THE ANTIGENIC POTENCY OF EPIDEMIC INFLUENZA VIRUS FOLLOWING INACTIVATION BY ULTRAVIOLET RADIATION*

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It has been shown repeatedly that bacteria and viruses, as well as enzymes and other proteins can be inactivated or denatured by ultraviolet radiation. Inactivation of many animal viruses has been reported (1–18), but in only a few studies have the antigenic properties of such preparations been investigated. Gordon and Hughes (18) observed that yellow fever virus when inactivated by ultraviolet radiation, heat, or formaldehyde, possessed no demonstrable immunizing property, and concluded that any immunity which occurred was the result of infection. McKinley and Holden (4) injected rabbits subdurally with herpes virus inactivated by ultraviolet radiation without producing infection. Such animals, when later tested by subdural injection of active virus, were not immune. In order to measure the infectivity of irradiated virus and active virus, Graham, Brandly, and Levine (3), using the virus of fowl pox cultivated on the chorioallantois of the hen's egg, applied irradiated and untreated virus to the skin of susceptible chickens. When the animals were subsequently tested for immunity, only those which had received the active virus were immune.

In contrast to these negative results is the report of Hodes, Lavin, and Webster (12) who found that the infectivity of rabies virus could be destroyed without totally destroying its antigenicity. Kidd (15) has shown that the Shope papilloma virus may be inactivated by ultraviolet radiation without losing its ability to function as antigen in the complement-fixation test, although other procedures such as treatment with strong acid or alkali abolished both infectivity and the capacity to react serologically.

This report deals with the capacity of ultraviolet radiation to destroy the infectivity of the virus of epidemic influenza and the effect of irradiation on the antigenic activity of the virus as measured by its capacity to immunize mice.

Materials and Methods

Strains of Virus.—Two strains of epidemic influenza virus were studied—the PR8 (19) and the Melbourne (20) strains. Virus obtained both from infected mouse lungs and tissue culture medium was used.

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Tissue Culture Virus.—The technique used for maintaining the virus in tissue culture is that described previously by Magill and Francis (21). Both strains have been well adapted to growth in a medium containing chick embryonic tissue minced in physiological salt solution and have maintained their virulence for mice.

The Melbourne strain used in these experiments was in its 43rd to 77th transfer in tissue culture medium since its last mouse contact. The virulence of the virus was such that 0.05 cc. of a 1:1000 dilution and often a 1:10,000 dilution of tissue-free supernatant fluid given intranasally to young Swiss mice produced a fatal infection. A similar dose of a 1:10,000 and often a 1:100,000 dilution resulted in definite pulmonary lesions seen at autopsy on the 6th to 10th day following infection.

The preparation of the PR8 strain was from the 496th transfer in culture since the last mouse passage. Fatal infection was caused by the 1:1000 dilution of culture and definite lesions resulted when the 1:10,000 dilution was given intranasally.

Mouse Passage Virus.—Suspensions of infected mouse lungs were prepared by grinding with the aid of sterile alundum, lungs removed aseptically from mice that had just died or had been sacrificed when moribund. Considering the weight of lung tissue as the unit of measure, a 10 per cent suspension was made in physiological saline or 0.05 M phosphate buffer of pH 7.8 by adding 1 cc. of fluid per 0.1 gm. of wet lung tissue. The suspension was centrifuged to remove gross particulate material and the opalescent supernatant fluid was decanted. In most experiments the supernatant fluid was diluted to form a 1 per cent suspension which was then irradiated. All virus preparations were made shortly before their use, usually the same day; rarely, after being kept frozen at −12°C., overnight. These suspensions were often free of bacterial contamination when made up in small amounts, that is, when few lungs were used. However, larger batches were frequently contaminated with organisms commonly found in the mouse's respiratory tract. Such contamination interfered in no demonstrable manner since it was found that the bacteria were extremely sensitive to ultraviolet radiation and were killed long before the virus was inactivated.

The Melbourne strain was transferred from tissue culture to mice and maintained through 19 pulmonary passages before use. Usually a dilution of 1:1,000,000 produced fatal infection when 0.05 cc. was given intranasally to mice in ether anesthesia. However, in Experiment 1, the virus suspension was prepared in physiological saline and caused death in a dilution of 1:10,000 but still produced lesions when given in a dilution of 1:1,000,000.

The PR8 strain was in its 407th to 424th mouse passages since last ferret contact. The maximum dilution causing a fatal infection was usually 1:1,000,000 and definite lesions resulted with the 1:10,000,000 dilutions.

The concentration of virus in the preparations employed is sometimes given in terms of both minimal lethal doses and minimal infective doses per cc. The minimal lethal dose (M.L.D.) is determined by the highest dilution, 0.05 cc. of which given intranasally produces fatal infection in mice within 10 days. The minimal infective dose (M.I.D.) is determined in the same way, using the development of visible lung lesions as the criterion of infection.

The concentration of inactive virus in irradiated preparations is given in terms of the minimal lethal units (M.L.U.) and minimal infective units (M.I.U.) corresponding to M.L.D. and M.I.D. of the original untreated material.

Method of Irradiation.—The source of ultraviolet radiation employed was the full light of a quartz mercury vapor lamp.[1]

Periods of exposure were timed with a stop watch. At the end of an interval, the lamp was screened and a sample of irradiated suspension removed with a sterile pipette. That the intensity of the radiation was constant in different experiments was demonstrated by measurements made with an ultraviolet doseometer consisting of a photronic cell with a filter and a milliammeter. Any variation in intensity was controlled by a series of resistance coils.

Suspensions to be irradiated were contained in sterile long necked quartz flasks plugged with sterile cotton and capped with tinfoil. The flasks were mounted on a shaking device (28), so arranged that each flask was equidistant from the source of irradiation. Approximately 30 to 50 cc. of suspension were contained in each flask. The flasks were at a distance of 18 cm. from the lamp. Under these conditions the temperature rise was seldom more than 2° above that of room temperature.

During the irradiation procedure, which involved considerable shaking, light, fibrinous particles formed. At first this precipitate was removed by centrifugation but was later disregarded when it was found that no active virus was contained therein.

Criteria Used to Determine Destruction of Infectivity of Material Used in Vaccination Experiments.—The irradiated preparation to be tested was given intranasally in 0.05 cc. doses to six mice anesthetized with ether. At the end of the 3rd or 4th day three of the mice were autopsied and their lungs removed with sterile precautions. If no lesions were present the lungs were ground and made up to a 50 per cent suspension in broth and passed to another group of three mice. Both these and the three remaining mice of the original group were killed and their lungs examined on the 10th day after the initial inoculation. If no lesions were present in either set, the preparation tested was considered free of infectious virus. This procedure was adopted after observing that if traces of infectious virus were present, one mouse to mouse passage was usually sufficient for its detection.

EXPERIMENTAL

Inactivation of Influenza Virus by Exposure to Ultraviolet Radiation

Experiment 1.—Undiluted tissue cultures and 1 per cent mouse lung suspensions of both the Melbourne and PR8 strains of epidemic influenza virus were irradiated for different periods of time up to 33 minutes. At the time intervals shown in Table I, 0.5 cc. samples were withdrawn to be tested for infectivity. This was done by instilling 0.05 cc. of the sample intranasally into each of three anesthetized mice. The mice were observed for 6 or 7 days and all deaths recorded. At the end of this period, all survivors were sacrificed and their lungs examined for gross lesions. The results are presented in Table I.

It will be noted from the data that the infectivity of both strains of virus in either tissue culture or mouse lung suspension can be destroyed by ultraviolet radiation. Cultures of the Melbourne strain of virus no longer

[1] Manufactured by the Hanovia Chemical Company.

produced lung lesions consistently after an exposure of 25 minutes and in 31 minutes inactivation was complete. A 1 per cent mouse lung suspension

TABLE I

Infectivity of Virus Preparations after Irradiation for Various Lengths of Time

Period of irradiation	Virus infectivity			
	Melbourne strain		PR8 strain	
	Undiluted tissue culture	1 per cent lung suspension	Undiluted tissue culture	1 per cent lung suspension
min.				
0	3, 4, 4	3, 3, 4	4, 4, +	5, 5, 6
2		++, ++, ++*	4, 4, 5	6, 6, +++†
4		+, 0, 0	5, 6, ±†	+, +, 0
5	4, 4, 4			
6		0, 0, 0	7, 7, +++	±, 0, 0
8		0, 0, 0	+++, +++, ++	0, 0, 0
10	6, ++, 0*		++, ++, +	0, 0, 0
13	6, +++, +			
14			±, ±, ±	
16	++, +, 0			
18			±, ±, +	
19	+, ±, ±			
21	±, ±, ±			
22	+, ±, ±		0, 0, 0	
23	+, ±, ±			
25	±, 0, 0			
27	+, 0, 0			
29	±, 0, 0			
31	0, 0, 0			
33	0, 0, 0			
Final dilution	Titration of preparations prior to irradiation			
Undiluted			4, 4, +†	
10^{-2}		3, 3, 4	5, 5, 5	5, 5, 6
10^{-4}	Not done	6, 6, 7	++, +, +	5, 5, 6
10^{-5}		++, ++, ++‡		+++, ++, ++†
10^{-6}		++, +, +		0, 0, 0

Numerals denote days of death of individual mice.

± to ++++ represent increasingly greater degrees of pulmonary involvement.

0 indicates no pulmonary involvement.

* Survivors were autopsied on the 6th day after intranasal inoculation.

† Survivors were autopsied on the 7th day after intranasal inoculation.

‡ Survivors were autopsied on the 11th day after intranasal inoculation.

of the same strain produced a minimal lesion in one of three mice after 4 minutes' irradiation, and was completely non-infectious after 6 minutes'

exposure. The infectivity of the PR8 strain of virus in culture was completely eliminated in from 18 to 22 minutes while a 1 per cent suspension

TABLE II

Effect of Concentration of Lung in Suspension on Time of Irradiation Necessary to Destroy Infectivity of Virus (PR8)

Period of irradiation	Concentration of lung in suspension		
	10 per cent	5 per cent	1 per cent
min.			
0	5, 5, 5	4, 4, 4	5, 5, 6
2			6, 6, +++†
4			+, +, 0
5	4, 5, +++*		
6			±, 0, 0
8			0, 0, 0
9	5, 6, 6		
10		4, 4, 6	0, 0, 0
12	4, 5, 5		
15	4, 6, ++++	++, ++, ++*	
18	4, 6, 6		
20		+, +, +	
21	5, 5, 6		
24		+, 0, 0	
27	5, 5, ++++		
28		0, 0, 0	
30	5, 6, +++		
32		0, 0, 0	
35	+++, +++, +++		
40	5, ++++, +++		
Final dilution	**Titration of preparations prior to irradiation**		
10^{-1}	4, 4, 4		
0.5×10^{-1}		4, 4, 4	
10^{-2}	4, 4, 4		5, 5, 6
10^{-4}	4, 4, 5	6, 6, 7	5, 5, 6
10^{-5}	7, 8, 8	8, 9, 9	+++, ++, ++†
10^{-6}	+, 0, 0‡		0, 0, 0

* Survivors were autopsied on the 6th day after intranasal inoculation.

† Survivors were autopsied on the 7th day after intranasal inoculation.

‡ Survivors were autopsied on the 10th day after intranasal inoculation.

of PR8 mouse passage virus required 8 minutes' exposure for complete inactivation.

There is a striking difference between the lengths of time required for rendering virus in tissue culture and lung suspension non-infectious. Whereas it required 31 minutes' irradiation to inactivate Melbourne culture

virus, only 6 minutes were necessary in the case of the mouse lung suspension. An explanation for this descrepancy is not yet certain. However, it is thought to be due to a difference in the constitution of the suspending medium rather than in the virus.

It has been observed among the many preparations irradiated that ordinary variations of virus titer (10- to 100-fold differences) did not influence to any measurable degree the amount of exposure required to effect complete inactivation. However, when the concentration of lung tissue in the suspension was varied, a distinct difference became apparent in the time required to render the mixture non-infectious. As is shown in Table II, there seems to be a direct linear relationship between the time required for inactivation and the concentration of lung in suspension; thus, a 1 per cent suspension is rendered almost completely non-infectious in 4 minutes, while a 5 per cent and a 10 per cent suspension are brought to approximately the same degree of diminished infectivity in about 20 and 40 minutes, respectively.

The Antigenicity of Virus Inactivated by Ultraviolet Radiation

Previous workers have reported results of attempted immunization of mice, ferrets, and human subjects with the use of non-infectious preparations of influenza virus. Fairbrother and Hoyle (22) have stated that suspensions of "elementary bodies" of influenza virus, deprived of infective properties by heating at 57°C. for 30 minutes, still retain their antigenicity for mice, but not for ferrets. On the other hand, the effect of heat on crude suspensions was found to be irregular and uncertain. They state further that the effect on immunizing potency was of the same degree as that produced by formaldehyde. Andrewes and Smith (23, 24) have reported the use of mouse passage virus made non-infectious by treatment with formaldehyde as an immunizing agent and found it to be somewhat less effective than fully active virus, but satisfactory for the immunization of mice. They do not mention whether or not the effect of formaldehyde on virus in tissue culture was tested but do note that heat completely destroyed the antigenicity of culture virus for mice. In attempting to immunize ferrets with preparations of non-infectious virus, these authors found that when the vaccine was made from tissue of the homologous species, some degree of immunity resulted, but always less than that obtained with active virus. Although fully active virus derived from mouse tissue was effective as an immunizing agent for the ferret, a similar preparation deprived of infectious properties was no longer effective in that animal but was still suitable for immunizing mice. Smith, Andrewes, and Laidlaw (25) failed to immunize ferrets with virus rendered non-infectious by the photodynamic action of methylene blue or by heat. Stock and Francis (26) have, however, demonstrated the high antigenic activity of influenza virus rendered non-infectious by treatment with fatty acids.

Since the preceding experiments of the present study had clearly shown that irradiation with ultraviolet light inactivated the infectivity of in-

fluenza virus, it was of interest to ascertain what effect the same procedure exerted upon the antigenic potency of the treated virus. The following experiment was designed, therefore, to compare the immunity induced in mice by intraperitoneal inoculation of untreated active and irradiated inactive virus preparations.

Experiment 2.—70 cc. of full strength culture of the Melbourne strain of influenza virus were irradiated. 18 cc. portions were removed at the end of 15, 25, and 35 minutes, respectively. Five groups, each containing 15 young Swiss mice, were selected. One was set aside for controls. On two occasions a week apart the mice in each of the other four groups were given 1.0 cc. intraperitoneally of undiluted culture either untreated

TABLE III

Preliminary Test of Effect of Ultraviolet Irradiation on the Antigenicity of Tissue Culture Virus (Melbourne)

Intranasal test dose of virus 0.05 cc. tissue culture (dilution)	Time of irradiation of culture used for vaccination of mice				
	Unirradiated	15 min.	25 min.	35 min.	Unvaccinated controls
Undiluted	0, 0, 0, 0, 0‡	3, ++, ++, ++, 0	4, ++, ++, ++, +	++, +, 0, 0, 0	2, 3, 3, 3, 4
10^{-2}	0, 0, 0, 0, 0	±, ±, 0, 0, 0	±, ±, ±, ±, 0	±, ±, 0, 0, 0	3, 4, 4, 4, 4
10^{-3}	0, 0, 0, 0, 0	0, 0, 0, 0, 0	±, ±, 0, 0, 0	±, 0, 0, 0, 0	5, 6, 7, 7, 7
	Infectivity of virus in material used for vaccination				
1st dose	3, 3, 3	++, +, +*	0, 0, 0	0, 0, 0	Nil
2nd dose	3, 3, 3	++++, ++++, +++	+, ±, 0	0, 0, 0	Nil

* Survivors were autopsied on the 6th day after intranasal inoculation.

‡ Survivors were autopsied on the 10th day after intranasal inoculation.

or irradiated for 15, 25, or 35 minutes. The material was also tested for infectious activity by intranasal instillations of 0.05 cc. in each of three ether-anesthetized mice. One week after the last intraperitoneal inoculation, each group of 15 was divided into three sets of five. While in ether anesthesia, the mice of a group were treated intranasally with 0.05 cc. of either the undiluted, 1:100, or 1:1000 dilution of active Melbourne culture virus. The mice were observed for 10 days and all deaths recorded. At the end of this period survivors of the immunity test were sacrificed and their lungs examined for pulmonary involvement. The results are recorded in Table III.

It is seen from the results that all 15 mice of the control group died within 7 days after intranasal infection. All the mice vaccinated with fully active virus survived without pulmonary involvement. Only two of the 45 mice receiving irradiated virus succumbed, while more than half of the survivors escaped without any lung lesions and the rest exhibited only minimal changes.

Despite the fact that the material irradiated for 35 minutes was devoid

of demonstrable infectivity, the immunity induced was closely comparable to that resulting from the use of fully active virus. It is also to be noted that although there were traces of infectious virus in the preparation irradiated for 25 minutes and there were approximately 20 M.L.D. per cc. in the preparation exposed for 15 minutes, the residue of active virus resulted in no greater degree of immunity than that induced by virus the infectivity of which had been completely eliminated. It is clear, therefore, that a small amount of active virus contributes in no significant manner to the immunizing potency of a preparation which consists for the most part of avirulent but antigenically active virus.

TABLE IV

Preliminary Test of Effect of Ultraviolet Irradiation on the Antigenicity of Mouse Lung Virus (PR8)

Intranasal test dose of virus 0.05 cc. lung suspension (dilution)	Mice vaccinated with 1 per cent lung suspension*		Unvaccinated controls
	Unirradiated	9 min. irradiated†	
10^{-1}	11, ++, +, 0, 0, 0	4, 4, 4, 7, ++, 0	3, 3, 3, 3, 3, +++
10^{-2}	++, +, ±, ±, ±, 0	4, ++, +, +, 0, 0	3, 3, 4, 4, 4, 4
10^{-3}	+, ±, ±, 0, 0, 0	+, ±, 0, 0, 0, 0	3, 4, 4, 4, 6, 7
10^{-4}	+, ±, ±, 0, 0, 0	±, 0, 0, 0, 0, 0	4, 4, 4, 7, 9, +++
10^{-5}	+, ±, ±, 0, 0, 0	±, 0, 0, 0, 0, 0	4, 5, 5, 6, 7, +++

Survivors were autopsied on the 11th day after intranasal inoculation.

* Infectivity of lung suspension used for vaccination:

10^{-2} dil.....4, 4, 4

10^{-4} dil.....4, 4, 4

† Infectivity test of irradiated suspension:

10^{-2} dil.....+, 0, 0

In view of the results obtained with influenza virus propagated in tissue culture medium, the next experiment was undertaken to determine the effect of ultraviolet radiation upon the PR8 strain of influenza virus in suspensions of mouse lung. The vaccination procedure used in this experiment differed from that employed in Experiment 2 in that a single immunizing dose was given and the immunity test made 2 weeks thereafter.

Experiment 3.—Three groups, each consisting of 30 young Swiss mice, were selected. One group was vaccinated intraperitoneally with 1.0 cc. amounts of a 1 per cent suspension of infected mouse lung tissue; the second was given a similar dose of the same suspension which was devoid of infectious properties following 9 minutes' exposure to the ultraviolet lamp; the third group was set aside to be used as a control. The infectious titer of the samples used for vaccination was tested by intranasal in-

oculation in mice. 2 weeks later, six mice of each group were anesthetized and given an intranasal test dose of 0.05 cc. of a 10 per cent suspension of infected PR8 mouse lung tissue. Other groups of six were given 0.05 cc. of decimal dilutions of the same suspension made up to final concentrations of 1:100, 1:1000, 1:10,000, and 1:100,000, respectively. The mice were observed for 11 days and all deaths during the interval were recorded. Survivors were then sacrificed and the extent of the pulmonary lesions noted. The results are charted in Table IV.

All the control mice died or had extensive pulmonary involvement by the 11th day following infection. Only one of the 30 mice vaccinated with the active virus preparation died, and of the survivors, 16 had minimal lesions. Five animals in the group vaccinated with the inactive virus preparation died as a result of intranasal infection; four of them had been infected with at least 10,000 M.L.D. and one with 1000 M.L.D. Of the survivors, seven had minimal pulmonary involvement; the others had none. It is clear, therefore, that a satisfactory degree of immunity can be induced by the intraperitoneal route with only one vaccinating dose of infectious or non-infectious virus.

The results of this experiment suggest that virus inactivated by ultraviolet radiation approaches the immunizing capacity of fully active virus. Before accepting this conclusion, however, it was necessary to gain further quantitative data, and subsequent experiments were performed for this purpose.

Quantitative Comparison of the Antigenic Potency of Active and Irradiated Inactive Virus

It has been demonstrated previously (27) by quantitative procedures that there is a direct relationship between the concentration of epidemic influenza virus used for intraperitoneal vaccination of mice and the degree of immunity which develops to intranasal infection. In the same manner the antigenic activity of different virus preparations can be compared. Accordingly an attempt has been made to obtain more accurate quantitative data concerning the effect of irradiation on the antigenic potency of influenza virus by comparing the immune response induced in mice by intraperitoneal vaccination with serial dilutions of both active and irradiated suspensions of virus.

Experiment 4.—The virus preparations employed in this experiment were 1 per cent suspensions of the lung tissue of mice infected with the PR8 strain of influenza virus in 0.05 M phosphate buffer at pH 7.8. A portion was irradiated for 9 minutes and tested for the presence of infectious virus by two successive passages through mice. As judged by the presence of small pulmonary lesions some active virus was apparently present in

the first vaccine but not in the second. In order that graded vaccinating doses might be administered, decimal dilutions in broth were made of both irradiated and untreated virus from the original 1 per cent suspension. Each dilution was prepared just before inoculation and used immediately. The higher dilutions were prepared first so as to avoid a possible deterioration in virus content while standing.

150 young Swiss mice were divided into six groups. Two 1.0 cc. doses of the 1:100 dilution of irradiated virus were given intraperitoneally to all mice in the first group. The two doses were given a week apart. Three other groups were given the 1:1000, 1:10,000, and 1:100,000 dilutions, respectively. The two remaining groups received the 1:1000 and 1:100,000 dilutions of the untreated virus suspension. A group of 25 mice was set aside as controls. Fresh virus was used for each of the two intraperitoneal inoculations; the first was from the 422nd, the second from the 424th mouse passage.

One week following the second injection, five mice of each group, while in ether anesthesia, were tested for immunity to an intranasal instillation of 0.05 cc. of a 1:10 suspension of infected mouse lung. The remaining mice in each of the original groups were divided into sets of five, each of which was anesthetized and treated intranasally with 0.05 cc. of the 1:100, 1:1000, 1:10,000 or 1:100,000 dilutions, respectively, of the same suspension. Thus, mice vaccinated with a given concentration of virus were tested for immunity with graded amounts of virus ranging from 10 to 100,000 fatal doses. Control mice were similarly infected. The day of death of mice succumbing to the test infection was recorded. On the 10th day, all survivors were sacrificed and their lungs examined for lesions. The results are recorded in Table V.

These results demonstrate that the parallel in immunizing potency of active and inactive virus suggested by Experiment 3 does not hold when subjected to a more complete quantitative analysis. Under the conditions of the present experiment, mice vaccinated with the 1:100 dilution of irradiated virus and the 1:1000 dilution of untreated virus, resisted death and exhibited signs of only minimal infection when tested with as much as 100,000 fatal intranasal doses. However, an examination of the results of vaccination with the higher dilutions reveals that the degree of immunity induced by the 1:1000 dilution of inactive virus was but slightly better than that following vaccination with the 1:100,000 dilution of active virus. The immunity resulting from vaccination with the 1:10,000 dilution of inactive virus was exhibited by a delay of death and by partial resistance to infection with 10 M.L.D. No immunity was detected in the mice vaccinated with the 1:100,000 dilution of irradiated material.

If it is considered that 0.05 cc. of a 1:1,000,000 dilution of the untreated virus suspensions used in this experiment contained 1 M.L.D., then each 1.0 cc. of the various dilutions of vaccinating material contained decimal multiples of 20 M.L.D. of active virus or 20 M.L.U. of inactive virus. Mice vaccinated with the 1:1000 and the 1:100,000 dilutions of active virus had then received a total of 40,000 and 400 M.L.D., respectively. The

mice vaccinated with the 1:100, 1:1000, 1:10,000, and 1:100,000 dilutions of inactive virus received a total of 400,000, 40,000, 4000, and 400 M.L.U., respectively.

It is seen that intraperitoneal vaccination with 400,000 M.L.U. of non-infectious virus results in a solid immunity to 100,000 M.L.D. of active

TABLE V

Quantitative Comparison of the Immunogenic Potency of Irradiated and Non-Irradiated Mouse Passage Virus (PR8)

Intranasal test dose of virus 0.05 cc. mouse lung suspension*		Unvaccinated	Mice vaccinated with active virus		Mice vaccinated with irradiated virus†			
			10^{-3}	10^{-5}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Dilution	M.L.D.		40,000 M.L.D.	400 M.L.D.	400,000 M.L.U.	40,000 M.L.U.	4000 M.L.U.	400 M.L.U.
10^{-1}	100,000	3,3,3,4,4	+,+,±,±,0	3,3,3,3,±	+,+,0,0,0	3,3,5,++,++	3,3,3,4,++++	3,3,3,4,++
10^{-2}	10,000	3,3,4,4,5	0,0,0,0,0	3,4,++,±,±	+,0,0,0,0	7,+,+,+,0	4,4,4,5,5	3,3,3,4,4,9
10^{-3}	1000	4,4,4,4,9	0,0,0,0,0	3,6,+,0,0	±,0,0,0,0	+,+,+,±,0	4,5,6,++,++	3,4,4,4,4
10^{-4}	100	4,4,4,5,5	0,0,0,0,0	4,+,+,+,0	0,0,0,0,0	3,+,+,+,0	4,5,5,10,+	4,4,4,5,5
10^{-5}	10	4,4,4,5,5	0,0,0,0,0	+,+,+,+,+	0,0,0,0,M	+,0,0,0,0	6,7,++,+,±	4,4,5,6,+++

Survivors were autopsied on the 10th day after intranasal inoculation.

M = mouse died in course of vaccination.

Titer of lung suspension used for vaccination:

Dilution	1st dose	2nd dose
10^{-4}	5,5,6	5,5,5
10^{-5}	4,5,6	4,5,5
10^{-6}	6,6,7	7,9,9
10^{-7}	8,+++,++	++,++,+

* Titer of lung suspensions used for immunity test:

10^{-6} dil.....6, 8, +++

10^{-7} dil.....+++, +++, ++

† Infectivity of suspension irradiated for 9 minutes:

First vaccinating dose:

10^{-2} dil.....±, ±, ± (mice were autopsied on 5th day)

Second vaccinating dose:

10^{-2} dil.....0, 0, 0 (mice were autopsied on 4th day and passed in a 50 per cent suspension to 3 other mice, none of which developed lesions by the 6th day)

virus given intranasally. Vaccination with 40,000 M.L.U. prevents fatal infection by 10,000 M.L.D. However, minimal pulmonary lesions in the majority of the survivors indicate that the immunity is not complete. The immune response elicited by 4000 M.L.U. of non-infectious virus was detectable only in mice infected with 10 M.L.D. indicating that the minimal effective antigenic dose was being approached.

Further analysis reveals that equivalent degrees of incomplete immunity are induced by somewhat less than 40,000 M.L.U. of non-infectious virus and 400 M.L.D. of active virus. It would seem, therefore, that coincidentally with the complete destruction of infectivity by ultraviolet radiation, there is approximately a 100-fold decrease in immunizing efficiency.

A similar experiment was performed, using tissue culture virus.

Experiment 5.—Tissue cultures of the 71st and 74th transfers of the Melbourne strain of virus were used for the two intraperitoneal vaccinating doses. The virus was inactivated by irradiation for 40 minutes. Six groups of 24 mice each were used. The mice in a group were given two intraperitoneal inoculations of 1.0 cc. each, one week apart, of either a 1:10 or 1:1000 dilution of unirradiated tissue culture or the undiluted, 1:10, 1:100, or 1:1000 dilutions of the irradiated culture of virus. One control group was set aside. One week following the last intraperitoneal injection the mice in each group were divided into four sets of six, which were anesthetized and treated intranasally with 0.05 cc. of either undiluted, 1:10, 1:100, or 1:1000 dilution of fresh Melbourne tissue culture virus. Following the intranasal test inoculation the mice were observed for 11 days and deaths recorded. At the end of the period all survivors were sacrificed and the lungs examined for lesions. The results are presented in Table VI *a*.

When measured by survival and death, it would appear that the undiluted non-infectious virus induced a degree of immunity equally as good as the 1:10 dilution of fully active virus, but at autopsy, pulmonary lesions were much greater in survivors of the former series. The 1:10 dilution of the irradiated virus was as effective as the 1:1000 dilution of the untreated virus. Vaccination with the 1:100 dilution of inactive material merely delayed the day of death after infection. The 1:1000 dilution of the same suspension induced no demonstrable immunity.

If it is considered that 0.05 cc. of the 1:1000 dilution of the untreated tissue culture used in this experiment contains 1 M.L.D., then each 1.0 cc. of the dilutions of vaccinating material contains decimal multiplies of 20 M.L.D. of fully active virus or M.L.U. of non-infectious virus. Reinterpreting the results in this manner, it is seen that partial immunity to 1000 fatal doses given intranasally was obtained by vaccination with a total dosage of 40,000 M.L.U. of non-infectious virus. Vaccination with 4000 M.L.U. prevented a fatal outcome against a test dose of 100 M.L.D. Not all mice vaccinated with 400 M.L.U. of non-infectious virus survived infection when tested with as little as 1 M.L.D., but the presence of some resistance is suggested by the fact that death was in general delayed. 40 M.L.U. induced no demonstrable immunity.

In Table VI *b* are recorded the data of another experiment using virus

TABLE VI*a*

Quantitative Comparison of the Immunogenic Potency of Irradiated and Non-Irradiated Culture Virus (Melbourne)*

Intranasal test dose of virus 0.05 cc. tissue culture		Unvaccinated	Mice vaccinated with active virus		Mice vaccinated with irradiated virus			
			10^{-1}	10^{-3}	Undiluted	10^{-1}	10^{-2}	10^{-3}
Dilution	M.L.D.		4000 M.L.D	40 M.L.D.	40,000 M.L.U.	4000 M.L.U.	400 M.L.U.	40 M.L.U.
Undiluted	1000	3, 3, 3, 3, 3, 3	+, +, +, +, ±, 0	3, 3, 4, 5, 5, M	6, +++, ++, +, +, +	3, 3, 3, 4, 5, M	3, 3, 3, 4, 4, 4	3, 3, 3, 3, 3, 4
10^{-1}	100	3, 3, 4, 4, 4, 4	++, ±, 0, 0, 0, 0	5, +++, +, ±, 0, 0	+++, ++, +, +, ±, 0	+++, +, +, +, +, ±	4, 4, 5, 5, 5, 5	3, 4, 4, 4, 4, 5
10^{-2}	10	5, 5, 5, 6, +++, ++	+, +, ±, ±, 0, 0	++, ++, +, +, ±, 0	5, 5, 6, +++, +++, +	+++, +++, +, +, +, ±	4, 6, 6, 7, 7, +	4, 4, 5, 5, 5, 6
10^{-3}	1	6, 7, 8, 8, 8, 8	6, ++, 0, 0, 0, 0	+, +, ±, 0, 0, 0	+++, ++, +, +, 0, 0	±, 0, 0, 0, 0, 0	8, 9, 11, ++++, ++++, ++	6, 7, 8, 8, 8, 8

Survivors were autopsied on the 11th day after intranasal inoculation. M = mouse died in course of vaccination.

Titer of tissue culture used for vaccination:

Dilution	1st dose	2nd dose
10^{-1}	4, 4, 4	7, 8, 10
10^{-3}	4, 5, 7	8, 8, +++

Infectivity of irradiated suspension:

1st dose.....0, 0, 0, (trace of virus in 2nd passage)

2nd dose .....0, 0, 0, (no virus in 2nd passage)

* 40 minutes of irradiation.

TABLE VI*b*

Quantitative Comparison of the Immunogenic Potency of Irradiated and Non-Irradiated Culture Virus (Melbourne)*

Intranasal test dose of virus 0.05 cc. tissue culture (dilution)	Unvaccinated	Mice vaccinated with active virus	Mice vaccinated with irradiated virus			
		10^{-1}	Undiluted	10^{-1}	10^{-2}	10^{-3}
Undiluted	3, 3, 3, 3, ++	+, +, +, ±, ±, 0	4, 4, ++, ±, 0, 0	3, 3, 3, 5, +, +	3, 3, 3, +, +, ±	2, 2, 2, 2, 3, 3
10^{-1}	3, 4, 4, 4, 4, 4	9, ±, 0, 0, 0, 0	6, 0, 0, 0, 0, 0	6, 11, ++, 0, 0, 0	3, 4, 4, 4, 6, 7	3, 3, 3, 3, 3, 4
10^{-2}	4, 4, 4, 5, 5, 5	±, 0, 0, 0, 0, 0	±, ±, ±, 0, 0, 0	8, +++, ±, 0, 0, 0	4, 4, 5, 6, 7, 9	4, 4, 4, 4, 5, 6
10^{-3}	5, 6, 7, 7, 8, 9	6, 8, 0, 0, 0, 0	+, 0, 0, 0, 0, 0	+++, +, ±, ±, 0, 0	5, 7, ++, ++, +, 0	6, 7, 8, 8, 11, ++++

All survivors were autopsied on the 12th day after intranasal inoculation.

* 35 minutes of irradiation.

irradiated for 35 minutes. The results emphasize the conclusions drawn from Experiment 5.

Examination of results of immunity experiments using non-infectious virus and fully active virus, reveals that the immunity resulting from vaccination with 4000 M.L.U. of inactive virus is comparable with that induced by 40 M.L.D. of active virus. It seems, therefore, that the complete inactivation of the Melbourne strain of influenza virus by ultraviolet radiation is associated with approximately a 100-fold reduction in antigenic potency. As in the previous experiment, an erroneous impression regarding the effect of an inactivating procedure on the antigenic potency of this virus was corrected by a more careful quantitative comparison.

DISCUSSION

The foregoing experiments have demonstrated that the infectivity of the virus of epidemic influenza is destroyed by exposure to ultraviolet radiation and further, that virus so treated is still capable of inducing an immune response in mice.

With the exception of the study of Hodes, Lavin, and Webster (12) on rabies virus, reports in the literature indicate that virus inactivated by ultraviolet radiation is incapable of functioning as an effective immunizing agent. In their study of the effect of ultraviolet irradiation of rabies virus, these authors found that infectivity could be destroyed without totally destroying the antigenicity. They used exposures of 45 and 60 minutes, since after 30 minutes traces of infectious virus were still detectable.

The negative results reported by other investigators, however, do not give a fair impression of the potentialities of this method of inactivation.

For example, McKinley and Holden (4), irradiated herpes virus and injected a single dose of the inactive material subdurally in rabbits. When later tested for immunity the animals were found not to be immune. Similarly, Graham, Brandly, and Levine (3) introduced fowl pox virus irradiated with ultraviolet light into the skin of susceptible chicks to test for infectious activity. They were able to demonstrate a subsequent immunity in only those animals which developed typical lesions. The fact that immunity did not result from inactive virus can hardly be called conclusive evidence that fowl pox virus inactivated by ultraviolet radiation is non-antigenic, since the observations were made entirely on groups of animals in which takes were not observed and in which no further attempts to vaccinate were made. Gordon and Hughes (18), in efforts to immunize animals by injection of inactive yellow fever virus preparations, found that neither solid nor partial immunity was induced by single injections of large amounts of virus inactivated by ultraviolet radiation, heat, or formaldehyde. When immunity occurred it was always found to be the result of infection. A possible reason for their

failure in the use of ultraviolet radiation is suggested by an analysis of their data. The preparations employed were usually inactivated in 30 minutes. However, traces of infectious virus were sometimes detected by inoculation of monkeys with 10 to 50 cc. of irradiated virus even after exposures of as long as 150 or 240 minutes. The irregular presence of traces of infective virus after 30 minutes may possibly have been due to variation in irradiation or in the virus suspensions. Since the destruction of infectivity of yellow fever virus proceeds as a function of time and seems to be essentially complete in 30 minutes, it is possible that if any antigenicity were retained at the end of this period continued exposure of the preparation for 150 to 240 minutes before use as a vaccine may have so altered the virus as to have completely destroyed antigenicity, as well as infectivity.

In the present study, lung suspensions were irradiated for 9 minutes and tissue cultures were irradiated for 35 or 40 minutes. The time chosen in each case was just beyond the inactivation point, so that little opportunity was afforded for the continued destruction of the antigenicity, which might occur during a longer period of irradiation after infectivity had been completely eliminated.

The rates of inactivation of 5 per cent and 10 per cent infected mouse lung suspensions were compared with the rate of inactivation of a 1 per cent suspension. The periods of exposure required for inactivation of the more concentrated preparations were approximately five and ten times that necessary to inactivate the 1 per cent preparation.

In the preliminary experiments of this study, in which comparisons were made of the immunogenic potencies of irradiated and unirradiated 1 per cent suspensions of infected mouse lung, no significant difference was apparent. Nor was any great difference between active and irradiated material noted when tissue culture virus was tested in the same manner. However, upon the application of a procedure for titrating antigenicity, involving a comparison of the antigenic response elicited by dilutions of active and inactive preparations, the quantity of antigenic material in the irradiated preparations was found to have diminished nearly 100-fold. Thus, mice vaccinated with 400,000 M.L.U. of non-infectious PR8 virus in lung suspension resisted infection with 100,000 M.L.D. given intranasally, whereas a similar degree of immunity is induced by 40,000 M.L.D. of infectious virus. Mice vaccinated with 40,000 M.L.U. of inactivated virus were protected from fatal infection with 10,000 M.L.D. given intranasally, results corresponding with those obtained following vaccination with 400 M.L.D. of active virus. However, 4000 M.L.U. failed to induce any appreciable immunity against infectious doses containing more than 10 M.L.D., thus marking a critical drop in efficiency with increasing dilution.

When inactivated tissue culture virus is used a similar sequence of

events is observed, but the fall in immunizing capacity is sharpest between the 4000 and 400 M.L.U. levels. As compared with active virus the efficiency of the lower doses is approximately 1 per cent while the large doses appear nearly as effective as active virus.

These results again emphasize the importance of quantitative evaluations in determining the antigenic potency of a vaccine. The relation of the degree of immunity to the size of the immunizing dose has been stressed previously, and it was pointed out that a threshold is reached below which vaccination with active virus does not elicit immunity. In the present experiments this minimal threshold is reached with equivalents of inactive virus approximately 100 times greater than in the case of active virus. When amounts of virus above the minimal requirement are used, a distinct rise in the degree of resistance engendered is seen.

The results obtained in the present study indicate that a second threshold occurs with both active and inactive virus. When progressively greater amounts of virus are used for vaccination a point is reached at which maximal immunity is induced. Since the vaccinating units required to induce that state are well below the maximum amount of virus that could be used, the suggestion arises that, so far as is measurable by the methods employed, a certain antigenic minimum is required to induce complete immunity. Increasing the vaccinating dose beyond that level would, therefore, add no demonstrable increment of protection. This would explain why the lower dilutions of irradiated virus produce effects comparable to those obtained with low dilutions of active virus, while smaller doses of inactive virus are distinctly less effective than their equivalents in active virus. It would also explain the rapidly broadening zone of immunity obtained with the medium sized doses of lung suspension of high titer and with virus inactivated by fatty acids (26). With mouse passage virus the upper threshold capable of protecting mice against 100,000 M.L.D. is reached with vaccinating doses of approximately 4000 M.L.D. of active virus and 400,000 M.L.U. of irradiated virus. With tissue culture preparations, where lower infectious titers prevail, the number of vaccinating units required is proportionate.

With these points in mind it would be an advantage to grade vaccines prepared from epidemic influenza virus in terms of the minimal number of M.L.U. required to induce complete immunity in mice against a maximal number of intranasal M.L.D. with two intraperitoneal doses. Using this procedure, the great number of animals required for quantitation would be reduced considerably and a standard established to which reference could readily be made.

SUMMARY

A study of the antigenic potency of influenza virus inactivated by ultraviolet radiation has been made. Virus so inactivated is still capable of functioning as an immunizing agent when given to mice by the intraperitoneal route.

In high concentrations inactivated virus appears to be nearly as effective as active virus but when quantitative comparisons of the immunity induced by different dilutions are made, it is seen that a hundredfold loss in immunizing capacity occurs during inactivation.

Virus in suspensions prepared from the lungs of infected mice is inactivated more rapidly than virus in tissue culture medium.

A standard for the comparison of vaccines of epidemic influenza virus is proposed.

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