THE REACTIVATION OF POXVIRUSES

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STATEMENT

The experiments described in part 1 were carried out under the supervision of Dr.W.K.Joklik. Those described in parts 2 and 3 were entirely the work of the candidate.

The study of the effects of enzymes and chemical treatments on H-RP and RP was carried out jointly with Dr.W.K. Joklik, leading to the results published in Virology 11, 202-218 (1960), a reprint of which is appended. The extension of this study to include M-RP and additional treatments which comprises part 4 of this thesis was the work of the candidate.

Ian H Holmes.

ACKNOWLEDGEMENTS

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CONTENTS

CONTENTS

CONTENTS

CONTENTS

ABBREVIATIONS

AET	2-aminoethylisothiuronium bromide hydrobromide
CAM	Chorioallantoic membrane
DEB	Diepoxybutane
DGE	Diglycidyl ether
DNA	Deoxyribonucleic acid
H-	Prefix designating virus inactivated by heat under the conditions specified in the APPENDIX
HN2	Nitrogen mustard, di(2-chloroethyl)methylamine
HQDGE	Hydroquinone diglycidyl ether
M	Molar concentration
M-	Prefix designating virus inactivated by nitrogen mustard
MEA	Mercaptoethylamine
PFU	Pock forming units
RNA	Ribonucleic acid
RP	Rabbitpox virus
7N	Vaccinia Lederle 7N
TAM	Transforming agent myxoma
U	Refers to pock character of vaccinia: opaque white
U^+	Pock character of vaccinia: haemorrhagic ulcerated
UV	Ultraviolet. As prefix, designating virus inactivated by ultraviolet irradiation
v/v	Proportion by volume

1. REACTIVATION OF HEAT-INACTIVATED POXVIRUSES BY RELATED ACTIVE VIRUSES

a) The Berry-Dedrick Phenomenon

The demonstration by Shope (1932) that the viruses of rabbit fibroma and myxoma were antigenically related, led Berry & Dedrick (1936) to suppose that they might be strains of a single virus. Griffith (1928) had found that it was possible to transform avirulent (R type) pneumococci into the virulent (S) form, by injecting live avirulent organisms plus heat-inactivated virulent ones into mice. Berry & Dedrick therefore attempted the analogous transformation of fibroma into myxoma.

They injected fibroma plus a heat-inactivated preparation of myxoma into rabbits, and found that the rabbits succumbed to typical, characteristic myxomatosis. Control rabbits injected with the heated myxoma alone or with various viruses other than fibroma never developed myxomatosis, nor could fibroma be transformed by anything

other than heated suspensions of myxoma. Myxoma suspensions heated for 30 minutes at 60° or 75° were active, but those heated 30 minutes at 90° failed to produce the transformation.

In addition Berry (1937) showed that the original observations could be confirmed with several different strains of fibroma, using either cottontail or domestic rabbits. Transformation occurred even if the fibroma and the heated myxoma were injected separately into different sites in the rabbit, either simultaneously or at different times. The interpretation which Berry favoured was that the more virulent myxoma was the more complex antigenically, and that some heat-stable component of the myxoma suspension caused a heritable increase in the virulence of fibroma, thereby transforming it into myxoma. On the other hand, he acknowledged that the experiments did not exclude the possibility that the active fibroma virus "reactivates" the heat-inactivated myxoma and that transformation in Griffith's sense does not occur.

In 1937 Hurst confirmed the original observation, and found that neuromyxoma (myxoma of reduced virulence obtained by serial intracerebral passage in rabbits) could not successfully be substituted for fibroma, nor was the transformation possible in fibroma-immune rabbits.

Smith (1952) claimed to have obtained transformation of fibroma with a protein-free supernatant obtained

by repeatedly shaking heated suspensions of myxoma with chloroform, and suggested that the transformation was mediated by deoxyribonucleic acid (DNA). Avery, MacLeod & McCarty (1944) had shown this to be the case in the pneumococcal transformation. Smith's finding has never been confirmed.

Most of the earlier authors reported that the occurrence of the Berry-Dedrick phenomenon was rather erratic, but Kilham (1957, 1958) showed that it occurred regularly in tissue culture. In his experiments cultures of trypsinized rabbit kidney cells were inoculated with fibroma and heat-inactivated myxoma and incubated at 36°. Every 2 or 3 days the medium was replaced by fresh medium containing heated myxoma equivalent to the amount originally added. The culture fluids removed were tested for the presence of myxoma by subcutaneous inoculation of 0.5 ml aliquots into test rabbits. If the rabbits showed systemic infection in 6 to 9 days, the fluids were considered positive for transformation. In negative cases the rabbit developed only local lesions even after 12 days.

Once culture fluids became positive in an experiment, subsequent fluids from the same culture were also found to be positive, and inoculation of a positive fluid into a number of rabbits gave rise to myxoma in all. Thus it was concluded that the transformation actually took place in the tissue culture and not in the test rabbit.

Kilham also found that squirrel fibroma could replace rabbit fibroma in experiments carried out in squirrel or rabbit kidney cultures.

Using the tissue culture system Kilham, Lerner, Hiatt & Shack (1958) were able to investigate the properties of the "transforming agent myxoma" or TAM. For the production of TAM, heating could be replaced by exposure to ether. Chloroform or ultraviolet irradiation destroyed the activity but trypsin or deoxyribonuclease did not. The activity was sedimented by centrifugation under conditions which sedimented active virus, indicating that the activity was associated with virus-sized particles. TAM was effective if it was added to the cells either 24 hours before or 24 to 48 hours after fibroma. They also reported that fibroma virus could not be replaced by vaccinia in this system. (Subsequently, Fenner & Woodroofe (1960) have shown that myxoma can reactivate heated vaccinia on the CAM, but demonstration of the reciprocal process seems to be prevented by interference, due to the growth advantage which vaccinia possesses in this system.) In the discussion Kilham et al. suggested that heat or ether treatment affected the protein-lipid coat of the virus, and that in the presence of fibroma the TAM might lose its outer coats, thus freeing its DNA for replication.

In 1959, Shack & Kilham reported that while dialysis of TAM against 10 M urea at 23° for 8 hours caused

no detectable loss of transforming activity, the activity was thus rendered susceptible to inactivation by deoxyribonuclease. Ribonuclease had no inactivating effect. This showed that the transformation depended on DNA, which was inaccessible to the enzyme until exposed by the protein denaturant, urea.

b) "Transformation" of Vaccinia Strains: Reactivation

Research on the fibroma-myxoma "transformation" was rendered slow and expensive by the necessity to carry out all assays in rabbits. Some confusion was also caused by the occurrence of "fibro-myxoma". The nature of fibro-myxoma was only elucidated in 1958 by Kilham, who found that the condition could be reproduced by inoculation of a rabbit with fibroma, followed by a similar dose of myxoma 2 or 3 days later. This would correspond to a small dose of myxoma inoculated together with a large excess of fibroma. The antibody response of the rabbit to the fibroma prevented development of fatal myxoma, and the rabbit appeared to have a disease of intermediate virulence. The occurrence of fibro-myxoma necessitated further passaging of the test material in order to confirm the occurrence of transformation.

Since in addition to these disadvantages, it is difficult to obtain and purify high-titre stocks of fibroma

or myxoma, attempts were made to demonstrate transformation using vaccinia virus. The study of genetic markers of vaccinia strains by Fenner (1958) made it easy to choose suitable strains for investigations. The strains vaccinia-Lederle-7N (7N) and rabbitpox-Utrecht (RP) are closely related serologically yet differ in five easily tested characters and for this reason had been selected for the demonstration of recombination (Fenner & Comben, 1958). They multiply readily in the mouse brain or rabbit skin, and may be titrated by means of pock counts on the chorioallantoic membrane (CAM). Whereas RP is highly virulent for the mouse, 7N causes only a transient infection. On the CAM, pocks produced by RP are haemorrhagic and ulcerated (U^+ type), and are thus easily distinguishable from the opaque, white (U type) pocks produced by 7N. Transformation of 7N to RP in mice should therefore be detectable either by the death of the test animal or the recovery of U^+ pock-producing virus from it.

When RP was inactivated by heat and injected with active 7N into the mouse brain, active virus having all the characters of RP could be recovered after 24 or 48 hours by homogenization of the brain and titration on the CAM (Fenner, Holmes, Joklik & Woodroofe, 1959). If the mice were not killed, they died of the infection about 6 days after inoculation. Mice inoculated with 7N or heated RP alone survived, and no U^+ virus was recoverable from either. Further

proof that the heated RP (H-RP) was completely inactivated was its failure to prcduce pocks even after passage on the CAM: this was shown to be the most sensitive test available (Joklik, Woodroofe, Holmes & Fenner, 1960c).

It was found that the "transformation" phenomenon was general within the poxvirus group, as defined by Fenner & Burnet (1957), but that viruses outside the group such as herpes simplex virus, influenza virus strain WSE, or Murray Valley encephalitis virus could not participate (Fenner & Woodroofe, 1960). Since progeny identical with the heat-inactivated viral component, but often very different from the active component, could always be obtained, it seemed likely that the process was reactivation of the heated component by the active one, and not transformation as Berry & Dedrick had thought.

Almost simultaneously with the first report of this work (Fenner et al., 1959), "transformation" of ectromelia into vaccinia virus was reported by Hanafusa, Hanafusa & Kamahora (1959a). In a subsequent publication (Hanafusa, Hanafusa & Kamahora, 1959b) they also demonstrated the generality of the phenomenon within, but not outside, the poxvirus group. Using various tissue culture systems, they showed that for transformation to occur, it was essential that the active virus should be able to multiply in the cells chosen.

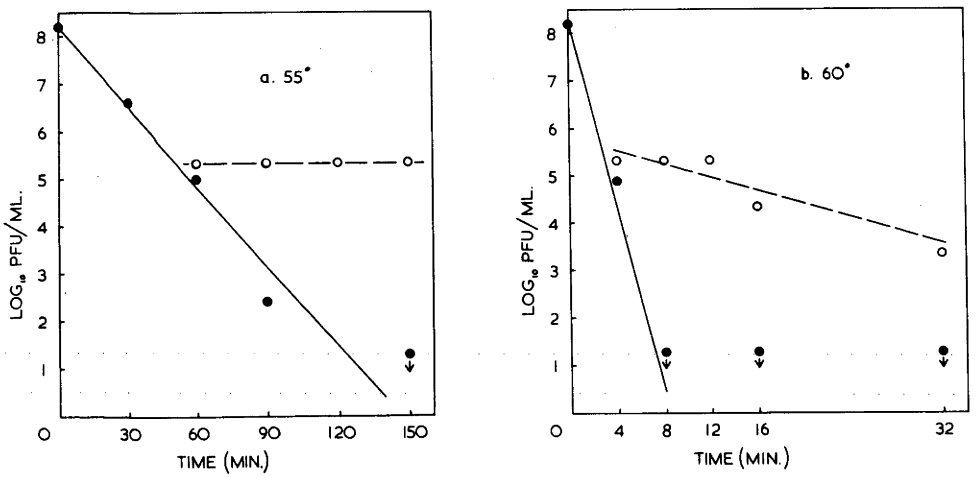

FIGURE 1.1. Inactivation of RP in citrate saline. a. 55°; b. 60°. ● , infective virus; ○ , reactivable virus.

c) Preparation of Reactivable Virus

The infectivity of RP in citrate-saline (0.1 M sodium chloride, 0.01 M sodium citrate, pH 7.0) is destroyed exponentially by heating at 55° or 60°, but if the heated suspension is assayed in the presence of a suitable active virus, such as ectromelia on the CAM, the titre of RP-type (U^+) pocks becomes stable at about one hundredth of the original infective titre. This is illustrated in figure 1.1. This activity which is detectable only in a cell system containing a different, multiplying poxvirus, is called the reactivability of the H-RP, or its reactivable titre.

Reactivable virus is also obtained by heating RP at 60° in gelatin McIlvaine's buffer (Appendix) and this product has the advantage that it flocculates less on storage. However the presence of soluble protein markedly influences the heat inactivation rate, so for reproducible results it is essential to standardise the preparation of virus stocks (described in the Appendix). No reactivable virus was obtained when the heating was carried out at pH<6, or when the RP was heated for 15 minutes at 70° or above.

Freshly-prepared virus was used for inactivation in order to abolish the tailing phenomenon found by Kaplan (1958). He observed an initial first order inactivation

of vaccinia by heat, changing abruptly to a slower inactivation at a survival of 10^{-4} to 10^{-5}. Woodroofe (1960) showed that this was demonstrable only if the virus was stored at 4° for several days before heating. Freshly prepared virus was inactivated exponentially down to a survival of 10^{-8}, and no "resistant fraction" was found.

The recommended method for the preparation of stocks of H-RP or other heat-inactivated poxviruses is described in the Appendix.

d) Assay Systems

The use of vaccinia has made it possible to assess reactivation by means of quantitative tests, instead of quantal tests which had to be used with myxoma. Reactivation experiments with vaccinia were at first performed in a variety of systems, and the results determined by subinoculation on the CAM. Subsequently it was found possible to assay the capacity of heated virus to be reactivated (its reactivability) directly on the CAM. This is the recommended system of assay and practical details of it are given in the Appendix. The various assay systems which were studied are described below. The mouse brain is still the most reliable system for the demonstration of vaccinia reactivation during the late summer months when the CAM becomes refractory.

Mouse brain with 7N

The plan of this assay was to inject into the mouse brain a standard dose of 7N with dilutions of H-RP and to use as a measure of the amount of H-RP injected, the number of reactivated U^+ virus particles found after a suitable period of growth. In practice, suspensions of H-RP were mixed with infective 7N and 0.03 ml aliquots of the mixtures were injected intracerebrally into 5-week-old mice, in groups of three. After harvesting, the brains were pooled and homogenized with 5 ml of Genetron (1-dichloro-fluoro-2-difluorochloroethane) and 10 ml of gelatin McIlvaine's buffer per group of three. After low speed centrifugation, the virus in the aqueous phase was titrated on the CAM. If a known aliquot of virus was mixed with 3 uninfected brains and extracted in this way, one third of the virus was recovered in the aqueous layer. A higher yield (fourfold) was obtained if the Genetron was omitted during the extraction, but the extract then flocculated on storage at 4°. In the experiments described below, Genetron was used during extraction and the total virus per brain was assumed to be three times that extracted.

Preliminary experiments were carried out first to ascertain the optimal dose of 7N, the optimal time of harvest and finally to determine whether the number of U^+ pocks recovered was an adequate measure of the amount of H-RP injected. In order to determine the effect of the dose of

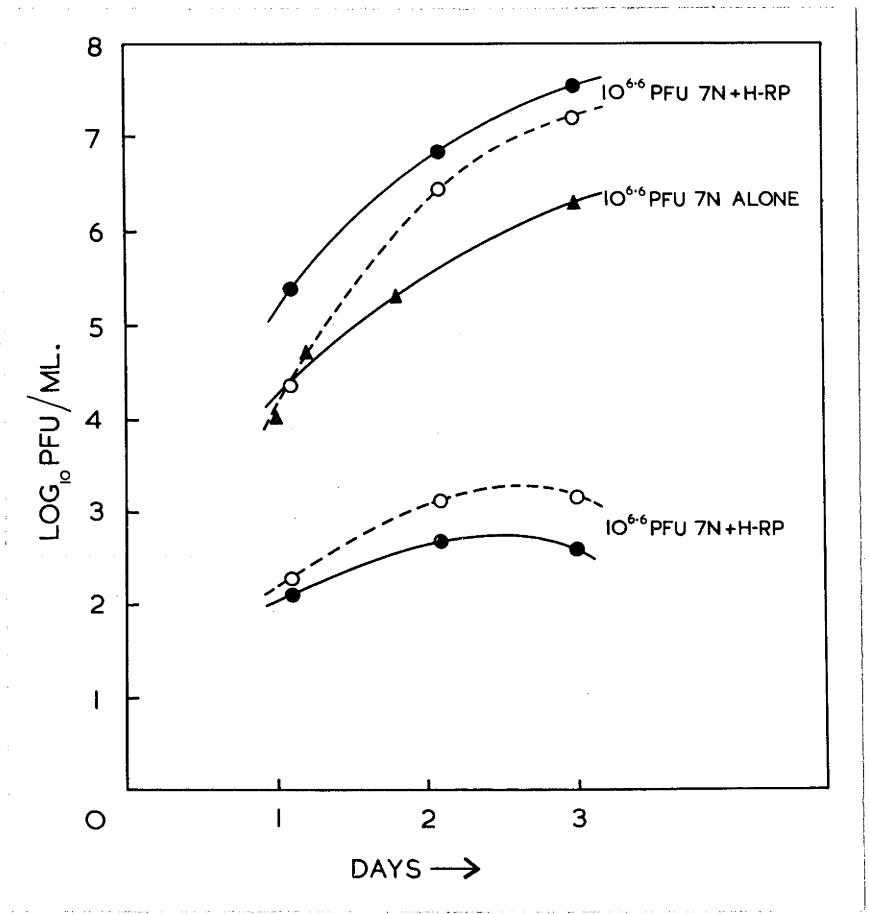

FIGURE 1.2. The growth of virus in the mouse brain following inoculation of $10^{3.6}$ or $10^{6.6}$ PFU of 7N with or without H-RP.

● , U pocks; ○ , U^+ pocks; ▲ , 7N pocks.

7N, and the optimal time for harvesting the mouse brains, mice were injected with an undiluted preparation of H-RP (see Appendix) and two concentrations of 7N, and the virus content of brains was determined after 1, 2 and 3 days. The growth curves obtained are shown in figure 1.2. The curve obtained with the higher concentration of 7N in the absence of H-RP is also illustrated.

It is seen that the lower dose of 7N provides the more sensitive system. The number of U^+ pocks approaches a maximum at 3 days, so it is preferable to harvest at this time. Also, longer periods before harvesting were inconvenient, since some deaths occurred.

It is also seen from figure 1.2 that the U pock count is higher in the presence of H-RP than in its absence. Although they have the same pock character as 7N, the virus strains obtained from many of these pocks are heat-resistant and highly virulent for mice, i.e. they are recominants between 7N and RP. All these white pocks interfere with the detection of RP, since U^+ pocks are not detectable on the CAM in the presence of more than about a thirtyfold excess of U pocks.

Since the actual titres of virus obtained vary considerably with different groups of mice, it appeared that the ratio of U^+ to U pocks was a better index of reactivation. In order to determine the effects of varying doses of H-RP and 7N on the U^+/U ratio, mice were injected

TABLE 1.1. Effect of the concentrations of H-RP and 7N inoculated on the ratio of U^+ to U pocks recovered from mouse brains after 3 days.

Dilution of H-RP	Dose of active 7N (PFU)			
	$10^{6.7}$	$10^{5.7}$	$10^{4.7}$	$10^{3.7}$
1	0.4	0.8	1.5	2.8
1/10	...[a]	0.4	0.6	0.8
1/100	...	0.2	0.5	0.4

[a] ... = not done.

with a series of tenfold dilutions of each. As table 1.1 shows, the haemorrhagic to white pock ratio increases as the inoculum of 7N decreases and as the concentration of H-RP increases. Considerable variation was found in the ratios obtained in different experiments, so the ratio gives only an indication of the reactivable titre. The variation probably arises from the difficulty of exactly standardising the 7N inoculum, and from the differences between groups of mice. Within a single experiment, however, the variation between ratios obtained from individual brains was much smaller, as is shown by table 1.2.

It was concluded that if the dose of 7N is controlled, the ratio gives a walid estimate of the dose of H-RP injected.

The sensitivity of this assay method is limited by the interference of 7N and recombinants with the growth

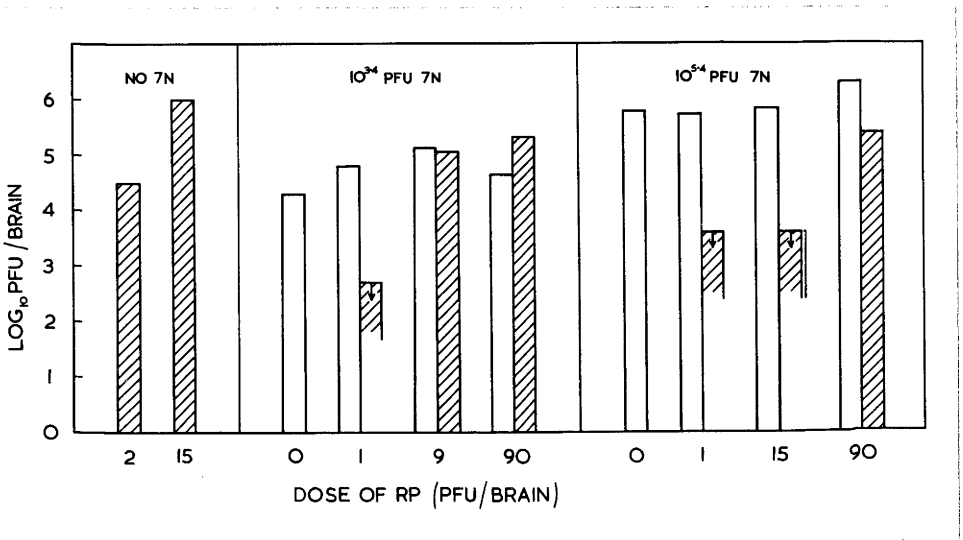

FIGURE 1.3. Interference between 7N and RP in the mouse brain. Brains were removed at 3 days. ▨, U^+ pocks; □, U pocks.

TABLE 1.2. Titres and ratios of pock types of virus recovered from brains of individual mice 3 days after injection of H-RP plus $10^{4.9}$ PFU of 7N.

Mouse	Log_{10}PFU/brain		U^+/U
	U pocks	U^+ pocks	ratio
1	6.93	6.37	0.28
2	7.11	6.62	0.32
3	6.93	6.51	0.38
4	6.32	6.11	0.62
5	7.36	6.88	0.32
6	7.10	6.72	0.42
7	6.66	6.23	0.38
8	7.15	6.75	0.40
9	6.54	6.24	0.50

of RP in the brain. The chart in figure 1.3 shows that whereas even 2 pock forming units (PFU) of active RP inoculated alone are easily detectable by their growth in 3 days, the threshold increases with the amount of 7N inoculated simultaneously. Thus with $10^{5.4}$ PFU of 7N even 15 PFU of RP are undetectable, since at 3 days, U^+ pock producing virus particles are outnumbered by U pock producing ones by more than 100 to 1.

Mouse brain with ectromelia

It was found that ectromelia virus reactivated H-RP satisfactorily, and the strain Dohi A was selected since it was of low virulence for the mouse when intracerebrally injected. Ectromelia has several advantages

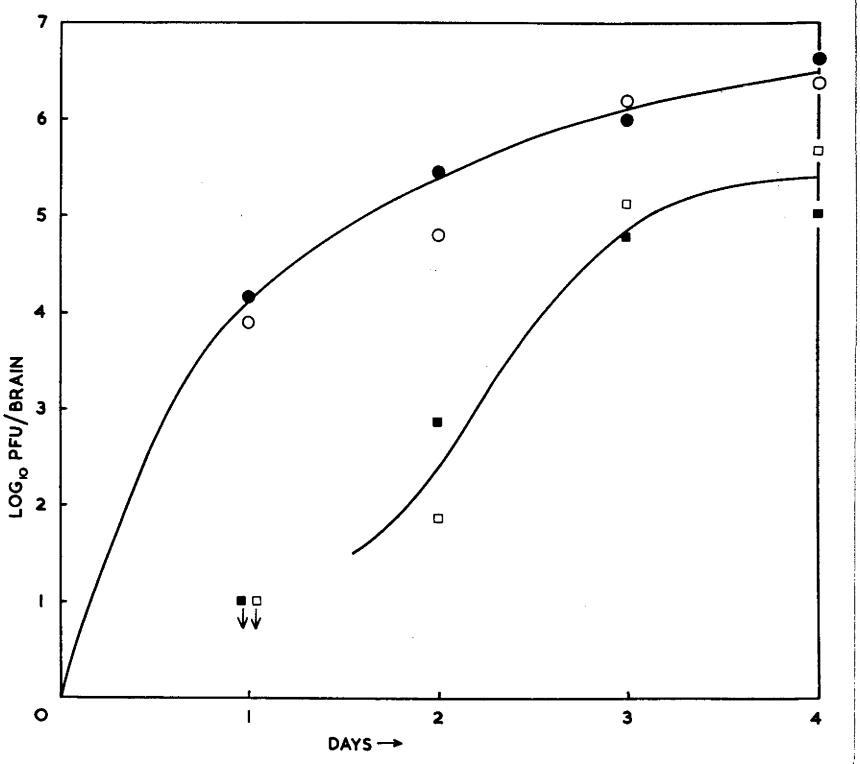

FIGURE 1.4. The growth of 20 PFU of RP in the mouse brain in the presence and absence of $10^{4.2}$ PFU of ectromelia.

● , ectromelia in the presence of RP;

○ , ectromelia in the absence of RP;

■ , RP in the presence of ectromelia;

□ , RP in the absence of ectromelia.

over 7N. In the doses used it does not interfere with the growth of RP in the brain, so that the progeny of 2 PFU of RP inoculated with 10^4 PFU of ectromelia were easily detectable after two days. Figure 1.4 shows that 20 PFU of RP multiplied to the same extent whether inoculated alone or with $10^{4.2}$ PFU of Dohi A, and that growth of the ectromelia was unaffected by the presence of RP. Counting RP pocks in the presence of even a large excess of ectromelia offered no difficulty, since on the CAM pocks of ectromelia are minute at 2 days, while those of RP are haemorrhagic and relatively large.

Not only is the assay of H-RP reactivability with ectromelia more sensitive than that with 7N, but it is also more reproducible. In particular, it is far less affected by variation in the dose of reactivating agent. Thus the yield of U^+ virus obtained from brains harvested after 2 days was found to vary with the amount of H-RP injected, yet changed only slightly when the inoculum of ectromelia was increased from 10^4 PFU to 10^6 PFU. The optimal amount was about 10^5 PFU.

For assaying different preparations of H-RP, two dilutions a hundredfold apart were injected intracerebrally into groups of three mice with about 10^5 PFU of ectromelia. The yields of U^+ virus found after 2 days were then referred to the reference curve shown in figure 1.5, which was compiled from the results of four experiments. The yield

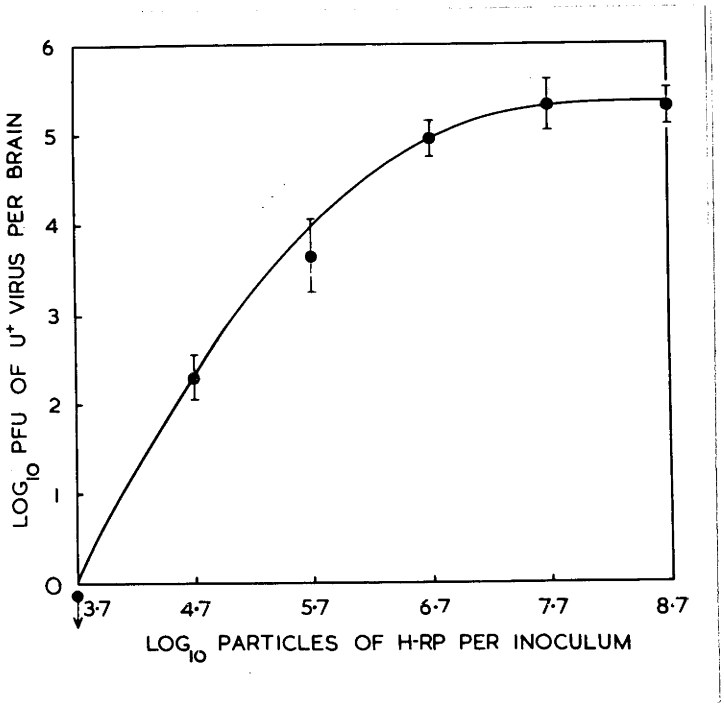

FIGURE 1.5. Yields of U^+ virus obtained after 48 hours from brains which received $10^{4.4}$ PFU of ectromelia and various doses of H-RP. Each point represents the mean of 4 separate experiments, in each of which 3 mice were used for each dilution. The limits indicated for each value are the standard deviations.

of U^+ virus increases approximately linearly with H-RP dose for low doses, but the response levels out to about $10^{5.6}$ PFU/brain with even the highest available doses of H-RP. If the brains were harvested after three days instead of two the yields were higher but the sensitivity of the assay was not increased. As can be seen from the curve the smallest amount of H-RP measurable corresponded to $10^{3.7}$ virus particles, or $10^{2.7}$ PFU in the RP suspension prior to heating.

KB cells with ectromelia

For some types of experiment, such as those on the effect of pre-inoculation with H-RP or reactivating agent, the development of a tissue culture assay system seemed desirable. Unfortunately it was found that for testing dilutions of H-RP, the cell system described is only about one hundredth as sensitive as the mouse brain. It proved possible to perform the pre-inoculation experiments in the mouse brain, but the tissue culture system is useful for other applications.

Monolayers of KB cells (Eagle, 1955) were grown in small bottles (cell area 2.3 cm^2) in a medium containing 0.5% lactalbumin hydrolysate and 15% human serum. After removal of the medium and a rinse with 1 ml of Hanks' balanced salt solution, the cells were exposed to 0.05 ml of dilutions of H-RP plus about 10^5 PFU of ectromelia, and virus was allowed to adsorb for 1 to 2 hours at 20°.

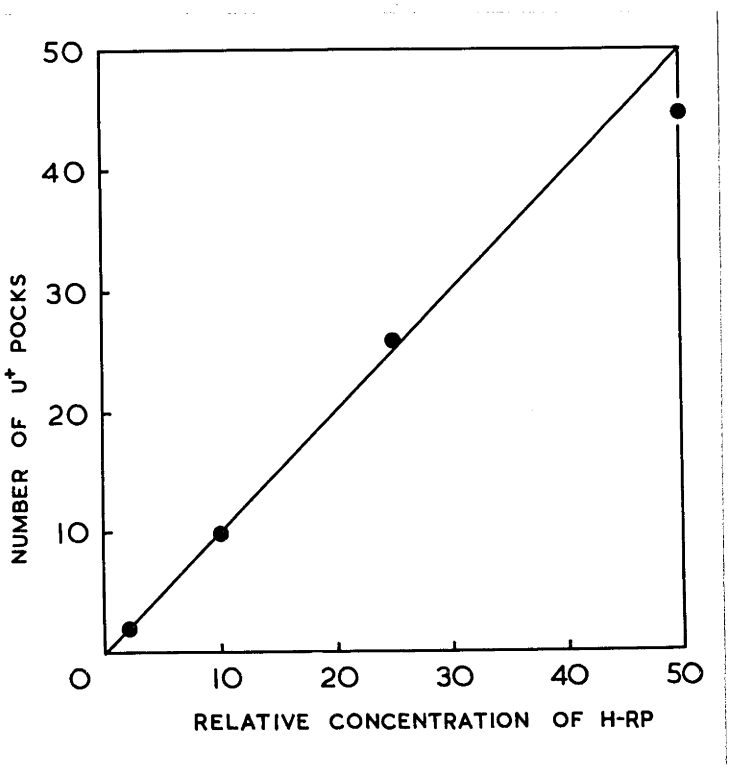

FIGURE 1.6. The relation between dose of H-RP and number of U^+ pocks on the CAM.

Medium was then replaced; after 2 days incubation at 36° the cells were sonicated and their contents titrated. Higher yields were obtained if the medium added after adsorption of virus contained 15% calf serum instead of human serum, because this reduced non-specific inactivation of the virus.

Chorioallantoic membrane with ectromelia.

The fact that RP produces large haemorrhagic pocks in 2 days on the CAM whereas ectromelia pocks are tiny, non-haemorrhagic and thus hardly visible at this time makes it possible to assay H-RP directly on membranes infected with a fairly large background dose of ectromelia. If a CAM is inoculated with H-RP plus $10^{4.5}$ to 10^{5} PFU of ectromelia, the number of U^{+} pocks observed after 2 days is a direct measure of the reactivable titre of the H-RP, as figure 1.6 shows. The assay is insensitive to variation in the dose of ectromelia within the limits of 10^{4} to $10^{5.5}$ PFU (Joklik, Woodroofe, Holmes & Fenner, 1960c). The highest ratio of reactivable to total particles in an H-RP preparation measured by this assay was 1/1000; thus the method is at least as sensitive as the mouse brain assay, as well as being quicker and more convenient.

e) Properties of H-RP

The reactivability of H-RP is relatively stable to heating under conditions which rapidly destroy the infectivity of RP, as figure 1.1 showed. At 55°, the reactivable titre decreases only after several hours. At 4°, preparations of H-RP retain their activity for at least 6 months. Activity was also stable to six rapid cycles of freezing and thawing. The activity was sedimented by centrifugation (90% by 90000g. minutes) and therefore can be assumed to be still associated with the virus particles. Heating causes no change in the morphology of the particles.

The effects of chemical and enzymic treatments on reactivability of H-RP are described in part 4 of this thesis.

f) Pre-Inoculation with H-RP or Reactivating Agent

Experiments carried out in the mouse brain using H-RP and either 7N or ectromelia Dohi A as reactivating agent confirmed Berry's (1937) observation, that cells need not be exposed to the two reagents simultaneously in order for reactivation to take place. Table 1.3 shows the results obtained with ectromelia. Brains were harvested and titrated 2 days after the second inoculation.

TABLE 1.3. The effect of inoculating H-RP and the reactivating agent at different times.

Time interval between inoculations		Log_{10}PFU of U^+ pocks per brain[a]
H-RP:	4 days before	---
	3 days before	2.9
	2 days before	3.6
	24 hours before	4.4
	16 hours before	3.9
	8 hours before	4.2
	4 hours before	4.7
	Simultaneous	6.3
	4 hours after	5.3
	8 hours after	5.2
	16 hours after	5.8
	24 hours after	6.1

[a] Reactivating agent: $10^{4.3}$ PFU of ectromelia. Brains were processed after 2 days.

From the titres of reactivated virus it can be seen that simultaneous inoculation is most efficient for reactivation, but that H-RP inoculated even 3 days before the ectromelia was still reactivable. Similar results were obtained in HeLa cells with 7N (Joklik et al., 1960a).

g) Poxvirus Reactivation in Perspective

The term "reactivation" has been applied to various processes in the field of virology, relating to the rescue of infectivity or genetic markers.

Photoreactivation is an example of reactivation by a physical process. Damage caused by ultraviolet irradiation of bacteriophage may be partially reversed by exposure of the phage (in the form of a virus-cell complex) to intense visible light.

Reactivation may also be accomplished by purely chemical means. For instance Krueger & Baldwin (1934) discovered that the toxicity of mercuric chloride for a staphylococcal bacteriophage could be reversed by treatment with hydrogen sulphide. Another example is the reactivation of formalin-treated phage T3 by incubation with asparagine, which was studied by Heicken & Spicher (1956).

Bacteriophage neutralised by antibody is also subject to reactivation by either chemical (enzymic) or physical means. Kalmanson & Bronfenbrenner (1943) found that incubation with papain restored to its original activity a coliphage 90% inactivated by antibody. Anderson & Doermann (1952) produced a similar reactivation by exposing the virus-antibody complexes to high frequency sonic vibration.

There are also two purely biological forms of

reactivation; both involve genetic recombination and take place only within infected cells. The first, multiplicity reactivation, is the cooperative multiplication of virus particles so damaged by ultraviolet irradiation that they are unable to produce progeny in single infections. Multiplicity reactivation of the T_{even} coliphages was described by Luria & Dulbecco (1949). It occurs also with heat- or X-ray-inactivated phage, but with a much lower efficiency. Mutsaars (1957) demonstrated multiplicity reactivation with formalin-inactivated phage alone or in mixtures with ultraviolet inactivated phage and found that in the latter case most of the progeny resembled the formalin-treated parent.

Active related virus multiplying in cells containing ultraviolet-inactivated virus can acquire genetic characters from the latter. This process was originally called cross reactivation, but is more accurately described as marker rescue. The hypothesis proposed to explain it by Doermann, Chase & Stahl (1955) in their work with bacteriophage was that at least part of the irradiation damage occurred in the DNA and that undamaged portions of genome carrying the markers could be rescued by recombination with DNA from superinfecting active virus. This type of reactivation may also be observed with strains of influenza virus (Burnet & Lind, 1954). In either case the result is the production of recombinant types, but not virus identical with the inactivated parent.

Since poxvirus reactivation can be accomplished only by related active virus multiplying in cells, it must be classed as a biological rather than a physical or chemical reactivation, and superficially it resembles marker rescue. The property which sets it apart from this is that virus identical in all known markers with the heat-inactivated parent is produced. Recombinants are found only if the viruses participating belong to the same serological sub-group and are thus capable of recombining secondarily (Woodroofe & Fenner, 1960). This suggests that different mechanisms underlie the two processes. The question of the mechanism of poxvirus reactivation will be taken up in the next part of this thesis.

2. REACTIVATION OF HEATED VIRUS BY ALKYLATED VIRUS

a) Principle

It was pointed out in part 1 that poxvirus reactivation results in the recovery of virus having all the known genetic markers of the virus originally inactivated by heat. The DNA of the virus thus seems to be stable to heating at 60°. In fact, if vaccinia DNA is comparable to the other types studied by Marmur & Doty (1959) its base composition (40.6% guanine + cytosine: Wyatt & Cohen, 1953) suggests that its denaturation temperature would be 85°. If the DNA of heat inactivated virus is intact, it seems most likely that the essential heat sensitive component supplied by the reactivating virus contains protein. In order to test this hypothesis, Miss P. Abel suggested that virus whose DNA had been selectively inactivated might be able to replace active virus as reactivating agent, in which case the genotype of the heat inactivated virus only should replicate.

The problem was to inactivate vaccinia DNA while causing a minimum of damage to protein. Ultraviolet irradiation was tried, but virus inactivated by this means gave inconsistent results when tested for ability to reactivate H-RP (Fenner, personal communication). It was then decided to try an alkylating agent, the nitrogen mustard di(2-chloroethyl) methylamine (HN2). It was found by Dr. W.K. Joklik that RP or 7N inactivated by HN2 could reactivate H-7N or H-RP, and that the product resembled the heated parent. At first, however, it was found difficult to inactivate RP reproducibly with HN2, and the reactivating activity of mustard treated RP was unstable, so a detailed study of the process was undertaken. As a result, variability was reduced by careful control of the conditions of preparation of viral suspensions and of stock solutions of HN2.

b) Effects of Mustards on Biological Systems

Mustards owe their name to a chemical relationship to di(2-chloroethyl) sulphide ("mustard gas") which in its crude state smells like oil of mustard. Mustards inactivate various micro-organisms, inhibit enzymes, induce chromosome abnormalities, induce mutations, and have a cytotoxic effect on certain neoplasms (Ross, 1953). In view of this wide range of activities, it is natural that much effort should

have been put into determining their mechanisms. Mustards can react with protein groups, but their biological effects appear more likely to be due to reactions with nucleic acids.

Herriott (1948) measured the effects on various biological activities of a fixed amount of mustard gas (di(2-chloroethyl)sulphide) and showed that Pneumococcus transforming principle was most sensitive, then phage T2, Escherichia coli, tobacco mosaic virus and lastly, enzymes. Also in 1948, Elmore, Gulland, Jordan & Taylor demonstrated the reaction of mustard gas with primary and secondary phosphate groups, and with amino groups, of nucleic acids. The amino groups may have been freed for reaction by the use of 35% ethanol in their experiments, since Alexander, Cousens & Stacey (1957) state that alkylating agents do not react with amino groups in undenatured DNA.

Butler, Gilbert, James & Ross (1951) found that treatment with HN2 caused a decrease in the sedimentation constant of thymus nucleic acid. They attributed this to actual chain breakage, and suggested that this might be due to the breakdown of trisubstituted phosphate esters.

The action of alkylating agents on Haemophilus transforming principle was studied by Zamenhof, Leidy, Hahn & Alexander (1956). The loss of biological activity was shown to be a far more sensitive index of reaction than change in viscosity, as complete inactivation was produced by 1/300th of the concentration of HN2 needed to cause the

first measurable drop in viscosity. It was also found that a sublethal exposure of transforming principle to certain reagents, for example 2-chloroethyldimethylamine, rendered it more sensitive to heating.

Alexander (1952) was able to estimate the total number of ionized phosphate groups in DNA by measuring the uptake of a basic dye (methylene blue) through a cellophane membrane into the DNA solution. By exposing the DNA to the action of alkylating agents and then measuring the number of phosphate groups remaining, he showed that a number of the reagents, including HN2, cause extensive esterification even under mild conditions. Monofunctional compounds, for example 2-chloroethyldimethylamine (HN1) cause about the same degree of esterification of phosphate groups as the related bifunctional compounds (in this case, HN2) but treatment with the bifunctional reagents interferes far more with the subsequent uptake of salmine by the DNA, i.e. nucleoprotein formation. It was suggested that this interference may account for the relatively much greater biological effects of bifunctional reagents.

In a discussion primarily concerned with mutagenesis, but dealing also with inactivation of biological systems Alexander, Cousens & Stacey (1957) came to the conclusion that the key reaction of HN2 (and that common to all the mutagenic alkylating agents) was combination with the acidic groups, carboxyl and phosphate. Owing to the high reactivity

of these alkylating agents other reactions are bound to occur, and may even predominate, but from the biological point of view these are comparatively unimportant. With DNA, phosphate was the only group reacting with the mustard. The resultant phosphate triesters were found to be unstable, and hydrolysis could cause breaks which, since they occurred in only one strand of the double DNA helix, would not result in scission of the whole molecule. A viscosity drop would show only when breaks occurred in close proximity but in opposite strands. These "hidden" breaks were demonstrable, however, if the strands of the treated molecules of DNA were dissociated by exposure to 4 M urea. Phosphate diesters, produced by alkylation of terminal (secondary) phosphate groups, and therefore far more common in RNA than DNA, were considerably more stable than the triesters. Crosslinking of the DNA was observed with bifunctional agents.

Loveless & Stock (1959a, b & c) studied the inactivation of the bacteriophages T2, T4 and T7 by a number of alkylating agents including HN2, and the properties of the inactivated phage. It was found that after a brief exposure of T2 or T7 to a high concentration of HN2, inactivation continued for some time after the suspension was diluted in buffer sufficiently to reduce the HN2 concentration to an innocuous level. This post-treatment decay was eliminated by diluting samples into 5% sodium thiosulphate, which reacts extremely readily with HN2, and incubating

them overnight. Loveless & Stock concluded that the lethal reaction preventable by thiosulphate must be that of the second reactive groups of attached molecules.

Inactivation of T2 by HN2 did not affect its adsorption to host cells or its antibody-blocking ability. Inactivated phage exhibited a very low efficiency of multiplicity or cross reactivation; in this respect particularly HN2 had a very similar effect to X-irradiation. Finally inactivation by ionized (aged) HN2 of a permeable mutant of T4 was found to be more rapid than inactivation of an osmotically shockable (therefore less permeable) mutant, which demonstrated clearly that the inactivating reaction occurred inside the head membrane, and thus presumably with the phage DNA.

All this evidence shows that there were good reasons to suppose that HN2 would inactivate the DNA of vaccinia. With this background, experiments were designed to show whether vaccinia behaved like phage T2 following exposure to HN2. If this were so, then it would be possible to stabilise preparations of mustard treated virus by treatment with thiosulphate.

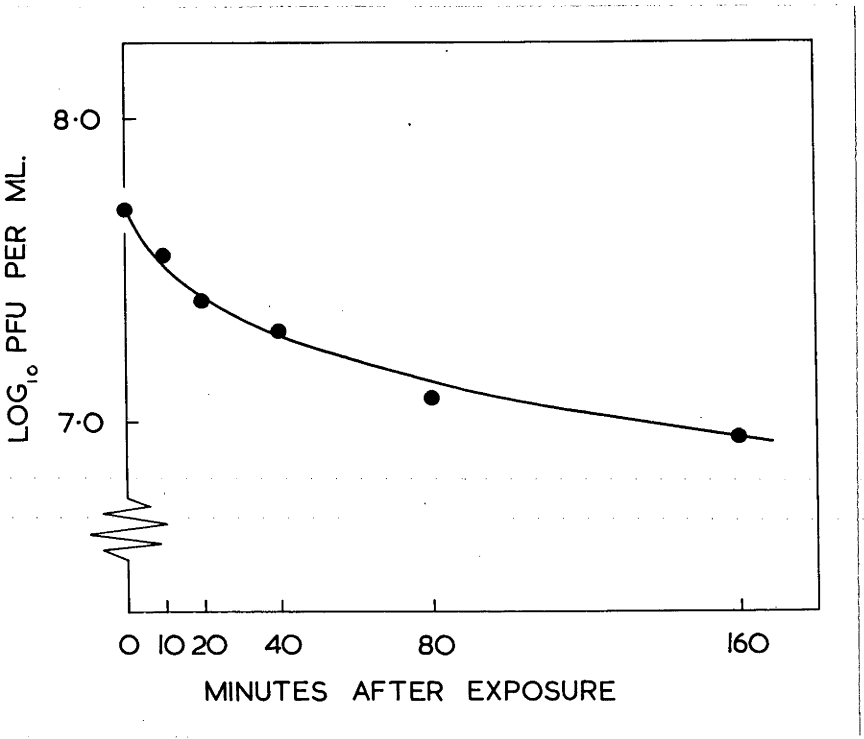

FIGURE 2.1. Loss of infectivity of RP diluted 1/1000 into gelatin saline after exposure to HN2 at 1 mg/ml for 2 minutes at 37°.

c) Inactivation of RP by HN2

Use of thiosulphate to stop the reaction

In order to test whether inactivation of vaccinia following exposure to HN2 continued after the excess HN2 was diluted out, and whether dilution into thiosulphate would stop this post-treatment decay, vaccinia was exposed to 1 mg/ml HN2 for 2 minutes at 37°, then diluted 1/1000 into gelatin saline and held at 37°. At various times, samples were removed, diluted tenfold into 5% sodium thiosulphate in gelatin saline, and incubated a further 2 hours at 37° before titration. The result is shown in figure 2.1. It is seen that in the absence of thiosulphate the titre of mustard treated virus continued to fall for at least 2 hours. On the other hand, incubation for 2 hours in 5% thiosulphate stopped the reaction completely, since the t = 0 titre was the same as that found in a control of virus not exposed to HN2. Evidently during the 2 minute exposure the mustard had attached itself to the virus by the reaction of only one functional group. This did not inactivate the vaccinia detectably; as with T2 (Loveless & Stock, 1959a) inactivation which ensued and was preventable by thiosulphate treatment was apparently caused by a combination of the second reactive groups of the attached mustard.

Effect of diluent and age of HN2 solution

On solution in water, HN2 ionizes, then rapidly

cyclizes to form ethyleneimonium ions (Golumbic, Fruton & Bergmann, 1946). From results quoted by Ross (1953) it can be estimated that at 37° this reaction is 50% complete in about 1½ minutes. These ions are the highly reactive form of the mustard, and react almost instantly with water or ionized anionic groups, including various anions used in buffers. Thus the amount of ionized HN2 available for reaction with the virus is conditioned by the composition of the virus diluent and the age and composition of the HN2 solution.

Phage T7 resembles vaccinia in that neither is osmotically shockable, and hence it may be assumed that both are permeable to small molecules. Loveless & Stock (1959c) found that T7 was considerably more sensitive to inactivation by "aged" solutions of HN2 than by fresh. The "aged" solutions had been incubated for 12 minutes at 37°, so in these the HN2 should be fully ionized. To test whether vaccinia was also more sensitive to ionized HN2, the following experiments, analogous to those of Loveless & Stock, were carried out. An HN2 solution in gelatin McIlvaine's buffer was incubated (aged) for 12 minutes at 37°, then portions of it and of a solution freshly prepared in iced buffer were added to vaccinia suspensions to give final HN2 concentrations of 0.1, 0.2, 0.4, 0.8 and 1.0 mg/ml. The suspensions were incubated at 37° for 2 minutes, then diluted 1/100 in gelatin saline and the incubation was

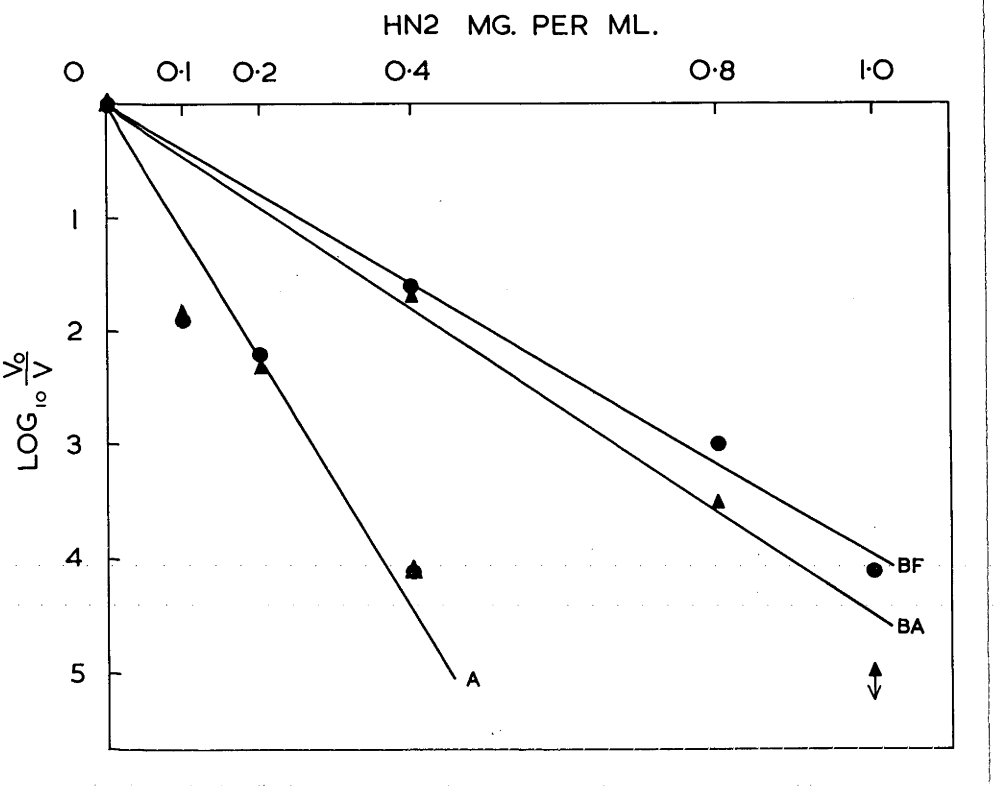

FIGURE 2.2. Inactivation of RP by fresh and aged (12 minutes at 37°) solutions of HN2 in gelatin McIlvaine's buffer (curve A) or gelatin M-9 medium (curves BF and BA). Exposure was for 2 minutes at 37°, and was followed by dilution 1/100 into gelatin saline or gelatin M-9 medium, respectively, and overnight incubation at 37°.

● , fresh HN2;

▲ , aged HN2.

continued overnight. When the residual virus was titrated, the results shown in figure 2.2, curve A, were obtained. Ageing of the HN2 is seen to have had no effect on its inactivation of vaccinia under these conditions.

In case this apparent difference between T7 and vaccinia was attributable only to the different diluents in which the experiments were carried out, vaccinia was retested in the diluent used by Loveless & Stock. This was M-9 buffer (Adams, 1959, p.446: M-9 medium with the omission of glucose), which contains considerably more phosphate than gelatin McIlvaine's buffer, 0.06 M in contrast to 0.004 M. 0.15% gelatin was added to the M-9 buffer to prevent inactivation of vaccinia at interfaces. The results obtained are illustrated by curves BF and BA of figure 2.2. In this case ageing of the HN2 did slightly increase the inactivation of vaccinia, but to a barely significant extent. Vaccinia thus does seem to differ from phage T7 in its response to fresh or aged HN2.

Figure 2.2 does, however, illustrate the marked effect of diluent, or more specifically of phosphate concentration, on inactivation rate. Inactivation of vaccinia in 0.004 M phosphate (curve A) is much more rapid than that observed in 0.06 M phosphate (curves BF and BA). Similar results were obtained when the HN2 reaction was stopped with thiosulphate: the inactivation rate varied with the amount of phosphate in the reaction mixture but not with

the age of the HN2 solution (at least over 12 minutes at 37°). Except in the experiment just mentioned, in all inactivations gelatin McIlvaine's buffer was used in the reaction mixture, as 0.004 M phosphate was ample to buffer the mixtures at the low concentrations of HN2 used. Also, virus which had been treated with HN2 was always incubated for 2 hours at 37° in 5% thiosulphate in gelatin saline before titration.

d) Non-Genetic Nature of Poxvirus Reactivation

It has been mentioned that Dr. W.K. Joklik found that HN2-inactivated RP or 7N (M-RP or M-7N) could reactivate H-7N or H-RP, and that the reactivated virus always had the pock character of the heated parent.

RP and 7N are not genetically closely related, so a more critical test was devised to show whether in fact the genome of the mustard-treated parent was absent from the progeny. For this wild type rabbitpox (RPu$^+$) and two white pock variants (RPu1 and RPu8) were used. These u mutants differ from the wild type at single genetic sites, and on the CAM the wild type outgrows either (Gemmell & Cairns, 1959; Gemmell & Fenner, 1960). Thus mustard treated RPu$^+$ with heat inactivated RPu1 or RPu8 provides a system in which the u$^+$ genome is sure to be detected if it survives, by the appearance of haemorrhagic ulcerated

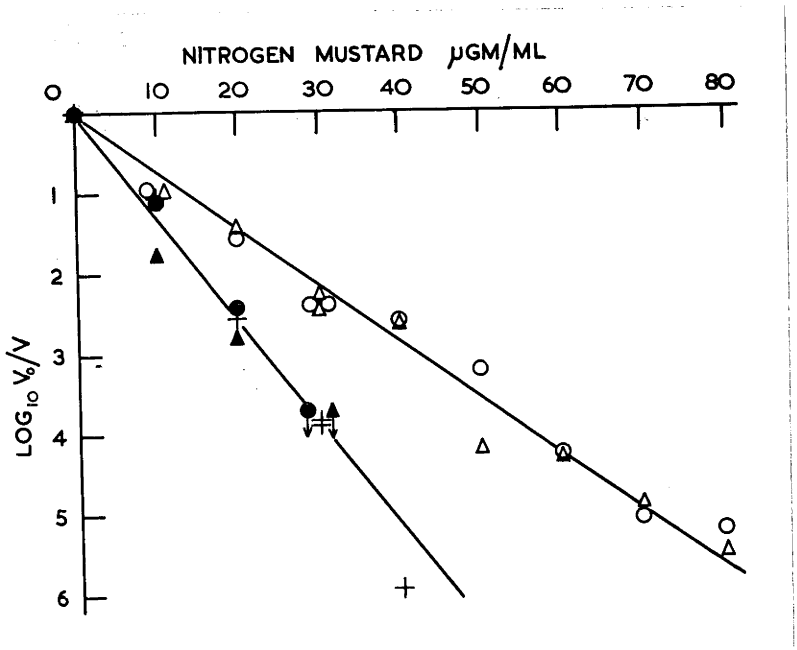

FIGURE 2.3. Inactivation of RPu$^+$ by HN2.

Assayed in the absence of heated virus: + , titre of U$^+$ pocks;

Assayed in the presence of heated RPu1: ● , titre of U$^+$ pocks; o , titre of U pocks;

Assayed in the presence of heated RPu8: ▲ , titre of U$^+$ pocks; Δ , titre of U pocks.

(U^+) pocks among the whites (U).

RPu$^+$ was inactivated by exposure to various concentrations of HN2 in gelatin McIlvaine's buffer for 20 minutes at 37°. The excess mustard was then destroyed by a tenfold dilution of the suspension into 5% sodium thiosulphate and further incubation for 2 hours. Particles capable of reactivating heated virus were assayed by inoculation of suitable dilutions of the material on the CAM together with about 5×10^8 particles of RPu1 or RPu8 heated at 60° for 12 minutes: the heated virus was completely non-infectious. Residual active particles of RPu$^+$ were also titrated in the absence of heated virus.

The results are shown in figure 2.3. Both the infectivity and the ability to reactivate heated virus are destroyed exponentially, but at different rates. A considerable proportion of particles whose infectivity is lost, retain the ability to reactivate heated virus. The u$^+$ genome disappears with the infectivity; it is not found in the reactivated virus. This supports the idea that the mustard inactivates the DNA primarily and that another constituent of the virus is responsible for the reactivation, which is therefore non-genetic in nature (Joklik, Abel & Holmes, 1960a). This essential constituent is also inactivated by the mustard, but at a considerably slower rate than the DNA of the genome. Experimental attempts to determine the chemical nature of the essential component of

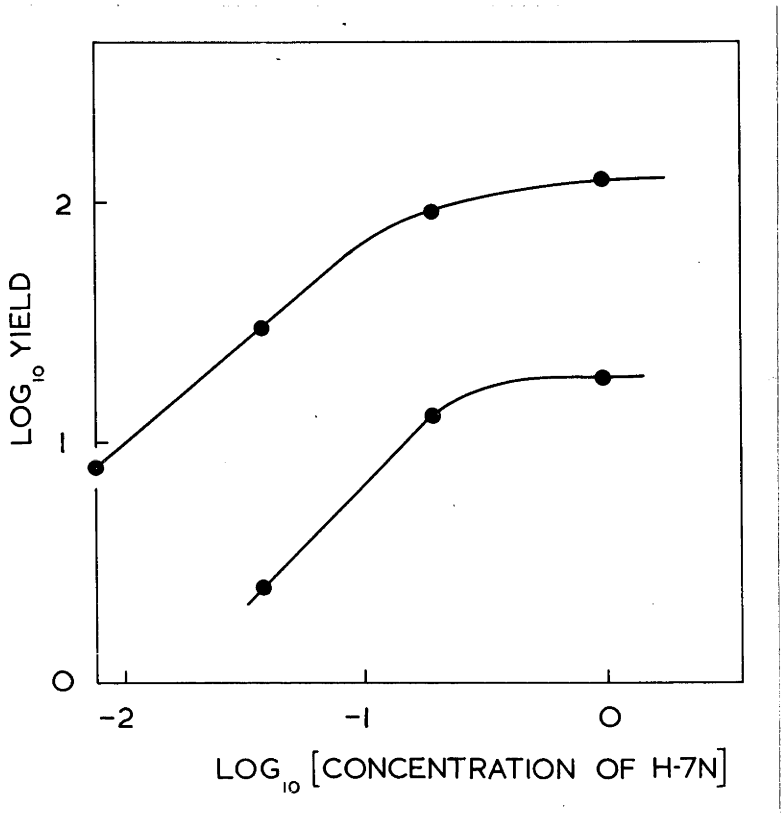

FIGURE 2.4. Effect of H-7N concentration on pock count in the assay of M-RP. The curves represent the yield of pocks obtained with two dilutions of an M-RP preparation.

mustard treated rabbitpox, or M-RP, are described in part 4.

e) Assay of M-RP

In assaying M-RP, the aim was to have every cell of the CAM containing particles of reactivable virus, so that every particle of reactivating agent adsorbed should produce a pock. Usually a white pock producing virus such as 7N was chosen as heated reagent because the "reactivated" pocks are frequently small and the large dose of heated virus necessary may cause some opacity in the CAM. Under these conditions white pocks are easier to count than haemorrhagic ones.

The effects on the pock count of varying concentrations of H-7N and M-RP are shown in table 2.1. For two concentrations of M-RP, the variation in pock count is plotted logarithmically against the relative concentration of H-7N in figure 2.4. It can be seen that not only is the count proportional to the dilution of M-RP, but also that with the highest concentrations of H-7N (undiluted stock, i.e. about 5×10^8 particles per CAM) the count appears to be reaching a maximum. There is also another line of evidence suggesting that the aim of the assay system has been attained. Figure 2.3 is drawn in such a way that both curves in it must pass through the origin. If, however, instead of showing changes in titre, it is drawn to show

TABLE 2.1. Effects of varying concentrations of M-RP and H-7N on pock counts obtained on the CAM.

		Dilution of H-7N			
		1/125	1/25	1/5	1
Dilution of M-RP	10^{-3}	8	30	96	130
	10^{-4}	0	2.5	13	19
	10^{-5}	not done		2.5	2.5

the actual infectivity and reactivating titres measured, the relationship of the curves is unchanged. The best-fitting line through the reactivating titres, when extrapolated back to zero concentration of HN2, coincides with the infectivity titre. Thus the efficiency of plating of reactivating particles in this system is equal to that of infectivity.

The experimental procedures adopted for the preparation and routine assays of M-RP are given in the Appendix.

f) Time Course of Reactivation by M-RP

It will be recalled that H-RP in the mouse brain or monolayer of HeLa cells retains its ability to be reactivated by ectromelia virus for up to 3 days. The persistence of the ability of M-RP in cells to reactivate H-7N was

determined in KB cells in the following way: KB monolayers in small tubes (cell area about 2.3 cm^2) were washed with Hanks' balanced salt solution and exposed to 0.05 ml inocula of suitable dilutions of M-RP, which were allowed to adsorb for 1 hour at 20°. At various later times 0.05 ml aliquots of H-7N (about 5×10^8 particles, non-infectious) were similarly adsorbed. After all adsorption periods the monolayers were washed with Hanks' balanced salt solution, covered to about 2 mm depth with medium, and replaced in the 37° incubator under 5% carbon dioxide. 24 hours after the second adsorption the medium was removed from the cells, replaced by 1 ml gelatin saline, and the tubes were frozen. After thawing and sonication to disrupt the cells, the yields of virus were assayed on the CAM.

Because of the low efficiency of adsorption in this cell system it was necessary to use a high-titre preparation of M-RP, which contained about 1% of residual active virus. Therefore replicate tubes were titrated individually, and any yielding U^+ (RP) pocks were excluded from the results. Only tubes yielding U type pocks alone were scored as positive for reactivation by M-RP.

The results are to be found in table 2.2. The effect of M-RP on the cell is shown to be a transitory one; the M-RP-cell complex remains capable of reactivating H-7N for only about 8 hours. Whereas heated virus appears to

TABLE 2.2. Time course of reactivation of H-7N by M-RP.

Adsorption of H-7N: (hours after M-RP)	0;	2;	4;	5;	6;	8;	10;	12;	24.
Fraction of tubes showing reactivation:	5/5;	2/2;	4/5;	3/3;	1/3;	1/3;	0/3;	0/5;	0/2.

remain passively awaiting reactivation, the entry of M-RP into a cell seems to initiate processes which will allow replication of viral DNA for a period of 8 hours. Whether the cell then returns to normal or is temporarily or permanently damaged, it is not possible to say; in any case it can no longer reactivate heated virus.

It would be interesting to know whether the events following entry into a cell of reactivable plus reactivating virus differ from those following normal infection. In his study of the latter, Cairns (1960) has shown that after a period of initiation and preparation, viral DNA and antigen are synthesised simultaneously. It remains to be determined whether M-RP can initiate formation of any viral components in the absence of heated particles. In the future reactivation may offer the possibility of resolving the infective process into parts for purposes of investigation.

g) Inactivation of H-RP by HN2

If the activity of H-RP is due to intact DNA, then H-RP should be as sensitive to inactivation by HN2 as active RP, and more sensitive than M-RP. (The upper curve in figure 2.3 represents not only the reactivating ability of mustard treated RP, but also the inactivation of M-RP by HN2.) In fact, the ability of H-RP to be reactivated by ectromelia is more sensitive to inactivation than active RP. Even when the reaction was terminated by the addition of thiosulphate, exposure for 20 minutes at 37° to 5 μg/ml destroyed 98% of the activity, and 10 μg/ml destroyed over 99.9%. It is possible that heating renders the particles more permeable to HN2 and thus more easily inactivated than those of unheated RP.

h) Discussion

With regard to the mechanism of HN2 inactivation it is notable that all trace of the viral genome disappears. This is not true only of vaccinia, since Loveless & Stock (1959b) remarked on the characteristic lack of ability to participate in cross reactivation of HN2-inactivated phage T2. This finding is consistent with the suggestion that the lethal reaction in HN2 inactivation is breakage of a DNA strand, since this would be expected to prevent replication

of the molecule as a whole.

Alexander et al. (1957) showed that the triesters formed when HN2 reacted with phosphate groups in DNA were unstable, but that hydrolysis nearly always occurred between the phosphate and the mustard residue, leaving the DNA undamaged. Very infrequently, splitting occurred at one of the sugar-phosphate linkages, causing a break in the DNA chain. It may be significant that with both vaccinia and T2, inactivation by HN2 appears to occur only after reaction of mustard molecules with two groups within the virus particle (cf. p.28), of which at least one seems likely to be a phosphate in a DNA chain. This observed result could be explained if reaction of the second functional group of the mustard made the phosphate triester more likely to split at one of the sugar linkages, thereby destroying the genetic activity of the DNA.

3. REACTIVATION BY VIRUS TREATED WITH ALKYLATING AGENTS OTHER THAN HN2

a) Aim

It was remarked previously, in connection with the results shown in figure 2.3, that HN2 destroyed both infectivity and reactivating ability of RP, though at different rates. If the assumption that reactivating ability depends on an intact, non-DNA viral component is correct, then destruction of reactivating ability by HN2 must be due to inactivation of this component. If a reagent could be found with a specificity for DNA greater than that of HN2, then it would be possible to inactivate virus without destroying so much of its reactivating activity, thus producing a non-infective reactivating agent with a higher reactivating titre than can be attained with M-RP.

b) Comparison of Mustards with Other Alkylating Agents

The alkylating agents considered by Ross (1953) and Alexander et al. (1957) to react similarly to mustards in biological systems included ethyleneimines, β-propiolactone, and epoxides. The reactions of epoxides in aqueous buffers were discussed in considerable detail by Ross in 1950. He pointed out that the mechanism of reaction of epoxides with anionic groups differed from that of mustards, in a way which might have biological significance.

With mustards, ionization is the rate limiting step, and combination with the anionic group or water ensues immediately. Since a change of covalency occurs in only one molecule, the mustard, this reaction is described as monomolecular, or of S_N1 type. The rates of S_N1 reactions (involving ionization) depend on the medium and are largely independent of the presence of groups with which the subsequent reaction will take place.

Epoxide reactions are of S_N2 type, or bimolecular. Reaction occurs only when the epoxide approaches the group with which it is going to react, so the rate of reaction depends on the concentration of these groups.

The reactions are summarised thus (Ross, 1953):

S_N1 $\quad RX \rightarrow R^+ + X^- \qquad R^+ + A^- \rightarrow RA$

S_N2 $\quad A^- + RX \rightarrow A..R..X \rightarrow RA + X^-$

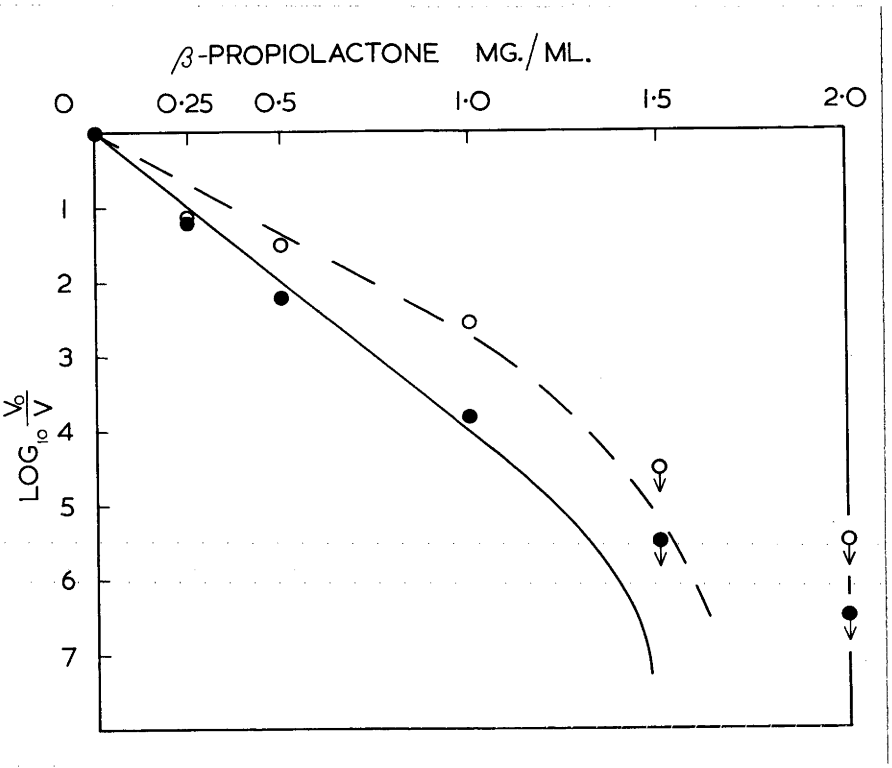

FIGURE 3.1. Inactivation of RP by β-propiolactone. Exposure 20 minutes at 37°, followed by dilution 1/10 into 0.1 M phosphate and 30 minutes further incubation at 37°.

● , infectivity titre;

o , reactivating ability, assayed in the presence of H-7N.

Since nucleic acids contain a high concentration of ionized phosphate groups, Ross suggested that reaction of epoxides with them might be "specially favoured".

Alexander et al. (1957) demonstrated the reaction of epoxides with DNA. They found that the proportion of epoxide reacting with water was greater than that of mustard, but it was still possible that epoxides would have less affinity than the mustards for protein groups.

c) Inactivation and Reactivating Activity of Virus Treated with Various Alkylating Agents

Reactivating ability was tested under the conditions given in the Appendix for titration of M-RP.

β-propiolactone

β-propiolactone has been shown to inactivate various viruses with little loss of antigenicity (LoGrippo, 1960). It is hydrolysed extremely rapidly in water forming propionic acid, and while this is an advantage in that no special treatments other than simple dilution and incubation are required to remove the excess after the desired reaction time, it makes accurate control of the inactivation rate difficult to achieve. Exposure of RP for 20 minutes at 37° to various concentrations of β-propiolactone in the presence of 0.05 M phosphate pH 7.0, followed by dilution 1/10 into 0.1 M phosphate and incubation for a further 30

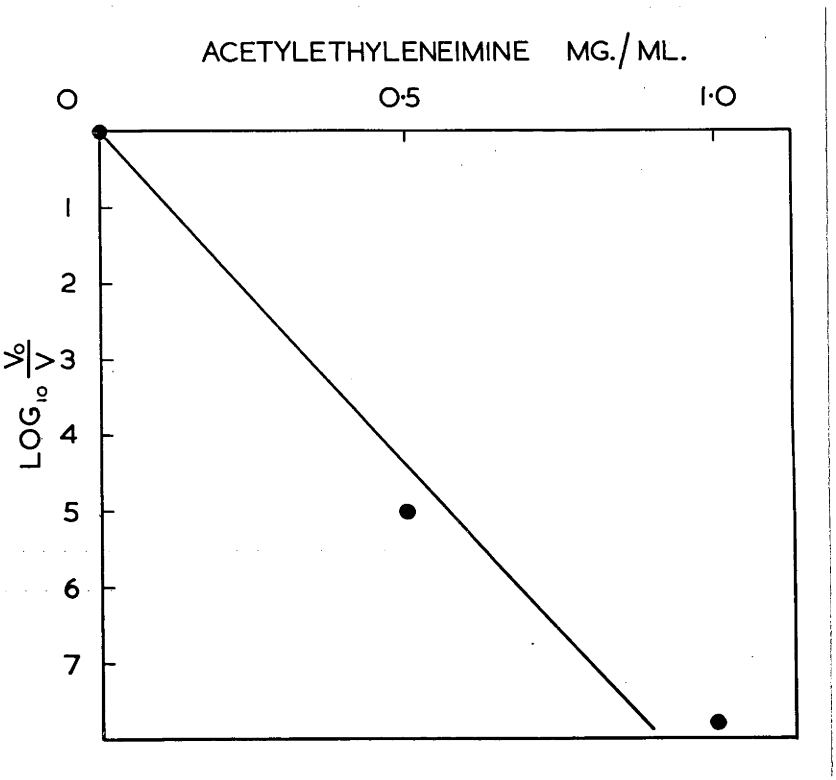

FIGURE 3.2. Inactivation of RP by acetylethyleneimine. Exposure 30 minutes at 37°. Samples freeze-dried before titration.

minutes, produced the inactivation shown in figure 3.1. The inactivation rate increases markedly at concentrations of β-propiolactone exceeding 1.0 mg/ml, suggesting that at concentrations below this level much of the reagent is hydrolysed before it can react with the virus. The treated virus possessed some reactivating activity, as the upper curve in figure 3.1 shows, but was inferior to M-RP.

Acetylethyleneimine

Difficulty was experienced in measuring inactivation due to this reagent because some product of its interaction with virus suspensions was highly toxic for the chick embryo. Acetylethyleneimine itself did not appear to be toxic when tested alone. The inactivation curve shown in figure 3.2 was obtained by exposing virus to the reagent for 30 minutes at 37°, then freeze-drying test samples before titrating them. Like washing by centrifugation, this reduced but did not eliminate the egg toxicity. Virus inactivated by acetylethyleneimine had about the same reactivating activity as that inactivated by β-propiolactone. Other conditions for inactivation were not tested, because of the troublesome toxicity.

Epoxides

(i) Epichlorohydrin

The inactivation of RP produced by incubation with various concentrations of epichlorohydrin was measured in the presence of 0.05 M phosphate, pH 7.0. Mixtures were

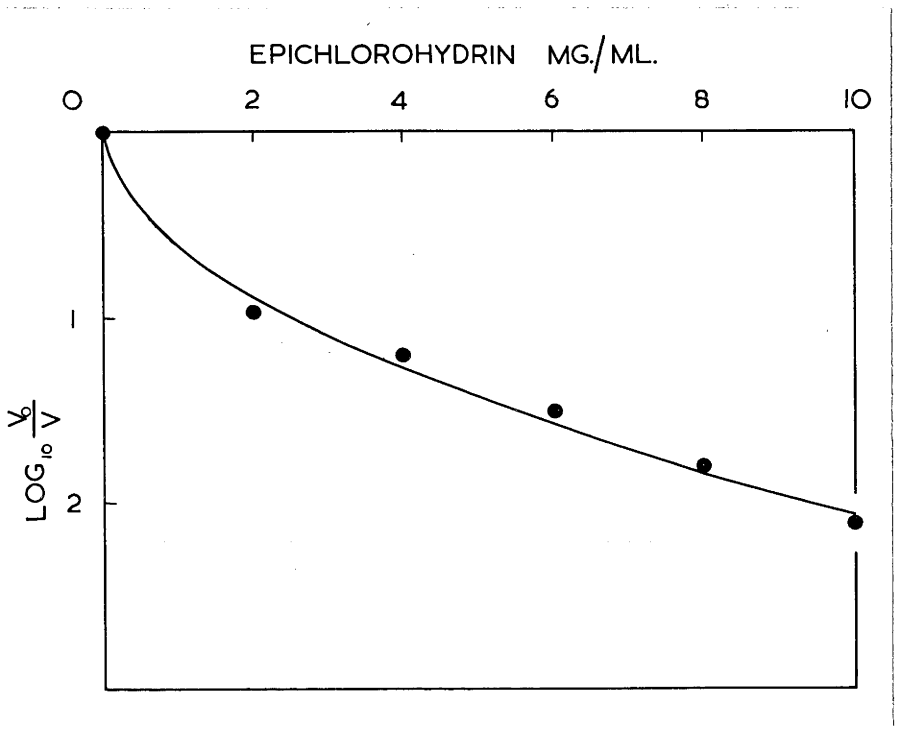

FIGURE 3.3. Inactivation of RP by epichlorohydrin. Exposure 30 minutes at 37°, followed by dilution 1/10 into gelatin saline and further incubation for 2 hours at 37°.

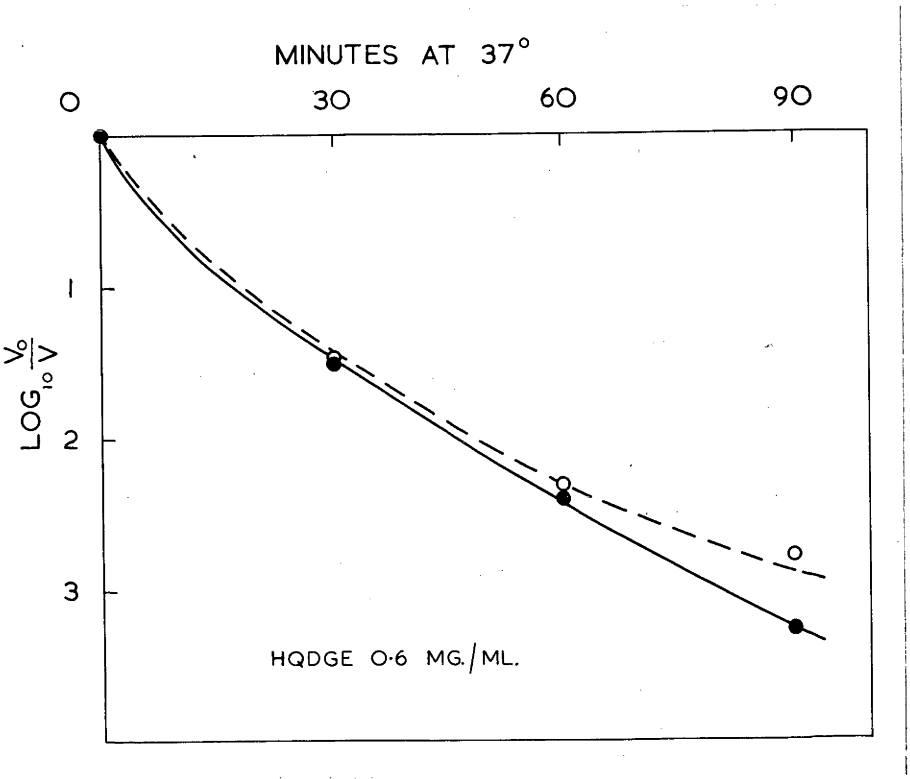

FIGURE 3.4. Inactivation of RP by hydroquinone diglycidyl ether, 0.6 mg/ml in 10% acetone. After exposure samples were diluted 1/100 in gelatin saline and incubated overnight at 37°.

● , infectivity;

o , reactivating ability, assayed in the presence of H-7N.

incubated at 37° for 30 minutes, then were diluted 1/10 into either gelatin saline or 5% sodium thiosulphate in gelatin saline and incubated a further 2 hours. The result is shown in figure 3.3. Inactivation was almost exponential, but low concentrations of epichlorohydrin caused proportionally more inactivation than higher ones; the reason for this is unknown.

Incubation with thiosulphate was found to have no effect on the degree of inactivation, though it would be expected to react with the excess (unattached) epichlorohydrin. Epichlorohydrin treated virus had no reactivating activity, whether or not it had been incubated with thiosulphate.

(ii) Hydroquinone diglycidyl ether

Owing to its low solubility in water, this reagent was dissolved in 10% acetone. Even so, 0.6 mg/ml was close to the limit of solubility. Virus exposed to this concentration at 37° for 30, 60 or 90 minutes, then diluted 1/100 in gelatin saline and incubated overnight at 37°, was inactivated approximately exponentially, as shown in figure 3.4. Treated virus had a very slight reactivating ability, shown in figure 3.4 by the broken curve.

(iii) Diglycidyl ether and diepoxybutane

These diepoxides can be considered together, since they are similar in structure and in reactivity. According to Ross (1953) the velocity constants of their

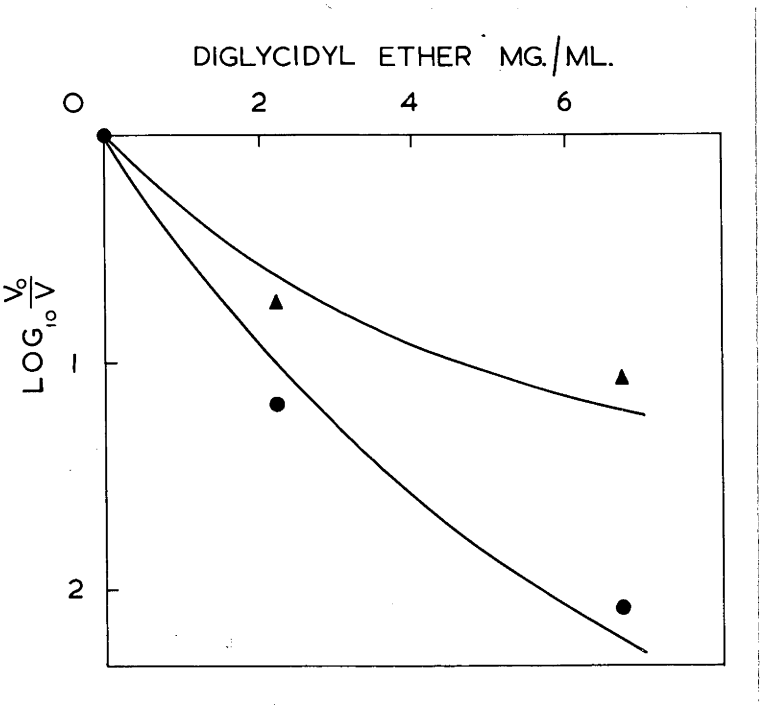

FIGURE 3.5. Inactivation of RP by diglycidyl ether. Exposure 30 minutes at 37°, followed by dilution 1/10 as below and incubation for 3½ hours at 37°.

▲ , dilution in 5% thiosulphate in gelatin saline;

● , dilution in gelatin saline.

reactions with water at 37° are identical. The solubility of diglycidyl ether in water is about 50 mg/ml, and diepoxybutane is completely miscible.

Exposure of RP to various concentrations of diglycidyl ether for 30 minutes at 37°, followed by dilution 1/10 and incubation for 3½ hours at 37° in gelatin saline alone or plus 5% sodium thiosulphate, produced the curves shown in figure 3.5. Little or no further inactivation was found if the incubation in thiosulphate was prolonged, but in plain gelatin saline the titre decreased about tenfold in about 6 hours. The sparing effect of thiosulphate with the diepoxide, in contrast to its lack of effect on inactivation by epichlorohydrin, is in agreement with the results Loveless & Stock (1959a) obtained with bacteriophage T2.

The decay of infectivity occurring after dilution of diepoxide-treated virus in gelatin saline was so slow that it seemed unlikely to seriously affect the results of inactivation experiments, provided that survivors were assayed within an hour or two of treatment. Accordingly in subsequent experiments dilution and incubation in thiosulphate or gelatin saline was omitted.

The curves in figure 3.6 were obtained by exposing RP for various times at 37° to diglycidyl ether at 10 mg/ml, or diepoxybutane at 8 mg/ml. Similar results were also obtained by incubating the virus for a fixed

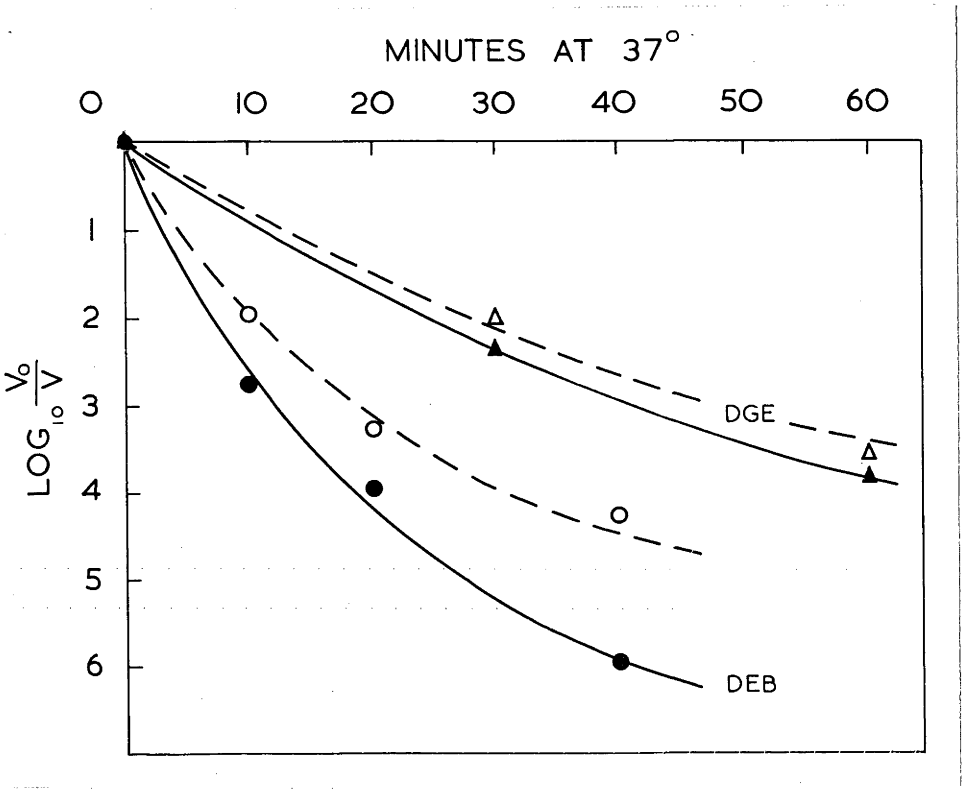

FIGURE 3.6. Inactivation of RP by diepoxides.
Diglycidyl ether at 10 mg/ml:
▲ , infectivity; Δ , reactivating ability.
Diepoxybutane at 8 mg/ml:
● , infectivity; o , reactivating ability.

time in various concentrations of the reagents. Residual active virus and reactivating activity were titrated immediately after the exposure. Slightly less reactivating activity was found when samples were incubated in 5% thiosulphate for 4 hours at 37° before titration.

Despite the identity of their chemical reactivities towards water, the two diepoxides differed in ability to inactivate RP. Diepoxybutane, being the smaller molecule, would be expected to have an advantage if inactivation depended on diffusion into the virus particle. The fact that 8 mg/ml diepoxybutane does have a significantly more rapid inactivating effect than 10 mg/ml diglycidyl ether, as figure 3.6 shows, suggests that the site of inactivation is within the particle.

These diepoxides also differed in their ability to produce viral reactivating agent. In this respect diglycidyl ether is comparable to hydroquinone diglycidyl ether, but inactivation of virus by diepoxybutane leaves a considerable proportion of particles capable of reactivating H-7N. All the same, even diepoxybutane is clearly less effective than HN2.

d) Discussion

Although it had been hoped that the epoxides at least might prove superior to nitrogen mustard for the preparation of a non-infectious reactivating agent, no other reagent even equalled its efficacy. The mustard treated product has a higher ratio of reactivating ability to infectivity than that produced by any other alkylating agent tested, and it can be rendered quite stable.

If, as has been assumed, the relationship between the rate of loss of infectivity and the rate of loss of reactivating ability reflects the relative affinity of the inactivating agent for DNA and for protein, then it definitely appears that HN2 has a greater specificity towards DNA than have the epoxides tested. The suggestion by Ross (1950) from theoretical premises that epoxides might be particularly specific is thus not supported by this experimental evidence.

4. CHEMICAL NATURE OF BIOLOGICALLY ACTIVE COMPONENTS : H-RP AND M-RP

In order to test the hypothesis that the biological activity of H-RP depends on intact DNA and that of M-RP upon an intact protein component, each type of preparation was exposed to the action of various physical and chemical agents. The effects of the treatments on the biological activities of H-RP and M-RP were compared with their effects on the infectivity of active RP, and differences which might provide an understanding of the essential chemical structures were sought.

a) Effects of Enzymes

As table 4.1 shows, deoxyribonuclease had no effect on the biological activities of M-RP, H-RP or RP.

During the work on H-RP and RP it was found that salt concentration affected their rate of inactivation by both trypsin and papain. The effect was most striking

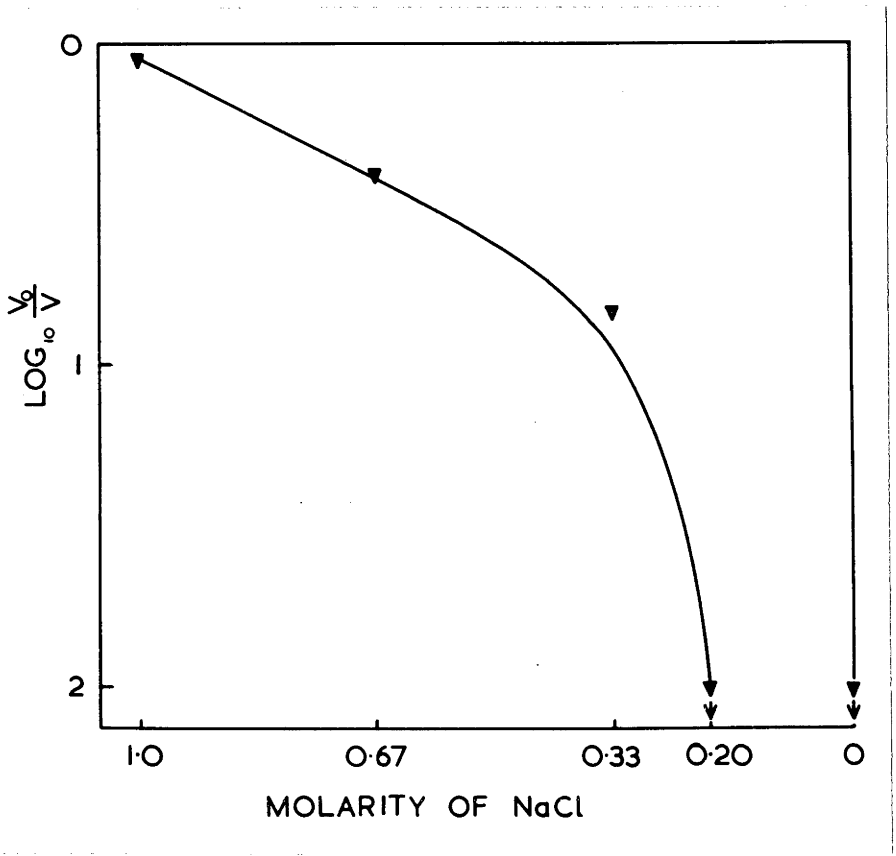

FIGURE 4.1. The effect of sodium chloride concentration on the inactivation of RP by papain. Reaction mixtures contained 0.02 M potassium cyanide, 0.02 M phosphate pH 7, 0.02% papain, 0.2% gelatin. Exposure 3 hours at 37°.

TABLE 4.1. Effects of enzymes on M-RP, H-RP and RP.

Enzyme	Concentration µg/ml	Time of incubation (min.)	Log_{10} control titre / residual titre		
			M-RP	H-RP	RP
Deoxyribonuclease[a]	100	30	0	0[b]	0
	10	30	0	...[c]	...
Trypsin[d]	200	60	0.7-2.0	0-1.0,0[b]	1.0-2.0,0.6[b]
	100	60	1.0	...	...
	10	60	0	...	...
Papain[e]	200	60	0.3-2.0	0.8-1.3	2.0,2.0[b]

All reactions were carried out at 37° in the presence of 0.15% gelatin.

[a] In 0.05 M acetate pH5.4, + 0.01 M Mg.

[b] Value from Joklik, Holmes & Briggs, 1960b.

[c] ... = not determined.

[d] In 0.05 M tris pH 8.5.

[e] In 0.02 M phosphate pH 7.0, + 0.02 M cyanide.

with papain; its action on RP in the presence of various concentrations of sodium chloride is represented in figure 4.1. 0.02% papain activated with cyanide caused no inactivation of RP in 3 hours in the presence of 1 M sodium chloride, whereas in salt concentrations below 0.2 M, inactivation was over 99%.

Although all later tests were carried out in dilute buffers to avoid this source of variation, reproducibility was poor. The results listed in table 4.1 were compiled from several experiments, and indicate the range of results

TABLE 4.2. Effects of protein reagents and denaturants

Compound	Molarity	pH	Buffer	Time (min.)	Temp. (°C)	Log_{10} control titre / residual titre M-RP	H-RP	RP
1-fluoro-2:4-dinitro-benzene	0.001	8.5	0.1 M tris	30	37	2.0	2.1	1.4
	0.0005	8.5		30	37	>4	>3	>4
p-hydroxy-mercuri-benzoate[a]	0.01	8.5	0.05 M tris	60	37	0, 0.4	2.4	3.8, 0.3[b]
	0.001	8.5		60	37	0	0	0
N-ethyl-maleimide	0.01	7.0	0.05M PO_4	30	37	0	0	0
	0.01	8.4	0.05 M tris	30	37	1.7	1.6	1.5
	0.01	8.7		30	37	>3.6	...[c]	>4.0
	0.001	8.5		30	37	0	0	0
thioglycol-ic acid[d]	0.01	7.0	0.01 M PO_4	60	37	0.3	0, 0[e]	0, 0[e]
	0.01	7.0		180	37	1.1	1.7, 1.7[e]	1.1, 2.0[e]
urea	6.0	7.0	0.02 M PO_4	90[f]	4	>4.9	>2.7	>6
	5.0	7.0		90	4	1.4	1.6	1.2
	4.0	7.0		90	4	0.95	0.5	0.9
guanidine hydrochlor-ide	2.0	8.5	0.05 M tris	90[f]	4	>4.9	3.8	>6
	1.0	8.5		90	4	1.3	1.8	0.6

[a] formerly known as p-chlormercuribenzoate.

[b] RP exposed to, but not inactivated by HN2, ie. residual active RP in an M-RP preparation.

[c] ... = not determined.

[d] under N_2 atmosphere.

[e] value from Joklik, Holmes & Briggs, 1960.

[f] afterwards dialysed 3 hours at 4° against 0.14 M NaCl, 0.02 M PO_4, pH 7.0.

obtained. No clear difference between the preparations emerged, though it may be said that the infectivity of RP was usually more susceptible to inactivation than the other two activities.

b) Protein Reagents and Denaturants

1-fluoro-2:4-dinitrobenzene

1-fluoro-2:4-dinitrobenzene reacts with free amino groups in proteins, though it can also react to some extent with thiol, phenolic hydroxyl and imidazole groups (Putnam, 1953). Amino groups in DNA are less likely to be accessible to it. As table 4.2 shows, it inactivates M-RP, H-RP and RP about equally, even at low concentrations.

p-hydroxymercuribenzoate

The results obtained with p-hydroxymercuribenzoate are noteworthy. This thiol reagent at a concentration of 0.01 M readily destroys the activity of H-RP and RP. M-RP, on the other hand, is almost unaffected under the same conditions (table 4.2). It was found that protection was afforded by even a sublethal exposure to HN2, since residual active virus in a preparation of M-RP was similarly resistant. Virus which had been exposed to β-propiolactone, hydroquinone diglycidyl ether, diepoxybutane or diglycidyl ether was not protected.

The protection of vaccinia by the alkylating

agent HN2 against p-hydroxymercuribenzoate can be explained by assuming that HN2 reacts with viral -SH groups, thereby preventing their combination with the mercurial. This involves the additional assumption that the infectivity of the virus is unaffected by alkylation of its available -SH groups whereas it is destroyed by their combination with mercuribenzoate. If this is the explanation, it is curious that none of the other alkylating agents tested provide similar protection.

There is some doubt, however, whether one is justified in assuming that inactivation of vaccinia by p-hydroxymercuribenzoate is due only to mercaptide formation. From the results given in table 4.2 it can be seen that while a concentration of 0.01 M causes considerable inactivation, 0.001 M is innocuous. For the purpose of demonstrating essential -SH groups, p-hydroxymercuribenzoate is usually employed at considerably lower concentrations. Sohler, Siebert, Kreke & Cook (1952) concluded that the inhibition of catalase caused by 3×10^{-4} M p-hydroxymercuribenzoate was nonspecific and due to protein denaturation, so this may well be the case with vaccinia also.

This latter conclusion is also supported by the fact that it did not prove possible to reverse the mercury toxicity by exposure of the treated virus to thiol compounds. It is a characteristic of enzyme inhibitions by mercaptide formation, that they are readily reversed (Dixon & Webb,

1958). Dialysis for 3 hours at 26° against 0.005 M cysteine or 0.0005 M 2:3-dimercaptopropanol (B.A.L.) brought about no recovery of infectivity of RP inactivated by 0.005 M p-hydroxymercuribenzoate. Higher concentrations of reversing agents could not be used, since even 0.005 M cysteine itself caused a small degree of inactivation, and 0.005 M B.A.L. inactivated control virus completely.

HN2 could prevent denaturation by crosslinking protein chains in strategic regions of the virus particle.

N-ethylmaleimide

At pH 7 N-ethylmaleimide has been said to be a specific reagent for thiol groups in proteins (Fraenkel-Conrat, 1957). More recently Smyth, Nagamatsu & Fruton (1960) have shown that it also reacts with the imidazole portion of histidine, which catalyses its polymerisation. The polymer has a deep red colour in alkaline solution. Gregory (1955) showed that the rate of decomposition of N-ethylmaleimide to N-ethylmaleamic acid, and also of its reaction with glutathione, increase with alkalinity. Gregory noted that at pH 9 inactivation of myokinase by N-ethylmaleimide was more rapid than at pH 7.5, and that the same degree of inactivation was achieved with a lower concentration of reagent.

The effects of pH on the inactivation of vaccinia by N-ethylmaleimide are to be found in table 4.2. 0.01 M N-ethylmaleimide at pH 7 caused no loss in activity of

M-RP, H-RP or RP, whereas all were inactivated considerably at pH 8.4, and completely at pH 8.7. At the end of the reaction time at pH 8.4 or over, the mixtures were distinctly red. Whether it is the polymer which inactivates the virus, or whether the increased reactivity of the N-ethylmaleimide in alkaline solution causes both the inactivation and the polymerisation, is not clear.

Thioglycolic acid

Table 4.2 also shows the inactivating effect of thioglycolic acid, which splits disulphide linkages. Though 0.01 M has little or no inactivating effect in the first hour of incubation, significant inactivation has occurred after 3 hours, with M-RP, H-RP and RP approximately equally affected.

Urea and guanidine hydrochloride

Urea and guanidine hydrochloride are protein denaturants which break hydrogen bonds and, in the case of urea at least, disulphide bridges (Resnick, Carter & Kalnitsky, 1959). Urea is of special interest because poxviruses inactivated by 6.7 M urea at 4° are reactivable in the same way as heated virus (Joklik, Holmes & Briggs, 1960b). The results in table 4.2 indicate that under the experimental conditions the amount of inactivation increases sharply with concentration of urea between 5 M and 6 M, and with concentration of guanidine hydrochloride between 1.M and 2 M. M-RP, H-RP and RP are equally susceptible to de-

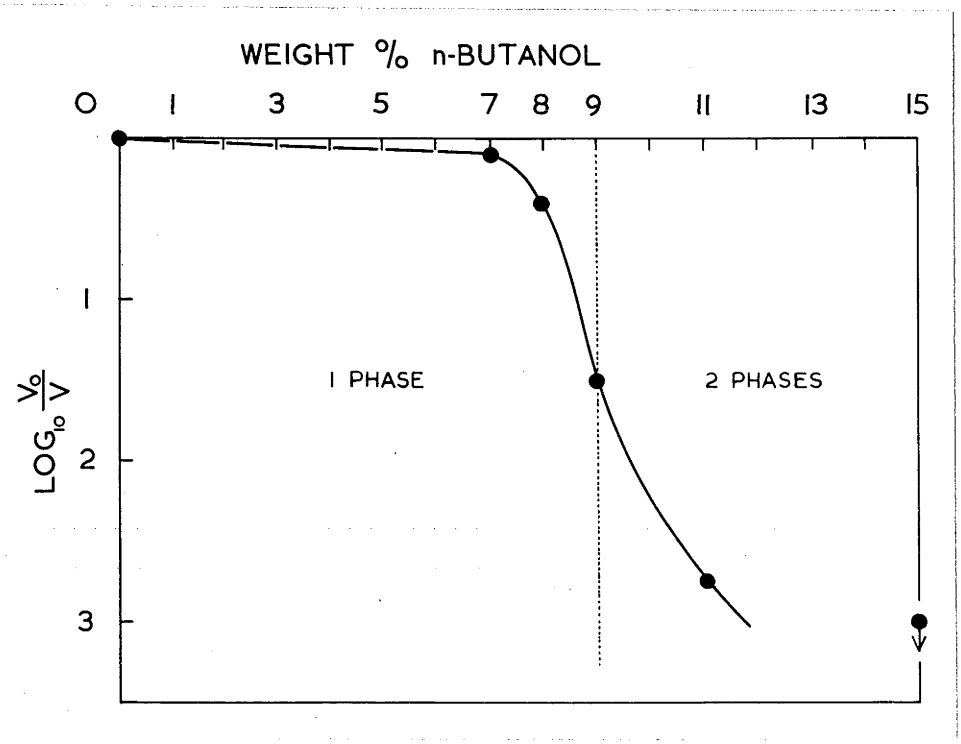

FIGURE 4.2. Inactivation of RP by exposure for 30 minutes at 0° to various concentrations of n-butanol.

naturation by these reagents.

c) Organic Solvents

The inactivation caused by exposure for 30 minutes at 0° to acetone and various alcohols is listed in table 4.3. All the solvents tested had similar effects on M-RP, H-RP and RP. While at 50% v/v iso-butanol inactivated strongly, at 15% v/v it was innocuous. Only the butanols caused considerable inactivation at 15% v/v. As this concentration exceeded their solubility, a butanol phase was present in the reaction mixture. The inactivating effect for RP decreased sharply as the concentration was reduced below the saturation point, as figure 4.2 shows.

n-butanol is known to have a specific disrupting effect on phospholipid-protein complexes, but causes very little protein denaturation at 0° (Morton, 1950). Morton mentions that for efficient extraction of phospholipids from wet preparations a two phase (alcohol/water) system is usually necessary, and the results in figure 4.2 show that the inactivation of vaccinia is also much more marked in the two-phase system. This virus-inactivating effect of n-butanol (and to a lesser extent of iso-butanol) under conditions where the lower alcohols were without effect is therefore good evidence for a phospholipid-containing component essential for the activity of M-RP, H-RP and RP.

TABLE 4.3. Effects of organic solvents on M-RP, H-RP and RP.

Compound	Log_{10} $\frac{\text{control titre}}{\text{residual titre}}$ after 30 min. at 0° in:					
	50% v/v[a]			15% v/v		
	M-RP	H-RP	RP	M-RP	H-RP	RP
Methanol	0.3	0.0	0.6	...[b]	...	0.1
Ethanol	1.1	0.1	1.1	0.0	0.0	0.2
iso-propanol	3.3	2.0	2.3	0.0	0.0	0.0
iso-butanol	...	...	...	1.1	2.3	2.1
n-butanol	2.3	2.7	3.0	2.5	3.5	3.0
Acetone	0.7	0.0	0.7	...	...	0.0

[a] The solubility of the butyl alcohols is about 12% v/v.

[b] ... = not tested.

Phospholipid has been found associated with vaccinia by McFarlane, McFarlane, Amies & Eagles (1939) but has not been shown to be an essential part. Since electron micrographs of butanol-treated virus showed particles of normal size and shape with slightly "blistered" outlines, and phospholipoproteins are frequently components of membranes, it is suggested that butanol destroys an outer membrane of the virus particle. This may prevent it from entering a cell or, once inside, may allow it to be completely digested. That an outer membrane exists has been demonstrated clearly in morphological studies by Epstein (1958) and Peters (1959).

d) Surface-Active Compounds

The lowest concentration at which sodium dodecyl sulphate will inactivate vaccinia or its derivatives varies to a slight extent with the salt content of the solution. The presence of sodium chloride would be expected to increase the formation of micelles of dodecyl sulphate which may inactivate more than the anion itself (Wills, 1954). In the absence of salt, H-RP appears to be more resistant than M-RP or RP to 0.0002 M sodium dodecyl sulphate, but at the same concentration in the presence of 0.5 M sodium chloride both H-RP and RP are considerably inactivated (table 4.4). At 0.001 M even in the absence of salt, all are completely inactivated.

Sodium dodecyl sulphate is classed as a very powerful protein denaturant; it dissociates plant viruses such as tobacco mosaic virus, tomato bushy stunt virus and potato "X" virus into protein and RNA, and remains electrostatically bound to the protein molecules (Putnam, 1948). Complete disintegration of bacterial cell walls and cytoplasmic membranes by sodium dodecyl sulphate may be attributable to a detergent action on lipid components (Pethica, 1958). Thus it cannot be said with certainty whether dodecyl sulphate inactivation of vaccinia is due to protein denaturation or loss of lipid, or both.

TABLE 4.4. Effects of surface-active agents on M-RP, H-RP and RP.

Compound	Molarity	Minutes at 37°	Log_{10} control titre / residual titre		
			M-RP	H-RP	RP
Sodium dodecyl sulphate[c]	0.001	60	3.0	3.0[a]	6.0
	0.0002	60	3.0[b]	0.1	2.0
	0.0002 + 0.5 M NaCl	60	...	2.0	3.0
	0.00004	60	0	0[a]	0
Sodium deoxycholate[d]	0.0014	120	2.2	1.4,1.3[a]	1.0,1.5[a]
	0.0007	120	0	0,0[a]	0,0[a]

[a] Value from Joklik, Holmes & Briggs, 1960b.

[b] ... = not determined.

[c] Tested in 0.01 M tris pH 7.0.

[d] Tested in 0.02 M phosphate pH 7.0 + 0.5 M NaCl.

Inactivation of vaccinia by sodium deoxycholate requires a salt concentration of about 0.5 M, and pH about 7.0. No inactivation occurs in 0.1 M sodium chloride, or at pH 5.8 or 8.5. At pH 5.8 the sparingly soluble deoxycholic acid (pK = 6.5) is precipitated, whereas the inactivity at pH 8.5 is presumably due to the effect of pH on the surface of the virus particle. M-RP, H-RP and RP are inactivated equally by an 0.0014 M concentration, as is shown in table 4.4.

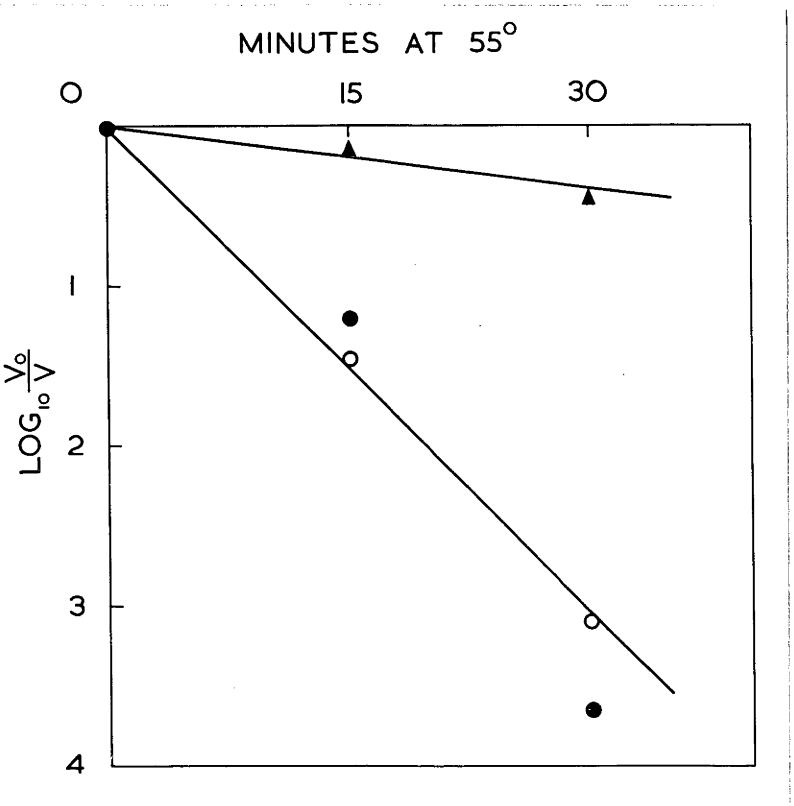

FIGURE 4.3. Effect of heating at 55° on the biological activities of H-RP, M-RP and RP.

▲ , reactivability of H-RP, assayed in the presence of ectromelia virus;

o , reactivating ability of M-RP, assayed in the presence of H-7N;

● , infectivity of RP.

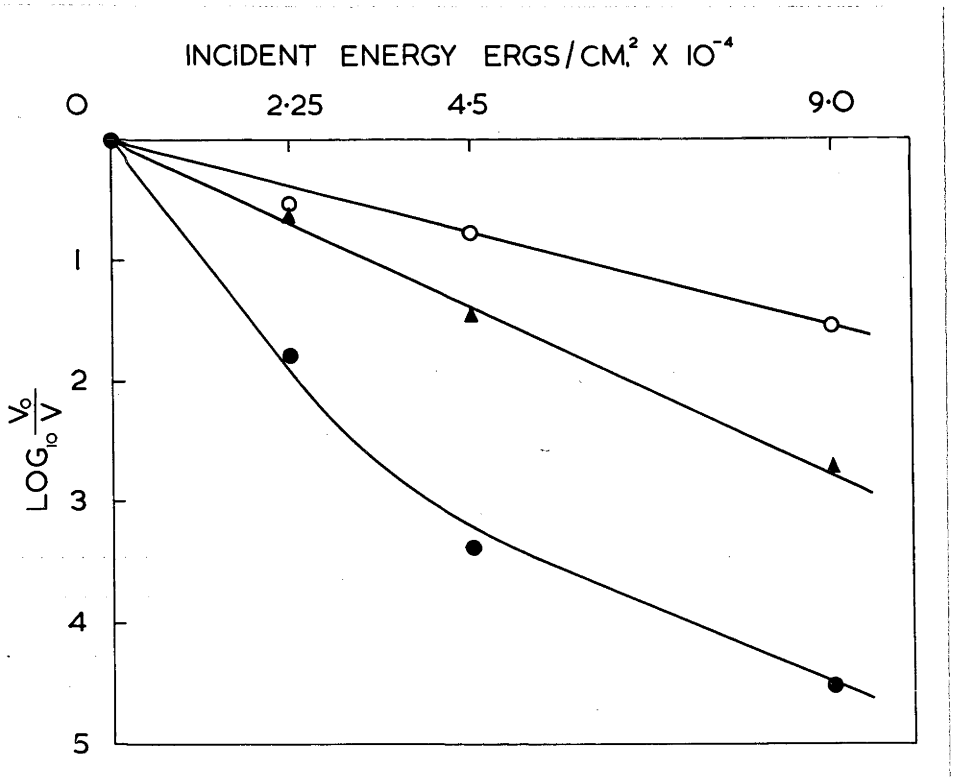

FIGURE 4.4. Inactivation of M-RP, H-RP and RP by ultraviolet irradiation.

○ , M-RP, reactivating ability;

▲ , H-RP, reactivability;

● , RP, infectivity.

e) Effects of Physical Agents

Heat

In figure 4.3 the sensitivity of M-RP to heating at 55° is compared with that of H-RP and RP which had been similarly incubated with thiosulphate and dialysed (see Preparation of M-RP, in Appendix). Each was made 0.05 M with respect to phosphate buffer, pH 7.0. The M-RP was inactivated at the same rate as RP, while H-RP was almost unaffected.

pH

When exposed to buffers for 30 minutes at 24°, M-RP, like H-RP and RP was stable within the pH range 4.5 to 10.5. At pH 3.6 or 11.4, all were at least 99% inactivated.

Ultraviolet irradiation

M-RP and H-RP proved to be only about half as sensitive as RP to inactivation by ultraviolet (UV) irradiation. The inactivation curves are shown in figure 4.4. Those of M-RP and H-RP are typical exponential curves but RP shows a change of inactivation rate at about 10^{-2} survival. This has also been observed consistently when RP was irradiated in other diluents (P. Abel, personal communication) but it is at present unexplained.

Hanafusa (1960) found no difference between the rates of UV inactivation of heated and active vaccinia

(strain IHD). This requires some explanation, since his other results are entirely in accord with those obtained here. The inactivation curves already given (figure 4.4) for H-RP and RP were obtained using gelatin McIlvaine's buffer as diluent, and were reproducible. It is possible, though it seems unlikely, that the difference in results is due to the use of different virus strains. Once again it is possible, though unlikely that Hanafusa's active virus preparation was protected by its diluent (see below) more than his heated preparation. The most probable cause of the divergence is that our assay system is quite different from those used by Hanafusa. The methods of assay have not been directly compared, and while they appear to give qualitatively similar results, there may be quantitative differences sufficient to account for the apparent divergence.

(i) Compounds protective against UV irradiation

The inactivation curves in figure 4.4 were obtained using gelatin McIlvaine's buffer as virus diluent. A different inactivation rate was observed in gelatin saline, so some study of the effect of suspending fluid on UV inactivation was undertaken.

The mechanism of inactivation of viruses by UV irradiation is not known. One cause of damage to DNA may be the crosslinking of adjacent thymine molecules to form dimers (Beukers & Berends, 1960). Influence of the suspending medium on inactivation by X-irradiation is well known,

TABLE 4.5. Inactivation of rabbitpox virus in various diluents by 2537 A^{o} ultraviolet irradiation (dose = 4.5×10^{4} erg.cm^{-2}) compared with the ultraviolet absorption of the diluents at the same wavelength.

DILUENT	CONCENTRATION	ABSORPTION DU/CM[a]	$\log_{10} \frac{Vo}{V}$	INACTIVATION % OF CONTROL
Gelatin McIlvaine's buffer	0.15%[b]	0.22	3.76	100
Same + adenine	10^{-3} M	9.4	1.62	0.8
	10^{-4} M	0.94	2.86	13
Same + AET[c]	10^{-2} M	0.92	2.80	11
Same + dimethyl-aniline	10^{-3} M	10.0	2.52	6
	10^{-4} M	1.0	3.02	19
Same + glutathione	10^{-3} M	0.08	3.50	55[d]
Same + MEA	10^{-2} M	0.15	3.36	40[d]
	10^{-3} M	0.02	3.90	100
Gelatin in water	0.5%	0.59	3.60	70
Gelatin saline	0.5%[b]	0.70	2.55	6
Gelatin saline[e]	0.5%	0.70	1.0	0.2

[a] UV absorption at 2537 A^{o}, measured in a Beckman Spectrophotometer.

[b] Concentration of gelatin. It is the only constituent which absorbs radiation at 2537 A^{o}.

[c] Inactivates virus at 10^{-1} M.

[d] These levels of protection are of doubtful significance.

[e] Virus stored in gelatin saline for 3 weeks at 4^{o}.

where the indirect inactivation mediated by short-lived radicals can be eliminated by the use of protective compounds. For protection of bacteriophage against X-irradiation damage even 0.01% gelatin is effective (Luria & Exner, 1941). Mercaptoethylamine (MEA) and 2-aminoethylisothiuronium bromide hydro-bromide (AET) are highly active against free radicals (Doherty & Burnett, 1955). MEA does not protect vaccinia against UV inactivation and AET is effective only at a concentration where its own UV absorption is considerable. Thus there is no evidence that UV irradiation is inactivating vaccinia by the X-ray type of indirect effect.

Alexander & Moroson (1960) reported that protection of oxygenated solutions of DNA against the effects of UV was provided by certain compounds which were also active against the indirect effects of X-rays, for example dimethylaniline (at 10^{-5} M) or gluthathione (at 10^{-4} M). With vaccinia protection was found only with greater concentrations of these agents, and the amount of protection was related to the UV absorption of the compounds at 2537 A^{o}. As table 4.5 shows, this was also true in the case of AET, MEA and adenine. In fact adenine, which was tested only because it had a high extinction coefficient at 2537 A^{o} and had not been listed as a chemical protective agent, proved to be the best of all in this system.

Though the evidence in table 4.5 suggests that simple shielding of the virus from the radiation by com-

TABLE 4.6. Inactivation by ultraviolet irradiation of rabbitpox in gelatin saline or its constituents.

DILUENT	$\log_{10}\frac{Vo}{V}$	% INACTIVATION OF GEL.McI. BUFFER
Gelatin McIlvaine's buffer	3.40	100
Gelatin saline	2.63	17
0.5% gelatin (in water)	3.60	100
0.5% gelatin + sodium chloride[a]	2.25	7
0.5% gelatin + + calcium & magnesium[a]	3.23	70
0.5% gelatin +borate[a]	3.43	100
0.5% gelatin + sodium chloride + calcium & magnesium	2.40	10
0.5% gelatin + sodium chloride +borate	2.17	6
0.5% gelatin + calcium & magnesium +borate	3.40	100
0.5% gelatin + sodium chloride + calcium & magnesium +borate	2.43	11

[a] Final concentrations as in gelatin saline (see Appendix).

pounds present in the suspending fluid is one mechanism of protection, this is not to say that others do not exist. Glutathione appears to have more protective effect than would be expected on the basis of mere absorption, and the protective effect of gelatin saline does not fit into the scheme at all.

Gelatin is the only constituent of gelatin saline which absorbs radiation appreciably at 2537 A^{o}, and even then part of its "absorption" may be only due to light scattering, which would not protect virus. Since gelatin alone seemed to afford little or no protection, it was tested with various combinations of the other constituents of gelatin saline, final concentrations being always those listed in the Appendix. It is clear from the results in table 4.6 that the essential protective combination was 0.5% gelatin plus 0.14 M sodium chloride. Later experiments showed that the virus was also protected in 0.5% or even 0.15% gelatin by the presence of 0.14 M sodium chloride, potassium chloride, calcium chloride, magnesium sulphate or sodium phosphate. The addition of these salts to 0.5% gelatin solutions caused only slight changes in the measured UV absorption.

Thus vaccinia is less susceptible to UV irradiation in the presence of a moderate concentration of various salts, than in gelatin McIlvaine's buffer, which has a very low ionic content. Since vaccinia is not osmotically shockable,

and hence is permeable to small molecules, its content of associated ions probably varies with its suspending fluid. This is known to occur with the permeable bacteriophages T3, T5 and P22 (Ames & Dubin, 1960). The difference found between virus diluted into gelatin saline and that which had been stored in it for some time (table 4.5) suggests that some time is required for the attainment of equilibrium. Some of the cations present are likely to be associated with the phosphate groups of the DNA, and it is conceivable that it is this factor which governs the UV sensitivity of the particle.

(ii) Biological activity of UV-RP

In view of the inactivation curves of M-RP, H-RP and RP (figure 4.4) it seemed necessary to reconsider the question of the biological activity of UV inactivated RP (UV-RP), which has previously been mentioned only in passing (p.23).

M-RP and H-RP are inactivated at approximately equal rates, and the rate of loss of infectivity of RP is approximately the sum of the two. This suggests a simple model such as the following: RP contains two components necessary for infectivity. The inactivation of one of these by heat or of the other by treatment with HN2 gives reactivable or reactivating reagents respectively. These reagents are equally susceptible to UV irradiation, hence it is likely that the components themselves are equally susceptible.

Since the inactivation of either component will destroy the infectivity of the virus, the inactivation rate of RP should be the sum of the inactivation rates of M-RP and H-RP, as observed.

On this model, however, UV irradiated suspensions of RP should contain many particles in which only one or the other component has been inactivated. Of these, about half should be reactivable, and the remainder should have reactivating activity. Their titres for a given dose of radiation should correspond to the original titre of RP less the observed inactivation of H-RP or M-RP. Thus for example after irradiation to the extent of 9×10^4 erg.cm^{-2} reactivable and reactivating particles should outnumber residual active ones by 100 to 1, yet none could be detected. It is possible that the efficiency of plating of reactivability is only 1/1000, but the assay of reactivating ability appears to be as efficient as that of infectivity (p.34) and M-RP-like activity should have been easily detectable. Hanafusa (1960) also tested for such activity without success.

The reason why the simple model does not hold remains obscure; possibly in the infective particles UV inactivation of either component is not independent of inactivation of the other.

f) Summary of Protein-DNA Hypothesis of Reactivation

It seems that in most of the experiments just described, superficial similarities of M-RP and H-RP have masked their essential differences. In M-RP and H-RP two biological functions of RP have been resolved, but the physical particles have not. Particles whose coats have been too grossly affected by chemical or enzyme may fail to gain access to the cell, or once inside, may be broken down, so losing their biological activity by a nonspecific process. This would account for the results obtained with proteolytic enzymes, protein reagents, organic solvents and surface-active agents. Deoxyribonuclease has no action on H-RP because as in RP, the DNA is inaccessible. UV irradiation scarcely distinguishes between H-RP and M-RP, besides which its relative specificity for inactivation of DNA or protein is an unknown quantity.

It has thus not been possible to add greatly to the evidence at hand at the outset: that of the nature of the treatments which yield H-RP or M-RP.

From the identity of the heat inactivation curves of RP and M-RP (figure 4.3) one can infer that a common component is responsible for infectivity of the former and for reactivating ability of the latter. Since it is inactivated by heating at 55° or 60° and this treatment does not destroy the viral genetic material, this component is

likely to be protein rather than DNA.

On the other hand, the known chemical properties of HN2, and the destruction of infectivity and disappearance of the viral genetic markers upon treatment with HN2 at levels comparable with those known to inactivate biologically active DNA (Haemophilus transforming principle; Zamenhof, Leidy, Hahn & Alexander, 1956) together suggest that in this case it is the DNA which is primarily attacked.

It therefore seems justifiable to conclude that reactivable virus contains an intact complement of DNA, and that reactivation is effected by the intervention of another component, probably protein in nature, supplied by infective or mustard inactivated particles.

APPENDIX

This includes only practical details, for example sources of materials, formulae and procedures recommended for the repetition of work described in this thesis. Details of other procedures etc. not listed here will be found in the text where they are first mentioned.

Diluents

Gelatin McIlvaine's buffer. Modified from McIlvaine (1921).

	g/l	M
$Na_2HPO_4.2H_2O$	0.65	0.0036
citric acid.H_2O	0.038	0.00018
gelatin	1.5	---

The final pH is about 7.2. Special gelatin (see Reagents) is used. After sterilization by autoclaving, this was used in the preparation of virus suspensions and in all treatments which called for a low concentration of phosphate.

Gelatin saline. From Fenner (1958).

	g/l	M
NaCl	8.0	0.14
$CaCl_2.6H_2O$	0.06	0.00027
$MgCl_2.6H_2O$	0.17	0.00084
H_3BO_3	1.2	0.019
$Na_2B_4O_7.10H_2O$	0.05	0.00013
gelatin	5.0	---

The pH is 7.2. For gelatin see Reagents. This was used as diluent in all virus titrations.

Hanks' balanced salt solution. From Dr.J.H. Hanks, cited by Weller & Enders (1948).

	g/l	M		g/l	M
NaCl	8.0	0.14	$CaCl_2$	0.14	0.0013
KCl	0.4	0.0054	Na_2HPO_4	0.06	0.00042
$MgSO_4.7H_2O$	0.1	0.00041	KH_2PO_4	0.06	0.00044
$MgCl_2.6H_2O$	0.1	0.00049	glucose	1.0	0.0056

For each litre, 5 ml of 0.4% aqueous phenol red solution is added and the solution is sterilized by autoclaving at 10 lb pressure for 10 minutes.

Experimental Animals

Chick embryos. Fertile eggs were obtained from local sources. After 11 or 12 days incubation at 38°, they were prepared for inoculation on the CAM by the method of Westwood, Phipps & Boulter (1957).

Mice. Mice were bred in the University Animal Breeding Establishment from stock originating from the Walter and Eliza Hall Institute, Melbourne. Intracerebral inoculation of 0.03 ml volumes of virus was carried out on 5-week-old mice under ether anaesthesia.

H-RP, (or H-7N), preparation

Virus, prepared and purified as described under the heading Infective virus, was sealed in 2 ml ampoules and submerged in a water bath held at 60°. After being heated for 14 minutes, ampoules were rapidly cooled and stored at 4°. Heating was carried out within a few hours of the preparation of stock suspensions to ensure exponential inactivation (see pp.8 & 9). Absence of infectivity was checked by inoculation of 0.05 ml samples on the CAM

H-RP (or H-7N), titration of reactivability

0.05 ml aliquots of dilutions of reactivable virus, plus $10^{4.5}$ to $10^{5.0}$ PFU of ectromelia Dohi A were inoculated on the CAM of 11 or 12 day chick embryos. Pocks were counted two days later.

Infective virus, preparation of stocks

Confluent membranes were prepared by inoculating about 2 x 10^4 PFU of active virus per CAM and harvesting after 2 days (for vaccinia) or 3 days (for ectromelia).

Extraction and purification of virus from the confluent CAM was carried out by the following modification of the fluorocarbon method of Gessler, Bender & Parkinson (1956). 20 CAM with 20 ml of Genetron were extracted successively with 40 ml, 40 ml and 30 ml of gelatin McIlvaine's buffer. Each time the mixtures were homogenized, centrifuged for 10 minutes at 600g, and then the aqueous (virus containing) phase was retained and the Genetron phase re-extracted. During extraction the homogenizer vessel was cooled by an ice bath, and refrigerated (4°) centrifuges were used. After the third extraction the Genetron phase was discarded and the aqueous phases were pooled.

The virus in the pooled supernatants was sedimented in 10 minutes at 15,700g, and the resulting supernatant fluid was discarded. The deposits plus 8 ml of Genetron were extracted twice as above with 16 ml portions of gelatin McIlvaine's buffer, and once again the aqueous phases were pooled and the Genetron phase discarded.

The virus-containing fluid was finally centrifuged for 15 minutes at 2250g and the small pink deposit was discarded. The supernatant is found to have a volume of about 35 ml and (with vaccinia) to contain about 3 x 10^9 PFU/ml.

Unless it was to be used for preparation of reactivable virus, the stock was ampouled, frozen in ethanol-dry-ice, and stored in a dry-ice box.

Infective virus, titration

0.1 ml amounts of suitable dilutions of virus were inoculated on the CAM of 11 to 12 day chick embryos, prepared as described above. Pocks of vaccinia were counted after 2 days further incubation at 36°, those of ectromelia after 3 days. Titres were expressed as pock forming units (PFU) per ml.

M-RP, preparation

Suspensions of RP to be converted into M-RP were prepared as described above under Infective virus. Di(2-chloroethyl)methylamine (HN2) hydrochloride was dissolved in iced distilled water to give a solution containing about 1 mg/ml. This was immediately added to the purified virus suspension to give a final concentration of 35 µg/ml (about 0.0002 M). The mixture was incubated at 37° for 20 minutes, then the unreacted mustard was destroyed by a tenfold dilution of the suspension into gelatin saline containing 5% sodium thiosulphate and further incubation at 37° for 2 hours. The thiosulphate was dialysed away at 4° against 0.01 M tris buffer pH 8.5 for 2 to 4 hours.

The resulting suspension is stable at 4° for months. It contains about $10^{5.7}$ PFU/ml of reactivating

ability and about $10^{3.7}$ PFU/ml of residual active virus. The thiosulphate is apparently not toxic to the CAM but unless it is removed the stability of the M-RP is reduced to a few days at 4^{o}.

M-RP, titration of reactivating ability

0.1 ml aliquots of suitable dilutions of M-RP, together with 0.05 ml of undiluted H-7N (about 5×10^{8} particles) were inoculated on the CAM of 11 or 12 day chick embryos. Pocks were counted after 2 days. Residual active virus was distinguishable qualitatively in this system, though not always quantitatively, as a proportion of the pocks it produced were white instead of haemorrhagic.

Reagents

All enzymes used were crystalline preparations from the Worthington Biochemical Corporation.

Acetylethyleneimine and β-propiolactone were obtained by courtesy of Dr. E. Weston Hurst, of Imperial Chemical Industries Ltd.

2-aminoethylisothiuronium bromide hydrobromide (AET) is obtainable from Schwarz Bioresearch, Inc.

Di(2-chloroethyl)methylamine (HN2) was obtained by courtesy of Dr. A.J. Roemmfeldt, Defense Standards Laboratories, Ascot Vale, Victoria.

Diepoxybutane and diglycidyl ether were from Bios Laboratories, Inc.

Dimethylaniline, epichlorohydrin and 1-fluoro-2:4-dinitrobenzene were manufactured by British Drug Houses, Ltd.

Gelatin was "Fineleaf Gelatine E" marketed by Société des Produits Chimiques, Coignet, Belgium, as specified by Fenner (1958).

Genetron 113 is supplied by the General Chemical Division of the Allied Chemical and Dye Corporation.

Hydroquinone diglycidyl ether was prepared as described by Werner & Farenhorst (1948). The higher melting isomer was separated by crystallisation from benzene and purified by treatment with decolourising charcoal and recrystallisation from ethanol. M.P. was 115-117°.

p-hydroxymercuribenzoate and N-ethylmaleimide are supplied by the Sigma Chemical Company.

Mercaptoethylamine (MEA) was from L.Light & Co. Ltd.

Sonication

Virus suspensions were exposed for 15 to 30 seconds to high frequency vibrations produced by a Mullard Ultrasonic Drill Generator (50 watt, peak output at 16 Kc). This treatment was used routinely for the resuspension of centrifuged, stored or heated virus preparations.

Tissue culture

KB cells (Eagle, 1955) were grown in the following medium:

Lactalbumin hydrolysate (enzymic)	5.0 g
Hanks' BSS (10x concentrated)	100 ml
10^5u/ml penicillin and streptomycin	1 ml
Human serum (heated 30 minutes at 55°)	150 ml
Distilled water to	1000 ml.

After sterilization by Seitz filtration, to each litre was added 15 ml of sterile 1.4% $NaHCO_3$. Medium was stored at -18°.

The cells were grown as monolayers in 250 ml medicinal half flat bottles and subcultured weekly. Medium was changed every 2 days. For subculture or the setting up of cultures in small tubes, cells were removed from the glass by exposure for 10 minutes at room temperature to 0.05% trypsin, 0.015% versene in saline, followed by pipetting. Bottles were inoculated with about 10^6 cells in 10 ml of medium, and small tubes received about 5×10^4 cells in 0.25 ml medium.

Ultraviolet irradiation

Gelatin McIlvaine's buffer was the diluent used in all irradiations. 5 ml samples of virus in 50 mm Petri dishes were exposed to UV radiation from a Philips TUV germicidal lamp. The incident energy was 750 $erg.cm^{-2}.sec^{-1}$,

and more than 95% of the output was at 2537 A^{o}. The samples were continuously agitated by a small magnetic stirrer.

Virus strains

Ectromelia Dohi A (Dohi, 1951) was kindly provided by Dr. S. Dohi.

The origins of rabbitpox-Utrecht (RPu^{+}) and vaccinia Lederle 7N (7N) are described fully by Fenner (1958), and samples were supplied by him.

RPu1 and RPu8 are white pock forming mutants of RPu^{+} isolated by Mrs. A. Gemmell (Gemmell & Cairns, 1959; Gemmell & Fenner, 1960) and kindly supplied by Prof. F. Fenner.

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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[4] 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[5] 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[6]. 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 *p*H 7,

Table 1. BIOLOGICAL CHARACTERS OF TWO STRAINS OF VACCINIA VIRUS (FROM REF. 6).

Designation of strain	Morphology of pock on chorioallantoic membrane	Production of hæm-agglutinin	Heat resistance	Mouse virulence	Rabbit virulence
RP	hæmorrhagic ulcerated (U^+)	nil (A)	high (T^+)	high (VM^+)	high (VR^+)
V-Led-7N	white non-ulcerated (U)	high (A^+)	low (T)	low (VM)	low (VR)

The appropriate symbols for phenotypes are shown in brackets.

suspensions which originally contained about 10^8 pock-forming units/ml. were shown to be non-infective when inoculated on the chorioallantoic membrane, with passage of the membrane two days later. All heated material used was inactive by this test, which was the most sensitive available. Mixtures of heated *RP* and active *V-Led-7N* were inoculated intracerebrally in mice, and brains were removed for assay on the chorioallantoic membrane at various intervals. Such brain suspensions yielded clones of virus indistinguishable from *RP* in all the characters listed in Table 1, as well as recombinants between *RP* and *V-Led-7N*. Both the recombinants and *RP* were recovered as early as 18 hr. after inoculation. Since *V-Led-7N* multiplies to a limited extent only in the mouse brain, it was found only in the early stages. This result is directly comparable to Berry's observations, for living virulent virus was recovered from an animal inoculated with a mixture of heat-inactivated virulent virus and a related active attenuated strain. It also showed something that could not be determined with myxoma-fibroma, namely, that some clones of virulent virus resembled the original heat-inactivated virus in all the characters that could be examined, and that others, although highly virulent, were recombinants.

The mouse brain was used to test the capacity of heated *RP* to be reactivated after further physical, chemical and enzymic treatments. Suspensions of *RP* could be heated at 55° C. for more than 7 hr. before the capacity for reactivation was greatly reduced, but this was destroyed in a few minutes at 60° C. In contrast to the report of Smith[7] (for which detailed support has never been published) attempts to dissociate protein and deoxyribonucleic acid have so far been unsuccessful. As Kilham and his collaborators[5] found with myxoma virus, various forms of enzymic digestion of the heat-killed virus (trypsin and deoxyribonuclease at *p*H 11, pepsin at *p*H 3·5, papain at *p*H 7·5 and above) leave the

capacity for reactivation intact and associated with particles deposited in the same centrifugal field as vaccinia virus.

Reactivation of heated *RP* was also observed after the inoculation of mixtures of heated *RP* and active *V-Led-7N* into cultures of HeLa cells, and on the chorioallantoic membrane. Use of the chorioallantoic membrane had the advantage that it allowed the direct demonstration of reactivation without the necessity of passage. When about 10^8 particles (originally 10^7 pock-forming units) of heated *RP* were inoculated on the chorioallantoic membrane together with 20–30 pock-forming units of active *V-Led-7N*, about half the pocks seen two days later were of the U^+ type, that is, they resembled those of *RP*. Analysis of 32 clones from U^+ and U pocks showed that nine resembled *V-Led-7N*, 11 resembled *RP* and 12 were recombinants. Using the chorioallantoic membrane, reactivation of heated *RP* has been induced not only by another strain of vaccinia (*V-Led-7N*) as the active agent but also by all the other members of the poxvirus group that have been tested, namely, cowpox, ectromelia, myxoma, fibroma and fowlpox. The reverse process, reactivation of heated *V-Led-7N* by active *RP* and by ectromelia virus, has also been accomplished. The phenomenon is probably a general one, demonstrable with many different combinations of pox viruses, although the ease of recognition of the reactivated component differs. Experiments with several other viruses which produce pocks on the chorioallantoic membrane yielded negative results; no reactivation of heated *RP* was observed when the active virus component was herpes simplex virus, influenza virus strain *WSE*, or Murray Valley encephalitis virus.

The search for recombinants between *RP* and reactivating viruses other than *V-Led-7N* is not yet completed, but reactivation of heated *RP* invariably yielded virus indistinguishable from *RP* in all the characters set out in Table 1 ($U^+ A T^+ V (M^+ R^+)$). The recovery of such clones from mixtures of viruses as dissimilar as *RP* and fibroma or fowlpox renders unlikely Berry's original idea that the heat-inactivated component 'transforms' the active virus. Reactivation of the heated virus by the active agent is a more plausible explanation. Whether reactivation is accomplished by some sort of genetic interaction, or whether the primary mechanism is repair of some non-genetic damage of the heated virus, with recom-

bination as a possible secondary event, cannot yet be decided.

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The Reactivation of Poxviruses

I. Demonstration of the Phenomenon and Techniques of Assay

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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 Woodroofe, 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 1½ 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 $10^{9.4}$ 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

COMPARABLE 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[a]

Dose of active RP (PFU)	Test system									
	CAM			Mouse brain				Rabbit skin		
	+ H-RP		+ Saline	+ H-RP		+ Saline		+ H-RP		+ Saline
	Pock count	Passage[b]	Pock count	Deaths[c]	Passage[b]	Deaths[c]	Passage[b]	Skin[d] lesion	Passage[b]	Skin[d] lesion	Passage[b]
0	0, 0	—	..	0	..			N	—	—	
1	0, 1	5.5	..	0	—	0	—	N	—	—	
2	..	..	..	0	—	1	—	N	—	N	..
4	1, 2	5.4	..	0	—	3	1.8	N	—	N	..
8	7, 4	..	10, 12, 4, 7, 2	0	—	6	2.2	N	—	N	0.6
16	11, 8	..	..	0	—	4	3.6	N	—	NPC	1.0
32	..	..	..	0	—	6	3.6	N	—	IPC	2.3
64	50, 32, 40, 40	..	..	0	0.6	6	..	N	—	IPC	3.3
128				0	1.8	6	6.0	N	—	IPC	..
256				0	2.5	6	6.0	N	—	IPC	..
512								N	—	IPC	..
1024								N	0.6	IPC	..

[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 10^9 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.

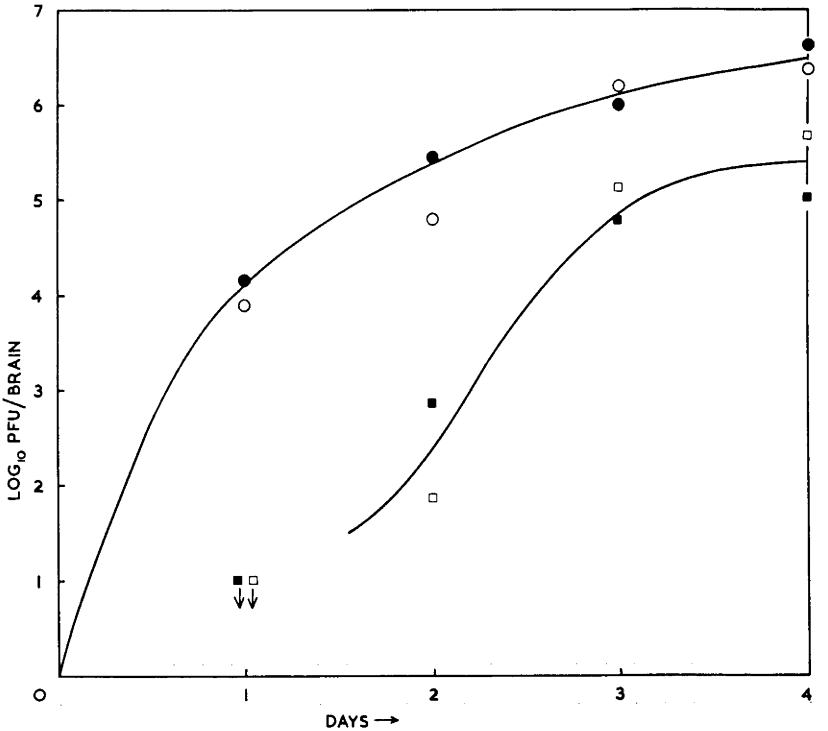

FIG. 1. The growth of 20 PFU RP in the mousebrain in the presence and absence of $10^{4.2}$ PFU ectromelia. ● = ectromelia in presence of RP; ○ = ectromelia in absence of RP; ■ = RP in presence of ectromelia; □ = RP in absence of ectromelia.

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 10^5 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

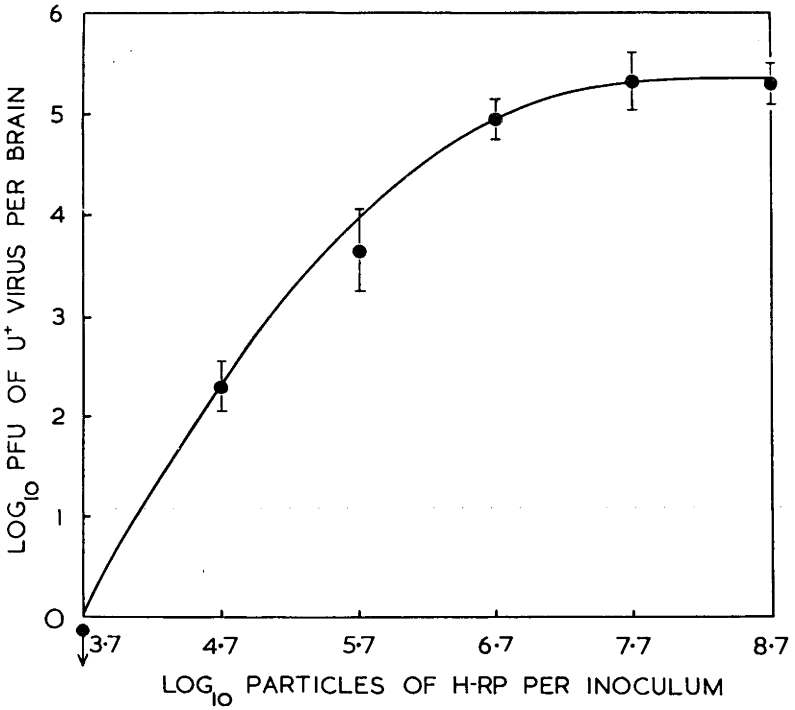

FIG. 2. Yields of U^+ virus obtained after 48 hours from brains which received $10^{4.4}$ PFU of ectromelia and various doses of H-RP. Each point represents the mean of four separate experiments, in each of which three mice were used for each dilution. The limits indicated for each value are the standard 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 $10^{5.6}$ 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 $10^{3.8}$ 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 Woodroofe, 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.

TABLE 2

COMPARISON OF ECTROMELIA, FIBROMA, AND MYXOMA FOR THE ASSAY OF H-RP ON THE CAM[a]

Active Virus	Dose of H-RP[b]	Pock Count
Ectromelia, 10^5 PFU	$10^{4.7}$	14, 6, 6, 6, 2
Fibroma, $10^{5.5}$ ID_{50}	$10^{5.7}$	9, 3, 5, 0, 2
Myxoma, 10^5 PFU	$10^{5.7}$	1, 3, 3, 3, 2

[a] 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

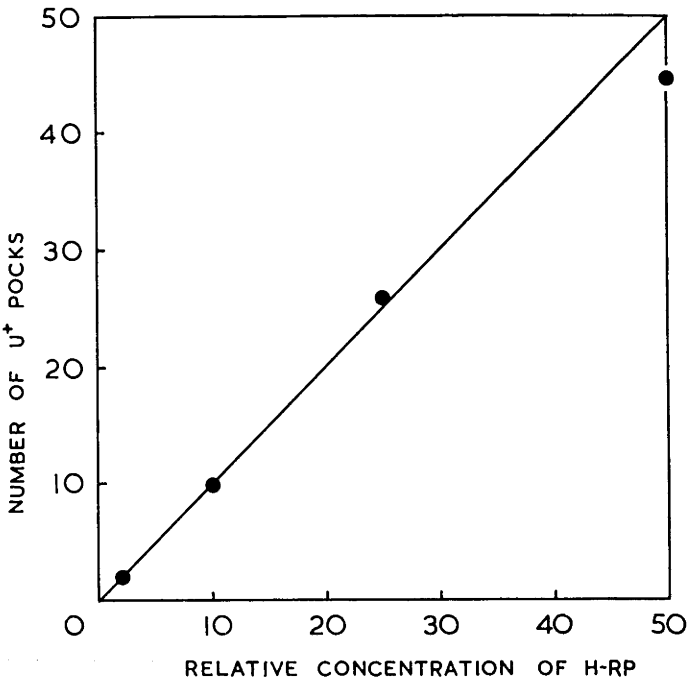

FIG. 3. The relation between dose of H-RP and number of U^+ pocks on the CAM.

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 $10^{6.8}$ 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 $10^{5.5}$ 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

EFFECT OF AMOUNT OF ECTROMELIA ON THE TITER OF H-RP ON THE CAM[a]

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

[a] 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^+ pocks 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

Time interval between inoculations	Experiments in mouse brain (reactivating agent, ectromelia) U^+ pocks per brain (log_{10} PFU)[a]	Experiments in HeLa cells (reactivating agent, 7N) Presence of reactivation[a]
H-RP: 4 Days before	−	−
3 Days before	2.9	++
2 Days before	3.6	++
24 hours before	4.4	+++
16 Hours before	3.9	+++
8 Hours before	4.2	+++
4 Hours before	4.7	+++
Simultaneous	6.3	+++
4 Hours after	5.3	++
8 Hours after	5.2	++
16 Hours after	5.8	++
24 Hours after	6.1	+
2 Days after	..	−

[a] − = 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.

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The Reactivation of Poxviruses

III. Properties of Reactivable Particles

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Rabbitpox virus has been converted to the reactivable form by controlled heat or urea treatment (resulting in H-RP and U-RP). H-RP is rapidly inactivated by urea, and U-RP is inactivated by heat. The effect of surface-active reagents and of trypsin and papain on the biological activity and morphology of active and reactivable virus has also been studied. Under certain conditions, notably in the presence of low concentrations of salt, the proteolytic enzymes digest away a large part of the virus particle leaving it flattened with a well-defined central body. This change may be accomplished under conditions that diminish the reactivability of U-RP and H-RP to a slight extent only. The results have been interpreted as suggesting that the property of reactivability resides in the central body of the particle.

INTRODUCTION

In the first paper of this series (Joklik *et al.*, 1960) we have shown that rabbitpox virus (RP), the infectivity of which has been destroyed by heating (H-RP), is reactivated in cells supporting the multiplication of other poxviruses. In this paper we report that another method of inactivation, namely, treatment with concentrated solutions of urea, also transforms active rabbitpox to the reactivable form (U-RP). The effects of certain surface-active substances and proteolytic enzymes on the active virus, and on H-RP and U-RP, have been studied in an effort to obtain information about the groupings or structures in the reactivable particles that differentiate them from active virus and from inactive virus that cannot be reactivated. Virus particles subjected to various treatments have been examined by means of the electron microscope.

TERMINOLOGY

The terminology used follows that set out in the previous paper (Joklik *et al.*, 1960).

MATERIALS AND METHODS

Virus strains. Rabbitpox (RP) was used as the source of the reactivable virus, and ectromelia Dohi A as the reactivating agent. Since virus was frequently examined in the electron microscope throughout this work, purified suspensions were employed in which the amount of debris had been reduced to a low level. Such suspensions were prepared by extraction of confluent CAM with Genetron (Joklik *et al.*, 1960) and purified by several cycles of differential centrifugation in swing-out buckets between 25,000 and 100,000 *g*-minutes. The final suspensions contained about $10^{9.3}$ pock-forming units (PFU) per milliliter.

Titration of reactivable virus was carried out on the CAM, using $10^{4.5}$ PFU of ectromelia virus as the reactivating agent (Joklik *et al.*, 1960). Titers are expressed in terms of pocks of reactivated virus present 42 hours later, representing the number of particles reactivated in the presence of reactivating virus.

Sonic treatment. A Mullard 50-watt ultrasonic drill generator was adapted for laboratory use. Peak output was at 16 kilocycles.

Enzymes. All enzymes were crystalline preparations purchased from Worthington Biochemicals.

Preparation of samples for electron microscopy. Virus suspensions were fixed in 0.01 % osmic acid, mixed with polystyrene latex, and sprayed onto collodion films on glass slides. The films were floated onto a water surface and there dialyzed for 4 hours or overnight to remove salts. The films were then collected on grids, shadow cast with uranium, and photographed with an RCA EMU-3B electron microscope at an instrumental magnification of 5000 ×.

EXPERIMENTAL RESULTS

1. The Preparation of Reactivable Virus

The Conversion of RP to H-RP. The heat stability of active RP is affected by the soluble protein content of the suspending medium. To eliminate the protective effect of soluble protein as far as possible, virus was suspended in 0.1 *M* NaCl buffered at pH 7 with 0.01 *M* sodium citrate. When such preparations are heated at 55°, there is a rapid decrease in titer, resulting in total loss of infectivity within 2½ hours. If, however, titrations are carried out in eggs simultaneously given ectromelia virus, the titer of U^+ pocks becomes stable at about 1 % of the original active virus titer (Fig. 1a). The activity thus measured is the reactivability of H-RP. At higher temperatures the decrease in both

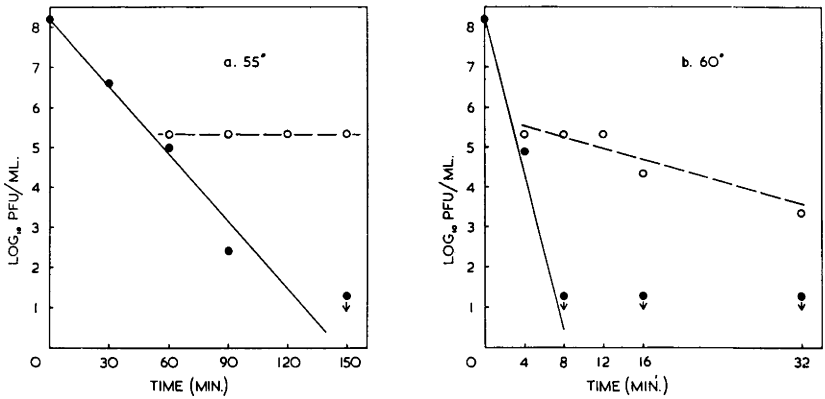

FIG. 1. Inactivation of RP in citrate-saline. a. 55°; b. 60°. ●——● Infective virus; O- - -O reactivable virus.

active and reactivable virus is more rapid, but reactivability is much more heat resistant than infectivity (Fig. 1b). Reactivable virus formation occurs when suspensions of RP are heated at temperatures up to 70°; above that it is too unstable for ready demonstration. Morphologically H-RP is indistinguishable from RP.

Material heated in the absence of protein, although stable in activity over six months or more at 4°, tended to flocculate. Nonflocculating stable preparations of H-RP, which were used for most of the work described below, were obtained by heating virus in 0.15% gelatin-0.005 *M* phosphate-citrate buffer (pH 7.2) at 60° for 12 minutes. This heat treatment was always carried out within 24 hours of preparing the virus suspension to prevent formation of heat-stable active virus (Woodroofe, 1960). Absence of infectivity was ensured by inoculation of the undiluted material on the CAM, with passage (Joklik *et al.*, 1960).

The conversion of RP to U-RP. Since RP was transformed into reactivable virus by careful heat treatment, other means of denaturing protein were sought that might have the same effect. Treatment with urea proved to be the most successful. When suspensions of RP are diluted 1:10 into 6.7 *M* urea in normal saline at 2°, there is a rapid decrease in infectivity and suspensions are completely noninfectious within 1 hour. When titrated in the presence of ectromelia virus, however, the titer becomes stable within half an hour, and then remains constant for at least 20 hours. This reactivable virus titer is about 1000 times lower than that of active virus.

In preparing stock suspensions of reactivable virus by treatment

with urea (U-RP), it is preferable to dialyze the virus against 6.7 *M* urea solution. U-RP preparations of highly reproducible titer were produced by 3 hours' urea dialysis, followed by 2 hours' dialysis against normal saline. Figure 2 shows the decrease in titer of active RP, the titer of reactivable virus reached, and the rate of dialysis of urea into the dialysis bag under the conditions used.

Dialysis against 6.7 *M* urea at 4° proved to be the optimal treatment for conversion of RP to U-RP. Dialysis against 4 *M* urea at 4° destroyed infectivity much more slowly; even after 16 hours the titer was reduced by less than 10,000-fold. On the other hand, treatment for 16 hours with urea saturated at 4° (about 9 *M*) or with 6.7 *M* urea at room temperature, not only completely destroyed infectivity, but also failed to yield reactivable virus.

Urea treatment produces some morphological change in RP (Fig. 7a).

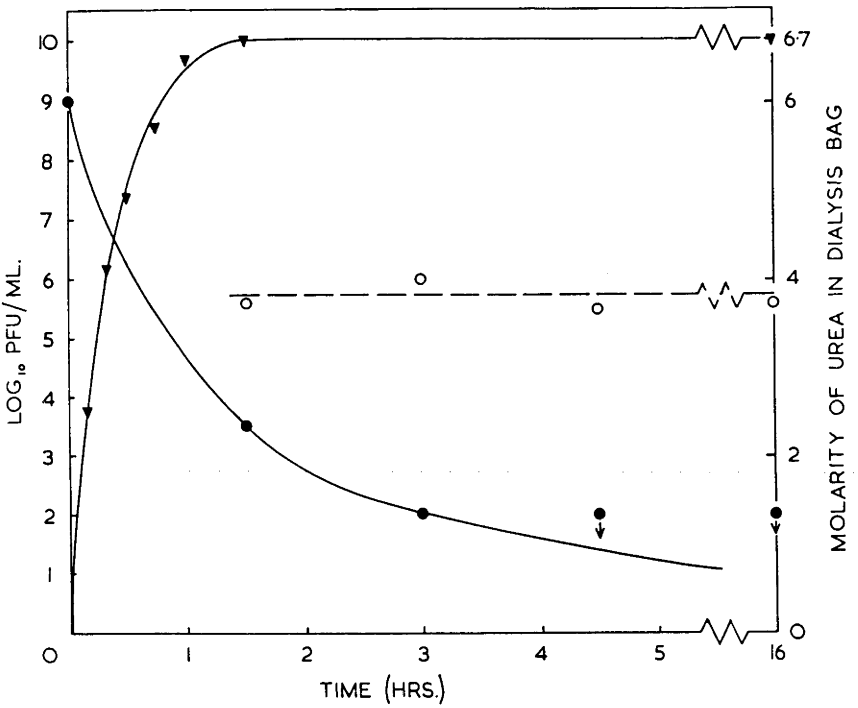

FIG. 2. The inactivation of RP by urea at 4° and the appearance of reactivable virus. ▼——▼ Molarity of urea in dialysis bag; ●——● infective virus; ○- - -○ reactivable virus.

Shadows cast by U-RP particles are shorter, indicating flattening (Fig. 8a), and the central body is sometimes more pronounced.

2. The Stability of H-RP and U-RP to Urea, Heating, and DNAase

Shack and Kilham (1959) prepared reactivable preparations of myxoma virus by urea treatment of heat-inactivated myxoma virus; and found that the product, unlike H-myxoma, was susceptible to the action of deoxyribonuclease (DNAase). We therefore made quantitative studies of the effects of heating on U-RP, urea on H-RP, and DNAase on both preparations.

H-RP was found to be rapidly inactivated by urea (Table 1). This is in contrast to the effect of urea on reactivable preparations of myxoma virus prepared by heat treatment, which were reported to be stable to dialysis against 10 *M* urea at room temperature for 8 hours (Shack and Kilham, 1959).

Conversely, U-RP preparations were considerably less stable to heat treatment than H-RP, although much more stable than active virus (Fig. 3). Since H-RP was inactivated by urea, and U-RP by heat, tests were carried out which showed that H-RP and U-RP did not cross-reactivate each other and ruled out the possibility that heat and urea inactivate different proteins of RP. It is more likely that the inactivation of U-RP by heat and of H-RP by urea is due to the fact that heat and urea differ in the manner and extent to which they denature protein. This will be further discussed below.

The possibility exists that by careful degradation of reactivable virus the DNA contained in the denser inner regions of the virus particle (Peters, 1957a) is exposed, leading to a reactivable form of the virus which is sensitive to the enzyme DNAase. Indeed, Shack and Kilham (1959) have reported that urea treatment of reactivable myxoma virus

TABLE 1

EFFECT OF 6 *M* UREA AT 4° ON H-RP

Time after addition to urea (min)	% Residual activity
0	100
10	10
20	1
40	0.2
80	0.2

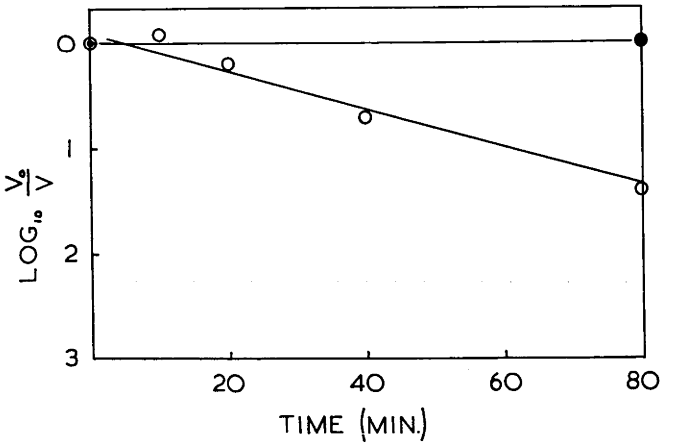

FIG. 3. Effect of heating at 60° on the reactivability of H-RP and U-RP. ●——● H-RP; ○——○ U-RP.

prepared by heating has this effect. This is not the case with RP; neither heat nor urea treatment separately or in combination resulted in the appearance of DNAase-sensitive biological activity.

Further degradation of the reactivable particles has been attempted along several lines, but as yet no "reactivable DNA" has been detected. In the process of the controlled degradation of the reactivable particles, however, several interesting differences in their behavior have been observed that permit certain conclusions to be drawn regarding the components of active and reactivable RP.

3. The Effect of Surface-Active Agents on RP, H-RP, and U-RP

Sodium deoxycholate. Table 2 shows the effect of sodium deoxycholate (SDC) on the activity of RP, H-RP, and U-RP. In 3 hours at 37°, 0.06% SDC inactivated all three more than tenfold; 0.03% SDC, on the other hand, inactivated U-RP considerably, whereas RP or H-RP were unaffected by this concentration. The action of SDC is very sensitive to the presence of salt and to the pH. Thus H-RP is unaffected by SDC in 0.1 *M* sodium chloride, although it is inactivated in 0.5 or 1 *M* sodium chloride; and in the presence of 0.5 *M* sodium chloride, SDC is active at pH 7, but not at pH 5.8 or 8.5. The inactivity at pH 5.8 is explained by the dissociation constant of deoxycholic acid (pK = 6.5), and the low solubility of the free acid, whereas the inactivity at pH 8.5 is presumably due to the effect of pH on the surface of the virus particle.

The inactivity of SDC at pH 8.5 made it impossible to use this compound in conjunction with trypsin, but it was used in the presence of papain (see below).

Electron micrographs of SDC-treated particles showed them to be surrounded by amorphous debris (Fig. 7f). This material was protein in nature since it was not evident in preparations treated with both SDC and papain. The particles themselves appeared little changed.

Sodium dodecyl sulfate. Treatment with sodium dodecyl sulfate (SDS) led to rapid inactivation. Table 3 shows the effect of varying concentrations of SDS in the presence or absence of 0.5 *M* sodium chloride on the activity of H-RP. At a concentration of 0.001 *M*, SDS inactivates rapidly. At 0.0003 *M* there is inactivation in the presence, but not in the absence, of sodium chloride; and at 0.0001 *M* there is no inactivation. The presence of salt would be expected to increase the formation of micelles of SDS which may inactivate more than the anion itself.

TABLE 2

EFFECT OF SODIUM DEOXYCHOLATE ON RP, H-RP, AND U-RP[a]

Concentration of SDC(%)	Time (hr)	RP	H-RP	U-RP
0.03	1	100	100	50
	2	100	100	30
	3	100	100	20
0.06	1	100		3
	3	3	5	3

[a] Each reaction mixture contained 0.5 *M* NaCl at pH 7. The figures represent the percentage residual activity.

TABLE 3

EFFECT OF SODIUM DODECYL SULFATE ON H-RP

Concentration of SDS	Concentration of NaCl	Residual activity after 60 min at 20° (%)
0.001 *M*	—	0
0.001 *M*	0.5 *M*	0
0.0003 *M*	—	80
0.0003 *M*	0.5 *M*	0
0.0001 *M*	—	100
0.0001 *M*	0.5 *M*	100

Digitonin. RP, H-RP and U-RP were found to be stable to as much as 1 % digitonin in 0.5 *M* sodium chloride at pH 7 for 2 hours at 37°.

4. The Effect of Proteolytic Enzymes on RP, H-RP, and U-RP

Pepsin. Pepsin has been used extensively by Peters and associates in morphological studies on vaccinia virus (Peters, 1957b). These studies were generally carried out on particles fixed histologically and at a pH of less than 4. At such low pH values, the biological activity of RP, H-RP, and U-RP was unstable. In fact, at all pH values tested in conjunction with pepsin, the predominating effect was that of the hydrogen ion concentration: at concentrations permitting pepsin to be active, loss of activity (infectivity or reactivability) was as rapid in the absence as in the presence of pepsin, but at lower concentrations, where the virus preparations were stable, pepsin no longer acted.

Trypsin. When suspended in dilute phosphate-citrate buffer (0.005 *M*

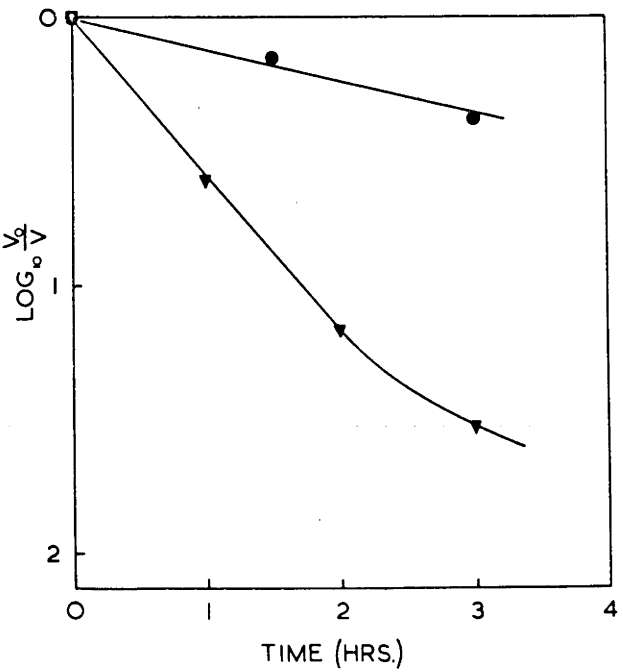

FIG. 4. The effect of sodium chloride on the inactivation of RP by 0.02 % trypsin at 37° (pH 8.5). ▼——▼ No sodium chloride; ●——● 0.5 *M* sodium chloride.

phosphate) RP is inactivated by 0.02% trypsin at 37°. The titer decreases fourfold per hour for the first 2 hours, and more slowly after that. In the presence of 0.15 *M* or more sodium chloride the loss in titer is much smaller (Fig. 4).

This result is of interest, as tryptic digestion has been used repeatedly as a step in the purification of vaccinia virus. Neither Smadel and Wall (1937) nor Kaplan and Valentine (1959) detected any loss in titer after 30 minutes' digestion. It appears, however, that longer treatment leads to a serious loss of infectivity.

Particles treated with trypsin in the absence of sodium chloride show extensive changes in morphology (Fig. 7b). An outer membrane is visible, apparently because digestion of material immediately inside it has occurred. There are great differences in the extent of digestion of different particles. This is observed generally when proteolytic enzyms act on vaccinia virus, as has been pointed out by Peters (1957a). In the presence of sodium chloride, trypsin induces only slight changes (Fig. 7c).

H-RP and U-RP lose little or no activity if incubated with 0.02% trypsin for 3 hours either in the presence or absence of sodium chloride. However, there are morphological changes. In the presence of salt, particles of H-RP and U-RP appear slightly flattened (Figs. 7d and 8c). In the absence of salt there is very considerable attack on the outer region of the particles, especially those of U-RP (Figs. 7e and 8b). In most particles a central body is clearly defined. This central body presumably consists of the *Ringstruktur* as well as the *zentralgelagerter Körper* (Peters, 1957a).

Papain. Cyanide was used as the activator of papain throughout this work since cysteine and thioglycolic acid strongly inactivate RP, H-RP,

TABLE 4

EFFECT OF CYSTEINE AND THIOGLYCOLIC ACID ON RP, H-RP, AND U-RP[a]

Compound	Concentration	Time (hr)	Percentage residual activity		
			RP	H-RP	U-RP
Cysteine	0.0005 *M*	3		20	
Thioglycolic acid	0.006 *M*	1	100	100	100
		3	1	2	2

[a] Reaction mixtures (buffered at pH 7) were gassed with nitrogen and incubated at 37°.

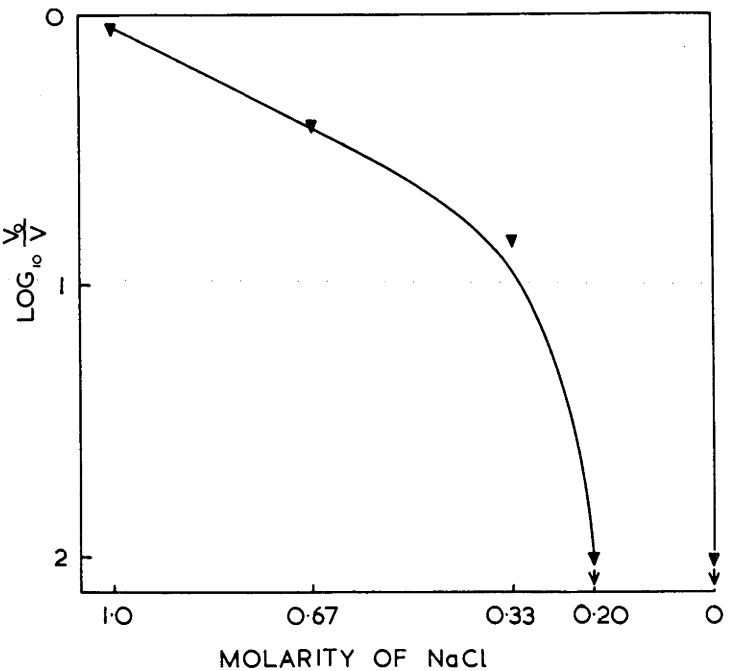

FIG. 5. The effect of sodium chloride concentration on the inactivation of RP by papain. Reaction mixtures contained 0.02 *M* potassium cyanide, 0.02 *M* phosphate pH 7, 0.02% papain, 0.2% gelatin. Time of incubation: 3 hours at 37°.

and U-RP (Table 4). The significance of this effect by —SH compounds will be discussed below.

The effect of sodium chloride on the inactivation of RP, H-RP, and U-RP by papain was even more pronounced than in the case of trypsin. Figure 5 shows the effect of sodium chloride concentration on the inactivation of RP in the presence of 0.2% gelatin. There was little loss of infectivity after 3 hours' incubation with 0.02% papain at 37° at sodium chloride concentrations of 0.5 *M* or higher. As the amount of sodium chloride was decreased inactivation increased rapidly, until at 0.2 *M* sodium chloride or less, there was over 99% inactivation. Inactivation was also influenced by the presence of gelatin, which exerted a protective effect. Peters (1957a) has drawn attention to the conflicting reports in the literature regarding the stability of vaccinia virus in the presence of papain (Weineck, 1940; Hoagland *et al.*, 1940). It appears now that the concentration of both soluble protective protein and salt markedly influence the inactivation of RP by papain.

H-RP was also inactivated in the absence, but not in the presence, of sodium chloride. U-RP was inactivated by papain even in the presence of 0.5 *M* sodium chloride (Fig. 6a). The titer fell to less than 5 % within 3 hours.

U-RP particles treated with papain in the presence of salt (Fig. 8d) showed signs of digestion of the outer region. In the absence of salt, particles were attacked far more extensively. There seemed to occur an initial loss of material surrounding the central body, causing it to stand out clearly (Fig. 8e), but after prolonged treatment most particles took on a greatly flattened appearance (Fig. 8f).

Experiments were carried out to determine the effect of papain and SDC acting in conjunction (Fig. 6b); 0.5 *M* sodium chloride was included in these reaction mixtures since SDC had no detectable effect on virus in its absence. Under these conditions RP is stable to either 0.03 % SDC or 0.02 % papain alone, but in the presence of both there is a greater than 100-fold decrease in titer in 3 hours. Similarly H-RP, which is only slightly attacked by either reagent alone, loses 99 % of its activity within 1½ hours. A comparison of micrographs of H-RP particles treated with SDC alone and in conjunction with papain (Fig. 7, f and h) shows that the amorphous material surrounding the particles treated with SDC is not present when both reagents are used. In this case the particles appear slightly digested at the periphery.

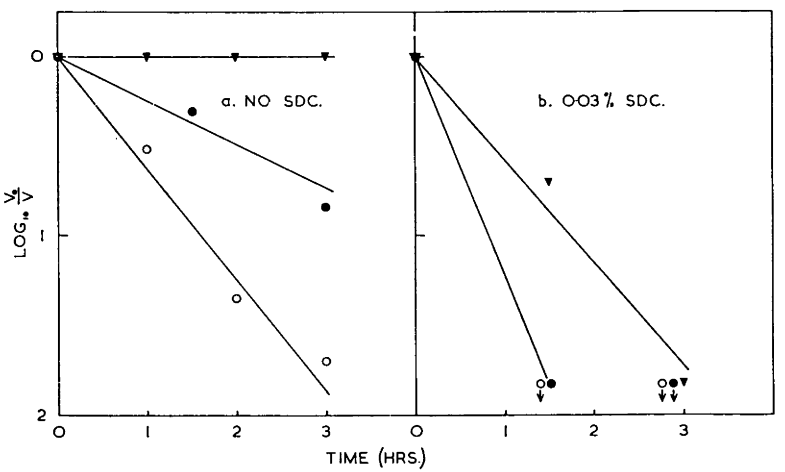

FIG. 6. Effect of papain on RP, H-RP, and U-RP. a. No sodium deoxycholate; b. 0.03 % sodium deoxycholate. All reaction mixtures contained 0.02 % papain, 0.02 *M* potassium cyanide, 0.02 *M* phosphate pH 7, 0.5 *M* sodium chloride, and 0.2 % gelatin; 37°. ▼——▼ RP; ●——● H-RP; ○——○ U-RP.

U-RP is less stable than RP or H-RP to either reagent alone and suffers a correspondingly more precipitous loss in activity when attacked by them in conjunction (Fig. 6b).

DISCUSSION

Before considering in some detail the relation between morphology of the reactivable rabbitpox particle and its biological activity, we may first summarize the data on the chemical basis of infectivity and reactivability. The work described in this paper relates to the properties of reactivable virus prepared from active virus by treatment with heat or urea. Other reagents have been tested, but although reactivable virus was sometimes detected after treatment, the efficiency of conversion was low. It would be expected that certain compounds, such as guanidine hydrochloride, might be as efficient as urea; a broad survey of such compounds is now being carried out.

The common denominator of the two most efficient conversion techniques, heat and urea treatment, is that they are mild protein denaturants. It is unlikely that they damage DNA in any way, and this is borne out by the finding (Fenner and Woodroofe, 1960) that, as far as can be tested at present, all genetic information present in the virus particle before conversion to the reactivable state is also present in the progeny of the reactivated virus. Furthermore, it has recently been found (Joklik and Abel, to be published) that when RP is inactivated with nitrogen mustard, which is known to combine with and inactivate nucleic acid rather than protein, the resulting particles are not reactivable. Reactivable particles, like active ones, contain a full complement of undamaged DNA.

They differ from active particles in the component, presumably protein, which is inactivated by heat or urea. Evidently multiplication of reactivable virus is not possible unless that component is introduced into the cell. It may be supplied, either by an infectious poxvirus particle, or by poxvirus particles rendered inactive by agents that damage DNA rather than protein (Joklik and Abel, to be published).

The biological activity of the reactivable particle is not, however, dependent merely on the integrity of its DNA. That protein, in the proper configuration, is also necessary is shown by the following lines of evidence: (1) a number of more severe protein denaturing agents which do not destroy DNA, notably SDS, completely destroy reactivability; (2) treatment with papain destroys reactivability; and (3) reactivability is not sensitive to DNAase, suggesting either an accessibility barrier, or combination and protection of DNA with protein.

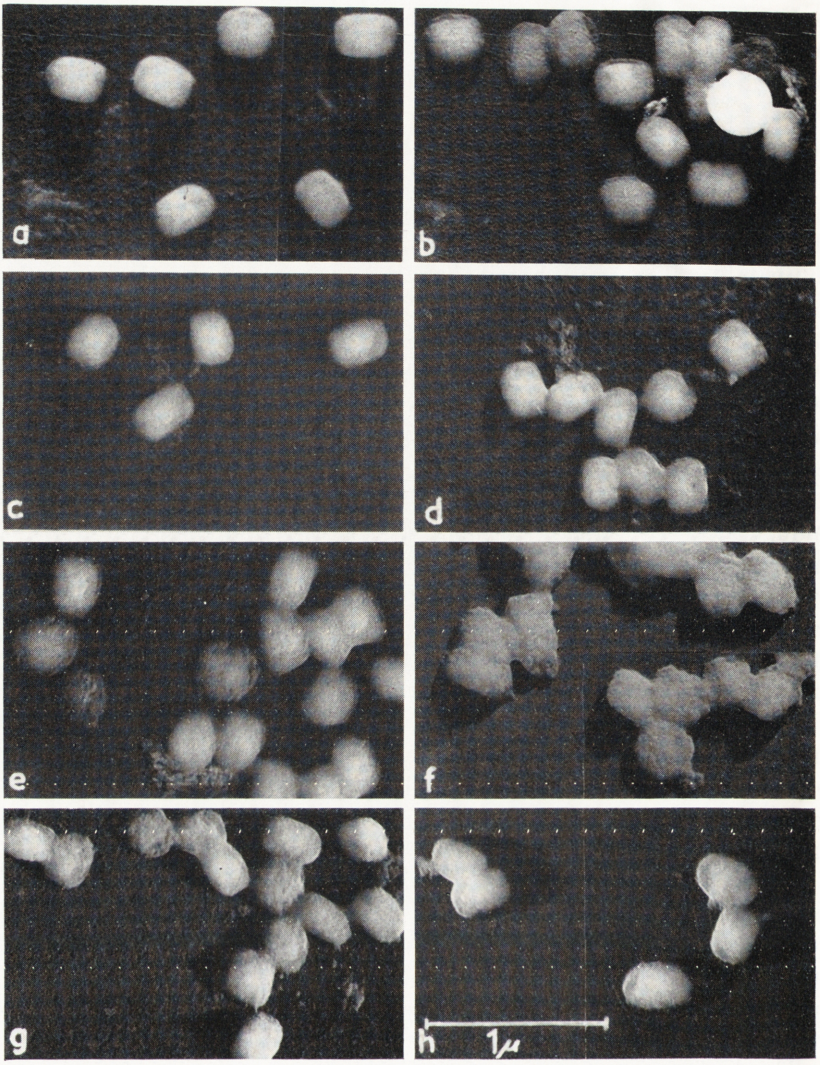

FIG. 7. Electron micrographs of rabbitpox virus subjected to various treatments. Incubation with enzymes: 3 hours at 37°. a. RP; b. RP, 0.02 % trypsin, pH 8.5; c. RP, 0.02 % trypsin, 0.5 *M* NaCl, pH 8.5; d. H-RP, 0.02 % trypsin, 0.5 *M* NaCl, pH 8.5; e. H-RP, 0.02 % trypsin, pH 8.5; f. H-RP, 0.06 % SDC, 0.5 *M* NaCl, pH 7, 1 hour at 37°; g. H-RP, 0.02 % papain, 0.02 *M* KCN, 0.5 *M* NaCl, pH 7; h. H-RP, 0.02 % papain, 0.02 *M* KCN, 0.03 % SDC, 0.5 *M* NaCl, pH 7.

Extensive trials have been carried out with lipases and lecithinases, as well as with organic solvents acting on freeze-dried preparations in efforts to minimize permeability barriers, but so far no treatment has been found that will yield reactivable material sensitive to DNAase. The evidence thus indicates that the reactivable virus is a form in which a protein necessary for infectivity has been denatured (this protein being supplied during reactivation by either infectious virus or virus in which only the DNA has been inactivated), but which still contains some

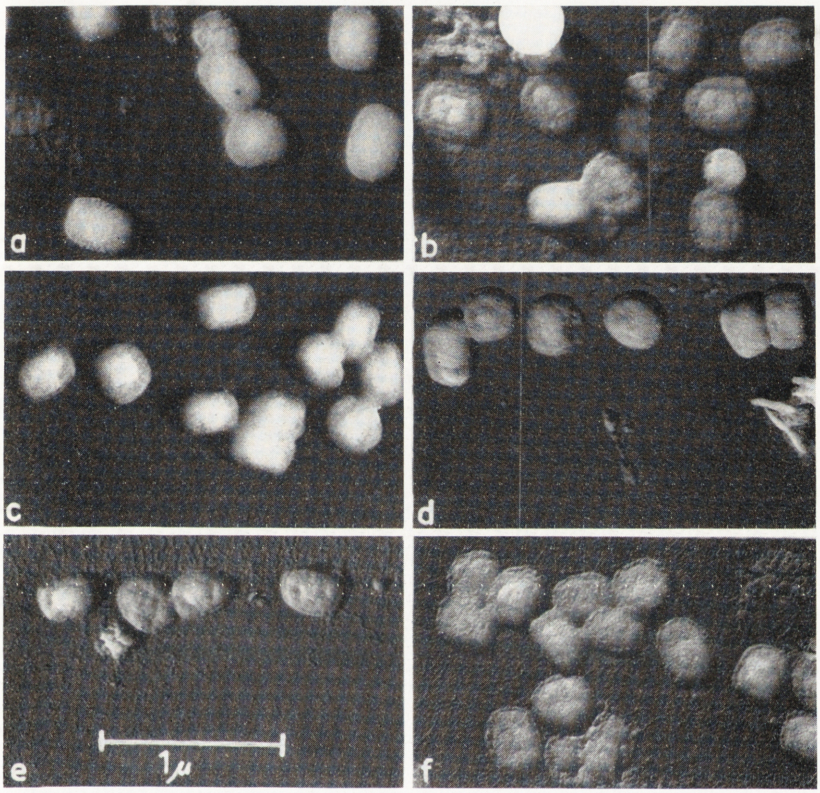

FIG. 8. Electron micrographs of rabbitpox virus subjected to various treatments. Incubation with enzymes: 3 hours at 37°. a. U-RP; b. U-RP, 0.02 % trypsin, pH 8.5; c. U-RP, 0.02 % trypsin, 0.5 *M* NaCl, pH 8.5; d. U-RP, 0.02 % papain, 0.02 *M* KCN, 0.5 *M* NaCl, pH 7; e. U-RP, 0.002 % papain, 0.02 *M* KCN, pH 7; f. U-RP, 0.01 % papain, 0.02 *M* KCN, pH 7.

TABLE 5

COMPARISON OF THE SUSCEPTIBILITY OF THE BIOLOGICAL ACTIVITY OF RP, H-RP AND U-RP TOWARD PROTEIN DENATURING REAGENTS AND PROTEOLYTIC ENZYMES[a]

Agent tested	RP	H-RP	U-RP
Heating at 55°	s	r	s
Urea (6.7 *M*)	s	s	r
SDC (0.03%)	r	r	s
SDS (0.001 *M*)	s	s	s
Trypsin (0.02%), no sodium chloride	s	r	r
Trypsin (0.02%) + 0.5 *M* sodium chloride	r	r	r
Papain (0.02%), no sodium chloride	s	s	s
Papain (0.02%) + 0.5 *M* sodium chloride	r	r	s
Papain (0.02%) + 0.5 *M* sodium chloride + 0.03% SDC	s	s	s

[a] *s* = sensitive; *r* = resistant.

other undenatured protein component(s) as well as the full complement of functional DNA.

H-RP and U-RP have the same biological activity and appear to differ only in the manner and extent of denaturation that has been effected. The differences in their behavior toward both protein denaturing agents and proteolytic enzymes (Table 5) is explicable on this basis. U-RP is in general more readily digested by proteolytic enzymes than H-RP, as judged by loss in activity and changes in morphology. The precise mechanism of denaturation of proteins by heat and urea is not yet fully understood, but it is probable that urea causes a more general unfolding of the ordered secondary noncovalent structure of protein than does heating. This disarrangement of macromolecular configuration evidently affects the structure responsible for reactivability, since U-RP is stable to urea whereas H-RP is inactivated and H-RP is stable to heat whereas U-RP is inactivated.

The inactivation of H-RP and U-RP as well as of RP by cysteine and thioglycolic acid is of interest. Since one of the major results of denaturation is exposure of —S—S— bonds, it might have been expected that RP would be more resistant to these reducing agents than H-RP and U-RP. Since this was not the case, it would appear that more or less exposed —S—S— bonds figure prominently in conferring structural stability on the protein necessary for infectivity.

Before discussing the electron micrographs the following observation is necessary. As assayed by the standard procedure (Joklik *et al.*, 1960),

not more than one active virus particle in 100 is converted to the reactivable form. Allowing for a ratio of active to physical particles of 1:10 in the original preparation (Fenner and Comben, 1958), this may mean that only 1 in 1000 of particles observed by the electron microscope is reactivable. It is likely that the efficiency of plating of reactivable virus by the method at present in use is low, rather than that only 1% of active particles become reactivable, but there is no evidence as to which of the particles pictured in the electron micrographs are reactivable. As in all studies of animal viruses by means of the electron microscope, it is assumed here that where a preparation of virus containing a majority of inactive particles has been treated in a certain manner, the morphological changes induced in inactive and active particles are the same.

With these reservations, the following observations may be made on the electron micrographs of RP, H-RP, and U-RP exposed to proteolytic enzymes. There is a gradation in susceptibility to trypsin and papain from RP through H-RP to U-RP. This may be due to the known greater susceptibility of denatured proteins to tryptic, and to a lesser extent, papain digestion. It is apparent both from changes in the appearance of the particles and from changes in the biological activity that both enzymes cause a much more extensive loss of viral material in the absence than in the presence of sodium chloride. The fact that the reactivability of both H-RP and U-RP is resistant to enzymatic digestion (except in the case of papain acting on U-RP), under conditions when there are considerable changes in morphology, may indicate that reactivability is closely associated with the central core of the particles, which appears to be resistant to attack by either enzyme.

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Reactivation of Poxviruses by a Non-Genetic Mechanism

It has recently been shown that poxviruses inactivated by heat are reactivated in cells in which another poxvirus multiplies[1]. The virus yield from cells infected with inactivated virus of one strain and active virus of another strain contains infectious particles of both strains, and also, if the viruses are sufficiently closely related, recombinants[2]. Inactivation by heat, therefore, does not inactivate the deoxyribonucleic acid of the virus particles, since all the known genetic markers are preserved. Since proteins are in general less stable to heat than deoxyribonucleic acid, the role of the reactivating virus may be to provide some essential native protein which has been destroyed by heating. On this hypothesis, it should be possible to use, as the reactivating agent, virus of which the deoxyribonucleic acid has been specifically inactivated. In this case the genotype of the heat-inactivated virus only should survive.

Such inactivation might be achieved by treatment with the nitrogen mustard, di(2-chloroethyl)methylamine, which, although capable of combining with protein, reacts preferentially with nucleic acid. Loveless and Stock[3] have shown that nitrogen mustard inactivates phage probably by combination with its deoxyribonucleic acid without affecting the protein structures responsible for adsorption to host cells and combination with specific antibody, and they particularly comment on the low efficiency of multiplicity and cross-reactivation shown by phage treated with mustard.

Preliminary experiments showed that rabbitpox virus (RPu^+), treated with nitrogen mustard, would reactivate heated RPu^+, and also heated vaccinia 7*N*, and that the product had the pock character of the heated parent. In order to provide a sensitive test for partial survival of the genome of the mustard-treated parent, the mutants RPu1 and RPu8 were chosen as the heated parent. The *u* mutants of rabbitpox virus, which produce white non-ulcerated pocks on the chorioallantoic membrane, differ from the wild type (RPu^+), which produces hæmorrhagic ulcerated pocks, at single genetic sites[4,5]. In mixed infections of the chorioallantoic membrane with RPu1 or RPu8 and RPu^+, the wild type outgrows both mutants[5], so that if there is survival of that part

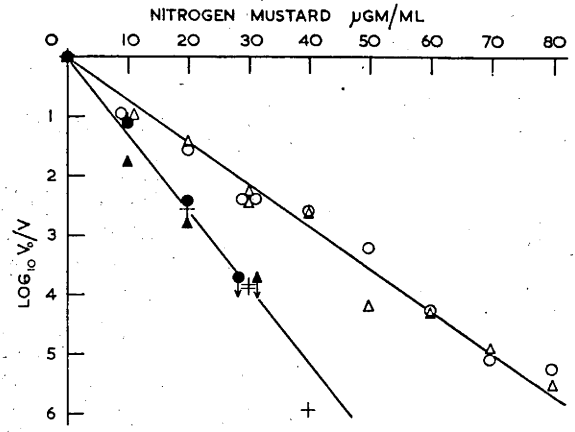

Fig. 1. Inactivation of RPu^+ by nitrogen mustard.
Assayed in the absence of heated virus: +, titre of U^+ pocks.
Assayed in the presence of heated RP*u*1: ●, titre of U^+ pocks; ○, titre of U pocks.
Assayed in the presence of heated RP*u*8: ▲, titre of U^+ pocks; △, titre of U pocks

of the genome which carries the wild locus it should be readily detected.

RPu^+ was inactivated by exposure to various concentrations of nitrogen mustard in 0·005 *M* phosphate buffer, *p*H 7·2, for 20 min. at 37°. The excess mustard was then destroyed by a tenfold dilution of the suspension into 5 per cent sodium thiosulphate and further incubation for 2 hr. Assay of particles capable of reactivating heated virus was carried out by inoculating suitable dilutions of the material on the chorioallantoic membrane of 11-day old chick embryos together with 5×10^8 particles of *RPu*1 or *RPu*8 heated at 60° for 12 min.: the heated virus was completely non-infectious[1]. Residual active particles of RPu^+ were also titrated in the absence of heated virus.

The results are shown in Fig. 1. The infectivity of RPu^+ is seen to be destroyed by nitrogen mustard by a one-hit process, and the surviving particles can be assayed with equal efficiency in the presence or absence of heated virus. The ability of RPu^+ to reactivate heated RP*u*1 or RP*u*8 is destroyed less readily, though again this appears to be a one-hit process.

It seems, therefore, that in rabbitpox virus all the lethal damages caused by nitrogen mustard prevent expression of the genotype (as judged by one marker), but only about half of them fall in that part of the virus which is necessary for reactivation of heated

virus. Thus the evidence is compatible with our hypothesis, namely, that reactivating virus provides some essential non-genetic material which is lacking in heated virus.

We are indebted to Prof. Frank Fenner for the *u* mutants.

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