)	+	(+)	+	—

+, Pairwise cross and its reciprocal present; (+), pairwise cross but not reciprocal present; 0, pairwise cross absent.

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tissue culture and at first difficulty was experienced in obtaining plaques from these viruses. It was, in fact, only after a study of the intermediate forms of plaques and particularly those of VC12, that the behaviour of variola was fully appreciated. It must be stressed that we did not use an agar overlay, because in our experience an overlay considerably delays the evolution of plaques. Our findings cannot therefore be said to conflict with those of Mika & Pirsch (1960), who claimed that variola did not produce plaques in chick embryo monolayers, but they used an agar overlay system and ceased observations on the 4th day.

It was recognized early in the study of the genetics of pox viruses that virulence characters were complex and unsuited for genetic analysis (Fenner & Comben, 1958). They have, however, continued to be of practical importance, and this has been shown yet again by the three virulence markers used in the present study. As in previous studies, hybrids of intermediate virulence have been relatively common, suggesting that each virulence character is controlled by multiple factors. In no case has this been more clearly demonstrated than with the VC hybrids and their virulence for the chick embryo. The drawbacks of this marker have already been discussed but it does have the advantage that it allows a comparative measure of virulence. It was this which made it possible to show quite clearly that some hybrids had a virulence which was outside the parental range.

The hybrids have also been of use in analysing the extent to which certain characters are interdependent. An example of this is shown by the VC hybrids and the characters chick virulence, speed of plaque formation and ceiling temperature. These three characters all relate to facets of the chick embryo host and it might not be unreasonable to expect some connexion between them. Indeed, this is the conclusion suggested by an examination of the 'natural' pox viruses, for, as is shown in Table 3, the three characters appear to be closely correlated. The data for chick virulence and ceiling temperature have been taken from Bedson & Dumbell (1961) and those for plaque formation from unpublished observations made in our department in collaboration with Mr D. P. McHugh. Yet, when one examines the VC hybrids (Table 1) neither ceiling temperature nor speed of plaque formation are correlated with chick virulence, and the pairwise crosses (Table 2) show quite convincingly that these markers segregate independently. In the same way rabbit and mouse virulence have been shown to be independent among the AR hybrids. It follows 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 underly virulence.

One further point which may be considered in the light of the VC hybrids is the often disputed question of the origin of vaccinia. Derivations from both smallpox and cowpox have been claimed but the histories of the many strains now in use are too obscure to be of any value. The drawback to both claims is that attempts to repeat the process have usually been completely unsuccessful and in the few instances in which success has been claimed the work has always been open to serious criticism (for discussion see Downie & Dumbell, 1956). There is, however, the possibility that vaccinia may have originated as a hybrid of variola and

cowpox. These viruses between them possess all the characters that we at present recognize in vaccinia, and the study of the VC hybrids has shown how readily viruses resembling vaccinia may be produced. It is most unlikely that the necessary mixed infection will have occurred purely by chance, but it is stated that in some quarters on the continent there has been an accepted practice of mixing variola with vaccine virus from time to time, in order to enhance its potency (Pettenkofer, Stöss, Helmbold & Vogel, 1962). Such an act performed at an earlier stage in the history of vaccinia could have provided the necessary conditions. It may be objected that none of the VC hybrids so far examined corresponds to vaccinia in all its characters, but only relatively few strains have been studied and there seems no reason why a hybrid with such properties should not eventually be found.

Table 3. *Virulence for the chick embryo, ceiling temperature and speed of plaque formation of the natural pox viruses*

Virus	Virulence (D4 value)*	Ceiling temperature (° C.)*	Speed of plaque formation (hr.)†
Rabbit pox	0·3	>41	36
Vaccinia	0·8–2·2	41	36
Cowpox	3·2–3·5	40	42
Monkey pox	3·3–3·7	39	72
Ectromelia	3·3–3·6	39	72
Variola major	4·6–5·5	38·5	96
Alastrim	6·8	37·5	96

* Data from Bedson & Dumbell (1961).

† Unpublished data obtained in collaboration with D. P. McHugh; the time given is in each case the optimum for counting primary plaques in the system without agar overlay.

SUMMARY

The characterization of 16 clones of virus derived from mixed infections with variola major and cowpox has been described. This work has involved the description of the plaques produced by variola major in chick embryo monolayers, and a more detailed study of the effects of raised temperature on pock production by pox viruses on the C.A.M.

Two of the variola major–cowpox clones were found to correspond to cowpox virus, while the other 14 shared various combinations of parental characters. Among them there were 10 distinct new types of virus. Evidence of the stability of these viruses has been presented.

Analysis of the combination of characters encountered among the hybrids has given good evidence that all the eight marker characters used segregate independently. A particular instance of this was the character of chick embryo virulence which, among the hybrid viruses, was shown to be unrelated either to ceiling temperature or speed of plaque formation.

The significance of these findings in relation to the general problem of virulence has been briefly discussed. The possibility that vaccinia may have originated as a hybrid of variola and cowpox has lso been considered.

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EXPLANATION OF PLATE 1

Examples of the plaques produced in chick embryo monolayers by variola major, cowpox and various hybrid (VC) viruses.

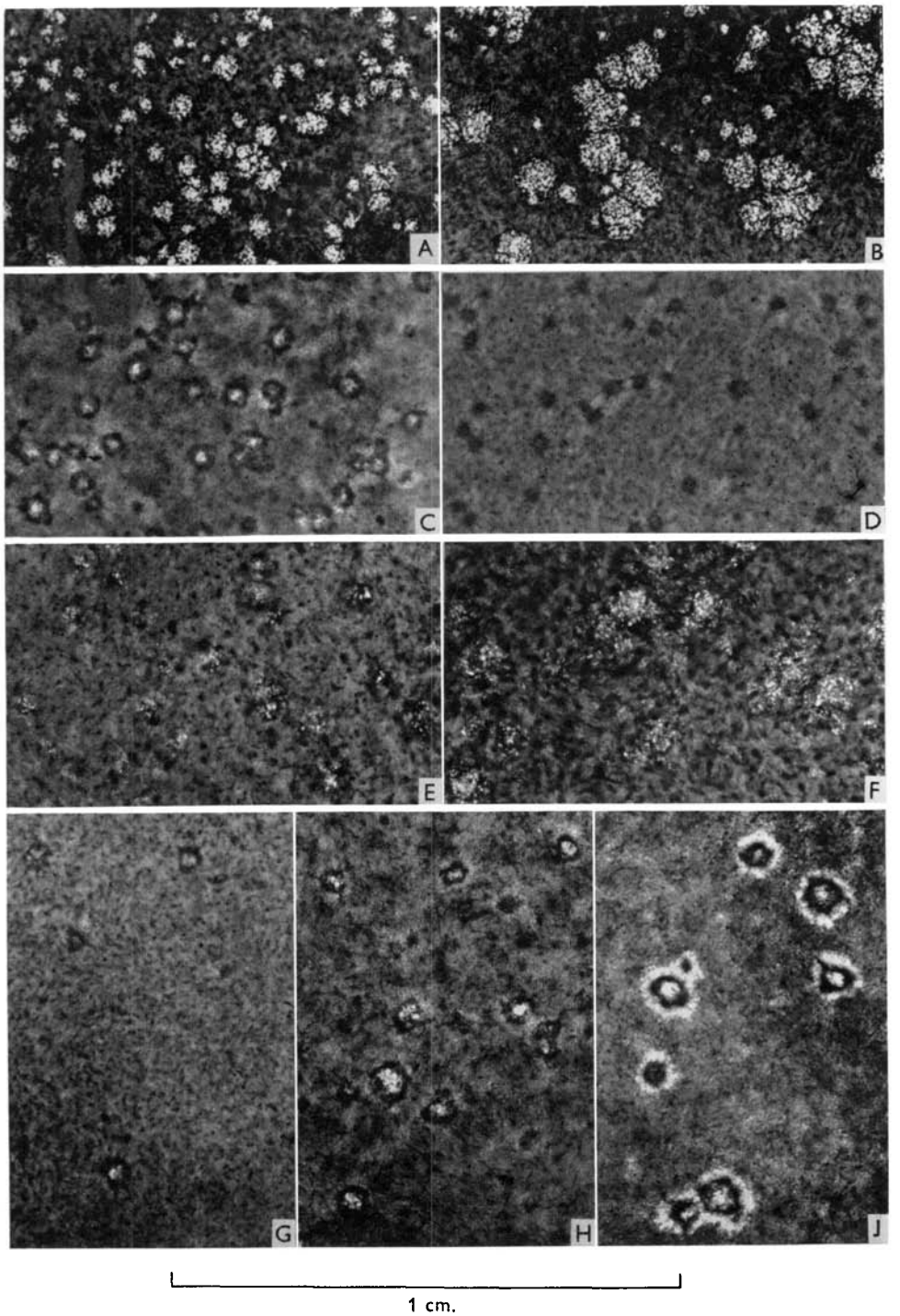
A and B. Plaques of cowpox at 2 days and at 3 days.

C and D. Plaques of variola major at 4 days and at 3 days.

E. Plaques of VC14 at 3 days.

F. Plaques of VC7 at 4 days.

G, H and J. Plaques of VC12 at 3, 4 and 5 days.



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