THE PREPARATION AND PROPERTIES OF INFLUENZA VIRUS VACCINES CONCENTRATED AND PURIFIED BY DIFFERENTIAL CENTRIFUGATION*

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Plates 6 and 7

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Since the discovery of swine influenza virus in 1931 (1), of human influenza virus in 1933 (2), and subsequently of numerous strains of the latter which can be grouped into at least two distinct types (3), many investigators have studied the immunization of human beings and animals with such viruses in either the active or inactivated state. For a time, extracts of diseased tissues, usually mouse or ferret lungs, were used (4–22); but following the discovery that the extra-embryonic fluids of chick embryos infected with influenza virus are unusually rich in virus (23–26), such fluids have been used in most of the immunization studies. Recently the trend has been to attempt immunization either by the administration of allantoic fluids containing active virus to the respiratory tract (27–30), or by vaccination, usually subcutaneously in the case of man and intraperitoneally in the case of animals, with allantoic fluids containing either active or inactivated virus (28, 31–39). In addition, immune sera have been used with varying degrees of success (40–44) and although the method can probably be developed satisfactorily for the temporary protection of small groups of individuals, it is unlikely that it can be used when large populations are involved; hence, it will not be considered further here. The use of active virus is fraught with dangers and presents problems that are best appreciated and coped with by pathologists, hence procedures involving the administration of active virus will also be eliminated from the present discussion.

It is possible that the great variation that has been reported in the effectiveness of vaccination with inactive virus was due largely to three interrelated factors, namely, differences in the ratio of virus to extraneous protein in the vaccine, variation in the amount of virus administered, and differences in the degree of destruction of antigenicity of virus during inactivation. No exhaustive study of these factors has been reported although there are many suggestions in the literature as to the importance of each. For example, Andrewes and Smith (45) in a study of the effect of foreign tissue extracts on the efficacy of influenza virus vaccines concluded that "the relative poorness of vaccines made from heterologous species is probably accounted for by the fact that foreign tissue extracts interfere with the immunizing response," and "that the antibody mechanism is so swamped by the large amount of foreign protein presented to it that it cannot deal so efficiently with the minute amount of virus it receives at

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the same time." There is a wide variation in the amount of virus present in tissue extracts and in infectious extra-embryonic fluids, and hence, in vaccines prepared from such materials. That the amount of virus in a vaccine is important was indicated in a study on the quantitative relationship between the immunizing dose of epidemic influenza virus and the resultant immunity by Francis (13) who concluded that "a direct proportion exists between the concentration of epidemic influenza virus used for intraperitoneal immunization of mice and the degree of immunity to intranasal infection which develops." The only comparative study in which vaccines were used that contained virus more concentrated than that occurring normally appears to be that of Hirst, Rickard, Whitman, and Horsfall (34). These workers concluded that "within certain limits the mean antibody response of human beings increased as the amount of virus injected was increased. When large amounts of influenza A virus were given, the antibody response was of the same order of magnitude as that which occurred following actual infection by this virus." The favorable results obtained with a vaccine concentrated by the red cell method may have been due to the fact that the virus content of this vaccine was somewhat greater than that which obtains in extra-embryonic fluids (39). Experiments bearing on the loss of antigenicity on inactivation range from the early results with formalinized preparations (12, 15, 46) which indicated an essentially complete loss of immunizing potency, to the results of Salk, Lavin, and Francis (47) who reported that a hundredfold loss in immunizing capacity occurs during inactivation and those of Eaton (17) which indicated a thirtyfold loss, to the results of Hirst and coworkers (34) who reported that "there was no significant difference in the antibody response of human beings given active virus, formalin-inactivated virus, heat-inactivated virus, or virus inactivated by the drying process." Although part of the variation in the results is probably due to differences in the kind of experiments conducted, it seems likely that differences in loss of antigenicity are responsible for a considerable part of the variation, since in most of the work special attention was not paid to the determination and use of minimal amounts of inactivating agents.

As a whole, the results just described, as well as those of other investigators (9, 20, 21, 38), provide ample evidence of the desirability of preparing concentrated and purified vaccines, of determining the minimal amounts of treatment necessary for inactivation, and of establishing the optimum dosage for optimum antibody response and persistence of that response. From a practical standpoint, such vaccines should be purified sufficiently so that the virus represents the major antigenic component, concentrated sufficiently so that the virus content is several times that present in infectious allantoic fluid, and inactivated in such a manner that appreciable loss of immunizing potency does not occur. The present report records the preparation and properties of influenza virus vaccines concentrated and purified by means of differential centrifugation and inactivated by different procedures.

Materials and Methods

Virus Strains.—The PR8, Lee, and Weiss strains of influenza virus were all obtained from Dr. T. Francis, Jr., of the University of Michigan. The recent passage history of the PR8

strain has been described (48). When received in this laboratory the Lee strain had been isolated from ferret B75 following 8 passages in ferrets and then passed 137 times in mice and 21 times in chick embryos. The Weiss strain had been passed 3 times in ferrets, 23 times in mice, and 4 times in chick embryos when received. Following 2 or 3 passages in chick embryos in this laboratory large pools of the three strains were prepared as described earlier (48).

Chicken Red Cells, Red Cell Agglutination Titrations, Chick Embryo Titrations, Mouse Titrations, Nitrogen Determinations, High-Speed Centrifugation, and Sedimentation Constants.—These have been described earlier (48–53). Unless otherwise noted all chicken red cell agglutination (CCA) activities are given in standardized units (50).

Ultraviolet Light Inactivation.—Solutions of virus to be inactivated were placed in open glass dishes in a hood under a Westinghouse Sterilamp in such a manner that the upper surfaces of ¼ inch layers of liquid were 4 inches from the tube of the lamp. The solutions were stirred, usually two times, during the period of inactivation.

Immunizing Potency.—The immunizing potency of the various vaccines was determined essentially according to the method recommended by Francis (13), and Salk, Lavin, and Francis (47). In most of the experiments, four groups of twenty-two to twenty-four four-week-old mice from the colony of the Department of Animal and Plant Pathology of the Institute were vaccinated intraperitoneally on two occasions 7 to 9 days apart with 0.5 cc. portions of dilutions of the vaccines in sterile 0.05 M sodium phosphate at pH 7 containing from 10^{-4} gm. to 10^{-7} gm. of virus material per cc. Seven to 9 days after the second intraperitoneal inoculation, four lots of five mice from each group were treated intranasally while under ether anesthesia with 0.05 cc. portions of four different concentrations of freshly harvested infectious allantoic fluid. The mice were observed for 10 days and all deaths from influenza were recorded, following which the survivors were sacrificed and their lungs examined for pulmonary involvement. At the time of the intranasal inoculation the test inoculum was titrated in four-week-old mice, usually in the dilution range of from $1:10^3$ to $1:10^7$, and the weighted 50 per cent end-point was calculated. Because four-week-old mice are somewhat more susceptible than mice held for an additional 2 weeks, this procedure tends to overestimate the actual number of 50 per cent mouse doses. However, it does serve to provide a standard for comparison of the potency of the test inocula used in the different experiments.

EXPERIMENTAL

Experiment 1.—The first experiment to be described, which was more or less exploratory in nature, differs from the other experiments in that in the tests for immunizing potency the intraperitoneal doses consisted of 1 cc. portions of serial one-hundredfold dilutions in 0.1 M sodium-potassium phosphate buffer instead of 0.5 cc. portions of serial tenfold dilutions in 0.05 M sodium phosphate buffer and in that serial one-hundredfold dilutions of purified active virus were used for the test inocula instead of serial tenfold dilutions of infectious allantoic fluids.

Four lots totaling 1395 ten-day chick embryos were inoculated with PR8 influenza virus and following 48 hours' incubation at 36° C. and 12 hours chilling at 4° the allantoic fluids were harvested. The four lots of allantoic fluids, which totaled 7830 cc., and possessed 260, 201, 212, and 208 chicken red cell agglutination (CCA) units per cc., respectively, were combined and subjected to two cycles of differential centrifugation. Periods of 15 minutes at 24,000 R.P.M. were used for the high-speed centrifugation. The purified material was dissolved in 202 cc. of 0.1 M phosphate buffer at pH 7 and following clarification at low speed this solution was found to contain 2 mg. of protein per cc. To a 60 cc. portion of this solution was added 0.15 cc. of a 37 per cent solution of formaldehyde, to a 30 cc. portion was added 0.225 cc. of chloroform, and to another 30 cc. portion was added 30 mg. of phenol. Another 30 cc. portion was subjected to 30 minutes' irradiation with ultraviolet light and the remaining 52 cc. portion

was left untreated. Portions of each of the five preparations were held at 4° C. and after different periods of time were tested for CCA activity and titered in chick embryos and in mice. Sedimentation constant determinations were made on these preparations after standing at 4° C. for 1 week. Other portions of each of the five preparations were sealed in glass vials and frozen in a CO_2 ice box and still other portions were frozen and dried. These preparations were also tested for CCA activity and titered in chick embryos.

TABLE I

CCA Activity of Vaccines Containing 2 Mg. of PR8 Virus Material per Cc. after Being Held for Different Periods of Time in Solution at 4° C., in a CO_2 Ice Box and in the Frozen and Dried State at Room Temperature

Storage conditions	Type of treatment	CCA activity				
		2 hrs.	3 days	2 wks.	8 wks.	15 wks.
		units per mg. of protein	*units per mg. of protein*	*units per mg. of protein*	*units per mg. of protein*	*units per mg. of protein*
4° C. in solution	0.1 per cent formaldehyde	3590	3200		3940	1920
	0.75 per cent chloroform	3060	2560		2880	1970
	0.1 per cent phenol	3240	3050		2850	3040
	Ultraviolet irradiation	2260	1580		1780	900
	Untreated	3550	3050		3500	3000
CO_2 ice box	0.1 per cent formaldehyde*		60	48	49	<1
	0.75 per cent chloroform		2310	750	853	277
	0.1 per cent phenol‡		270	30	18	35
	Ultraviolet irradiation‡		1440	1300	1415	486
	Untreated		3000	2880	3070	1210
Frozen and dried	0.1 per cent formaldehyde§			<15	<2	<1
	0.75 per cent chloroform*			790	565	80
	0.1 per cent phenol*			37	5	<1
	Ultraviolet irradiation*			133	57	<1
	Untreated*			560	413	87

* Contained large amount of insoluble material following treatment and storage.

‡ Contained small amount of insoluble material following treatment and storage.

§ Preparation was insoluble following treatment and storage.

The four treatments did not appear to affect the sedimentation constant of the purified virus material, for, in each case, the material was found to show a single boundary in the ultracentrifuge which sedimented at a rate not significantly different from that of the untreated material. The sedimentation constants corrected to water at 20° C. but not corrected for solution viscosity were found to be 676 S, 688 S, 697 S, 684 S, and 688 S for the untreated, formalinized, chloroformized, phenolized, and ultraviolet light-treated materials, respectively, at a concentration of 2 mg. per cc. The fourth of a series of photographs taken at 5 minute intervals during sedimentation at 11,100 R.P.M. of the Svensson schlieren diagrams of each of the preparations is shown in Figs. 1, 2, 3, 4, and 5, respectively.

The results of the CCA tests are presented in Table I. It can be seen that the CCA activity of the untreated and phenolized solutions held at 4° remained

essentially unchanged, whereas that of the remaining solutions decreased measurably. Freezing caused almost complete loss of CCA activity in the cases of the formalinized and phenolized preparations and some loss in the cases of the chloroform- and ultraviolet light-treated preparations. A decrease in the CCA activity of the untreated frozen preparation was not detected until the test at 15 weeks. Freezing and drying caused almost complete loss of CCA activity in the cases of formalinized, phenolized, and irradiated preparations, and over a 70 per cent reduction in CCA activity in the cases of the chloroformized and untreated samples.

The results of the virus activity determinations in chick embryos and in mice of the vaccines following storage for different periods of time and under different conditions are presented in Table II. It can be seen that the chloroformized, phenolized, and untreated solutions held at 4° C. were essentially fully active, whereas the formalinized and ultraviolet light-treated samples were essentially inactive. Freezing appeared to cause about a 90 per cent loss of activity in the case of the phenolized vaccine but did not appear to have any further effect on the remaining vaccines. Drying from the frozen state appeared to result in a 90 to 99 per cent loss of virus activity.

After standing for 3 days at 4° C. or in a CO_2 ice box, the immunizing potency in mice of the five vaccines was determined. The initial intraperitoneal inoculation of 1 cc. portions of the untreated samples at the highest concentration tested, 0.2 mg. per cc., was found to result in the death of a large number of mice; hence, it was found necessary to administer these inocula in two doses of 0.2 cc. and 0.8 cc., respectively, about 3 days apart. In this experiment in which both the vaccine and the test inocula consisted of purified PR8 virus material, as well as in all later experiments, the purified material is considered to possess approximately 10^{14} 50 per cent chick embryo doses and approximately 10^{10} 50 per cent weighted mouse doses per gram of protein. In each test the three lots of mice were vaccinated with a total of 4×10^{-4}, 4×10^{-6}, and 4×10^{-8} gm. of material, respectively, and were tested with active virus at concentrations of 10^{-3}, 10^{-5}, and 10^{-7} gm. per cc. respectively. It can be seen from the results presented in Table III that all five vaccines held in solution at 4° C. possessed about the same immunizing potency. All protected against 50 mouse doses at all three levels of vaccination but protected against 5000 mouse doses only following vaccination with the equivalent of 4,000,000 mouse doses. None of the vaccines was found to protect against 500,000 mouse doses. The results obtained with the vaccines that had been frozen in a CO_2 ice box were similar except that in most instances there was an indication that the immunizing potency had been decreased somewhat by the freezing process. The indication of a decreased potency was especially marked in the cases of the formalinized and phenolized vaccines, for vaccination with the equivalent of 400 mouse doses failed to protect the mice against intranasal inoculation with 50 mouse doses. It can be seen from Table I that these two vaccines were the only ones which showed large initial losses in CCA activity on freezing.

As a whole the results indicate that suitable inactivation of influenza virus for the preparation of vaccines can be accomplished by the use of formaldehyde or ultraviolet light, but not by the use of chloroform or phenol, and more importantly, that such inactivation can be accomplished without causing a measurable decrease in immunizing potency. The results also provide a definite indi-

cation that the freezing of formalinized or phenolized purified vaccines should be avoided and at least a suggestive indication that the freezing of other types of purified vaccines should be avoided.

TABLE II

Virus Activity of Vaccines Containing 2 Mg. of PR8 Virus Material per Cc. after Being Held for Different Periods of Time in Solution at 4° C., in a CO_2 Ice Box and in the Frozen and Dried State at Room Temperature

Storage conditions	Type of treatment	Amount of material giving a 50 per cent infectivity end-point in chick embryos			Amount of material giving a weighted 50 per cent end-point in mice		
		2 hrs.	3 days	14 days	2 hrs.	4 days	14 days
		gm.	gm.	gm.	gm.	gm.	gm.
4° C. in solution	0.1 per cent formaldehyde	$<10^{-9.8}$	$>10^{-5.3}$	—	$10^{-7.3}$	$>10^{-3.5}$	
	0.75 per cent chloroform	$<10^{-9.8}$	$<10^{-9.8}$	—	$<10^{-8.0}$	$10^{-9.0}$	
	0.1 per cent phenol	$10^{-14.0}$	$10^{-13.8}$	—	$10^{-9.6}$	$10^{-10.0}$	
	Ultraviolet irradiation	$10^{-6.0}$	$>10^{-5.3}$	—	$>10^{-4.0}$		
	Untreated	$10^{-13.8}$	$10^{-13.8}$	$10^{-13.5}$	$10^{-9.5}$		
CO_2 ice box	0.1 per cent formaldehyde		$>10^{-5.8}$				
	0.75 per cent chloroform		$<10^{-9.8}$				
	0.1 per cent phenol		$10^{-12.2}$				
	Ultraviolet irradiation		$>10^{-4.8}$				
	Untreated		$10^{-13.4}$				
Frozen and dried	0.1 per cent formaldehyde			—			—
	0.75 per cent chloroform			$10^{-10.9}$			$>10^{-6.0}$
	0.1 per cent phenol			$>10^{-6.4}$			$>10^{-6.0}$
	Ultraviolet irradiation			—			—
	Untreated			$10^{-13.2}$			$10^{-7.5}$

Experiment 2.—The results of Experiment 1 provided an indication that there might be some correlation between CCA activity and immunizing potency. It should be noted in this connection that the CCA activity can be measured with an accuracy of 10 per cent, when necessary, whereas measurements of immunizing potency are probably far less accurate. However, if a correlation between CCA activity and immunizing potency actually exists, sufficiently large differences in CCA activity should be reflected in measurable differences in immunizing

TABLE III

Immunizing Potency in Mice of Different Vaccines Containing 2 Mg. of PR8 Virus Material per Cc.

Storage conditions	Type of treatment of vaccine	Test dose* No. of 50 per cent mouse doses in inoculum	Mice vaccinated twice intraperitoneally a week apart with 1 cc. portions equivalent to total dosages listed below			Mice not vaccinated
			4×10^{-4} gm. or 4,000,000 mouse doses	4×10^{-6} gm. or 40,000 mouse doses	4×10^{-8} gm. or 400 mouse doses	
4° C. in solution	0.1 per cent formaldehyde	500,000	D_2,‡D_2,D_2,D_2,1	D_2,D_2,D_2,D_2,D_2	D_2,D_2,1, 0, 0	
		5,000	0, 0, 0, 0, 0	D_2,D_2,D_7,0, 0	D_4,1, 1, 1, 0	
		50	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 1, 1, 0, 0	
	0.75 per cent chloroform	500,000	D_2, D_2,D_2,D_2,D_2	D_2,D_2,D_2,D_2,0	D_2,D_2,D_2,D_2,D_2	
		5,000	1, 0, 0, 0, 0	1, 1, 0, 0, 0	D_2,D_2,0, 0, 0	
		50	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 0, 0, 0, 0	
	0.1 per cent phenol	500,000	D_2, D_2,D_2,D_2,D_2	D_2,D_2,D_2,D_2,0	D_2,D_2,D_2,D_2,0	
		5,000	0, 0, 0, 0, 0	D_2,D_2,0, 0, 0	D_7,1, 1, 1, 1	
		50	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	
	Ultraviolet irradiation	500,000	D_2, D_2,D_2,D_2,0	D_2,D_2,D_2,M, M	D_2,D_2,D_2,D_2,D_2	
		5,000	0, 0, 0, 0, 0	D_2,D_2,D_6,1, 0	D_2,D_2,D_2,0, 0	
		50	0, 0, 0, 0, M	0, 0, 0, 0, 0	D_7,1, 1, 0, 0	
	Untreated	500,000	D_2, D_2,D_2,0, 0	D_2,D_2,D_2,D_2,D_2	D_2,D_2,D_2,0, M	D_2,D_2,D_2,D_2,D_2
		5,000	0, 0, 0, 0, 0	D_2,D_2,D_6,0, 0	D_2,D_2,1, 1, 0	D_2,D_2,D_2,D_2,D_2
		50	0, 0, 0, 0, M	0, 0, 0, 0, 0	1, 0, 0, 0, 0	D_2,D_6,D_7,D_2,2
CO_2 ice box	0.1 per cent formaldehyde	500,000	D_2, D_2,D_2,D_2,D_2	D_2,D_2,D_2,1, M	D_2,D_2,D_2,D_2,D_2	
		5,000	D_2, D_2,0, 0, 0	D_2,D_2,D_2,0, 0	D_2,D_2,D_4,D_4,1	
		50	0, 0, 0, 0, 0	0, 0, 0, 0, 0	D_4,D_6,D_2,D_2,0	
	0.75 per cent chloroform	500,000	D_2, D_2,D_2,D_2,0	D_2,D_2,D_2,D_2,0	D_2,D_2,D_2,D_4,D_2	
		5,000	D_2, D_2,0, 0, 0	D_2,D_2,D_2,D_2,D_2	D_2,D_2,D_2,0, 0	
		50	0, 0, 0, 0, 0	0, 0, 0, 0, 0	D_2,0, 0, 0, 0	
	0.1 per cent phenol	500,000	D_2, D_2,D_2,D_2,D_2	D_2,D_2,D_2,D_2,D_2	D_2,D_2,1, 1, M	
		5,000	0, 0, 0, 0, 0	D_2,D_2,D_2,1, 0	D_2,D_2,D_2,D_6,3	
		50	0, 0, 0, 0, 0	0, 0, 0, 0, 0	D_2,D_6,D_7,3, 1	
	Ultraviolet irradiation	500,000	D_2, D_2,D_4,D_2,0	D_2,D_2,D_2,D_2,D_2	D_2,D_2,D_2,M, M	
		5,000	D_4, D_2,0, 0, 0	D_2,D_2,D_2,0, 0	D_4,D_4,D_2,D_7,1	
		50	0, 0, 0, 0, 0	0, 0, 0, 0, 0	D_{10},0, 0, 0, 0	
	Untreated	500,000	D_2, D_2,D_2,D_2,M	D_2,D_2,D_2,D_2,D_2	D_2,D_2,D_2,D_2,D_2	D_2,D_2,D_2,D_2,D_2
		5,000	D_2, D_2,0, 0, 0	D_2,1, 1, 0, 0	D_2,D_4,D_2,D_{10},0	D_2,D_2,D_2,D_2,D_2
		50	D_2, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	D_4,D_7,D_7,D_2,D_2

* This preparation of purified PR8 virus material was found to have a weighted 50 per cent end-point in 4-week-old mice at $10^{-9.9}$ gm. of protein at the time of administration of the test doses.

‡ In this and in the following similar tables the mice which died are listed as D, with a subscript denoting the day of death. The degree of pulmonary involvement in the animals surviving on the 10th day is indicated by numerals, with 4 = lung completely consolidated, 3 = ¾ consolidated, etc.

potency. It appeared worthwhile, therefore, to compare the immunizing potency of the frozen, and of the frozen and dried formalinized vaccines, described in Experiment 1 which possessed less than 1 CCA unit per mg. of protein, with that of the frozen ultraviolet light-treated vaccine of Experiment 1 which possessed 486 CCA units per mg. of protein after standing for 15 weeks. In addition, there was included, for purposes of comparison, a preparation of purified PR8 virus material which had been inactivated by standing at pH 4.8 for some time at 4° at a concentration of 2 mg. per cc. This material, which originally possessed 2800 CCA units per mg. of protein, was found to possess only 67, 34, 18, and 1 CCA units per mg. of protein after standing at pH 4.8 for 3 hours, 1, 2, and 12 days, respectively. It was titered in chick embryos at the end of the 12 day period and found to possess a 50 per cent infectivity end-point at more than 10^{-4} gm. of protein. It is interesting to note that another portion of the same virus preparation which was held under the same conditions except at pH 3.2 was found to possess 64, 44, 37, and 45 CCA units per mg. of protein, respectively, at the end of similar periods of time. These results indicate that at hydrogen ion concentrations near or more acid than the isoelectric point of the virus material there is an immediate loss of about 98 per cent of the CCA activity but that the residual activity is more stable at pH 3.2 than at pH 4.8.

The immunizing potency in mice of the four preparations just described was determined by means of two intraperitoneal doses, 8 days apart, of 0.5 cc. portions containing 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} gm. of material per cc. followed, 8 days later, by test doses of appropriate dilutions of infectious PR8 allantoic fluid. It can be seen from the results which are presented in Table IV that the frozen and dried formalinized preparation having less than 1 CCA unit per mg. of protein possessed no immunizing potency and that the frozen formalinized preparation gave only a small amount of protection to the mice. The acid-inactivated sample and the frozen ultraviolet light-treated sample, which possessed about 20 and 486 CCA units per mg. of protein, respectively, provided somewhat better protection to the mice.

As a whole, the results indicate that there is at least a rough correlation between CCA activity and immunizing potency.

Experiment 3.—It has been noted in experiments conducted with Dr. C. A. Knight that purified PR8 virus preparations at a concentration of 0.1 mg. per cc. lost chick embryo and CCA activities rapidly on irradiation with ultraviolet light and that the rate of loss of embryo infectivity was greater than that of CCA activity. It appeared desirable, therefore, to determine the rates of loss of these activities at other concentrations of virus and especially to compare the immunizing potency of an irradiated preparation having considerable CCA activity with that of an irradiated preparation having no demonstrable CCA activity. Accordingly, a portion of a preparation of purified PR8 virus at a concentration of 9 mg. per cc. was diluted with 0.1 M phosphate buffer at pH 7 to give a solution containing 1 mg. of virus material per cc., and 2 cc. portions of this solution as well as 2 cc. portions of the original solution were placed in small porcelain dishes $\frac{5}{8}$ inches in diameter at the bottom and about 1 inch in diameter at the top. These dishes were placed under a Sterilamp in such a manner that the upper surfaces of the liquid layers, which were $\frac{1}{4}$ inch in depth, were 4 inches from the tube of the lamp. The solutions were irradiated for the periods of time listed in Table V and each solution was stirred twice during each time period. The temperature of the liquid was 25° at the start of the experiment and 27.5° at the end of 4 hours' irradiation. Immediately following the designated periods of irradiation the samples were removed, placed in sterile test tubes, and tested for CCA and virus activities.

It can be seen from the results presented in Table V that in each case the rate of loss of virus activity greatly preceded that of CCA activity and, more importantly, that the greatest decrease in virus activity with the least change

TABLE IV

Immunizing Potency in Mice of Vaccines Containing 2 Mg. of PR8 Virus Material per Cc. and Possessing Different CCA Activities

Nature of vaccine	Test dose* No. of 50 per cent mouse doses in inoculum	Mice vaccinated twice intraperitoneally 8 days apart with 0.5 cc. portions equivalent to total dosages listed below			
		10^{-4} gm. or 1,000,000 mouse doses	10^{-5} gm. or 100,000 mouse doses	10^{-6} gm. or 10,000 mouse doses	10^{-7} gm. or 1,000 mouse doses
Frozen and dried formalinized preparation of Exp. 1 <1 CCA unit per mg. of protein	240,000	D_2,D_2,D_2,D_3,D_3	D_2, D_2,D_3,D_3,D_6	D_2,D_2,D_2,D_3,D_3	D_2,D_3,D_3, D_3, D_4
	24,000	D_4,D_4,D_5,D_6,D_6	D_2, $D_4,D_5,D_6,1$	D_2,D_3,D_5,D_4,D_5	D_2,D_3,D_5, D_4, D_7
	2,400	$D_4,D_4,D_6,D_9,0$	D_5, $D_5,D_7,3$, 1	D_4,D_4,D_6,D_7,D_7	D_5,D_5,D_6, $D_{10},3$
	240	$D_4,D_5,3$, 1, 0	D_4, $D_9,1$, 0, 0	$D_5,D_5,D_6,D_5,3$	$D_5,D_7,D_{10},3$, 1
Frozen formalinized preparation of Exp. 1 <1 CCA unit per mg. of protein	240,000	$D_2,D_2,D_3,D_3,3$	D_2, D_2,D_3,D_3,D_3	D_2,D_3,D_3,D_5,D_5	D_2,D_3,D_3, D_3, D_9
	24,000	$D_2,D_2,D_5,2$, 0	D_2, $D_5,D_5,D_5,1$	$D_2,D_3,D_7,2$ 1	D_2,D_3,D_4, D_4, D_4
	2,400	0, 0, 0, 0, 0	$D_{10},1$, 1, 0, 0	$D_3,D_4,3$, 2, 1	D_4,D_5,D_7 ,2, 0
	240	0, 0, 0, 0, 0	D_7, $D_9,0$, 0, 0	3, 0, 0, 0, 0	D_7,D_7,D_8, D_9, D_9
Purified PR8 virus material inactivated at pH 4.8, *ca.* 20 CCA units per mg. of protein	240,000	D_2,D_2,D_2,D_2,D_3	D_2, D_2,D_2,D_2,D_2	D_2,D_3,D_2,D_2,D_4	D_2,D_2,D_2, D_3, D_4
	24,000	0, 0, 0, 0, 0	D_3, $D_9,1$, 1, 0	$D_3,1$, 0, 0, 0	$D_3,2$, 1, 0, 0
	2,400	0, 0, 0, 0, 0	0, 0, 0, 0, 0	$D_4,2$, 0, 0, 0	$D_4,D_5,0$, 0, 0
	240	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, M	0, 0, 0, 0, 0
Frozen ultraviolet irradiated preparation of Exp. 1 486 CCA units per mg. of protein	240,000	$D_2,D_2,D_2,D_2,1$	D_2, D_2,D_2,D_2,D_5	D_2,D_2,D_2,D_2,D_2	D_2,D_2,D_2, D_2, D_2
	24,000	1, 1, 0, 0, 0	D_3, D_4,D_5,D_6,D_9	$D_2,D_3,D_4,D_5,1$	D_3,D_2,D_3, D_4, D_6
	2,400	0, 0, 0, 0, 0	1, 0, 0, 0, 0	$D_2,0$, 0, 0, 0	D_6,D_6,D_7, D_7, 0
	240	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 0, 0, 0, 0	$D_6,D_7,2$, 1, 1
Unvaccinated	240,000	D_2,D_2,D_2,D_2,D_3			
	24,000	D_3,D_3,D_4,D_4,D_6			
	2,400	$D_3,D_7,D_7,D_8,3$			
	240	$D_5,D_5,D_6,D_6,3$			

* This PR8 allantoic fluid was found to possess 220 CCA units per cc. and to have a weighted 50 per cent end-point in 4-week-old mice at a dilution of $1:10^{5.88}$ at the time of administration of the test doses.

TABLE V

CCA Activity and Chick Embryo Activity of Preparations Containing 1 and 9 Mg. of Purified PR8 Virus Material per Cc. in 0.1 M Phosphate Buffer at pH 7 Following Irradiation with Ultraviolet Light for Different Periods of Time

Time of irradiation	1 mg. purified PR8 virus material per cc.		9 mg. purified PR8 virus material per cc.	
	CCA activity	50 per cent chick embryo infectivity end-point	CCA activity	50 per cent chick embryo infectivity end-point
min.	*units per mg. of protein*	*gm. of protein*	*units per mg. of protein*	*gm. of protein*
0	2975	$10^{-12.2}$	2975	$10^{-12.2}$
15	2230	$>10^{-4}$	—	—
30	506	$>10^{-3}$	2550	$10^{-8.2}$
60	<1	$>10^{-3}$	2430	$10^{-8.2}$
120	<1	—	1830	—
240	—	—	435	$10^{-8.4}$

in CCA activity was achieved with the virus preparation at a concentration of 1 mg. per cc. Irradiation of the preparation containing 1 mg. of material per cc. for only 15 minutes was sufficient to destroy the virus activity and yet this treatment caused only a small decrease in CCA activity.

The immunizing potency in mice of the preparation containing 1 mg. of protein per cc., which following irradiation for 60 minutes possessed no demonstrable chick embryo or CCA activity, was compared with that of the preparation containing 9 mg. of protein per cc., which following irradiation for 60 minutes possessed 2430 CCA units per mg. of protein and a 50 per cent infectivity end-point in chick embryos at $10^{-8.2}$ gm. of protein. It can be seen from the results presented in Table VI that the immunizing potency of the preparation possessing 2430 CCA units per mg. of protein was measurably superior to that of the preparation possessing no CCA activity. It seems unlikely that this difference can be due to the small residual virus activity possessed by the former preparation since the amount of this activity, even at the highest dosage level administered, was only about one 50 per cent mouse dose.

TABLE VI

A Comparison of the Immunizing Potencies of Ultraviolet Light Irradiated PR8 Virus Vaccines Possessing 2430 and Less than 1 CCA Unit, Respectively, per Mg. of Protein

Nature of vaccine	Test dose* No. of 50 per cent mouse doses in inoculum	Mice vaccinated twice intraperitoneally 8 days apart with 0.5 cc. portions equivalent to total dosages listed below			
		10^{-4} gm. or 1,000,000 mouse doses	10^{-5} gm. or 100,000 mouse doses	10^{-6} gm. or 10,000 mouse doses	10^{-7} gm. or 1,000 mouse doses
Irradiated with ultraviolet light and possessing 2430 CCA units per mg. of protein	44,000	$D_2,D_2,D_2,D_2,2$	$D_2, D_2,D_2,D_2,2$	D_2,D_2,D_2,D_2,M	$D_2,D_2,D_2,D_2,2$
	4,400	0, 0, 0, 0, 0	$D_3, D_2,1, 1, 1$	$D_2,D_2,D_2,D_2,0$	1, 1, 1, 0, 0
	440	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 1, 0, 0, 0	1, 0, 0, 0, 0
	44	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, M	0, 0, 0, 0, 0
Irradiated with ultraviolet light and possessing less than 1 CCA unit per mg. of protein	44,000	$D_2,D_2,D_2,D_2,0$‡	D_2, D_2,D_2,D_2,D_2	D_2,D_2,D_2,D_2,D_2	$D_2,D_2,D_2,D_2,1$§
	4,400	$D_4,D_2,2, 1, 0$	$D_2, 1, 1, 1, 0$	$D_4,2, 2, 1, 1$	D_2,D_4,D_2,D_7,D_2
	440	0, 0, 0, 0, 0	$D_{10},1, 0, 0, 0$	1, 1, 0, 0, 0	2, 2, 1, 1, 0
	44	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	2, 1, 1, 1, 0
Unvaccinated	44,000	D_2,D_2,D_2,D_2,D_4			
	4,400	D_2,D_2,D_4,D_4,D_2			
	440	$D_2,D_2,D_7,D_7,2$			
	44	$D_7,2, 2, 2, 1$			

* This PR8 allantoic fluid was found to possess 272 CCA units per cc. and to have a weighted 50 per cent end-point in 4-week-old mice at a dilution of $1:10^{4.64}$ at the time of administration of the test doses.

‡ Three mice immunized at the same time but tested 20 days later with the same inoculum gave readings of D_2, D_4, and D_4.

§ Four mice immunized at the same time but tested 20 days later with the same inoculum gave readings of D_2, D_3, D_3, and D_4.

The results obtained with the irradiated vaccine listed in Table III, together with the results just described and those of other investigators (47), provide

evidence that fully potent non-infectious influenza vaccines can be prepared by ultraviolet light irradiation, but that too much irradiation can result in a decrease in immunizing potency. These results provide a practical approach for the preparation of irradiated influenza virus vaccines for they indicate that fully potent vaccines can be obtained by the administration of an amount of irradiation sufficient to cause loss of virus activity but insufficient to cause a marked decrease in CCA activity and that this can be accomplished satisfactorily at a concentration level of about 1 mg. of virus material per cc.

Experiment 4.—In view of the results obtained on exposure of PR8 influenza virus to different amounts of ultraviolet irradiation it appeared necessary to determine the minimum concentration of formaldehyde that is required to cause suitable inactivation of virus and also the concentration that will cause measurable changes in CCA activity. Accordingly, aliquots of solutions of purified PR8 virus material in 0.1 M phosphate buffer at pH 7 containing 1 and 10 mg. of protein, respectively, per cc. were treated with different concentrations of formaldehyde and the CCA and chick embryo activities were determined following standing at 4° for different periods of time. It can be seen from the results presented in the bottom half of Table VII that even 0.01 per cent formaldehyde in a solution containing 1 mg. of virus material per cc. was sufficient to cause a reduction in chick embryo titer from 10^{-14} gm. to $10^{-4.2}$ gm. in 4 days. This concentration of formaldehyde, as well as a concentration five times greater, did not appear to affect the CCA activity after 4 days, but there was indication that a concentration of 0.1 per cent formaldehyde causes a decrease in CCA activity of virus material at a concentration of 1 mg. per cc. This result is similar to that obtained in Experiment 1 in which 0.1 per cent formaldehyde in a solution containing 2 mg. of virus material per cc. resulted in a decrease in CCA activity at the end of 15 weeks.

TABLE VII

CCA and Chick Embryo Activities of Purified PR8 Virus Material at 1 and 10 Mg. per Cc. Containing Different Amounts of Formaldehyde after Standing at 4° C. in 0.1 M Phosphate Buffer for Periods of Time Listed Below

Concentration of purified PR8 virus material	Concentration of formaldehyde	CCA activity					Amount of material giving a 50 per cent infectivity end-point in chick embryos				
		1 day	2 days	4 days	1 mo.	6 mos.	1 day	2 days	4 days	1 mo.	6 mos.
mg. protein per cc.	*per cent*	*units per mg. of protein*	*units per mg. of protein*	*units per mg. of protein*	*units per mg. of protein*	*units per mg. of protein*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
10	0.00	3200	2570		2510	3340		10^{-14}		$10^{-13.9}$	$10^{-11.8}$
	0.05	3070	2990	3310	2720	2000	$>10^{-10.2}$	$>10^{-8.2}$	$10^{-4.2}$	$10^{-4.2}$	
	0.10	3020	3280	3200	3340	2560	$>10^{-9.2}$	$>10^{-7.2}$	$10^{-4.2}$	$10^{-4.2}$	
	0.20	3170	3130	3240	3460	1560	$>10^{-8.2}$	$>10^{-6.2}$	$10^{-4.2}$	$10^{-4.2}$	
	1.00	2690	2470	1790	1620	280	$>10^{-7.2}$	$>10^{-5.2}$	$10^{-4.2}$	$>10^{-3.2}$	
1	0.00	2910		3200		2780		10^{-14}	$10^{-14.2}$		$10^{-11.6}$
	0.01	3470	3220	3130			$10^{-11.2}$	$10^{-8.7}$	$10^{-4.2}$		
	0.05	3080	2890	3070			$>10^{-9.2}$	$10^{-7.2}$	$10^{-4.2}$		
	0.10	2800	2630	2410			$>10^{-8.2}$	$10^{-6.2}$	$10^{-4.2}$		

The results given in the upper half of Table VII indicate that during a period of 1 month 0.05, 0.1, and 0.2 per cent formaldehyde in the presence of 10 mg. of virus material per cc. causes suitable inactivation of virus without altering the CCA activity. However at the end of 6 months the formalinized preparations showed significant decreases in CCA activity and this was especially marked in the case of 1.0 per cent formaldehyde. As a whole, the results with formaldehyde demonstrate that suitable inactivation of virus without marked

TABLE VIII

Immunizing Potency in Mice of a Vaccine Containing 2 Mg. of Purified PR8 Virus Material per Cc. and 0.1 Per Cent Formaldehyde after Standing for 2 Months at 4°, 18–25°, and 37° C., Respectively

Storage conditions	Test dose* No. of 50 per cent mouse doses in inoculum	Mice vaccinated twice intraperitoneally 8 days apart with 0.5 cc. portions equivalent to total dosages listed below			
		10^{-4} gm. or 1,000,000 mouse doses	10^{-5} gm. or 100,000 mouse doses	10^{-6} gm. or 10,000 mouse doses	10^{-7} gm. or 1,000 mouse doses
4° C.	80,000	$D_2,D_2,D_4,2,\ 1$	D_2,D_2,D_2,D_2,D_4	D_2,D_2,D_2,D_4,D_8	D_2,D_2,D_2,D_2,D_4
	8,000	0, 0, 0, 0, 0	$D_2,0,\ 0,\ 0,\ 0$	1, 1, 1, 1, 0	$D_2,D_2,1,\ 0,\ 0$
	800	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 0, 0, 0, 0	0, 0, 0, 0, 0
	80	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 0, 0, 0, 0	0, 0, 0, 0, 0
18 to 25° C.	80,000	D_2,D_2,D_2,D_2,D_4	$D_2,D_2,D_2,1,\ M$	$D_2,D_2,D_2,D_4,0$	$D_2,D_2,D_2,D_4,2$
	8,000	0, 0, 0, 0, 0	$D_2,D_2,1,\ 1,\ 1$	$D_2,D_4,1,\ 1,\ 1$	$D_4,D_4,D_6,2,\ 1$
	800	0, 0, 0, 0, 0	0, 0, 0, 0, 0	$D_4,1,\ 0,\ 0,\ 0$	$D_7,D_7,3,\ 1,\ 0$
	80	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	$D_6,0,\ 0,\ 0,\ 0$
37° C.	80,000	$D_2,1,\ 1,\ 1,\ M$	D_2,D_2,D_2,D_2,D_2	$D_2,D_2,D_2,D_2,2$	D_2,D_2,D_2,D_2,D_2
	8,000	$D_2,0,\ 0,\ 0,\ 0$	$D_2,0,\ 0,\ 0,\ 0$	$D_2,D_2,1,\ 0,\ 0$	$D_2,D_2,D_2,1,\ 0$
	800	0, 0, 0, 0, M	1, 0, 0, 0, 0	$D_2,1,\ 0,\ 0,\ 0$	$D_4,D_6,D_7,2,\ 1$
	80	0, 0, 0, 0, M	0, 0, 0, 0, 0	0, 0, 0, 0, 0	$D_6,D_2,1,\ 0,\ 0$
Unvaccinated	80,000	D_1,D_2,D_2,D_2,D_6			
	8,000	D_2,D_4,D_4,D_6,D_7			
	800	$D_2,D_2,D_7,3,\ 2$			
	80	D_7,D_7,D_7,D_7,D_8			
	8	$D_6,D_7,D_7,3,\ 2$			

* This PR8 allantoic fluid was found to possess 123 CCA units per cc. and to have a weighted 50 per cent end-point in 4-week-old mice at a dilution of $1:10^{4.9}$ at the time of administration of the test doses.

effect on CCA activity can be achieved over a fairly wide range of formaldehyde concentration.

Experiment 5.—The stability of the immunizing potency of a formalinized PR8 virus vaccine following storage at different temperatures was studied by holding aliquots of the solution of formalinized vaccine, described in Experiment 1, at 4°, 18–25°, and at 37°, respectively, for 2 months and determining the comparative immunizing potencies of the three preparations. In addition, the CCA activities of the three preparations were determined at the end of 2, 4, and 6 months of storage. It can be seen from the results presented in Table VIII that storage at 18–25° and at 37° resulted in a measurable deterioration of immunizing potency. Vaccination of mice with 10^{-7} gm. of the material held at 4° protected against 80 and 800 weighted 50

per cent mouse doses, whereas from 10^{-5} to 10^{-6} gm. of the materials held at the higher temperatures were required for similar protection of mice. A comparison of the results recorded in Table VIII for the solution held at 4° with those listed in Table III for the same material at the start of the experiment indicates that no measurable loss in immunizing potency occurred on storage for 2 months at 4°.

The results of the CCA tests following storage for different periods of time at different temperatures are presented in Table IX. It can be seen that at 4° a measurable decrease in CCA activity had taken place by the end of 4 and 6 months. At 18° to 25° and at 37° the decrease in CCA activity was noticeable at 2 months and was quite marked at 4 and 6 months. It is possible that the relatively high concentration of formaldehyde of 0.1 per cent with 2 mg. of virus material per cc., which was used in this vaccine preparation, may have been responsible for part of the decrease in CCA activity. As can be seen from the

TABLE IX

CCA Activity of a Vaccine Containing 2 Mg. of Purified PR8 Virus Material per Cc. and 0.1 Per Cent Formaldehyde after Standing for Different Periods of Time at 4°, 18–25°, and 37° C., Respectively

Storage conditions	CCA activity			
	At start	2 mos.	4 mos.	6 mos.
	units per mg. of protein	*units per mg. of protein*	*units per mg. of protein*	*units per mg. of protein*
4° C.	3500	3860	1920	1500
18–25° C.	3500	2310	570	7
37° C.	3500	1260	350	81

results of the preceding experiment, this concentration of formaldehyde is considerably higher than is necessary to secure suitable inactivation of virus. It is possible that a formaldehyde concentration of considerably less than 0.1 per cent will be found to be much more suitable for vaccines containing influenza virus material at concentration levels near 1 mg. per cc.

Experiment 6.—In order to secure a relationship between the present purified and concentrated vaccines and vaccines that have been used earlier for the immunization of man and animals a comparison was made of the immunizing potency in mice of three different preparations. The first consisted of the solution containing 2 mg. of purified PR8 virus material per cc. and 0.1 per cent formaldehyde described in Experiment 1; the second was a sample of freshly harvested untreated infectious PR8 allantoic fluid; and the third was a preparation of three strains of influenza virus purified and concentrated by the red cell elution method. The latter material, which was kindly supplied by Dr. T. Francis, Jr., represents a portion of the vaccine that was used extensively in tests in man (39). Although each cubic centimeter of this vaccine represents the amounts of Lee, PR8, and Weiss virus materials obtained from 5, 2.5, and 2.5 cc. of the respective infectious allantoic fluids, the actual amounts of the three virus materials present in the vaccine are unknown because of uncertainties regarding the recovery of virus by the red cell elution method. In order to gain some idea of the approxi-

mate amount of virus material present, 15 cc. of the vaccine were subjected to purification and concentration by differential centrifugation. The original vaccine was found to possess 470 CCA units per cc. and to contain 0.11 mg. of protein nitrogen per cc. of which 0.01 mg. was removed on centrifugation at low speed. The clarified solution containing 0.10 mg. of

TABLE X

Comparison of the Immunizing Potency of Formalinized Purified PR8 Virus Material, Untreated PR8 Allantoic Fluid, and a Vaccine Prepared by the Red Cell Elution Method

Nature of vaccine	Test dose* No. of 50 per cent mouse doses in inoculum	Mice vaccinated twice intraperitoneally 8 days apart with 0.5 cc. portions equivalent to total dosages listed below			
		10^{-4} gm. or 1,000,000 mouse doses	10^{-5} gm. or 100,000 mouse doses	10^{-6} gm. or 10,000 mouse doses	10^{-7} gm. or 1,000 mouse doses
Purified PR8 virus material at 2 mg. per cc. and 0.1 per cent formaldehyde	160,000	D_2,D_2,D_2,D_2,D_2	D_2,D_2,D_2,D_2,D_2	D_2,D_2,D_2,D_2,D_2	D_2,D_2,D_2,D_2,D_2
	16,000	$D_2,0,\ 0,\ 0,\ 0$	$D_2,D_2,D_2,D_6,1$	$D_2,D_2,D_4,1,\ 0$	$D_2,D_2,3,\ 2,\ 1$
	1,600	$0,\ 0,\ 0,\ 0,\ 0$	$1,\ 0,\ 0,\ 0,\ 0$	$1,\ 1,\ 1,\ 0,\ 0$	$1,\ 1,\ 0,\ 0,\ 0$
	160	$0,\ 0,\ 0,\ 0,\ 0$	$0,\ 0,\ 0,\ 0,\ 0$	$1,\ 1,\ 1,\ 0,\ 0$	$1,\ 0,\ 0,\ 0,\ 0$
		Undiluted or 5,000,000 mouse doses	1:10 dilution or 500,000 mouse doses	1:100 dilution or 50,000 mouse doses	1:1000 dilution or 5,000 mouse doses
Untreated PR8 allantoic fluid‡	160,000	D_2,D_2,D_2,D_2,D_4	D_2,D_2,D_2,D_2,D_2	D_2,D_2,D_2,D_2,D_2	$D_2,D_2,D_2,D_2,1$
	16,000	$D_2,0,\ 0,\ 0,\ 0$	$D_2,D_2,D_2,0,\ 0$	$D_2,D_2,D_6,D_7,0$	$D_2,D_2,1,\ 0,\ 0$
	1,600	$0,\ 0,\ 0,\ 0,\ 0$	$D_2,0,\ 0,\ 0,\ 0$	$0,\ 0,\ 0,\ 0,\ M$	$0,\ 0,\ 0,\ 0,\ 0$
	160	$0,\ 0,\ 0,\ 0,\ 0$	$2,\ 1,\ 0,\ 0,\ 0$	$0,\ 0,\ 0,\ 0,\ 0$	$0,\ 0,\ 0,\ 0,\ 0$
		Undiluted or 1,000,000 mouse doses	1:10 dilution or 100,000 mouse doses	1:100 dilution or 10,000 mouse doses	1:1000 dilution or 1,000 mouse doses
Vaccine prepared by red cell elution method§	160,000	D_2,D_2,D_2,D_2,D_2	D_2,D_2,D_2,D_2,D_2	$D_2,D_2,D_2,D_2,1$	D_2,D_2,D_2,D_2,D_2
	16,000	D_2,D_2,D_7,D_7,D_7	D_2,D_2,D_4,D_6,D_6	$D_2,D_2,D_2,D_4,2$	$D_2,D_2,D_4,D_6,1$
	1,600	$D_6,0,\ 0,\ 0,\ 0$	$0,\ 0,\ 0,\ 0,\ 0$	$0,\ 0,\ 0,\ 0,\ 0$	$D_6,D_6,1,\ 1,\ 0$
	160	$0,\ 0,\ 0,\ 0,\ 0$	$0,\ 0,\ 0,\ 0,\ 0$	$0,\ 0,\ 0,\ 0,\ 0$	$D_6,1,\ 1,\ 1,\ 0$
Unvaccinated	160,000	D_2,D_2,D_2,D_2,D_6			
	16,000	D_2,D_2,D_7,D_2,D_4			
	1,600	D_6,D_6,D_6,D_6,D_7			
	160	$D_6,D_6,D_7,D_7,3$			

* This PR8 allantoic fluid was found to possess 249 CCA units per cc. and to have a weighted 50 per cent end-point in 4-week-old mice at a dilution of $1:10^{5.2}$ at the time of administration of the test doses.

‡ This PR8 allantoic fluid was found to possess 222 CCA units per cc. and to have a weighted 50 per cent end-point in 4-week-old mice at a dilution of $1:10^{5.4}$ at the time of administration of the first vaccination.

§ Kindly supplied by Dr. T. Francis, Jr., and used about 8 months after preparation.

protein nitrogen per cc. was centrifuged at 24,000 R.P.M. for 15 minutes and the supernatant liquid was found to contain 8 CCA units and 0.08 mg. of protein nitrogen per cc. Of the 0.02 mg. of protein nitrogen per cc., which was sedimented, 0.014 mg. was found to be soluble. This fraction possessed 2700 CCA units per mg. of protein and, at a concentration of 2.1 mg. per cc., gave a rather diffuse boundary in the analytical centrifuge with a sedimentation constant of about 700 S. The fourth of a series of photographs taken at 5 minute intervals during

sedimentation at 11,100 R.P.M. of the Svensson schlieren diagrams of this material is shown as Fig. 6. The actual isolation by differential centrifugation of 0.14 mg. of protein per cc. of vaccine can be regarded as an indication that the vaccine contains approximately 0.2 mg. of virus material per cc. For purposes of the present experiment it was assumed that one-half of this material, or 0.1 mg. per cc., is Type A virus material and, hence, that 1 cc. of the vaccine represents the equivalent of 10^6 weighted mouse doses of Type A virus.

It can be seen from the results presented in Table X that there is little difference in immunizing potency of 0.1 mg. of formalinized purified PR8 virus material, 1 cc. of undiluted infectious PR8 allantoic fluid, and 1 cc. of the vaccine prepared by the red cell elution method and the corresponding dilutions of these preparations.

There is some indication that the protection afforded by the red cell vaccine against PR8 virus was somewhat less secure than that provided by the other two materials, since all five mice vaccinated with 1 cc. of the undiluted red cell vaccine died following intranasal instillation of 16,000 weighted mouse doses, whereas only one of five mice died in each of the two comparable groups vaccinated with 1 cc. of undiluted allantoic fluid or with 0.1 mg. of formalinized purified PR8 virus. Similarly, of the mice vaccinated with 1 cc. of a 1:1000 dilution of the red cell vaccine, two died following the administration of 1600 weighted mouse doses whereas none of the mice died in comparable groups vaccinated with the other two materials. However, it is difficult to be certain that such differences are significant due to uncertainties in the actual amounts of virus material present in the red cell vaccine and in the allantoic fluid. The results do indicate that the immunizing potency of the red cell vaccine against active PR8 virus is not greater than that of untreated PR8 allantoic fluid of good potency or purified PR8 virus material at a concentration of 0.1 mg. per cc. This result is in accordance with expectation since in each instance the amount of immunizing antigen administered was essentially the same.

The results demonstrate that the 95 per cent of extraneous protein present in infectious allantoic fluid and the 80 per cent of extraneous protein present in the red cell vaccine are not necessary for the effective immunization of mice.

Experiment 7.—In order to determine, by means of clinical tests, the optimum amount of influenza virus necessary to produce optimum protection in man, it appeared that it would be necessary to prepare a much more concentrated polyvalent vaccine than any heretofore prepared. The vaccines described earlier in this report contained 2 mg. of PR8 virus material per cc. or an amount of immunizing antigen approximately ten times that contained in the vaccine, prepared by red cell elution, which was used in extensive tests in human beings and demonstrated to afford considerable protection against the natural disease (39). In order to increase the probability of securing a concentration greater than that actually required for the optimum protection in man, a polyvalent vaccine containing 10 mg. of virus material per cc. was prepared and subjected to laboratory tests. Lee, PR8, and Weiss virus materials produced in chick embryos and concentrated and purified by means of two cycles of differential centrifugation were used in this vaccine. To 14 cc. of 0.05 M sodium phosphate solution at pH 7 containing 140 mg. of purified Lee virus material were added 7 cc. of a similar solution containing 70 mg.of purified PR8 virus material and 7 cc. of a similar solution containing 70 mg. of purified Weiss virus material. The Lee virus material possessed 1270 CCA units per mg. of protein, a 50 per cent infectivity end-point in chick embryos at $10^{-12.6}$ gm., and a sedimentation constant corrected for solution viscosity of 832 S. The PR8 virus mater-

ial possessed 2800 CCA units per mg. of protein, a 50 per cent infectivity end-point in chick embryos at $10^{-13.2}$ gm., and a sedimentation constant corrected for solution viscosity of 719 S. The Weiss virus material possessed 1280 CCA units per mg. of protein, a 50 per cent infectivity end-point in chick embryos at $10^{-13.2}$ gm., and a sedimentation constant corrected for solution viscosity of about 700 S. The third of a series of photographs taken at 5 minute intervals during sedimentation at 11,100 R.P.M. of the Svensson schlieren diagrams of each of the strains at a concentration of 2.5 mg. per cc. is shown as Figs. 7 to 9. It can be seen that the PR8 and Lee boundaries are quite sharp whereas the Weiss boundary is diffuse. A similar photograph for the formalinized mixture of strains at a concentration of 10 mg. per cc. is shown as Fig. 10. The mixture shows the presence of two components with sedimentation constants corrected for solution viscosity of 790 S and 518 S.

To 26 cc. of the mixture of the three virus materials were added 0.13 cc. of 3.7 per cent formaldehyde and 0.13 cc. of a solution containing 2 mg. of phenyl mercuric nitrate per cc. The finished vaccine thus contained 5, 2.5, and 2.5 mg. of Lee, PR8, and Weiss virus materials per cc., respectively, or a total of 10 mg. of virus materials per cc. in 0.05 M sodium phosphate at pH 7 in the presence of 1:2000 formalin and 1:100,000 phenyl mercuric nitrate. After standing for 3 days at 4° the vaccine was found to be non-infectious for chick embryos when tested at 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} gm. per cc., and in tests conducted after an additional 10 days the vaccine was found to cause no lesions in mice when instilled intranasally at concentrations of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} gm. per cc. However, when administered undiluted to five mice, equivalent to a concentration of 10^{-2} gm. per cc., two of the mice were found to have 3+ lesions at autopsy and the remainder were negative. Fourteen days after the preparation of the vaccine, four groups of 66 mice were vaccinated by means of two intraperitoneal doses a week apart with a total of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} gm. of material, respectively, per mouse. In addition, one group of sixteen mice was immunized with the vaccine at a concentration level of 10^{-8} gm. per cc. Nine days after the second vaccination dose the mice were divided into three groups and tested by means of intranasal instillation of serial tenfold dilutions of infectious allantoic fluids containing Lee, PR8, and Weiss viruses, respectively. The Lee allantoic fluid possessed 62 CCA units per cc., a 50 per cent embryo infectivity end-point at $10^{-7.3}$ cc., and a weighted 50 per cent mouse end-point at $10^{-5.0}$ cc. The PR8 allantoic fluid possessed 331 CCA units per cc., a 50 per cent embryo infectivity end-point at $10^{-9.1}$ cc., and a weighted 50 per cent mouse end-point at $10^{-5.9}$ cc. The Weiss allantoic fluid possessed 92 CCA units per cc., a 50 per cent embryo infectivity end-point at $10^{-9.0}$ cc., and a weighted 50 per cent mouse end-point at $10^{-6.0}$ cc.

It can be seen from the results presented in Table XI that vaccination with the polyvalent vaccine over the range of 10^{-4} to 10^{-7} gm. provided good protection to the mice against several thousand mouse doses of the respective viruses. In general, the results are similar to those reported in earlier experiments with PR8 virus except that the mice appeared to be somewhat more solidly protected against the Weiss virus.

The administration of 50,000 mouse doses of Weiss virus caused no deaths among mice immunized with 10^{-4}, 10^{-5}, or 10^{-6} gm. of virus material and two of the five mice vaccinated with 10^{-7} gm. of material survived. It should be noted that 10^{-7} gm. of material is about the minimum amount that gives a fair amount of protection to mice for in each case vaccination with 10^{-8} gm. of material gave only a little protection against 50 to 500 mouse doses. A comparison of the results obtained with the present polyvalent vaccine against PR8 influenza virus with those obtained with the polyvalent vaccine prepared by the red cell elution method, presented in Table X, indicates that at equivalent dosages there is no great difference in im-

TABLE XI

Immunizing Potency in Mice of a Vaccine Containing Purified Lee, PR8, and Weiss Virus Materials at Concentrations of 5.0, 2.5, and 2.5 Mg., Respectively, per Cc. in 0.05 M Sodium Phosphate at pH 7 and Containing 1:2000 Formalin and 1:100,000 Phenyl Mercuric Nitrate

Vaccinated mice tested with the following allantoic fluids	Test dose No. of 50 per cent mouse doses in inoculum	Mice vaccinated twice intraperitoneally a week apart with 0.5 cc. portions equivalent to total doses listed below					Mice not vaccinated
		10^{-4} gm.	10^{-5} gm.	10^{-6} gm.	10^{-7} gm.	10^{-8} gm.	
Lee*	5,000	$D_3, D_7, 2, 0, 0$	$D_3, D_8, D_8, D_4, 1$	$D_3, 2, 1, 1, 1$	$D_3, D_6, D_6, 3, 2$		D_2, D_2, D_2, D_2, D_4
	500	0, 0, 0, 0, 0	2, 0, 0, 0, 0	$D_7, 1, 0, 0, M$	$D_6, 1, 1, 0, 0$		D_4, D_4, D_6, D_6, D_6
	50	0, 0, 0, 0, 0	0, 0, 0, 0, 0	2, 0, 0, 0, 0	$D_5, 3, 0, 0, 0$	$D_8, D_9, 2, 1, 0$	$D_6, D_7, D_7, D_8, 1$
	5	0, 0, 0, 0, 0	0, 0, 0, 0, 0	2, 1, 0, 0, 0	1, 1, 1, 0, 0		$D_6, 2, 1, 1, 0$
PR8‡	40,000	$D_2, D_3, D_3, D_3, 0$	$D_2, D_2, D_2, 1, 0$	$D_4, D_4, D_5, D_7, 2$	$D_2, D_3, D_3, D_3, 0$		D_2, D_2, D_2, D_2, D_3
	4,000	0, 0, 0, 0, 0	$D_3, 0, 0, 0, 0$	$D_6, 1, 0, 0, 0$	$D_3, 1, 0, 0, 0$		D_3, D_4, D_6, D_6, D_6
	400	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 1, 0, 0, 0	$D_3, D_7, 3, 1, 0$	$D_6, D_7, D_7, D_9, D_{10}$
	40	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 1, 0, 0, 0		$D_7, D_7, D_7, 2, 1$
Weiss§	50,000	1, 0, 0, 0, 0	1, 1, 1, 0, 0	1, 1, 0, 0, 0	$D_2, D_3, D_4, 2, 0$		D_3, D_3, D_3, D_3, D_3
	5,000	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0		D_3, D_4, D_6, D_6, D_9
	500	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 0, 0, 0, M	1, 1, 0, 0, 0	$D_7, D_7, D_9, 0, M$	D_6, D_6, D_7, D_7, D_7
	50	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 0, 0, 0, 0	0, 0, 0, 0, 0		$D_8, D_9, 3, 2, 1$

* This Lee allantoic fluid was found to possess 62 CCA units per cc. and to have a weighted 50 per cent end-point in 4-week-old mice at a dilution of $1:10^{3.7}$ at the time of administration of the test doses.

‡ This PR8 allantoic fluid was found to possess 331 CCA units per cc. and to have a weighted 50 per cent end-point in 4-week-old mice at a dilution of $1:10^{4.6}$ at the time of administration of the test doses.

§ This Weiss allantoic fluid was found to possess 92 CCA units per cc. and to have a weighted 50 per cent end-point in 4-week-old mice at a dilution of $1:10^{4.7}$ at the time of administration of the test doses.

munizing potency. The fact that the vaccine prepared by differential centrifugation protected fourteen out of fifteen mice against intranasal dosages of up to about 4,000 50 per cent weighted mouse doses following vaccination with only 10^{-7} gm. of material, whereas the red cell vaccine at a dilution of 1:1000, corresponding to 2×10^{-7} gm. of virus material, protected only eight out of fifteen mice against intranasal dosages of up to 16,000 mouse doses could be due to the fact that the red cell vaccine was used after several months storage.

As a whole, the results can be regarded as providing an indication that, at equivalent dosages of virus material, the vaccines prepared by the red cell elution method and by differential centrifugation are approximately equivalent with respect to immunizing potency in mice. However, since the vaccine prepared by the red cell elution method contains only about 0.2 mg. of virus material per cc., whereas the vaccine prepared by differential centrifugation contains 10 mg. of virus material per cc., the latter is about 50 times more concentrated than the former. In immunizing potency, therefore, 1 cc. of the vaccine prepared by differential centrifugation would be equivalent to about 50 cc. of the vaccine prepared by the red cell elution method. The greater concentration of virus materials afforded by the centrifuge product may make it possible to secure better protection in man, especially if used in conjunction with adjuvants (54), than would be possible with less concentrated vaccines. Additional advantages may be provided by the fact that in the case of the centrifuge product the virus materials make up essentially all of the protein content of the vaccine, whereas in the case of the red cell product the virus materials make up only about 20 per cent of the protein content of the vaccine. The remaining 80 per cent of the protein content of the vaccine prepared by the red cell elution method consists of proteins of low molecular weight which, as indicated by the experiments reported above, possess little or no capacity to induce immunity against influenza in mice.

A vaccine prepared by differential centrifugation and similar to the one described in this experiment has been prepared commercially under the auspices of the Committee on Medical Research.

In tests in this laboratory the PR8, Lee, and Weiss components of this vaccine were found to possess 2900, 2000, and 1050 CCA units, respectively, per mg. of protein. The completed vaccine was found to possess 2050 CCA units per mg. of protein. The third of a series of photographs taken at 5 minute intervals during sedimentation at 11,100 R.P.M. of the Svensson schlieren diagrams of the PR8, Lee, and Weiss components and of the completed vaccine at concentrations of 4.6, 3.5, 4.2, and 4.0 mg. per cc. is shown in Figs. 11 to 14, respectively. In each instance there appears to be a single major component although in the cases of the Weiss component and the completed vaccine there is evidence for the presence of some material of lower molecular weight. The sedimentation constants not corrected for solution viscosity were calculated to be 620 S, 755 S, about 698 S, and 699 S for the PR8, Lee, and Weiss components and for the completed vaccine, respectively.

The immunizing potency in mice of the commercially prepared vaccine was determined in this laboratory and the results are presented in Table XII. It

can be seen that the immunizing potency is essentially the same as that of the vaccine prepared in this laboratory. The commercially prepared vaccine is now being subjected to clinical tests in man at dosage levels ranging from 0.01 mg. to 10 mg. The latter corresponds to a level approximately 100 times that

TABLE XII

Immunizing Potency in Mice of a Commercially Prepared Vaccine Containing Purified Lee, PR8, and Weiss Virus Materials at Concentrations of Approximately 4.0, 2.2, and 2.2 Mg., Respectively, per Cc. in 0.05 M Sodium Phosphate at pH 7 and Containing 1:2000 Formalin and 1:100,000 Phenyl Mercuric Nitrate

Vaccinated mice tested with the following allantoic fluids	Test dose No. of 50 per cent mouse doses in inoculum	Mice vaccinated twice intraperitoneally a week apart with 0.5 cc. portions equivalent to total doses listed below				Mice not vaccinated
		10^{-4} gm.	10^{-5} gm.	10^{-6} gm.	10^{-7} gm.	
Lee*	32,000	$D_6,D_7,3,\ 1,\ 1$	$D_4,D_6,3,\ 1,\ 0$	$D_3,D_3,D_3,D_3,2$	$D_3,D_4,D_4,1,\ 0$	$D_2,D_2,D_3,D_4,0$
	3,200	1, 0, 0, 0, 0	0, 0, 0, 0, 0	2, 1, 1, 0, 0	$D_7,D_3,2,\ 1,\ 0$	D_3,D_4,D_8,D_9,D_{10}
	320	1, 0, 0, 0, M	0, 0, 0, 0, 0	1, 1, 0, 0, 0	2, 1, 0, 0, 0	$D_4,D_5,D_6,D_7,3$
	32	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 0, 0, 0, 0	1, 0, 0, 0, 0	$D_5,D_7,D_8,D_9,4$
PR8‡	100,000	D_2,D_2,D_2,D_2,D_2	$D_2,D_2,D_2,1,\ 1$	D_2,D_2,D_2,D_2,D_2	$D_2,D_2,D_2,D_2,2$	$D_2,D_2,D_3,D_3,1$
	10,000	$D_2,1,\ 0,\ 0,\ 0$	2, 0, 0, 0, 0	$D_2,D_2,1,\ 0,\ 0$	$D_4,D_5,1,\ 1,\ 0$	$D_2,D_2,D_4,D_7,2$
	1,000	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 0, 0, 0, 0	3, 1, 1, 1, .	$D_5,D_5,D_6,\ 3,\ 0$
	100	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 0, 0, 0, 0	1, 1, 1, 0, 0	$D_8,D_9,D_{10},4,\ 2$
Weiss§	6,300	1, 0, 0, 0, 0	1, 0, 0, 0, 0	$D_2,1,\ 0,\ 0,\ 0$	$D_2,D_2,D_2,1,\ 0$	$D_2,D_2,D_4,\ D_6,D_7$
	630	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	$D_8,0,\ 0,\ 0,\ 0$	$D_7,D_8,D_8,\ 3,\ 1$
	63	0, 0, 0, 0, 0	0, 0, 0, 0, 0	3, 0, 0, 0, 0	3, 1, 0, 0, 0	$D_7,D_7,D_8,\ 4,\ 2$
	6	0, 0, 0, 0, 0	0, 0, 0, 0, 0	0, 0, 0, 0, 0	1, 0, 0, 0, 0	$D_7,3,\ 2,\ 2,\ 2$

* This Lee allantoic fluid was found to possess 98 CCA units per cc. and to have a weighted 50 per cent end-point in 4-week-old mice at a dilution of $1:10^{4.5}$ at the time of administration of the test doses.

‡ This PR8 allantoic fluid was found to possess 262 CCA units per cc. and to have a weighted 50 per cent end-point in 4-week-old mice at a dilution of $1:10^{5.0}$ at the time of administration of the test doses.

§ This Weiss allantoic fluid was found to possess 85 CCA units per cc. and to have a weighted 50 per cent end-point in 4-week-old mice at a dilution of $1:10^{3.8}$ at the time of administration of the test doses.

of infectious allantoic fluid. The results will be described in later communications.

The Production of a Concentrated and Purified Influenza Virus Vaccine on a Large Scale.—A great many problems intervene when a vaccine is manufactured commercially on a large scale that do not intervene when the vaccine is prepared on a small scale for experimental purposes. The maintenance, during the manufacture of the vaccine, of conditions as nearly bacteriologically sterile as possible and the cost of the vaccine are two such problems. It is relatively

easy to harvest the allantoic fluids of a few hundred embryos and to centrifuge a few hundred cubic centimeters of the fluid under absolutely sterile conditions, whereas when the operations are carried out on a large scale involving thousands or hundreds of thousands of embryos, bacterial contamination frequently occurs. Since the purification process involving differential centrifugation is especially suited for the removal of organized bacteria and their soluble components of low molecular weight from the virus material, the contamination of the allantoic fluid usually causes no serious consequences provided the bacterial contamination is light and the fluid is worked up without delay. However, it is not always possible to obtain such conditions in large scale production; hence, it appeared desirable to determine if it would be possible to secure and maintain bactericidal or at least bacteriostatic conditions in the fluid containing the virus immediately after harvesting and throughout the purification and concentration process. Although inactivation of bacteria and virus by ultraviolet light irradiation immediately after harvesting, followed by the carrying out of all open operations under an ultraviolet lamp, was found suitable, such a processing on a large scale would require the installation of considerable irradiation equipment; hence, the present studies were directed towards the use of chemical inactivating agents.

In a preliminary experiment with 3600 cc. of PR8 allantoic fluid possessing 235 CCA units per cc., one portion was made up to contain 1:1000 formaldehyde and the remainder left untreated. After standing overnight at 4° C. the two portions were processed by differential centrifugation. The formalinized preparation yielded 0.04 mg. of purified material per cc. of allantoic fluid and this possessed 1210 CCA units per mg. of protein, whereas the untreated portion yielded 0.07 mg. of purified material per cc. of allantoic fluid and this possessed 2460 CCA units per mg. of protein. Although the formalinized preparation was sterile and the other lightly contaminated the lower yield obtained in the case of the former, both with respect to protein and CCA activity, indicated that too much inactivating agent had been used. In another experiment in which 1:2000 formalin was used with PR8 allantoic fluid containing 252 CCA units per cc. the yields in the case of the treated and untreated were 0.10 and 0.11 mg., respectively, per cc. of allantoic fluid. However, the purified material from the untreated sample possessed 2420 CCA units per mg. of protein, whereas that from the formalinized fluid possessed 1710 CCA units per mg. of protein. Hirst (55) has noted that formaldehyde has a slow inactivating effect on CCA activity.

An experiment was conducted with freshly harvested PR8 allantoic fluid possessing 245 CCA units per cc. in which four aliquots of 300 cc. each were used to determine the effect of formalin at a 1:10,000 dilution, of phenyl mercuric nitrate at a dilution of 1:100,000, and of phemerol at a 1:100,000 dilution. After standing for 22 hours at 4° the untreated sample was found to possess 245 CCA units per cc. and to be lightly contaminated with bacteria as indicated by the presence of 35 colonies on an agar slant after 48 hours' incubation, whereas the preparations containing formalin, phenyl mercuric nitrate, and phemerol possessed 250, 276, and 250 CCA units per cc., respectively, and gave no bacterial colonies on agar slants on 48 hours' incubation. The four preparations were subjected to two cycles of differential centrifugation and in each case the yield of purified material was 0.10 mg. per cc. of allantoic fluid. The untreated purified preparation was found to possess 3120 CCA units per mg. of

protein and a 50 per cent infectivity end-point in chick embryos at $10^{-14.4}$ gm. The formalin–, phenyl mercuric nitrate–, and phemerol treated–purified samples possessed 3350, 3550, and 3020 CCA units per mg. of protein and 50 per cent chick embryo infectivity end-points at $10^{-11.5}$, $< 10^{-13.5}$, and $< 10^{-13.5}$ gm., respectively.

The results indicate that concentrations of 1:10,000 of formalin or of 1:100,000 of either phenyl mercuric nitrate or phemerol are sufficient to achieve and maintain at least bacteriostatic and very probably bactericidal conditions in freshly harvested allantoic fluids lightly contaminated with bacteria without affecting the yield or the CCA activity of the virus. However in subsequent experiments, in which fairly heavily contaminated allantoic fluids were purposely employed, it was found that occasionally the bacteria continued to increase in number.

The increase occurred most frequently when phenyl mercuric nitrate was used and least frequently when formaldehyde was used. Since it appeared undesirable to increase the concentration of the bacteriostatic agents, tests were made to determine the effect of combining formalin plus phenyl mercuric nitrate at concentrations of 1:10,000 and 1:100,000, respectively, in the allantoic fluid followed by the suspension of the sedimented pellets of virus in a solution of 0.05 M sodium phosphate containing a part per 2,000 of formalin and a part per 100,000 of phenyl mercuric nitrate. In view of the rather high cost in time and materials of a vaccine containing 10 mg. of virus materials per 1 cc. dose and the fact that a measurable degree of protection in man was obtained with a vaccine containing about 0.2 mg. of virus material per 1 cc. dose (39), it appeared that a vaccine containing an intermediate amount of from 1 to 2 mg. of virus material per cc. might suffice for vaccination on a large scale. Virus preparations at this level of concentration are obtainable readily following a single cycle of differential centrifugation; hence, such preparations were used in the present tests.

In a typical experiment 2400 cc. of PR8 allantoic fluid were harvested by pouring from the opened eggs through a funnel containing a glass wool mat. The fluid was found to contain 181 CCA units per cc. and 0.1 cc. applied to a nutrient agar slant gave a confluent growth of bacteria after 24 hours' incubation. Immediately following the harvesting one portion of 1400 cc. was clarified by centrifugation at 3000 R.P.M. for 10 minutes and the liquid, which then contained 160 CCA units per cc., was sedimented at 24,000 R.P.M. The pellets were suspended in 57 cc. of 0.05 M sodium phosphate buffer at pH 7 and this solution was clarified by 10 minutes' centrifugation at 3000 R.P.M. The clarified solution was found to contain 5360 CCA units and 2 mg. of protein per cc., hence the yield was 0.08 mg. of virus material per cc. of allantoic fluid. A 0.1 cc. portion of this solution was found to yield a confluent growth of bacteria on an agar slant following 24 hours' incubation. There was added to the 1000 cc. portion of allantoic fluid immediately following harvesting, 0.1 cc. of a 37 per cent solution of formaldehyde and 10 cc. of a 0.1 per cent solution of phenyl mercuric nitrate. The mixture was held at 4° C. for 24 hours. At the end of 4 and 24 hours 0.1 cc. portions of the mixture gave 80 and 30 bacterial colonies, respectively, on agar slants following 24 hours' incubation. The solution was then subjected to a single cycle of differential centrifugation after the manner just described for the untreated allantoic fluid, except that the pellets of sedimented virus material were dissolved in 37 cc. of 0.05 M sodium phosphate buffer containing a part per 2000 of formalin and a part per 100,000 of phenyl mercuric nitrate. The final preparation was found to contain 75 mg. of virus material possessing 2220 CCA units per mg. of protein; hence the yield was 0.075 mg. per cc. of allantoic fluid. Portions of 0.1 cc. and 1 cc. of the purified solution on agar slants and in 10 cc. portions of nutrient broth, respectively, failed to show the presence of bacteria following 24 and 48 hours' incubation. Similar results were obtained

with other samples of PR8 and Lee allantoic fluids. Sedimentation diagrams of influenza virus preparations purified and concentrated by a single cycle of differential centrifugation, two of which are shown in Figs. 15 and 16, failed to show the presence of material of low molecular weight, hence it can be concluded that materials of low molecular weight probably make up less than 5 per cent of such virus preparations. An idea of the degree of purification that can be achieved by differential centrifugation can be obtained from Figs. 17 to 20 which show electron micrographs of preparations of normal allantoic fluid, PR8 allantoic fluid, PR8 virus purified by a single cycle of differential centrifugation, and PR8 virus purified by two cycles of differential centrifugation, respectively. The characteristic particles having a diameter of about 115 mμ are, of course, not present in the normal allantoic fluid but show quite clearly in the micrograph of the infectious allantoic fluid. The materials of low molecular weight, which make up much of the materials shown in the micrograph of infectious allantoic fluid, are largely absent in the micrograph of the virus preparation purified and concentrated by a single cycle of differential centrifugation.

The results described indicate that infectious allantoic fluid, which usually has a protein content consisting of less than 10 per cent of virus protein and over 90 per cent of protein of low molecular weight, can be converted by a single cycle of differential centrifugation to a product in which the virus material comprises the major antigenic component and in which there appears to be less than 5 per cent of proteins of low molecular weight. The results also indicate that the bacterial contamination, which invariably accompanies work on a large scale, can be controlled by the addition of a part per 10,000 of formalin plus a part per 100,000 of phenyl mercuric nitrate to the allantoic fluid immediately after harvesting followed by the solution of the final product in 0.05 or 0.1 M sodium phosphate at pH 7 containing a part per 2000 of formalin plus a part per 100,000 of phenyl mercuric nitrate without affecting appreciably the yield or CCA activity of the virus. Influenza virus vaccines have been prepared by the procedure just described by means of the vacuum type air turbine centrifuge as well as by means of a commercially available Sharples Laboratory Super-Centrifuge and are being subjected to tests for immunizing potency and for stability. Although these vaccines are somewhat less pure and less concentrated than the vaccines described earlier in this paper, which were prepared by two cycles of differential centrifugation, their compositions and properties are so similar that no marked differences in immunizing potency and in stability can be anticipated. It is probable, therefore, that through the use of the Sharples centrifuge and the procedure just described a suitable influenza virus vaccine can be prepared on a very large scale with considerable ease and efficiency.[1]

[1] Since the submission of the manuscript for publication a vaccine has been manufactured commercially, by the procedure described, under the auspices of the Committee on Medical Research. The vaccine was prepared by means of a single cycle of differential centrifugation in the Sharples centrifuge and contains 1.0, 0.5, and 0.5 mg. of Lee, PR8, and Weiss virus materials per cc. respectively, in 0.1 M sodium phosphate buffer at pH 7 containing 1:2000 formalin and 1:100,000 phenyl mercuric nitrate. The average

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SUMMARY

Influenza virus vaccines containing from 1 to 10 mg. of virus materials per cc. concentrated and purified from infectious allantoic fluids by means of one or two cycles of differential centrifugation and inactivated by different treatments have been prepared and subjected to laboratory tests. Suitable inactivation of the virus preparations with retention of full red cell agglutinating activity and immunizing potency in mice was achieved by treatment with minimal amounts of formaldehyde or ultraviolet light. Treatment with phenol or chloroform failed to cause adequate loss of virus activity. Excessive amounts of formaldehyde or of ultraviolet light were found to cause a loss in red cell agglutinating activity and in immunizing potency. Freezing resulted in the immediate loss of red cell agglutinating activity of the formalinized vaccine. Storage of the vaccines in the frozen state was accompanied by a gradual decrease in red cell agglutinating activity. Drying of the vaccines from the frozen state resulted in a loss of red cell agglutinating activity and, in the case of the formalinized vaccine, in a loss in immunizing potency. There appeared to be at least a rough correlation between red cell agglutinating activity and immunizing potency. The immunizing potency and red cell agglutinating activity of a purified formalinized vaccine containing 2 mg. of virus material per cc. were unchanged following 2 months' storage at 4° but were measurably decreased following storage for 2 months at 18 to 25° and at 37°. At equivalent dosages of virus material the immunizing potency of formalinized centrifugally purified virus, of formalinized virus purified by the red cell elution method, and of infectious allantoic fluid was not measurably different. The immunizing potency of a formalinized polyvalent vaccine containing centrifugally purified Lee, PR8, and Weiss influenza virus materials at concentrations of 5, 2.5, and 2.5 mg. per cc., respectively, was found to be essentially the same as that of a similar vaccine prepared commercially. In both cases the protection afforded against the Weiss strain appeared to be better than that against the Lee and PR8 strains. The commercially prepared vaccine is being subjected to clinical tests in man at dosage levels ranging from 0.01 mg. to 10 mg. The latter corresponds to a level approximately 100 times that of infectious allantoic fluid.

It was found that the bacterial contamination that frequently accompanies operation on a large scale can be controlled by the addition of one part per 10,000 of formalin plus one part per 100,000 of phenyl mercuric nitrate to the allantoic fluid immediately following harvesting, without affecting the quality

over-all yield of virus materials was 0.065 mg. per cc. of allantoic fluid, hence one embryo yielded about 0.5 mg. of virus material. The vaccine is being subjected to laboratory and clinical tests.

of the vaccine. This procedure and the use of virus materials purified and concentrated by a single cycle of differential centrifugation by means of the Sharples centrifuge were found to be suitable for the production of influenza virus vaccines on a large scale. By means of this method influenza vaccines possessing 20 or more times the immunizing potency of infectious allantoic fluid and 10 or more times the immunizing potency of the usual commercial vaccine prepared by the red cell elution method can be manufactured rapidly on a very large scale with considerable ease and efficiency.

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EXPLANATION OF PLATES

PLATE 6

The figures show one, usually the fourth, of a series of photographs taken at 5 minute intervals during sedimentation at 11,100 R.P.M. of the Svensson schlieren diagrams of preparations of influenza virus purified by differential centrifugation and subjected to different treatments. The sedimentation constant determinations were kindly made by Dr. M. A Lauffer and Mr. H. K. Schachman.

FIG. 1. Untreated PR8 virus material at a concentration of 2 mg. per cc. in 0.1 M phosphate buffer at pH 7. From Experiment 1.

FIG. 2. Formalinized PR8 virus material at a concentration of 2 mg. per cc. in 0.1 M phosphate buffer at pH 7. From Experiment 1.

FIG. 3. Chloroformized PR8 virus material at a concentration of 2 mg. per cc. in 0.1 M phosphate buffer at pH 7. From Experiment 1.

FIG. 4. Phenolized PR8 virus material at a concentration of 2 mg. per cc. in 0.1 M phosphate buffer at pH 7. From Experiment 1.

FIG. 5. Ultraviolet light-treated PR8 virus material at a concentration of 2 mg. per cc. in 0.1 M phosphate buffer at pH 7. From Experiment 1.

FIG. 6. Virus material from vaccine prepared by red cell elution method following further purification and concentration by one cycle of differential centrifugation. 2.1 mg. per cc. in 0.1 M phosphate buffer at pH 7. From Experiment 6.

FIG. 7. Untreated purified Lee virus material used for vaccine described in Experiment 7 at a concentration of 2.5 mg. per cc. in 0.05 M sodium phosphate buffer at pH 7.

FIG. 8. Untreated purified PR8 virus material used for vaccine described in Experiment 7 at a concentration of 2.5 mg. per cc. in 0.05 M sodium phosphate buffer at pH 7.

FIG. 9. Untreated purified Weiss virus material used for vaccine described in Experiment 7 at a concentration of 2.5 mg. per cc. in 0.05 M sodium phosphate buffer at pH 7.

FIG. 10. Formalinized mixture containing Lee, PR8, and Weiss virus materials at concentrations of 5, 2.5, and 2.5 mg. per cc., respectively, in 0.05 M sodium phosphate buffer at pH 7. From Experiment 7.

FIG. 11. Untreated commercially prepared PR8 virus material at a concentration of 4.6 mg. per cc. in 0.05 M sodium phosphate buffer at pH 7.

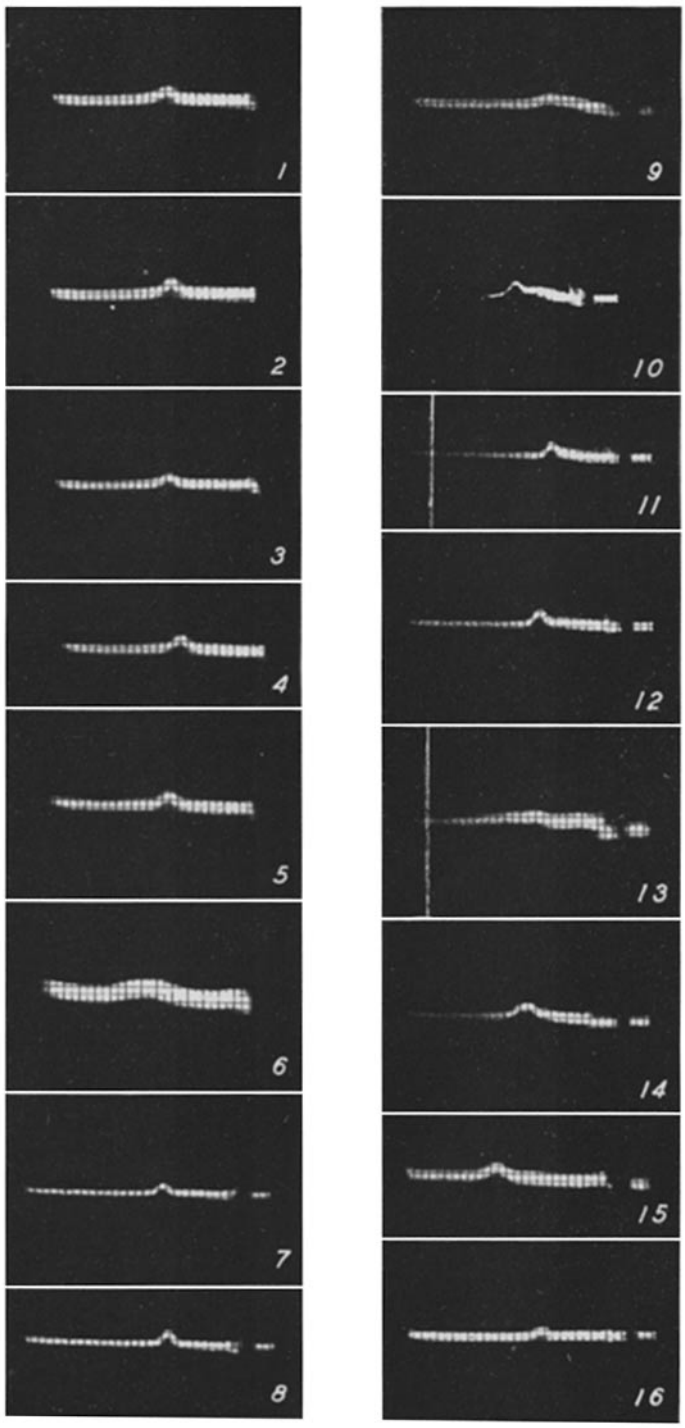
FIG. 12. Untreated commercially prepared Lee virus material at a concentration of 3.5 mg. per cc. in 0.05 M sodium phosphate buffer at pH 7.

FIG. 13. Untreated commercially prepared Weiss virus material at a concentration of 4.2 mg. per cc. in 0.05 M sodium phosphate buffer at pH 7.

FIG. 14. Commercially prepared formalinized polyvalent influenza vaccine at a concentration of 4 mg. per cc. in 0.05 M sodium phosphate buffer at pH 7. The Lee, PR8, and Weiss components are present in the ratio of 2, 1, and 1, respectively.

FIG. 15. Untreated PR8 influenza virus purified and concentrated by means of one cycle of differential centrifugation. The concentration is 5 mg. per cc. and the solvent is 0.1 M phosphate buffer at pH 7. The picture is the third of the series and the sedimentation constant is 657 S.

FIG. 16. A preparation of Lee influenza virus purified and concentrated from allantoic fluid containing 1:10,000 formalin plus 1:100,000 phenyl mercuric nitrate by means of one cycle of differential centrifugation. The concentration is 2.6 mg. per cc. and the solvent is 0.05 M sodium phosphate buffer at pH 7 containing 1:2000 formalin and 1:100,000 phenyl mercuric nitrate. The picture is the third of the series and the sedimentation constant is 752 S.



(Stanley: Concentrated purified influenza virus vaccines)

PLATE 7

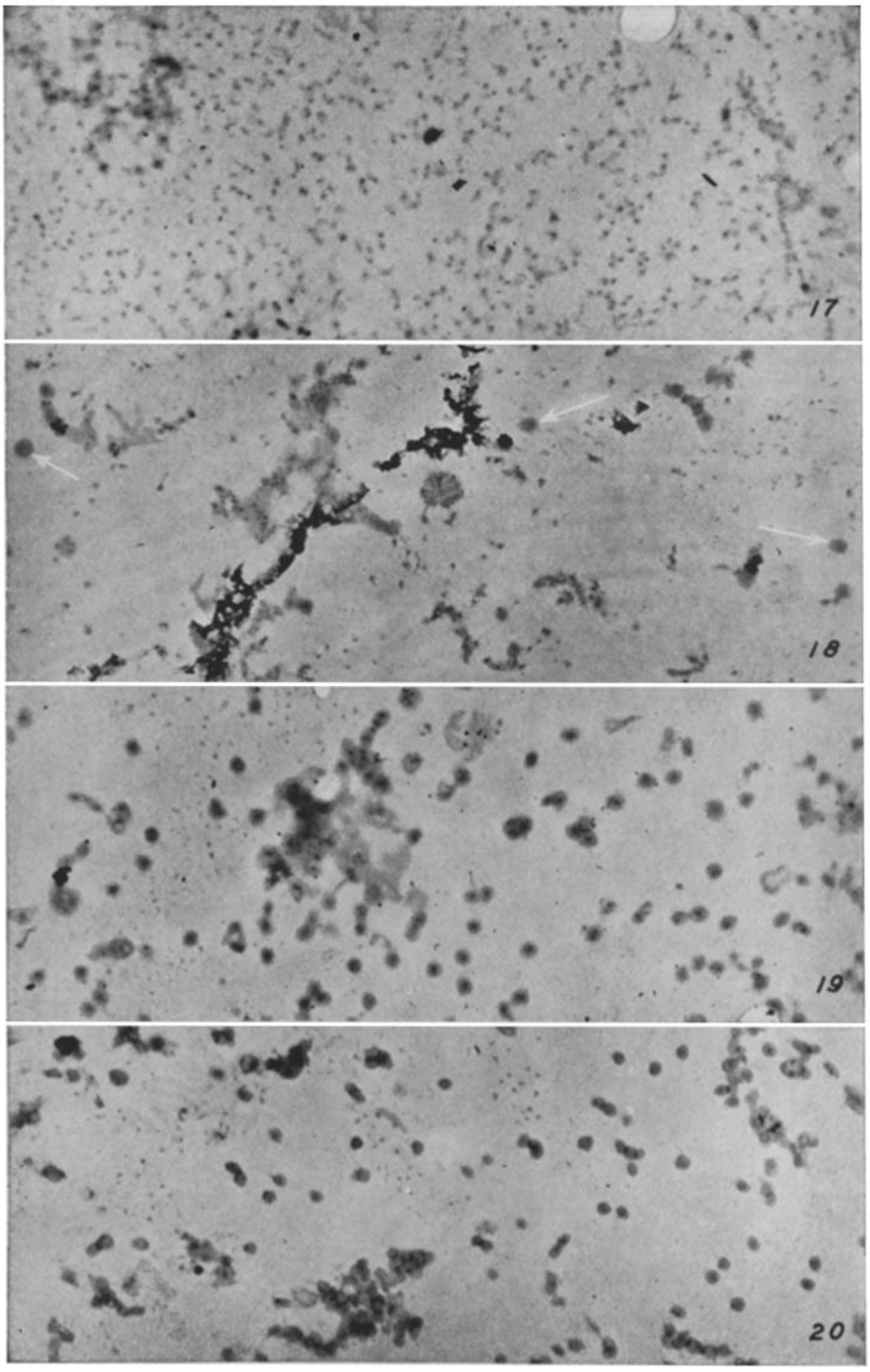
The figures show electron micrographs of different preparations taken at 55 kv. with an RCA type B instrument at a magnification of 7100 and enlarged to 18,400. The virus particles are about 115 mμ in diameter. The preparations were applied to the collodion mounts, allowed to dry, then washed with a small volume of distilled water, and allowed to dry. The micrographs were made in the RCA Laboratories in Princeton with the generous cooperation of Dr. James Hillier.

FIG. 17. Undiluted allantoic fluid from normal embryos.

FIG. 18. Undiluted allantoic fluid from embryos infected with PR8 influenza virus. Arrows indicate some of the virus particles.

FIG. 19. Virus particles obtained from the infectious allantoic fluid shown in Fig. 18 by means of a single cycle of differential centrifugation. The preparation was diluted with 0.1 M phosphate buffer at pH 7 to give a concentration of 10^{-4} gm. per cc. and mounted immediately.

FIG. 20. A preparation of PR8 influenza virus concentrated and purified by means of two cycles of differential centrifugation. The preparation was diluted with distilled water to give a concentration of 10^{-4} gm. per cc. and mounted immediately.



(Stanley: Concentrated purified influenza virus vaccines)