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The use of ceiling temperature and reactivation in the isolation of pox virus hybrids

By K. R. DUMBELL and H. S. BEDSON
Department of Bacteriology, University of Liverpool

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INTRODUCTION

Genetic recombination among the mammalian pox viruses was first described in 1958 when it was observed by Fenner and Comben in a study of mixed infection with two different strains of vaccinia. It has since been demonstrated between the white mutants of rabbit pox (Gemmell & Cairns, 1959; Gemmell & Fenner, 1960) and between a number of different viruses of the variola–vaccinia group (Woodroofe & Fenner, 1960). In the latter instance where different viruses and not separate strains of the same virus were involved, it was suggested that the new viruses produced should be called *hybrids* rather than recombinants.

In this paper we describe a method for the clonal isolation of hybrid pox viruses which differs from those used previously. Our method introduces some degree of selection, though we have not achieved a selective system in which the growth of both parental types is suppressed. The novel feature of our method is that it employs a temperature of incubation which has been raised sufficiently to inhibit one parental component while yet permitting the growth of the other parental component. It is based on the observation that viruses of the variola–vaccinia group differ in the temperatures at which their growth on the chick chorioallantois (C.A.M.) is inhibited (Bedson & Dumbell, 1961). The maximum temperature at which lesions are produced (ceiling temperature) is a constant character of each of the viruses tested. We are concerned here with two properties of virus inhibited by raised temperature. The inhibited virus will grow again if the temperature is lowered, and, in its inhibited state, the virus behaves as a 'reactivating agent' (Joklik, Woodroofe, Holmes & Fenner, 1960).

We propose to describe, as 'heat-tethered', virus which, although not inactivated, has been prevented from growth on the C.A.M. by a raised temperature of incubation. This will avoid confusion when describing experiments which also involve virus which has been inactivated by heating at 55° C. *in vitro*. The term 'heat-tethered' connotes restraint and reversibility and is appropriate to virus in this particular condition.

The preliminary data on heat-tethered virus are given in the first part of this paper. There follows a short account of the use of reactivation with heat-tethered virus to obtain clones of presumptive hybrids. Alastrim has been crossed with rabbit pox virus, and variola major with cowpox virus. The detailed characters of these hybrid viruses are given in the two accompanying papers (Bedson & Dumbell, 1964*a*, *b*).

MATERIAL AND METHODS

Virus strains

The viruses used were the international reference strains of alastrim (Butler), variola major (Harvey), cowpox (Brighton) and rabbit pox (Utrecht) which were listed by Fenner & Burnet (1957). In addition to these, a few experiments were made with vaccinia (Connaught Labs.) and with white variants of the strains of cowpox and rabbit pox mentioned above. The characters of these additional viruses are given by Fenner (1958).

Eggs

Inoculations were made on the C.A.M.S of 12-day chick embryos, prepared for inoculation by the technique of McCarthy & Dumbell (1961). After inoculation the eggs were incubated at the required temperatures as previously described (Bedson & Dumbell, 1961).

Virus suspensions

Heavily infected C.A.M.S were harvested after 3 days' incubation at 35° C., shaken with glass beads in McIlvaine's phosphate–citrate buffer (0·004 M-phosphate, pH 7·2) and centrifuged at 2000 r.p.m. to deposit cellular debris. The supernatant was mixed with an equal volume of sterile glycerol and stored at − 20° C. for use as stock suspension.

Heat-inactivated virus suspensions

The low-speed supernatant of an extract of virus-infected C.A.M.S was centrifuged at 8000 r.p.m. for 30 min. in a Spinco Model L centrifuge (Rotor No. 40) to deposit the virus. The pellet was resuspended in 0·1 M-sodium chloride in McIlvaine's phosphate–citrate buffer (0·01 M phosphate pH 7·2). This suspension was sealed in a thin glass ampoule and completely immersed in a water bath at 55° C. for $2\frac{1}{2}$ hr. to inactivate the virus. The inactivation was done on the same day that the suspension was prepared (Woodroofe, 1960). The suspension was tested after heating to ensure that no residual active virus was present. Two successive passes on the C.A.M. at 35° C. produced no lesions (Joklik *et al.* 1960).

Isolation of virus clones

The methods used to prepare clones of virus were essentially those of Fenner (1959). Initial mixtures of viruses were inoculated on the C.A.M. at a dilution giving discrete pocks. These pocks were sampled with finely drawn Pasteur pipettes and the material thus obtained was again inoculated on the C.A.M. at 35° C. at a dilution to give not more than 30 pocks. If these latter pocks were all alike in appearance, a single pock was excised, extracted with buffer and inoculated on the C.A.M. of 2–3 eggs at 35° C. Stock suspensions in 50 % glycerol buffer were prepared from these C.A.M.S.

Reversibility

PROPERTIES OF HEAT-TETHERED VIRUS

Alastrim virus produces no lesions on the C.A.M. of eggs incubated at or above 38° C. (Bedson & Dumbell, 1961). This inhibition is removed if, 16 hr. after inoculation, the temperature is lowered to 35° C. Growth curves have been determined for alastrim virus in eggs incubated at 35, 38·5 and 40° C., and also in eggs incubated at 38·5° C. for the first 16 hr. after inoculation and then transferred to 35° C. In these experiments the inoculum for each egg was 10^3 pock-forming units (p.f.u.) and three membranes were harvested for each sample. The results are plotted in Fig. 1. There was no evidence of progressive multiplication at either 38·5 or 40° C. In the controls at 35° C., an exponential increase in titratable virus was found from the 10th hr. onwards. When eggs were transferred from 38·5 to 35° C. after 16 hr. incubation at the higher temperature, virus almost immediately began to increase at approximately the same rate as in the controls.

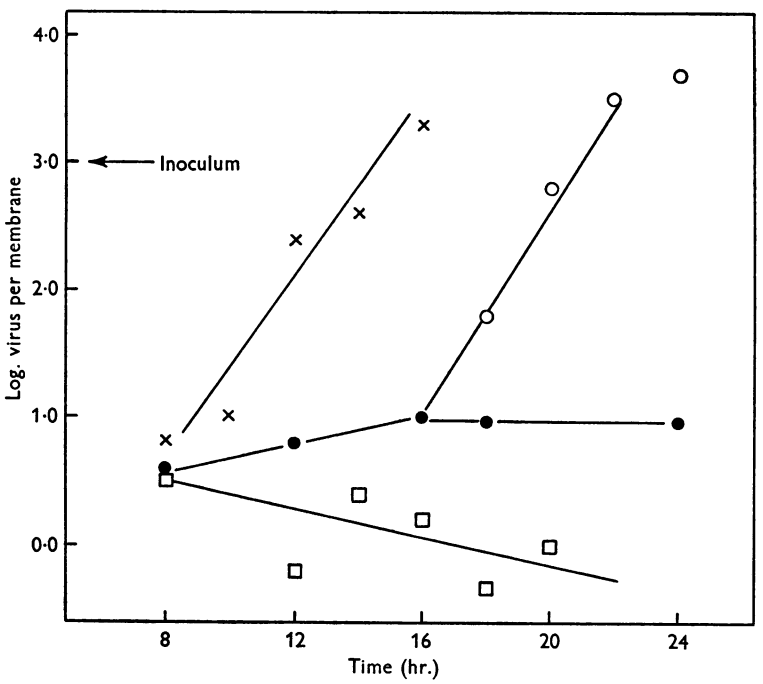

Fig. 1. Growth of alastrim virus in chick chorioallantois at 35° C. (×—×), at 38·5° C. (●—●), at 40° C. (□—□) and at 35° C. after 16 hr. at 38·5° C. (○—○).

Experiments were also made with variola major virus. Hahon, Ratner & Kozikowski (1958) had shown that the multiplication of variola major in the C.A.M. was reduced at 37° C. and inhibited at 39° C. We have found that variola major does not multiply in the C.A.M. at 39·5° C. If eggs were incubated at this temperature for 16 or 18 hr. after inoculation and then transferred to 35° C., a rapid increase in virus began almost immediately.

The inhibition imposed on the growth of variola minor virus at 38·5° C., and on variola major at 39·5° C., thus appears to be reversible, at least within certain limits. The reversibility of the inhibition shows that the growth potential of the inhibited virus was not seriously affected by the amount of restraint imposed in these experiments.

The short interval between the lowering of an inhibitory incubator temperature and the increase of titratable virus suggested that the inhibition occurred at a fairly late stage in the intracellular cycle of virus development. Reactivation is associated with early stages in the virus growth cycle (Fenner, 1962; Joklik, 1962). This encouraged us to hope that heat-tethered virus might behave as a reactivating agent.

Reactivation by heat-tethered virus

In the first series of experiments the Utrecht strain of rabbit pox was chosen as the virus to be reactivated, because it had the highest ceiling temperature of those tested by Bedson & Dumbell (1961).

A heat-inactivated suspension of rabbit pox was prepared as described above. Eggs were inoculated with mixtures of the heated rabbit pox suspension and 10^3–$10^{4.5}$ p.f.u. of active alastrim, variola major or cowpox. The eggs were incubated above the ceiling temperature of the active virus chosen. Controls showed that none of the active viruses produced lesions on the C.A.M. when used at the same dose and temperature as in the mixed inoculations. Every mixture tested produced lesions, most of which resembled those produced by rabbit pox. A small proportion of atypical lesions was found; these will be discussed later. The results of these experiments are set out in Table 1A. A second series of experiments, listed in Table 1B, showed that heat-tethered alastrim virus reactivated heated suspensions of vaccinia, cowpox and variola major, and also white variants of cowpox and rabbit pox. These heated suspensions were prepared in a manner similar to that used for the rabbit pox suspension already mentioned. All were tested by serial passage on the chick C.A.M. and produced no lesions.

Heat-tethered virus may be used to titrate heat-inactivated suspensions. Table 1C illustrates the linear increase in pock count as the concentration of heat-inactivated virus is raised. An advantage of the use of heat-tethered virus in studies on reactivation is that there are no background pocks, so that all pocks that appear have arisen following reactivation. In genetic work this means that one parent is apparently inhibited at a late stage in its developmental cycle. The intracellular growth of the other parent is possible only after it has been reactivated by the heat-tethered parent. The virus progeny from such a cell, and later from the resulting pock, might contain a proportion of hybrids. A higher proportion of hybrid viruses could be expected from pocks whose appearance was unlike that of either parent virus. This supposition was put to practical test in the experiments now to be described.

Table 1. *The reactivation of heated pox virus suspensions by heat-tethered pox viruses*

	Inactivated virus			Active virus				
	Virus	log p.f.u. per egg*	Ceiling temperature† (° C.)	Virus	log p.f.u. per egg	Ceiling temperature† (° C.)	Temperature of incubation‡ (° C.)	Average no. reactivated pocks§
A	Rabbit pox	7·0	> 41	Alastrim	3·0	37·5	38·5	89
	Rabbit pox	7·0	.	Alastrim	3·0	37·5	40	5
	Rabbit pox	7·0	.	Variola major	3·0	38·5	40	70
	Rabbit pox	7·0	.	Cowpox	4·5	40	41·3	10
B	Rabbit pox W.	6·7	> 41	Alastrim	4·0	37·5	38·5	100
	Vaccinia	6·6	41	Alastrim	3·0	.	38·5	51
	Cowpox	6·0	40	Alastrim	5·0	.	38·5	15
	Cowpox W.	5·7	40	Alastrim	4·0	.	38·5	34
	Variola major	6·7	38·5	Alastrim	5·0	.	38·5	3
C	Vaccinia	4·1	41	Alastrim	4·5	37·5	38·5	9
	Vaccinia	4·4	.	Alastrim	4·5	.	38·5	16
	Vaccinia	4·7	.	Alastrim	4·5	.	38·5	33
	Vaccinia	5·0	.	Alastrim	4·5	.	38·5	64

* Titre of heat-inactivated virus given as the equivalent number of pock-forming units before inactivation.
† Data from Bedson & Dumbell (1961) and unpublished data.
‡ Incubator temperature controlled to ± 0·25° C.
§ Pock count given as arithmetic mean of total counts on 3–6 membranes.

THE PRODUCTION OF HYBRID STRAINS

Crosses between alastrim and rabbit pox

Eggs were inoculated on the C.A.M. with mixtures of $10^{3\cdot4}$ p.f.u. of active alastrim virus (ceiling temperature, 37·5° C.) and heat-inactivated rabbit pox (ceiling temperature > 41° C.) equivalent to 10^6 p.f.u. before inactivation. The eggs were incubated at 40° C. for 3 days and then examined. There were altogether 32 pocks, of which 20 looked like rabbit pox and 12 were white. The white pocks were themselves not uniform in appearance. Some were ulcerated and others smaller, whiter and domed. Material from two white pocks, one of each kind, was sampled. The material from the ulcerated pock was passed on eggs incubated at 38·5° C. The resulting pocks were well separated and clones were developed from three of them. These were labelled AR 1, AR 2 and AR 3. Material from the non-ulcerated pock was passed at 35 and 38·5° C. Three clones, AR 4, AR 5 and AR 6, were developed from pocks in the eggs incubated at 35° C. One clone, AR 7, was grown from a pock appearing at 38·5° C. The properties of these seven clones will be described in a subsequent paper (Bedson & Dumbell, 1964*a*).

Crosses between variola major and cowpox

Active variola major virus (C.T. 38·5° C.) was used to reactivate heat-inactivated cowpox virus (C.T. 40° C.). This was carried out on the C.A.M. of eggs incubated at 39·5 °C.

Several eggs were inoculated with approximately 10^4 p.f.u. of variola virus and heated cowpox, equivalent to 10^6 p.f.u. before inactivation. After incubation at 39·5° C. for 3 days, there were about 20–40 pocks on each membrane. These varied in appearance and were almost all unlike those of either cowpox or variola major; the majority were white and ulcerated and some had a small central area of haemorrhage. Material was collected from six of the pocks for the isolation of virus clones. During the preparation of these, it became apparent that at least five of the six pocks had originally contained a mixture of viruses. From the six samples, 16 clones of virus were prepared. These were selected on the basis of their pock appearance. Two had haemorrhagic, ulcerated pocks, like those of cowpox, but the remaining 14 were chosen because their pocks differed from those of cowpox. The properties of these clones will be described in a subsequent paper (Bedson & Dumbell, 1964*b*).

DISCUSSION

The experiments reported here show that hybrid pox viruses may be isolated with relatively little labour. The method used ensures that growth of virus is initiated only in cells which have been infected with both parental types. This is achieved without technical difficulty. Temperature has been used to prevent the growth of one parental type but the method is not doubly selective. This is because the other parental virus can grow freely as soon as it has been reactivated.

The C.A.M. has some advantages over a cell monolayer for picking out variants. Pock appearance allows for more individual variation than plaque type and also the sampling process is much easier. The clones whose isolation has been described

are presumed to be hybrids on the basis of pock appearance alone. Their detailed characterization for several markers is presented in the accompanying papers (Bedson & Dumbell, 1964*a*, *b*).

The novel feature of our method of obtaining hybrids has been the use of virus in the heat-tethered state. Two properties of heat-tethered virus have been described. The reversibility of the state of inhibition suggests that the virus genome is not damaged by constraint of this degree and kind. The rapidity with which new virus appears when the inhibition is released suggests that the block occurs at a late stage of the viral developmental cycle. As yet we have not attempted to determine directly whether any viral DNA synthesis occurs in the heat-tethered state. The ability of the heat-tethered genome to take part in genetic mixing would suggest that it underwent replication but we cannot exclude the possibility that the other parental virus is able, after it is itself reactivated, to release in some way the state of inhibition of the heat-tethered parent. It is obvious that heat-tethered virus is sufficiently interesting to warrant further study. A more detailed report of this phenomenon is in preparation. Here we have been concerned only with those aspects which relate directly to experiments with pox virus hybrids.

SUMMARY

A simple method for the isolation of pox virus hybrids on the C.A.M. has been described. One parental virus was used as a heat-inactivated suspension. The other parent was used in the active state, but at a temperature higher than its ceiling temperature. Under these conditions the inactive parent was reactivated so that pocks resulted only from the cells infected with both parental viruses. Many of these pocks were unlike those of either parent. Such lesions were found to contain a high proportion of hybrids. In these experiments, alastrim was crossed with rabbit pox and variola major with cowpox.

The term 'heat-tethered' has been used to describe virus whose intracellular cycle of development has been arrested by incubation at too high a temperature. Heat-tethered virus has interesting properties and two of these have been described. When the temperature is lowered, heat-tethered virus will start to grow again. Its reactivating potential has been mentioned above. A more detailed account of the properties of heat-tethered virus is being prepared.

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