BIOLOGICAL ASPECTS OF THE REACTIVATION OF POXVIRUSES

by

Gwendolyn M. Woodroffe

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STATEMENT

The work reported in this thesis was carried out in the Department of Microbiology of the Australian National University during the years 1959-1961 inclusive.

Paper I, "Heat Inactivation of Vaccinia Virus", is entirely my own work.

I carried out an appreciable amount of the experimental work involved in the three papers (II, III, IV of the thesis) on the reactivation of poxviruses. In particular, I was responsible for the development of the direct method of demonstrating reactivation on the chorioallantoic membrane, which was of basic importance in Paper IV, and in work on reactivation published by other authors (e.g. Joklik, Abel and Holmes 1960, Nature 186, 992).

Most of the experimental work in Papers V, "Hybridization Between Several Different Poxviruses", and VI, "Serological Relationships Within the Poxvirus Group" is my own work.

Gwendolyn E. Woodroofe.
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INTRODUCTION

Although the first observation of the reactivation of poxviruses was made by Berry and Dedrick in 1936, when they recovered active myxoma virus from rabbits injected with mixtures of heat-inactivated myxoma and active fibroma, twenty-five years elapsed before the present studies established that the basic mechanism of the process was reactivation of the heated-component, rather than "transformation", which had been evoked to explain the phenomenon.

Elucidation of the mechanism was not possible using the fibroma-myxoma system, owing to technical deficiencies, but emerged from studies with vaccinia virus. The latter has many advantages — purified high-titre stocks of virus are readily obtainable, many of the strains exhibit well-defined genetic markers, and reactivation occurs in a number of different host-cell systems including mouse brain, the chorioallantoic membrane and in tissue culture, each of which can be used to elucidate special problems. Individual clones of virus are readily isolated by plating on the chorioallantoic membrane, and this greatly facilitated the subsequent recognition of parental and recombinant types in the progeny.

The thesis is divided into two parts. The first, in the
form of published papers (I – V) or papers submitted for publication (VI), presents the experimental work on reactivation and recombination of poxviruses, together with newly-developed ideas on the serological relationship between different members of the poxvirus group. The relation of the six papers to each other, and to the wider context of the subject of reactivation and recombination in animal viruses, is discussed in the second part of the thesis.
ORIGINAL WORK PRESENTED AS PAPERS EITHER PUBLISHED

OR ACCEPTED FOR PUBLICATION
The Heat Inactivation of Vaccinia Virus

Kaplan (1) observed that thermal inactivation of vaccinia virus at temperatures between 55° and 60° was not a simple first-order process, and he interpreted this as indicating that suspensions of vaccinia virus were heterogeneous in their heat sensitivity. There was an initial temperature-dependent inactivation of the heat-sensitive virus, followed by inactivation at a constant slow rate, unrelated to temperature, of the heat-resistant survivors.
just before heating, on the other hand, showed characteristic flattening (Fig. 2). The concentrated freshly prepared suspension was completely inactivated only after 15 hours' heating at 50°, whereas after storage large amounts of active virus remained even after 30 hours at 50°. Hundredfold dilution of this material rendered it much more susceptible to heat inactivation (Fig. 2), showing that a considerable degree of protection was afforded by impurities in the original suspension.

Pohjanpelto (6) found that L-cystine greatly increased the heat stability of poliovirus heated at temperatures of 50° and less. L-Cystine (50 µg per milliliter) had no effect on the heat stability of vaccinia virus when concentrated semi-purified suspensions (10⁸ PFU per milliliter) were incubated at 37° for 12 hours and then heated at 55°. Similar pretreatment of dilute suspensions (10⁵ and 10⁶ PFU per milliliter) led to a slight increase in their heat stability at 50°. Cystine does not appear to be the responsible agent for the heat stabilization of stored vaccinia virus suspensions described in this letter.

REFERENCES

Reactivation of Heat-inactivated Poxviruses: a General Phenomenon which includes the Fibroma–Myxoma Virus Transformation of Berry and Dedrick

In 1936, Berry and Dedrick\(^1\) reported that active myxoma virus could be recovered from rabbits injected with mixtures of heat-inactivated myxoma and active fibroma virus. Because their experiments were suggested by Griffith's\(^2\) studies on the transformation of pneumococcal types, Berry and later investigators called heated myxoma virus the "transforming agent" and the phenomenon "fibroma–myxoma virus transformation". However, Berry recognized that reactivation of the heat-inactivated myxoma rather than transformation of the active fibroma virus was a possible alternative mechanism.

The term 'transformation', as used in bacterial genetics, has been defined as the heritable modification of the properties of one bacterial strain by an extract derived from cells of another strain. The active material responsible for transformation is deoxyribonucleic acid. There is no evidence that the phenomenon described by Berry and Dedrick is an example of transformation, in this sense. The process is better interpreted as reactivation of the inactivated virus, and we shall use that term henceforth.

Berry's results have been repeatedly confirmed, but further analysis of the mechanism was precluded by the irregular results obtained in intact animals. Recently, Kilham\(^3\) showed that the Berry–Dedrick phenomenon could be demonstrated with regularity with heat-inactivated myxoma virus and active fibroma virus, in cultures of rabbit, monkey and squirrel kidney cells. With collaborators\(^4\) he has described the effects on the heated myxoma virus component of some physical and enzymic treatments.

The definition of several independent genetic markers of vaccinia virus suggested that it might be a more suitable agent for the study of the mechanism of the Berry–Dedrick phenomenon than myxoma and fibroma viruses. The strains of vaccinia virus used for most experiments discussed here were those employed previously for recombination experiments\(^5\). Their biological characters are set out in Table 1.

The virulent strain, \(RP\), was used as the inactivated agent. After heating for $2\frac{1}{2}$ hr. at 55°C. in 0.1\(M\) sodium chloride, 0.01\(M\) citrate, at pH 7,
bination as a possible secondary event, cannot yet be decided.

FRANK FENNER
I. H. HOLMES
W. K. JOKLIK
GWENDOLYN M. WOODROOFFE

Department of Microbiology,
John Curtin School of Medical Research,
Australian National University,
Canberra. March 6.

2 Griffith, F., J. Hyg., 27, 113 (1928).
The Reactivation of Poxviruses

I. Demonstration of the Phenomenon and Techniques of Assay

W. K. Joklik, Gwendolyn M. Woodroofe, I. H. Holmes, and Frank Fenner

Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia

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When heat-inactivated rabbitpox virus is present in cells in which another poxvirus is multiplying, it is reactivated, that is, it is represented among the yield. Techniques are described for the demonstration of this phenomenon in the chorioallantoic membrane of the developing chick embryo, rabbit skin, mouse brain, and HeLa cells. Inactivated virus can be introduced into cells of the mouse brain or into HeLa cells as long as 3 days before the reactivating virus and still appear in the yield.

The preparations of reactivable virus used contained less than $10^{-9}$ infective units, and it has been shown that by inoculation on the chorioallantoic membrane and subsequent passage one infective particle can be detected in the presence of $10^9$ heat-inactivated particles. Likewise it has been shown that heat-inactivated virus does not undergo multiplicity reactivation. The reactivability of heat-inactivated virus suspensions may be assayed either in the mouse brain or on the chorioallantoic membrane; titers of reactivable particles are generally one-hundredth of infective virus titers before heating.

INTRODUCTION

Berry and Dedrick (1936) showed that active myxoma virus could be recovered from rabbits inoculated with a mixture of heat-inactivated myxoma virus and active fibroma virus. We recently suggested (Fenner et al., 1959) that this was a special case of what appeared to be a general phenomenon among the poxviruses; for any active poxvirus has the capacity to reactivate any other member of the poxvirus group which has been inactivated by heating. This series of papers amplifies various aspects of our preliminary communication. The first paper describes the criteria for inactivation, and methods of demonstrating and assaying reactivation. The second paper illustrates the generality of the process among poxviruses; and the third describes the production of reactivable particles by urea treatment as well as by heating, and compares the
resistance of infectivity and reactivability to various chemical treatments.

Studies of reactivation by procedures that utilize high concentrations of the inactivated virus depend upon the certainty with which inactivation can be carried out, and a satisfactory method of demonstrating the noninfectious nature of the inactive component is described in this paper. Different methods of achieving reactivation are useful for different purposes. We have used the mouse brain, the chorioallantoic membrane (CAM), the rabbit skin, and cultured cells to provide the host cells within which reactivation occurs, and the production of pocks on the CAM, or lesions in susceptible animals, as methods for its demonstration. Investigations can be put on a quantitative basis by assay of the reactivability of the inactivated component and two assay methods, involving the use of the mouse brain and the CAM, or the CAM alone, are described.

TERMINOLOGY

In order to facilitate discussion it is necessary to define some of the terms to be used in these papers.

1. “Active virus” is fully infectious virus.
2. “Reactivating agent” is material the addition of which to cells containing appropriately inactivated virus causes its reactivation. This may be active virus, virus inactivated by certain chemical or physical agents, or possibly a defined fraction of the virus.
3. “Reactivable virus” is virus which by itself is noninfectious, even under conditions of multiple infection of cells, but which can be reactivated by the addition under the proper conditions of the reactivating agent.
4. The “reactivability” of an inactivated virus preparation is its capacity to be reactivated by a chosen reactivating agent in a chosen host cell system.

ABBREVIATIONS

The following abbreviations are used. CAM = chorioallantoic membrane; PFU = pock-forming unit; SP = single pock; heat-inactivated virus is designated by the prefix H-, thus H-RP and H-7N.

MATERIALS AND METHODS

Virus strains. The origins and biological characters of rabbitpox virus (RP) and vaccinia-Lederle-7N (7N) have been described previously (Fenner, 1958, 1959). The hemorrhagic ulcerated pocks produced by
RP on the CAM are referred to as phenotypically U⁺, the white pocks of 7N being designated U.

The “Dohi A” strain of ectromelia virus (Dohi, 1951) was kindly provided by Dr. S. Dohi.

Stocks of the Boerlage strain of fibroma virus (Fenner and Woodroffe, 1954) and the MSD (Californian) strain of myxoma virus (Fenner and Marshall, 1957) were prepared in rabbits and assayed by the intradermal inoculation of rabbits (for fibroma) or by pock counts on the CAM (for myxoma).

**Virus titrations.** One-tenth-milliliter amounts of suitable dilutions of virus in gelatin saline were titrated on the CAM of 11-day-old chick embryos by the method of Westwood *et al.* (1957). The pocks were counted after incubation for 2 days (for vaccinia) or 3 days (for ectromelia and myxoma).

The titers of virus stocks stored frozen were often found to be reduced when determined after thawing, but could be restored to the original value after 30 seconds’ exposure to high frequency vibrations produced by a Mullard Ultrasonic Drill Generator (50 watts, peak output at 16 kilocycles). This treatment was used routinely for the resuspension of centrifuged, stored, and heated virus preparations.

**Mice.** Intracerebral inoculations were carried out on 5-week-old mice under ether anesthesia. Unless otherwise indicated, mice were always used in groups of three, and the brains were pooled before the virus was extracted.

Fluorocarbon extraction, as described by Gessler *et al.* (1956), was used to separate virus from brains: 5 ml of Genetron (1-dichlorofluoro-2-difluorochloroethane) and 10 ml of McIlvaine’s phosphate-citrate buffer (0.005 M phosphate, pH 7.2) containing 0.15% gelatin were used per group of three mouse brains. After a single cycle of homogenization followed by low speed centrifugation, the virus in the aqueous layer was titrated.

**Tissue culture systems.** For tissue culture experiments a cloned line of HeLa cells was grown in Eagle’s medium with 20% human serum.

**Characterization of virus clones.** Pocks were sampled by needling, purified by SP passage, and characterized as described by Fenner and Comben (1958) and Fenner (1959).

**EXPERIMENTAL RESULTS**

1. **Preparation of Suspensions of Heat-inactivated Virus**

Stock suspensions of RP and 7N were obtained by extracting confluent CAM by means of the fluorocarbon procedure of Gessler *et al.* (1956).
Twenty-four CAM were homogenized with 48 ml phosphate-citrate buffer containing 0.15% gelatin and 24 ml Genetron. The Genetron phase was then extracted with 1 1/2 volumes of buffer, and the pooled aqueous extracts spun at 12,000 g for 10 minutes to deposit the virus. The operation was repeated on the pellet resuspended in 15 ml buffer. The final volume was 24 ml and had a titer of about $10^{8.4}$ PFU per milliliter.

The conversion to reactivable virus was always carried out within a few hours of the preparation of stock suspensions in order to prevent the tailing effect noted by Kaplan (1958) and elucidated by Woodroofe (1960). The virus was heated in totally submerged ampules for 12-15 minutes at 60° and was then ready for use.

2. Demonstration of the Complete Inactivation of Heated Virus

For the rigorous demonstration of reactivation by procedures utilizing high concentrations of the inactivated agent it is essential to use heated preparations that contain no residual infective virus. Immediate heating, instead of heating after storage at 4°, eliminated one cause of residual infectivity (Woodroofe, 1960), but it was necessary to be sure that apparent complete inactivation was not due to the masking of a small residual amount of active virus by a great excess of inactive material. The sensitivity of different methods of detecting residual active virus was therefore determined with RP, which is highly infective for the CAM, the rabbit skin, and the mouse brain (Fenner, 1958).

Artificial mixtures were made of a preparation of H-RP containing $10^{10}$ particles per milliliter (pock count before heat inactivation, $10^9$ PFU per milliliter) and twofold dilutions of active RP, such that the mixtures contained an estimated 1, 2, 4, 8, . . . . 512 PFU of active virus and $10^9$ particles of H-RP per 0.1 ml. Control preparations contained gelatin saline in place of H-RP. Test and control preparations were inoculated in mice (0.05-ml volumes intracerebrally), in rabbits (0.1-ml volumes intradermally), and on the CAM (0.1-ml volumes). Eggs were examined for pocks on the second day, when two membranes were removed, ground in chilled mortars, suspended in gelatin saline, and subinoculated on another group of eggs. The brains were removed from two mice of each group of eight on the third day, and after extraction, these brains were subinoculated on the CAM. Lesions on the rabbits' backs were examined on the third, the fifth, and the seventh days after inoculation, and suspensions of skin slices taken on the third day were assayed for infective virus on the CAM, as described by Day et al. (1956). The results are set out in Table 1.
The rabbit skin was useless for the detection of residual infectivity, for at all inoculation sites, including the control sites containing only H-RP, small flat nodules developed which reached their maximum size on about the fifth day and had almost disappeared by the seventh. Skin slices taken from these on the third day yielded virus only with concentrations greater than $10^3$ PFU of active RP, when these were mixed with $10^{8.7}$ particles of H-RP. In contrast, control inoculation sites showed lesions of graded severity, positive to the level of 2 PFU of RP.

In the mouse brain, also, large amounts of heated virus interfered

**TABLE 1**

**Comparative Sensitivity of Different Test Systems for the Detection of Small Amounts of Active Virus (RP) in the Presence of Large Amounts of Heat-inactivated Virus**

<table>
<thead>
<tr>
<th>Dose of active RP (PFU)</th>
<th>Test system</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAM</td>
</tr>
<tr>
<td></td>
<td>+ H-RP + Saline</td>
</tr>
<tr>
<td></td>
<td>Pock count</td>
</tr>
<tr>
<td>0</td>
<td>0, 0</td>
</tr>
<tr>
<td>1</td>
<td>0, 1</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>1, 2</td>
</tr>
<tr>
<td>8</td>
<td>7, 4</td>
</tr>
<tr>
<td>16</td>
<td>11, 8</td>
</tr>
<tr>
<td>32</td>
<td>—</td>
</tr>
<tr>
<td>64</td>
<td>50, 32, 40, 40</td>
</tr>
<tr>
<td>128</td>
<td>—</td>
</tr>
<tr>
<td>256</td>
<td>—</td>
</tr>
<tr>
<td>512</td>
<td>—</td>
</tr>
<tr>
<td>1024</td>
<td>—</td>
</tr>
</tbody>
</table>

$^a$ Dose of H-RP = $10^9$ particles ($10^{8.0}$ PFU before heating) for egg and rabbit; half as much in the mouse. Doses of active virus as shown.

$^b$ Pock count on passage expressed as log$_{10}$ PFU; not done; — = no pocks seen. Passage of CAM on second day; of mouse brain and rabbit skin lesion on third day.

$^c$ Deaths out of six mice inoculated.

$^d$ N = nodule; IPC = indurated swelling with purple center; — = no lesion. Readings made 5 days after inoculation.
with the multiplication of the added active virus. Virus was recovered from the brains of the control mice on the third day after the inoculation of doses of 4 PFU or more, the amount of virus increasing with dosage. The great majority of control mice inoculated with 8 PFU or more of RP died within 9 days. None of the mice inoculated with mixtures of active and inactive virus died, and virus was obtained from brains only with doses of 64 PFU of RP or more; and then only in concentrations about a thousandfold lower than in the corresponding control animals.

The CAM, on the other hand, proved to be a highly sensitive indicator of the presence of active virus in the presence of high concentrations of inactive material. Direct observation of membranes on the second day revealed the expected numbers of pocks, and passage of such membranes yielded large amounts of active virus. The pocks seen on membranes inoculated with mixtures of active and inactive virus varied in appearance; sometimes they were the typical U+ pocks of RP, but usually they were small white pocks, which yielded much smaller amounts of virus than “non-interfered” pocks. Virus obtained from these by passage was always typical RP.

This experiment showed that inoculation on the CAM, with passage of the inoculated membranes on the second day, is a highly sensitive assay method for residual infectivity. Further, its value is the same for all viruses of the vaccinia-variola group, since the CAM is a sensitive host for all of these (Fenner, 1958; Overman and Sharp, 1959). In all subsequent experiments “complete inactivation” involves failure to demonstrate active virus by this method.

The negative results obtained with very concentrated suspensions of heated virus (containing $10^8$ particles per 0.1 ml inoculum) also indicate that heat-inactivated virus does not undergo multiplicity reactivation.

3. Demonstration of Reactivation of H-RP

It will be shown in a subsequent paper that all poxviruses tested have been found to possess the capacity to reactivate heat-inactivated RP or 7N (Fenner and Woodroofe, 1960). The choice of the active agent is therefore dictated by the ease with which reactivation can be demonstrated and quantitated with different active viruses. Much of the preliminary work was carried out with H-RP and the vaccinia strain 7N, using mouse brain, CAM, and cultured cells as the growth medium, and the CAM for the demonstration of the occurrence of reactivation. Later, ectromelia virus was used for the routine assays of reactivability both in the mouse brain and on the CAM.
Fig. 1. The growth of 20 PFU RP in the mousebrain in the presence and absence of 10^{4.2} PFU ectromelia. • = ectromelia in presence of RP; O = ectromelia in absence of RP; ■ = RP in presence of ectromelia; □ = RP in absence of ectromelia.

**Reactivation in the mouse brain.** Although reactivation of H-RP by active 7N could be easily demonstrated by subinoculation of mouse brains inoculated with appropriate mixtures (Fenner et al., 1959), these viruses are not a suitable combination for assay purposes because 7N interferes with the growth of RP in the mouse brain. Following observations on the efficiency with which ectromelia virus produced reactivation (Fenner and Woodroofe, 1960), we used as the reactivating virus ectromelia Dohi A, a strain of low intracerebral virulence for mice, which produces pocks on the CAM which are scarcely visible on the second day, and are small and nonhemorrhagic on the third day. In the doses used this virus does not interfere with RP in the mouse brain. Figure 1 shows that 20 PFU of RP multiplied to the same extent whether inoculated alone or together with 10^{4.2} PFU of ectromelia virus.
In the course of experiments on reactivation of H-RP by ectromelia in the mouse brain it was observed that the titer of $U^+$ pocks varied with the dose of H-RP injected, suggesting that it might be possible to measure the titer of reactivable virus by employing standard inocula of ectromelia and assaying mouse brains for $U^+$ pocks after a standard time. The best results were obtained by harvesting mouse brains after 48 hours, and using an ectromelia dose of about $10^6$ PFU. At this concentration small variations in the amount of ectromelia inoculated had no effect on the yield of $U^+$ pocks, but deviations of more than tenfold from this amount led to decreased recoveries of virus with the $U^+$ phenotype. For assaying different preparations of H-RP, tests were carried out at two dilutions. If the preparations were likely to be very active, they were diluted 1/10 and 1/1000; if the preparations were less active, lower

![Graph showing yields of $U^+$ virus](image)

**Fig. 2.** Yields of $U^+$ virus obtained after 48 hours from brains which received $10^{4.4}$ PFU of ectromelia and various doses of H-RP. Each point represents the mean of four separate experiments, in each of which three mice were used for each dilution. The limits indicated for each value are the standard deviations.
dilutions were used. The titers of U+ virus obtained were then referred to a reference curve (Fig. 2), which demonstrates that at lower doses there is a linear relationship between the dose of H-RP injected and the yield of U+ virus. With high doses of reactivable virus a plateau is reached (the system becomes insensitive) and maximum titers of U+ virus recovered from the brains 48 hours after injection are about $10^{6.6}$ PFU per brain. Longer incubation periods did not significantly extend the range of the assay procedure. By the inoculation of closely spaced high dilutions of a preparation of H-RP which contained $10^{10}$ particles per milliliter ($10^9$ PFU per millimeter before heating), it could be calculated that the minimum reactivable dose of H-RP detectable by this procedure was equivalent to $10^{2.8}$ particles. If it is assumed that only 10% of the inoculated virus lodged in the mouse brain (Cairns, 1950; Mims, 1960) the ratio of reactivable to total particles is 1:600.

Reactivation on the CAM. Reactivation in mouse brain is demonstrable only by subculture of brain suspensions on the CAM, on which the characteristic pocks of the reactivated RP can be counted. Since we wished to test the reactivating capacity of viruses which did not multiply in the mouse brain, experiments were carried out on the CAM, initially with 7N. When undiluted suspensions of H-RP were mixed with small doses (approximately 30 PFU) of 7N and inoculated on the CAM, a large proportion of the resulting pocks were of the U+ type, and these proved to consist predominantly of virus indistinguishable from RP.

Two methods were available for testing for reactivation on the CAM. If the reactivation of a virus of distinctive pock type was highly efficient the typical pocks could be counted directly. The phenotypes of the dominant viral populations of such pocks could be determined by needling, purification by SP passage, and characterization by the methods described earlier (Fenner, 1959). In doubtful cases the CAM was removed, ground up, and passaged either on eggs, or in an animal resistant to the active virus used but susceptible to the reactivated virus. Reactivation on the CAM has proved of value in the experiments described in the next paper (Fenner and Woodroffe, 1960) dealing with reactivation by homologous and heterologous viruses.

In a typical direct experiment a mixture of $10^8$ particles (originally $10^7$ PFU of active virus) of H-RP and 30 PFU of 7N was inoculated on the CAM. Two days later there were 66 U and 31 U+ pocks on the five membranes examined. Needling and subculture of some of these pocks showed that the majority viral population of about one-half the pocks was identical in all characters tested with either parental 7N or RP while the rest were recombinants.
THE REACTIVATION OF POXVIRUSES. I

TABLE 2
COMPARISON OF ECTRROMELIA, FIBROMA, AND MYXOMA FOR THE ASSAY OF H-RP ON THE CAM

<table>
<thead>
<tr>
<th>Active Virus</th>
<th>Dose of H-RP</th>
<th>Pock Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectromelia, 10^6 PFU</td>
<td>10^4.7</td>
<td>14, 6, 6, 6, 2</td>
</tr>
<tr>
<td>Fibroma, 10^5.5 ID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>10^5.7</td>
<td>9, 3, 5, 0, 2</td>
</tr>
<tr>
<td>Myxoma, 10^5 PFU</td>
<td>10^4.7</td>
<td>1, 3, 3, 3, 2</td>
</tr>
</tbody>
</table>

* Mixtures of H-RP with ectromelia, fibroma, and myxoma viruses were inoculated on the CAM and the numbers of pocks produced by the reactivated virus counted 2 days later. The results shown are the best observed with each combination.

b Expressed in terms of particles of H-RP. The pock count of the original preparation, before heating, was 10% of the particle count.

Assay on the CAM. The reactivability of a preparation of heated virus could not be assayed by the method just described, which involved the inoculation of a concentrated suspension of heated virus and a very dilute suspension of active virus. The reverse procedure was therefore examined, using active agents which grew slowly and produced small pocks, usually not visible until the third day after inoculation. Three poxviruses, ectromelia, fibroma, and myxoma MSD were used as the reactivating virus and H-RP as the inactivated component. Falling tenfold dilutions of heated virus were inoculated on the CAM, together with varying large doses of the reactivating virus. After 2 days, when control eggs inoculated with the reactivating virus showed only a slight granularity or opacity, discrete RP pocks were seen on eggs inoculated with the virus mixtures (Table 2).

Ectromelia virus was easier to prepare in high titer from infected CAM than myxoma or fibroma and gave better results than the other two viruses. Further experiments were therefore carried out to determine the optimum conditions for the assay of reactivability. Figure 3 shows that an accurate assay of the titer of H-RP preparations can be made by inoculating various dilutions of H-RP together with 10^4.5 to 10^5 PFU of ectromelia on the CAM of several eggs. The titer of reactivable virus preparations can thus be expressed in terms of the pock count obtained in the presence of ectromelia. Table 3 shows that the titer of H-RP is independent of the amount of ectromelia used over the range of 10^4 to 10^6 PFU. It is likely that the larger amounts of ectromelia interfered with pock formation by the reactivated virus, whereas the efficiency of reactivation decreased with small inocula of ectromelia. Under optimum conditions, the ratio of the titer of reactivable to total particles was
Fig. 3. The relation between dose of H-RP and number of U+ pocks on the CAM.

1:1000; i.e., this method was as sensitive as assay by mouse brain inoculation and passage on the CAM, and much less laborious.

Reactivation in tissue culture. Monolayers of cultured cells that support the multiplication of RP and reactivating virus can be used as the system in which reactivation occurs, in the same way as the mouse brain. They are of use in the study of the reactivating capacity of viruses that will multiply in cultured cells but not in the mouse brain or on the CAM, and they allow greater flexibility in the design of experiments.

In a typical experiment monolayers of HeLa cells containing $10^5$ cells, grown on the bottom of small bottles, were inoculated with $10^8$ particles of H-RP and $10^8$ PFU of 7N. After 2 hours' adsorption the monolayers were washed, growth medium was added, and the cells were reincubated at 36°. At varying times from 8 to 48 hours later, the cells were disrupted by treatment with the ultrasonic drill, heated for 15 minutes at 55° to destroy most of the virus with the low heat resistance of 7N, and inoculated on the CAM. Of 50 clones picked off and charac-
terized after this selective procedure 14 resembled parental RP, 10 resembled parental 7N, and 26 were recombinants.

Reactivation in the rabbit skin. Reactivation of H-RP could occur also in the rabbit skin. Mixtures were made of twofold dilutions of H-RP ($10^{10}$ particles per milliliter) with tenfold dilutions of fibroma virus (titer $10^6.8 \text{ ID}_{50}$ per milliliter), and twenty-four mixtures were inoculated intradermally into separate skin areas on the backs of two rabbits. Three days later skin slices were taken from the resulting lesions and assayed on the CAM. U+ pocks were produced from sites inoculated with $10^{5.5} \text{ ID}_{50}$ of fibroma virus and doses of H-RP varying from $10^8.7$ to $10^7.5$ particles. Yields were highest with the most concentrated mixture.

Time relationship of the reactivation process. In all the foregoing experiments the inactivated virus and the reactivating agent were added simultaneously to the host cells in which reactivation and growth occurred. Experiments were carried out in the mouse brain, and in cultures of HeLa cells, to ascertain the effects of inoculating the reactivating agent at intervals before or after the inoculation of H-RP.

Doses of $10^{4.5}$ PFU of ectromelia virus were inoculated intracerebrally into groups of mice at various intervals before and after the inoculation of doses of $10^7.5$ particles of H-RP containing $10^{4.5}$ reactivable particles.

**TABLE 3**

<table>
<thead>
<tr>
<th>Dose of Ectromelia per CAM (PFU)</th>
<th>Number of U+ pocks</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^6$</td>
<td>62</td>
<td>Poocks difficult to count; CAM semiconfluent with ectromelia.</td>
</tr>
<tr>
<td>$10^{5.5}$</td>
<td>53</td>
<td>Slightly less ectromelia; U+ poocks more distinct.</td>
</tr>
<tr>
<td>$10^{5.0}$</td>
<td>60</td>
<td>U+ poocks standing out well against background of ectromelia.</td>
</tr>
<tr>
<td>$10^{4.6}$</td>
<td>68</td>
<td>Ectromelia does not interfere with count of U+ poocks.</td>
</tr>
<tr>
<td>$10^{4.1}$</td>
<td>65</td>
<td>Very good CAM; equivalent to counting active RP on its own.</td>
</tr>
<tr>
<td>$10^{3.6}$</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

*Each egg received 0.1 ml of the required dilution of ectromelia and 0.1 ml of a suitable dilution of H-RP and was incubated for 42 hours. The number of U+ poocks represents the average of the two membranes showing the highest counts out of groups of five.*
TABLE 4
THE EFFECT OF INOCULATING H-RP AND THE REACTIVATING AGENT AT DIFFERENT TIMES

<table>
<thead>
<tr>
<th>Time interval between inoculations</th>
<th>Experiments in mouse brain (reactivating agent, ectromelia)</th>
<th>U+ pocks per brain (logio PFU)</th>
<th>Experiments in HeLa cells (reactivating agent, 7N)</th>
<th>Presence of reactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-RP:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Days before</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Days before</td>
<td>2.9</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Days before</td>
<td>3.6</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hours before</td>
<td>4.4</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 Hours before</td>
<td>3.9</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 Hours before</td>
<td>4.2</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Hours before</td>
<td>4.7</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simultaneous</td>
<td>6.3</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Hours after</td>
<td>5.3</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 Hours after</td>
<td>5.2</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 Hours after</td>
<td>5.8</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 Hours after</td>
<td>6.1</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Days after</td>
<td>..</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* = no evidence of reactivating even after passage of CAM; + = U+ pocks present on passage; ++ and +++ = few or many U+ pocks seen on direct assay; .. = not done.

Pools of three brains were processed in the usual way 2 days after the second inoculation and titrated on the CAM, with the results shown in Table 4. Reactivation occurred when H-RP was inoculated 3 days before ectromelia, but not with longer time intervals. The efficiency of reactivation, as judged by the yield of U+ pocks, was maximal if the two reagents were inoculated simultaneously, and was greater when H-RP followed ectromelia virus than when the inactivated virus was inoculated first.

In the mouse brain it was impossible to infect all susceptible cells with the dose of ectromelia virus used, and in the 2 days allowed after the second inoculation the viruses went through several growth cycles. An experiment was therefore set up in cultures of HeLa cells, which were inoculated with doses of virus large enough to ensure infection of all cells.

Cell monolayers containing $10^5$ HeLa cells were inoculated with $10^8$ particles of H-RP and $10^8$ PFU of 7N at various intervals, as shown in Table 4. Twenty-four hours after the addition of the second component the cells were disrupted, the fluids treated at 55° for 15 minutes to reduce the titer of virus with the low heat resistance of 7N, and inoculated on the CAM either undiluted or at appropriate dilutions.
Reactivation was most efficient in cells inoculated simultaneously with the two viruses, but it could be demonstrated with decreasing efficiency when H-RP was inoculated up to 3 days before 7N, or up to 24 hours after the active virus. The monolayer showed confluent cytopathic changes by the second day after the inoculation of 7N, so that it is not surprising that addition of H-RP at this stage failed to produce reactivation.

DISCUSSION

The reactivation of heat-inactivated RP by a variety of reactivating viruses occurs in a number of host cell systems, indeed it is likely that reactivation will occur in any cells that will support the multiplication of both the reactivating and the reactivated viruses. Each of the host cell systems described in this paper has been used for the elucidation of special problems, as will be evident from the work presented in the next papers of this series. Host animals highly susceptible to the one virus but resistant to another may be used when it is necessary to confer a selective advantage on the reactivated virus; the CAM is useful for the assay of reactivability and for the demonstration and quantitation of phenotypes of the pock-type character. Cultured cells, in which input multiplicities can be more accurately measured than in intact animals, are being used for quantitative studies of reactivation.

Just as different host cell systems are appropriate for different types of investigation, so also is there a wide choice of both the inactivated virus, and the reactivating agent. It will be shown in the next paper (Fenner and Woodroofe, 1960) that any poxvirus will serve as a reactivating agent for any other suitably inactivated member of the poxvirus group. H-RP is the preferred inactive agent for many experiments because of its high infectivity and rapid growth rate in many hosts, including mouse brain and the CAM. However, the U pocks of 7N, which has a similar growth rate to RP on the CAM, are somewhat easier to count than the U+ pocks of RP, and for quantitative assays on the CAM H-7N it is probably a more suitable agent. Ectromelia virus of any strain is satisfactory as the reactivating agent in direct assays on the CAM, for all strains of this virus produce minute pocks on the CAM on the second day, when assays of the reactivated H-RP or H-7N are carried out. Ectromelia Dohi A is the most suitable agent for mouse brain assay because it is of lower intracerebral virulence for the mouse than other strains of ectromelia virus, and it does not interfere with the growth of RP in the mouse brain.

The assay procedures described in this paper provide a convenient
means for quantitating the reactivability of inactivated virus suspensions, and they have been extensively used in quantitative work on the properties of inactivated virus suspensions (Joklik et al., 1960). The chief advantage of the mouse brain for the assay of reactivability is that it is very tolerant to chemical and enzymatic reagents. The assay is, however, a slower and more laborious one because it involves successive inoculation of mouse and CAM, and incubation in each host for 2 days. It has been used only when direct assay on the CAM was impossible.

The sequence of events in the CAM or mouse brain during reactivation is complex. With the lower concentrations of inactivated virus a reactivable particle may be lodged in a cell some distance from the nearest reactivating virus particle. During the early growth cycles in the CAM or mouse brain, the cell concerned may be invaded by active virus, and this initiates reactivation. The reactivated particle may then proceed to multiply at a much faster rate than the reactivating agent, and, depending upon the availability of uninfected cells, may produce a characteristic pock. We have calculated that under optimum conditions about 1% of the originally infectious virus in a preparation of H-RP may be reactivable. We do not yet know whether this figure is much too low, since there may be interference with the production of a typical pock, or with the growth of the reactivated virus in the mouse brain, due to the presence of the reactivating virus.

Kilham et al. (1958) found that heated myxoma virus could be reactivated by fibroma virus in tissue cultures when the inactive virus was inoculated 24 hours before or 48 hours after the reactivating agent. We have obtained similar results with H-RP and 7N in tissue cultures, and with H-RP and ectromelia virus in mouse brain.

Except when simultaneous infection of all cells with the active agent occurred, as in the experiment in HeLa cells, the results involving infection with active virus before addition of the inactivated component are of little significance. In the HeLa cell experiment it was remarkable that under conditions which ensured single cycle infection with active 7N reactivation occurred, although with low efficiency, when H-RP was introduced as long as 24 hours later, when multiplication of 7N would be well advanced in all cells.

H-RP can remain in a reactivable form in both mouse brain and HeLa cells for at least 3 days, suggesting that some reactivable particles resist digestion by the host cell enzymes for this period.

Many experiments on reactivation can be carried out with virus suspensions that are not completely noninfectious, if suitable dilutions are
used. However, it is convenient, for other experiments, to use high concentrations of inactivated virus, and this is practicable only if the inactivated material can be shown to be completely noninfectious. Experiments with mixtures of RP and H-RP demonstrated that rigorous proof of the absence of infectious RP from such mixtures could not be obtained by the inoculation of mice or rabbits, but could be ensured by assay on the CAM, with passage of the membranes 2 days after inoculation.

REFERENCES


The Reactivation of Poxviruses

II. The Range of Reactivating Viruses

FRANK FENNER AND GWENDOLYN M. WOODROOFE

Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia

Accepted February 12, 1960

Heat-inactivated poxviruses can be reactivated by any other active poxvirus. Thus, H-RP, H-7N, and H-CPR can be reactivated by single-step mutants (RPu1 and CPu16), by other members of the mammalian poxvirus group (homoreactivation) and by myxoma, fibroma, fowlpox, and contagious pustular dermatitis virus (heteroreactivation). Under similar conditions the same heated preparations cannot be reactivated by active agents belonging to other groups, such as Rickettsia australis, psittacosis virus, infectious laryngotracheitis virus, herpes simplex virus, influenza virus, Rous sarcoma virus, or Murray Valley encephalitis virus.

Analysis of clones of virus obtained from pocks appearing on membranes showing evidence of reactivation showed that many pocks contained a majority population of virus indistinguishable in the several markers examined from the heat-inactivated agent. This occurred irrespective of whether the reactivating virus belonged to the same subgroup (homoreactivation) or was an unrelated poxvirus (heteroreactivation).

In homoreactivation, but not in heteroreactivation, recombinants, as well as the phenotypes of the reactivating and the reactivated virus, were recovered.

INTRODUCTION

The preceding paper (Joklik et al., 1960a) describes several methods for demonstrating the reactivation of heat-inactivated rabbitpox virus (H-RP). We have already reported briefly on the reasons for regarding this sort of reactivability as a general phenomenon in the poxvirus group (Fenner et al., 1959); independent proof of this has recently been furnished by Hanafusa et al. (1959). The present paper records detailed results showing the range of active viruses that will or will not reanimate selected heat-inactivated poxviruses. Detailed characterization of the reactivated clones provides a basis for the view that the phenomenon with which we are concerned, which is the same as that first reported
by Berry and Dedrick (1936), and recently elaborated by Kilham (1958), is best regarded as reactivation of the inactivated virus rather than "transformation" of the active by the inactivated agent.

**TERMINOLOGY**

Reactivating agent, reactivable virus, and reactivability are used in the sense described earlier (Joklik et al., 1960a).

*Homoreactivation* refers to the reactivation of a particular inactivated poxvirus preparation by a closely related active virus. This may be either a mutant form of the inactivated virus (e.g., a u mutant of RP), or another member of the same subgroup of the poxvirus group.

*Heteroreactivation* refers to the reactivation of a particular inactivated poxvirus preparation by an active poxvirus belonging to another subgroup, e.g., the reactivation of H-RP by fowlpox virus.

**MATERIALS AND METHODS**

Virus strains used, the methods of preparation of virus stocks, and the methods of titration used are set out in Table 1.

**Preparation of heat-inactivated virus.** For convenience, heat-inactivated viruses will be symbolized by the letter "H" used as a prefix for the abbreviation of their name—thus H-RP = heat-inactivated rabbitpox virus.

The method of preparation of H-RP and the demonstration of its lack of infectivity were described earlier (Joklik et al., 1960a). The preparations of H-RP and H-7N used in all experiments described in this paper had been heated in phosphate-buffered saline at 60°C for 15 minutes. Comparable results were obtained with preparations heated at 55° for 2½ hours. Details of the conditions for inactivation of other viruses are given in appropriate sections.

Complete lack of infectivity of the heated poxviruses was ensured by inoculating heated preparations on the CAM and passing the inoculated membranes on a further group of CAM. This is the most sensitive available test for residual infective virus (Joklik et al., 1960a).

**Demonstration of reactivation.** Reactivation can be demonstrated by mixed infection of cells that support the growth of the active forms of both the viruses employed. In the previous paper it was pointed out that mouse brain, the chorioallantoic membrane, or cultured cells all provided a satisfactory milieu for reactivation. Most of the experiments reported in this paper were carried out on the CAM, for it was found that when concentrated H-RP and a dilute suspension of another pox-
<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Reference</th>
<th>Abbreviation</th>
<th>Source of stock virus</th>
<th>Method of infectivity titration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbitpox-Utrecht (wild type)</td>
<td>Fenner (1958)</td>
<td>RP</td>
<td>CAM</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td>Rabbitpox-Utrecht (white mutant)</td>
<td>Gemmell and Fenner (1960)</td>
<td>RPs, RPs2</td>
<td>CAM</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td>Vaccinia-Lederle-7N</td>
<td>Fenner (1958)</td>
<td>7N</td>
<td>CAM</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td>Cowpox-Brighton-Red</td>
<td>Fenner (1958)</td>
<td>CPR</td>
<td>CAM</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td>Cowpox-Brighton (white mutant)</td>
<td>Fenner (unpublished)</td>
<td>CP-W10</td>
<td>CAM</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td>Cowpox-Brighton-White</td>
<td>Fenner (1958)</td>
<td>ECT</td>
<td>CAM</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td>Ectromelia-Moscow</td>
<td>Fenner (1949)</td>
<td>MYX</td>
<td>Rabbit skin and testis</td>
<td>CAM: pock count, Intradermal titration in rabbits</td>
</tr>
<tr>
<td>Myxoma-Lausanne</td>
<td>Fenner and Marshall (1957)</td>
<td>FIB</td>
<td>Rabbit skin and testis</td>
<td>CAM: pock count, Intradermal titration in rabbits</td>
</tr>
<tr>
<td>Fibroma-Boerlage</td>
<td>Fenner and Woodroffe (1954)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contagious pustular dermatitis</td>
<td>Commonwealth Serum Laboratory vaccine</td>
<td>CPD</td>
<td>Scarification of sheep</td>
<td>CAM: pock count, Intradermal titration in sheep</td>
</tr>
<tr>
<td>Fowlpox</td>
<td>From Walter and Eliza Hall Institute</td>
<td>FP</td>
<td>CAM</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td>Murray Valley encephalitis</td>
<td>French (1952)</td>
<td>MVE</td>
<td>Mouse brain</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td>Rous sarcoma virus</td>
<td>From Walter and Eliza Hall Institute</td>
<td>RSV</td>
<td>Chicken tumor</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td>Herpes simplex, HFEM</td>
<td>From Walter and Eliza Hall Institute</td>
<td>HERP</td>
<td>CAM</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td>Infectious laryngotracheitis</td>
<td>Commonwealth Serum Laboratory vaccine</td>
<td>ILT</td>
<td>CAM</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td>Influenza WSE</td>
<td>Dr. S. Fazekas de St. Groth</td>
<td>FLU</td>
<td>Allantoic fluid</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td>Psittacosis Blount</td>
<td>From Walter and Eliza Hall Institute</td>
<td>PSIT</td>
<td>Yolk sac of chick embryo</td>
<td>CAM: pock count</td>
</tr>
<tr>
<td><em>Rickettsia australis</em></td>
<td>From Queensland Institute of Medical Research</td>
<td>NQTT</td>
<td>Yolk sac of chick embryo</td>
<td>CAM: pock count</td>
</tr>
</tbody>
</table>
virus were inoculated together on the CAM some of the pocks were of the \( U^+ \) (rabbitpox) type. Sampling of these by needling and SP passage showed that most of the virus clones thus recovered resembled RP in all characters \((U^+ A T^+ V(M^+ R^+))\) (Fenner, 1959). Various recombinants were also obtained, and these will be described fully elsewhere. The direct demonstration of reactivation on the CAM was found to apply to a wide variety of combinations of viruses.

This method was also used to exclude the occurrence of reactivation with certain combinations of inactive and "reactivating" viruses. For this purpose concentrated heated virus and various concentrations of the agent under test were inoculated together. If the membranes showed no evidence of reactivation 2 days later they were ground up, and the suspensions obtained were subinoculated on the CAM, and also into host animals that were susceptible to the active form of the heat-inactivated virus but naturally resistant (or actively immunized) to the reactivating agent.

Characterization of virus clones. Marker characters of the viruses most extensively used in the current experiments have already been described (Fenner, 1959). Virus clones were purified by needling and single-pock selection. The characterization of virus clones followed the procedures described earlier (Fenner and Comben, 1958), modified where necessary for viruses other than RP and 7N. They will not be described in detail here, but where heat-inactivated RP, 7N, CPR, or CPW are said to have been reactivated, this means that in all detectable characters the reactivated clones resembled the appropriate parental phenotypes.

**EXPERIMENTAL RESULTS**

**Homoreactivation**

1. **Homoreactivation of H-RP and H-CPR by Their Respective \( u \) Mutants**

Different white variants of rabbitpox virus (RP) have been shown to be due to single mutations at several different loci (Gemmell and Cairns, 1959). Two of these mutants, RPu1 and RPu2, were selected for tests of homologous reactivation because they produced small white (U) pocks on the CAM (Gemmell and Fenner, 1960), whereas RP produces hemorrhagic ulcerated \((U^+)\) pocks. There is a similar situation with the white variants of cowpox virus (Fenner, unpublished), and CPu16 was selected for the reactivation of the wild-type H-CPR.

**Homoreactivation of H-RP by RPu1 and RPu2.** Volumes of 0.1 ml containing \(10^8\) particles of H-RP and about 20 PFU of RPu1 or RPu2
were inoculated on the CAM, with appropriate controls. Pock counts 2 days later gave the results shown in Table 2. Pocks were of three types; some were small white pocks identical with those produced by RPu1 or RPu2, others were large, pale, ulcerated pocks of the type produced by RPu+ (wild type), and there were a few larger white pocks with irregular edges. Subinoculation of the latter showed that they were produced by RPu+ under conditions of partial interference, and they have been classified as RPu+ in Table 2.

In both experiments about half of the pocks seen were of the type associated with the reactivated virus, and half of the type produced by the reactivating agent. In this, as in all other experiments in which high concentrations of heated virus were used, there was great variation in the response of individual eggs. Some membranes showed considerable thickening and opacity due to the H-RP inoculum alone, and this sometimes interfered with the development of characteristic pocks; and on other membranes there was little nonspecific opacity.

Homoreactivation of H-CPR by CPu16. The pocks of cowpox and its white variants develop more slowly on the CAM than those of vaccinia (Fenner, 1958), and readings were therefore made on the third day.

Reactivation of the preparation of H-CPR used was consistently less frequent than with H-RP, and in three separate experiments only about a quarter of the pocks were of the U+ type (Table 2).

2. Homoreactivation between Different Members of the Vaccinia-Variola Group

Four "species" of closely related mammalian poxviruses have been recognized (Fenner and Burnet, 1957). These are vaccinia (of which rabbitpox virus may be considered a variety), variola, cowpox, and ectromelia viruses. Contagious pustular dermatitis virus (CPD) exhibits some antigenic relationships with vaccinia virus (Webster, 1958), but it will be considered in the next section. Variola virus is not available in Australia, but the interactions of the other three members of the mammalian poxvirus group were tested. Several different viruses served as the heat-inactivated agents.

The simplest method of demonstration of homoreactivation was the inoculation of the CAM with concentrated heat-inactivated virus and suitable dilutions of the active agent. The membranes, and suitable controls, were examined 2 or 3 days later, with the results shown in Table 2. This method gave unequivocal results when there were pronounced differences between the pocks produced by the reactivated and
<table>
<thead>
<tr>
<th>Heat-inactivated virus</th>
<th>Active virus</th>
<th>Pock type</th>
<th>Inoculum</th>
<th>Day of examination</th>
<th>Pock count</th>
<th>Ratio &quot;reactivated pocks&quot;/total pocks</th>
<th>Clones characterized</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-RP</td>
<td>RPw1</td>
<td>Small white</td>
<td>H-RP + RPw1</td>
<td>2</td>
<td>18, 17, 3, 6, 2, 5</td>
<td>10/97</td>
<td>8 U⁺ pocks, all RP</td>
</tr>
<tr>
<td>H-RP</td>
<td>RPw2</td>
<td>Small white</td>
<td>H-RP + RPw2</td>
<td>2</td>
<td>2, 4, 11, 1, 5, 2, 2</td>
<td>9/18</td>
<td>6 U⁺ pocks, all RP</td>
</tr>
<tr>
<td>H-CPR</td>
<td>CPw16</td>
<td>Small white</td>
<td>H-CPR + CPw16</td>
<td>3</td>
<td>6, 6, 0, 1, 3, 5, 3, 6</td>
<td>40/70</td>
<td>10 U⁺ pocks, all CPR</td>
</tr>
<tr>
<td>H-RP</td>
<td>7N</td>
<td>Large white</td>
<td>H-RP + 7N</td>
<td>2</td>
<td>17, 9, 8, 2, 21, 30</td>
<td>31/97</td>
<td>32 pocks: 7—7N, 11—RP, 14—recomb.</td>
</tr>
<tr>
<td>H-RP</td>
<td>CPw16</td>
<td>Small white</td>
<td>H-RP + CPw16</td>
<td>2</td>
<td>6, 5, 4, 4, 1, 10, 19, 5</td>
<td>40/70</td>
<td>—</td>
</tr>
<tr>
<td>H-RP</td>
<td>CPW</td>
<td>Large white</td>
<td>H-RP + CPW</td>
<td>2</td>
<td>12, 14, 2, 4, 10</td>
<td>36/72</td>
<td>7 U⁺ pocks: 6 U⁺, 1 recomb.</td>
</tr>
<tr>
<td>H-RP</td>
<td>ECT</td>
<td>Very small white</td>
<td>H-RP + ECT</td>
<td>2</td>
<td>35, 30, 38, 70, 38</td>
<td>223/223</td>
<td>10 U⁺ pocks, all RP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ulcerated red</td>
<td>H-7N + RP</td>
<td>Saline + RP</td>
<td>H-7N + CPR</td>
<td>Saline + CPR</td>
<td>H-7N + ECT</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
<td>---------------</td>
<td>-----------</td>
<td>-------------</td>
<td>------------</td>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>H-7N</td>
<td>RP</td>
<td>Ulcerated red</td>
<td>2</td>
<td>42, 11, 43, 7, 61, 122</td>
<td>34/43</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>H-7N</td>
<td>CPR</td>
<td>Ulcerated deep red</td>
<td>2</td>
<td>114, 9, 181, 5, 10</td>
<td>53/58</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>H-7N</td>
<td>ECT</td>
<td>Very small white</td>
<td>3</td>
<td>22, 25, 14, 14, 10, 8</td>
<td>113/113</td>
<td>20 Large white pocks, all 7N</td>
<td></td>
</tr>
<tr>
<td>H-CPR</td>
<td>ECT</td>
<td>Very small white</td>
<td>3</td>
<td>44, 29, 33, 512, 32, 11</td>
<td>21/69</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>

* Volumes of 0.1 ml concentrated heated virus and diluted reactivating virus were inoculated on the CAM of 11-day-old chick embryos. H-RP contained $10^{9.7}$ particles per milliliter (initially $10^{8.0}$ PFU per milliliter), and had been heated at 60°C for 15 minutes. H-7N contained $10^{9.5}$ particles per milliliter (initially $10^{9.3}$ PFU per milliliter), and had been heated at 60°C for 15 minutes. H-CPR contained initially $10^{8.4}$ PFU per milliliter, and had been heated at 55°C for 2½ hours. Control membranes were inoculated with the diluted reactivating agent and saline. Membranes were examined after 2 or 3 days' incubation at 36°C. Pocks ascribed to reactivated virus are set in boldface type.
the reactivating virus, as for example, with vaccinia and ectromelia. Clones of virus recovered from several of these “reactivated” pocks were fully characterized (Table 2). The common association of virus indistinguishable from the inactivated agent with these pocks justified the direct counting of reactivation. Recombinants were recovered from only a small proportion of such pocks (Woodrooife and Fenner, 1960).

The results set out in Table 2 show that homoreactivation can be clearly demonstrated on the CAM with a variety of members of the variola-vaccinia group. The examples described were chosen because they included members of the major recognized “species” of this subgroup (except variola); and combinations were selected that allowed a clear differentiation of the pocks produced by each agent. The number of “reactivated” pocks produced by ectromelia virus inoculated with H-RP and H-7N was greater, and the pock counts were more consistent, than on control membranes inoculated with ectromelia alone.

Several combinations were also inoculated intracerebrally in the mouse, or into HeLa cell cultures, with subsequent examination of virus from the mouse brain or cell cultures on the CAM. Positive results were invariably obtained with homologous viruses of the variola-vaccinia group.

**Heteroreactivation**

Within the poxvirus group (Fenner and Burnet, 1957), there are several subgroups, each comprising several serologically related viruses. There is no serological crossing between members of these different subgroups. However, it was found that heat-inactivated viruses of the vaccinia-variola subgroup could be reactivated by active viruses of these other subgroups—thus H-RP was reactivated by myxoma, fibroma, fowlpox, and CPD viruses.

As with homoreactivation, heteroreactivation of members of the vaccinia-variola subgroup could usually be demonstrated directly on the CAM. The results of several such experiments are shown in Table 3. If no pocks of the type associated with the reactivated virus were seen on the CAM, the membranes were removed aseptically and passaged in eggs and on the rabbit skin. Sometimes positive results were obtained on passage although no pocks were seen on the membrane, but usually the direct pock assay gave the same end point as passage of the CAM.

As can be seen from Table 3 all combinations tested gave positive results, although the efficiency varied. Membranes inoculated with H-RP or H-7N plus myxoma (MYX) always showed only “reactivated” pocks on the second day, and the average pock count was only slightly
lower than that of the control membranes inoculated with the same dilution of myxoma and examined on the third day. There was great variation in the response of different membranes. Reactivation of H-CPR by myxoma virus was less efficient than reactivation of H-RP or H-7N by this active agent.

Fibroma virus (FIB) multiplies on the CAM and can be passaged in series on the membrane (Smith, 1948), but it produces no pocks that can be recognized macroscopically, although large doses cause a “ground glass” opacity. When various dilutions of a stock preparation of fibroma were inoculated with H-RP or H-7N characteristic “reactivated” pocks were produced out to a dilution of 10^{-3}, which corresponded to 10^{2.8}\text{ ID}_{50}\text{ of fibroma virus, titrated by the intradermal inoculation of rabbits. With a given dilution of fibroma, there were great variations between replicate membranes and great deviation from linearity in the relationship between the mean pock count and the dilution of fibroma virus.}

Fowlpox virus (FP) produces large, pale, nonulcerated pocks on the CAM but grows much more slowly than members of the vaccinia-virola group. By examining membranes for reactivated pocks 2 days after inoculation and control membranes after 4 days, a clear differentiation could be made, for fowlpox produces no recognizable pocks on the second day. The efficiency of reactivation of H-RP and H-7N by fowlpox appeared to be considerably lower than by myxoma.

Contagious pustular dermatitis virus (CPD) produces minute pocks on the CAM but cannot be passaged in this host (Webster, 1958). When various dilutions were inoculated on the CAM together with H-RP or H-7N, positive results were obtained on passage with the 10^{-1} and 10^{-2} dilutions, but reactivated pocks were seen only with the 10^{-2} dilution. Titrated by the intradermal inoculation of sheep, the CPD preparation used contained 10^{6}\text{ ID}_{50}\text{ per milliliter.}

Pocks were picked off by needling from suitable membranes of most of the combinations shown in Table 3, purified by SP passage, and characterized for pock morphology, heat resistance, hemagglutinin production, and mouse and rabbit virulence. In all cases all the clones selected resembled the reactivated parent in all those characters [U^{+}A^{+}T^{+}V(M^{+}R^{+}) for RP; U A^{+}T V(M R) for 7N].

**Attempts to Demonstrate Reactivation of Heated Poxviruses by Active Viruses of Other Groups**

Since reactivation was demonstrable by all members of the poxvirus group that could be tested, further experiments were carried out with
<table>
<thead>
<tr>
<th>Heat-inactivated virus</th>
<th>Designation</th>
<th>Active virus</th>
<th>Inoculum</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-RP</td>
<td>MYX</td>
<td>Very small white</td>
<td>H-RP + MYX</td>
<td>Direct pock count</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Subinoculation of ground membranes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Clones characterized</td>
</tr>
<tr>
<td>H-7N</td>
<td>MYX</td>
<td>Very small white</td>
<td>H-7N + MYX</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0, 1, 2, 15, 9, 4, 27, 0, 0, 4</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7 U pocks, all 7N</td>
</tr>
<tr>
<td>H-CPR</td>
<td>MYX</td>
<td>Very small white</td>
<td>H-CPR + MYX</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20, 4, 0, 0, 2, 0, 8, 8, 7, 1</td>
</tr>
<tr>
<td></td>
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<td>14, 11, 8, 8, 3</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>8, 9, 7 4, 12 8, 15, 1 26</td>
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<td></td>
<td></td>
<td></td>
<td>70, 50, 24, 34, 36</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>H-RP</td>
<td>FIB</td>
<td>No definite poocks (10⁶.8 ID₅₀ per ml)</td>
<td>H-RP + FIB 10⁴</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
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<td></td>
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<td></td>
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<td>36, 31, 48, 20, 3, 125, 200, 38</td>
</tr>
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<td></td>
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<td></td>
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<td>69, 30, 100, 200, 29, 36</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>37, 38, 11, 109, 5, 2, 16</td>
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<td></td>
<td></td>
<td>14, 2, 0, 0, 3, 1, 6, 5</td>
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<td></td>
<td></td>
<td></td>
<td>Negative</td>
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<td></td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>H-7N</td>
<td>FIB</td>
<td>No definite poocks (10⁶.8 ID₅₀ per ml)</td>
<td>H-7N + FIB 10⁴</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>180, 250, 14, 500, 170, 52, 6, 106</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>94, 100, 22, 40, 25, 58, 104, 33</td>
</tr>
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<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0, 17, 2, 0, 5, 8, 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0, 1, 0, 0, 0, 0</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>FIB</td>
<td></td>
<td></td>
<td></td>
<td>4 U pocks, all 7N</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negative</td>
</tr>
</tbody>
</table>

TABLE 3
HETEROACTIVATION

Note: The table provides results of heteroreactivation experiments involving various combinations of heat-inactivated viruses and active viruses. The results include direct pock counts and clones characterized.
<table>
<thead>
<tr>
<th></th>
<th>FP</th>
<th>Large pale white on 4th day</th>
<th>H-RP + FP 10^-2</th>
<th>6, 0, 35, 39, 51, 29, 77, 31</th>
<th>—</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>H-RP + FP 10^-3</td>
<td>2</td>
<td>2, 0, 0, 19</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H-RP + FP 10^-4</td>
<td>2</td>
<td>0, 0, 1, 0</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H-RP + FP 10^-5</td>
<td>2</td>
<td>0, 0, 2, 0, 0</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H-RP + FP 10^-6</td>
<td>2</td>
<td>0, 0, 0, 0, 0</td>
<td>Negative</td>
</tr>
</tbody>
</table>

| H-7N     | FP  | Large pale white on 4th day | H-7N + FP 10^-2  | 2                             | 57, 0, 9, 108 | — |
|----------|-----|-----------------------------| H-7N + FP 10^-3  | 2                             | 6, 5, 0, 22, 44, 16, 27 | — |
|          |     |                             | H-7N + FP 10^-4  | 2                             | 17, 1, 0, 2, 2, 15, 0, 1 | — |
|          |     |                             | H-7N + FP 10^-5  | 2                             | 1, 0, 0, 0, 0 | Positive |
|          |     |                             | H-7N + FP 10^-6  | 2                             | 0, 0, 0, 0, 0 | Negative |
|          |     |                             | Saline + FP 10^-6 | 4                             | 13, 7, 9, 8, 6, 9 | |

|          | CPD | Very small on third day (10^8 ID50 per ml) | H-RP + CPD 10^-1 | 2                             | 0, 0, 0, 0, 0 | Positive |
|----------|-----|--------------------------------------------| H-RP + CPD 10^-2  | 2                             | 3, 10, 0, 0, 0 | Positive |
|          |     |                                            | H-RP + CPD 10^-3  | 2                             | 0, 0, 0, 0, 0 | Negative |

| H-7N     | CPD | Very small on third day (10^8 ID50 per ml) | H-7N + CPD 10^-1 | 2                             | 0, 0, 0, 0, 0 | Positive |
|----------|-----|--------------------------------------------| H-7N + CPD 10^-2  | 2                             | 3, 7, 1, 14, 1 | Positive |
|          |     |                                            | H-7N + CPD 10^-3  | 2                             | 0, 0, 0, 0, 0 | Negative |
|          |     |                                            | Saline + CPD 10^-2 | 3                             | 30, 42, 8, 27, 7 | — |

*Procedure and heated virus preparations as in Table 2. If no "reactivated pocks" were seen on the CAM, the membranes were aseptically reaped, ground up, and subinoculated on fresh CAM; and into rabbits. Pocks ascribed to reactivated virus are set in boldface type.*
TABLE 4

FAILURE TO DEMONSTRATE REACTIVATION OF HEAT-INACTIVATED POXVIRUSES BY ACTIVE VIRUSES BELONGING TO OTHER GROUPS

<table>
<thead>
<tr>
<th>Active agent</th>
<th>Concentration of stock virus (PFU per ml)</th>
<th>Inoculum (equal parts in 0.1-ml volumes)</th>
<th>Direct examination</th>
<th>Passage of CAM and subinoculation at various dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Designation</td>
<td></td>
<td>In rabbit</td>
<td>On CAM</td>
</tr>
<tr>
<td>MVE</td>
<td>10⁰</td>
<td>H-RP + MVE 10⁻⁴</td>
<td>2 Days: scattered small pocks</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-7N + MVE 10⁻⁴</td>
<td>2 Days: general opacity only</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Saline + MVE 10⁻⁶</td>
<td>3 Days: 7, 28, 11 small pocks</td>
<td>Negative</td>
</tr>
<tr>
<td>RSV</td>
<td>10⁴.³</td>
<td>H-RP + RSV 10⁻⁰.³</td>
<td>2 Days: general opacity</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-7N + RSV 10⁻⁰.³</td>
<td>2 Days: general opacity</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Saline + RSV 10⁻³</td>
<td>6 Days: 2, 4, 1, 0 pocks</td>
<td>Negative</td>
</tr>
<tr>
<td>HERP</td>
<td>10⁴.²</td>
<td>H-RP + HERP 10⁻¹</td>
<td>2 Days: semiconfluent herpes pocks</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-7N + HERP 10⁻¹</td>
<td>2 Days: semiconfluent herpes pocks</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Saline + HERP 10⁻².³</td>
<td>3 Days: 84, 74</td>
<td>Negative</td>
</tr>
<tr>
<td>ILT</td>
<td>10⁴.²</td>
<td>H-RP + ILT 10⁻¹</td>
<td>2 Days: general opacity</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-7N + ILT 10⁻¹</td>
<td>2 Days: general opacity</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Saline + ILT 10⁻²</td>
<td>3 Days: 5, 9, 5 pocks</td>
<td>Negative</td>
</tr>
<tr>
<td>WSE</td>
<td>10⁷.²</td>
<td>H-RP + WSE 10⁻⁴</td>
<td>2 Days: general opacity</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H-7N + WSE 10⁻⁴</td>
<td>2 Days: general opacity</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Saline + WSE 10⁻⁴.³</td>
<td>2 Days: 124, 57, 42</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
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<td>----</td>
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<td>----</td>
<td></td>
</tr>
<tr>
<td>PSIT</td>
<td>$10^6.3$</td>
<td>H-RP + PSIT $10^{-2}$</td>
<td>3 Days: general opacity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H-7N + PSIT $10^{-3}$</td>
<td>3 Days: general opacity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saline + PSIT $10^{-4.3}$</td>
<td>3 Days: 3, 2, 20, 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>No RP pocks</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>No 7N pocks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NQTT</td>
<td>$10^4.7$</td>
<td>H-RP + NQTT $10^{-1}$</td>
<td>2 Days: general opacity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H-7N + NQTT $10^{-1}$</td>
<td>2 Days: general opacity</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Saline + NQTT $10^{-3}$</td>
<td>3 Days: 28, 64, 45</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>No RP pocks</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>No 7N pocks</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Preparations of H-RP and H-7N that gave positive results in homoreactivation and heteroreactivation tests (Tables 2 and 3), in the same dosages as in those tests. Procedure similar to that outlined in Table 3.
viruses belonging to unrelated groups, to see whether they could reacti-
vate H-RP or H-7N.

The basic technique with these active agents, all of which belong to
different virus families, and all of which grow on the CAM, was the
same. Doses of the active agent that alone would produce about 100
and about 1000 pocks on the CAM were mixed with stock preparati-
s of H-RP or H-7N and, with the appropriate controls, inoculated on the
CAM of groups of eggs. The membranes were examined 2 days later
for RP or 7N pocks; then they were ground up, suspended in diluent,
and tested for the presence of active RP or 7N by subinoculation on eggs
and into the skin of the rabbit. Results were uniformly negative, whereas
the control groups of eggs inoculated with heated RP or 7N and ecto-
menia virus gave the expected positive results. Details of the agents
tested and the procedure used are shown in Table 4.

DISCUSSION

These experiments demonstrate that heat-inactivated vaccinia virus
can be reactivated by all the active poxviruses tested: by one-step
mutants of the inactivated viruses, by other members of the vaccinia-
variola subgroup, and by various serologically unrelated poxviruses,
such as myxoma, fibroma, contagious pustular dermatitis, and fowlpox.
On the other hand, all our attempts to reactivate heated poxviruses by
active viruses of other groups were unsuccessful.

With other viruses no evidence of reactivation of the heat-inactivated
component has been obtained with influenza, poliovirus, or herpes
simplex. Fazekas de St. Groth (personal communication, 1959) carried
out tests with several dilutions of both reagents, but found no evidence
of reactivation of either influenza A (PR8) or influenza B (LEE) by the
heterologous virus after heating at 56° for 30, 60, or 120 minutes.
Infertility was reduced to less than 10⁻⁹ by the shortest period of
heating. With poliovirus, Howes (personal communication, 1959) failed
to demonstrate reactivation of poliovirus type 1 heated at 50° for 1 hour
(with a reduction of infectivity of 10⁻⁴) by active poliovirus type 2.
With herpes simplex virus we failed to reactivate heated HFEM (which
produces relatively large pocks on the CAM) with an active preparation
of a recently isolated strain of herpes virus that produced minute pocks.

Although reactivation has not been observed with influenza virus,
recombination between heated and active influenza A virus strains has
been reported (Burnet and Lind, 1954; Lind and Burnet, 1957; Frazer,
1959). Ribonucleic acid is much less heat-resistant than deoxyribonucleic
acid, so that heat inactivation may always damage the genome too greatly
for reactivation, although marker rescue by closely related strains may occur.

As we pointed out previously (Fenner et al., 1959) reactivation is a more suitable term than "transformation" to describe this phenomenon. In bacterial genetics, transformation has been defined as the heritable modification of the properties of one bacterial strain by an extract derived from the cells of another strain. Analysis of "reactivated" pocks produced after the addition of agents as diverse as vaccinia, ectromelia, fibroma, and fowlpox showed that in all cases many pocks contained virus particles indistinguishable from the heat-inactivated agent in the several characters tested. It is much simpler to regard this as a consequence of the reactivation of the heated agent than as the "transformation" of each of these different active viruses into something indistinguishable from the heat-inactivated virus. As yet, however, we have no evidence to indicate whether reactivation is a consequence of genetic interaction or of some nongenetic mechanism.

It is probable that reactivation of any suitably heat-inactivated poxvirus can be effected by any other active poxvirus, but this may be difficult to demonstrate unless the active agent is rapidly overgrown by the reactivated agent. Thus although myxoma virus reactivated vaccinia virus very efficiently, attempts to reactivate heated myxoma virus with active vaccinia virus in tissue cultures (Kilham, 1959) or on the CAM, were unsuccessful. On the CAM, and in tissue culture, vaccinia grows much more rapidly, and to much higher titer, than does myxoma virus. The negative results obtained in mass cell cultures or in experimental animals are presumably due to overgrowth of reactivated myxoma virus by vaccinia, with consequent interference. By contrast, myxoma virus always outgrows fibroma and squirrel fibroma viruses in tissue culture, so that within this group reactivation of heated myxoma is readily demonstrated; reactivation of heated fibroma by active myxoma is difficult or impossible to recognize.

The experiments described in this paper reveal the whole range of poxviruses as suitable agents for the experimental investigation of reactivation, allowing a wide choice in the selection of the agents, or combinations of agents, best suited for particular purposes. Thus, the assay procedures described in the previous paper (Joklik et al., 1960a) and extensively used in the succeeding paper (Joklik et al., 1960b) are based on the high efficiency of reactivation of heated vaccinia virus by ectromelia and the rapid overgrowth of the reactivating ectromelia by the reactivated vaccinia virus.

In the course of experiments upon reactivation recombinants were
obtained from all combinations within the vaccinia-viara group (homoreactivation), but not from heterologous pairs. Recombinants between rapidly growing viruses like vaccinia and slowly growing viruses like ectromelia are much more easily obtained using heat-inactivated vaccinia than with both viruses in the active form. The recombinants found in the current experiments are described elsewhere (Woodroffe and Fenner, 1960).

Reactivation may be used as a taxonomic tool. All the viruses that have been regarded as members of the poxvirus group on other grounds (Fenner and Burnet, 1957) participate in reactivation, and on this basis they are justifiably regarded as members of one large group of viruses. It would be of value to extend this criterion to other viruses which on morphological grounds have been described as poxviruses, such as molluscum contagiosum and various animal "poxviruses."

Addendum

Since the preparation of this paper H. Hanafusa, T. Hanafusa, and J. Kamahora [Biken's J. 2, 85–91 (1959)] have independently reported results in complete agreement with our own. They found reactivation to be a general phenomenon among the poxviruses and showed that viruses of other groups were unable to produce reactivation of heat-inactivated poxviruses.

REFERENCES


Genetic Studies with Mammalian Poxviruses

IV. Hybridization between Several Different Poxviruses

Gwendolyn M. Woodroofe and Frank Fenner

Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia

Accepted July 3, 1960

During experiments on reactivation of heat-inactivated poxviruses, seven clones of virus were recovered which appeared to be hybrids between the two types of poxvirus used. Hybrids were recognized only with viruses belonging to the same subgroup. Within the vaccinia subgroup hybridization appeared to be more common with the more closely related viruses.

A probable hybrid was recovered from a reactivation experiment with myxoma and fibroma. The difficulties of using virulence as a genetic marker are stressed.

INTRODUCTION

The major aim of our current work on poxvirus genetics is to develop a system which can be used for a quantitative study of recombination. For this purpose the μ mutants of rabbitpox virus (Gemmell and Fenner, 1960), some of which, in pairwise crosses, recombine to produce wild type, currently furnish the most suitable material.

It is also of interest to know what range of parental types can give rise to viable hybrids. Observations made during recent experiments on the reactivation of heat-inactivated poxviruses (Fenner and Woodroofe, 1960) furnished some information on this point. The present paper describes in detail hybrids recovered both during these experiments and from other experiments made with various combinations of poxviruses.

MATERIALS AND METHODS

Except where noted, virus clones described in this paper were obtained from the experiments on reactivation described by Fenner and Woodroofe (1960).

Particulars of the origins of the strains of virus used were published there; the abbreviations by which they will be designated are set out in Table 1.
TABLE 1

The Phenotypic Characters of Viruses of the Vaccinia Subgroup

<table>
<thead>
<tr>
<th></th>
<th>Abbreviation</th>
<th>Phenotypic characters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbitpox</td>
<td>RP</td>
<td>U⁺ A⁺ T⁺ V(M⁺ R⁺)</td>
</tr>
<tr>
<td>Dermal vaccinia</td>
<td>7N</td>
<td>U A⁺ T V(M R)</td>
</tr>
<tr>
<td>Red cowpox</td>
<td>CPR</td>
<td>U⁺ A⁺ T⁺ V(M⁺ R⁺)</td>
</tr>
<tr>
<td>White cowpox</td>
<td>CPW</td>
<td>U A⁺ T⁺ V(M⁺ R⁺)</td>
</tr>
<tr>
<td>Ectromelia Moscow</td>
<td>ECT-M</td>
<td>Uᵐ A⁺ T V(M⁺ R⁺)</td>
</tr>
<tr>
<td>Ectromelia Pittsburgh</td>
<td>ECT-P</td>
<td>Uᵐ A⁺ T V(M⁺ R⁺)</td>
</tr>
<tr>
<td>Myxoma Lausanne</td>
<td>MYX</td>
<td>—</td>
</tr>
<tr>
<td>Fibroma Boerlage</td>
<td>FIB</td>
<td>—</td>
</tr>
</tbody>
</table>

See Fenner (1959).

The pock of CPR, though hemorrhagic and ulcerated (U⁺) differs considerably from that of RP (see illustrations in Fenner, 1958).

The pock of CPW is nodular and nonulcerated (U) but develops more slowly than that of 7N. The heat resistance is intermediate between that of RP and 7N.

Ectromelia strains produce minute nonulcerated pocks on the CAM, scarcely visible until the third day. Uᵐ = minute, nonulcerated pock. Heat resistance is much lower than that of 7N, no infective virus being found after 40 minutes at 55°C (drop in titer, 10⁶ PFU). ECT-M is highly lethal after the peripheral inoculation of small doses of virus into mice; ECT-P is pathogenic, but much less virulent. Ectromelia virus fails to produce a lesion in the rabbit skin (R⁰) after the inoculation of standard doses of virus (10⁸ PFU).

Selection of virus clones and their purification followed the methods described earlier (Fenner, 1959), and characters were tested by the methods described in Fenner and Comben (1958). Symbols for phenotypic characters follow Fenner (1959) with modifications necessitated by the use of viruses other than RP and 7N, for which these symbols were originally designed.

Tissue cultures. Monolayers of HeLa cells and chick embryo fibroblasts were grown as in a previous study by Gemmell and Fenner (1960). Monolayers of rabbit kidney cells were grown as described by Kilham (1958).

TERMINOLOGY

The poxvirus group can be divided into several subgroups, each of which contains one or more species. Thus the vaccinia subgroup includes vaccinia, variola, cowpox, and ectromelia viruses; and the myxoma subgroup, myxoma, fibroma, and squirrel fibroma. All species belonging to a subgroup are closely related serologically. The exact
status of the Dutch strain of rabbitpox virus is uncertain (Moeljono, 1958), but for the present it will be regarded as a variety of vaccinia virus, not as a distinct species within the vaccinia subgroup.

Viable products of genetic exchange between different species of pox virus will be called hybrids; in keeping with previous reports (Fenner and Comben, 1958; Fenner, 1959), hybrids between varieties of one species will be called recombinants.

Definitions used in reactivation experiments have been set out previously (Joklik et al., 1960; Fenner and Woodroofe, 1960).

EXPERIMENTAL RESULTS

Material from Experiments on Reactivation of Heated Rabbitpox (H-RP) by Dermal Vaccinia (7N)

RP and 7N have been used extensively in previous experiments on recombination (Fenner and Comben, 1958; Fenner, 1959) and reactivation (Joklik et al., 1960). In the present experiments mice were inoculated intracerebrally with a mixture of H-RP (10^7 particles, originally 10^6 PFU) and 7N (10^6 particles, 10^5 PFU). Figure 1 shows the growth

![Figure 1](image_url)  
**Fig. 1.** Growth curve of active RP and active 7N in mouse brain, after the intracerebral inoculation of 10^6 PFU; and of virus produced after the intracerebral inoculation of heat-inactivated RP (10^7 particles, originally 10^6 PFU) and active 7N (10^6 PFU). The latter included reactivated RP and recombinants between RP and 7N.
curves of \( U \) and \( U^+ \) pocks in such brains, and those obtained after the intracerebral inoculation of \( 10^5 \) PFU of RP or 7N alone.

Each day from the first to the seventh, selected pocks on CAM used for the growth curve experiment were picked off by needling and the clones of virus thus obtained were tested for their biological characters. After the first day, there was a selection of strains of high mouse virulence, either reactivated RP or recombinants between 7N and RP, for these multiplied to a much higher titer than 7N (Fig. 1). In addition to thirteen clones of reactivated RP, twenty-nine clones falling into thirteen different recombinant phenotypes were recognized.

Thirty-two clones were obtained directly by needling pocks which appeared on CAM inoculated with H-RP (\( 10^8 \) particles, originally \( 10^7 \) PFU) and 30 PFU of 7N. Eleven had all the characters of RP, 7 all the characters of 7N, and the other 14 were recombinants. Eleven different types of recombinant phenotype were recognized.

Monolayers of HeLa cells (\( 10^5 \) cells on the bottoms of small flat-bottomed tubes) were infected with H-RP (\( 10^9 \) particles, originally \( 10^7 \) PFU) and 7N (\( 10^8 \) particles, \( 10^7 \) PFU). At various intervals after adsorption tubes were removed, frozen and thawed, treated for 30 seconds with a Mullard ultrasonic drill and titrated after heating at 55° for 15 minutes (to reduce the concentration of parental 7N). Forty-seven clones obtained from tubes incubated for 8–60 hours included 26 recombinants. Reactivated RP was first found 10 hours after inoculation; during the next 2 hours the titer of reactivated virus and of recombinants rose a hundredfold.

### TABLE 2

**Occurrence of Recombinants and Parental Phenotypes among 121 Clones**

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>CAM experiment</th>
<th>Mouse brain experiment</th>
<th>Tissue culture experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live parent (7N): U pock</td>
<td>7</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Heat-inactivated parent: U(^+) pock</td>
<td>11</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>U Pock recombinants</td>
<td>6</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>U(^+) Pock(^b) recombinants</td>
<td>3</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^a\) The clones were recovered directly from CAM inoculated with H-RP and 7N, from mouse brains inoculated with H-RP and 7N and then plated on CAM, and from cultures of HeLa cells inoculated with H-RP and 7N and then plated on CAM.

\(^b\) U\(^+\) pocks = white pocks with hemorrhagic center.
Mixed infections of HeLa cells, mouse brain, or CAM with H-RP and 7N yielded many recombinants, and a variety of characters were exchanged. Details of the phenotypic characters are omitted from this paper since they were similar in many respects to those described earlier (Fenner, 1959). U+ recombinants were found with about the same frequency as U and U recombinants, in contrast to their rarity in earlier experiments with active RP and 7N.

Analysis of the total yield of HeLa cells mixedly infected with large doses of H-RP and 7N showed that reactivated virus indistinguishable from RP could be detected 10 hours after addition of the virus suspension to the cell monolayers, and recombinants appeared at about the same time. The titer of both the reactivated virus and the recombinants rose a hundredfold during the next 2 hours.

Material from Experiments on Reactivation of Heated Rabbitpox (H-RP) by Cowpox (CPW)

In order to simplify the recognition of reactivation of H-RP by cowpox virus the white variant CPW was used. Two days after the inoculation of CAM with H-RP (10^8 particles, originally 10^7 PFU) and 20 PFU of CPW, eleven pocks were sampled by needling and the virus clones were characterized as before (Table 3). Five were hybrids, four of U, and one of U+, pock type. Characters exchanged included hemag-
glutinin production, heat resistance, mouse virulence, and rabbit virulence.

Reactivation of H-RP and the production of hybrids also occurred in the mouse brain. Mice were inoculated intracerebrally with H-RP (10⁶ particles, originally 10⁸ PFU) and CPW (10⁵ PFU). Brain suspensions harvested 4 days later were inoculated on the CAM, and 13 pocks were sampled. Five of these were hybrids (Table 3). Clones with the combination of characters UA (i.e., nonulcerated pocks, no hemagglutinin) of which there were three, could be u mutants of RP, although the chance of finding such a high proportion of u mutants among reactivated H-RP particles would be small. In addition, each of the three clones had other characters probably derived from the cowpox parent (T¹, R).

Thus using a white variant of cowpox virus, hybridization was demonstrated between cowpox and rabbitpox viruses. Several characters were exchanged, and hybrids (as well as reactivated RP) were recovered from mouse brain as well as the CAM.

**Material from Experiments on Reactivation of Heated Rabbitpox (H-RP) by Ectromelia (ECT-M and ECT-P)**

Experiments similar to those just described were set up on the CAM with ECT-M as the active agent and in the mouse brain with active ECT-P. The recovery of U+ pocks from the inoculated CAM was much more difficult than in the previous experiments because of their minute size. However 20 U+ and 8 U pocks were sampled and characterized, with the results shown in Table 4. None of these was unequivocally a hybrid, for clones with U pocks and similar combinations of the other characters have previously been recognized among the U mutants of RP⁺⁺ (Gemmell and Fenner, 1960).

Five clones were obtained from CAM inoculated with mouse brain suspension 3 days after the inoculation of H-RP and ECT-P. Three of these were reactivated RP; the other two, of U+ pock type but avirulent from rabbits, were clearly hybrids.

Small doses of all except those exhibiting all the characters of RP were inoculated into the footpads of mice. The five classified in Table 4 as ectromelia all produced typical foot swelling and liver lesions, whereas none of the others produced such symptoms.

The slow growth of ectromelia on the CAM made recovery of ectromelia and hybrid clones more difficult than in the crosses described earlier. Nevertheless a few clones which are probably correctly classed
as hybrids were recovered from mixed infection experiments involving heated rabbitpox and active ectromelia viruses.

**Material from Experiments on Reactivation of Heated Cowpox (H-CPR) by Ectromelia (ECT-M and ECT-D)**

Downie and MacDonald (1950) showed that ectromelia and cowpox were serologically more closely related to each other than to other poxviruses. Because of the difficulty of recovering recombinants from H-RP and ECT, similar types of experiment were carried out on the CAM and in the mouse brain with H-CPR and ECT. The distinguishing characters tested were pock type, plaque type on chick embryo fibroblasts, and type of lesion produced in the rabbit skin. On chick fibroblast monolayers CPR produces large plaques with an irregular edge, best seen on the fifth day after inoculation, white (\(u\)) variants of CPR produce smaller plaques with well-defined edges, clearly visible on the fourth or fifth day, and ectromelia produces a moderate-sized plaque with a central "pimple."

Fourteen isolates from CAM and seven from mouse brain inoculated with H-CPR and ECT-M were examined, with the results shown in Table 5. Judging from these characters and others not recorded in Table 5 seven of the clones recovered directly from the CAM were reactivated CPR and three were ECT-M. The four clones characterized by large \(U^+\) pocks, nodular lesions on the rabbit skin, and moderate-sized plaques on chick embryo fibroblasts could not be distinguished from white variants of cowpox, which occur with a frequency of about 1% in most clones of CPR. However, the seven clones characterized by small \(U^+\) pocks, large or small plaques on chick embryo fibroblasts, and

### Table 4

**Detailed Analysis of 33 Clones Recovered Directly from CAM Inoculated with H-RP and ECT-M; and from Mouse Brains Inoculated with H-RP and ECT-P and Subinoculated on the CAM 3 Days Later**

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>CAM experiment</th>
<th>Mouse brain experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>(U^m) A+ T V(M+ R°):ECT</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>U+ A T+ V(M+ R°):RP</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>U A T+ V(M+ R°)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>U A T+ V(M R°)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>U A T+ V(M+ R°)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>U+ A T+ V(M+ R°)</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>U+ A T+ V(M+ R°)</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>CAM experiment</th>
<th>Mouse brain experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>(U^m) A+ T V(M+ R°):ECT</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>U+ A T+ V(M+ R°):RP</td>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>U A T+ V(M+ R°)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>U A T+ V(M R°)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>U A T+ V(M+ R°)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>U+ A T+ V(M+ R°)</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>U+ A T+ V(M+ R°)</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>
TABLE 5
Properties of 21 Clones Recovered Directly from CAM Inoculated with H-CPR and ECT-M; and from Mouse Brains Inoculated with H-CPR and ECT-D

<table>
<thead>
<tr>
<th>Clones studied</th>
<th>Pock type</th>
<th>Appearance of plaque on chick embryo fibroblasts</th>
<th>Reaction in rabbit skin</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Number</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAM</td>
<td>7</td>
<td>Large U+</td>
<td>Large, irregular edges</td>
<td>Large, hemorrhagic</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Minute U</td>
<td>Moderate, central &quot;pimple&quot;</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Large U</td>
<td>Moderate, regular edges</td>
<td>Nodular</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Small U+</td>
<td>Large, irregular edges</td>
<td>Nodular</td>
</tr>
<tr>
<td>Mouse brain then CAM</td>
<td>6</td>
<td>Small U+</td>
<td>Very small</td>
<td>Nodular</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Large U</td>
<td>Moderate, regular edges</td>
<td>Nodular</td>
</tr>
</tbody>
</table>

Nodular lesions on the rabbit skin were clearly distinguishable from CPR, ECT, and the white variants of CP; and may reasonably be regarded as hybrids between cowpox and ectromelia viruses.

THE MYXOMA SUBGROUP
Kilham (1958) has shown that heat-inactivated myxoma (H-MYX) can be reactivated by either rabbit or squirrel fibroma virus.

At present we are concerned with attempting to find out whether viable hybrids between fibroma and myxoma can occur. This is made difficult by the paucity of markers, rabbit virulence being the only character on which reliance can be placed, and by the difficulty of recovering clones of virus because of the inadequacy of the plating method. The procedure adopted was the following. Myxoma Lausanne, which is invariably lethal in laboratory rabbits, with a mean survival time of 12 days after the intradermal injection of small doses of virus (Fenner and Marshall, 1957), was heated at 60° for 12 minutes. This completely destroyed its infectivity. Fibroma Boerlage was used as the active agent.

A mixture of H-MYX (10^6.7 PFU before heating) and FIB (10^6
TABLE 6

<table>
<thead>
<tr>
<th>No virus present</th>
<th>Fibroma only</th>
<th>Myxoma-Lausanne</th>
<th>Myxoma of reduced virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11</td>
<td>4</td>
<td>37</td>
</tr>
</tbody>
</table>

ID$_{50}$) was inoculated into small bottles containing monolayers of rabbit kidney cells. Four and 6 days later monolayers were frozen and thawed and the cells disrupted by sonic treatment. Assay on the CAM yielded $10^{3.7}$ myxoma pocks per milliliter. Fifty-three single pocks were cut out, ground up, and inoculated into rabbits, with the results shown in Table 6. The occurrence of unmodified fibroma in a few rabbits, no reaction in several others, and rapidly lethal myxomatosis of Lausanne type in the majority, showed that plating of the contents of the tissue culture bottles on the CAM and the subsequent selection of single pocks avoided the production of "fibromyxoma," as described by Kilham (1958).

One rabbit survived for 30 days, a situation without precedent in our extensive experience with myxoma Lausanne. Subinoculation of small doses of virus obtained from this animal into fifty-one other rabbits showed that this isolate was indeed of slightly reduced virulence (mean survival time 16.3 days; range 11–34 days). It could be either a mutant of myxoma or a hybrid between myxoma and fibroma viruses.

DISCUSSION

The present report shows that viable hybrids can be produced under conditions that allow mixed infections of cells with several different pairs of members of the vaccinia subgroup. The recognition of clones as hybrids is sometimes rendered difficult by the similarity of some of them to $u$ mutants of rabbitpox and cowpox viruses, in experiments in which the latter viruses were used.

The paucity of satisfactory marker characters makes genetic work with viruses like ectromelia, fibroma, and myxoma, almost impossible. The difficulties presented by the use of virulence as a marker is evident from the experiment with myxoma and fibroma. It is not even certain that the one slightly attenuated strain of virus recovered from this experiment was a myxoma-fibroma hybrid, for it could equally well be a myxoma mutant of slightly reduced virulence. The fact that Berry...
(1938) obtained two myxoma strains of reduced virulence from experiments comparable to those we have reported makes it somewhat more likely that hybridization rather than mutation was responsible, but the inadequacy of marker characters makes it impossible to distinguish between these alternatives.

Myxoma and fibroma viruses readily reactivate heat-inactivated viruses of the vaccinia subgroup (Fenner and Woodroffe, 1960), but characterization of some sixty clones obtained from such experiments with H-RP and H-7N failed to yield any which could be recognized as other than reactivated RP or 7N.

We have already shown (Fenner and Woodroffe, 1960) that all tested viruses currently regarded as members of the poxvirus group can participate in reactivation, although unpublished experiments with "monkey tumour poxvirus" of Bearcroft and Jamieson (1958) have given negative results. Hybridization appears to be much more common with closely related viruses, such as RP and 7N, than with more distantly related viruses like RP and ectromelia; and to occur rarely if at all with serologically unrelated viruses like RP and myxoma. However, the experiments described are in no sense quantitative and major technical difficulties currently render attempts at quantitation unprofitable. All we can record is the impression that hybridization between two varieties of vaccinia (RP and 7N) is common, between rabbitpox or cowpox and ectromelia rare, and between vaccinia and myxoma very rare, if possible. It is probable that viable hybrids can be derived from myxoma-fibroma crosses.

REFERENCES


SEROLOGICAL RELATIONSHIPS WITHIN THE POXVIRUS GROUP:

AN ANTIGEN COMMON TO ALL MEMBERS OF THE GROUP.

GWENDOLYN M. WOODROOFE & FRANK FENNER

Department of Microbiology,
The John Curtin School of Medical Research,
The Australian National University,
Canberra, Australia.
SUMMARY

The serological relationships between several members of the poxvirus group were investigated by a variety of techniques, including complement-fixation, gel-diffusion and ring precipitation, hemagglutinin inhibition, pock and plaque neutralization and staining with fluorescein-coupled antibody.

The existence of close relationships between vaccinia, cowpox, rabbitpox and ectromelia; and between myxoma and fibroma; was confirmed. No crossing could be demonstrated between these subgroups, or between either of them and fowlpox or monkey tumour poxvirus, except when an alkaline extract of vaccinia or myxoma viruses (the "NP antigen") was used. The "NP antigen" appears to contain a group antigen common to all viruses of the poxvirus group. In addition to fixing complement and precipitating when tested against both homologous and heterologous "NP antigens", antisera to the "NP antigen" of vaccinia and myxoma neutralized the homologous but not the heterologous virus, when tested by pock and plaque neutralization tests.

INTRODUCTION

It is generally believed (Downie and Dumbell, 1957; Fenner and Burnet, 1957; Mayr, 1959) that the poxvirus group comprises several
II.

subgroups of serologically related viruses and several viruses which are serologically unrelated to each other or to members of the subgroups. Recently, however, Takahashi et al. (1959a) claimed that there were strong cross-reactions between members of the three major subgroups (vaccinia-variola, fowlpox, myxoma-fibroma), demonstrable by complement-fixation tests and fluorescent antibody staining. This claim derives special significance from the demonstration that reactivation of heat-inactivated poxviruses is a general phenomenon throughout the poxvirus group (Hanafusa et al. 1959; Fenner and Woodroffe, 1960), and can be produced by a poxvirus which is itself inactivated by nitrogen-mustard (Joklik et al. 1960).

Using convalescent antisera and intact virus and/or the soluble antigens no such serological cross-reactions were found. However, a group antigen which reacted with antisera to a wide variety of poxviruses was obtained from vaccinia and myxoma viruses by alkaline extraction of the virus particles.

MATERIALS AND METHODS

Virus. The virus strains used and their origins have been described previously (Fenner and Woodroffe, 1960) with the exception of cowpox-Amsterdam-white (Fenner, 1958) and monkey tumor poxvirus (Bearcroft and Jamieson, 1958).
Antisera. Convalescent sera were obtained from rabbits which had recovered from infection with vaccinia, cowpox, myxoma and fibroma viruses, and from a monkey infected with monkey tumor poxvirus. Ectromelia antisera were obtained from convalescent mice and fowlpox antisera by hyperimmunization of fowls.

Vaccinia anti-LS serum was obtained from rabbits hyperimmunized with LS antigen (Shedlovsky and Smadel, 1942). Vaccinia anti-NP and myxoma anti-NP sera were prepared in rabbits hyperimmunized with the appropriate "NP antigens" (Smadel et al., 1942). In these cases rabbits were given an initial intramuscular inoculation of 1 ml of antigen mixed with an equal volume of Freund's complete adjuvant, followed 40 days later by an intravenous or intramuscular injection of 1 ml of the antigen alone. Serum was obtained 3, 5 and 7 days after the last inoculation.

Antiserum to myxoma virus was also prepared as described by Takahashi et al. (1959b). Two rabbits were inoculated intraperitoneally with a 10% suspension of the standard laboratory strain of myxoma virus prepared from tumor tissue. This was UV-irradiated for 20, 10, 5 and then 2 minutes using a 15 w UV lamp at a distance of 10 cm., and 1 ml doses were given at weekly intervals. Active virus (0.5 ml of a 1/100 dilution, containing 5,000 pfu) was given intravenously after a further 10 days. One rabbit died with signs of modi-
IV.

fied generalized infection after the third dose (UV for 5 minutes),
the other showed no symptoms. Serum was obtained two weeks after
the last inoculation.

Dr. E.L. French kindly provided pre-inoculation and convalescent sera from cattle experimentally infected with bovine papular
stomatitis virus.

Antigens. Confluent chorioallantoic membrane (CAM) prepara­
ations, ground in a pestle and mortar with freezing, suspended in
1 ml calcium magnesium saline per CAM and spun free of sediment,
were used as antigens for the complement fixation, gel diffusion
precipitation and hemagglutinin-inhibition tests.

Extraction of 'NP' Antigen from Vaccinia and Myxoma Viruses

Potent suspensions of vaccinia and myxoma viruses were
prepared by scarification of shaved rabbits. After preliminary clari­

cation, the suspensions were centrifuged at 12,000 rpm for 15 min­
utes. The supernatants constituted the 'soluble antigens' used in
ring precipitin tests, and the sediment was resuspended in dilute
McIlvaine's buffer, treated with an ultrasonic drill for one minute,
and freeze dried. The dried powder was extracted with ethyl ether,
rehydrated in distilled water overnight, and extracted with N NaOH
at 56°C for 15 minutes. It was then partially neutralized with
N/2 HCl and centrifuged at 30,000 rpm for 30 minutes. The water clear supernatant fluid was brought to pH 9.0 with N/2 HCl and dialysed overnight against M/100 borate buffer at pH 8.6. This constituted the 'NP' antigen of Smadel et al. (1942).

**Complement-fixation.** Complement-fixation tests, using the drop technique of Donnelley (1951), were set up with antigens at a dilution of 1:8, a range of serial two-fold dilutions of inactivated antisera, and 3 M.H.D. of complement. Complement was fixed overnight at 4°C, the indicator system of 3% sensitized red cells was added, and the complement fixing titers were determined after 1/2 hour at 37°C. The titers, equivalent to the 50% lysis end-point, are expressed as reciprocals. Appropriate controls including normal sera from the various animals used, and antigen prepared from normal chorioallantoic membranes, were always included. Neither direct complement-fixation nor complement-fixation inhibition tests were attempted with the fowl antisera.

**Precipitation.** Gel diffusion precipitation tests were done in plates using the method of Mansi (1957). Antisera were used undiluted and unheated. Antigens were usually undiluted preparations of confluent chorioallantoic membranes, but pieces of infected monkey tumor poxvirus tissue were also used. Plates were left at room temperature and precipitin bands appeared within 24 hours. Normal tissues,
uninfected chorioallantoic membranes and normal sera were included as controls.

Ring precipitin tests were carried out with the 'soluble' and 'NP' antigens, according to standard procedure. Undiluted ice-cold antiserum in capillary tubes was overlaid carefully with the appropriate antigens warmed to 37°C. The interface was examined for precipitin rings after six hours at 4°C, the result scored according to the density of the precipitate. Appropriate serum and antigen controls were always included.

Hemagglutinin inhibition. Serial two-fold dilutions of antiserum were tested for antihemagglutinin activity against vaccinia-Lederle-7N using the method described by Fenner (1958). Partial agglutination was taken as the end point.

Neutralization. Vaccinia, myxoma and fowlpox antisera were used in neutralization tests. These were carried out by the pock counting method on the chorioallantoic membrane of the developing chick embryo or by plaque counting on chick fibroblast monolayers. Undiluted serum was heated at 56°C for 30 minutes before mixture with the virus dilution. The virus-serum mixture was allowed to stand for half an hour at room temperature before inoculation onto eggs or onto chick fibroblast monolayers. The latter were overlaid with agar (Franklin et al. 1957) after two hours absorption, and were then
incubated at $36^\circ$ for 4-6 days in a humidified incubator flushed with 5% $CO_2$ in air. The degree of neutralization is expressed as the ratio between the pock or plaque count obtained with antisera compared with those obtained with normal serum. Ratios are expressed in $\log_{10}$ units. Values smaller than $-1.0$ are positive and those greater than $-0.3$ are negative.

**Fluorescence microscopy.** KB cells supporting the growth of vaccinia, cowpox, ectromelia and myxoma viruses, were grown in slide cultures (Cairns, 1960) and several separate cultures were infected with a high multiplicity (5 PFU per cell) of each virus. Ten hours later the cells infected with vaccinia, cowpox and ectromelia were fixed and stained with fluorescein-coupled antisera, using the direct method. The myxoma-infected cells were stained 24 hours after infection. Frozen sections (Mims, 1959) of infected chorio-allantoic membranes were used to study the reactions of fowlpox virus. Uninfected cells and sections of uninfected tissue were included as controls, and a conjugated anti-influenza rabbit antiserum was also used to confirm the specificity of staining. Rhodamine bovine albumen was used as a counter stain.

**RESULTS**

The results can be divided into two groups; those obtained with crude virus suspensions, which contained both viral particles
### TABLE I

Complement-fixation tests

<table>
<thead>
<tr>
<th>ANTIGENS</th>
<th>rabbitpox</th>
<th>cowpox</th>
<th>ectromelia</th>
<th>vaccinia-NP</th>
<th>myxoma</th>
<th>fibroma</th>
<th>myxoma-NP</th>
<th>monkey tumour poxvirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>vaccinia</td>
<td>1280</td>
<td>80</td>
<td>80</td>
<td>..</td>
<td>-</td>
<td>-</td>
<td>..</td>
<td>-</td>
</tr>
<tr>
<td>rabbitpox</td>
<td>640</td>
<td>120</td>
<td>120</td>
<td>80</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cowpox</td>
<td>480</td>
<td>320</td>
<td>80</td>
<td>120</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ectromelia</td>
<td>240</td>
<td>60</td>
<td>640</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>vaccinia-NP</td>
<td>60</td>
<td>30</td>
<td>10</td>
<td>120</td>
<td>-</td>
<td>-</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>myxoma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2560</td>
<td>40</td>
<td>30</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>myxoma-NP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30</td>
<td>80</td>
<td>40</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>fowlpox</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30</td>
<td>80</td>
<td>40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>monkey tumour poxvirus</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>40</td>
<td>-</td>
</tr>
</tbody>
</table>

---

- **a** antigens consist of extracts of infected CAM, except monkey tumour poxvirus antigen, which was a 10% emulsion of infected tumour tissue used at a dilution of 1/8, and vaccinia-NP and myxoma-NP which were alkaline extracts of virus.
- **b** antisera were from rabbits, mice, fowls or monkeys convalescent after infection with the appropriate viruses; antisera to vaccinia-NP and myxoma-NP were from hyperimmunized rabbits.
- **c** titers expressed as reciprocal of dilution of serum showing 50% fixation; — indicates no fixation at 1/10.
- **d** indicates test not done.
and soluble antigens, and those obtained with the 'soluble' and 'NP' antigens.

The results of all types of serological test made with crude virus suspensions confirmed the view that the poxviruses could be divided into several subgroups, within which strong cross-reactions occurred but between which no crossing could be demonstrated.

Table 1 here

In the complement fixation tests, convalescent sera from animals infected with viruses of the vaccinia–variola subgroup, for example, showed extensive crossing with antigens derived from members of that subgroup. Convalescent anti-myxoma and anti-fibroma sera, on the other hand, reacted only with myxoma and myxoma 'NP' antigens, and no serum reacted with fowlpox antigen. Monkey tumor poxvirus antiserum reacted with the homologous antigen only.

Hyperimmune serum produced against vaccinia–NP, on the other hand, reacted with the crude antigens of the vaccinia–variola subgroup, with vaccinia–NP, and to a lower titre with myxoma–NP. Hyperimmune serum produced against myxoma–NP showed the reciprocal crossing with vaccinia–NP antigen, as well as with myxoma and myxoma–NP.
Gel diffusion precipitin tests were carried out only with crude antigens and convalescent sera, and showed complete crossing within the subgroups and no crossing between subgroups. Inhibition of hemagglutination by vaccinia was exhibited only by convalescent antisera against viruses of the vaccinia-variola subgroup. Hyperimmune antisera to vaccinia-LS and vaccinia-NP possessed no hemagglutinin-inhibitory capacity.

The results of neutralization tests carried out on the CAM (pock reduction) and on chick embryo fibroblast monolayers (plaque reduction) are shown in Table 2. Convalescent sera neutralized the viruses belonging to the appropriate subgroups, but no others.

Hyperimmune anti-vaccinia-LS serum had no neutralizing capacity, even for vaccinia virus, but the vaccinia anti-NP and myxoma anti-NP sera neutralized vaccinia and myxoma respectively. Neutralization tests thus showed no crossing outside the recognized subgroups.

Tests with fluorescent-antibody staining of cells infected with several poxviruses gave results analogous to those obtained in complement-fixation tests. The conjugated convalescent sera stain the viruses belonging to the appropriate subgroups, but no others (Table 3). Tests were carried out with the antisera to vaccinia-NP
## TABLE 2

Pock and plaque neutralization tests

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>Antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vaccinia</td>
</tr>
<tr>
<td></td>
<td>Pock a</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>-1.4 c</td>
</tr>
<tr>
<td>Cowpox-red</td>
<td>-2.7</td>
</tr>
<tr>
<td>Ectromelia</td>
<td>-2.2</td>
</tr>
<tr>
<td>Myxoma</td>
<td>-0.1</td>
</tr>
<tr>
<td>Fowlpox</td>
<td>0</td>
</tr>
</tbody>
</table>

* a Pock counts were read on the second day with vaccinia, on the third day with cowpox, ectromelia and myxoma, and on the fourth day with fowlpox virus.

* b All plaque counts (with the exception of myxoma) were made on the fifth day. Myxoma plaques were counted on the sixth day.

* c Figures express ratios of pock and plaque counts, obtained with normal sera and the antisera indicated, in log₁₀ units. Values of -1.0 or less (heavy type) indicate neutralization; values greater than -0.3 indicate no neutralization.

* d indicates test not done.
TABLE 3

Staining with fluorescent antibody

<table>
<thead>
<tr>
<th>Virus</th>
<th>Time of growth before fixation</th>
<th>Fluorescein-conjugated antibody</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vaccinia</td>
<td>Myxoma</td>
<td>Fowlpox</td>
<td>Influenza</td>
</tr>
<tr>
<td>KB cells infected with</td>
<td>10 hours</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>vaccinia-7N</td>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KB cells infected with</td>
<td>10 hours</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cowpox-red</td>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KB cells infected with</td>
<td>10 hours</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ectromelia</td>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KB cells infected with</td>
<td>24 hours</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>myxoma</td>
<td></td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CAM infected with fowlpox</td>
<td>4 days</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* + specific fluorescence in cytoplasm of infected cells
- no specific fluorescence
TABLE 4

Ring precipitin tests with 'NP' and soluble antigens

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Vaccinia</th>
<th>Myxoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NP</td>
<td>soluble</td>
</tr>
<tr>
<td>vaccinia</td>
<td>++++ a</td>
<td>+++</td>
</tr>
<tr>
<td>cowpox</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>ectromelia</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>myxoma</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>fibroma</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>fowlpox</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>monkey tumour</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>poxvirus</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>vaccinia anti-LS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>vaccinia anti-NP</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>myxoma anti-NP</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>influenza A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>normal sera</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>bovine papular</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>stomatitis</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a  ++++  +++ etc. indicate varying densities of precipitate
-
no precipitate
and myxoma-NP, but staining was relatively weak and was evident only with cells infected with the homologous virus.

The viruses used clearly fell into four subgroups. Vaccinia, cowpox, ectromelia and rabbitpox viruses cross-reacted in all tests. Myxoma and fibroma viruses cross-reacted with each other but not with the other viruses tested, while fowlpox and monkey tumor poxvirus reacted only with their homologous antisera.

The antisera produced against the 'NP' antigens of myxoma and vaccinia, however, reacted with both homologous and heterologous NP-antigens in the CF test. The relationships between the NP antigens and a variety of antisera were explored in greater detail by ring precipitin tests, the results of which are shown in Table 4. The soluble antigens reacted only with antisera to viruses of the appropriate subgroups. The 'NP' antigens of both vaccinia and myxoma, on the other hand, reacted with antisera to all the poxviruses, as well as with both myxoma anti-NP and vaccinia anti-NP antisera. Vaccinia anti-LS antiserum failed
to precipitate either 'NP' antigen, but produced strong precipitation of the vaccinia soluble antigen.

It was thought possible that the myxoma antiserum used by Takahashi et al. (1959a) might contain a high content of anti-NP antibody, and that this might partly explain their results. However, antiserum produced by multiple inoculations of UV-irradiated myxoma virus produced, in our hands, results entirely in accord with those obtained with myxoma-convalescent serum.

**Adsorption Tests with Antisera to 'NP antigens'**

The location of the LS antigen on the surface of the virus particle was conclusively demonstrated by the removal of antibodies reacting with this antigen after adsorption with purified viral particles (Craigie and Wishart, 1934). A similar procedure was used to determine the location of the group-reacting and the subgroup-specific components of the 'NP antigens'.

Antisera to myxoma-NP and vaccinia-NP were adsorbed with suspensions of virus particles (about $10^9$ particles of myxoma virus and $10^{11}$ particles of rabbitpox virus to 1.5 ml of undiluted unheated serum). The virus was added to serum, and the mixture incubated at 37° for 60 minutes, with stirring. The virus particles were then deposited by centrifugation at 15,000 rpm for 15 minutes, and the
TABLE 5

Adsorption of Antisera to Vaccinia-NP and Myxoma-NP

with Homologous Virus Particles

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Adsorbed with</th>
<th>Antigens</th>
<th>Neutralization Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vaccinia-NP</td>
<td>Myxoma-NP</td>
</tr>
<tr>
<td>vaccinia anti-NP</td>
<td>nil</td>
<td>+++^a</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>vaccinia</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>myxoma anti-NP</td>
<td>nil</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>myxoma</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

a density of precipitate in ring precipitin test
b results of pock neutralization tests, expressed as ratio of pock counts obtained in mixtures of virus and normal serum or adsorbed and non-adsorbed antisera, in log_{10} units
c test not done
adsorption repeated. After a second centrifugation residual
infective virus was destroyed by heating at 60° for 15 minutes.
The unadsorbed serum was heated in the same way. The sera
were then tested for precipitation with the vaccinia-NP and
myxoma-NP antigens, and for neutralization of the homologous
viruses on the chorioallantoic membrane, with the results shown in
Table 5. Adsorption with the homologous virus particles removed

Table 5 here

the neutralizing capacity of these sera, slightly reduced the density
of the precipitate obtained with the homologous 'NP antigen', and
had no effect on the density of the precipitate obtained with the
heterologous 'NP antigen'.

DISCUSSION

The results of complement-fixation, precipitation, hemagglu-
tinin-inhibition, neutralization and fluorescent antibody tests with
antisera produced in animals which had recovered from infection with
eight different poxviruses completely confirmed those of other investi-
gators who had reported the existence of serological crossing between
viruses of the vaccinia-variola, myxoma-fibroma and birdpox subgroups;
but not between subgroups, nor with certain currently ungrouped pox-
viruses. Even when myxoma antiserum produced in exactly the same way as that apparently used by Takahashi et al. (1959a) was used, we were unable to confirm their claim that there was a high degree of cross-reactivity between vaccinia, myxoma and fowlpox viruses, demonstrable by complement-fixation and fluorescent-antibody tests.

However, using as the antigens in ring precipitin tests the 'NP antigens' of vaccinia and myxoma viruses, produced by alkaline extraction of dried virus particles, serological crossing was observed between all the poxviruses examined. The 'NP antigen' comprises 50% of the mass of the virus and contains all its DNA (Smadel et al. 1942). There are probably several distinct proteins in this fraction, but the harsh method of extraction appears to destroy the antigenicity of all except a component which reacts in precipitin tests with convalescent antisera to a wide range of poxviruses, and a component which is responsible for the production of low titer homologous neutralizing capacity in antisera prepared to the 'NP antigens'. The demonstration of homologous agglutination of virus particles by such antisera (Smadel et al. 1942) and the existence of homologous neutralizing capacity in anti-'NP' sera which can be removed by adsorption with suspensions of homologous virus, indicates that the subgroup specific component of the 'NP antigen',
like the LS antigen, occurs on the surface of the virus particle.

The major antigen extracted from dried virus by treatment with N NaOH, however, is precipitated by antibodies to a wide range of poxviruses. Adsorption of antisera produced against these 'NP antigens' with purified virus failed to remove the precipitin, showing that the responsible antigen is not present on the surface of intact virus particles. We suggest that this group antigen is associated with the internal protein which Epstein (1958) and Peters (1960) have shown is associated with the viral DNA. Antisera to the myxoma 'NP antigen' should provide a highly specific tool for identifying the location of the group antigen in vaccinia virus particles, using the ferritin-coupling technique for electron microscopy recently applied to vaccinia virus by Morgan et al. (1961).

The group antigen has obvious significance in taxonomy, and the poxviruses may now be defined as large brick-shaped deoxy viruses (Cooper, 1961) which have a common group antigen. The presence of antibody to the group antigen in serum from cows which had recovered from bovine papular stomatitis (Snowden and French, 1961) confirms French's contention (French, to be published), based on cross-reactivation experiments, that this virus is a member of the poxvirus group. Antiserum to monkey tumor poxvirus also contains precipitating antibody to the group antigen, thus confirming
the suggestion of Niven et al. (1961) that this virus belongs to
the poxvirus group.

This internal protein may also be involved in the reactions
leading to non-genetic reactivation, since this probably involves
a protein rather than DNA (Joklik et al. 1960), and it is a general
reaction throughout the poxvirus group (Fenner et al. 1959).
REFERENCES


DISCUSSION - REVIEW

"REACTIVATION AND RECOMBINATION IN ANIMAL VIRUSES"
I. THE FIBROMA- MYXOMA "TRANSFORMATION"

Since its discovery by Berry and Dedrick in 1936, the phenomenon whereby active myxoma is recovered from rabbits injected with mixtures of heat-inactivated myxoma and active fibroma has been called "transformation". For reasons set out in the second, third and fourth papers of this thesis and again in the next section of this discussion-review, it is probable that the basic mechanism is reactivation of the heated myxoma rather than "transformation" in the sense that this term is used in bacterial genetics. In this section the term "transformation" will be used where it was used by the authors quoted.

Bacterial Transformation

One of the major advances in biology over the last few decades has been the identification of deoxyribonucleic acid (DNA) as the chemical substance concerned with the transfer of genetic information. This discovery stems from the experiments of Griffith (1928) on the transformation of pneumococcal types which set the pattern for all the subsequent work. While investigating the serological reaction of pneumococci, Griffith vaccinated mice with heat-killed, virulent type III organisms and later challenged them with a rough, avirulent culture of type II organisms. On their own neither the vaccinating nor the challenging
pneumococci had any effect on the mice, but together they produced a fatal disease and living, virulent type III pneumococci were recovered from the heart blood. These experiments provided the first example of the transformation of an avirulent bacterium into a highly virulent one of another serotype — the mouse providing a selective environment for the growth of the virulent organism.

The next important discovery came from Avery's laboratory at the Rockefeller Institute. Using the technique of Dawson and Sia (1931), of growing rough (R) pneumococci in a fluid medium containing anti-R serum and heat-killed, encapsulated smooth (S) cells, Avery and his colleagues were able to reproduce Griffith's phenomenon in vitro. This approach permitted a much more penetrating analysis of the basic mechanism of transformation. In experiments which are now classical Avery, MacLeod and McCarty (1944) identified the transforming principle as DNA. All subsequent work reviewed by Hotchkiss (1952, 1957) has confirmed this. Avery's group isolated a DNA fraction from type III pneumococci and found it capable of transforming, unencapsulated, rough variants of type II into fully-encapsulated type III strains. The DNA was isolated in a highly purified form and was able to induce transformation in exceedingly minute amounts. It was shown by various chemical, physicochemical and serological tests to contain no protein, lipid or polysaccharide. At first there was some criticism of the identifi-
cation of the transforming principle with protein-free DNA (Mirsky, 1947). However, Zamenhof et al. (1952, 1953) purified the transforming principles of Haemophilus influenzae and Hotchkiss (1952) that of Pneumococcus to the point where their protein nature could be excluded, and it was accepted that the transforming principle was DNA (Zamenhof, 1957).

Since then the transformation of pneumococci by DNA has become one of the most active fields in molecular genetics. Excellent reviews by Hotchkiss (1953-54, 1955, 1957), Ephrussi-Taylor (1955, 1958, 1960), and Zamenhof (1956, 1957) summarize progress up to the present time.

Similar transformations of other bacterial species such as Haemophilus influenzae, Escherichia coli, Shigella dysenteriae and Meningococcus occur and have been adequately reviewed by Austrian (1952), Ephrussi-Taylor (1955) and Ravin (1958). Recently Spizizen (1958) has described the phenomenon for Bacillus subtilis. The closely allied phenomenon of transduction has been described for Salmonella by Zinder and Lederberg (1952). Much of our present knowledge of the biological and physical properties of nucleic acids has stemmed from studies by Goodgal and Herriott (1957) and Zamenhof (1957) with H. influenzae; and from Lerman and Tolmach (1959) and Ephrussi-Taylor (1960) with Pneumococcus.
All available evidence shows that in bacterial transformation a fragment of biologically active DNA obtained from donor bacteria is incorporated, by a process akin to recombination, into the genome of the acceptor bacterium. These fragments are equivalent to bacterial genes (Colter, 1958) and it has been shown by Lerman and Tolmach (1957) and Fox (1957) using \( P^{32} \) that the number of bacterial cells transformed is directly proportional to the quantity of transforming DNA incorporated into cells of the acceptor strain.

The Fibroma-Myxoma Transformation

The "transformation" of viruses was first accomplished by Berry and Dedrick (1936a). These workers recognizing the close immunological relationship between fibroma and myxoma and noting the behaviour of the two viruses in laboratory rabbits, (in which fibroma is benign and myxoma highly virulent) concluded they might be different strains of the same virus. So stimulated by Griffith's studies (1928) with avirulent and virulent pneumococci, they attempted the analogous "transformation" of fibroma into myxoma. They succeeded in recovering active myxoma virus from rabbits injected with mixtures of heat-inactivated myxoma and active fibroma viruses. On analogy with Griffith's findings they called the heated myxoma virus, the "transforming agent" and the phenomenon the fibroma-myxoma virus "transformation". However,
Berry (1937a) also Gardner and Hyde (1942), realized that reactivation of the heat-inactivated myxoma virus, rather than "transformation" (in the Griffith's sense) of the active fibroma virus was a possible alternative mechanism. As will be shown in the next section, recent experiments in Australia (Fenner et al., 1959, Fenner and Woodroffe, 1960) and Japan (Hanafusa et al. 1959a, 1959b, 1959c) have shown unequivocally that this interpretation is the correct one.

Several different strains of fibroma virus (Shope's (1932) original A strain; Shope's B, C, D and E strains; Andrewes' (1936) mutant IA and OA strains; the "Cayuga" strain (Berry, 1937a) and the closely related squirrel fibroma (Kilham et al., 1953) have been "transformed", either in the presence of purified elementary body suspensions (Berry, 1937b; Gardner and Hyde, 1942; Smith, 1952) or the more usual tissue homogenate of myxoma virus. If the myxoma suspensions were inactivated for 30 minutes at 60° and 75° and 90°, successful "transformations" occurred with the 60° and 75° material only, not with the 90° material (Berry and Dedrick, 1936a). Considerable latitude in technique was consistent with the demonstration of the phenomenon and Berry (1937a) observed it when the active fibroma and heat-inactivated myxoma were injected separately into different sites of the rabbit.

The phenomenon, however, did not occur in fibroma immune rabbits (Berry and Dedrick, 1936b; Hurst, 1937). Also the replacement of either the heated myxoma or active fibroma virus by a variety of biological
materials such as other viruses, bacteria, gastric mucin, various animal sera and the subsequent injection of these mixtures into normal, hypersensitive, immunized or benzol-poisoned rabbits failed to induce the phenomenon (Berry, 1937a). So it was proved conclusively that rabbits developed infectious myxomatosis only when heat-inactivated myxoma suspensions and active fibroma virus were injected together.

Many times since its initial discovery, the fibroma-myxoma transformation has been confirmed by workers such as Hurst (1937), Gardner and Hyde (1942), Houlihan (1942) and Smith (1952). The results have always been irregular with myxomatosis developing sometimes in only 1/21 rabbits (Houlihan, 1942). Hurst (personal communication) had no difficulty in effecting "transformation" while working at the Lister Institute in London, but was unable to repeat these experiments later in Australia. Meek and Acree (1939) obtained no "transformations".

"Transformation" in Tissue Culture

More recently Kilham (1957, 1958) has shown the fibroma-myxoma transformation to occur in tissue culture and, in contrast to the findings in rabbits, experiments in tissue culture proceed in a regular and predictable fashion. This in vitro demonstration of the phenomenon parallels Avery's (1944) in vitro demonstration of the transformation of pneumococcal types, and such techniques certainly ensure greater control over
experimental conditions.

Kilham's "transformations" were carried out in cultures of rabbit, squirrel and monkey kidney tissues, a good growth of supporting cells being an essential prerequisite for successful "transformation". With such favourable conditions 100% efficiency of *in vitro* experiments was claimed. Kilham (1959) also firmly believed that many factors contributed to this increased efficiency of tissue cultures to act as a substrate for the "transformation" process, the most important being that the two reacting components — the heat-inactivated myxoma and live fibroma virus — had a greater chance of entering the same cells in the confined environment of a tissue culture system while, in the animal body, the injected particles may be dispersed by circulating body fluids and/or be removed by macrophages — the natural scavengers of the body. Therefore greater control over the input of viral components is possible using a tissue culture system. Also trypsinized kidney and testicular cells cultured on glass may become phagocytic in this habitat, and absorb the heated and active viral particles more readily.

However, if good growth of supporting cells is the main prerequisite one would expect the chorioallantoic membrane of the developing chick embryo to provide an ideal site for "transformation" since both fibroma and myxoma grow well in this tissue (Smith, 1948; Lush, 1937), but so far only negative findings on the chorioallantoic membrane have
been reported (Hoffstadt and Pilcher, 1941; Smith, 1952).

Kilham (1958) successfully "transformed" Shope's (1932) rabbit fibroma into myxoma virus by adding heat inactivated myxoma and live fibroma virus to cultures of rabbit kidney cells. The supernatant fluids from these cultures were tested at various intervals in rabbits. Usually only fibroma virus was recovered after 2-3 days incubation at 36°, but after 5-7 days all fluids tested contained myxoma virus and caused fatal, systemic infections in the rabbits. The appearance of myxoma virus in tissue culture was concomitant with a marked increase in virus titre from $10^{-2}$ to $10^{-5}$ and a noticeable, cytopathogenic effect on the cells.

The "transformation" worked equally well if the closely related squirrel fibroma (Kilham et al., 1953) replaced the rabbit fibroma virus. However, Kilham (1959, 1960) reported repeated failures when using vaccinia virus, another poxvirus in place of the live fibroma virus. Reactivation studies with poxviruses (Fenner et al., 1959; Hanafusa et al., 1959d), implying as they do the generality of this phenomenon, would refute this finding on theoretical grounds; but, in practice, overgrowth of a slow growing virus like myxoma by a rapidly growing vaccinial strain may preclude successful demonstration of "transformation". Certainly reactivation studies (Fenner and Woodroffe, 1960; Hanafusa et al., 1959d) show the reverse situation to be true i.e., that myxoma virus
reactivates heated vaccinia virus very efficiently.

A tissue culture system is an ideal environment for the study of the various properties of the main reactants of a "transforming" system and, as such, was fully exploited by Kilham's group. In particular they examined the heated myxoma component — the "transforming agent myxoma" or TAM, as they called it — and identified its active constituent as DNA (Kilham et al. 1958; Schack and Kilham, 1959). Their TAM was usually prepared from a concentrated suspension of ground myxoma tumours by heating for 12-40 minutes at 65⁰ or following exposure to ether at room temperature for a 1/2 to 2 1/2 hours. These preparations they called heat-TAM or ether-TAM respectively, and both were noninfectious and active in "transformation". The activity of the TAM particles was sedimented in the Spinco under conditions which sedimented active virus, therefore probably the TAM consisted of myxoma particles that had lost infectivity due to denaturation of their outer protein coat (Schack and Kilham, 1959). Both types of TAM were very stable being resistant to prolonged heating, to trypsin and to DNAse, but they were rapidly destroyed by light of certain wavelengths, ultraviolet light and by the photodynamic action of toluidine blue (Kilham, Lerner, Hiatt and Schack, 1958). All these agents have a specific poisoning effect on nucleic acids (Zamenhof, 1957).

Still thinking too rigidly in terms of bacterial transformations
mediated by DNA, and not visualizing simple reactivation of the heated component (with its DNA functionally intact) by some constituent of the active virus, Schack and Kilham (1959) directed considerable effort towards removing the outer protein coat of the heated myxoma virus to reveal an inner core of DNA. No doubt Smith (1952) had also thought along similar lines when she suggested that TAM was probably DNA — but her statement was never substantiated.

Mild digestion of the heated myxoma virus with urea exposed an inner core of DNA (Schack and Kilham, 1959). This urea-TAM, was simply prepared by dialyzing heated-TAM against 10 volumes of 10 M urea in buffered saline for 8 hours at room temperature (higher temperatures gave less consistent preparations), and then further dialysis at 4°C. Urea-TAM led to 100% "transformations" but, unlike heated TAM, was specifically destroyed by DNase, but not by RNase. This confirmed that urea-TAM consisted of particles of myxoma virus whose outer coats of protein had been denatured and partially removed thus exposing their inner core of DNA.

The urea-TAM or free myxoma DNA was not infectious by itself (Kilham, 1960) in contrast to other infectious DNA from T2 bacteriophage (Fraser et al. 1957; Spizizen, 1957) and polyoma virus (Di Mayorca et al. 1959; Smith et al. 1960); or infectious RNA from TMV (Fraenkel-Conrat, 1956; Gierer and Schramm, 1956), poliomyelitis (Colter et al. 1957a;
Alexander et al. (1958), MVE (Ada and Anderson, 1959), encephalomyocarditis virus (Huppert and Sanders, 1958), foot and mouth disease (Brown et al. 1958), Mengo (Colter et al. 1957b), West Nile (Colter et al. 1957a) and E.E.E. virus (Wecker and Schafer, 1957). To explain this, Kilham (1960) postulated that myxoma nucleic acid consisted of a loose structure of several molecules of DNA and, exposure to urea, affected the protein component and disrupted the structure sufficiently to destroy its capacity for replicating new virus by itself, but not enough to effect the role the DNA played in the "transformation" process.

Conclusions

Thus in summary we have the analogy between bacterial transformations, especially with pneumococci, stimulating investigations of what at first appeared to be its counterpart in viral "transformation", the so called fibroma-myxoma "transformation" or the Berry-Dedrick phenomenon. However, the mechanism of the transformation differs considerably in each instance and clearly differentiates the two phenomena.

In bacterial transformations there is a direct transfer of DNA to the recipient, and subsequently transformed, cells. The use of $^{32}$P-labelled DNA carrying the specific marker for streptomycin resistance has completely substantiated this fact by showing that incorporation of the tracer into the recipient cells is concomitant with genetic modification.
(Lerman and Tolmach, 1957; Fox, 1957). Further elucidation of the mechanism comes from Ephrussi-Taylor (1960).

On the other hand the fibroma-myxoma "transformation", while superficially resembling bacterial transformation, depends on an entirely different mechanism suggested as an alternative, but later discounted, by Berry (1937a), the discoverer of the phenomenon. He advanced the hypothesis that the active fibroma virus reactivates the heat-inactivated myxoma virus and that transformation, in the sense used in bacterial genetics, does not occur. But despite this suggestion all workers in the field for the past twenty-five years have accepted the idea of "transformation" per se.

Elucidation of the possible reactivation mechanism has not come from the fibroma-myxoma system, but has only recently emerged from studies with vaccinia virus (Fenner et al. 1959; Hanafusa et al. 1959a, 1959b), which will be reviewed in the following section.
II. REACTIVATION OF POXVIRUSES

The term reactivation has been applied to various phenomena in the field of virology concerned with the rescue of infectivity and/or genetic markers from viral particles previously exposed to various types of lethal damage.

Certain types of damage are reversible by light (photoreactivation), by certain chemicals (chemoreactivation) or by the intervention of some biological component (poxvirus reactivation). Since these reactivation processes do not involve recombination of any kind, they are classed as non-genetic in origin. With other types of lethal damage, reactivation of inviable viral particles only occurs under conditions of multiple infection (multiplicity reactivation) or by rescue of markers during mixed infection with active virus (cross reactivation). The latter types of reactivation involve genetic interaction between two or more viral particles within an infected cell, thus implying that the mechanism of reactivation is genetic in origin.

The majority of these reactivation processes were first observed during studies on the radiobiology of bacteriophage (Adams, 1959).

Photoreactivation

Photoreactivation (Adams, 1959; Stahl, 1959) is an example of
reactivation by a physical process in which damage caused by ultraviolet irradiation can be repaired by exposure to intense visible light. Dulbecco (1950) first described the phenomenon after observing that phage inactivated by ultraviolet can be reactivated, and made to produce plaques, if exposed to bright light. Photoreactivation however only occurs when the bacterium-phage complex is illuminated, and not with extracellularly illuminated phage or with bacteria illuminated before infection. The T2 phages show the greatest degree of photoreactivation. The phenomenon can best be interpreted in one of two ways, either the visible light performs some function which in the dark must be carried out by the phage particle, or the visible light erases a fraction of the ultraviolet hits in the phage population. Stahl (1959) maintains that the widespread occurrence of the event favours the second alternative and the experiments of Lennox, Luria and Benzer (1954) directly support the idea that the reactivation is a direct repair of ultraviolet damage. The phenomenon can occur with plant viruses (Bawden and Kleczkowski, 1952, 1959), but so far has not been demonstrated with an animal virus.

Chemoreactivation

Reactivation can be accomplished by chemical means. Krueger and Baldwin (1934) found that the inactivation of Staphylococcus phage K
by mercuric chloride could be reversed with hydrogen sulphide.

Kalmanson and Bronfenbrenner (1943) reported that incubation with
papain restored a coliphage, almost completely neutralized with anti­
body, to its original titre. A similar effect was obtained with sonic
vibration by Anderson and Doermann (1952) with phage T3 neutralized
with antibody. They concluded that the high frequency sonic vibra­
tions broke the bonds linking the antibody molecules to the phage
particles.

Multipliclity Reactivation

Multiplicity reactivation of ultraviolet-inactivated phage was
discovered by Luria (1947). He noticed that a bacterium infected with
a single UV-inactivated phage particle did not lyse or yield active phage.
However, when bacteria were infected with large numbers of UV-inactivated
phage particles so that two or more particles were absorbed to the same
host cell, the multiply infected bacteria lysed and yielded active phage
particles. Thus UV-irradiated phage particles which individually were
unable to replicate, could cooperate in multiple infections to produce
viable phage progeny.

Multiplicity reactivation occurs with phage T2, T4, T5 and
T6, is rarely detected with T1 and does not occur with T3 and T7
(Adams, 1959). The major damage caused by UV-irradiation involves
phage nucleic acid rather than phage protein (Bowen, 1953) and is therefore genetic damage. Repair of this damage in multiply infected cells by genetic recombination forms the basis of the various theories advanced by Luria (1947), Luria and Dulbecco (1949) and Barricelli (1956, 1960) to explain the mechanism and high efficiency of the reactivation process.

Phage preparations inactivated by X-ray irradiation (Watson, 1950; Weigle and Bertani, 1956; Harm, 1958) can be multiplicity reactivated to the same extent as UV-inactivated phages. The phenomenon also occurs with phage T2 inactivated by gamma-irradiation (Weigle and Bertani, 1956), following formalin-inactivation (Mustaars, 1957) but not after P32-inactivation (Stent and Fuerst, 1955). That multiplicity reactivation should occur following multiple infection with phages inactivated by such a variety of treatments all of which act in different ways, argues strongly against a direct chemical reversal of the damage and favours the recombination hypothesis to explain multiplicity reactivation.

The phenomenon of multiplicity reactivation occurs also with animal viruses and has been encountered by Henle and Liu (1951) and Barry (1961) with UV-irradiated influenza virus, and possibly with poliomyelitis virus (Drake, 1958). Abel (1961) consistently demonstrated multiplicity reactivation of UV-irradiated vaccinia virus in chick fibroblasts in experiments essentially similar in design to those of Luria
(1947) with the T-even phages, when he first observed the phenomenon. However she was unsuccessful, at first, in demonstrating multiplicity reactivation in KB cells which contain ten times the volume of chick fibroblasts. This she interpreted as due to a topographic factor, shown by Bresch (1959) to be of importance in phage replication. She argued that since Cairns (1960) had shown that multiple infection of cells with vaccinia virus resulted in the formation of discrete factories which merge later in the growth cycle, and since the effective cooperation necessary for multiplicity reactivation can occur only if the irradiated particles are close together, such a situation would arise more readily in small than large cells. This hypothesis received support when multiplicity reactivation was induced in the larger KB cells when they were infected with virus clumped with MgCl$_2$, to ensure that the irradiated particles were lodged in close proximity to each other within the cell.

**Cross Reactivation**

Like multiplicity reactivation, cross reactivation of UV-irradiated phage was originally described by Luria (1947). The process however involves the rescue of genetic markers from non-viable particles during mixed infection with viable particles and is more accurately described as "marker rescue". The work of Doermann, Chase and Stahl (1955) and Kreig (1959) with T4 phage provides good evidence that the irradiation
damage is located in the phage genome. Markers hit directly cannot be rescued but undamaged markers can be rescued by recombination with live phage particles.

Cross reactivation in the animal virus field was first observed by Burnet and Lind (1954) using heat-inactivated and active influenza A virus, and has since been confirmed by Baron and Jensen (1955) and Hirst and Gotlieb (1956) using virus inactivated by ultraviolet light. Subsequent work by Lind and Burnet (1957a) and Fraser (1959) leaves little doubt that marker rescue of inactivated influenza virus by closely related active virus results in the production of recombinant types, but not of virus identical with the inactivated parent.

Recently Abel (1961) demonstrated cross reactivation in vaccinia and, using chick fibroblast monolayers, followed the rescue of the $\mu^+$ marker from UV-irradiated, wild type rabbitpox by two of the $\mu$ mutants of rabbitpox, $\mu_1$ and $\mu_2$ (Gemmell and Fenner, 1960).

Poxvirus Reactivation

The first observation of reactivation of poxviruses was made by Berry and Dedrick (1936a) when they recovered active myxoma virus from rabbits injected with mixtures of heat-inactivated myxoma and active fibroma. This "transformation", as it was called, was repeatedly confirmed by other workers. It has also been discussed at some length in
However using this system, further analysis of the mechanism of the reaction was precluded by the irregular results, including the occurrence of "fibroma-myxoma" (Smith, 1952; Kilham, 1958) obtained with intact animals. With Kilham's (1957, 1958) demonstration that "transformation" could be effected in tissue culture in a predictable fashion, results were much more reproducible. But even with this technique an exact quantitative approach to the problem was impossible.

About this time Fenner (1958, 1959) commenced genetic studies using vaccinia, another poxvirus. With this system there was no difficulty in obtaining purified, high titre stocks of virus and in studying it quantitatively. Also the definition (Fenner, 1958) of at least five different marker characteristics for vaccinia suggested that it might be a more suitable agent for the study of the mechanism of the Berry-Dedrick phenomenon than the myxoma and fibroma viruses.

a. Reactivation of vaccinia strains. With the above idea in mind we attempted (Fenner et al. 1959; Joklik, Woodroofe, Holmes and Fenner, 1960) to demonstrate reactivation using strains of vaccinia virus. The strains originally selected for investigation were vaccinia-Lederle-7N (7N) and rabbitpox Utrecht (RP) as they were closely related serologically, but differed consistently in the morphology of pocks produced on the chorioallantoic membrane, haemagglutinin
production, heat resistance, lethality for mice and the production of skin lesions in rabbits (Fenner, 1958). For the above reasons also these two strains were used by Fenner and Comben (1958) to demonstrate recombination of poxviruses and, in this context, will be discussed in a subsequent section of this discussion-review.

The virulent strain, rabbitpox, was used as the heat-inactivated agent in the majority of reactivation experiments. For the preparation of heated-rabbitpox high titre virus purified by genetron extraction (Gessler et al. 1956) was inactivated with the minimal amount of heat to destroy infectivity. This was accomplished by heating freshly prepared suspensions at 60° for 12 minutes or at 55° for 2 1/2 hours.

A detailed investigation of the heat inactivation of vaccinia virus (Woodroofe, 1960) showed that the above procedure ensured exponential inactivation and avoided the 'tailing effect' reported at length by Kaplan (1958). He observed that thermal inactivation of vaccinia virus over the temperature range of 55° to 60° was not a simple first-order process and he interpreted this as indicating that the viral particles were heterogeneous in their heat sensitivity. The heat-sensitive fraction showed first order kinetics, but the heat-resistant fraction was inactivated at a constant slow rate over a period of hours. Judging by these results it would have been virtually
impossible for us to obtain minimally heated virus free of residual infectivity.

Occasionally we saw evidence of a heat-resistant, viral fraction and obtained results similar to those of Kaplan. However, this anomalous effect only appeared with virus suspensions stored in the refrigerator for several days before heating. Further experimentation showed that, during storage of concentrated suspensions of virus in the liquid state, some physical or chemical change occurred which greatly increased the heat stability of a small proportion of the viral particles.

On the other hand, freshly prepared virus when heat-inactivated always showed first order kinetics and no resistant fraction was ever found. Confirmation of these findings was reported by Hahon and Kozikowski (1961). While attempting to characterize the thermal inactivation of variola virus they noted a first-order reaction with virus stored at $-60^\circ$ and reported a two-component curve with virus stored at $4^\circ$.

Similar two-component inactivation curves were found during the heat-inactivation of poliovirus at $54^\circ$ by Koch (1960). In these experiments heating at $54^\circ$ denatured the protein coat of the viral particle but left the viral RNA intact. The 'tailing effect' was therefore due to the release of free RNA with low infective efficiency.
Subsequent treatment of the heated preparations with RNAse resulted in the complete loss of infectivity and first order inactivation curves resulted. In a similar vein, further experiments were carried out with the vaccinia system but the 'tailing effect' observed here with virus stored at 4°C could not be explained as due to free DNA (Woodroffe, unpublished results).

Before being used in reactivation experiments, the heated-rabbitpox preparations were checked for lack of infectivity by inoculation directly onto the chorioallantoic membrane and after passage of the membranes. This is the most sensitive test for the detection of any residual virus (Joklik, Woodroffe, Holmes and Fenner, 1960).

The reactivation of heat-killed vaccinia by active virus was first accomplished in mouse brain (Joklik, Woodroffe, Holmes and Fenner, 1960). Following the inoculation of heated RP and active 7N intracerebrally into mice, active virus indistinguishable from RP in all its genetic markers was recovered, as early as 18 hours after inoculation, from homogenates of brain tissue assayed on the CAM. A few clones of virus indistinguishable from the active parent (7N) were also recovered in the early stages of infection, and later highly virulent recombinant clones were isolated. This reactivation of heated-RP by active 7N in mouse brain was directly comparable to the fibroma-myxoma 'transformation' for living, virulent virus was recovered from an animal injected with a mixture of heat-inactivated virulent virus and an active, closely-related, attenuated strain —
the animal providing a selective environment for the growth of the virulent organism. However, the vaccinia system has the great advantage over the fibroma-myxoma system of permitting isolation of individual clones of virus by plating on the CAM with the subsequent recognition of parental and recombinant types.

Mouse brain is not the only environment in which reactivation of heated virus can occur, and the phenomenon has been successfully demonstrated in tissue culture using many different types of cells, such as HeLa, KB or chick fibroblasts which support the growth of both viruses. Reactivation also occurs directly on the chorioallantoic membrane.

To digress a little, this last technique has been used extensively in all our investigations, as the chorioallantoic membrane has the advantage of permitting the direct observation of reactivation without the necessity of passage. Following the simultaneous inoculation of about $10^7$ pfu of heated-rabbitpox and about 20-30 pfu of active 7N onto the chorioallantoic membrane, about half the pocks developing two days later were the $u^+$ type characteristic of reactivated, heated-rabbitpox, the other half were parental 7N or recombinants. From the direct platings, clones of virus were readily isolated and their marker characteristics identified with a minimal amount of effort. This simple, direct technique was therefore used very extensively
for demonstrating the reactivation of heated virus by all other poxviruses, and later to establish the generality of the phenomenon of poxvirus reactivation. This procedure facilitated the isolation and identification of the progeny virus from hybridization studies between several different poxviruses. It also formed the basis of direct assay methods for determining the reactivability of different batches of virus inactivated in various ways by exposure to heat, urea, nitrogen mustard and other alkylating agents.

Almost simultaneously with our first report (Fenner et al., 1959) of the reactivation of heat-inactivated poxviruses, a similar result was independently reported from Japan. Hanafusa et al. (1959a, 1959b, 1959c) reported the reactivation of heat-killed vaccinia by live ectromelia in cultures of L cells, HeLa, FL cells and on the chorioallantoic membrane. Like us they found reactivation occurred in any cells supporting the growth of both viruses. The live vaccinia virus, which appeared within 48 hours, was identified by its pathogenicity for mice and rabbits, its relative heat stability, its cytopathogenic effect in tissue culture and the size and appearance of its plaques.

b. The range of reactivating viruses. Following the initial demonstration of the reactivation of heated RP by active 7N, extensive
experiments (Fenner and Woodroofe, 1960), predominantly on the CAM and using a wide variety of poxviruses, were carried out to confirm the generality of the phenomenon. These established the fact that any heat-inactivated poxvirus can be reactivated by any other poxvirus if placed together in an environment where both can multiply. Thus the reactivation of heated-rabbitpox, heated-vaccinia and heated-cowpox was induced by single-step mutants (Gemmell and Fenner, 1960), by other mammalian poxviruses and by the more distant members of the poxgroup such as myxoma, fibroma, fowlpox and contagious pustular dermatitis virus. However, the same heated preparations were not reactivated by active agents belonging to other groups such as rickettsia, psittacosis virus, infectious laryngotracheitis virus, herpes simplex virus, influenza virus, Rous sarcoma virus or Murray Valley encephalitis virus. These results were independently confirmed by Hanafusa et al. (1959d) using the unrelated viruses influenza NWS and HVJZ, herpes, measles and poliomyelitis viruses. Thus reactivation was established as a general phenomenon confined to the poxvirus group.

In the course of some of the above reactivation experiments recombinants were obtained with great regularity (Woodroofe and Fenner, 1960), but discussion of this aspect of the work will be dealt with in the next chapter.
c. The mechanism of poxvirus reactivation. The successful demonstration of reactivation with so many different combinations of poxviruses and the observation that the phenomenon was confined to the poxvirus group led to the next important question: how is reactivation accomplished? Is it a consequence of genetic interaction or is it due to some non-genetic mechanism?

The fact that reactivation was confined to the poxgroup automatically suggested that it was a genetic phenomenon and that genetic homology of the reactants was essential. In keeping with this idea was the implication (Burnet, 1959b, 1960) of some sort of genetic interaction to explain the fibroma-myxoma transformation.

On the other hand, the analysis of reactivated pocks produced from mixtures of heated-rabbitpox and active viruses as diverse as myxoma and fowlpox, invariably yielded virus indistinguishable from the heated-component, rabbitpox, in all the characters tested. No marker of the reactivating agent was ever found. This implied that reactivation was not due to recombination but was accomplished by some non-genetic mechanism, which simply repaired the damage sustained by the heated virus.

Joklik, Abel and Holmes (1960) produced experimental proof that the reactivation of heat-inactivated poxviruses was by a non-genetic mechanism and possibly due to protein. This concept was
a complete volte face from the historical idea of "transformation" usually evoked to explain the Berry-Dedrick phenomenon. The recovery of clones of virus from reactivation experiments having all the known genetic markers of the heat-inactivated agent suggested that inactivation by heat does not destroy the DNA of the viral particles. The studies of Marmur and Doty (1959) with DNA derived from many different sources also confirm the relative heat stability of DNA. On the other hand, proteins are usually less stable than DNA to heat, so it seemed most likely that the active virus supplied an essential protein which had been destroyed in the heated component. To test this hypothesis, Abel (personal communication) suggested that virus whose DNA was selectively inactivated by ultraviolet-irradiation might replace active virus as the reactivating agent, in which case the genotype of the heat-inactivated virus only should replicate. In practice however, reactivation was inconsistent (Fenner and Holmes, personal communication) probably because ultraviolet light is not a specific DNA poison but partially inactivates the protein as well (Sinsheimer, 1955). Hanafusa (1960) obtained no evidence of reactivation using ultraviolet-inactivated virus in place of active virus.

In an elegant series of experiments, Joklik, Abel and Holmes (1960) used nitrogen mustard, di(2-choroethyl)methylamine
to inactivate DNA and found that RP or 7N inactivated with this agent could reactivate heated-7N or heated-RP, and the progeny virus retained all the genetic markers of the heated parent. Nitrogen mustard was a good choice of agent to inactivate DNA specifically because, although the mustards can combine with protein, they tend to react preferentially with nucleic acids. For this reason, too, they have been widely used in phage work (Loveless and Stock, 1959a, 1959b) and in studying the inactivation of the transforming principle of bacteria (Herriott, 1948; Zamenhof, Leidy, Hahn and Alexander, 1956). Holmes (1961) in an extensive series of experiments also found nitrogen mustard much superior to other alkylating agents such as the ethyleneimines, B-propiolactone and epoxides for the specific inactivation of DNA. His preparations treated with nitrogen mustard still retained a high reactivating efficiency, despite lack of infectivity.

More sophisticated proof that during reactivation the reactivating virus provides some essential non-genetic material lacking in the heated virus came from experiments (Joklik, Abel and Holmes, 1960) using the closely related, white mutants of rabbitpox (Gemmell and Fenner, 1960) RPu1 and RPu8 as heated parents and the wild type (RPu+) as the mustard-treated parent. Use of these single-step mutants provided the most sensitive method available for detecting
whether the genome of the mustard-treated parent was completely absent from the progeny. Gemmell and Cairns (1959) showed that these \( u \) mutants, with their characteristic white pock, differ only at a single genetic site from the wild type (\( RPu^+ \)). On the chorioallantoic membrane, the wild type, with its typical red pock, will outgrow both mutants (Gemmell and Fenner, 1960); therefore, if used together, such a system should detect the \( u^+ \) marker if it survives. However, when either heated-\( RPu_1 \) or heated-\( RPu_8 \) were reactivated with mustard-treated \( RPu^+ \) on the chorioallantoic membrane, the \( u^+ \) marker failed to appear and all the progeny virus resembled the heated parent. The production of active virus identical with the heated component from mixtures of two inert particles, one with its protein coat destroyed by heat and the other with its DNA destroyed by nitrogen-mustard, supports the view that the reactivating virus provides some essential non-genetic material, possibly protein, which is lacking in heated virus.

The Japanese workers (Hanafusa et al. 1959c, 1959d; Hanafusa, 1960) also suggested that reactivation of poxviruses was not accomplished by genetic interaction but was brought about by a non-genetic mechanism. They visualized the active virus supplying some protein component which the inactive component had lost during heat inactivation. Like us, they considered the possibility that the
agent capable of reactivating heated virus need not always be active virus. To prove this point, Hanafusa (1960) unsuccessfully attempted to reactivate heated-vaccinia virus with UV-irradiated ectromelia virus. However, he maintained that these results did not exclude the possibility that some agent other than active virus might be capable of reactivating heat-inactivated virus.

The effects of various physical, chemical and enzymic treatments on the heated virus was studied by Joklik, Holmes and Briggs (1960) and Hanafusa (1960) in order to obtain some information about the specific groupings present in the heated or reactivable component. Joklik, Holmes and Briggs (1960) showed that heated virus was relatively heat stable and could be heated at 55° for more than 7 hours before the capacity for reactivation was markedly reduced but this was destroyed in a few minutes at 60°. The capacity for reactivation was also unaffected by enzymic digestion with trypsin and DNAse at pH 11, pepsin at pH 3.5 and papain at pH 7.5, despite the fact that electron micrographs of some of these particles showed considerable morphological changes associated with the removal of a large part of the outer region of the virus. While mild protein denaturants such as heat and urea were very efficient in converting virus to the reactivable form, more severe protein denaturing agents such as sodium dodecyl sulphate completely destroyed reac-
tivability. Thus heated but reactivable virus is probably in a form in which the protein necessary for infectivity has been de-natured, but the virus probably still contains some unaltered protein constituents as well as its functionally intact DNA. During reactivation it is the protein associated with infectivity which is supplied either by active virus or by virus in which only the DNA has been inactivated. Hanafusa's (1960) experiments with various enzyme treatments and ultraviolet irradiation also confirmed that the viral genome is unchanged by heat-inactivation and it is mainly the protein portion that is damaged.

d. Use of the term 'reactivation'. The previous discussion leaves little doubt that reactivation of heat-killed virus by active virus is a general phenomenon so far only demonstrable with pox-viruses (Fenner and Woodroffe, 1960). It also implies that the fibromamyxoma "transformation", which was the first example of the event, is a special case of this general phenomenon amongst poxviruses and not merely due to a specific interaction between the two viruses. Despite Kilham's (1960) retention of the term "transformation" on historical grounds we maintain, and the Japanese workers (Hanafusa, 1960) now agree, that the term reactivation describes the phenomenon more accurately. Complete reproduction of the genome of the heated virus can be brought about by a diverse array of active viruses.
Therefore it is more plausible and simpler to regard this as the reactivation of the heated virus by the active agent, rather than "transformation" of each of the different active viruses in turn into something indistinguishable from the heated component.

The demonstration of the reactivation of heated virus by mustard-treated virus (Joklik, Abel and Holmes 1960) would also support the view that the phenomenon is one of reactivation of the heated component rather than "transformation" as used by the bacterial geneticists.

e. A component of the 'NP antigen' common to all members of the poxvirus group. It is interesting to speculate about the reason for the sharp cleavage between members of the poxvirus group which are capable of reactivating heated virus and the many other strains of virus incapable of participating in the event. Is it possible that all the poxviruses possess some factor in common, perhaps an antigenic component, which may be the operative component in reactivation?

A possible correlation between serological crossing and reactivation was inferred by Hanafusa et al. (1959a, 1959b) following the report of Takahashi et al. (1959) that the poxviruses capable of reactivating heat-killed vaccinia virus were antigenically related and strong cross-reactions were demonstrable by complement fixation tests and fluorescent antibody staining. However, it is generally
believed (Downie and Dumbell, 1956; Fenner and Burnet, 1957; Webster, 1958; Mayr, 1959) that, while there are several subgroups of serologically related viruses within the poxvirus group, there are no cross reactions between the different subgroups.

The last paper presented in this thesis (Woodroffe and Fenner, 1961) attempts to clarify the discrepancy in these findings and to point to a common component which may be responsible for initiating reactivation. A comprehensive series of immunological tests including complement fixation, gel-diffusion precipitation, haemagglutinin-inhibition, plaque and egg neutralizations and fluorescent antibody staining were carried out. Since these tests were primarily concerned with the reactions of the soluble and surface antigens of the various viruses, they demonstrated the usual, and accepted, cross reactions between vaccinia, cowpox and ectromelia, and those between myxoma and fibroma, but indicated no cross-reactions between the different subgroups. These findings did not confirm the experimental results of Takahashi et al. (1959). However, electron micrographs of poxviruses (Morgan et al., 1954; Flewett, 1956; Epstein, 1958; Peters, 1960) show a complex structure which would imply the possession of many different antigens, some being surface components others being more deepseated, possibly nucleoprotein in origin. By following the method of Smadel et al.
(1942) the nucleoprotein or NP antigen was extracted from both vaccinia and myxoma viruses and was shown to cross react in ring precipitin tests with all available poxvirus antisera. Thus, an antigen known for a considerable time was shown to be common to all members of the poxvirus group. The possession of this common component is probably an important prerequisite determining the success of the reactivation of heated virus by all other members of the poxvirus group.

f. Reactivation of azide-inhibited virus. Recently the reactivation of vaccinia virus inactivated in the presence of sodium azide by active, closely-related strains of the vaccinia-virola group was reported by Easterbrook (1961b). He showed that superinfection with RPU+ of KB cells infected with Vaccinia-Mill Hill, and incubated with azide, resulted in the 'rescue' of all the azide-blocked virus. Using the more distant myxoma virus 'rescue' of the azide-blocked virus did not occur. Since 'rescue' was only possible by closely-related strains, the mechanism of the reactivation process was probably comparable to marker-rescue by recombination. He also noted that the addition of heat-inactivated virus to cells containing azide-blocked virus resulted in reactivation of the heated virus and the live virus in turn rescued the azide-blocked virus.
g. Taxonomic considerations. In conclusion, it might be pertinent to point out how the reactivation of heated virus may be used as a taxonomic tool. All the work reviewed in this section supports the view that all poxviruses participate in reactivation and, on this criterion and on others outlined by Fenner and Burnet (1957), they can be regarded as members of one large group of viruses. The reactivation criterion could be extended to other viruses described as poxviruses on morphological grounds, e.g. monkey tumour poxvirus, molluscum contagiosum, and the various animal and bird 'poxviruses'. Recently Snowden and French (1961) isolated a virus from Australian cattle suffering from a papular stomatitis. It was subsequently identified unequivocally as a poxvirus, using the reactivation technique on the chorioallantoic membrane (French, personal communication). In our hands (Woodroffe and Fenner, 1961) convalescent sera from the infected animals reacted strongly with extracts of NP antigen prepared from vaccinia and myxoma viruses. This confirmed the presence of the poxvirus group antigen and completed the identification of the virus as a member of the poxvirus group.
III. RECOMBINATION OF POXVIRUSES

The demonstration that both bacteria (reviews by Hartman and Goodgal, 1959; Ravin, 1960) and bacterial viruses (reviewed by Bresch, 1959 and Levinthal, 1959) could exchange genetic material has stimulated similar work in the field of animal virology.

Technical Prerequisites for Genetic Studies

An effective study of genetic recombination between animal viruses is dependent upon certain important technical developments outlined by Burnet (1960). Firstly, it is essential to work with pure clones of virus. This prerequisite is readily fulfilled when working with viruses such as poxviruses and herpes. These produce discrete, focal lesions or pocks when inoculated, according to the method of Lush (1937), onto the chorioallantoic membrane of the developing chick embryo. Since a linear relationship exists between the dilution of virus inoculated and the number of lesions produced (Beveridge and Burnet, 1946), it is a relatively simple matter to inoculate a number of eggs with a dilution of virus calculated to yield one pock, and then isolate pure clones of virus by subinoculation from the single pocks that develop (Fenner and Comben, 1958). A less exacting method (Fenner, 1959), used with membranes that contain several
pocks, is to cut out a well-isolated one and grind it in a small glass grinder. Alternatively, the pock may be pricked with a sterile needle and the cells dislodged into a small amount of diluent. All techniques yield pure clones of virus.

The isolation of individual pocks has its counterpart in the technique of Dulbecco (1952) for plaque production by animal viruses on a monolayer of cells in tissue culture. Here subinoculation from a well-isolated plaque provides a source of pure clone virus, especially since Dulbecco and Vogt (1954) have shown that each plaque is produced by a single virus particle. This method has been used successfully for the isolation of pure clones of vaccinia (Noyes, 1953), poliomyelitis (Dulbecco and Vogt, 1954), herpes (Kaplan, 1957) and meningopneumonitis virus (Higashi and Tamura, 1960).

With viruses such as influenza and psittacosis, which produce neither pocks nor plaques, pure clones of virus are obtained by the more tedious method of transfer at limit infecting dilution (Burnet and Bull, 1943). Here, three successive isolations from limit dilution virus with less than 10% infectivity yields a population which can be regarded as a pure clone of virus (Burnet, 1957; 1959a).

Secondly, for effective work on virus genetics, strains of virus must be available which consistently differ from each other in several independent marker characteristics. Definitive markers may
distinguish differences in viral morphology, virulence, antigenic structure, production of enzymes and haemagglutinin, resistance to heat and/or various drugs (Burnet, 1959b). If such markers are applicable to large scale screening tests it is advantageous. Well established markers exist for strains of influenza virus (Burnet and Lind, 1952), vaccinia, (Fenner, 1958), herpes (Wildy, 1955) and psittacosis virus (Gordon et al. 1957).

The last prerequisite for an adequate study of genetic recombination demands a cell system which will support the growth of the two parental virus strains. The progeny virus resulting from genetic interaction should be recoverable as pure clones of virus from the mixedly infected cells. Analysis of the yield can be accelerated if selective methods are available to differentiate any minority components of the viral population. This can be done by the use of appropriate antisera (Burnet and Lind, 1951b), by preferential heating to destroy a heat-sensitive component (Woodroffe and Fenner, 1960), or by the inoculation of a particular host which is susceptible to the virus in question (Burnet and Lind, 1951a; Joklik, Woodroffe, Holmes and Fenner, 1960).

All the above requirements were fulfilled for bacterial viruses by 1946 when genetic recombination between different strains of T2 phage was simultaneously announced by Hershey and by Delbruck
and Bailey. However, a similar approach to animal viruses came considerably later but, by 1958, genetic recombination had been successfully demonstrated with influenza viruses, poxviruses, herpes and psittacosis. Details of each animal virus system will be discussed in the appropriate sections of this review.

**Marker Characteristics of Vaccinia Virus**

To provide a basis for genetic studies with the mammalian poxviruses, Fenner (1958) made a detailed investigation of the biological characteristics of 24 different strains of vaccinia, cowpox and rabbitpox viruses. He found that reproducible differences could be demonstrated in respect to six different marker characteristics. These included differences in the viral content and the morphology of the pocks produced on the chorioallantoic membrane. Pock types varied from the opaque-white pock of the dermal vaccinia through the transparent red of rabbitpox to the intense haemorrhagic lesions of cowpox. Differences in haemagglutinin production were marked between strains of rabbitpox which failed to agglutinate susceptible fowl cells and most of the vaccinia strains which produced large amounts of haemagglutinin. The cowpox strains were intermediate in haemagglutinin yield. Differences also occurred in heat stability. Most strains were consistently heat resistant, the titre dropping only ten to fifty-fold after heating for 40 minutes at 55°.
However, Vaccinia-Lederle-7N proved exceptionally heat-sensitive and the titre fell 100,000 fold with the same heat treatment. Differences were demonstrated in virulence on intra-cerebral inoculation in mice and intra-dermal inoculation in rabbits. In mice, following the intra-cerebral inoculation of $10^5$ pfu, the majority of dermal vaccinia and cowpox strains caused few deaths, while rabbitpox and certain neuro-vaccinia strains were highly lethal. The skin lesions in rabbits receiving comparable doses of virus varied from a small, raised nodule characteristic of dermal vaccinia to the large, indurated lesions with a haemorrhagic, necrotic centre produced by rabbitpox, cowpox and some strains of neuro-vaccinia.

**Genetic Studies with Vaccinia Strains**

Several pairs of strains with different characters could be selected from among the twenty-four tested for the demonstration of genetic recombination in the poxgroup. From among these strains Fenner and Comben (1958) selected two for further investigation — a dermal strain of vaccinia, Vaccinia-Lederle-7N (7N) and the Utrecht strain of rabbitpox (RP). These strains differ in pock character (from the opaque-white of 7N to the transparent red of RP), in haemagglutinin production (7N producing large amounts, RP none), in heat stability (7N being markedly heat-sensitive; RP heat-resistant)
and in virulence on intra-cerebral inoculation in mice and intra-dermal inoculation in rabbits (7N non-pathogenic in both hosts, RP being fatal for mice and producing spreading, necrotic lesions in rabbit skin).

Before experiments on genetic interaction were undertaken, extensive experiments (Fenner and Comben, 1958) with thirty-three clones of 7N and nineteen of rabbitpox established that the two strains were homogeneous for all five markers and that the markers were stable on passage.

Four methods of allowing genetic interaction to occur between RP and 7N were investigated — two involved mixed infection of cells on the chorioallantoic membrane and two the use of HeLa cells in mass culture or as single cells (Fenner and Comben, 1958; Fenner, 1959). In the first, the chorioallantoic membrane was infected with a mixture containing large amounts of the two viruses. The newly formed virus of the first cycle yield was obtained by reaping the chorioallantoic membrane 12 hours later. The chorioallantoic membrane was washed, ground and titrated. Single pocks were selected from membranes with only one pock present and were tested for the various marker characteristics. After 12 hours of mixed growth 2/81 recombinants were isolated. Reaping the products of double infection at 24 hours, with more than one cycle of growth,
yielded 4/31 recombinants. In the second, the chorioallantoic membrane was inoculated with 20-40 pfu of each strain and 2 days later the exact areas of overlapping pocks of different type were excised and their virus content analysed. This method while involving an unknown number of cycles of multiplication yielded a high proportion (6/21) of recombinant clones. In the third, large amounts of both viruses were inoculated onto HeLa cell monolayers, 8 hours was allowed for absorption and the products of double infection were harvested at 18 hours and yielded 5/16 recombinant clones. In the fourth, the virus content of 33 single HeLa cells mixedly infected with a very high multiplicity of the two viruses, was analysed 36 hours after infection when yields per cell were maximal. A variety of phenotypes were obtained. From one cell in which the total yield of 132 clones was examined, 20 different recombinant types occurred, together with both parental types.

These experiments showed unequivocally that, in mixed infections with poxviruses, genetic recombination occurred. From all four methods permitting genetic interaction between 7N and RP, the two parental types were recovered with great regularity, together with a variety of recombinant types. Amongst the latter there was a predominance of white, pock-producing strains which showed a variety of combinations of the other parental characters. Recombinant strains
producing red pocks were in a vast minority (5/474) and were only isolated from mixedly-infected, single HeLa cells. Haemagglutinin production and heat resistance appeared to be all or none characters and no intermediates were found amongst the recombinants. The reaction of the recombinants in mice and rabbits resembled those of the less virulent parent or were intermediate. No correlation was evident between changes in mouse and rabbit virulence. Some instability was noticed on passage of some of the recombinant clones, a few showing variability of one marker such as haemagglutinin production, heat resistance or pock morphology from the original single pock isolates.

Recombination in the poxvirus group was not an uncommon event as judged by the recovery of 123/474 recombinant clones from the above mixed-infection experiments with 7N and RP. Likewise the finding is in agreement with Adams (1959) who states "the occurrence of genetic recombination implies that the two infecting strains are similar enough in genetic constitution that genetic determinants may be exchanged between them". Further experiments also bear out this statement and are concerned with hybridization between several different poxviruses (Woodroofe and Fenner, 1960).
Hybridization between Different Poxviruses

As stated in the previous section of this review, in the course of certain experiments on reactivation of heat-inactivated poxviruses, recombinants were recovered as well as the phenotypes of the reactivating and reactivated viruses. However, it was soon evident that while the reactivation of, say, heated-rabbitpox was accomplished equally well by all strains of the closely related vaccinia-varioila group or by more distant unrelated poxviruses, it was only in the first instance with the closely related strains that recombinants were isolated. So it was of interest to clarify the conditions under which viable hybrids might arise from interaction between different poxviruses.

Reactivation experiments between heated-rabbitpox and 7N whether carried out in mouse brain, on the chorioallantoic membrane or in HeLa cells, yielded a rich crop of recombinants. In the characters exchanged, these recombinants were comparable to those recovered from experiments with active RP and 7N (Fenner, 1959). However, with one inactive component, red pock-producing recombinant strains were no longer a rarity and occurred with high frequency.

Hybridization (or genetic exchange between different species of poxviruses) was demonstrated between cowpox and rabbitpox viruses in reactivation experiments on the chorioallantoic membrane, and in
mouse brain using heated-rabbitpox and a readily distinguished white mutant of cowpox. The characters exchanged included haemagglutinin production, heat resistance and virulence markers.

With greater difficulty viable hybrids were isolated from mixtures of heated-rabbitpox or heated-cowpox and ectromelia inoculated onto the chorioallantoic membrane and into mouse brain. These experiments established the fact that viable hybrids can be produced from cells mixedly infected with several different members of the vaccinia-variola group.

On the other hand, myxoma and fibroma viruses will reactivate heat-inactivated viruses of the vaccinia-variola group very efficiently, but such combinations of poxviruses failed to yield viable hybrids. However, myxoma-fibroma crosses yield viable hybrids if full weight can be placed on the use of the complex virulence marker.

These experiments showed conclusively that hybridization between two varieties of vaccinia (RP and 7N) is a common occurrence, between rabbitpox or cowpox and ectromelia rare, and between vaccinia and myxoma exceedingly rare, if possible. They also substantiate Adam's (1959) view concerning a similar genetic constitution as the main prerequisite for genetic recombination.
Genetic Studies with the White (u) Mutants of Rabbitpox

All the previous work has demonstrated conclusively that recombination can take place between poxviruses but the approach to the problem as a genetic one was purely qualitative. An exact quantitative approach to the problem of poxvirus genetics may be possible using the white (u) mutants of rabbitpox (Fenner, 1958) or cowpox (Downie and Haddock, 1952), which produce characteristic white pocks on the chorioallantoic membrane.

Such a possibility follows the demonstration by Gemmell and Cairns (1959) and Gemmell and Fenner (1960) that certain combinations of u mutants of rabbitpox recombine to produce wild type (u+)
progeny. These results were analogous to those obtained with different r mutants of coliphage T2 (Hershey and Rotman, 1948) and suggested that, in vaccinia, mutations to white pock production might occur at a number of different loci.

Using 18 different RP u mutants pairwise crosses were set up in HeLa cells and, after 24 hours, the yields were examined on the chorioallantoic membrane for the presence or absence of wild type recombinants, producing ulcerated haemorrhagic u+ type pocks. On the basis of the presence or absence of u+ recombinants in the various crosses, the 18 u_ mutants were arranged in a linear, ordered manner. It was established that the pock morphology of rabbitpox was controlled
by a number of different genes located at different sites on the genome, and that the \( u \) mutants arose following mutation at these loci. By using Benzer's (1959) recombination matrix, the 18 \( u \) mutants of rabbitpox were allotted to four groups. In the matrix several pairs of mutants were indistinguishable, but the biological characteristics of the strains (Gemmell and Fenner, 1960) readily differentiated them even if recombination failed to do so. Thus all 18 \( u \) mutants were different.

Such a system looked ideal for the calculation of recombination frequencies but inherent difficulties made this impossible. Gemmell and Cairns (1959) reported considerable egg to egg variation which prevented the accurate scoring of the relative number of \( u \) and \( u^+ \) pocks from the various crosses. Likewise plaque morphology on chick fibroblast monolayers, while reducing variability in the actual count, failed to distinguish between most of the \( u \) mutants and \( RPU^+ \). Evidence of intracellular selection during mixed growth of two \( u \) mutants was also apparent (Gemmell and Fenner, 1960). This could effect the yields of \( u^+ \) and \( u \) virus and provided another difficulty.

**Genetic Studies with the White (\( u \)) Mutants of Cowpox**

Failure to calculate recombination frequencies from the rabbitpox data led to the investigation of the \( u \) mutants of cowpox
(Fenner, Woodroofe and Greenland, to be published). A genetic analysis of this system was thought, at first, to be entirely comparable with that of rabbitpox except for considerable advantages which would permit quantitative assessment of the data. In preliminary experiments Fenner had noted excellent differentiation between the plaque type of $u$ and $u^+$ mutants of cowpox on chick fibroblast monolayers. This, allied with very distinctive pock morphology of $u$ and $u^+$ pocks on the chorioallantoic membrane, suggested an overall advantage of the cowpox system for the calculation of recombination frequencies. But extensive preliminary experiments (in HeLa cells, on the chorioallantoic membrane, in mouse brain and in experiments using reactivation on the chorioallantoic membrane) with nine independently isolated $u$ mutants of cowpox showed that, under no circumstances, would the various pairwise crosses produce wild type ($u^+$) progeny. Utilizing the hybridization technique between cowpox and rabbitpox described by Woodroofe and Fenner (1960), Greenland (to be published) carried out pairwise crosses between the cowpox $u$ mutants and selected $u$ mutants of rabbitpox with positive results. With this approach she hopes to analyse the data in terms of the known linkage groups of rabbitpox. If successful, this should lead towards the development of a more precise approach to the genetics of poxviruses.
IV. RECOMBINATION IN OTHER GROUPS

"Recombination will be found wherever it is adequately looked for in the larger viruses" states Burnet (1959a). This belief has been well substantiated by the demonstration of genetic inter-change among poxviruses, and between different strains of influenza virus, herpes and psittacosis. So far it has not been established that true recombination can occur with the smaller viruses such as the enteroviruses. However, interaction as evidenced by phenotypic mixing has been reported frequently.

1. MYXOVIRUSES

Influenza

Burnet and Lind in 1949 were the first to demonstrate recombination between animal viruses. They used two related type A influenza strains. Since that time active research groups in Australia, England and America have added greatly to our knowledge of influenza virus genetics, as evidenced by the extensive literature on the subject. In a review such as this, however, it is virtually impossible to cover all facets of such a broad subject, so this discussion will be limited to the major advances in the field of influenza genetics as outlined in recent reviews by Burnet (1959a, 1959b, 1960), by Hirst, Gotlieb
and Granoff (1957) and by Hirst (1959).

Recombination between influenza strains was initially observed in interference experiments (Burnet and Lind, 1951a) involving mixed infection of mouse brain with related type A strains, the neurotropic NWS and non-neurotropic WSM. Extracts of the mouse brains, harvested several days later yielded variant types of virus, neuro-WSM strains. These were stable on limit dilution passage and the new property of neuropathogenicity for mice was presumably derived from NWS. These findings were analogous to those of Delbrück and Bailey (1946) and Hershey (1946a, 1946b) with related strains of bacteriophage, and those of Tatum and Lederberg (1947) with coliform bacteria. They strongly suggested that the neuro-WSM strains arose through genetic interaction of the two parental strains.

This was the starting point for all further work on recombination in influenza viruses, which was studied consistently in Burnet’s laboratory at the Walter and Eliza Hall Institute from 1949-1956. Substantial support for the recombination theory came from similar experiments (Burnet and Lind, 1951b; Burnet and Edney, 1951) undertaken with neurotropic NWS and MEL. Later MEL was replaced by two other serologically distinct, non-neurotropic A strains — Swine 15 and an A prime strain from Ocean Island. In all cases
stable, neurotropic, variant strains were isolated with the serological character of the non-neurotropic parent. However, these experiments were all carried out in mouse brain, which could conceivably select out neuropathogenic mutants. To exclude this possibility, the same strains, NWS and MEL, were set up in a "neutral host", either in allantoic cells of intact embryos or de-embryonated eggs. As previously, similar neuro-MEL or N-M strains were isolated (Fraser and Burnet, 1952). The reciprocal recombinant, a non-neurotropic NWS, was not obtained in these experiments but later Fraser (1955) isolated it from the brains of day-old mice infected with mixtures of NWS and MEL. All these findings were convincing proof of recombination.

All further work in Burnet's laboratory on recombination with influenza virus was carried out in chick embryos. Using this technique the NWS strain proved unsuitable and was replaced by the embryo pathogenic strain, WSE. A great amount of work was done with the readily distinguished MEL and WSE strains, and eventually the concept of two linkage groups in the MEL/WSE system was developed (Burnet and Lind, 1952).

a. Interchange of linkage groups. The recombination experiments between MEL and WSE resulted in the harvest of four different types of virus during the first cycle of multiplication in
doubly-infected, allantoic cells. Two types were identical with parental MEL and WSE and the other two were the reciprocal recombines, subsequently called M+ and WS-. Back recombination experiments using the stable M+ and WS- strains produced virus with the characteristics of the original MEL and WSE strains (Lind and Burnet, 1953).

If the characteristics of MEL are represented by the symbols ABCDEF and WSE by the symbols abcdef, the characteristics of M+ can be expressed as ABcDeF and WS- as abCdEf. The symbols Aa represent differences in serology

Bb  "  "  heat resistance of haemagglutinin
Cc  "  "  indicator status for ovomucin and meconium
Dd  "  "  indicator status for sheep mucin
Ee  "  "  chick embryo pathogenicity
Ff  "  "  mouse lung pathogenicity

Examination of the recombinants from both the original MEL/WSE experiment and the back recombination M+/WS- experiment showed a close association between certain groups of characters. This suggestion of linkage groups between genetic determinants is exemplified if the interactions, in the two experiments are represented as follows: -
(1) MEL (ABDF-CE) + WSE (abdf-ce) → M+ (ABDF-ce) + WS- (abdf-CE).
(2) M+ (ABDF-ce) + WS- (abdf-CE) → MEL (ABDF-CE) + WSE (abdf-ce).

These experiments with the MEL/WSE system leave no doubt that reciprocal, genetic interaction between two influenza A strains can occur, and from them evolved the concept of the interchange of linkage groups.

Further evidence for the interchange of two linked groups of characters is obtained if the marker-characteristic of mouse neurotropism (g) is examined (Lind and Burnet, 1954). Here the character "g" is associated with the "ce" linkage group and not with "abdf". If neuro-MEL (ABDF-ceg) strains are crossed with WS- (abdf-CEG) strains, a neuro-WSE strain is obtained. The exchange of markers can be represented as follows:


However, variation in linkage relationships has been observed in studies with WSE and CAM (Burnet and Lind, 1955). Here the character for lung pathogenicity (f) is associated with the "ce" linkage group and not with "abd" as above, providing the exception to the rule. This is seen more clearly if the interaction between the WSE and CAM strains is stated as follows:

Thus in recombination with MEL, WSE behaves as if it contained two linkage groups (abdf and ce), but in recombination with CAM it behaves as if the linkage groups were "abd" and "fce". Strains used by Baron and Jensen (1955) behaved similarly. This appears to be the one exception because further experiments (Burnet and Lind, 1956) between WSE and several other type A strains yielded recombinants similar to the original M+ type and possessing the same linkage relationships.

In the influenza system recombination has been demonstrated with a great variety of influenza A strains and with two type B strains (Perry and Burnet, 1953; Perry et al. 1954). As will be seen later, only phenotypic mixing not true recombination is demonstrable between type A and type B influenza strains (Gotlieb and Hirst, 1954).

b. Phenotypic mixing and heterozygosis. Many workers have observed evidence of phenotypic mixing and heterozygosis during recombination experiments with influenza strains. In 1953 Fraser noted that the primary fluids harvested from allantoic cells mixedly infected with MEL and WSE contained virus neutralized by antisera against the two parental strains. He also observed that only fluids which showed this double neutralization yielded recombinants.
Subsequent work by Burnet and Lind (1955, 1956) established this fact unequivocally.

A clearer picture of the above phenomena comes from the work of Hirst and his collaborators. From mixed infection experiments with MEL and WSN, Hirst (1953) and Hirst and Gotlieb (1953a, 1953b) obtained doubly neutralizable virus which they termed $X_1$. On repeated passage, $X_1$ virus gave rise to $X_1$, MEL and WSN. Continued passage of $X_1$ virus yielded a similar, unstable form ($X_2$) but later a doubly neutralizable virus ($X_3$) was isolated that bred true.

In interpreting these results, Hirst and his colleagues (Hirst, 1953; Gotlieb and Hirst, 1954; Hirst et al., 1957) concluded that the $X_1$ and $X_2$ virus arose as a result of phenotypic mixing. The phenomenon of phenotypic mixing was originally encountered with bacteriophage and has been studied in much detail by Streisinger (1956). It implies that the surface of doubly neutralizable particles is made up of a mosaic of antigens derived from the two parental strains. On passage such particles are unstable and give rise to offspring corresponding to one or other of the parental types. The same group of workers observed phenotypic mixing following mixed infection with influenza A and B strains (Gotlieb and Hirst, 1954) and with influenza A and Newcastle disease virus (Granoff and Hirst, 1954). The $X_3$ virus was considered to be a stable recom-
binant of MEL and WSN (Hirst et al. 1957).

Another important point to emerge from these studies (Gotlieb and Hirst, 1954) was that most viable particles showing phenotypic mixing were heterozygotes and, as such, each segregated to give rise to two or more genetically distinct particles. Further studies by Hirst and Gotlieb (1955) suggested that heterozygote formation was a necessary preliminary to the formation of recombinants in the influenza system. In fact they were convinced that all recombination occurred via a heterozygous condition. On the other hand, Lind and Burnet (1957b), while acknowledging the occurrence of heterozygotes, failed to establish a consistent relationship between heterozygote formation and recombination using their N-WSE/MEL system. They assumed a fundamental difference between the systems used in the two laboratories. They noted too that heterozygotes were less viable than homozygotes, which rapidly replaced them.

c. Redistribution of virulence. Despite its complexity virulence has been used extensively as a marker in recombination studies with influenza viruses. Most workers in the field have used neurotropic strains of WS and, by crossing them with other non-neurotropic strains have transferred neuropathogenicity. However, all workers found great variability in the levels of transferred virulence amongst the recombinant clones. Typical of all these
experiments showing this redistribution of virulence, was the finding of Burnet and Edney (1951) using the MEL/NWS system. They found that the recombinant clones of neuro-MEL showed a wide range of neuropathogenicity, from the ability to affect only suckling mice to almost full pathogenicity of the parental NWS. Similar results occur with the transfer of mouse-lung virulence. Perry et al. (1954) found a wide scatter of intermediate virulence amongst the progeny of crosses between the mouse-pathogenic, influenza B strain, LEE and the non-pathogenic MIL strain. Similar variations in chick embryo pathogenicity have been observed by Burnet and Edney (1951).

d. Genetic interchange with one inactivated parent. Genetic interchange between influenza viruses using one component killed by heat or UV-irradiation and the second in the form of active virus has been mentioned in an earlier section (Chapter II) in the discussion of cross-reactivation or marker rescue by recombination. The use of non-viable virus as an effective parent has been confirmed repeatedly (Burnet and Lind, 1954; Baron and Jensen, 1955; Hirst and Gotlieb, 1956). Burnet and Lind (1954) were able to show, by using various combinations of heated and active virus, that the contribution of the inactivated parent was in the form of linkage groups. Virus identical with the heated component was never recovered. This discounted
reactivation of the inactivated virus as such. The rescue of various groups of markers from their experiments with heated and active M+/W- and MEL/WSE is best seen from the following formulae:

\[
\begin{align*}
\text{Heated MEL (ABDF-CE) + WSE (abdf-ce) } & \rightarrow M^+ (ABDF-ce) \\
\text{MEL (ABDF-CE) + heated WSE (abdf-ce) } & \rightarrow WS^- (abdf-CE) \\
\text{Heated M}^+ (ABDF-ce) + WS^- (abdf-CE) & \rightarrow \text{MEL (ABDF-CE) + WSE (abdf-ce)} \\
\text{M}^+ (ABDF-ce) + \text{heated WS}^- (abdf-CE) & \rightarrow \text{WSE (abdf-ce)}
\end{align*}
\]

These results would also confirm their belief that, with influenza virus, the linkage group rather than the individual genetic determinant is the fundamental replicating unit of the virus.

In more recent experiments, Lind and Burnet (1957a) made a closer analysis of virus heat-inactivated at different temperatures and obtained the best yield of recombinants by slow thermal degradation at 37°C for 20 days. Nothing suggested that the type of recombinant obtained from non-viable/viable crosses differed significantly from those from normal crosses, but the yield of recombinants was sometimes lower (Burnet and Lind, 1954). On the other hand, Hirst and Gotlieb (1956) were more successful in obtaining M+ virus with high mouse pathogenicity from W+/M- crosses when the M- component was inactivated by UV-light than when active virus was used. All workers
have found that the inactive viral component can be added to allantoic cells one or more days before the addition of active virus, without affecting the yield of recombinants. Recently Kilbourne and Murphy (1960) suggested that genetic interchange with one inactivated partner could be put to practical use in vaccine production to yield well-adapted, recombinant virus of desired antigenicity and good growth potential. They recommended using heat-inactivated PR8 for the rapid adaptation of recently isolated, filamentous strains of Asian influenza. Such techniques could prove useful during epidemics with new antigenic types of influenza virus.

As mentioned previously (in Chapter II) multiplicity reactivation of UV-irradiated influenza virus can occur (Henle and Liu, 1951; Barry, 1961) this being another example of reactivation by recombination.

e. Limitations of the influenza system. The demonstration of recombination with influenza viruses established the phenomenon amongst animal viruses. The system has its limitations as the technique of limiting dilution for the purification and isolation of clones of virus is very restrictive, as it is both cumbersome and time-consuming. Even when the technique is used in a rigid manner several virus types may be found in one clone (Hirst, 1959), and it is virtually impossible to differentiate between faulty purification and segregation of a recombinant. Another difficulty is the facility with which natural
variations and mutations arise within the influenza group. Results in quantitative experiments can be so variable that the data is useless for the recognition of such things as an ordered, linear sequence of the genetic determinants of certain characters (Burnet, 1960). For the data to be usable, experiments would be needed of a magnitude beyond the resources of most laboratories.

Despite these drawbacks a number of important facts has emerged from the recombination studies regarding the genetic behaviour of influenza viruses. These include the existence of heterozygotes and phenotypic mixing, the interchange of linkage groups, genetic interchange with one inactivated parent and the redistribution of virulence amongst recombinant clones.

Newcastle Disease Virus

Unfortunately most of the data on which our knowledge of influenza virus genetics is based is qualitative in character. This stimulated Granoff (1959a, 1959b, 1961a, 1961b) to investigate the problem of recombination between strains of Newcastle Disease virus (NDV), another myxovirus. Hoping to approach the problem quantitatively, he used two strains of NDV, the Beaudette and Ro strains, which differed from each other in three distinct characteristics — plaque type on chick fibroblast monolayers, heat stability of the
haemagglutinin and virus particle, and the serotype (Granoff, 1959a). Chick fibroblast monolayers were mixedly infected with high multiplicities (7–20 pfu/cell) of the two mutants. The progeny was harvested 18–20 hours later, after multiple cycles of infection when the yields were maximal. The marker characteristics of 399 plaques were examined but no recombinants were found. Subsequently the lack of recombination was verified by the pairwise crossing of eight closely-related, small plaque-type mutants of NDV, induced by treatment with nitrous acid. These crosses yielded no recombinant, wild-type plaques indicating a frequency of recombination, if it did occur, of less than $5 \times 10^{-5}$ (Granoff, 1961a).

However 10% of the plaques obtained from mixed infections with different strains of NDV showed evidence of phenotypic mixing both in respect to serotype and thermal stability (Granoff, 1959b). These mixed plaques were initiated by heterozygous particles which were probably diploid. However the heterozygotes of NDV segregated into particles indistinguishable from parental particles, in contrast to influenza heterozygotes which provide the main source of recombinants (Hirst and Gotlieb, 1955). Rather surprisingly, mixed infections with NDV at low multiplicities (0.1 or less) yielded 5–10 times the expected amount of phenotypically mixed virus. This excess virus was shown by Granoff (1961b) to result from the activation of nonplaque
2. HERPES SIMPLEX

Wildy (1955) established conclusively that recombination can occur within the Herpes group. From chorioallantoic membranes he isolated three stable recombinant clones from the progeny of mixed infection with two well-differentiated strains of Herpes, H,FEM and WC. The parental strains were clearly differentiated by several stable marker characteristics. These included differences in the type of pock formed on the chorioallantoic membrane from the large, opaque lesions of H,FEM to the small lesions of the WC strain. Strain differences in the rate of attachment onto the chorioallantoic membrane distinguished the WC strain, which attached slowly, from H,FEM. Marked differences were evident in the behaviour of strains inoculated intracerebrally into mice, where only the H,FEM strains caused encephalitis and death. On the cornea of rabbits, only H,FEM produced a noticeable keratitis in 2–4 days.

Recombination experiments were carried out by mixedly infecting chorioallantoic membrane with large amounts of the two viral strains. Unabsorbed virus was neutralized with Herpes antiserum. Virus from the first cycle of intracellular growth was harvested at 7 hours. The infected membranes were ground up and titrated on the chorioallantoic
membrane and single clones were prepared by excising well-isolated pocks from membranes showing less than 5 pocks. Ten clones were isolated of which six were parental types. The other four produced pocks which were characteristic of one or other of the parental types, but all possessed degrees of virulence for mice and rabbits intermediate between those of the parents. Differences in the rate of attachment of virus to the chorioallantoic membrane were less clear cut. On subculture three of the recombinant clones were stable after 3-5 passages and, since they exhibited marked differences from either parent, it was concluded they arose by recombination.

3. PSITTACOSIS

According to Bedson (1959) who has reviewed the taxonomic position of members of the psittacosis group, these agents are intermediate between bacteria and viruses possessing properties of both groups but resembling bacteria more closely than viruses. Their bacterial affinities include the presence of both DNA and RNA, enzymes and coenzymes and bacteria-like cell walls. They multiply by binary fission and, like bacteria, are sensitive to penicillin, sulphonamides, chloramphenicol and the tetracyclines. It is mainly in their obligate intracellular parasitism that the Psittacosis group resemble viruses. However, since recombination between different bacterial strains (Luria and Burrous, 1957; Baron et al. 1959a, 1959b)
is well established and this thesis has shown the phenomenon to be widespread amongst viruses, let us consider the possibility of genetic interaction between members of this intermediate group of organisms.

The ease with which Psittacosis virus and other viruses in the same group developed resistance to certain chemotherapeutic agents (Gordon et al. 1957), and the existence of several stable drug resistant strains led Gordon and his colleagues (1957, 1960a, 1960b) to undertake genetic studies in this group of organisms. Preliminary experiments (Gordon and Mamay, 1957) were carried out with two strains of psittacosis virus, one resistant to sulphonamide and the other to chlortetracycline. Each strain was fully susceptible to the other drug. These two strains were grown in the yolk sac of the chick embryo in the presence of 1) sulphonamide, 2) chlortetracycline, 3) sulphonamide and chlortetracycline together, and 4) without the addition of drug. The expected embryo deaths occurred on the fourth day in the first, second and last group, as in each of these groups at least one strain of psittacosis could grow. The same mixture in the presence of both drugs showed no embryo deaths for the first week. This was anticipated as each virus in the mixture encountered a drug to which it
was susceptible. However unexpected late embryo deaths occurred after 8-10 days in this group and psittacosis strains were isolated that possessed resistance to both drugs. This character of dual drug resistance remained stable for at least two limit dilution passages. Since the resistance to various drugs is usually acquired by gradual adaptation and not by spontaneous mutation, the acquisition of dual drug resistance after single passage points strongly to some type of genetic interaction.

More extensive experiments (Gordon et al. 1960a, 1960b) using mixtures of the same drug-resistant strains of psittacosis virus confirmed the earlier findings of the emergence of strains with dual drug resistance. Later studies, however, by Greenland and Moulder (1961) failed to demonstrate recombination. Using four members of the psittacosis group bearing double drug-resistance markers, they set up eleven different crosses in yolk sac and on the chorioallantoic membrane. Enrichment passage in the presence of suitable combination of drugs was used to select possible recombinants and fifty-seven clones of virus were isolated by limit dilution passage. None of these showed evidence of dual drug resistance. They suggested that topographical isolation of the infecting particles within the cell might preclude genetic interchange.
4. ENTEROVIRUSES

Sprunt et al. (1955a, 1955b) investigated the problem of whether true recombination could occur with the smaller viruses by analysing the progeny from the mixed growth of type I and type II poliomyelitis viruses in monkey kidney cultures. A large proportion of the yield was neutralized by both type I and type II antisera suggesting the presence of a "combination form" of poliomyelitis virus, similar to the $X_2$ particles of Hirst and Gotlieb (1953a, 1953b). The new forms were capable of reproducing as such but were not stable.

Confirmation of phenotypic mixing with type I and type II polioviruses comes from the work of Ledinko and Hirst (1961). They examined the progeny from HeLa cells (in mass culture and as single cells) mixedly infected with the two types of poliovirus, and frequently found 100% phenotypic mixing. Often they found their mixedly infected cells produced 2–14 times the expected amount of doubly neutralizable virus. They explained this on analogy with NDV (Granoff, 1961b) by assuming that plaque forming virus may activate non-plaque forming virus.

The results of mixed infections between echo and polioviruses have been reported by Melnick (1957) and by Benyesh et al. (1957). They simultaneously infected monkey kidney cells with equal amounts
of type I poliomyelitis virus and type I echovirus in high concentration, and examined the progeny at limiting dilution 24 hours later. Clones of virus picked from well-isolated plaques showed that 1/129 were neutralized by a mixture of both parental antisera. Further investigation showed this clone consisted of a mixture of particles of which 52% were neutralized by poliomyelitis type I antiserum, and 48% by echo type I antiserum. This clone of doubly neutralizable particles was repassaged and 10/171 plaques tested yielded mixed progeny. At the third passage "mixed" particles were still present.

Itoh and Melnick (1959) examined the yield of single monkey kidney cells mixedly infected with echo 7 and coxsackie A9 virus. The antigenic character of the viral progeny was determined by means of differential plaque counts in the presence of the parental antisera. Fifteen per cent of the single cells contained doubly neutralizable particles. On passage the doubly neutralizable particles yielded progeny of both parental types suggesting phenotypic mixing of the two viruses but not stable, genetic recombination.

So far it has not been established that true recombination can occur with the smaller viruses. However, the available evidence suggests that phenotypic mixing can occur between strains of poliovirus, between echo and polioviruses and between echo and coxsackie viruses.
V. THE MECHANISM OF THE RECOMBINATION
AND REACTIVATION PROCESSES

It will have been apparent throughout the preceding
discussion that, apart from some qualitative observations with
influenza virus, and the present studies with vaccinia virus, virus
genetics is virtually bacteriophage genetics. This is especially
ture for genetic interactions. Any attempt to understand the mech­
anism of genetic interaction in animal viruses must therefore draw
heavily on the large corpus of knowledge which has been built up
with the bacteriophages T2, T4 and lambda. Being deoxyvirus which
multiplies in the cell cytoplasm, analogies may be more reasonably
drawn between vaccinia virus and the T-even phages than between
the latter and influenza virus. We must, nevertheless, maintain some
reservations on the validity of the analogies we will draw, for the
animal cell is functionally and morphologically, vastly different from
a bacterium.

Recombination in Bacteriophage

Genetic recombination in bacteriophage was first observed
by Delbruck and Bailey (1946) and by Hershey and Rotman (1948) who
showed that cells simultaneously infected with two different mutants
of phage T2 and T4 yielded, in addition to the two infecting types, both double mutant and wild type progeny phages. Since then extensive studies have been made and genetic recombination has been found in every phage examined (Adams, 1959). Pfeifer (1961) even obtained evidence of genetic recombination with phage X174 whose DNA is single-stranded, not double-stranded as in all other phages. Hundreds of mutant strains from phages T1, T2, T4 and lambda have been isolated and crossed and, from the proportion of the parental and recombinant types present in the yields, recombination frequencies have been calculated. Thus the degree of linkage between different genetic loci can be determined for certain phages and can be expressed diagrammatically, on a single, linear genetic map (Hershey and Rotman, 1949; Doermann and Hill, 1953; Bresch and Menningmann, 1954; Benzer, 1955, 1957; and Kaiser, 1955).

The Mechanism of Genetic Recombination in Phage

The basic facts concerning the genetic aspects of the recombination process in phage have been reviewed by Hershey (1953, 1957), Doermann (1953), Doermann, Chase and Stahl (1955), Stent (1958) and Levinthal (1959). They may be summarized as follows. When two genetically distinct phages are adsorbed to a susceptible bacterium the phage DNA but not the protein is injected into the cell. Upon entering
the host cell the replication of the genome of each phage begins.

Even though the sites of entry of the DNA may be situated at opposite ends of the bacterial cell the two pools of replicating phage DNA soon coalesce and become a single pool. Phage at this stage of its developmental cycle is called vegetative phage, and this can be regarded as a pool of multiplying and interacting, non-infective viral components (Doermann, 1953). During this period of thorough mixing of the DNA from both parents, recombination occurs. Phage DNA is then withdrawn from the pool, condenses, and is coated with protein to form mature phage particles. Mature particles never re-enter the DNA pool, but are ultimately liberated as infective phage by lysis of the host cell.

a. Visconti-Delbrück theory. The above facts led Visconti and Delbrück (1953) to formulate a quantitative theory of genetic recombination in T2 and T4 phages. According to this theory whole vegetative phage particles mate pairwise and at random with respect to partner and to time in a mating pool. Soon after the onset of mating, particles are withdrawn at random and enter the maturation cycle. Thus replication and recombination, which in phage are indistinguishable events, precede the maturation of the particles. Visconti and Delbrück interpreted these findings in terms of the genetics of small populations. They derived equations to describe the results of mixed infection
experiments involving parental phage differing by two or three genetic factors and, for phage T2, decided that each progeny particle resulted from an average of five, pairwise, mating encounters among the vegetative phage particles.

The Visconti-Delbruck theory satisfactorily explained the kinetics of genetic recombination in phage but it said nothing about the mechanism of recombination. It failed also to account for the occurrence of heterozygous phage particles in bacteria mixedly infected with different phages. These heterozygous or mixed particles were first observed by Hershey and Chase (1951) and were studied in greater detail by Levinthal (1954). Since the heterozygous particles segregated to produce both parental types and recombinants they were ultimately accepted as the precursors of all recombinant phage particles. An acceptable theory of genetic recombination in phage must therefore account for the production of such particles.

b. Partial-replica or copy-choice hypothesis. The concept of heterozygosis as the probable mechanism of genetic recombination in phage led to the suggestion of yet another model of recombination involving the synthesis of new genetic material first along one and then partly along the second of two parental templates. Such a model maintains both the chemical and genetic integrity of the parental structures while still giving rise to recombinants. This process
favoured by the majority of phage geneticists beginning with Levinthal (1953, 1954), forms the basis of the partial-replica hypothesis (Hershey, 1952) or the copy-choice mechanism (Lederberg, 1955) of recombination.

The formation of recombinants as partial-replicas or by copy-choice immediately suggested to Levinthal (1954) an analogy with the structure of DNA as proposed by Watson and Crick (1953). In their model they visualize DNA as a double helix, in the words of Crick (1957) "consisting of two polynucleotide chains running in opposite directions and twined around one another. The two chains are held together by hydrogen bonds between the bases, each base being joined to a companion base on the other chain. The pairing of bases is specific, adenine going with thymine, and guanine with cytosine". The sequence of nucleotides is responsible for the genetic information carried by the structure. These main features of DNA are supported by physical and chemical studies (reviewed by Jordan, 1960). Recent X-ray analyses by Hamilton et al. (1959) and Langridge et al. (1960) have confirmed the validity of this structure in DNA derived from a variety of different sources, including bacteriophage. Watson and Crick also suggested that in replicating the complementary chains of the DNA molecule separate at one end and each chain then serves as a template for the orderly assembly
of new component molecules to give a new chain. As the assembly continues, further separation of the parental chains occurs until the whole pattern has been duplicated along the full length of each parental chain.

c. Nucleic acid as the primary genetic material. By this time it was well established that the primary genetic material in all living systems was nucleic acid. In bacteria and phage DNA was accepted as the carrier of genetic information. This followed the classical studies of Avery and his colleagues (1944) showing that DNA could transfer genetic information from one bacterium to another, and those of Hershey and Chase (1952) demonstrating that phage DNA and protein were separable at the time of infection, the DNA acting as the carrier of the genetic information for replication. In all known plant viruses, most of the smaller animal viruses and in a few small bacterial viruses, the genetic material is RNA (Lederberg, 1959; Gierer, 1960; Loeb and Zinder, 1961). With the elucidation of the structure of the DNA molecule by Watson and Crick (1953) and their suggestions for its duplication, high hopes were held for the establishment of a molecular basis for replication and genetic recombination.

d. The mechanism of DNA replication. Many theories (Delbruck and Stent, 1957; Stent, 1958; Levinthal, 1959; Penrose,
1960) were advanced suggesting modifications or alternative mechanisms for the duplication of DNA consistent with the experimental findings, but so far none has replaced the self-complementary mechanism of duplication originally proposed by Watson and Crick (1953). This scheme is the accepted one at the present time.

Extensive and ingenious isotopic studies were also devised (Levinthal, 1956; Delbrück and Stent, 1957; Levinthal and Thomas, 1957) using labelled DNA to check the hypotheses which developed from the molecular level, by following the distribution of parental DNA among the progeny particles. Probably the most definitive of these isotopic experiments were the recent ones of Meselson and Weigle (1961) and those of Kellenberger, Zichichi and Weigle (1961). These latter experiments with phage utilized the technique of density gradient centrifugation, used so successfully to follow the replication of DNA in bacteria (Meselson and Stahl, 1958).

e. Isotopic studies with DNA. Since it is well-established that the genetic material of phage is DNA and it is usually assumed that the genetic information resides in the linear sequence of nucleotides which comprise the DNA molecule, Meselson and Weigle (1961) argued that genetic recombination in phage corresponds to the production of a nucleotide sequence derived partly from one parental line and partly from another. They visualized this occurring in either of two ways:
1) by copy-choice (described earlier) in which the recombination sequence is synthesized de novo by copying first one parental sequence and then another, or 2) by breakage in which the recombination sequence is formed by association of DNA fragments from different parental lines. Under these conditions, recombinant phage produced by copy-choice would be free of parental DNA, whereas breakage could result in the appearance of portions of parental DNA in the recombinant phage.

To find out whether there was parental DNA in the recombinant phages, experiments were carried out with phage lambda. One unlabelled lambda strain was crossed with another, heavily labelled with the isotopes C$^{13}$ and N$^{15}$. Density gradient centrifugation was used to determine the distribution of labelled parental DNA amongst parental and recombinant types in the progeny. The results unequivocally demonstrated the presence of discrete amounts of the original parental DNA in the recombinant phage. They also implied that the entire DNA complement of phage lambda was a single, conservatively replicating structure which could be regarded as its chromosome. The structure and mode of replication of lambda DNA was in accord with the scheme proposed by Watson and Crick (1953).

The presence of parental DNA in the recombinant phage suggested to Meselson and Weigle (1961) that recombination occurred
by breakage of parental chromosomes followed by reunion of fragments to form genetically complete chromosomes. From the study of the distribution of parental DNA amongst the recombinants, several additional conclusions emerged regarding the recombination process. These established that recombination by chromosome breakage occurs without separation of the two subunits of the parental chromosome. For recombination by breakage, the chromosome does not need to replicate in order to recombine.

Kellenberger, Zichichi and Weigle (1961) were able to demonstrate the physical exchange of DNA in genetic crosses between closely-related, mutant strains of phage lambda of varying densities by labelling the DNA of one parent with $^{32}$P and measuring the distribution of the isotope among the progeny. On centrifugation of the yield from such a cross in a density gradient four bands appeared, two corresponding to the parental densities and two new ones containing only recombinants. The presence of the $^{32}$P label in recombinant phage different in density from the labelled parent showed conclusively that recombination involves a physical exchange of DNA, and that the recombinants were formed by breaks of the two parental materials. The results also indicated that breakdown and re-utilization of parental-infecting DNA were probably negligible.

These recent findings by Meselson and his colleagues
strongly suggest that chromosome breakage is the mechanism of recombination in phage, thus presenting a more optimistic view on the status of the problem than that held by Hershey (1958). The broken fragments can be reconstructed into complete chromosomes by the joining up of appropriate fragments (break and join) or by copying missing regions from other chromosomes (break and copy). Their experiments do not distinguish between these alternatives and therefore do not completely rule out some copy-choice recombination.

Speculations on the Mechanism of Recombination in Poxviruses

With this background of information on growth and recombination in phage, we will attempt to formulate, by analogy, a scheme of replication for vaccinia virus. We are encouraged in this by the facts that it is a "deoxyvirus" (Cooper, 1961) and its genetic determinants can be arranged in a linear, ordered sequence (Gemmell and Cairns, 1959).

Following the absorption and penetration of vaccinia virus into susceptible cells, there is a marked fall in infectivity of the absorbed virus and a true eclipse phase ensues (Easterbrook, 1961a), during which the DNA becomes susceptible to DNAse (Joklik and Roderick, 1959; Cairns, 1960). This stage could be regarded as
equivalent to the vegetative phase during phage reproduction. Concomitant with these changes, autoradiographic studies and fluorescence microscopy of vaccinia-infected cells (Cairns, 1960) have shown that individual viral particles establish discrete factories of viral growth. These may merge late in the growth cycle to form large replicating pools. At this stage there is a thorough mixing of the DNA from several infecting particles, and we can postulate that the DNA helices interact by breaking and rejoining, so that there is opportunity for an exchange of genetic material and the formation of recombinants.

Cairns (1960) has shown that, when cells are infected by dispersed single virus particles, it is only late in multiplication when there has already been much DNA synthesis that the separate areas of DNA synthesis coalesce. Topography may be a much more important factor in recombination in vaccinia than it is in phage (Bresch, 1959), since the animal cell is so much larger than a bacterial cell. With phage T2, Kellenberger et al. (1959) have shown that the centres of DNA synthesis only fuse at about the time maturation begins.

As the DNA synthesis is proceeding, a protein pool develops. Protein membranes develop in the replicating pool and appear to enclose fragments of the viroplasm from the pool, presumably inclu-
ding both protein and DNA components. This stage can be recognized in electron micrographs by the occurrence of large "developmental" forms limited by a double membrane and containing relatively undifferentiated "viroplasm" (Ozaki and Higashi, 1959; Dales and Siminovitch, 1961). Maturation appears to consist of the "condensation" of these developmental forms with the production of the complicated structure of membrane and nucleoid which characterizes the mature particle (Epstein, 1958; Peters, 1960).

Speculations on the Mechanism of the Reactivation of Heat-Killed Poxviruses

Although several different types of reactivation such as marker-rescue, multiplicity reactivation and reactivation of heat-killed and azide-inhibited virus can occur with poxviruses (see Chapter II), they all utilize one of two basically different mechanisms. All must occur by the interaction of two or more viral particles within an infected cell but, in marker-rescue, multiplicity and azide reactivation, the reactivation process is genetic in origin and there is direct repair of the lethal damage by recombination. In contrast the reactivation of heat-inactivated virus is effected by a non-genetic mechanism and the damage sustained is reversed by the intervention of some biological component, probably protein.
Since the main part of the experimental work in this thesis has been directly concerned with biological aspects of the reactivation of heat-inactivated poxviruses, only the mechanism of this type of reactivation process will be considered here.

The experiments in which heated-rabbitpox virus was reactivated by other active poxviruses with the recovery of clones of virus indistinguishable from the heated-component, suggested that this treatment ($60^\circ$ for 12 minutes) had obviously not destroyed the viral genetic material but had probably denatured a more heat-sensitive protein component responsible for the infectivity of the virus. Active virus could reverse this damage by supplying the missing protein component, not directly, but by initiating poxvirus multiplication within the mixedly-infected cell. This was followed by replication of the functionally-intact DNA present in the heated-virus. The same reversal of the damage was accomplished by poxviruses rendered inactive by treatment with nitrogen-mustard in which the DNA of the virus was destroyed preferentially, leaving some protein. Thus the protein component was specifically implicated in causing the reversal, and the mechanism of the reactivation process was established as non-genetic (Joklik, Abel and Holmes, 1960).

The most recent experiments of Woodroofe and Fenner (1961)
suggest that the protein associated with the DNA in the "nucleoid" of the virus, which is common to all members of the poxvirus group, may be the operative protein in this reactivation process. The experimental findings that members of the poxvirus group which possess this common component are the only viruses capable of reactivating heated-poxviruses, while so many other viruses are incapable of participating in the event, support this concept.

A study of the time relationships of the reactivation process (Joklik, Woodroofe, Holmes and Fenner, 1960; Woodroofe and Fenner, 1960) were carried out in mouse brain and in HeLa cells using heated-rabbitpox and either 7N or Ectromelia dohi as the reactivating agent. These indicated that cells need not be exposed to the two reagents simultaneously for reactivation to take place. Simultaneous inoculation of the two components undoubtedly resulted in more efficient reactivation, but heated-rabbitpox inoculated even three days before and up to 24 hours after the active agent was still reactivable. Hanafusa et al. (1959c) also noted that the heated component inoculated 24 hours before the active virus was not degraded by the cell but was subsequently reactivated. In contrast to the relative intracellular stability of heated-virus, Holmes (1961) observed that, once mustard-treated rabbitpox was absorbed to cells, its capacity to reactivate heated-virus was very transient and only lasted a few hours.
Hanafusa et al. (1960) investigated the time of appearance of reactivated virus in cells superinfected with active ectromelia and heat-killed vaccinia virus. When active ectromelia and heated vaccinia were simultaneously inoculated into L cells, reactivated virus appeared 12 hours after inoculation, whereas on their own, vaccinia and ectromelia virus multiplied logarithmically after a latent period of 4–6 hours. The reactivated virus multiplied rapidly after 12 hours as if all the heated virus had been reactivated simultaneously. Preinfection with ectromelia 4 hours prior to the inoculation of heated-vaccinia produced the same picture — the synchronous appearance of reactivated virus 12 hours after inoculation with inactivated virus. However, when active virus was added 4 hours or more after the inoculation of heated-virus, reactivated vaccinia appeared 8 hours after infection with the active virus and increased asynchronously, as did the titre of ectromelia. Hanafusa et al. therefore suggested that the phenomenon of reactivation could be analysed in two parts — 1) the development of conditions in the inactivated vaccinia in which reactivation can occur, and 2) maturation of the active ectromelia virus. To them it seemed likely that the appearance of reactivated vaccinia coincides with the maturation of ectromelia virus, even when the change of the inactivated virus into a form which can be reactivated has been accomplished.
From Cairns' (1960) studies on the initiation of vaccinia infection one could imagine that the role of the protein component (supplied either by active or mustard-inactivated virus) in accomplishing multiplication of heat-killed virus may be to initiate or trigger some preliminary reaction to virus synthesis, so that DNA replication can begin. This idea would fit well with Hanafusa's observation that the appearance of reactivated particles always coincides with the maturation of the active virus. With such a sequence of events, and the intermingling of the pools of DNA, it is very easy to visualize how recombinants can appear at the same time as reactivated particles (WoodrooFe and Fenner, 1960).

In order to build up a theory of vaccinia replication and to explain the mechanism of the various reactivation processes, this section of the discussion has borrowed heavily from the experimental findings with phage. This, surely, is legitimate since it is well-established that both vaccinia and phage contain DNA, both groups of viruses include strains with well-defined genetic markers which are capable of mixedly infecting cells with the production of recombinants, and in both systems the isolation of pure clones provides a firm basis for genetic studies. The great volume of work which has appeared on phage has been invaluable to our understanding of the fundamental issues of recombination, the process of DNA duplication
and virus-host-cell relationships. Many analogies have already been drawn between the phage and animal virus systems, and there is no doubt that they have clarified our thinking on many important issues.
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