BIKEN'S JOURNAL 3, 41-56 (1960)

Reactivation Phenomena in the Pox Group Viruses III. Some Properties of Heat-inactivated Vaccinia Virus

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(Received for publication, March 16, 1960)

SUMMARY

Reactivation of heat inactivated vaccinia (IV) by superinfection of active ectromelia virus in L cells has been investigated. Studies on some properties of IV have revealed that it is unable to produce either specific antigen or its progeny virus in the cells, but it has the same characteristics as the active virus in particle size, specific antigenicity, UV-sensitivity and the ability to be adsorbed onto the cells. UV-irradiation experiments and enzyme treatment have shown that the genetic units of the virus may be unchanged by heat inactivation and suggest that the mechanism of this phenomenon differs from that of cross-reactivation of UV-inactivated bacteriophages.

This paper describes another distinct property of IV. It is interference with reproduction of active homologous virus. In all experiments, inactivation of the "capacity of interference" (CI) was closely associated with the inactivation of "capacity to be reactivated" (CR). Therefore these two capacities are due to an entity in the heated virus in which viral DNA may play a part in establishing the interference. Methods for the quantitative estimation of CR and CI are also presented.

INTRODUCTION

The Berry-Dedrick phenomenon with rabbit fibroma and myxoma virus is known as a unique example of genetic interactions between animal viruses. It has been called transformation in this field of virus research (Smith, 1952; Kilham, 1957; Burnet, 1958), for it is apparently analogous to the transformation of bacteria in mice (Griffth, 1928). Transformation of fibroma into myxoma virus was also accomplished in cell cultures of rabbit tissues by Kilham (1957). He and his collaborators investigated several properties of the heat killed myxoma virus inducing the Berry-Dedrick phenomenon (Kilham *et al.*, 1958), and more recently found that upon treatment with urea it is susceptible to deoxyribonuclease (Shack *et al.*, 1959).

It was demonstrated independently by us (1959 a, b, c, d,) and Fenner *et al.* (1959) that this phenomenon applies to almost all the members of the pox group viruses including myxoma and fibroma virus. For example, inoculation of heat killed vaccinia together with active ectromelia in L cells produced a reactivated virus among the progenies, which was indistinguishable from vaccinia virus in four characters tested. Our experiments in tissue cultures indicated that the process of this reactivation took place within the cells infected with active and inactive viruses. Therefore, the situation in this case is quite different from bacterial

"transformation" in that the latter is associated with deoxyribonucleic acid and recipient bacteria. The "reactivation" has been known as a genetic interaction between active and inactive bacteriophages (*e. g.* cross-reactivation; Doermann *et al.*, 1955) and of myxovirus (*e. g.* reactivation by recombination; Burnet *et al.*, 1954; Gotlieb *et al.*, 1956). In these instances, some of the genetic markers of the inactive parents may manifest themselves among the progeny resulting from a mixed infection with heat- or UV-inactivated virus and the active one which are closely related in their genetic characters. It has been pointed out that the cross-reactivated loci are those which reside in healthy resions of the irradiated DNA and reproduce only after their "rescue" from the moribund genome by genetic recombination with the unirradiated parent (Doermann *et al.*, 1955).

To avoid a confusion in terminology, in this paper the author proposes to give up the term "transformation" used previously and call this genetic change of pox-viruses "reactivation", though it remains uncertain whether the mechanism of this reactivation involves any process of genetic interaction.

Since it can occur in vitro and the virus can be precisely titrated by a plaque method, a system composed of ectromelia and vaccinia virus in L cells was used for the quantitative study of the mechanism of this reactivation. The first approach to this problem is to see the degree of alteration in the virus by heat inactivation. As described previously, a clone of the reactivated virus obtained from a mixed infection with heat killed vaccinia and active ectromelia behaves in the same manner as the original vaccinia in the following four characters; plaque size (*p*), heat stability (*h*) and pathogenicity to rabbit (*r*) and mice (*m*). By the use of these differences in characters between ectromelia and reactivated virus, the "capacity to be reactivated" (abbreviated as CR; it may correspond to the phenotypic action of UV-inactivated bacteriophage or transforming activity of heated myxoma virus of Kilham) of heat inactivated virus was determined. The changes in CR of heated preparations after various physical and chemical treatments were compared with the infectivity of active virus.

This paper describes another distinct property of heat killed vaccinia virus, that is its "capacity of interference" (CI) with the multiplication of homologous virus. In order to see whether the two properties of this inactivated virus, i. e. CR and CI, are derived from the same or from separate entities and whether there is some relationship between these two activities, the effect of various treatments on these capacities was also investigated.

MATERIALS AND METHODS

1. *Tissue culture*

Monolayer cultures of Earle's L cells were prepared and cultivated as previously described (Hanafusa *et al.*, 1959c). Most of the experiments were carried out with monolayers grown in 200 ml prescription bottles, using La-Ye (cf. Media) with 5% calf serum as the growth medium.

2. *Media and salt solutions*

The following media and salt solutions were used: HS—Hanks' saline, La-Ye—Hanks' saline with 0.5% lactalbumin hydrolysate and 0.1% yeast extract, PBS—phosphate-buffered saline (Dulbecco and Vogt, 1954).

3. *Virus*

The IHD strain of vaccinia virus and the Biken strain of ectromelia virus derived from L cell culture were prepared as reported previously. Heat-inactivated vaccinia virus (IV) was obtained by heating the virus suspension of the L cell supernatant in an ampoule at 56°C usually for an hour. It had been well confirmed that the IV preparation contained no infectious surviving virus (Hanafusa *et al.*, 1959c).

4. *Virus assay*

The method of infectivity titration of the virus was given in detail in an earlier report (Hanafusa *et al.*, 1959c). In each titration, two bottles of culture were routinely assayed at each dilution.

5. *UV-irradiation*

As the source of ultraviolet light (UV) a germicidal lamp (Toshiba Electrics, 15-watt) was employed. A 2 ml aliquot of samples was placed in a petri dish (87 mm I.D.) and irradiated at the distance of 40 or 75 cm from a UV tube. Samples were shaken manually during the exposure.

6. *Standard infection procedure*

All the infections were carried out on monolayer of L cells, which were made up of approximately 5×10^6 cells. The standard procedure for infecting the culture was as follows. One ml of each sample was deposited on the cell layer, and the culture was incubated at 37°C for 2 hours and then washed three times with 4 ml of HS to remove unadsorbed material. After adding 8 ml of fresh medium the culture was reincubated at 37°C. However, in certain experiments in which the cells were subsequently exposed to a second inoculum of active virus, similar procedures were followed after washing off the first inoculum.

7. *CR assay*

In this study the determination of CR was carried out by two methods based on different principles. One of them was a dilution method, which uses the difference in *r* marker, i.e. pathogenicity to rabbits, of the reactivated and active ectromelia virus. One ml aliquots of ten fold dilutions of IV preparation were inoculated in L cells together with 1 ml of 5×10^5 PFU (plaque forming units) active ectromelia virus. After 48 hours the virus was harvested from the infected cells and passaged on another L cell culture. 0.2 ml of the virus suspension obtained from the first passage was inoculated intradermally into the rabbit. A skin lesion indicated the occurrence of reactivation of IV at that dilution.

While this dilution method was reliable for titration of CR and gave an estimate of the reactivated *r* marker, a still better result could be obtained by the following technique. It was based on the difference in the size of plaques (p) produced by the two viruses. One ml of the IV preparation to be determined was inoculated in L cells as described in the *Standard infection procedure*. After washing free of unadsorbed IV preparation, the cells were superinfected with 1 ml of 1×10^3 PFU active ectromelia virus. On the 3rd day, large plaques of the reactivated virus made their appearance among the small plaques due to ectromelia. It was easy to count the number of these large plaques.

If one assumes that reactivation takes place always in those cells which are infected with both ectromelia and IV and, that the distribution of IV is Poissonian, the CR (R) of the IV preparation may be expressed as a function of the number of large plaques produced (P), in the following manner,

$$P / E = 1 - e^{-R/n} \qquad (1)$$

or

$$R = -n \cdot \ln (1 - P / E) \qquad (2)$$

where E is the number of adsorbed ectromelia virus and n is the number of cells. The titer of CR was calculated from the count of large plaques by equation (2) and expressed as CRU

(units of the capacity to be reactivated). As shown in Fig. 1, a linear correlation was shown between the CRU and the relative concentration of IV. If the number of P to be

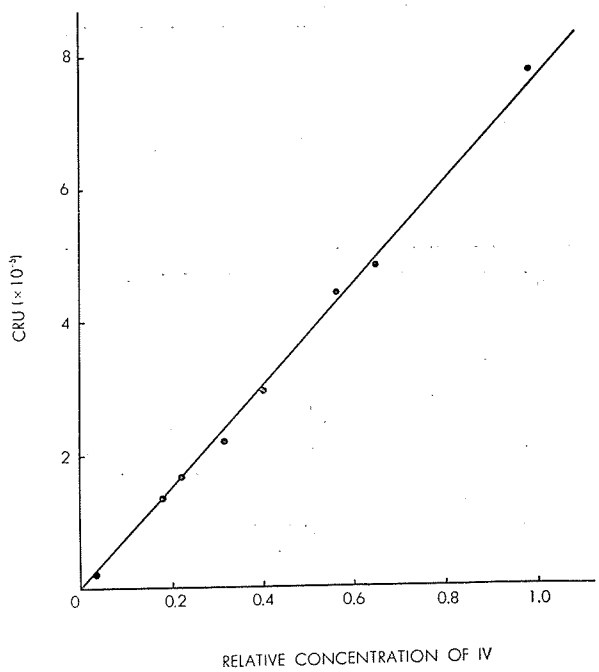

Fig. 1. Relationship between Doses of IV and Units of Capacity to be Reactivated (CRU).

counted is too small, the error of the determination will mostly come from the standard deviation of the count of P. Therefore, the determination was carried out in the range of $2\sim6 \times 10^6$ CRU. Since P is also proportional to E as shown in Fig. 2. the deviation of R derived from the second cycle-infection of ectromelia virus was assumed to be negligible. Although the absolute number of particles having CR could not be determined by either of the two procedures described above, the dilution method or the plaque method, its relative amount could be estimated by the latter method with a standard error not exceeding 20 per cent. Although parallel assays by the two methods did not give exactly the same values, the supernatant fluids of the culture producing large plaques always caused pathogenic lesions in the rabbit skin. This indicates at least that reactivation of an identical nature may take place simultaneously with these two genetic characters.

8. *CI assay*

A general aspect of the phenomenon of homologous interference by the IV preparation is presented in the section of Results. In this section, only procedures for determination of CI are dealt with. The interfering capacity of IV was revealed from the fact that the number of plaques produced by active vaccinia were reduced, when IV was inoculated to the culture at least 2 hours prior to the infection of active virus. Therefore, the CI assay of the IV preparation could be carried out by a procedure analogous to the plaque method for the CR assay. After being exposed to IV for 2 hours, the cell cultures were superinfected with 1 ml of 200~400 PFU of active vaccinia virus. As control cultures, the same doses of active vaccinia were inoculated into untreated cell cultures. On the assumption that the

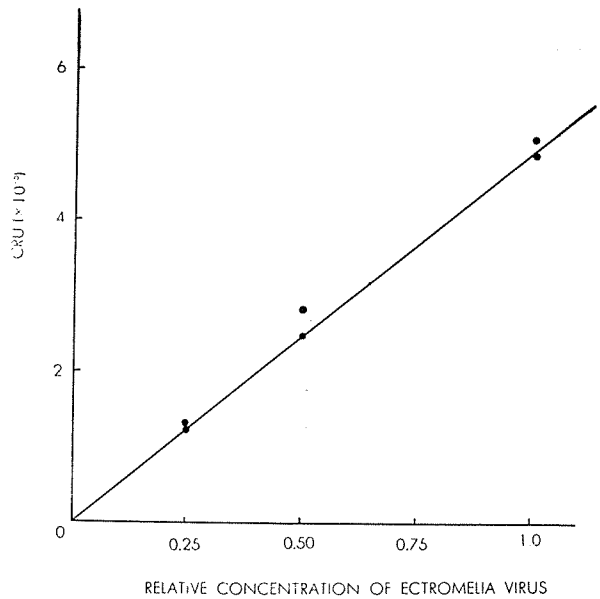

Fig. 2. Relationship between Doses of Active Ectromelia Virus and Units of Capacity to be Reactivated (CRU).

uptake of one IV particle is enough to bring about the interference in the cell itself, the CI (I) may be expressed as a function of the difference in the number of plaques of IV-inoculated and control cultures by the following equation,

$$(C - P) / C = 1 - e^{-I/n} \qquad (3)$$

where P and C are the numbers of plaques produced in IV-inoculated and control culture respectively and n is the number of cells.

The titer of CI was calculated from the data of plaque count by this equation and expressed as CIU (units of the capacity of interference). As described later, about ten per cent of the original capacity still remained in the cells after 24 hours, but no correction was made with regard to the effect of this residual interference on the plaque count of active virus.

By this assay, for example, IV prepared by heating 2×10^6 PFU/ml of vaccinia virus has 1×10^6 CIU/ml. The ratio of CRU to CIU was always 0.36~0.40 for every lot of IV. Since this constant value was obtained even after the capacities had been reduced by further treatment, it was supposed that the uptake of one IV particle was enough to bring about the interference in a cell but could not always cause reactivation. Presumably, the value may express the probability of interaction between two particles in those cells which are infected with both ectromelia and IV.

RESULTS

1. *Some characteristics of the biological activities of IV*

Reactivation

Previous experiments on the reactivation of poxviruses indicated that IV may enter the cells by itself and may be reactivated as a result of some interaction

with active heterologous virus simultaneously infected. (Hanafusa *et al.*, 1959, c, d). In preliminary experiments, however, no morphological changes including the formation of inclusion bodies which is characteristic of pox virus infection (Kamahora *et al.*, 1958) was seen, nor was there any increase in the titer of complement fixing antigen in IV-inoculated cells.

Interference

As described in an earlier report, IV does not interfere with the multiplication of active vaccinia if both are added to a cell culture at the same time. However inoculation of IV prior to infection with active virus caused a marked decrease in the number of plaques produced by the superinfecting virus, as compared with the control cultures (Table 1). From ultracentrifugation experiments, the entity to which this interfering effect is attributable seems to be the heat inactivated virus particle itself and not secondary products other than virus particles as the interferon of Isaacs *et al.* (1957 a, b). Other characteristics of this interference phenomenon were as follows.

Table 1. Interference of IV with Active Viruses

First inoculum	Second inoculum	Number of plaques
La-Ye	Voccinia	260
—	IV + Vaccinia	255
IV	Vaccinia	130
La-Ye	Ectromelia	201
IV	Ectromelia	197

The first inoculum was adsorbed for one hour. Then the cells were exposed to the second inoculum for two hours.

a) *Specificity*

The interfering effects of IV on active vaccinia and ectromelia virus are shown in Table 1. IV interfered strictly with the multiplication of active homologous virus only.

b) *Time intervals.*

In order to examine the effect of time intervals on the establishment of interference, the active virus was superinfected at different times after IV-inoculation and the degree of interference was estimated by the method of CI assay. The interference could be established 1 hour after IV-inoculation but late the degree of interference decreased with time (Tables 1 and 2). Thus, this interference differs from that of UV-irradiated NDV where the interfering effect is manifested on the adsorption of active NDV after as short a time as 15 minutes (Baluda, 1957, 1959) and from that of UV or heat inactivated influenza virus, whose interfering activities increase gradually until 24 hour postinfection and still remains unchanged after 48 hours (Henle and Paucker, 1958).

Table 2. Effect of Time Intervals on the Interference of IV

First inoculum	Time of second inoculation	Second inoculum	Number of plaques	CIU
La-Ye	2 hours	Vaccinia	265	—
IV	2	Vaccinia	130	2.1×10^6
IV	6	Vaccinia	140	1.9×10^6
IV	11	Vaccinia	173	1.3×10^6
IV	24	Vaccinia	240	2.1×10^5

The first inoculum was adsorbed for two hours and at the various times ndicated after the first inoculation the cells were exposed to the second inoculum.

Table 3. Adsorption of Superinfecting Virus onto Cells previously Treated with IV

Exp.	State of cells during adsorption	Agent	Active virus	Number of plaques on plate	Titer of unadsorbed virus	Fraction of unadsorbed virus
1	Monolayer	La-Ye	Vaccinia	294	238	0.448
		IV	Vaccinia	174	258	0.460
2	Suspension	La-Ye	Vaccinia	420	1010	0.705
		IV	Vaccinia	212	910	0.685
3	Suspension	La-Ye	Ectromelia	370	670	0.643
		IV	Ectromelia	370	720	0.660

One ml of active virus was added on the monolayer and suspended-cells previously treated with La-Ye and IV for 2 hours. After the inoculum had been removed, the cells were washed and the degree of infection was assayed either by direct plaque count on the monolayer (Exp. 1) or by plating of infected cells on another monolayer (Exp. 2 and 3). The amounts of unadsorbed virus were also determined by plaque count.

c) *Effect on the adsorption of active virus.*

The effect of IV on the rate of adsorption of active virus to cells was investigated in order to determine whether IV interferes with the process of adsorption of active virus or the subsequent intracelluar process of viral growth. About 1×10^6 L cells were suspended in 1 ml of IV preparation, incubated at 37°C for 2 hours under shaking occasionally and centrifuged at 1000 rpm for 5 minutes. The supernatant fluids were washed twice with La-Ye and resuspended in a suspension containing about 10^3 PFU of active virus. After a 2 hour's adsorption period the cells were centrifuged and washed. The titer of unadsorbed virus in the supernatant fluids was determined. As controls IV preparation was replaced by La-Ye solution. As shown in Table 3, the adsorption of active virus was not affected by pretreatment with IV.

2. *Effects of various treatments on CR and CI*

The IV preparation was subjected to various treatments, enzyme, antibody,

sedimentation, heat and UV-irradiation, and the effects of these treatments on the capacities were determined by CR and CI assay and compared with the effects of these agents on the infectivity of active vaccinia virus.

Action of Enzyme

It is known that vaccinia virus can not be destroyed by chymotrypsin, trypsin, carboxypeptidase and ribonuclease, but undergoes a remarkable change on treatment with papain (Hoagland *et al.*, 1940). Kilham and coworkers reported that the transforming activity of heat killed myxoma virus was not destroyed by the action of trypsin or deoxyribonuclease (Kilham *et al.*, 1958), but their experiments gave only a qualitative estimate. Even if the enzyme destroys 90 per cent of the transforming activity, this corresponds to the decline of its titer to one tenth, and therefore the heat killed virus might retain its capacity for reactivation even after enzyme treatment.

Table 4. Action of Enzymes on CR and CI of IV Preparation

Enzyme	μg/ml	Buffered medium	% Inactivation		
			IV		Vaccinia
			CR*	CI	Infectivity
DNase	100	La-Ye (pH 6.8)	56.8	55.0	0
	10	(plus 0.02M $MgSO_4$)	50		0
	1		51		0
	(100)		(10^{-1})**		
Trypsin	50	PBS (pH 6.6)	0	0	0
Trypsin DNase	50 and 100	PBS (pH 6.6)	55.0	—	0

A stock solution of IV and active vaccinia virus were mixed with the enzyme and buffered medium and kept at 37°C for 1 hour. For treatment with trypsin, the reaction mixture was heated at 56°C for a further 20 minutes to prevent degeneration of cells by trypsin. Then the CR and CI of IV and the infectivity of vaccinia virus were assayed. The per cent inactivation was determined by comparison with parallel controls. * Plaque method was used for CR assay. ** Dilution method was used; the degree of inactivation was expressed as the decline in titer of ten fold dilutions.

The IV preparation was treated with deoxyribonuclease (DNase) and trypsin and its titers for CR and CI were determined. Crystalline pancreatic DNase and trypsin (Worthington Biochemical Corp.) were allowed to act on IV at 37°C for one hour. Preliminary tests showed calf thymus DNA and casein were rapidly hydrolyzed in the presence of these enzymes under the conditions used. The results obtained are summarized in Table 4. Evidently, the capacities of IV decline to about a half of the original values after incubation with DNase but are not affected by trypsin, whereas the infectivity of active vaccinia virus are not affected by these enzymes. The inactivation of IV by DNase was rather

incomplete even at a higher enzyme concentration. It might have been due to a structural barrier of the heat denatured proteins which coat the inner DNA core of the virus particle, but the action of DNase was not accerelated by pretreatment with trypsin. Since IV may contain heat-denatured protein, the stability of CR on tryptic digestion is somewhat curious. However, the inner protein which is essential for reactivation might be resistant to this protease or be protected by outer protein coat. Therefore one cannot deny some role of protein in reactivation.

Action of antibody

Anti-vaccinia serum was obtained from hyperimmunized rabbits with vaccinia-IHD and heated at 56°C for 30 minutes.

Table 5. Action of Anti-Vaccinia Serum on IV and Active Vaccinia Virus

State of virus during the reaction with antibody	Relative concentration of antibody	% Inactivation				
		IV			Vaccinia	
		CR		CI	Infectivity	
Free*	10^{-1}	92.6	93.2	90.0	95.3	97.0
	10^{-2}	38.5	44.3	42.0	84.0	86.0
Adsorbed**	10^{-1}	85.5	90.0			0

* Raboit anti-vaccinia serum diluted in La-Ye was mixed with the active vaccinia (3×10^{2} PFU) and the IV preparation (1×10^{5} CRU), and the mixture was incubated at 37°C for 1 hour. Samples were cooled and the survivors were determined by the standard techniques of infectivity, CR and CI assay.
** In the course of the standard procedure for infectivity titration and CR assay, the anti-vaccinia serum was placed on the infected cells for 30 minutes after adsorption of the active virus or IV. The per cent inactivation was calculated on the basis of a similarly treated control containing La-Ye in place of antibody.

The actions of antiserum on the active virus and IV were compared by determining the residual activities after treatment with the same concentration of antiserum for 1 hour. In Table 5, the results were represented as per cent inactivation. The degree of inactivation of IV was less than that of active virus at either concentration, but it was noted that a considerable portion of the specific antigenicity still remains after the heat inactivation. On these preparations the antibody acted quite differently in the adsorbed state. After 2 hour's adsorption on the cells, IV could still be inactivated to almost the same extent as in the unadsorbed state, whereas the infectivity of the active virus was not affected. The difference in susceptibility to antibody between IV and active virus in the adsorbed state may be due to differences in the rates of penetration of these particles, IV being slower than the active virus so that the former gets in touch with the antibody for a longer period.

Sedimentation

The vaccinia virus has a sedimentation constant of about 5000 S and conse-

quently more than 90 per cent can be sedimented by centrifugation at 34, 850×g for 40 minutes. The heat killed virus suspended in La-Ye was spun for 40 minutes at 34,850×g in the Spinco Preparatory L centrifuge. The titers of CR and CI in the upper layer of this supernatant fluid were less than 10 per cent of original capacities, and most of the titers were found in the sediment. Therefore, it may be concluded that the dimension of the IV particle is about the same magnitude as the active virus, and the two capacities of IV are due to the heat inactivated virus particle itself, and not to smaller elements such as interferon.

Heat

The infectivity of vaccinia virus is decreased to less than 10^{-6} by heating at 56°C for 30 minutes, (Hanafusa *et al.*, 1959 c), whereas the CR and CI of the heat killed virus seem to be more resistant to heat. However, on further heating, these capacities were also inactivated.

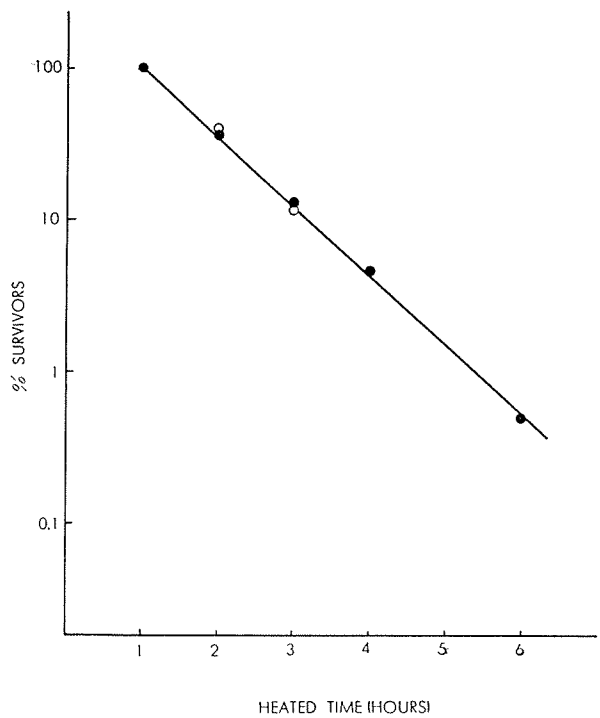

Fig. 3. Thermal Inactivation of IV at 56°C.
A stock solution of vaccinia virus (about 2×10^6 PFU/ml) was exposed to 56°C. At the times indicated, a sample was removed, stored in the cold and assayed by the CR and CI assay procedures. Survival of the capacities are calculated as percent of the one hour values.
●, CR; ○, CI.

In all experiments, the samples were suspended in La-Ye containing 5 % calf serum, sealed in ampoules and completely immersed in a water bath at the

indicated temperature for various periods. Then they were chilled in ice-water and stored in a deep-freezer until they were assayed. The time courses of inactivation of CR and CI both showed apparent first order kinetics with the same rate constant of inactivation (Fig. 3).

It takes 5 minutes for CR to be destroyed at 70°C and about 30 days at 37°C, in the latter case the decline of CR is parallel with that of infectivity of active virus.

UV-irradiation

The UV-inactivated vaccinia can not be reactivated by a mixed infection with active ectromelia (Hanafusa *et al.*, 1959 c). Experiments on marker rescue of bacteriophage have clearly shown that UV-induced damages are of limited extent within the genome and the different UV-sensitivities have been found for the different phenotypes (Doermann *et al.*, 1955; Krieg, 1959). If the genetic substance necessary for reactivation are localized within the entire genome of the virus and process of reactivation involves some genetic interaction between active and inactive virus, CR of IV may be more resistant to UV-irradiation than infectivity of active virus. Therefore, a precise analysis of UV-inactivation was attempted.

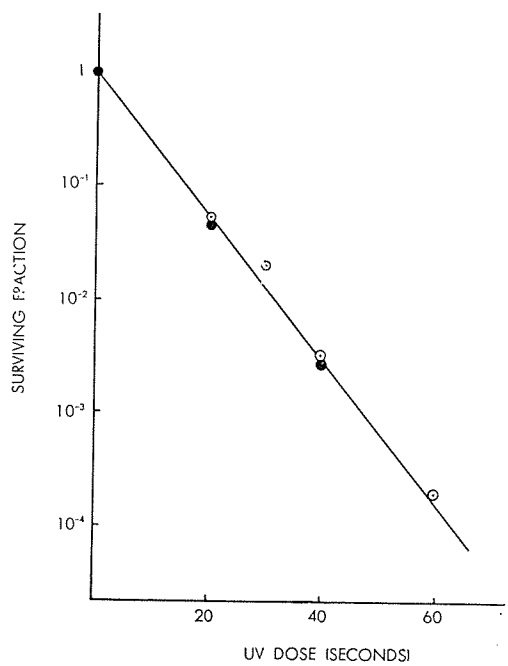

Fig. 4. Inactivation of Infectivity of Active Virus and CR of IV by Ultraviolet Light.
2 ml-samples containing infective titer 2×10^6 PFU per ml for active virus and 5×10^5 CRU per ml for IV were exposed to ultraviolet light at a distance of 40 cm for various time intervals. Then the suspensions were diluted suitably for titration in La-Ye.
⊙, infectivity; ●, CR.

In Fig. 4 the fraction of residual infectivity and CR after exposure is plotted against the UV dose. The results indicate single hit kinetics for inactivation of these activities, and the curves can be completely superimposed. Moreover, the inactivation of CR runs essentially parallel by the two assay procedures : the plaque and dilution methods. Since these methods are based on different marker characteristics : plaque morphology and pathogenicity for rabbits, the results show that the genetic units of these markers have the same sensitivity to UV as the infectivity of active virus.

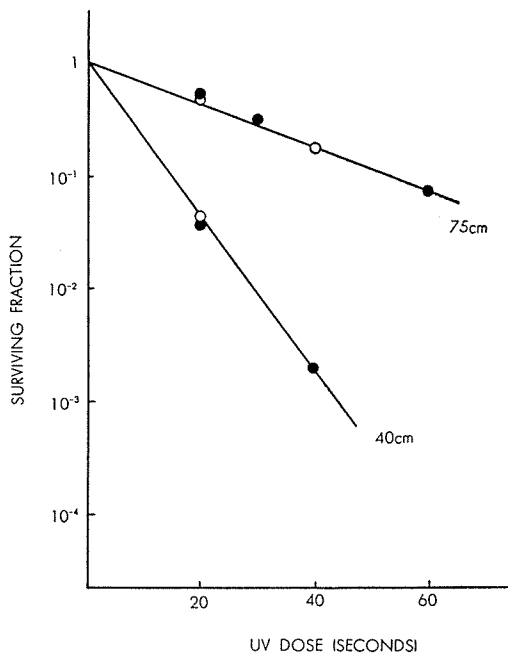

Fig. 5. Inactivation of CR and CI by Ultraviolet Light.
Experimental procedures were identical to Fig. 4 except that the height of the UV lamp was 40 and 75 cm above the sample.
●, CR; ○, CI.

By a similar procedure the inactivation of CI was compared with that of CR. In order to determine the CI-units precisely, irradiations were carried out at two different UV intensities. It will be seen in Fig. 5 that the two capacities have the same UV-sensitivity. If one assumes that the inactivation of the capacities is due to the inactivation of functions of nucleic acids in the IV particle, the finding may suggest the role of nucleic acid in establishment of interference.

3. *Loss of capacities of IV after inoculation to cells*

The reactivation of IV was accomplished by superinfection of active ectromelia even after 24 hours, as reported already. On the other hand, after inoculation the capacities of interference decreased gradually as seen in Tabls 2. In order to

estimate the relationship between CR and CI, an attempt was made to compare the rate of loss of these capacities after inoculation to cells.

After IV had been adsorbed on the cells for 2 hours according to the standard assay procedure, unadsorbed IV was removed by repeated washing. Then 8 ml of fresh medium were added. At various times the cells were superinfected with active ectromelia or active vaccinia and the titers of CR and CI were determined by counting the large vaccinia plaques. The results shown in Fig. 6 indicate that these capacities are lost at the same rate.

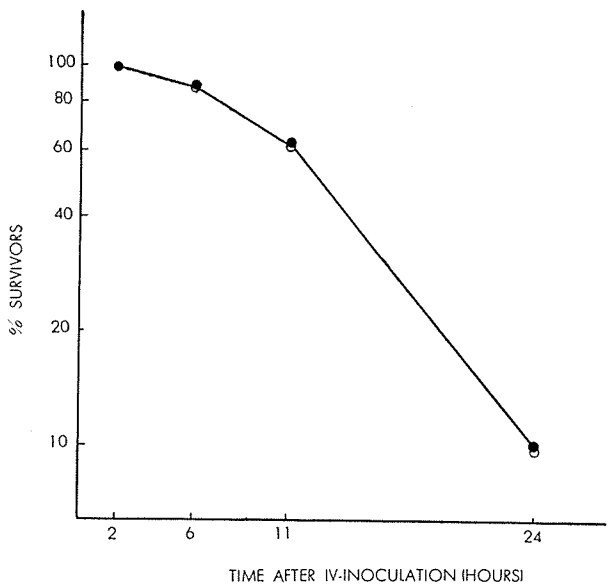

Fig. 6. Loss of CR and CI of IV After Inoculation to Cells.
Procedures were identical with that of the standard CR and CI assays except that the superinfecting virus was added at the various times indicated. The values obtained are calculated as per cent of the 2 hour value.
●, CR; ○, CI.

Although it seems likely that IV particles enter the cells themselves, no definite information has been obtained about the fate of IV after adsorption. However the above facts do prove that some sort of inactivation of CR on the cell surface or at some intracellular site might be closely associated with the inactivation of CI.

4. *Active virus as reactivating agent*

One probable hypothesis for the process of reactivation is that IV is not reactivated through any genetic interaction with active virus, but indirectly through supply or repair with some deficient metabolic function or some physiological circumstance, which may be brought about only by infection of active virus.

According to this hypothesis the agent capable of reactivating IV may not always be the active virus. Experiments were performed to examine this possibility. Partial UV-inactivation of ectromelia virus caused a simultaneous decrease in infectivity and in reactivating ability and completely UV-inactivated ectromelia could not reactivate IV. The same results were obtained by UV-irradiation of ectromelia-infected cells. Five hours after infection with active ectromelia the cells were suspended and exposed to UV for various periods. Then the irradiated cells were mixed with IV preparation and plated on L cell monolayers. There was no increase in the infective center on addition of IV for any irradiation period and the number of plaques of reactivated virus decreased proportionally to the number of surviving ectromelia.

Although one can not exclude the above possibility from these few experiments, up to now active virus is the only known reactivating agent.

DISCUSSION

On the mechanisms of reactivation of animal viruses, little information has been obtained. Kilham and his coworkers have investigated the nature of heat killed myxoma virus as an transforming agent and shown that the transforming agent is susceptible to DNase only after treatment with urea (Kilham *et al.*, 1958 ; Shack *et al.*, 1959). On the other hand, the reactivation of the heat-inactivated influeza virus was demonstrated by the presence of recombinants in the progenies after mixed infection with related strains of the influenza virus, where a process similar to that of cross-reactivation in the bacteriophage was postulated (Gotlieb *et al.*, 1956 ; Burnet *et al.*, 1954). To clarify the process of reactivation of poxviruses, the properties of heat killed vaccinia virus were investigated with the aid of quantitative assay procedures. From the data available, it seems that heat killed vaccinia virus possesses the same characteristics as the active one in particle size, specific antigenicity, UV-sensitivity and the ability to be adsorbed on the cells. However, the former can not produce specific anitgen for its progeny virus in the cells. The genetic unit of the virus may remain unchanged or be little modified by heat and it may be mainly the protein portion that is damaged. For example, experiments by UV-irradiation indicated that both the infectivity of the active virus and the capacity of reactivation of the heat killed virus were destroyed to the same degree and the capacity of the heat killed virus was susceptible to the action of DNase. However, there is no evidence about the role of other constituents, especially viral proteins in reactivation.

As mentioned by Fenner, an important problem in the mechanism of reactivation consists in whether reactivation is accomplished by some sort of genetic interaction or whether the primary event is repair of some non-genetic damage of the heated virus (Fenner *et al.*, 1959). The results of UV-irradiation seem to indicate that the latter possibility is more plausible, though the former can not be ruled out. Recent advance in virus research reveals that RNAs extracted with phenol from some animal viruses are also themselves infectious, but so far no infectious nucleic acid has been prepared from DNA-containing virus. Another

assumption may arise from these facts that IV cannot enter the cells by itself but the infection of active virus may promote the penetration of IV and once IV is incorporated into the cells, it can induce the complete reaction for virus synthesis. However, various experiments suggest the entry of IV into the cells and neither the Biken strain of vaccinia, which cause an abortive infection on L cells, nor UV-inactivated virus are able to cause reactivation. Moreover, the fact that IV interferes with multiplication of the homologous virus at some intracellular site might be an indication of the presence of an initial reaction for viral growth in IV-inoculated cells.

The parallelism between CR and CI in their behaviour to physical and chemical agents is very remarkable. All enzymes tested, antibodies, heat and UV-irradiation treatment rendered these capacities inactive to the same extent. Even after IV was inoculated to the cells, the degree of loss of these capacities coincide with each other. These results show clearly that the two capacities are due to an entity in the heat killed virus and therefore viral DNA as representative of the genetic function may participate in establishment of this interference. Concerning the role of nucleic acid as an interfering agent some suggestive evidence has been presented. Powell *et al.* (1956 a, b) have examined the effect of ionizing- and UV-irradiation on the interfering capacity of the influenza virus. Their data suggest that the interfering capacity resides in a very small portion of the viral particle and that the active moiety may be a protein associated with the viral RNA. By study of the interference by incomplete forms of influenza virus, Paucker and Henle (1958) have shown that their results were compatible with the hypothesis that S-antigen, of its RNA part, constitutes the actual agent responsible for inducing interference. However, no evidence has been found to indicate whether the RNA acts as a bearer of genetic information.

From the limited data described here, it is hard to draw any coordinated interpretation on the reactions induced in the IV-inoculated cells, which bring about the interference with the growth of the homologous virus in the cells. Nor can any clear idea be formed of the reactions which lead to reactivation of IV by some co-operation with the heterologous virus. More definite information on the mechanism of multiplication of pox-viruses will give a clue to the more precise analysis of these phenomena.

ACKNOWLEDGMENTS

I am greatly indebted to Dr. J. Kamahora for encouragement, advice, and discussion throughout this work. I also want to express my thanks for many valuable discussions with Dr. S. Akabori and Dr. T. Amano.

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