THE PRODUCTION OF A PERSISTENT ALTERATION IN INFLUENZA VIRUS BY LANTHANUM OR ULTRAVIOLET IRRADIATION

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(Received for publication, July 12, 1948)

The capacity of influenza virus to combine with and agglutinate red blood cells and thereafter to be eluted from them (1, 2) has stimulated numerous investigations. As a result of early work it was assumed that adsorption of the virus to RBC and subsequent dissociation of the complex were closely associated phenomena and it was suggested that the reactions could be considered as analogous to those between an enzyme and its substrate (2). Shortly after the discovery that influenza virus could cause hemagglutination it was found (2) that two different virus strains, *i.e.* Lee and PR8, showed different rates of elution from RBC. Recently it was shown (3) that by heating the virus the elution phenomenon could be almost completely abolished without affecting the capacity of the agent to combine with RBC and cause hemagglutination. Heat, however, inactivates the virus; the property of multiplication in susceptible hosts is lost and probably other properties also are altered. It was decided to investigate the elution of influenza virus from RBC more thoroughly and particularly to attempt to find means whereby the rate of elution of the virus could be altered without destroying the capacity of the agent to induce infection.

The results obtained in the present study provide evidence that it is possible by means of certain physical or chemical agents, *i.e.* treatment with lanthanum acetate or ultraviolet irradiation, to produce a marked alteration in the elution rate of the Lee strain of influenza virus without causing demonstrable alterations in the other properties of the virus tested. It will be shown that strains modified by the procedures employed retained their altered state on serial passage in the chick embryo in the absence of the agent which originally effected the alteration.

Materials and Methods

Viruses.—The PR8 strain (4) of influenza A virus and the Lee strain (5) of influenza B virus were used in this study. Both strains had previously been passed many times in mice and in chick embryos. Between experiments allantoic fluids infected with either virus were stored frozen in sealed ampoules in a CO_2 storage cabinet at $-70°C$.

Chick Embryos.—White Leghorn eggs were incubated at 39°C. for 9 to 12 days. Embryos of the desired age were inoculated into the allantoic sac through a paraffin-sealed hole drilled in the shell. The inoculum consisted of 0.1 cc. of infected allantoic fluid diluted 10^{-3} in sterile

broth unless otherwise stated. After inoculation chick embryos were incubated for 48 hours at 35°C. and candled daily. Embryos which died were discarded. Eggs containing living embryos then were held at 4°C. for approximately 2 hours and thereafter the allantoic fluid was harvested in the usual manner. Fuids which gave positive hemagglutination with chicken RBC were pooled and used as virus source material. Depending upon the experiment the pooled allantoic fluids were stored either at 4°C. or at −70°C.

Virus Titrations.—Hemagglutination titrations were done according to the technique described by Hirst (6). Serial twofold dilutions of allantoic fluid pools were prepared in saline buffered at pH 7.2. To 0.4 cc. of each dilution was added 0.4 cc. of a 1 per cent suspension of washed chicken RBC. The tubes were shaken and the degree of hemagglutination recorded after 1 hour at room temperature. The patterns of the sedimented erythrocytes were graded from 4+ to ± in the customary manner and the end point was taken as the highest dilution which gave a 2+ pattern.

Virus infectivity titrations in chick embryos were done by the intra-allantoic technique (7) in 9 to 12 day embryos. Serial tenfold dilutions of allantoic fluid pools were prepared in sterile broth. A group of 4 embryos was inoculated with each dilution; each embryo received 0.1 cc. Allantoic fluid was harvested from each embryo after 48 hours' incubation at 35°C. and tested by the hemagglutination technique. The end point was taken as the highest dilution which induced demonstrable infection in 2 or more embryos in a group.

Virus infectivity titrations in mice were carried out by the intranasal technique. Serial tenfold dilutions in sterile broth were employed. A group of 6 Swiss mice was used for each dilution. The procedure was identical to that previously described (8) and the 50 per cent maximum score end point (M.S.50) was used.

Immune Serum.—Rabbits were immunized by the intravenous injection of infected allantoic fluid pools. A single injection of 10 cc. of undiluted fluid was given. Serum was obtained 14 to 21 days later and was stored without preservative at 4°C. Immediately before use in hemagglutination-inhibition tests serum was diluted 1:2 with saline and heated at 65°C. for 30 minutes. This procedure markedly reduces the so called non-specific inhibitory capacity of serum but does not significantly diminish the antibody titer of immune serum (9, 10).

Identification of Virus Strains.—Serological identification of the virus strains studied was carried out by means of the hemagglutination-inhibition technique (6). Serial twofold dilutions of infected allantoic fluid were prepared in saline. Each dilution was mixed with constant amount serum which was diluted so as to contain a quantity of antibodies just capable of inhibiting hemagglutination by 256 units of virus. To each mixture was added an equal quantity of a 1 per cent suspension of chicken RBC. The end point was taken as the highest dilution of infected allantoic fluid which caused 2+ hemagglutination. As a routine in hemagglutination-inhibition tests both anti-PR8 and anti-Lee immune as well as normal rabbit serum were employed.

Determination of Eluted Virus.—Inasmuch as the chief objective of this study was to investigate possible alterations in the capacity of influenza virus to dissociate from RBC after combination with them, it was essential to devise a technique by means of which both the rate of elution of the virus and the quantity of virus eluted could be determined. The following technique was found to be suitable: The RBC in 5 cc. of a 1 per cent suspension were packed by a few minutes' centrifugation at 4,000 R.P.M. The supernate was poured off and discarded. To the packed cells 1 cc. of an infected allantoic fluid pool was added and the mixture was shaken vigorously. Small volumes were employed to facilitate very rapid sedimentation of the RBC. Only pools with hemagglutination titers of 1:1024 or more were used. The final concentration of erythrocytes in the mixture was 5 per cent. The mixture was held at room temperature for 10 minutes in order for virus-erythrocyte combination to occur. In every instance hemagglutination occurred almost immediately after the mixture was made and the agglutinated cells settled very rapidly. At the end of 10 minutes the mixture was reshaken

and the cells sedimented by centrifugation at 4,000 R.P.M. for approximately 1 minute. The supernate was poured off and its hemagglutination titer determined subsequently. Immediately 1 cc. of buffered saline was added to the packed cells; this mixture was shaken vigorously and then held at room temperature. Identical cycles of centrifugation, removal of supernate, and resuspension of the sedimented cells in 1 cc. of fresh saline were carried out 30, 60, 90, 120, and 180 minutes later. Each supernate was kept so that its hemagglutination titer could be determined. In certain experiments even shorter periods were employed in each successive elution step.

This technique has made it possible to obtain fairly reproducible virus elution curves relative to time and has facilitated the present study. It should be pointed out that the resuspension of the agglutinated cells in fresh saline after each centrifugation makes it possible to determine the dissociation of relatively small quantities of virus during each time interval which is technically very difficult if the initial cell-virus mixture is retained and supernate aliquots are removed successively. It should be mentioned also that it is important to employ a relatively large amount of virus so that all RBC "receptors" rapidly combine with the agent; *i.e.*, are saturated. Otherwise, virus particles which dissociate from one RBC could promptly combine with another and only appear in the supernate subsequently thus leading to an inexact estimation of elution rate. It was found that under the conditions of these experiments allantoic fluid pools with hemagglutination titers of 1:1024 or more consistently saturated the quantity of cells employed as judged by the finding that demonstrable amounts of virus remained unadsorbed 10 minutes after the mixtures were prepared.

Ultraviolet Irradiation.—In all experiments the same ultraviolet source and distance were used. The lamp, which was kindly provided by Dr. George I. Lavin, was a "cold arc" resonance mercury lamp which operated at 15,000 volts A.C. The emission spectrum showed one main line at 2537 Å. Irradiation of infected allantoic fluid pools was performed in an open Petri dish with an inner diameter of 5 cm. Five cc. of fluid in this dish gave a depth of 5 mm. The distance from the fluid to the disc-shaped coil (diameter = 8 cm.) of the lamp, which was parallel to the fluid surface, was 9 cm. Irradiation was carried out for periods ranging from 15 to 60 minutes. Allantoic fluids were not dialyzed before irradiation.

Treatment with Lanthanum.—Lanthanum acetate ($LaAc_3$) was used for the treatment of infected allantoic fluid pools. The amorphous compound was dissolved in distilled water. Two stock concentrations, 0.1 and 0.01 M, were employed and the pH of the solutions used varied only between 6.5 and 7.0. When tested intra-allantoically in chick embryos, it was found that injections of 0.5 cc. of 0.1 M solutions did not kill the embryos. Solutions of $LaAc_3$ were mixed with equal quantities of infected allantoic fluid pools so as to give final concentrations ranging from 0.005 to 0.00005 M. The mixtures were held at 4°C. for 3 to 5 hours. Flocculent precipitates developed promptly. The precipitate was removed by centrifugation and discarded. The supernate was employed and excess lanthanum contained in it was precipitated by the addition of sufficient Na_2HPO_4 to give 0.5 M. This was done because lanthanum acetate even in very low concentrations causes agglutination of chicken RBC.

EXPERIMENTAL

Elution of Lee and PR8 Strains from RBC.—In order to determine whether persistent alterations in the rate of elution of influenza virus from RBC could be produced by experimental procedures, it was first necessary to devise a technique by means of which relatively small quantities of eluted virus could be measured. It was found that, when the RBC were sedimented from the mixture at fixed time intervals and resuspended in fresh saline after each centrif-

ugation, it was possible to measure with fair reproducibility the amount of virus which was eluted during each successive time interval. It appeared that in this manner the information desired could be obtained.

A number of experiments were carried out to determine the rates of elution of the Lee and PR8 strains from chicken RBC under these conditions. Hirst (2) showed previously, under different experimental conditions, that the Lee strain was eluted more rapidly than the PR8 strain.

The stepwise technique employed for the determination of the rate of elution of influenza viruses from RBC is described in detail above. In the present experiments successive 30 minute elution intervals were employed.

The results of a series of such experiments with a number of allantoic fluid pools containing either the Lee or the PR8 strain are presented in summary form in Table I. It can be seen that elution of the Lee strain is characterized by very rapid dissociation during the first 30 minute step and thereafter during successive 30 minute intervals by progressively less rapid dissociation. It is evident that after the second step (70 minutes) only very small quantities of virus were released into the supernatant fluids. The progressively decreasing rate of elution becomes especially evident when the percentage of virus eluted during each step is calculated relative to that combined with the RBC at the beginning of each step. As is shown in Table I, this quantity diminishes rapidly from an average value of 27.3 per cent during the first 30 minute step to no more than 0.3 per cent during the final 60 minute step.

The elution of the PR8 strain showed a very different pattern. Only relatively small amounts of virus were released from the RBC during each 30 minute interval and at no time did the titer of the supernates reach a high level as was the case with the Lee strain during the first 30 minute step. Moreover, it will be noted that the percentage of combined virus which was eluted during each step remained almost constant. This indicates that with the PR8 strain the rate of elution did not vary significantly during the time interval studied. In Figs. 1 and 2 the amounts of each strain which were eluted in successive steps are presented graphically. The logarithms of the geometric mean of the hemagglutination titers of the supernates are plotted against the time after the virus was mixed with RBC.

Similar experiments in which the supernatant fluid was changed at shorter intervals were carried out with both the Lee and PR8 strains. In every instance analogous results were obtained. Although it was possible by employing very short elution periods, *e.g.* 5 minutes or less, to obtain even more rapid elution rates with the Lee strain, it was not possible to increase the rate of elution of the PR8 strain as can be seen from the data shown in Fig. 3.

It is apparent that a number of factors might be responsible for the results obtained with the Lee strain. However, in the light of the findings presented

below, it seems of most interest to consider the possiblity that the progressively decreasing rate of elution obtained with the Lee strain as not with PR8, may be an indication of an inhomogeneous population of Lee virus particles in infected allantoic fluid.

Effects of Lanthanum Acetate upon Influenza Viruses.—Hammarsten, Hammarsten, and Teorell (11) showed that lanthanum reacts with nucleic acids to

TABLE I

Results of Stepwise Virus Elution Experiments with Lee and PR8 Strains

Virus strain	No. of allantoic fluid pools	Time after mixing with RBC	Hemagglutination titer* of successive supernates			Adsorbed virus eluted per step
			Highest	Lowest	Mean Geometric	
		min.				*per cent*
Lee	9	0 (Control)	8192	1024	2757	—
"	"	10	256	16	34	—
"	"	40	2048	256	745	27.3
"	"	70	512	64	161	8.1
"	"	100	256	16	32	1.7
"	"	130	32	4	10	0.5
"	8	190	8	4	5	0.3
Total virus eluted					953	37.9
PR8	5	0 (Control)	4096	1024	2665	—
"	"	10	32	16	28	—
"	"	40	128	32	64	2.4
"	"	70	128	64	112	4.3
"	"	100	256	32	74	3.0
"	"	130	128	32	56	2.3
"	"	190	128	32	97	4.2
Total virus eluted					403	16.2

* Expressed as the reciprocal.

form an insoluble complex. It seemed possible that lanthanum could also react with influenza viruses and probable that, if it did, it might cause some alteration of the agents. This possibility was investigated.

When an aqueous solution of lanthanum acetate was added to normal allantoic fluid, a flocculent precipitate developed. The more concentrated the $LaAc_3$ solution, the more abundant was the precipitate. It was found that even in high dilution, *e.g.* 10^{-5} to 10^{-6} M, $LaAc_3$ caused agglutination of chicken RBC. Consequently, it was impossible to determine the hemagglutination titer of the virus in the presence of free lanthanum. When, however, a few

drops of 5 M Na_2HPO_4 solution were added to the mixture, the excess of $LaAc_3$ was precipitated and satisfactory virus hemagglutination titrations could be performed.

The addition of $LaAc_3$ to allantoic fluids containing influenza virus caused a prompt reduction in the hemagglutination titer of the virus and the reduction in titer appeared to be proportional to the concentration of lanthanum. Thus, a concentration of 0.0001 M did not

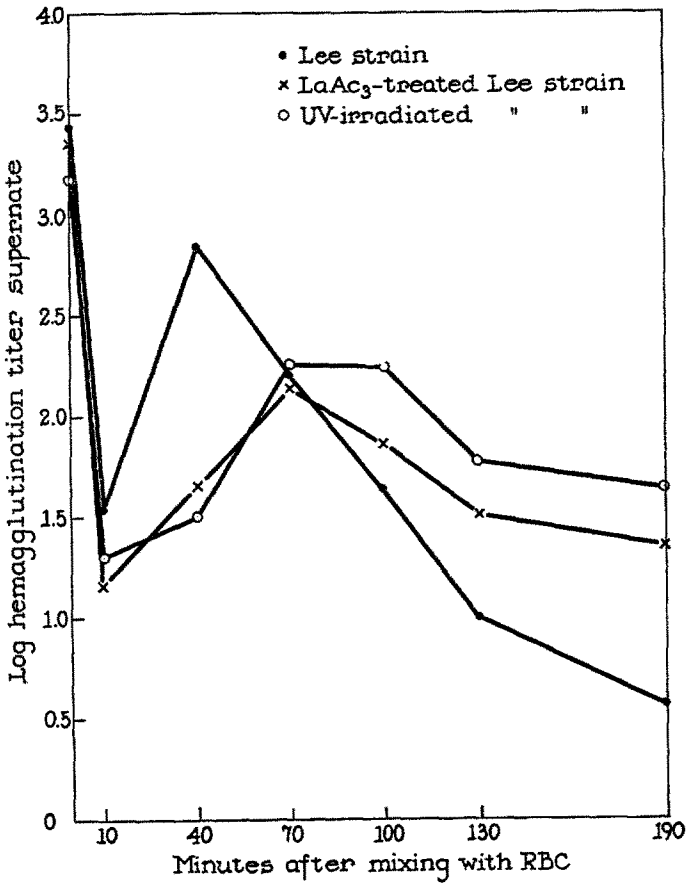

FIG. 1. Stepwise elution curves obtained with the Lee strain as well as with $LaAc_3$-treated and ultraviolet-irradiated strains derived therefrom. The logarithm of the geometric mean of the hemagglutination titers of successive supernates (*cf.* Tables I, II, and IV) is plotted against the time at which the supernate was removed.

demonstrably affect the hemagglutination titer, concentrations of 0.01 to 0.001 M decreased the titer 100- to 1000-fold, while a concentration of 0.1 M abolished the hemagglutination reaction completely. The reduction in titer occurred immediately after the lanthanum was added and prolonged treatment, *e.g.* 10 hours, did not lead to any further decrease in titer.

The effect of $LaAc_3$ upon the infectivity of influenza virus also was studied. It was found that a mixture of equal parts of allantoic fluid infected with the

Lee strain and a 0.01 M solution of $LaAc_3$ was capable of infecting chick embryos. The virus infectivity titer of such a mixture, however, was lower by about 3 logarithmic units than that of the untreated allantoic fluid. Prolonged

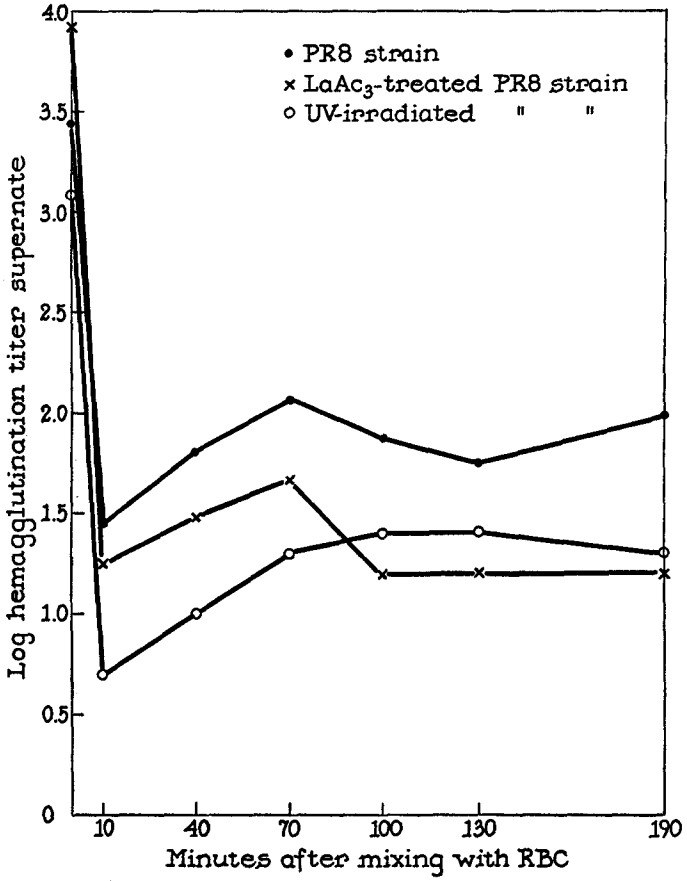

FIG. 2. Stepwise elution curves obtained with the PR8 strain as well as with $LaAc_3$-treated and ultraviolet-irradiated strains derived therefrom. The logarithm of the geometric mean of the hemagglutination titers of successive supernates (*cf.* Tables I, II, and IV) is plotted against the time at which the supernate was removed.

contact, *e.g.* 8 hours, with $LaAc_3$ did not cause any further decrease in the virus infectivity titer.

Elution of Lanthanum-Treated Virus from RBC.—To determine whether treatment with $LaAc_3$ caused an alteration in the rate of elution of the Lee or PR8 strains was technically difficult with most of the original mixtures because of their markedly reduced hemagglutination titers. However, when a concentration of 0.00025 M was employed with the Lee strain, the hemagglutination

titer was reduced only two- to fourfold and it was possible to demonstrate directly that elution of this treated strain occurred at a definitely slower rate than that of the original strain.

Because virus treated with $LaAc_3$ retained infectivity for the chick embryo, it was of obvious interest to determine the rate of elution of lanthanum-treated virus from RBC after serial passage in the allantoic sac. A number of experiments were carried out with both the Lee and PR8 strains.

Allantoic fluid pools infected with the desired virus were mixed with $LaAc_3$ solution so as to yield a concentration of 0.005 M. After 3 to 5 hours at 4°C. and light centrifugation to

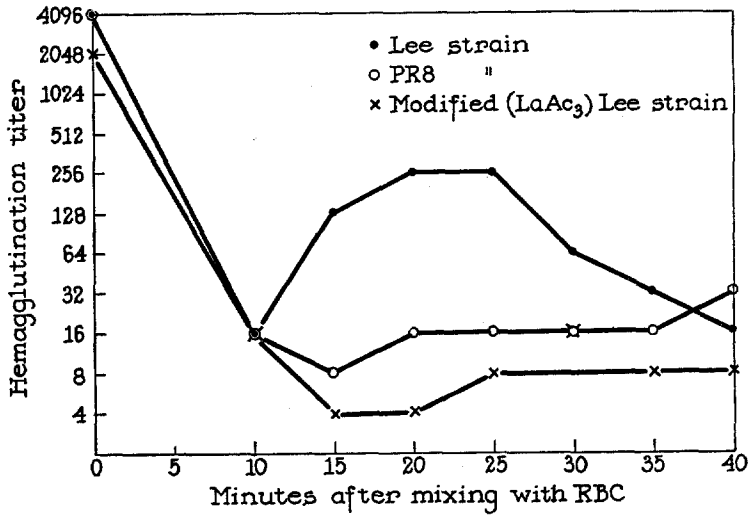

FIG. 3. Stepwise elution curves obtained by removal of the supernate at 5 minute intervals with the Lee and PR8 strains as well as a modified ($LaAc_3$-treated) Lee strain.

remove the precipitate, groups of 6 embryos were inoculated intra-allantoically with a 10^{-2} or 10^{-3} dilution of each supernate. The embryos were incubated and their allantoic fluids removed as described above. All fluids from each group which gave positive hemagglutination tests were pooled and used either in elution rate experiments or as inocula for additional groups of embryos. In passages the inoculum was diluted 10^{-3}. No further treatment with $LaAc_3$ was employed during the serial passage experiments. After a single treatment with $LaAc_3$ the Lee strain was carried through 15 serial passages in the allantoic sac; the PR8 strain through 7.

The results of stepwise elution rate experiments with $LaAc_3$-treated strains of virus after serial passage in the chick embryo are shown in Table II as well as in Figs. 1 and 2. With the Lee strain the allantoic fluid pools employed ranged from the 1st through the 15th serial passage. With the PR8 strain pools obtained at each passage from the 1st through the 7th were used. It

is seen that the rate of elution of the $LaAc_3$-treated Lee strain from RBC was strikingly different from that of the original Lee strain (*cf.* Table I). The very rapid elution rate during the first 30 minute step was no longer demonstrable and the progressive decrease in the elution rate, which characterized the original strain, was no longer evident. Moreover, the total quantity of virus which was eluted during successive steps was approximately ⅛ of the

TABLE II

Results of Stepwise Elution Experiments with Lanthanum-Treated Lee and PR8 Strains after Serial Passage in the Chick Embryo

Virus strain ($LaAc_3$-treated)	No. of allantoic fluid pools	Time after mixing with RBC	Hemagglutination titer of successive supernates			Adsorbed virus eluted per step
			Highest	Lowest	Mean Geometric	
		min.				*per cent*
Lee	9	0 (Control)	8192	1024	2352	—
"	"	10	128	4	15	—
"	"	40	512	4	35	1.4
"	"	70	512	8	138	5.6
"	8	100	256	32	70	3.0
"	9	130	128	8	32	1.4
"	"	190	64	8	24	1.1
Total virus eluted					299	12.5
PR8	7	0 (Control)	4096	1024	2470	—
"	"	10	64	8	18	—
"	"	40	512	8	24	1.0
"	"	70	256	16	29	1.2
"	"	100	256	16	35	1.5
"	"	130	128	16	39	1.6
"	"	190	128	16	39	1.7
Total virus eluted					166	7.0

amount obtained with the original strain. In these respects, the elution of the $LaAc_3$-treated Lee strain closely resembled that of the original PR8 strain (*cf.* Table I). It seems important to point out that prolonged serial passage with infected allantoic fluid inocula diluted only 10^{-3}, did not cause reversion in the peculiar elution characteristics of the $LaAc_3$-treated Lee strain; after 15 passages in the absence of lanthanum, the elution curve remained grossly abnormal and was not significantly different from that obtained after but a single passage of the modified strain in the allantoic sac.

It also is seen from the results shown in Table II that the rate of elution of

the $LaAc_3$-treated PR8 strain from RBC was somewhat slower than that of the original PR8 strain. It should be noted, too, that only about ½ as much of the treated strain as of the original strain was eluted during successive elution steps in the time period studied. Even after 7 serial passages in the allantoic sac in the absence of lanthanum these alterations in the elution curve of the $LaAc_3$-treated PR8 strain remained demonstrable; no evidence for reversion to the characteristics of the original strain was obtained.

Elution of Ultraviolet-Irradiated Virus from RBC.—Henle and Henle (12) showed that ultraviolet irradiation inactivated various potentialities of influenza virus at different rates. Under the experimental conditions which they employed there was but little effect upon elution of the virus unless irradiation was prolonged sufficiently to affect markedly the capacity of the virus to cause hemagglutination.

In the light of the unexpected and apparently persistent modifications produced by a single treatment with $LaAc_3$ in the strains under study, it appeared of interest to study further the effects of ultraviolet irradiation upon the Lee and PR8 strains.

Undiluted allantoic fluid pools infected with the desired virus were irradiated with high intensity ultraviolet for varying periods under the conditions described above. After irradiation the hemagglutination titers of the fluids were determined. The longer periods (45 to 60 minutes) of irradiation employed caused a marked reduction in the hemagglutination titer of previously non-irradiated strains. Irradiated fluids were diluted 10^{-3}, and each was inoculated intra-allantoically into a group of 6 embryos. The embryos were incubated and their allantoic fluids harvested as described above. These allantoic fluids were used in elution rate experiments as well as for the inoculation of additional groups of embryos. In passages the inoculum was diluted 10^{-3}. With two irradiated Lee strains 6 serial passages of each, after a single period of irradiation, were carried out in the allantoic sac. With another irradiated Lee strain 11 serial passages were performed and several additional irradiations were carried out between certain passages. With the PR8 strain 7 serial passages were performed and further irradiation was carried out between each passage.

The results of stepwise elution rate experiments with two ultraviolet-irradiated Lee strains after serial passage in the chick embryo are shown in Table III. With these two strains, which were irradiated only once, allantoic fluid obtained after each passage from the 2nd through the 6th was studied. It will be noted that the elution rate of these two irradiated Lee strains was markedly slower than that of the original Lee strain (*cf.* Table I) even though the total amount of virus eluted during all the successive steps was not greatly different. As with the $LaAc_3$-treated Lee strain (*cf.* Table II), the most striking reduction was demonstrated during the first 30 minute elution period.

The results of similar experiments with a repeatedly irradiated Lee strain are shown in Table IV and in Fig. 1. These experiments were made in an attempt to reduce even more markedly the elution rate of the irradiated Lee strain by means of further irradiation of allantoic fluid between embryo pas-

TABLE III

Results of Stepwise Elution Experiments with Lee Strains after a Single Ultraviolet Irradiation and Serial Passage in the Chick Embryo

Virus strain (ultraviolet-irradiated)	No. of allantoic fluid pools	Time after mixing with RBC	Hemagglutination titer of successive supernates			Adsorbed virus eluted per step
			Highest	Lowest	Mean Geometric	
		min.				*per cent*
Lee	10	0 (Control)	8194	1024	1684	—
"	"	10	256	16	42	—
"	"	40	1024	4	73	4.4
"	"	70	1024	16	157	10.0
"	"	100	256	32	91	6.4
"	"	130	128	8	45	3.4
"	"	190	256	4	42	3.3
Total virus eluted					408	27.5

TABLE IV

Results of Stepwise Elution Experiments with Lee and PR8 Strains Repeatedly Ultraviolet-Irradiated after Serial Passage in the Chick Embryo

Virus strain (ultraviolet-irradiated)	No. of allantoic fluid pools	Time after mixing with RBC	Hemagglutination titer of successive supernates			Adsorbed virus eluted per step
			Highest	Lowest	Mean Geometric	
		min.				*per cent*
Lee	10	0 (Control)	2048	1024	1520	—
"	"	10	128	0	21	—
"	"	40	128	16	32	2.1
"	"	70	512	64	181	12.3
"	"	100	512	32	179	13.9
"	"	130	256	16	60	5.4
"	"	190	128	8	42	4.0
Total virus eluted					494	37.7
PR8	7	0 (Control)	4096	1024	2030	—
"	"	10	32	0	5	—
"	"	40	32	0	10	0.5
"	"	70	64	4	20	1.0
"	"	100	128	8	26	1.3
"	"	130	64	8	26	1.3
"	"	190	64	8	20	1.0
Total virus eluted					102	5.1

sages. It is evident that the altered elution rate of the irradiated Lee strain was not additionally affected by further irradiation.

It can be seen also from the results shown in Table IV and Fig. 2 that the elution rate of a repeatedly irradiated PR8 strain was somewhat slower than that of the original PR8 strain (*cf.* Table I) and that only approximately ⅓ as much of the irradiated strain as of the original strain was eluted during the time interval studied. The elution rate of the irradiated PR8 strain corresponded very closely to that of the $LaAc_3$-treated PR8 strain (*cf.* Table II).

With the irradiated Lee strains no evidence of reversion to the elution characteristics of the original strain was obtained despite serial passage in the embryo with no further irradiation. The elution rates obtained with fluids from the last serial passages, *i.e.* the 6th, appeared not to differ significantly from those obtained with fluids from the 2nd embryo passages. It appears, therefore, that the modifications induced by ultraviolet irradiation, with respect to elution of the strains studied from RBC, persisted on serial passage in the absence of the original agent, as was the case after treatment with $LaAc_3$.

Infectivity of Modified Strains.—After serial passage in the chick embryo the infectivity titer of $LaAc_3$-treated Lee and PR8 strains as well as ultraviolet-irradiated Lee and PR8 strains was determined in parallel with that of the original strains. Titrations were carried out both in mice and in chick embryos according to the techniques described above.

In every instance it was found that there was no significant difference between the virus titration end points obtained with the modified and original strains in either mice or chick embryos. It appears, therefore, that both $LaAc_3$-treated and ultraviolet-irradiated strains retained in undiminished degree the capacity to induce infection in either species.

Immunological Properties of Treated Strains.—After serial passage in the chick embryo cross hemagglutination-inhibition titrations were carried out with $LaAc_3$-treated, ultraviolet-irradiated, and the original Lee and PR8 strains. The immune sera employed were anti-Lee, anti-PR8, and anti-ultraviolet-irradiated Lee.

In each case it was found that there was no significant difference between the results of quantitative hemagglutination-inhibition titrations with the original strain and with modified strains derived from it. It thus appears that both the $LaAc_3$-treated and the ultraviolet-irradiated strains retained the immunological specificity which characterizes the strain from which they were derived.

Effect of Increased NaCl Concentration upon Elution Rate.—In this laboratory Davenport (13) recently found that a decrease of electrolyte concentration in RBC-influenza virus systems leads to a reduction in the elution rate of the virus. In the present study, when the NaCl concentration of mixtures was reduced in experiments in which the stepwise elution technique was employed,

a decrease in elution rate also was noted. It was, therefore, of interest to determine what effect an increased salt concentration would have upon the elution rate under similar conditions.

TABLE V

Results of Stepwise Elution Experiments with Lee, PR8, and Ultraviolet-Irradiated Lee Strains in 3 Per Cent NaCl Solution

Virus strain	Time after mixing with RBC	Hemagglutination with successive supernates* diluted as indicated											Total virus eluted in	
		4	8	16	32	64	128	256	512	1024	2048	4096	3.0 per cent NaCl	0.9 per cent NaCl
	min.													
Lee	0 (Control)	2	2	2	3	3	3	3	3	2	2	2		
"	10	2	2	3	2									
"	40	0	0	±	2	2	2	2	3	2	2	2		
"	70	3	3	3	3	2	2							
"	100	3	2	2										
"	130	2												
"	190	2											104	55
Ultraviolet-irradiated Lee	0 (Control)	4	4	4	3	3	3	3	3	3	2	2		
" "	10	3	3	2										
" "	40	4	3	3	3	2								
" "	70	4	4	3	3	3	2							
" "	100	4	3	3	3	3	2							
" "	130	3	3	3	3	2								
" "	190	3	3	3	3	2							11	13
PR8	0 (Control)	4	4	4	3	3	3	3	3	3	2	2		
"	10	4	3	2										
"	40	4	4	4	4	4	4	3	2					
"	70	4	4	4	4	3	3	2	2					
"	100	4	4	4	4	3	3	2	2					
"	130	4	4	3	3	2	2	2						
"	190	4	4	4	3	3	2	2					50	10

* Dilutions prepared in 0.9 per cent NaCl solution.

A buffered solution containing 3 per cent NaCl, instead of 0.85 per cent, was used. When this solution was added to sedimented RBC and adsorbed virus, it was found that the RBC promptly formed a solid gel. If, however, the RBC were first gradually adapted to an increasing salt concentration by washing successively in 1.5, 2, 2.5, and 3 per cent NaCl solutions, no such difficulty was encountered. The experiment, therefore, was carried out with such NaCl-adapted RBC and 3 per cent buffered NaCl solution was added as an elution medium at each step. The supernates were diluted in 0.9 per cent NaCl in the usual manner.

As can be seen from the results shown in Table V the presence of 3 per cent NaCl in the mixtures increased the elution rate of both the Lee and PR8 strains. However, the modified (ultraviolet-irradiated) Lee strain did not show any significant change in elution rate under these conditions. It should also be noted that in the lower dilutions of the supernate obtained from the Lee strain after the first 30 minute elution step there was no evidence of hemagglutination at 1 hour. With the other supernates from the Lee strain as well as with each of the supernates from the PR8 and the modified Lee strain, the usual results were obtained.

Simultaneous hemagglutination titrations were performed with the Lee and PR8 strains in 3, 2, 0.9, and 0.45 per cent, respectively, buffered NaCl solutions. In 3 per cent NaCl solution the Lee strain causes agglutination rapidly, but after about 25 minutes aggregates disappeared, and at the end of 1 hour only 0 readings were obtained throughout the series of dilutions. In 2 per cent NaCl solution the lowest four dilutions showed 0 readings. No similar disappearance of hemagglutination occurred in either 0.9 or 0.45 per cent NaCl solutions. Identical experiments with the PR8 strain failed to show any such effect of NaCl concentration on the hemagglutination reaction.

It appears that it was possible to increase the elution rate of both the Lee and PR8 strains but not of the modified Lee strain by increasing the NaCl concentration of the elution medium. The disappearance of hemagglutination in the lower dilutions of the supernate from the first 30 minute step is undoubtedly the result of the very rapid elution rate of this fraction which is further accelerated by the increased NaCl concentration. It is of interest that both the PR8 strain and the modified Lee strain, both of which have relatively slow elution rates, failed to show a similar phenomenon.

DISCUSSION

That it is possible to cause certain alterations in a property of influenza virus by well defined and readily controlled laboratory procedures is apparent from the results obtained in this study. A single brief period of treatment with lanthanum acetate in low concentration or a single period of irradiation with ultraviolet light resulted in the development of virus strains which possessed a demonstrable modification as compared with the original strain. Of most interest and importance is the fact that the modified strains retained their unusual character on serial passage in the chick embryo in the absence of the agent which originally produced the alteration.

Insofar as was determined but a single property of the modified strains was altered. That other properties also may have been altered is, of course, possible. However, infectivity for both the mouse and the chick embryo, hemagglutinating capacity, rate and degree of adsorption on RBC, antigenicity and immunological specificity all appeared to be identical with those of the original

strain. Of the properties studied only elution from combination with RBC was demonstrably different, both as to rate and extent, and served to distinguish the modified strains of Lee virus from the original strain.

It is noteworthy that it is the Lee strain which yielded modified strains with the more striking reductions in elution rate. It has been known for some time (2) that the Lee strain elutes from RBC considerably more rapidly than the PR8 strain. The alteration was sufficiently marked to make it impossible to distinguish between modified Lee strains and the PR8 strain on the basis of their rates of elution alone.

As for the agents which were employed for the development of modified influenza virus, it is known that lanthanum forms insoluble complexes with nucleic acids (14) and the wave length of ultraviolet employed appears to be identical with that at which nucleic acids show maximal adsorption (15). Whether either or both agents exert their effects as a result of action on nucleic acid components of the virus particles is hypothetical. However, it appears possible that either agent might deleteriously affect such components.

The fact that the original Lee strain shows a progressively decreasing rate of elution with increasing time suggests that the individual virus particles are not entirely identical as regards this one property. There is, in fact, no good reason for thinking that all virus particles of a given strain are identical in all respects. There is some evidence which strongly suggests that influenza viruses, like numerous other infectious agents, may show variation relative to various properties; *i.e.*, pathogenicity for unnatural hosts (16, 17), capacity to agglutinate erythrocytes from various species (18), and immunological characteristics (19, 20). It seems probable that an infected allantoic fluid can be considered to contain an enormous population of virus particles all fundamentally similar in most respects but differing slightly, one from another, in certain respects. If such an inhomogeneity in a given population of virus particles exists, it would not be surprising that chemical or physical agents might affect individual particles in somewhat different ways. Thus, the deleterious action of lanthanum or of ultraviolet might vary in an inhomogeneous population, and those particles which, following treatment, retained the capacity to multiply in a susceptible host would be in all probability the particles least affected by either agent. Passage of such a treated strain should result in the development of a virus population closely similar to or identical, in distribution of properties, with that of the original strain only if the treated strain were as capable as the original strain of leading to the development of virus particles with slightly diverse properties. If, however, a treated strain contains less than the original distribution of slightly different virus particles, and leads in the susceptible host to the development of a virus population with an abnormally restricted distribution of properties, an altered strain would emerge. Such an altered strain could continue on serial passage to show the same unusual

property, although reversion with the eventual acquisition of the properties of the original strain might occur if passages were continued sufficiently long.

It seems likely that the alteration produced in influenza virus in the present study is best explained on the basis of selection of naturally occurring variants. It is, of course, possible that variants might actually have been induced by the procedures employed. With the modified Lee strains it appears that the relatively large proportion of the virus population of the original strain, which shows a very rapid elution rate, is much reduced. The slower elution rate of the modified strains may be explained by the reproduction in the susceptible host of a higher than normal proportion of virus particles with a slow elution rate due presumably to the selective inactivation of virus particles with a high elution rate by the agents initially employed. Whether variants were selected or induced by the experimental procedures, the final results would be similar and a modified strain could be evolved under either circumstance.

SUMMARY

The rates of elution from RBC of the Lee and PR8 strains of influenza virus were studied by means of a step-wise elution technique. By means of a single treatment with lanthanum acetate or irradiation with ultraviolet and subsequent passage in chick embryos, it was possible to alter the elution rate of the Lee strain so that it was similar to that of the PR8 strain. This alteration proved to be persistent on serial passage in the absence of the agent which caused it. As far as was determined, the elution rate of the virus appeared to be the only property which was altered. The phenomenon can be most readily understood on the assumption that the difference in elution rates of the two strains is due to a heterogeneous population of virus particles in the Lee strain with respect to elution rate.

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