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THE REACTIVATION OF ANIMAL VIRUSES*

BY

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It is still a common practice among medical men to speak of "killed" and "live" viral vaccines, and the everyday meanings of the terms are clear enough. But, as I shall demonstrate, virologists now recognize a variety of situations in which "killed" virus may multiply and produce new infectious virus. They have therefore discarded the term "killed" and adopted the word "inactivated" to replace it. Even "inactivated," however, is used in a restricted sense; it refers to the loss of viral infectivity—that is, to the inability of virus particles to multiply and produce new infectious virus in susceptible cells, when these cells each receive only single particles of the inactivated preparation, and no other virus particles or derivates thereof.

Purely practical considerations have led to a great deal of work being carried out on the inactivation of viral infectivity. There were three main objectives in this work: (*a*) the necessity to sterilize objects contaminated with viruses; (*b*) the understanding of the mode of action of antibodies and other naturally occurring components of biological systems which could play a part in recovery from viral infections or protection against them; and (*c*) the preparation of vaccines composed of non-infectious but antigenically potent viral material.

More recently, inactivation has been used as a method of studying the structure and function of viruses. This approach received its principal stimulus from the discovery that inactivation was sometimes reversible—that is, that "inactivated" virus could be "reactivated" or rendered infectious again. This was first recognized with virus inactivated by treatment with antibody. Apart from reversal of neutralization by simple dilution, virus "neutralized" by antibody can be rendered infectious again by treatment at low *p*H (Mandel, 1960), by ultrasonic vibration (Anderson and Doermann, 1952), by papain digestion (Kalmanson and Bronfenbrenner, 1943), and by treatment with fluorocarbon (Hummeler and Ketler, 1958). Other examples of extracellular inactivation and reactivation are the reversal of the toxic effects of mercuric chloride by hydrogen sulphide (Krueger and Baldwin, 1934) and the reactivation of formalin-treated phage T3 by incubation with asparagine (Heicken and Spicher, 1956).

Of much greater interest, from the point of view of the analysis of viral structure and function, was the discovery by Luria (1947) that bacterial viruses inactivated by ultra-violet (U.V.) irradiation could undergo reactivation. Ionizing and non-ionizing radiation and radiomimetic chemicals were shown to inactivate the infectivity of viruses primarily by damaging their genetic materials (although they all affect other components of the virion also). Sometimes the damage to the genetic material could be directly repaired, as in photoreactivation of U.V. damages (Dulbecco, 1950; Lennox *et al.*, 1954), but usually reactivation involved the intracellular participation of genetic material from more than one virion. Reactivation of U.V.-irradiated phage by multiple infection of susceptible bacteria has been termed multiplicity reactivation; the rescue of markers from irradiated phage of simultaneous infection of the same cell with U.V.-damaged phage and an active unlike phage was called cross-reactivation (Luria, 1947; Doermann *et al.*, 1955) or marker-rescue.

Many types of physical and chemical treatment inactivate phages by damage to their protein components rather than their genetic material. This may be the protein coat of the head—for example, osmotic shock (Anderson, 1950)—or may involve the complex mechanism by which bacterial viruses attach to susceptible bacteria and inject their genetic material. Phages inactivated by these treatments have not been reactivated in intact bacterial cells, but some progress has been made in the infection of protoplasts with them (Spizizen, 1957; Mahler and Fraser, 1959). The detailed investigation of genetic reactivation of phages has thrown considerable light on their replication (Stahl, 1959).

Although a great deal of information has been accumulated on the inactivation of animal viruses (see, for example, Pollard, 1960), little attention has been given to their intracellular reactivation. This stems principally from the lack of sufficiently precise quantitative methods, and to some extent from the lack of suitable genetically marked viruses. Multiplicity reactivation of U.V.-irradiated influenza virus has been demonstrated by Henle and Liu (1951) and by Barry (1961), and cross-reactivation has been shown to occur with the same virus by Gotlieb and Hirst (1956). Recently irradiated vaccinia virus has been shown to undergo both multiplicity and cross-reactivation (Abel, 1962b).

What Burnet (1960) described as "the first example of what may be called genetic interaction between animal viruses" was the demonstration by Berry and Dedrick (1936) that some rabbits inoculated with a mixture of heat-inactivated myxoma virus and active fibroma virus died of myxomatosis. After a period of neglect this phenomenon has been intensively studied

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during the last five years by three groups of workers: Kilham and his collaborators in U.S.A. (review: Kilham, 1960); the Hanafusas and Kamahora in Japan; and our group in Canberra (review: Fenner, 1962). Japanese and Australian workers independently demonstrated that the Berry–Dedrick phenomenon occurred with all pox viruses, and both groups now accept the view that it is basically due to reactivation of the heat-inactivated virus. We believe, however, that this is a novel kind of "non-genetic" reactivation, so that the Berry–Dedrick phenomenon is not to be regarded as an example of genetic interaction between animal viruses.

Genetic and Non-genetic Reactivation

For clarity of treatment of the subject we will consider separately two types of intracellular reactivation. Genetic reactivation includes multiplicity- and cross-reactivation, which involve co-operation between the genetic material of more than one virion. In non-genetic reactivation, which has so far been demonstrated only with the pox viruses (Table I), the intact genome of

TABLE I.—*Occurrence of Genetic and Non-genetic Reactivation, and Genetic Recombination, Among Animal Viruses*

Virus Group	Non-genetic Reactivation	Genetic Reactivation	Genetic Recombination
Pox virus	+	+	+
Myxovirus :			
Influenza virus	−	+	+
Newcastle disease virus	..*	−	−
Herpes virus	−	..	+
Poliovirus	−	−	−

*Not tested.

a virus inactivated by damage to some non-genetic component is enabled to express itself owing to the activity of a non-genetic component of another pox virus. This definition excludes consideration of extracellular non-genetic reactivation (such as reversal of neutralization by antibody) and of infectious nucleic acids.

Genetic Reactivation with Myxoviruses

Using intact chick embryos, Henle and Liu (1951) found that preparations of U.V.-irradiated influenza virus produced larger yields than would have been expected from their residual infectivity, and did so more rapidly than expected. Some of this effect was probably due to rapid elution, a characteristic of large inocula (Cairns, 1955), but the increased yield could not be entirely explained thus, and was probably due to multiplicity reactivation.

By measuring the first-cycle yields of pieces of surviving allantois-on-the-shell (Fazekas de St. Groth and White, 1958) Barry (1961) found that with active influenza virus there was a straight-line relation between dose and yield, corresponding to a "one-particle" curve, with input multiplicity $\leqslant 1$. With U.V.-irradiated virus, however, the yield rose much more steeply with increasing dosage, and was much greater than could be accounted for by the surviving infectious virus (Fig. 1). Barry ascribed this to multiplicity reactivation, which he found to be a highly efficient process. There was an interesting relationship between the irradiation dose, the multiplicity of infection, and the yield of incomplete influenza virus.

Newcastle disease virus (N.D.V.) is also a myxovirus, which has a rather different morphology from influenza virus (Horne *et al.*, 1960) and is regarded by Waterson (1962) as belonging to another subgroup. Barry (1962) found that in contradistinction to influenza virus multiplicity reactivation did not occur with N.D.V.

These results agree with observations on genetic recombination and cross-reactivation with influenza and N.D.V. Both these genetic interactions are readily demonstrated with viruses of the influenza group (Burnet, 1960; Simpson and Hirst, 1961); neither could be demonstrated with N.D.V. (Granoff, 1959).

Exploratory experiments failed to reveal evidence of non-genetic reactivation between influenza A and influenza B (Fazekas de St. Groth, quoted by Fenner

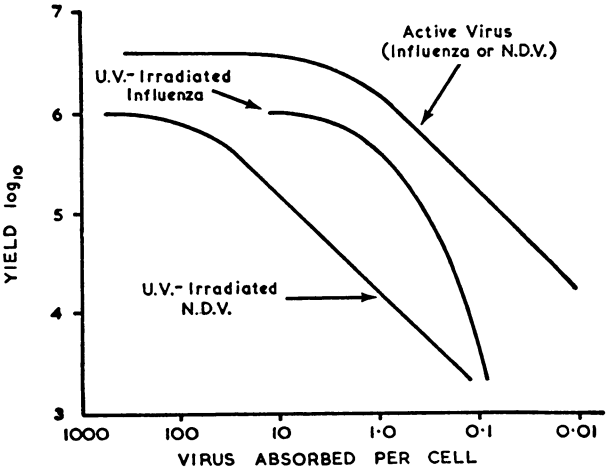

FIG. 1.—Multiplicity reactivation of U.V.-irradiated influenza virus, and failure of similarly irradiated Newcastle disease virus (N.D.V.) to undergo multiplicity reactivation. The response curves obtained in chick-allantoic cells for U.V.-irradiated influenza virus and N.D.V. (approximately six hits) are compared with the standard response curves obtained from unirradiated virus. The irradiated N.D.V. curve represents the yield of the residual infective virus in the irradiated preparation. The irradiated influenza-virus curve would have had the same slope had not multiplicity reactivation occurred. (Modified from Barry, 1961, 1962.)

and Woodroofe, 1960). However, in experiments with active and U.V.-irradiated influenza virus Simpson and Hirst (1961) found, in addition to cross-reactivation of the genetic type, suggestive evidence that some sort of non-genetic reactivation might occur. With one particular combination of viruses (U.V.-Mp^t/Jp^-) there were only three recombinants among 56 reactivants. The problem needs further study.

Genetic Reactivation with Polioviruses

In spite of intensive efforts by both American and Japanese investigators with what appeared to be satisfactory systems, no evidence has been produced of genetic recombination among polioviruses, either by using two active parents or by cross-reactivation.* Drake (1958) has reported that multiplicity reactivation of U.V.-irradiated poliovirus does occur, but the effects are slight and the evidence is equivocal. After inactivation with hydroxylamine under conditions which showed extensive multiplicity reactivation with fowl-plague virus (Schaefer and Rott, 1962), there was no evidence of multiplicity reactivation of poliovirus even at survivals of 10^{-9} and with adequate assay methods (Schaefer, personal communication, 1962).

The only work reported on non-genetic reactivation of poliovirus was an exploratory experiment by Howes (quoted by Fenner and Woodroofe, 1960), who found no evidence of reactivation of heat-inactivated type 1 poliovirus by active poliovirus type 2.

*Recently Ledinko (*Cold Spr. Harb. Symp. Quant. Biol.*, June, 1962) has reported conclusive evidence that recombination does occur with polioviruses.

Genetic Reactivation Among the Pox Viruses

The demonstration that genetic recombination occurred between unrelated strains of vaccinia virus (Fenner and Comben, 1958 ; Fenner, 1959) and between mutants derived from a single clone (Gemmell and Cairns, 1959 ; Gemmell and Fenner, 1960) suggested that genetic reactivation would occur with this system if suitable combinations of host cells, U.V.-irradiated virus, and active virus were used. Abel (1962b), working in Canberra, has now shown that this prediction was correct, and her experiments have highlighted the importance of the spatial distribution of infective particles within the cell in such genetic interactions (Abel, 1962a).

Multiplicity Reactivation

In the course of experiments on the kinetics of U.V. inactivation of rabbit-pox virus (a member of the vaccinia-variola subgroup) and of several strains of vaccinia virus Abel (1962b) found that when suspensions of virus irradiated for different periods were assayed on chick-embryo fibroblasts or on the chorio-allantoic membrane the inactivation curve consisted of three linear components. She was unable to demonstrate genetic inhomogeneity of the viral populations used, and the first change of slope remains unexplained. It may be due to host-cell reactivation. The flattening of the curve at a survival of 10^{-4} recalls the result obtained with phage by Luria (1947), and Abel has proposed the same explanation for the phenomenon as that developed by Luria—namely, multiplicity reactivation. She demonstrated this by inoculating chick-embryo fibroblast plates with doubling dilutions of unirradiated and heavily irradiated virus, with the results shown in Table II. With unirradiated virus the plaque

TABLE II.—*Effects of Doubling the Volume of the Inoculum on the Assay of Rabbit-pox Virus on Chick Embryo Fibroblasts. Before and After U.V. Irradiation. (From Abel, 1962b)*

U.V. Irradiation	Dilution	Volume of Inoculum	Plaque Count
0	10^{-5}	0·05 ml.	8
		0·1 ml.	15
		0·2 ml.	25
420 seconds	10^{-1}	0·1 ml.	4
		0·2 ml.	19
480 seconds	10^{-1}	0·1 ml.	1
		0·2 ml.	20

count increased in proportion to the dilution, but with concentrated suspensions of the heavily irradiated virus doubling the size of the inoculum (and in other experiments doubling the concentration with constant inoculum) led to a fivefold to twentyfold increase in the plaque count. Similar results were obtained with chick-embryo fibroblasts infected in suspension, but experiments with suspended KB cells gave no evidence of multiplicity reactivation.

Earlier experiments by Cairns (1960) on the initiation of vaccinia infection in KB cells had shown that in multiply-infected cells each infective particle produced a separate cytoplasmic "factory" where new viral deoxyribonucleic acid (D.N.A.) and protein were formed. The usual wide separation of these foci and their discrete character, for at least the first 10 hours of the multiplication cycle, led Cairns to suggest that topography might play an important part in genetic interactions of vaccinia virus. KB cells are at least 10 times larger than chick-embryo fibroblasts, and Abel postulated that in these larger cells the chance that two virions with U.V.-damaged genomes would be close enough to co-operate, so that multiplicity reactivation might occur, would be very small. In the small chick-embryo fibroblasts there was a greater chance of propinquity leading to reactivation. The hypothesis was tested by infecting suspended KB cells with clumped and redispersed normal and irradiated virus, with the results shown in Table III. As predicted by

TABLE III.—*Effect of State of Aggregation of Virus Particles on Infective Centre Assays of Suspended KB Cells Infected with Normal and U.V.-irradiated Rabbit-pox Virus. (From Abel, 1962a)*

U.V. Dose	Plaque Count with Virus		Ratio a/b
	Clumped (a)	Dispersed (b)	
0	100	160	0·62
60 seconds	66	10	6·6

the hypothesis, clumping reduced the infectivity of unirradiated virus but greatly increased the plaque count with irradiated virus.

Cross-reactivation

Since genetic recombination occurs in cells mixedly infected with active pox viruses and under suitable conditions multiplicity reactivation occurs with U.V.-irradiated preparations, it was to be expected that cross-reactivation would also occur. This was successfully demonstrated by Abel (1962b) in KB cells infected with U.V.-irradiated RPu^+ (wild type rabbit-pox virus), which produces large plaques on chick-embryo fibroblasts, and RP$u1$ or RP$u2$, two mutants which produce minute plaques (Gemmell and Fenner, 1960). The rate of inactivation of the u^+ marker, assayed in cells mixedly infected with U.V.-irradiated RPu^+ and active RP$u1$ or RP$u2$, was much slower than the rate of inactivation of the infectivity of RPu^+ assayed alone. As yet no attempt has been made to assess the role of topography in this interaction. *A priori*, the situation might be intermediate between those found in multiplicity reactivation and in genetic recombination between two active viruses.

Reactivation of Azide-blocked Vaccinia Virus

Easterbrook (1961) showed that, in the presence of 3×10^{-3} M sodium azide, vaccinia virus adsorbed normally to suspended KB cells, and underwent eclipse, but he could detect no new infectious virus and no new viral components in these cells 24 hours later. Using a more sensitive method, Appleyard and Westwood (1962) have shown that certain new antigens are produced in such cells. They confirmed the absence of new infectious virus.

Removal of the inhibitor and further incubation of these cells failed to reverse the inhibition, but if washed cells were superinfected with another virus of the vaccinia-variola subgroup both the superinfecting virus and the azide-blocked virus multiplied ; the latter only in cells in which the superinfecting virus also multiplied. In one experiment myxoma virus (which will effectively reactivate heated vaccinia virus by a non-genetic mechanism—see next section) was shown to multiply itself, but it failed to reactivate the azide-blocked virus (Table IV). This phenomenon merits more sophisticated genetic analysis, but it may tentatively be regarded as more like cross-reactivation than non-genetic reactivation.

TABLE IV.—*Reactivation of Azide-inhibited Vaccinia Virus. (Data from Easterbrooke, 1961)*

Superinfecting Virus		Virus Yield	
Strain	Pock Type	Size	Pock Type
Nil	—	0	—
Vaccinia	U	Large	U
Rabbit-pox	U+	,,	U, U+
Myxoma	U^m	,,	(U^m)*
U.V.-rabbit-pox	U+	0	—
N.M.-rabbit-pox	U+	0	—
H-rabbit-pox	U+	Small	U, U+

N.M. = Nitrogen mustard inactivated. H = Heat inactivated.

*Infected cells were stained by myxoma-immune fluorescent antibody, but not by vaccinia-immune coupled antibody. Egg assay revealed no vaccinia pocks.

Suspended KB cells were infected with vaccinia virus (U pocks) in the presence of $10^{-3.5}$ M sodium azide. After 18 hours at 37° C. they were washed, superinfected, and reincubated in normal growth medium for 24 hours. The virus yield was assayed on the chorio-allantoic membrane, particular attention being paid to pock morphology.

No reactivation occurred when the superinfecting virus had itself been inactivated with nitrogen mustard or U.V., but superinfection with heat-inactivated virus led to some degree of reactivation of both components. It is not yet possible to interpret this result, except to suggest that in some cells the azide-blocked virus might reactivate the heated virus by a non-genetic mechanism and this reactivated virus could then reactivate the azide-blocked virus by a genetic mechanism.

Non-genetic Reactivation

In the introduction it was pointed out that the Berry–Dedrick phenomenon, originally demonstrated with heat-inactivated myxoma virus and active fibroma virus (Berry and Dedrick, 1936), was a general feature of the pox viruses and that the most likely mechanism was the intracellular non-genetic reactivation of the heat-inactivated virus.

It will be recalled that Berry's experiments were based on the demonstration of bacterial transformation by Griffith (1928). Berry (1937) realized that his results could be interpreted either as "transformation" of the active fibroma by a component of the heated myxoma virus or as reactivation of the latter. He, and later Kilham (1960), adopted the term "transformation" to describe the phenomenon.

Terminology

The terminology seemed important to us, since it greatly affected the experimental approach to a study of the mechanism (Fenner *et al.*, 1959). As the term is used in bacterial genetics (from which it was borrowed by Berry) transformation describes the heritable modification of the properties of one bacterial strain by an extract (D.N.A.) derived from cells of another strain. Several features of the interaction of heated and active pox viruses precluded this interpretation. (1) The phenomenon is general to viruses of the pox virus group, but is confined to that group (Table V). (2) With pairs of viruses which do not undergo genetic recombination (such as members of different subgroups), all recognizable characters in the revived agent are identical with those of the virus which has been heat-inactivated; there is no evidence of transfer of genetic information between the two components (Fenner and Woodroofe, 1960). (3) Joklik *et al.* (1960a) showed that reactivation could be achieved by a virus whose genetic material was damaged with nitrogen mustard, again without evidence of genetic transfer.

Several lines of evidence indicate that reactivation is due to an intracellular event, and is not merely due to the facilitation of the uptake of the inactivated virus. Vaccinia virus enters cells by phagocytosis (Dales and Siminovitch, 1961; Mercer and Fenner, unpublished observations). Heat-inactivated virus appears to be taken up as readily as active virus, and in the absence of reactivation it has been shown to exert profound effects on the cell (H. Hanafusa, 1960b; T. Hanafusa, 1960; Galasso and Sharp, 1961). Smith and Sharp (1961) found that heat-inactivated virus underwent "eclipse" in the same fashion as active virus—that is, it disappeared as a morphological entity.

We will therefore adopt the view that the Berry–Dedrick fibroma–myxoma virus "transformation" is a special case of a phenomenon of general occurrence among the pox viruses, and that it is essentially a process of intracellular non-genetic reactivation of virus which has been inactivated by a method which leaves its genetic material intact.

TABLE V.—*Specificity of Non-genetic Reactivation and Genetic Recombination. Any Active Pox Virus (and Pox Viruses Inactivated in Certain Ways) Will Reactivate Heat-inactivated Rabbit-pox Virus —RPu− (or Any Other Heat-inactivated Virus). Closely Related Pox Viruses May Also Undergo Genetic Recombination as a Secondary Event. No viruses Other than Pox Viruses will Induce Non-genetic Reactivation. (Data from Fenner and Woodroofe, 1960; Woodroofe and Fenner, 1960)*

Reactivating Agent	Yield	
	RP*u*+	Recombinants
RP*u*1(rabbit-pox mutant)	+++	−
Vaccinia 7N	+++	−/+
CP*u*16 (cowpox mutant)	+++	−/+
Ectromelia	+++	+
Fowlpox	+++	−
Myxoma	+++	−
Fibroma	+++	−
MVE, herpes, Rous virus, psittacosis, influenza A, ILT	−	−
Nitrogen mustard 7N	+++	−

Demonstration of Non-genetic Reactivation

In describing and analysing this phenomenon certain new terms were proposed (Joklik *et al.*, 1960c), and these are shown in Table VI. Although we believe that any active pox virus can, under appropriate conditions, reactivate by this non-genetic mechanism any suitably inactivated pox virus, a number of practical considerations govern the ease of demonstration of reactivation. It will occur only in cells which will support at least the initial stage of the growth cycle of the reactivator (in its active form), and the complete growth cycle of the active form of the reactivable agent.

TABLE VI.—*Terminology Used in Discussion of Non-genetic Reactivation, Nature of Agents Used, and Combinations Used in Experimental Work*

Reactivable agent	Heat-inactivated virus Urea-inactivated virus
Reactivating agent or reactivator	Another active pox virus Nitrogen-mustard-treated virus
Favourable combinations	Fibroma and H-myxoma Ectromelia and H-vaccinia or H-rabbit-pox N.M.-rabbit-pox and H-vaccinia

Demonstration of reactivation is difficult if the growth rate of the reactivated agent is lower than that of the reactivator, and easy if its growth rate is much higher. This has led to the selection of particular combinations as model systems (Table VI).

The heat-inactivated component remains associated with cells in a reactivable stage for a prolonged period (Kilham *et al.*, 1958; Joklik *et al.*, 1960c). In this respect it resembles inactivated influenza virus, which can be cross-reactivated several days after it has entered

susceptible cells (Baron and Jensen, 1955; Gotlieb and Hirst, 1956).

The direct demonstration of reactivation by the simultaneous inoculation of large doses of heated vaccinia virus and small doses (usually 20–40 P.F.U.) of the reactivating agent on the chorio-allantoic membrane proved a convenient way of testing the reactivating capacity of the many viruses which produce pocks on the chorio-allantoic membrane (Fenner and Woodroofe, 1960). Vaccinia produces large and distinctive pocks which develop rapidly, and reactivated vaccinia virus therefore produces distinctive pocks at a time (two days after inoculation) when the reactivating agent—for example, myxoma, fibroma, or ectromelia virus—produces hardly recognizable lesions. The same principle was applied in cultured cells by Hanafusa *et al.* (1959). With suitable modifications it also provided a method of assay of reactivability (Joklik *et al.*, 1960b; H. Hanafusa, 1960a).

Properties of the Reactivable Agent

All three groups of workers have investigated the properties of the reactivable agent (Kilham *et al.*, 1958; H. Hanafusa, 1960a; Joklik *et al.*, 1960b) with results which show a large measure of agreement. No one has yet dissociated reactivability from the viral particle, and a variety of physical and chemical treatments of infectious virus will destroy infectivity but leave reactivable particles. Other treatments destroy both properties (Table VII). In general, agents which denature proteins

TABLE VII.—*Response of Active Virus and Reactivable and Reactivating Agents to Various Physical and Chemical Treatments. All Treatments Destroy the Infectivity of Active Virus (+). Some Treatments Convert Active Virus to the Reactivable State (→Ra); Some Treatments Leave Active Virus With the Reactivating Capacity (→Rg). For Reactivable and Reactivating Agents "+" Indicates that the Property is Destroyed as Rapidly as the Infectivity of Active Virus; "−" Indicates that it is Destroyed Less Rapidly. (Data from Joklik et al. (1960b), H. Hanafusa (1960b), and Holmes (1961)*

	Active Virus Infective	Heated Virus Reactivable (Ra)	Mustard-treated Virus Reactivating (Rg)
Heat	+→Ra	−	+
Urea	+→Ra	−	+
Nitrogen mustard	+→Rg	+	−
U.V.	+(→Rg)	+	−
n-Butanol	+	+	+
Sodium dodecyl sulphate	+	+	+

but have little effect on D.N.A. (heat, urea, etc.) destroy infectivity and produce reactivable particles. Agents which act primarily on viral D.N.A. (U.V., nitrogen mustard, photodynamic inactivation) destroy reactivability and infectivity at about the same rate but may spare the reactivating capacity (see next section).

Working with vaccinia and rabbit-pox virus, neither H. Hanafusa (1960a) nor Joklik *et al.* (1960b) were able to produce reactivable particles that were susceptible to DNase, but Shack and Kilham (1959) reported that the reactivability of heated myxoma virus which was subsequently treated with 8 M urea was destroyed by DNase. Unfortunately the method of assay these investigators used (rabbit inoculation) did not lend itself to quantitation. It will be recalled that the infectivity of myxoma virus is destroyed (Andrewes and Horstmann, 1949) but its reactivability spared (Kilham *et al.*, 1958) by treatment with ethyl ether, to which the infectivity of vaccinia is resistant. It is not altogether surprising that it is possible to produce reactivable myxoma which differs from reactivable vaccinia in its DNase susceptibility, but the problem requires further study.

Experiments with proteolytic enzymes, combined with electron microscopy of the treated particles, suggested that reactivability could survive fairly extensive changes in the protein coat of rabbit-pox virions (Joklik *et al.*, 1960b).

In experiments with virus labelled with ^{14}C Joklik (1962) found that conversion to the reactivable state by heating or treatment with urea was associated with the loss of about 5% of the viral protein. There is as yet no evidence that this loss is the cause of the inactivation nor that the protein component lost is the one which may be concerned with non-genetic reactivation.

Work on the reactivable agent suffers from the absence of an assay method which can be related to adsorbed viral particles, although such a method could be devised, using Sharp's (1960) methods of particle-counting. Nevertheless we can make the generalizations that reactivability is a property (*a*) which cannot be separated from the virus particle, (*b*) which is dependent upon the integrity of the viral D.N.A., and (*c*) which results when virus is inactivated by methods which affect primarily certain protein components of the virus.

Properties of the Reactivating Agent

Experiments by Hanafusa *et al.* (1959) and by Fenner and Woodroofe (1960) (see Table V) showed that any active pox virus (but no other active virus) was potentially capable of reactivating any reactivable pox virus. The only other properties which are common but peculiar to all members of the pox virus groups are size and shape, and the common internal antigen of Woodroofe and Fenner (1962). Table VIII summarizes the properties of the pox viruses as we understand them at present.

TABLE VIII.—*The Pox-virus Group*

Shape and size	Ovoid; approximately 300–350 by 200–250 by 100 mμ
Nucleic acid	D.N.A.
Antigens	Several, but common internal antigen
Site of multiplication	Cytoplasm
Non-genetic reactivation	Confined to members of group; active between subgroups
Genetic recombination	Only within subgroups

Subgroups			Ungrouped
Vaccinia Variola Ectromelia Rabbit-pox Cowpox	Fowlpox Canary-pox Pigeon-pox etc.	Myxoma Fibroma Squirrel fibroma	Molluscum contagiosum Monkey-tumour pox virus Swinepox Bovine papular stomatitis Sheep-pox Contagious pustular dermatitis

Joklik *et al.* (1960a) showed that the reactivating agent could survive treatment with nitrogen mustard, but reactivability is destroyed by this treatment (Holmes, 1961). Their results, illustrated in Fig. 2, showed that virus whose infectivity was destroyed by nitrogen mustard treatment was able to reactivate heated-inactivated virus. U.V. irradiation was found to produce an effect similar to that of nitrogen mustard, but the results were extremely irregular (Fenner, unpublished results). Fig. 2 shows that the infectivity of wild-type rabbit-pox virus (RPu^+) is destroyed by nitrogen mustard by a one-hit process. Its ability to reactivate RPu1—a mutant which is outgrown by RPu^+ in mixed

infections on the chorio-allantoic membrane (Gemmell and Fenner, 1960)—is destroyed at about half this rate, again by a one-hit process. It seems, therefore, that all the lethal damages caused by nitrogen mustard prevent the expression of the genotype (as judged by one marker), but only about one-half of them fall into that part of the virus which is responsible for the reactivation of heat-inactivated virus. Thus the reactivating agent contains some essential non-genetic material which is destroyed in heated virus.

Some clue to the nature of this material comes from an experiment of Holmes (1961), who found that the reactivating capacity of nitrogen-mustard-treated virus was as susceptible of destruction by heat as was the infectivity of active virus, and very much more sensitive than the reactivability of heated virus (Fig. 3). It is

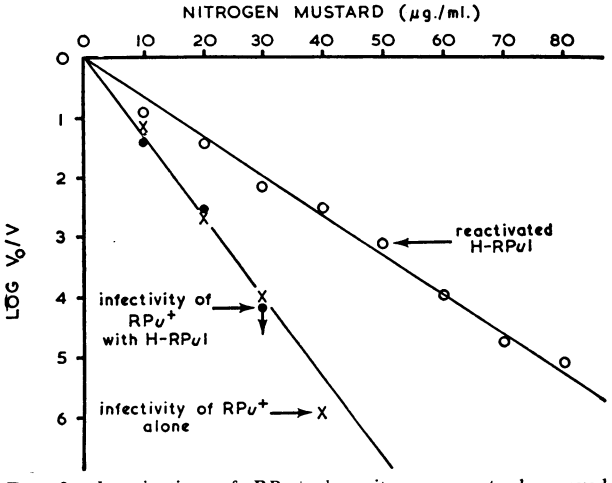

FIG. 2.—Inactivation of RP*u*+ by nitrogen mustard assayed alone (×), or in the presence of heated RP*u*1 (●), and the reactivation of heated RP*u*1 (○). (Modified from Joklik *et al.*, 1960a.)

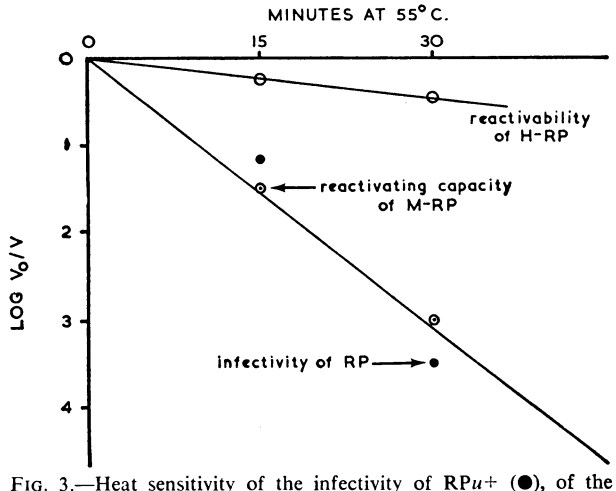

FIG. 3.—Heat sensitivity of the infectivity of RP*u*+ (●), of the reactivability of heated RP*u*+ (○), and of the reactivating capacity of nitrogen-mustard-treated RP*u*+ (⊙). (From Holmes, 1961.)

tempting to relate this heat-sensitive material to the protein which forms the common internal antigen of all members of the pox-virus group (Woodroofe and Fenner, 1962).

Discussion

The work I have described raises more problems than it answers. Apart from their special interest to virologists concerned particularly with animal viruses, experiments on the reactivation of influenza and vaccinia viruses pose three matters of general importance. These are (1) the role in genetic recombination of the spatial distribution within the cell of pools of replicating viral nucleic acid (the topographic factor); (2) the mechanism of non-genetic reactivation and its relation to initiation; and (3) the implications of reactivation for the preparation and testing of viral vaccines.

Topography in Virus Genetics

The possibility that topography might be important in phage genetics has been raised by Kellenberger *et al.* (1959) from a consideration of electron micrographs of thin sections of *E. coli* infected with T2, and by Bresch (1959) during a discussion of the mechanism of genetic recombination in phage. With the T-even phages the recombination frequency is not multiplicity-dependent, and recombination is not dependent upon replication. Here the role of topography depends upon the nature of the mating process. With bacteriophage T1 Trautner (1960) found that the recombination frequency rose with increasing multiplicity of infection. After excluding other possible causes Trautner concluded that this was due to the operation of a topographic factor.

The problem of topography is presented in a more clear-cut fashion with viruses affecting animal cells, for here the disparity of size between virus and cell is much greater. If the viruses multiply in the nucleus the genetic material from different virions in multiply-infected cells may be "focused" into a localized area. The multiplication of papilloma virus, for example, appears to occur initially in the nucleolus (Stone *et al.*, 1959). The nuclear location of ribonucleic acid (R.N.A.) replication in influenza virus infections may be responsible for the frequent occurrence of genetic co-operation with this virus (Barry, 1961).

With viruses which multiply in the cytoplasm, like vaccinia, it has long been known that multiple infection may produce multiple foci within the cytoplasm of single cells. Cairns's (1960) autoradiographic studies showed clearly that each infective virus particle produces its own discrete D.N.A. pool, and unless they were by chance quite close to each other different pools did not fuse or mix until quite late in the growth cycle. Cairns appreciated the implications of his observations so far as the role of topography in vaccinia genetics was concerned, but quantitative methods have until recently been too crude to allow the necessary kinetic experiments to be performed.

Multiplicity reactivation of U.V.-inactivated vaccinia virus bypassed this difficulty, for here there were no expanding D.N.A. pools but genetically damaged particles which could co-operate and ultimately replicate only if they were very close together in the cell. Abel's (1962a) experiments on the effect of clumping on multiplicity reactivation in KB cells appear to provide an unequivocal demonstration of the importance of the topographic factor in at least one genetic interaction.

Mechanism of Non-genetic Reactivation

Reactivable pox viruses can be produced by several methods of treatment of active pox viruses—mild heating, urea, guanidine (Holmes, 1961), and, with myxoma virus, ether. They are genetically intact in that reactivation results in full expression of their genetic potential, and their capacity for reactivation (reactivability) is destroyed by agents which act primarily on D.N.A. with much the same kinetics as the destruction of the infectivity of active virus by these treatments.

Reactivating agents, on the other hand, are either active pox viruses or pox viruses inactivated by methods which primarily damage their D.N.A. (such as nitrogen mustard). Their capacity to reactivate is destroyed by heat with the same kinetics as the infectivity of active virus.

Woese (1960) has suggested that the primary factor in thermal inactivation of animal viruses is damage to their nucleic acid. Experiments in which nucleic acid is extracted from heated virus have always shown that infectious nucleic acid could be obtained from virus suspensions which were completely inactive—Murray Valley encephalitis virus (Ada and Anderson, 1959); Shope's papilloma virus (Ito, 1961)—suggesting that there must be a component other than damaged nucleic acid which contributes to the inactivation of the intact virions. Vaccinia virus contains double-stranded D.N.A. with a molecular weight of about 180 million (Joklik, unpublished). Infectious D.N.A. has never been obtained from a pox virus, and it seems more likely that the component of a pox virus whose destruction by heat, urea, etc., inactivates infectivity but yields reactivable virus is a protein and not D.N.A.

Heat-inactivated vaccinia virus retains its characteristic morphology (Joklik *et al.*, 1960b) and is normally phagocytosed by susceptible cells, so that its inactivation is not the result of damage to its surface and consequent difficulties of adsorption and uptake. Three different activities, with somewhat different heat-sensitivities, have been demonstrated in heat-inactivated vaccinia virus: (*a*) in doses as low as one particle per cell it may interfere with the multiplication of active virus added two hours later (Sharp, personal communication, 1961); (*b*) it produces characteristic changes in the nucleic acid metabolism (T. Hanafusa, 1960) and the viability (H. Hanafusa, 1960b) of cells into which it enters; and (*c*) it may be reactivated by a mechanism which does not involve genetic interaction with another pox virus. All these properties are as susceptible to destruction by agents which act primarily on D.N.A. (U.V., nitrogen mustard) as is the infectivity of active virus.

We suggest, therefore, that methods of inactivation which produce reactivable virus destroy viral infectivity not by damage to viral D.N.A., or by damage to the surface protein and consequent loss of adsorptive properties, but by damage to some other protein component of the virus. The resistance of reactivability to tryptic digestion suggests that it is an internal protein, and the fact that all pox viruses, but only pox viruses, can act as reactivators suggests that it may be related to the internal antigen which Woodroofe and Fenner (1962) found to be common to all viruses of the pox-virus group. Cells in which the replication of a pox virus has been blocked with sodium azide may possess, for a limited period, the capacity to reactivate heated pox viruses (Easterbrook, 1961). Such cells may prove the best source for recovery of the component responsible for non-genetic reactivation free of the virus.

The evidence derived from non-genetic reactivation suggests that the pox viruses may require more than intact D.N.A. for replication, and it will be recalled that all attempts to obtain infectious D.N.A. from them have failed. The demonstration of the susceptibility to proteases of protoplast-infecting particles derived from T2 bacteriophages (Spizizen, 1957; Sekiguchi, 1958; Mahler and Fraser, 1959) raises the possibility that with these large viruses also the infected host cell of some intact protein, as well as intact D.N.A., may be a prerequisite for replication. The "infectious D.N.A." obtained from λ bacteriophage, on the other hand, appears to consist almost entirely of D.N.A., with less than 5% protein (Kaiser and Hogness, 1960). It consists of the intact phage chromosome (Kaiser, 1962), but it is not infectious in the absence of infection of the host bacteria by "helper phage." The exact function of the helper phage is unknown, but it may well supply one or more proteins necessary for early steps in the intracellular replication of the D.N.A.

Reactivation and Viral Vaccines

Reactivation, both genetic and non-genetic, is potentially important to those who manufacture viral vaccines. It is now widely recognized that cultures of primary cells may frequently be contaminated with viruses derived from the host animal. Perhaps the best example is the many simian viruses which have been recovered from primary monkey-kidney-cell cultures. If non-genetic reactivation can occur in groups other than pox viruses one can visualize the complications of assay that could occur when a nominally inactivated virus preparation was assayed in such cells, which happened to contain a reactivating virus as a contaminant.

One of the major requirements of an effective viral vaccine is that its antigenic potency should be high. Methods of inactivation have therefore been sought which might ensure this. Such methods—for example, U.V. irradiation, hydroxylamine—act primarily by damaging the genetic material of the virion, with much less effect on the antigenic coat. The foregoing discussion has shown that it is with such materials that multiplicity reactivation will occur if the virus concerned is capable of genetic reactivation. With influenza and vaccinia viruses, for example, U.V.-irradiated virus could be expected to undergo multiplicity reactivation if a large dose was injected in such a way as to allow multiple infection of cells. In human vaccination with these viruses this result would be irrelevant, but it is a difficulty encountered in attempts by virologists to obtain antisera without viral multiplication. With poliovirus multiplicity reactivation probably does not occur, but in planning the production of inactivated vaccines with other viruses the possibility of reactivation must not be overlooked.

Summary

Viruses may be treated by physical or chemical methods which render them non-infectious when cells are "infected" with single particles of the inactivated preparations. However, there is a variety of circumstances under which infection of cells with more than one virus particle may lead to reactivation of the inactivated virus.

Reactivation may be genetic—that is, due to co-operation between the genetic material from more than one virus particle. Examples are multiplicity reactivation, in which two or more particles of the same virus, each inactivated—for example, by ultra-violet irradiation—in a different part of the genetic material, co-operate to produce infectious virus; and cross-reactivation, where an active virus recombines with a genetically related U.V.-inactivated virus. Among the animal viruses these two phenomena have been demonstrated with influenza virus and vaccinia virus. Work with

vaccinia virus has underlined the importance, in genetic interactions of the animal viruses, of the spatial distribution of particles within infected cells.

Another kind of reactivation is known from studies with the pox viruses—namely, non-genetic reactivation. When first described this process was called "transformation," but it differs from bacterial transformation in so many respects that non-genetic reactivation is a more appropriate term. In essence, pox viruses inactivated by treatments (like heat and urea) which damage an essential viral protein while leaving intact the genetic material of the virus can be induced to multiply by introducing into the same cells another active pox virus or a pox virus in which the essential protein is intact even though the genetic material is damaged. This phenomenon has so far been observed only with the pox viruses.

Some general and special implications of viral reactivation are described, including its bearing on vaccine production.

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COMPARATIVE TRIAL OF BRITISH AND AMERICAN ORAL POLIOMYELITIS VACCINES

A REPORT OF THE PUBLIC HEALTH LABORATORY SERVICE TO THE POLIOMYELITIS VACCINES COMMITTEE OF THE MEDICAL RESEARCH COUNCIL*

Plan of the Trial

The purpose of this trial was twofold: (*a*) to compare the infectivity and antigenic potency of oral poliomyelitis vaccines prepared in Britain and the United States from Sabin's living attenuated strains of virus, and (*b*) to determine whether a single dose of oral vaccine could be relied upon to reinforce immunity in children who had previously received a full course of Salk inactivated vaccine.

The trial was carried out in five centres in England and Wales between May and August, 1961. Sixty children were vaccinated with British vaccine and 58 with American vaccine, and the poliovirus excretion and antibody response in the two groups of children were investigated.

The Vaccines.—The British and American vaccines were trivalent and contained the same attenuated strains of poliovirus. The British vaccine was prepared by the Wellcome Research Laboratories and the American vaccine by Merck Sharp & Dohme. Each vaccine was diluted and made up in ampoules containing a single dose of 0.5 ml. by the Medical Research Council's Immunological Products Control Laboratory. This dose contained $10^6 TCID_{50}$ of each type of poliovirus. The vaccine was given by mouth to the children either undiluted or mixed with syrup or honey.

The Children.—The children taking part in the trial were aged 5 to 7 years. All of them had been vaccinated

*The information was collated and analysed by Dr. N. S. Galbraith (Colindale). Those taking part in the investigation included Dr. P. G. Mann (Bath), Dr. A. D. Evans (Cardiff), Dr. F. T. Perkins (Hampstead), Dr. L. Robertson (Preston), Dr. J. A. Rycroft (Southend), and Dr. E. M. Mackay-Scollay (Stafford).