STUDIES ON HOST-VIRUS INTERACTIONS IN THE CHICK EMBRYO-INFLUENZA VIRUS SYSTEM*

VI. Evidence for Multiplicity Reactivation of Inactivated Virus

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It has been shown by Luria and Dulbecco (1, 2) that bacteriophage subjected to certain doses of ultraviolet light will lose its capacity to propagate under the condition of single infection; *i.e.*, when one bacterial host cell does not adsorb more than one irradiated virus particle. However, in the event of multiple infection, when a bacterial cell has adsorbed 2 or more irradiated phages propagation may occur. This indicates that the inactive virus particles may complement each other in some fashion so that reproduction can take place. This phenomenon has been termed "multiplicity reactivation."

In subsequent experiments employing "mixed infection" with two biologically distinct though related strains of irradiated phage evidence of an interchange of properties between the two agents was obtained (2). That a similar transfer of biological properties may occur in mixed infections of mice with two distinct strains of fully active influenza virus has been indicated recently by Burnet and Lind (3). Whether or not "multiplicity reactivation" and the interchange of properties are related phenomena has not been definitely established.

Evidence is presented in the present communication that "multiplicity reactivation" does occur with influenza viruses irradiated by ultraviolet light or heated to 56°C. for short periods of time. Although the quantitative aspects of this reaction are less readily ascertained in the influenza virus-chick embryo system the results indicate that reactivation occurs only with large doses of irradiated virus (presumably multiple infection of host cells) and not with more dilute preparations (presumably single infection). Furthermore, prolonged irradiation of virus prevents reactivation and the individual virus particles can no longer complement one another.

Methods and Materials

Virus.—The PR8 strain of influenza A and the Lee strain of influenza B were used throughout these studies. The source of virus was allantoic fluids obtained from chick embryos in-

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fected at the 10th to 11th day of incubation with dilute seed (0.2 ml. of 10^{-6} to 10^{-7}) and incubated for 48 hours at 36 to 37°C. The infected fluids were dialyzed against 20 volumes of phosphate-buffered saline solution of pH 7.0 and aliquots were then irradiated by ultraviolet light for various periods of time as indicated in the text. A General Electric germicidal lamp served as the source of ultraviolet light. The materials were exposed at a distance of 7 inches from the lamp in 15 to 20 ml. amounts in open sterile Petri dishes under continuous gentle rocking (4). Undialyzed fluids were used for the experiments with virus heated to 56°C.

The methods of virus titrations as to infectivity for chick embryos and hemagglutinating capacity have been described in detail in previous reports (5, 6). Likewise, the technic of growth curves and the collection of allantoic fluids and membranes has been discussed previously (6). Further experimental details are given in the text where indicated.

EXPERIMENTAL

Preliminary Experiments

Dialyzed preparations of PR8 or Lee viruses were irradiated for various periods of time; *i.e.*, from 20 seconds to 3 to 4 minutes. To the latter preparations, which were non-infectious as established by 2 successive passages in chick embryos, non-irradiated virus was added to correspond to a dilution of 1:10 to 1:20. Thus, assuming that all virus in the original preparation was active, the ratio of inactive to active virus was of the order of 10:1 to 20:1. Infectivity titrations of these mixtures showed that the active virus added could be demonstrated without significant loss. In the preparations irradiated for shorter periods of time the viruses were only partly inactivated, the infectivity decreasing logarithmically with the increase in ultraviolet dosage. In these cases the difference between the titer of non-irradiated and the irradiated preparation was used to determine the ratio of inactive to active virus, which were anywhere from about 40:1 to almost 100,000:1. These various materials were then injected without dilution into groups of 12 or more chick embryos each and allantoic fluids were collected from some of these after 2 hours of incubation in order to determine the level of residual non-adsorbed hemagglutinins of the seed virus, and from the remainder of the eggs after 48 hours for determination of viral propagation in terms of increase in the hemagglutinating activity.

Results of 2 such experiments are summarized in Table I. It can be seen that in the artificial mixtures of completely inactivated virus with the original allantoic fluid the ratio of inactive to active virus was lowest yet the hemagglutinin titers produced by this seed in 48 hours of incubation were less than those obtained with several of the preparations, which were irradiated to such an extent that the ratio of inactive to active virus was substantially higher. This discrepancy was particularly striking in the experiments with Lee virus. In the example presented in the table the seed irradiated for 20 seconds with a ratio of inactive to active virus of log 2.10 produced a hemagglutinin titer of 1:4096, whereas injection of the mixture of virus irradiated for 3 minutes and

original allantoic fluid with a ratio of log 1.27 resulted in a titer of only 1:192. Even material irradiated for 45 seconds with a ratio of log 3.15 still induced somewhat higher hemagglutinin formation.

The implications of these experiments are several-fold. They indicate that interference, which is easily demonstrated when injection of the inactivaten virus precedes the active agent (7–10), is not quite as readily obtained whed both are administered simultaneously and that in the latter case an increased dose of challenge virus may counteract interference, in contrast to observations

TABLE I

Development of Hemagglutinins Following Injection of Mixtures of Active and Inactivated Virus

Strain	Time of irradiation	LD_{50} injected	Ratio inactive/active virus	Hemagglutinin titer	
				2 hrs. (residual) seed	48 hrs.
	sec.	*log*	*log*		
PR8	0	9.46	0.00	1:16	1:3072
	20	7.87	1.59	1:12	1:768
	30	6.80	2.66	1:16	1:384
	45	5.80	3.66	1:16	1:192
	60	4.93	4.53	1:16	1:64
	240	(1.30)*	>8.16	1:16	1:12
	240 + 0	8.46	1.00	1:16	1:192
Lee	0	8.90	0.00		1:8192
	20	6.80	2.10		1:4096
	30	6.06	2.84	1:32	1:3072
	45	5.75	3.15		1:512
	60	3.93	4.97		1:16
	180	<(2.00)*	>6.90		1:32
	180 + 0	7.63	1.27	1:64	1:192

* Residual hemagglutinin from normal adsorbed seed.

made with the former experimental setup (9). This question deserves further analysis. The data would seem to suggest that the interfering property of irradiated virus increases with the dose of ultraviolet light or that reactivation may occur when individual virus particles have sustained only few hits of radiation. Since the experiments presented measured only the end-results it was felt that a continuous record of the development of the virus as obtained in growth curves would offer a more rewarding approach to these problems.

Growth Curve Experiments with Irradiated Seed. Hemagglutination Data

The general technic employed in these experiments was as follows:—

Dialyzed influenza A or B viruses were irradiated for various short periods of time and the preparations titrated for infectivity using 10 chick embryos per dilution. These results became available in 72 hours. Aliquots of the original allantoic fluid were then diluted sufficiently to correspond in infectivity to the irradiated preparations. Groups of adequate numbers of 12 to 13 day old chick embryos were then injected with original and irradiated preparations of approximately equal infectivity and incubated at 36 to 37°C. The allantoic fluids and membranes from 5 to 6 eggs of each group were collected at hourly intervals according to the established technics (6) and assayed for hemagglutinin concentration and infectivity. The infectivity titrations of the seed materials were, as a rule, repeated with the growth curve experiment or shortly thereafter, in order to establish the stability of the active virus component.

In the earlier experiments only hemagglutination was used for assay. It was known that the constant periods of *infectivity* with PR8 virus do not vary significantly regardless of the dose of seed virus used. 5 to 6 hours of incubation were required before new infective virus was liberated into the allantoic fluid (11, 12) and a period of 4 to 5 hours before an increase of infectivity could be measured in the allantoic tissue (6, 13). On the other hand, the *hemagglutination* test is less sensitive and considerable amounts of virus material are required before this test becomes positive. Consequently, the latent periods both in the fluids and membranes increase with the decrease in concentration of virus in the inoculum (6). The use of the hemagglutination technic promised, therefore, more decisive results. Fig. 1 summarizes the data of one such experiment. The amounts of active virus injected are given in the chart in the upper left-hand corner. The original virus preparation had been diluted $10^{-0.5}$, $10^{-1.5}$, and $10^{-2.5}$ and a dilution of $10^{-0.5}$ of the same seed irradiated for 20 seconds corresponded in infectivity to the last dilution of the original. It can be seen that with increasing 10-fold dilutions of the original virus preparation as seed, measurable levels of hemagglutinins appeared with increasing delay, *i.e.*, by the 4th, 6th, and 9th hour, respectively, both in the allantoic fluid and membrane suspensions. The irradiated virus, according to its infective titer, should have produced hemagglutination not before the 9th hour. Instead, measurable levels of this activity were noted in 5 hours; *i.e.*, the irradiated seed behaved as if it contained more than 10 times as much infective virus as had been actually determined.

A similar experiment with the Lee strain of influenza B virus is shown in Fig. 2. It has been shown in the preceding paper (14) that the quantity of B virus in the inoculum influences markedly the time required for one infectious cycle in contrast to the experience gained with influenza A strains. If undiluted allantoic fluid is used as seed, new generations of infective virus are liberated after 4 to 5 hours of incubation, with 2 or 3 successive 10-fold dilutions of the inoculum this period increases gradually to 8 to 10 hours and it remains at this range on further dilution of the seed. The constant periods in the membrane suspensions are less variable in that they extend only over 4 to 6 hours.

In view of these variations in cycle as measured by infectivity titrations it is not surprising to find that the appearance of hemagglutinins becomes even more strikingly delayed with influenza B virus than with the PR8 strain when increasingly diluted inocula are employed. Hemagglutinins rose in 6, 9, and 14 hours in the allantoic fluids, and in 5, 8, and 14 hours in the tissues, de-

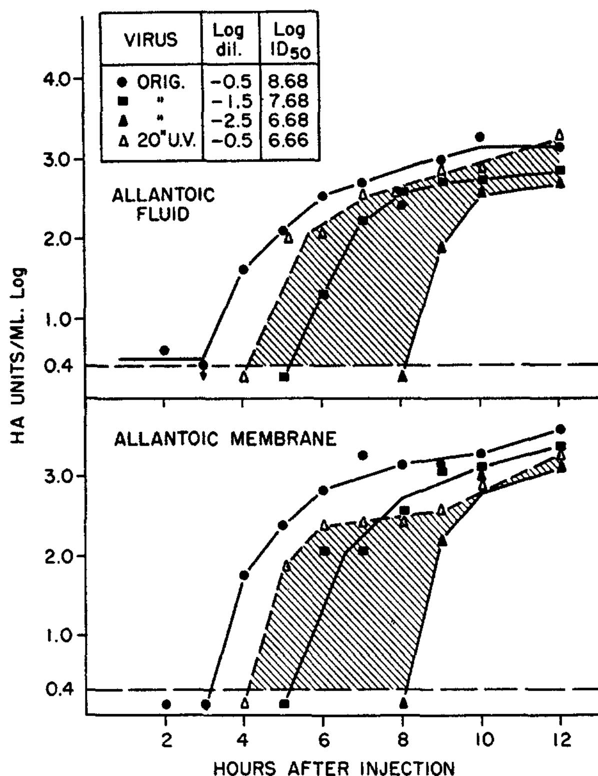

FIG. 1. Comparison of hemagglutinin curves in allantoic fluid and membrane obtained by injection of 3 concentrations of PR8 virus and by the same seed irradiated for 20 seconds. The infectivity of the latter corresponded to the lowest concentration of the former. The curves resulting from these two preparations are linked by cross-hatching.

pending on the dilution of the inoculum. Injection of the irradiated virus, diluted $10^{-0.5}$, produced measurable levels of hemagglutinin 5 to 6 hours earlier than the non-irradiated seed in dilution 10^{-2} which contained comparable quantities of infective virus. Thus, the irradiated virus preparation again appeared to contain at least 10 times as much active virus as was actually measured. It is seen in this experiment that the hemagglutinin titers of the irradiated series did not show a continuous rise during the experimental period

but that the curve leveled off and remained then at a relatively low level for the period of the experiment.

It was found in these and in additional experiments that demonstration of the phenomenon already described depended upon a number of factors: (*a*) It was best shown when a 100- to 1000-fold decrease in infectivity had been pro-

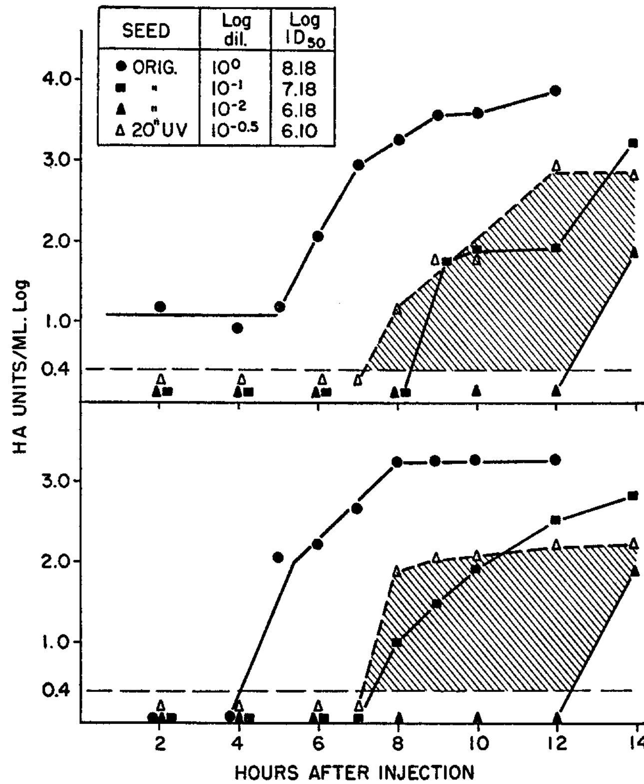

FIG. 2. Comparison of hemagglutinin curves in allantoic fluid and membrane obtained by injection of 3 concentrations of Lee virus and by the same seed irradiated for 20 seconds. The infectivity of the latter corresponded to the lowest concentration of the former. The curves resulting from injection of these two preparations are linked by cross-hatching.

duced in the seed by irradiation (20 to 45 seconds); (*b*) If inactivation was less pronounced (10 seconds of irradiation) the difference in the hemagglutinin curves produced by irradiated and control inocula of comparable infectivity became relatively less; (*c*) If irradiation resulted in too great a decrease in infectivity the seed did not produce measurable levels of hemagglutinin during the experimental period; (*d*) If the original seed had initially a relatively low infective titer the point mentioned under (*c*) was more readily observed; and, finally (*e*), if preparations of the type mentioned under (*a*) were diluted 10-fold

or higher prior to injection into chick embryos measurable levels of hemagglutinins failed to develop during the experimental periods of 12 to 16 hours. These various points will be discussed more extensively below in relation to infectivity titrations.

The data on the allantoic membrane suspensions presented in Figs. 1 and 2 were obtained without treatment by receptor-destroying enzyme (RDE) as obtained in culture filtrates of *V. cholerae* (15). When the membrane suspensions in subsequent experiments were treated with RDE the principal facts were not altered. The action of RDE advanced the detectability of hemagglutinins by 1 to 2 hours, in all instances, and increased the height of the titers to some extent in the first few preparations where some hemagglutinins were measurable without treatment, in agreement with previous observations (16).

When the membrane suspensions from such an experiment with Lee virus were heated to 70°C. for 30 minutes and subsequently tested for their capacity to inhibit hemagglutination, curves were obtained showing the rate of destruction of inhibitor in the tissues as a result of virus action (16). The inhibitory activity of the membranes remained unaltered until hemagglutinins made their appearance. Thereafter the concentration of inhibitor decreased rapidly. In this experiment the seeds irradiated for short periods of time again did not act in accordance with their infectivity titers but behaved as if they contained more than 10 times the amount of infective virus. In other words, a decrease in inhibitor was noted first after 3, 9, and 14 hours in the membranes derived following inoculation of undiluted, or 10- and 100-fold diluted non-irradiated seed, respectively. The irradiated inoculum, which corresponded in infectivity to the last dilution of the original seed, caused a decrease of inhibitor in the tissues by the 7th hour. In studying the allantoic fluid preparations it was found that the irradiated virus acted as rapidly as the undiluted non-irradiated seed, as was expected from the data obtained with non-infective virus irradiated for 3 to 4 minutes as previously reported (16). Upon dilution of the non-irradiated seed destruction of inhibitor in the allantoic fluids required increasingly longer periods of time depending on the concentration of virus in the inoculum.

In summarizing these data, there is no doubt that following injection of virus irradiated for short periods of time inhibitor is destroyed more rapidly and hemagglutinins are produced faster in the allantoic membrane, and they are liberated in measurable quantities into the allantoic fluid sooner than would be expected from the amount of infective virus in the seed. Upon dilution of such irradiated virus or upon prolonged exposure to irradiation the phenomenon is no longer observed under the conditions of the experiments, possibly because of the relative insensitivity of the hemagglutination test. In the light of this limitation and the lack of correlation between the hemagglutinating and infective properties of influenza viruses observed under certain conditions (6, 17–19) the phenomenon was analyzed further with the aid of infectivity titrations.

Growth Curve Experiments with Irradiated Virus. Infectivity Data

The technic used for these experiments was essentially the same as that used in the previous section detailing the results of the hemagglutination tests, except that some of the materials had to be stored for some time until all the infectivity titrations could be performed. It had been shown previously (13, 20) that the infectivity titers of allantoic fluids as well as of membrane suspensions remained constant for several weeks at 4°C. However, when the number of preparations to be tested was too large to handle within 1 week aliquots were transferred to ampuls, shell-frozen in a dry-ice–alcohol mixture, and stored until used in the dry-ice chest at −65°C. Comparative titrations of frozen samples and materials stored at 4°C. always fell well within the range of variations reported by Knight (21) for titrations employing 5 chick embryos per dilution, as were used here.

Main emphasis was placed in these tests on studies with the Lee strain. This strain seemed to offer more suitable critera for measuring reactivation because of the marked variations in the extent of the constant periods depending on the dose of seed virus (14). As seen in Fig. 3, upper chart, quite striking differences in the constant periods were noted in the allantoic fluids with irradiated and original virus seed of comparable concentrations of infective virus. In the experiment summarized in the figure allantoic fluids in dilution $10^{-0.5}$ were used as seed which had been irradiated for 10, 20, or 45 seconds. The residual infectivity titers of these preparations were comparable to those of the original fluid diluted to 10^{-1}, 10^{-2}, and $10^{-3.5}$, respectively, as shown in the upper left-hand chart of the figure. It is noted that the constant periods of the curves obtained with irradiated seed were all of the order of 5 hours, whereas they extended over 6, 7, or 9 hours with the comparable non-irradiated virus preparations. With the seed irradiated for the longer periods (20 and 45 seconds) the liberation of hemagglutinin progressed at such a rate that the titers exceeded for several hours even those obtained after injection of non-irradiated seed with 10 to 30 times the amount of active virus. The seed irradiated for 10 seconds, likewise, gave a somewhat shorter constant period and higher titers after onset of liberation than the corresponding dilution of non-irradiated virus. However, in this case, the curve did not reach that obtained with 10 times the amount of native seed virus (undiluted allantoic fluid). At the end of the experimental period of 12 hours the titers in all the curves obtained with irradiated seed were still 10 to 100 times higher than those achieved with non-irradiated seed of comparable infectivity.

Considering the data obtained with allantoic membrane suspensions it is seen (Fig. 3, lower chart) that essentially the same results were noted except that the constant periods in all instances were of the same order; *i.e.*, 4 hours. However, at the end of this period the increase in infectivity was strikingly more rapid with the irradiated materials than with the corresponding dilutions

of the native seed, and the difference was maintained up to the 9th, 10th, and 12th hour, depending on which of the paired curves are studied. The differences

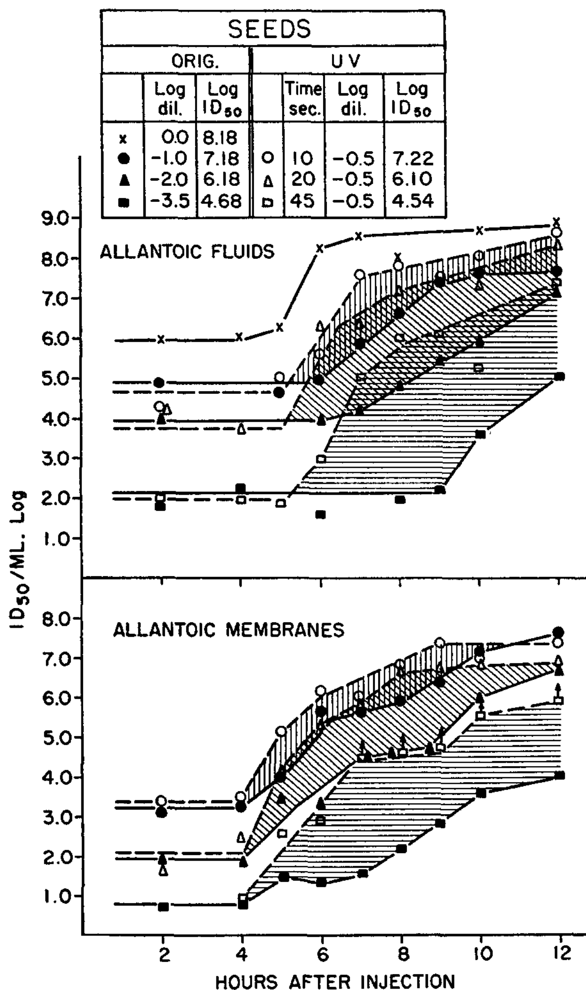

FIG. 3. Infectivity curves in allantoic fluid and membrane obtained by injection of Lee virus irradiated for various periods of time and of non-irradiated seed containing comparable concentrations of infective virus. The corresponding curves are linked by vertical, diagonal, or horizontal cross-hatching.

in titers were up to 250-fold and may have been greater if endpoints had been reached in all the titrations of the curve obtained with the seed irradiated for 45 seconds.

These results of the infectivity titrations confirmed those obtained by the

hemagglutination technic. It seems warranted to conclude that some of the virus inactivated by ultraviolet light can be reactivated in the chick embryo so that it is able to increase in quantity. It is apparent from Fig. 3 that the phenomenon is most strikingly observed with the seed irradiated for 45 seconds. However, the total amount of virus which can be reactivated decreases with increase in irradiation. This is demonstrated by the fact that the infectivity curves resulting from injection of the irradiated virus preparations reached successively lower levels with increased doses of ultraviolet light. In another experiment where irradiation was extended to 1, 2, and 3 minutes, the phenomenon was no longer demonstrable. After 1 or 2 minutes of irradiation, some active virus was still measurable and growth curves obtained with these seeds ran closely parallel to those recorded with comparable concentrations of active virus during the experimental period of 14 hours. 3 minutes of exposure to ultraviolet light completely inactivated the virus and in this case the titrations were negative in dilution 10^{-1} throughout the period of observation.

These data indicated that reactivation is possible only within a certain range of exposure to ultraviolet light. After prolonged irradiation it no longer occurs. Reactivation likewise could be prevented by dilution of the inactivated seed as demonstrated in Fig. 4. In the experiment summarized in the figure both the original and irradiated seeds (20 seconds exposure) were used in several 10-fold dilutions, 10^{-1}, 10^{-2}, and 10^{-3}, on the one hand, and $10^{-0.5}$, $10^{-1.5}$, and $10^{-2.5}$, on the other. The last 2 dilutions of the non-irradiated inoculum match in infective titer the first 2 of the irradiated seed (upper left-hand chart). As can be seen the irradiated preparation could be diluted $10^{-1.5}$ and reactivation was still quite obvious. Both in the allantoic fluid and membrane titrations the curves, after onset of liberation or increase in infective virus became apparent, matched those obtained with the original seed containing 10 times the amount of active virus. However, when the irradiated seed was diluted $10^{-2.5}$ the infectivity curve ran parallel to that resulting from 10 times the amount of non-irradiated virus at about one log distance. Although unfortunately no curve was obtained with a 10^{-4} dilution of the non-irradiated virus preparation, it is obvious from previous experiments (14) that this curve would have been closely similar to that recorded for the $10^{-2.5}$ dilution of the irradiated seed.

In Fig. 5 an experiment with PR8 virus is shown which was similar to that summarized in Fig. 4 for the Lee strain. It has been pointed out above that the constant periods with the influenza A strain were not significantly influenced by the dose of seed virus (6). Correspondingly the demonstration of reactivation was expected to be less striking. It is unfortunate that for technical reasons a number of titrations had to be omitted in this experiment and the choice of the materials to be omitted has not always been the best. Yet the results recorded in the figure leave no doubt that reactivation of irradiated PR8 virus can be demonstrated by infectivity tests, as well as by hemagglutinin titrations

as recorded in the preceding section, and that the limitations are similar to those recorded for the Lee virus. The data show that the constant periods in the allantoic fluid were of the order of 5 hours and that liberation in the ir-

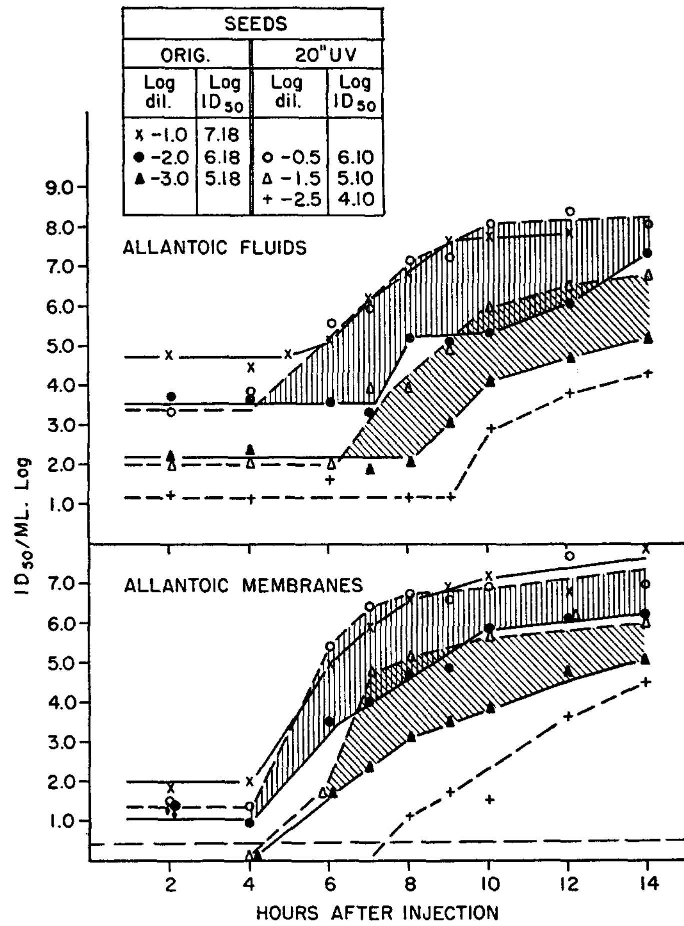

FIG. 4. Infectivity curves in allantoic fluid and membrane obtained with various dilutions of irradiated and non-irradiated Lee virus. The curves resulting from injection of preparations of comparable infectivity are linked by vertical or diagonal cross-hatching.

radiated series exceeded that in the comparable controls. Similarly, in the membrane titrations, the constant periods were about 3 hours and the increase in active virus thereafter was about 10 times higher in the curves produced by irradiated than in those resulting from non-irradiated seed of comparable infectivity. However, this difference did not last as long as in the Lee series and

the titers of the corresponding active and irradiated seed curves were closely similar by the 8th hour in the allantoic fluid, and somewhat earlier in the mem-

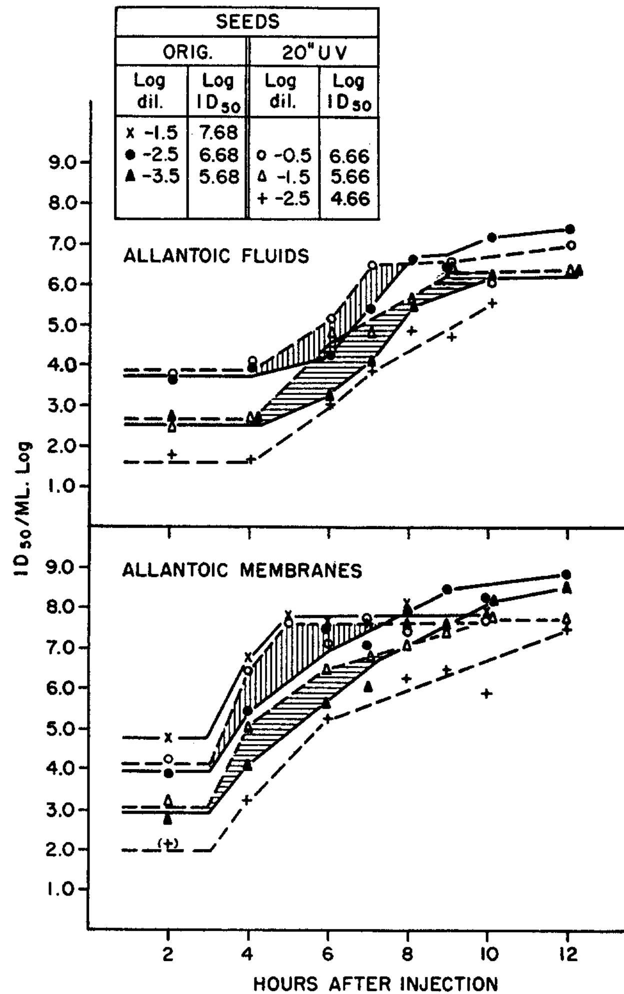

FIG. 5. Infectivity curves in allantoic fluid and membrane obtained with various dilutions of irradiated and non-irradiated PR8 virus. The curves resulting from injection of preparations of comparable infectivity are linked by vertical and horizontal cross-hatching.

brane suspensions. The data show that the irradiated seed could be diluted somewhat higher ($10^{-2.5}$) and still present slight evidence of reactivation since this curve ran closer than at a one log distance (6) from that obtained with non-irradiated seed containing 10 times the amount of active virus. This would

not be unexpected in consideration of the fact that the infectivity titer of the original PR8 seed was about 10 times higher than that of the Lee preparation used in the experiment recorded in Fig. 4.

In further experiments it was established that reactivation failed to occur when irradiated PR8 of Lee viruses were added to normal membrane suspension *in vitro*. One part of virus material was added to 10 to 20 parts of the tissue preparations and the mixtures were incubated at 37°C. for 2 to 8 hours prior to titrations in the chick embryo. The results are shown in Table II.

It was also considered that the original preparations of seed virus might possibly consist of a mixture of fast and slow growing variants, the former being less susceptible to ultraviolet light and therefore gaining the upper hand

TABLE II

Attempts to Reactivate Irradiated Influenza Virus by Normal Membrane Suspension

Experiment No.	Strain	Time of irradiation	Reactivation *in vivo*	Period of incubation at 37°C.	LD_{50}/ml.	
					Virus + Broth	Virus + normal membrane suspension
		sec.		*hrs.*	*log*	*log*
1	Lee	30	+	2	7.84	7.81
2	Lee	0		2	8.13	7.80
		20	+	2	6.68	6.13
		45	+	2	5.68	5.30
		60	–	2	5.47	5.08
3	PR8	45	+	0	5.80	5.30
				2	5.30	5.47
				6	5.64	5.77
				8	5.20	5.81

on subculture. Consequently, an allantoic fluid was selected from a growth curve experiment with irradiated Lee virus (30 seconds). This curve had been similar in every respect to those shown in Fig. 3. The fluid chosen had been collected several hours after onset of liberation and contained $10^{5.83}$ ID_{50}/ml. This material was employed undiluted as inoculum for a new growth curve and the results revealed a constant period of infectivity of 9 hours. This was in accordance with the usual experience following injection of stock Lee virus diluted to similar infectivity. Thus, no evidence was obtained of a fast growing variant.

Finally, it was thought that the presence, in the irradiated seed, of a large amount of non-infective virus, with, as far as could be ascertained, unimpaired inhibitor-destroying activity (16), might lead to a more rapid adsorption of

infective virus and thereby shorten the growth cycle. In contrast, the non-irradiated inoculum diluted to contain comparable amounts of infective virus would possess substantially less capacity to reduce inhibitor, in the allantoic fluids, and therefore adsorption might be delayed. Previous reports did not support such a possibility since the degree of adsorption appeared to be independent of the amount of seed virus injected (5). Nevertheless, an experiment was performed in which RDE was injected 1 hour after infection of chick embryos with 100-fold diluted non-irradiated Lee virus. The inhibitor-destroying activity of the RDE preparation used, matched that of the undiluted Lee virus. This had been determined by incubation of various concentrations of the 2 agents with normal allantoic fluid at 37°C. for 3 hours and assaying the residual inhibitor in the mixtures, after heating to 70°C. for 30 minutes, in hemagglutination-inhibition tests. The injection of RDE subsequent to infection had no effect upon the length of the growth cycle under the conditions cited.

Reactivation of Heated Virus

A few experiments were conducted with influenza A virus partially inactivated by heating to 56°C. The preparations were placed in a water bath at 56°C. for 8 and 10 minutes, which reduced the infectivity of the allantoic fluid from $10^{9.56}$ ID_{50}/ml. to $10^{7.20}$ and $10^{5.56}$, respectively. Growth curves were obtained with the undiluted heated seeds, as well as with the original fluid diluted to 10^{-2} and 10^{-4}, the latter two approximating the infectious titer of the former two preparations. It was seen that with the heated preparations hemagglutinins became measurable 3 to 4 hours in advance of the corresponding curves obtained with the unheated seed diluted to comparable infectious titers. The infectivity curves were likewise in agreement with the results obtained with irradiated virus. It was noted that with the shorter period of heating the curves reached higher levels than with the seed heated for longer time.

DISCUSSION

It has been shown previously (4) that upon irradiation of influenza viruses with increasing doses of ultraviolet light the infectivity of the preparations decreased rapidly at a logarithmic rate resembling "single hit curves." The data suggested that a single hit of radiation is required to render a virus particle non-infectious. According to this interpretation a decrease in infectivity of a virus preparation from its original titer in the order of $10^{9.0}$ ID_{50}/ml. to $10^{7.0}$ ID_{50}/ml. would denote that most of the inactivated virus particles have sustained a few hits at most. With prolonged irradiation, leading eventually to complete loss of infectivity, all virus particles will have adsorbed numerous hits.

When a partially inactivated virus preparation, in which the majority of

virus particles sustained only few hits of radiation, was used as inoculum the resulting growth curves did not conform with those obtained with non-irradiated preparations of comparable infectivity. The results obtained with the irradiated inoculum both by hemagglutination and infectivity titrations, resembled those noted after injection of more than 10 times the amount of non-irradiated infective virus. The hemagglutinins reached measurable and higher levels earlier in the allantoic membranes and fluids than was the case with comparable quantities of active virus in non-irradiated seed, both with the PR8 strain of influenza A and the Lee strain of influenza B virus. The infectivity reached at least 10 times higher levels in a given time, in both media in the case of PR8 virus, and in that of the Lee strain, in addition, the infective virus increased in the tissues and was liberated into the fluid earlier in accordance with the shortening of the constant periods observed with this virus with an increase in the amount of the infective agent inoculated (14).

In considering explanations for this phenomenon a number of possibilities had to be explored. Normal allantoic membrane suspensions were without effect upon irradiated virus. This would not exclude that the virus damaged by ultraviolet irradiation may be "repaired" in the intact host cell. However, if that were the case the phenomenon should be demonstrable also with highly diluted irradiated seed, which it was not. There was no evidence of fast growing variants of the influenza viruses with a greater resistance to ultraviolet light, nor would the data suggest the production of such variants by irradiation. It was more difficult to exclude any interaction of inhibitor of hemagglutination or adsorption (22, 23), in this phenomenon. The inhibitor in the membranes is destroyed only by active virus but not by virus preparations irradiated for 3 to 4 minutes which are non-infective but still cause interference (16). Nevertheless, adsorption of the seed onto the membrane conceivably may be delayed by inhibitor in the allantoic fluid. However, the results of injection of RDE in the present experiments and the data on adsorption of virus at 37°C. onto the tissue *in vivo* (5) or onto red cells *in vitro* (16), did not support such a view and it is likely that much greater quantities of inhibitor would be required to effect such a delay of adsorption *in vivo* at 37°C. This problem would bear further analysis.

The available evidence points to the conclusion that some of the virus particles rendered non-infective by irradiation for short periods of time may regain their infectivity in the host organism. Although the quantitative aspects of viral multiplication in the case of influenza virus are handicapped by many technical difficulties as discussed in an earlier study (13) inspection of the data presented clearly indicated that the virus liberated following injection of irradiated seed reached levels in excess of the amount of infective virus injected within 1 or 2 hours after onset of release. The phenomenon described therefore is not based upon elution of the adsorbed infective seed virus, nor

would one deal with a breakdown of seed virus, as the hemagglutination data might suggest, in view of the results of the infectivity assays.

Reactivation in the host can be obtained only with virus irradiated for short periods of time. With an increase in the dose of ultraviolet irradiation increasingly more hits are sustained by individual virus particles and the degree of reactivation becomes less until the phenomenon is no longer observed. Correspondingly, the yield of virus as a result of one infectious cycle decreases with the dose of ultraviolet irradiation. This would indicate that with prolonged irradiation the inactivated virus particles gradually lose their capacity of reactivation and the interference phenomenon comes increasingly to the fore (7–10). Whether completely non-infective preparations of virus may be reactivated by infective virus injected simultaneously is not known at present although breaks in interference have been observed under conditions in which large quantities of active virus were added to the interfering agent, as seen in the preliminary experiments reported in this study. This problem requires elucidation.

The phenomenon of reactivation can be shown only with relatively concentrated inocula of irradiated virus. Upon 100-fold dilution of such seeds it is no longer observed. With respect to this experience the quantitative aspects of the host-virus system under study need to be considered. It has been estimated (24) that the number of cells lining the allantoic cavity amount to about 10^8 based upon 100 $cm.^2$ of surface of the allantoic cavity and a diameter of 10 μ of the cells. This rough estimate is supported by other indirect evidence. In growth curve experiments with non-irradiated PR8 virus covering one infectious cycle it was noted that the yields of the agent were closely similar when undiluted or 10-fold diluted seeds were used corresponding to approximately 10^9 and 10^8 ID_{50} per inoculum, respectively. This suggested that with the smaller inoculum all susceptible cells had been infected. Even with a 100-fold diluted seed the production of virus during the first cycle was, on occasion better than 10 per cent of that resulting from 10 times the amount of seed virus. Likewise, in experiments on the interference phenomenon with inactivated influenza A and B viruses it was noted that the interfering agent could be diluted no more than 10- to 100-fold and still give almost complete protection against subsequently injected challenge virus (9). In other words, the equivalent of about 10^8 ID_{50} or slightly less in the inactive state were required to render all cells resistant to infection. These data all point to the conclusion that the number of susceptible entodermal cells is of the order of 10^8 and may be slightly less. In the reactivation experiments with PR8 virus the non-irradiated seed contained more than 10^9 ID_{50} per inoculum. The irradiated materials gave no conclusive evidence of reactivation when diluted $10^{-2.5}$ and no trace of the phenomenon on higher dilution. It would seem reasonable to assume that in the latter case the host cells adsorbed at most one virus particle, whereas with

the more concentrated seed several virus units became attached to individual cells. Consequently, the data would seem to be in agreement with those reported by Luria and Dulbecco concerning reactivation of irradiated bacteriophage (1, 2), in that this phenomenon depends upon multiple adsorption of inactivated virus particles onto individual bacterial cells.

The infectivity of the non-irradiated preparations of Lee virus, as a rule, is somewhat lower than that of PR8 seed; *i.e.*, it contains between 10^8 and 10^9 ID_{50} per inoculum. Yet after irradiation the material could be diluted $10^{-1.5}$ (the equivalent of $10^{6.7}$ ID_{50} in one of the experiments) and still present evidence of reactivation. Thus, the total amount of virus particles injected appears to be somewhat too low to suggest multiple adsorption onto the available host cells. With higher dilutions, where single infection of host cells would seem to be assured no reactivation occurred. In the light of the inaccuracies of all attempts at quantitation it is felt that reactivation in the case of irradiated Lee virus is also based upon multiple adsorption of virus particles onto host cells. In order to explain the somewhat discrepant results between the PR8 and Lee series of experiments as to the quantitative aspects it has to be pointed out that the assay of virus content in the original seeds is based upon infectivity and does not take into consideration any inactive virus that may have accumulated during the growth period of these materials. The hemagglutinin titers of the PR8 and Lee seeds are usually of similar height. One may assume, also, that the inocula do not spread rapidly throughout the allantoic cavity and that the cells close to the site of inoculation may have a chance of multiple adsorption of irradiated virus particles.

In spite of the greater difficulties encountered in evaluation of the reactivation phenomenon in the influenza virus-chick embryo system it is apparent that the conditions are closely similar to those encountered in the phage experiments reported by Luria and Dulbecco (1, 2). It is obtained when the influenza virus particles have sustained only a few hits of irradiation and according to all indications only upon multiple adsorption of irradiated virus onto host cells. This phenomenon of multiplicity reactivation would be more strikingly established if it could be shown, as in the phage system, that 2 strains with different biological properties may interchange these properties in the process of reactivation. That this is a distinct possibility may be gleaned from the recent report of Burnet and Lind (3) showing the development of neurotropic tendencies in certain heretofore non-neurotropic influenza viruses when inoculated together with a neurotropic variant of the WS strain. This possibility obviously will be explored in further experimentation.

SUMMARY

Evidence has been presented that influenza viruses both of type A and B partially inactivated by ultraviolet irradiation may regain their capacity to

propagate in the allantoic membrane of the chick embryo. In using such irradiated preparations as inocula for growth curve experiments it could be shown that the development of hemagglutinins as well as of infectivity proceded at rates resembling those noted with more than 10 times the amount of infective virus actually found in the irradiated seed. Partial inactivation of the inocula by heating to 56°C. gave similar results.

The phenomenon was observed only with seed irradiated for short periods of time so that the virus particles sustained only few hits of radiation. On prolonged exposure resulting in numerous hits per virus particle the capacity of reactivation was lost. Likewise, an irradiated preparation capable of reactivation in the allantoic membrane, could not be diluted more than about 30-fold and still clearly produce this phenomenon. This indicated that reactivation is obtained only when one host cell adsorbs more than one non-infective virus particle but not upon adsorption of a single particle.

These data are in striking agreement with the phenomenon of "multiplicity reactivation" observed in the bacteriophage-*E. coli* system by Luria and Dulbecco.

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