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

VIII. An Experimental Analysis of Various Deembryonation Technics

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Previous analyses of the growth cycle of influenza virus have been based largely upon data derived from allantoic infection of the intact chick embryo (1). By collecting and analyzing allantoic fluids and membranes from groups of embryos at regular intervals following inoculation growth curves were obtained of virus in conjunction with host cells (membrane suspensions) and of free extracellular virus (allantoic fluids). In the course of these studies it became apparent that the growth curve technic in the intact chick embryo offers certain handicaps which are particularly noticeable in efforts to obtain accurate evaluation of the time sequences in the development of various viral properties.

The difficulties encountered in the allantoic fluid series of growth curves may be listed as follows: (*a*) The measurements at any given time interval of a growth curve are derived from different embryos or groups of embryos. Thus, individual variations in susceptibility of the embryos, in the volumes of allantoic fluid they harbor, in the quantity of inhibitors of hemagglutination in the allantoic fluids, and possibly in other factors may influence to some extent the results. (*b*) Not all of the seed virus appears to be adsorbed onto the entodermal cells and a considerable proportion (30 per cent in the average) remains free in the allantoic fluids of the inoculated eggs (2, 3). This amount of seed virus represents a threshold level which has to be at least equalled by progeny liberated from the infected cells before such release can be detected. Thus it is not possible under these conditions to determine the beginning of liberation. (*c*) By the same token, the end of the liberation period in one-step growth curves cannot be determined accurately. After considerable amounts of newly formed virus have been liberated, new threshold levels are established in the allantoic fluid which prevent ready detection of additional liberation of virus. (*d*) Some of the virus

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in the allantoic fluid will lose its infectivity in the course of the incubation period at 37°C. Similar problems arise with respect to the allantoic membranes. Some of the seed virus is apparently adsorbed superficially and presumably does not enter into the reproductive process (3). This amount of virus (1 to 5 per cent of the seed) represents again a threshold level beyond which multiplication must proceed before it can be detected and the individual points of the growth curves are derived, as in the allantoic fluid series, from separate groups of embryos.

Efforts have been made to overcome some of these handicaps with respect to the extracellular part of growth curves. Washing of the allantoic cavity has been employed by Cairns and Edney (4), but this technic would seem to be cumbersome. Using tissue cultures of minced chorio-allantoic membranes (5–7) or of large pieces of it (8) in a balanced salt solution would offer the prospect of removing the medium at various intervals and replacement with fresh salt solution. However, in that case at least 2 different types of cells are available for infection, *i.e.* the ectodermal (chorionic) and the entodermal (allantoic) side of the tissue and possibly also some mesodermal cells. That this complicates the system has been shown recently (9) in that the infectious process in the chorionic side of the allantoic membrane apparently is different from that in the allantoic surface. The use of the deembryonation technic, on the other hand, has been useful in solving some of the problems mentioned. This technic, described by Bernkopf (10, 11) had been in use also in this laboratory by the time it was first reported. All structures are removed from the egg except the part of the chorio-allantoic membrane which adheres to the shell membrane. A standard volume of a balanced salt solution is added to bathe the tissue and the eggs are then slowly rotated by a mechanical device. In this system, only the entodermal layer of the tissue presumably becomes infected; most of the non-adsorbed seed virus can readily be removed by washing; serial determinations can be obtained on individual preparations; and the medium can readily be exchanged at stated intervals for separate analyses. With respect to the growth curves obtained with allantoic membrane suspensions the deembryonation technic offers no particular advantages. It has been shown that the superficially adsorbed seed virus can be removed to a large extent by the receptor-destroying enzyme of *Vibrio cholerae* (RDE) in the intact chick embryo (12, 13). The same technic is equally if not more readily applicable to DE preparations. Obviously no serial determinations on individual membranes are possible.

The deembryonation technic (DE) has been employed under a number of different conditions. It is the purpose of this paper mainly to present technical details of it and to compare results obtained in deembryonated eggs with those derived from intact chick embryos under comparable conditions. Applications of the technics to the study of various phases of the infectious cycle have been summarized recently (14) and will be presented in papers to follow.

Methods and Materials

Virus.—The PR8 strain of influenza A virus was used solely in these experiments. For preparation of seeds, small amounts of infected allantoic fluid (10^3 to 10^5 ID_{50}) were injected allantoically into 6 or more 10 to 12 day old chick embryos. After incubation of the eggs at

36 to 37°C. for 18 to 48 hours (the shorter incubation periods were employed more recently), the allantoic fluids were harvested and pooled. As a rule they contained 10^9 or more ID_{50}/ml.; 10^8 or more hemagglutinating (HA) units per ml.; and showed ID_{50}/HA ratios in excess of 10^6. The seeds were used after storage at 4°C. for not more than 48 hours, or in the dry-ice chest in sealed ampules for longer periods.

Growth Curves in Ovo.—The technics employed for inoculation and harvests have been fully described (2, 3, 15, 16).

Deembryonation (DE) Technic.—For this technic as a rule 14 to 15 day old chick embryos were used. With younger embryos (12 day old or less) the chorio-allantoic membrane frequently became detached from the underlying shell membrane, either during deembryonation or during subsequent incubation. In earlier experiments the membranes were infected after deembryonation by adding the desired amount of seed virus to the medium. Later it was found to be more convenient and satisfactory to infect eggs with seed virus in the usual way by the allantoic route and then to perform deembryonation at any desired time thereafter.

For deembryonation the *pointed* end of the egg was cleansed with 70 per cent ethyl alcohol and a circular cut was made through the shell by means of a diamond doughnut drill of 1 inch diameter, rotating at 2000 R.P.M. This technic was developed by Himmelweit (17). By means of a sleeve around the cutting edge of the drill, the depth of the cut was controlled and thus injury to the shell membrane and the underlying chorio-allantois could readily be avoided. While the egg was held horizontally the shell membrane and the chorio-allantois were cut with sterile scissors along the edge of the severed cone of the shell, and the albumen, yolk sac, amnion with the embryo, and the "visceral" part of the allantois were gently decanted while the link to the "parietal" allantois was cut with scissors. The latter remained firmly attached to the shell membrane and thus lined the whole inside of the egg, including the area under the air sac. The shell was then inverted on a sterile cardboard egg tray so that most of the remaining allantoic fluid drained from the surface of the allantoic membrane. Deembryonation from the tip of the egg has two advantages, (*a*) the albumen, rich in inhibitor of hemagglutination, is easily and almost completely removed in contrast to the experience with deembryonation from the air sac end; and (*b*) there are no cut edges of tissue within the shell where the medium would gradually diffuse between the ectodermal side of the allantois and the shell membrane which readily would lead to detachment of the tissue during rotation.

After draining, the shells were filled completely with M/100 phosphate buffered saline solution of pH 7.0, and warmed to 37°C. The fluid was decanted and the shell drained again and this procedure was repeated once more before the final medium was added. The effectiveness of the washing procedure was attested by the fact that in deembryonation of infected eggs the final medium contained, as a rule, 0.1 per cent or less of the infectious virus which was originally present in the allantoic fluid.

In earlier experiments, the actual medium added to the deembryonated eggs appeared to be of no great concern. Phosphate buffered saline or Tyrode solutions, 10 per cent chicken serum in saline, normal allantoic fluid, or even physiological salt solution all gave similar results when large inocula and relatively short incubation periods were used. However, when longer incubation periods are required glucose appears to be necessary to maintain the viability of the tissue and increases the yield of virus after inoculation of smaller amounts of seed virus, as has been shown also recently by Daniels *et al.* (18). A mixture of modified glucosol and phosphate buffer (pH 6.8) was adopted as the medium of choice. The glucosol solution was prepared according to Fulton and Armitage (8). It contained per liter 8.0 gm. NaCl; 0.2 gm. $CaCl_2$; 0.5 gm. $MgCl_2 \cdot 6H_2O$; 1.0 gm. glucose; and distilled water to 1 liter. The phosphate buffer consisted of 4.73 gm. Na_2HPO_4 and 4.54 gm. KH_2PO_4 per liter. Equal volumes of the 2 solutions were mixed shortly before use and penicillin and streptomycin were added

to concentrations of 500 units and 100 μg. respectively, per ml. 10 ml. of this medium, warmed to 37°C., was added to the washed and drained deembryonated eggs. The shells were closed by means of sterile rubber caps. The eggs were placed in the appropriate holes of the circular disk of the rotating machine and fixed in position by a lid having individual springs, or more recently a foam rubber cushion pressing down on the rubber caps. The preparations were then put in the warm room at 37°C. and rotated at a rate of 20 revolutions per hour with the long axis of the eggs inclined at a 20° angle from the horizontal plane, so that at each revolution of the machine the whole inner surface of the membranes was bathed with the medium. The speed of rotation was varied from 8 to 150 rotations per hour without significant changes in results.

Harvests from the deembryonated eggs were made according to one of several procedures which for convenience have been given special names. In all experiments presented here the fluids (and on occasion membranes) from at least 3, and more often 4 to 6 parallel preparations were pooled. In others, to be presented later (19), individual deembryonated eggs were studied.

(*a*) *"Cumulative" Harvests.*—At the desired times after deembryonation (or infection) small samples of the medium were removed while in the warm room and pooled according to groups. Usually not more than 0.3 ml. were taken at any one time. Further collections were made from the same eggs after continued rotation at 37°C. for various periods of time. The total amount of fluid removed during an experiment did not exceed 20 per cent, and usually was less. All the remaining medium was decanted when the experiment was terminated.

(*b*) *"Differential" Harvests.*—At suitable intervals after deembryonation, usually every 1 or 2 hours, the media were decanted and pooled according to groups. The inside of the eggs was washed twice with warm buffered saline solution and then 10 ml. of warm modified glucosol medium was added to each preparation. Thereafter they were replaced on the rotating machine and returned to the incubator for further incubation. For certain experiments washing of the deembryonated eggs at each exchange of medium was found not to be essential (19) and thus the medium was just decanted, the shell drained, and fresh medium was added. All these manipulations were carried out in the warm room in the more recent experiments.

(*c*) *Combined in Ovo-DE Technic.*—Groups of eggs were deembryonated at increasingly longer intervals following infection of the allantois of the intact egg, using as a rule a 1 to 2 hour spacing. The deembryonated eggs were then incubated on the rotating machine for 1 hour (occasionally for 2 or 3 hours) when the media (and the membranes in some instances) were harvested. Thus the liberation of virus during the 1st hour (or the first 2 or 3 hours) following deembryonation at successive stages of the infectious cycle could be analyzed.

The *allantoic membranes* of the deembryonated eggs were usually collected at the end of the experiments. If data on the membranes were desired at given periods during the experimental periods larger groups of deembryonated eggs were prepared and certain numbers of these were removed at the stated intervals for harvesting of the tissue. Before removal of the membranes the eggs were drained on sterile cardboard trays. The tissue was then washed twice exactly as at the time of deembryonation (see above). Following removal from the shells the membranes were pooled according to groups and washed twice more in Petri dishes containing approximately 50 ml. of buffered saline solution. After draining off of the excess fluid the tissue was kept at 4°C. overnight, or stored at −20°C. if longer storage was necessary. Suspensions of the membranes were made by blending in chilled semimicro Waring blendors for 3 minutes, using per membrane 5 to 10 ml. of M/100 phosphate buffered saline solution of pH 7.0 containing penicillin and streptomycin. The suspensions were clarified by centrifugation at 2000 R.P.M. for 20 minutes. Those obtained early after infection were as a rule treated with receptor-destroying enzyme (RDE) in order to remove any remaining inhibitor of hemagglutination according to the technic described (20).

The media and membrane suspensions obtained were titrated for virus activity either within 1 or 2 days of storage at 4°C. or, if infectivity titrations had to be delayed, after rapid shell freezing in sealed ampules in a dry-ice–alcohol bath and storage in the dry-ice chest at −65°C. Since virus in glucosol medium was found to be somewhat less stable than in allantoic fluid, a 10^{-1} dilution of the preparation in 10 per cent horse serum–saline was made immediately after harvest. In this way the virus was found to be quite stable. Membrane suspensions did not require addition of horse serum.

Infectivity Titrations.—This technic has been fully described in previous reports (3). The results are expressed as ID_{50}/ml.

Hemagglutination Tests.—The basic technic also has been described previously (16). However, in many experiments, after the range of titers had been established by the usual technic, the tests were repeated by diluting the test preparations initially in one step sufficiently so that the end-point of agglutination was reached within 2 to 4 further 2-fold dilution steps. This technic was considered to avoid some of the titration errors inherent in long 2-fold dilution series. The results are expressed as hemagglutinating (HA) units per millililiter.

ID_{50}/HA Ratio.—The ratio between the titers of infectious virus and of hemagglutinins provides information as to the presence of non-infectious virus material in a given virus preparation. The actual value of the ratio obviously depends on the sensitivity of the particular titration technics employed, and is reproducible within the limits of their accuracy. With the titration technics used in this laboratory, and particularly with the modification of the HA test described above, the virus found in allantoic fluid or in DE-media 18 to 24 hours after infection with small doses of seed virus (such as 10^4 ID_{50} per embryo) revealed ratios close to 10^7. Under other circumstances, such as inoculation of large doses of virus, or prolonged incubation periods, the yield may show considerably lower ratios, indicating the presence of non-infectious hemagglutinins (21).

Receptor-Destroying Enzyme of V. cholerae (RDE).—The RDE preparations used were derived from the 35A3 strain. Earlier batches were prepared according to the technic described previously (20). Later, the method of Ada and French was adopted (22), except for the final steps of purification after the methanol precipitation of the enzyme. One batch of RDE was kindly supplied by Dr. John Spizizen of Sharp & Dohme, Inc. The preparations were standardized according to the technic of Burnet and Stone (23). They contained between 640 and 2560 units per ml.

A few other technical details are presented in the experimental section.

EXPERIMENTAL

Deembryonation Prior to Infection

In earlier experiments, eggs were deembryonated and seed virus was added thereafter to the medium in varying concentrations. The infectivity and hemagglutinin titers attained after 24 hours of incubation were on the whole lower than those expected in the intact chick embryo. In further experiments cumulative growth curves were obtained with or without removal of the non-adsorbed seed virus by washing of the DE preparations ½ to 1 hour after infection. When the free seed virus was not removed the results obtained with the aliquots of media removed at given intervals resembled those recorded in *in ovo* growth curves (2); *i.e.*, the ID_{50} titers remained constant during the first 5 to 6 hours (non-adsorbed seed) before a rise in infectivity became apparent. If the DE preparations were washed after infection the results differed

to some extent as seen in Fig. 1. The ID_{50} and HA values of the medium at the time of infection are marked on the ordinate. After washing the infectivity titers decreased considerably, usually by more than 3 $\log_{10}$ units and the HA test became negative. The infectivity remained at this low level up to the 3rd or 4th hour and definite rises were seen by the 4th or 5th hour, distinctly earlier than in the *in ovo* experiments. More extensive washing of the DE preparations did not markedly decrease the residual infectivity titers and it is possible that the virus found during the early period represents seed virus eluted from the tissue after "superficial" adsorption. However, addition to the medium of large amounts of virus rendered non-infectious by irradiation with ultraviolet light but which had retained its hemagglutinating and eluting ac-

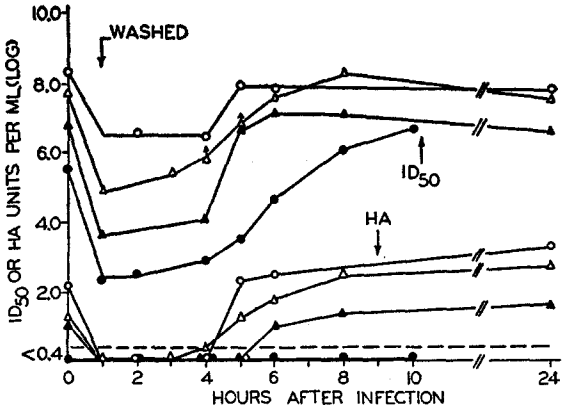

FIG. 1. Cumulative growth curves in eggs infected with varying doses of virus after deembryonation. 1 hour following infection the medium was removed and replaced by fresh medium after 2 washings of the tissue.

tivities failed to reveal elution under these conditions as measured by HA tests. Liberation of hemagglutinins became apparent either simultaneously with that of infectious virus or slightly later depending on the concentration of seed virus used.

These data indicate that both infectious and hemagglutinating virus materials are beginning to be liberated simultaneously after incubation periods of 4 hours or possibly less. In the intact chick embryo release of HA could be detected earlier than that of ID_{50} (16, 24) following injection of large doses of virus. Since under these conditions the progeny contains relatively less infectious virus than HA units (lower ID_{50}/HA ratio) (21) the liberation of infectious virus in the face of a high threshold level of non-adsorbed infectious seed virus cannot be detected in the earlier hours. Similar conclusions were presented by Bernkopf (10, 11).

With the more concentrated inocula maximal ID_{50} titers were attained

by the 6th to 8th hour, whereas for smaller inocula obviously longer incubation periods were required for reaching the peak. In the former group the ID_{50} titers remained constant or showed slight declines up to the 24 hour period. In contrast, the HA levels still increased over this period. In the light of evidence to be presented below and in subsequent papers (19, 25), it is likely that liberation of infectious virus continued over this period, but inactivation of liberated virus during the incubation period at 37°C. prevented its detection by infectivity titrations.

Deembryonation after Infection

Although infection after deembryonation has yielded answers to certain problems, this technic was found to give irregular results on occasion and suggestive evidence has been obtained that this was due possibly to variable extents of adsorption of the seed virus under these conditions. When chick embryos were infected prior to deembryonation more reproducible results were obtained. In addition, in view of the possibly deleterious effect of this technic on the viability of the tissue deembryonation could be performed at various stages after inoculation when infection had been established. This procedure was therefore adopted for all further studies.

(*a*) *Cumulative Growth Curves.*—Table I summarizes the results obtained in a number of experiments in which the eggs were deembryonated from 5 minutes to 11 hours after infection with varying dilutions of seed virus. Small amounts of medium were removed from the DE preparations at intervals up to 36 hours after infection. As can be seen the infectivity titers were generally lower than in the intact embryos studied concurrently. Similarly, the HA levels found in the DE series were lower than those in the *in ovo* series and the differences were of a similar order. Correspondingly, the ID_{50}/HA ratios produced under the 2 conditions were practically the same.

A few experiments were designed to see whether rotation of the DE preparations during incubation would be required. Stationary incubation had been employed previously by Schlesinger (26). In order to keep the tissue moist during stationary incubation and to collect any virus liberated from the membrane the eggs had to be nearly filled with the medium; *i.e.*, 50 ml. was required. In order to compare the results of the two technics, corrections had to be made for the different volumes. In Table II the data are presented as the total number of ID_{50} or HA units produced. It can be seen that the yield of virus in the stationary eggs was significantly less than in those rotated and hemagglutinins could not be detected in some of the stationary preparations because of the dilution factor. That these results are not based on the differences in the volumes of medium present during the experimental periods is seen by the fact that when a 50 ml. volume was used with rotation the total yield was of the same order as when 10 ml. was employed. No systematic com-

TABLE I

Cumulative Yield of Virus in Eggs Deembryonated after Infection and Comparison with Results Obtained in the Intact Chick Embryo

Experiment No.	Dilution of seed	Time of DE	Time of harvest (after infection)	A			B			A/B		
				DE series			*In ovo* series					
				ID_{50}/ml.	HA/ml.	ID_{50}/HA	ID_{50}/ml.	HA/ml.	ID_{50}/HA	ID_{50}/ml.	HA/ml.	ID_{50}/HA
			hrs.	*log*	*log*	*log*	*log*	*log*	*log*	*log*	*log*	*log*
1	10^0	5 min.	12	7.7	2.9	4.8						
			24	7.9	3.3	4.6						
			36	8.0	3.7	4.3						
2	10^0	5 min.	24		3.1			3.4			−0.3	
			44		3.4			4.0			−0.6	
3	10^0	30 min.	12	8.3	2.7	5.6						
			24	8.6	3.3	5.3						
			36	7.7	3.3	4.4						
4	10^0	1 hr.	14	7.7	2.5	5.2	8.8	3.7	5.1	−1.1	−1.2	0.1
			25	8.3	3.1	5.2	9.1	3.7	5.4	−0.8	−0.6	−0.2
5	10^{-3}	5 min.	12	6.5	<0.7							
			24	8.1	3.1	5.0	9.1	4.2	4.9	−1.0	−1.1	−0.1
			36	8.5	3.3	5.2	9.1	3.9	5.2	−0.6	−0.6	0.0
6	10^{-5}	11 hrs.	17	8.6	1.9	6.7	9.3	2.3	7.0	−0.7	−0.4	−0.3

TABLE II

Comparison of Yields from Deembryonated Eggs with or without Rotation

Experiment No.	Inoculum (ID_{50})	Time of DE	Volume of medium	Method of incubation	Time of harvest (after infection)	Total yield (ID_{50}/ml. × volume)		
						ID_{50}	HA	ID_{50}/HA
		hrs.			*hrs.*	*log*	*log*	*log*
1	$10^{6.5}$	2	10	Rotation	18	9.8	3.2	6.6
			50	Stationary	18	9.2	2.7	6.5
2	$10^{9.5}$	3	10	Rotation	24	7.9	3.8	4.1
			50	Stationary	24	7.3	3.6	3.7
3	$10^{3.5}$	2	10	Rotation	18	8.1	1.1	7.0
			50	Stationary	18	7.7	<2.1	>5.6
			10	Rotation	24	9.3	2.6	6.7
			50	Rotation	24	9.2	2.7	6.5
			50	Stationary	24	8.2	<2.1	>6.1

parison has been made between yields of virus under the two conditions after shorter incubation periods. However, in a few experiments it was seen that up to 4 hours after deembryonation (8 or more hours after infection) both types of preparations gave nearly identical results. In later harvests differences in yield became apparent and gradually more pronounced.

TABLE III

The Effect of RDE in the Medium on the Yield of Virus in Eggs Deembryonated 90 Minutes after Infection

Experiment No.	Dilution of seed	Time of harvest (after infection)	Concentration of RDE in medium									
			50 per cent		10 per cent		3 per cent		1 per cent		0	
			ID_{50}/ml.	HA/ml.	ID_{50}/ml.	HA/ml.	ID_{50}/ml.	HA/ml.	ID_{50}/ml.	HA/ml.	ID_{50}/ml.	HA/ml.
		hrs.	*log*	*log*	*log*	*log*	*log*	*log*	*log*	*log*	*log*	*log*
1	10^{-1}	2		<0.4		<0.4						<0.4
		24		2.5		2.7						2.9
	10^{-3}	24		<0.4		<0.4						2.8
	10^{-5}	24		<0.4		<0.4						1.5
2	10^{-3}	2	<2.3	<0.4	<2.3	<0.4					<2.3	<0.4
		24	5.2	<0.4	4.7	<0.4					8.8	2.8
3	10^{-2}	2				<0.4		<0.4		<0.4		<0.4
		24				1.0		1.9		2.2		2.4
	10^{-3}	24				<0.4		0.9		1.8		2.5
	10^{-4}	24				<0.4		<0.4		<0.4		2.4
4	10^{-4}	24			3.5	<0.4	4.4	<0.4	4.9	<0.4	8.6	1.6
		36				<0.4	4.6	<0.4		<0.4	9.2	2.4
		48			3.1	<0.4	4.6	<0.4	6.2	<0.4	9.0	2.7
5	10^{-4}	24					3.7	<0.4			8.2	1.6
		48					3.1	<0.4			8.9	2.6

Stationary incubation would thus seem to be satisfactory for short term experiments except for the difficulties in detecting hemagglutinins under these conditions.

The effect of RDE in cumulative growth curves: Experiments in intact chick embryos recorded by Stone (27) indicated that a considerable degree of protection may be obtained by injection of RDE prior to infection. This protection has been ascribed to removal of receptors from susceptible cells. The data showed, however, that both the enzyme and virus have to be carefully balanced. Furthermore, host cells may regenerate receptors so that protection is transitory (27).

In the experiments of Table III, eggs were infected with varying doses of seed virus 90 minutes prior to deembryonation. RDE was then added in varying concentrations to the medium (1 to 50 per cent) and samples of the fluid were withdrawn for assay after 2 to 48 hours of incubation. Under these conditions no effect of the enzyme was expected with respect to the first infectious cycle already in progress, but subsequent cycles should at least in part be inhibited. Indeed, when the largest dose of seed virus was used (10^{-1} or approximately 10^{8} ID_{50}) which presumably was sufficient to infect all the available cells, RDE even in the strongest concentration had little effect upon the yield of HA (Experiment 1). When the amount of seed virus was decreased in 10-fold steps the effect of the enzyme, as measured by the HA levels in the yield, became increasingly more pronounced and successively less RDE was required to prevent the appearance of detectable HA titers in the medium. Thus with a 10^{-4} inoculum and 1 per cent RDE no HA could be detected even after 48 hours (Experiment 4). Using a 10^{-3} dilution of the seed, 10 per cent RDE was needed for this effect, but with 3 or 1 per cent concentrations of the enzyme increasingly more HA was found (Experiment 3). Infectivity titrations were included in some of the experiments. They, likewise, showed that the first set of cells released virus but that further cycles apparently could be prevented by the enzyme. However, it is also seen (Experiment 4, 10^{-4} inoculum) that in the presence of large concentrations of RDE (10 per cent) the yield of virus was distinctly smaller than when the medium contained 3 per cent of the enzyme. This suggests that RDE preparations in large concentrations may have an effect on virus production in the tissue. If the amount of RDE was reduced to 1 per cent the yield of virus in 24 hours was of a similar order as in the 3 per cent series but the ID_{50} level rose significantly after further incubation to 48 hours. Thus, it appears that when less than a critical amount of RDE was present the remaining uninfected cells regained susceptibility presumably by restitution of cell receptors (27). Since the medium was not changed it was assumed that RDE was present throughout the experimental period. However, at the end of the test little of its activity could be detected in the medium. Therefore in further experiments the infected tissue was exposed for varying periods to RDE in order to study "recovery" of the tissue. The pertinent technical details of these tests as well as the results are shown in Table IV. It can be seen that the longer the contact with RDE the more pronounced and enduring its effect. When the RDE medium was removed at an early period by washing of the DE preparations and replaced by RDE-free medium some recovery of the tissue became apparent and both the ID_{50} and HA levels increased later in the incubation period. However, the titers ultimately attained never equalled those of the controls. The latter either did not contain any RDE in the medium or enzyme heated to 100°C. for 10 minutes in the same concentration as used for the native material.

These results indicated that with small enough inocula and a sufficient concentration of RDE in the medium one-step growth curves could be obtained and indeed, Fig. 2 represents evidence to that effect. In this experiment the embryos were inoculated with a 10^{-3} dilution of seed virus or approximately

TABLE IV

Cumulative Yields of Virus in Eggs Deembryonated after Infection and Exposed to RDE for Various Periods of Time

Experiment No.	Initial medium			Period of incubation					
	RDE			24 hrs.		36 hrs.		48 hrs.	
	Per cent	Status	Period of contact*	ID_{50}/ml.	HA/ml.	ID_{50}/ml.	HA/ml.	ID_{50}/ml.	HA/ml.
			hrs.	*log*	*log*	*log*	*log*	*log*	*log*
1	1	Native	2½		<0.4		1.0		1.8
		"	48	4.9	<0.4		<0.4	6.2	<0.4
	3	"	2½	7.5	<0.7	8.2	1.1	8.7	1.5
		"	48	4.5	<0.4	4.6	<0.4	4.6	<0.4
		10 min. 100°C.	48	8.95	2.0	9.1	2.2	8.9	2.8
	10	Native	2½	5.9	<0.4	7.0	<0.4	7.4	0.9
		"	48	3.5	<0.4		<0.4	3.1	<0.4
		10 min. 100°C.	48	9.4	2.2	9.5	2.6	8.5	2.8
2	0	—	—	8.2	1.7		2.0	8.9	2.6
	3	Native	1	6.5	<0.4		<0.4	7.6	1.1
		"	4	6.5	<0.4		<0.4	7.0	0.9
		"	8	4.2	<0.4		<0.4	7.1	0.9
		"	24	4.2	<0.4		<0.4	4.5	<0.4
		"	48	3.7	<0.4		<0.4	3.1	<0.4

Inoculum: 10^{-4} or approximately $10^{6}ID_{50}$.

* After the indicated period the initial medium was removed, the membranes were washed twice, and medium without RDE was added.

$10^{6}ID_{50}$. After deembryonation RDE was added to the medium to form either a 50 or 10 per cent solution, and samples were withdrawn at 2 hourly intervals for assay. The results were closely similar and combined therefore for presentation in the figure.

From the above data it is apparent that RDE can be used to advantage in deembryonated eggs provided proper relationships between the infecting virus and the concentration of the enzyme are maintained.

(*b*) *Differential Growth Curves.*—An example of the differential type of growth

curve in deembryonated eggs is shown in Fig. 3. In this experiment undiluted seed virus was injected into 4 embryos and deembryonation was performed 5

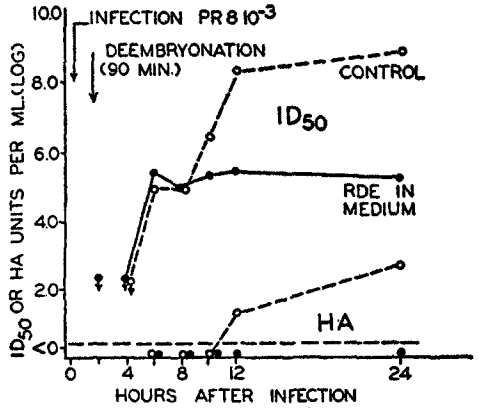

FIG. 2. One-step growth curve obtained in deembryonated eggs by addition of RDE to the medium.

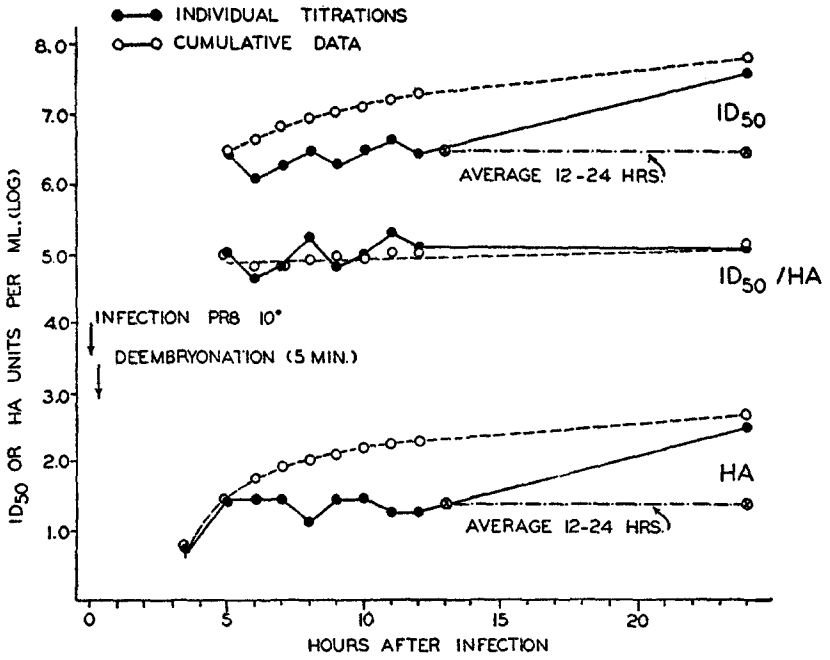

FIG. 3. Showing hourly liberation of infectious virus and of hemagglutinins in deembryonated eggs. Differential growth curve.

minutes thereafter. From the 3rd to the 12th hour following infection the medium was exchanged at hourly intervals and the liberated virus was permitted to accumulate thereafter up to the 24th hour. The results of the ID_{50}

and HA titrations on the pools of the hourly harvests indicated that the yields of both activities were nearly constant and if the amounts accumulated from the 12th to the 24th hour were divided by the number of intervening hours the average yields were of the same order as those seen in the preceding hourly harvests. This unexpected result has been analyzed further and the data will be presented in the paper to follow (19).

TABLE V

Comparison of Yields of Virus in Cumulative and Differential Deembryonation Experiments

Experiment No.	Inoculum		Time of DE	Time of harvest (after infection)	A			B			A/B		
					Differential series (calculated cumulative)*			Cumulative series					
	ID_{50}	Dilution of seed			ID_{50}/ml.	HA/ml.	ID_{50}/HA	ID_{50}/ml.	HA/ml.	ID_{50}/HA	ID_{50}/ml.	HA/ml.	ID_{50}/HA
				hrs.	*log*	*log*	*log*	*log*	*log*	*log*	*log*	*log*	*log*
1	$10^{8.6}$		2 hrs.	9	6.9	2.4	4.5	6.7	2.5	4.2	0.2	−0.1	0.3
2	$10^{8.3}$		1 hr.	10	6.6	1.8	4.8	7.4	2.2	5.2	−0.8	−0.4	−0.4
3	$10^{8.3}$		1 "	11	7.5	1.9	5.6	7.5	2.2	5.3	0.0	−0.3	0.3
4		10^{0}	5 mins.	12	8.1	2.7	5.4	7.7	2.9	4.8	0.4	−0.2	0.6
				24	8.3	3.1	5.2	7.9	3.3	4.6	0.4	−0.2	0.6
				36	8.4	3.2	5.2	8.0	3.6	4.4	0.4	−0.4	0.8
5		10^{0}	30 mins.	12	8.4	2.5	5.9	8.3	2.8	5.5	0.1	−0.3	0.4
				24	8.6	2.9	5.7	8.6	3.2	5.4	0.0	−0.3	0.3
				36	8.7	3.0	5.7	7.7	3.2	4.5	1.0	−0.2	1.2
6		10^{-3}	5 mins.	12	8.1	2.7	5.4	7.8	2.9	4.9	0.3	−0.2	0.5
				24	8.3	3.1	5.2	7.9	3.3	4.6	0.4	−0.2	0.6
				36	8.4	3.2	5.2	8.0	3.6	4.4	0.4	−0.2	0.8

* The values obtained in the individual 1 or 2 hour harvests were added.

If the amounts of ID_{50} and HA liberated in differential growth curves at hourly or 2 hourly intervals are added the cumulative titers at any given time interval can be calculated. This has permitted comparison of the yields from differential growth curves with those obtained in cumulative series performed at the same time. Such comparisons are given in Table V. On the whole the yields do not differ to a large extent. However, it is apparent that the calculated total yield of HA in the differential series as a rule is slightly lower (average 0.25 $\log_{10}$ units) than that obtained at the same time intervals in the cumulative series. On the other hand, the infectivity titers in the differential set were usually somewhat higher than in the cumulative experiments, particularly when the tests were carried over longer periods of time. This would seem to

indicate that in the differential series somewhat less total virus is produced and liberated (smaller yield of HA) but virus is removed at regular intervals and thus there is less chance of inactivation of infectivity which is likely to occur in the cumulative sets on prolongation of the incubation at 37°C. The apparent, though relatively slight, decrease in virus production in the differential type of experiment may in part be based on the more frequent handling of the eggs, in part on some loss of virus during the washing procedures, although the same differences were noted when the medium was just exchanged without additional washing of the membranes. The greater yields of virus in intact chick embryos as compared to those obtained in deembryonation experiments of the cumulative type have been discussed above.

(*c*) *Combined in Ovo-DE Growth Curves.*—The results obtained with the technics described above indicated that the yield of virus in deembryonated eggs is less than in the intact chick embryo. This decrease in part may be ascribed to the facts that there are fewer host cells in the DE preparations than in the allantoic cavity of the intact embryo, and that the volume of DE medium (10 ml.) is somewhat larger than that of allantoic fluid (5 to 8 ml.). However, the difference in yield (Table I) would seem to be greater than accounted for by these facts. Thus, it is suggestive that the viability of the tissue in DE series is impaired and that virus production may be decreased or delayed correspondingly. In order to obtain some information as to whether this interpretation is correct, or whether virus reproduction may take an abnormal course in deembryonated eggs, a technic was devised which permitted the infectious process to reach various stages in the intact embryo before deembryonation, and the medium was collected 1 or 2 hours thereafter for study. The short period of study after deembryonation was thought to minimize any deleterious effects on continued virus production and liberation that might become apparent in the DE preparations on prolonged incubation. The results obtained with these "1 hour" samples were then compared with those derived from *in ovo* growth curves. Such an experiment is shown in Fig. 4.

Chick embryos were infected with $10^{4.4}ID_{50}$. With such an inoculum liberation of HA becomes detectable after the 12th hour of incubation. Correspondingly, the experiment extended from the 11th to 22nd hour. One series of the infected eggs was left intact and allantoic fluids were collected from groups of 6 at 2 to 3 hour intervals, and the volume of fluid was estimated. Other groups of eggs were deembryonated at hourly intervals from the 11th hour on and handled as described above.

All preparations thus obtained were titrated in parallel for infectivity and hemagglutinins and the results were corrected for the differences in volumes of fluids present. The ID_{50} and HA titers in the allantoic fluid series fell on a smooth curve (Fig. 4 A). From this curve the virus titers in the allantoic fluid at any intermediary interval could be obtained. Also the amount of

virus liberated into the allantoic fluid during any 1 hour at least up to the 20th hour could be calculated by subtracting the value found at the beginning of the period from that observed 1 hour later. These values are plotted in Fig. 4 B (solid dots). The data derived from the DE series at the corresponding 1 hour intervals are shown in Fig. 4 B as circles. It is seen that the liberation of infectious virus into the medium of DE preparations was of the same order as the amounts calculated to have been released into the allantoic fluids of the chick embryos, although usually somewhat less.

In 2 other experiments of this kind similarly slight differences in yield were

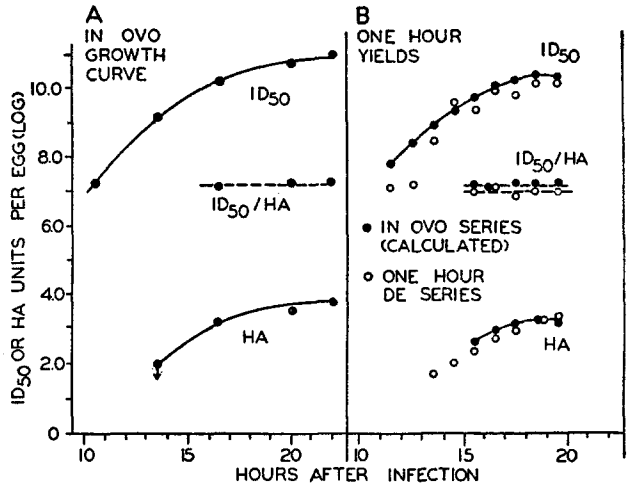

FIG. 4. Combined *in ovo*–deembryonation experiment after inoculation of $10^{4.4}$ ID_{50}. A, *In ovo* growth curve from which the hourly yields were calculated as charted in B; B, Comparison of the yields obtained 1 hour after deembryonation (circles) with the calculated 1 hour yields from the *in ovo* growth curve.

noted. The average differences in 18 comparable infectivity titrations being 0.27 $\log_{10}$ units. On the other hand, the quantities of hemagglutinins liberated under the 2 sets of conditions were often slightly greater in the DE series than in the intact chick embryo, with a mean difference of 0.13 $\log_{10}$ units in 13 comparable assays. As a result of these differences in liberation of ID_{50} and HA, the ID_{50}/HA in the allantoic fluids was up to 0.3 $\log_{10}$ units greater than in the DE medium.

On the whole the combined *in ovo*–DE technic has given results closely similar to those obtained in the chick embryo as far as total virus production and liberation are concerned (HA titers) but perhaps a little less infectious virus is found. It is unlikely that this is due to a rapid loss of infectivity at 37°C. since the rate of inactivation of virus in glucosol medium *in vitro* was found to be 0.06 to 0.07 $\log_{10}$ units per hour (25).

In the type of experiment just described, additional information was obtained by setting further groups of DE preparations at the 11th, 14th, and 17th hours after infection. From each of these, cumulative harvests were made after each 3 hour interval on the roller machine up to the 20th hour after infection. The ID_{50} and HA values obtained with these harvests could then be compared with the calculated sums of the titers derived from the "1 hour" series during the corresponding intervals. These comparisons are presented in Table VI. It can be seen that in every instance the calculated amounts of infectious virus liberated during any of the 3, 6, or 9 hour periods in the combined *in ovo*–DE series were larger (by 0.1 to 0.7 $\log_{10}$ units) than those found in the cumulative DE series. The HA values likewise were greater in the

TABLE VI

Comparison of Yields of Virus in Combined in Ovo-DE and Cumulative DE Experiments

Intervals	Period after infection	A			B			A/B		
		Combined *in ovo*-DE (1 hr.) series (calculated cumulative)			Cumulative DE series					
		ID_{50}/ml.	HA/ml.	ID_{50}/HA	ID_{50}/ml.	HA/ml.	ID_{50}/HA	ID_{50}/ml.	HA/ml.	ID_{50}/HA
hrs.	*hrs.*									
3	11–14	7.4	0.7	6.7	7.4	0.7	6.7	0.0	0.0	0.0
	14–17	9.1	1.9	7.2	9.0	1.7	7.3	0.1	0.2	−0.1
	17–20	9.5	2.6	6.9	9.0	2.0	7.0	0.5	0.6	−0.1
6	11–17	9.1	1.9	7.2	8.5	1.8	6.7	0.6	0.1	0.5
	14–20	9.6	2.7	6.9	8.9	2.2	6.7	0.7	0.5	0.2
9	11–20	9.6	2.7	6.9	8.9	2.2	6.7	0.7	0.5	0.2

former by 0.1 to 0.6 $\log_{10}$ units. It is apparent then that with an increase in incubation in the DE state virus production and liberation decrease to some extent, but as may be deduced from the differential type of experiment, liberation is stabilized rapidly at a constant rate which is maintained for many hours.

DISCUSSION

The primary purpose of the studies presented was an analysis of the deembryonation technic (10, 11) and comparison of the results obtained under a variety of conditions with those derived from parallel experiments in intact chick embryos. The use of the DE technic has certain advantages over *in ovo* experiments in that the fluid medium can be sampled or exchanged easily and repeatedly at stated intervals and thus cumulative or differential curves of viral liberation can be obtained on individual eggs. Various agents such as RDE can readily be added to the medium and removed from the DE prepara-

tions at any given time. Thus, the quantitative aspects of RDE treatment of the membrane can be analyzed more conveniently than in the intact egg (27). Under properly controlled conditions one-step growth curves can be obtained which are similar in appearance to those observed in the intact chick embryo, except that in the latter case the remaining cells were blocked by the interfering action of heterotypic virus inactivated by ultraviolet irradiation (2) whereas in the DE experiments the receptors of the remaining susceptible cells were presumably removed by action of RDE so that adsorption of virus to these cells was prevented. The DE technics have the further advantage that the time relationship in the growth cycle of influenza virus in the entodermal layer of the allantois can be determined more accurately than *in ovo* (1, 2). By removal of the major portion of non-adsorbed seed virus shortly after infection liberation of newly formed virus can be measured 1 to 2 hours earlier than in the intact chick embryo. This is in agreement with recent observations in infected tissue cultures (28). However, in spite of numerous washings of the membrane at the time of deembryonation immediate sampling of the final medium added still revealed some infectious virus and thus a low threshold level remains, beyond which liberation must proceed before it can be detected. It is possible, therefore, that some virus is liberated even earlier than 3 or 4 hours after infection. Similarly, removal of liberated virus at any given stage of the infectious process permits detection of late liberation of virus. On the basis of *in ovo* experiments it had been previously assumed that liberation in one infectious cycle is accomplished within a relatively short period of time; *i.e.*, 3 to 5 hours (2, 4). However, in deembryonation experiments, particularly by using the technic of frequent exchanges of the medium (differential growth curve) it has become apparent that liberation proceeds over a considerably longer period of time than previously suspected. This phase of the cycle will be analyzed further in the succeeding paper of this series (19). Frequent exchange of the fluid medium of the allantoic cavity of the intact chick embryo has been described by Cairns (4), but it is obvious that such exchanges are technically more easily and quantitatively accomplished in DE preparations.

In comparing the results of DE experiments under various conditions it became apparent that more reproducible results were obtained when deembryonation was performed *after* infection rather than *prior* to it. This in part may be due to differences in the degree of adsorption of seed virus under the 2 sets of conditions. In the intact chick embryo the allantoic cavity forms a rather narrow sac and seed virus delivered into the allantoic fluid may more readily make contact with susceptible cells. In the DE preparations on the other hand, the 10 ml. of medium is not confined to such a narrow space and some of the seed virus will have to move over longer distances before it can attach itself to cells. Thus, adsorption may require more time. No efforts have been made to analyze this problem further and infection before deembryonation was adopted as the method of choice.

The cumulative and differential types of experiments gave results closely similar as to total production and liberation of progeny. However, these yields were less than those observed in the intact chick embryo. The smaller yields may be ascribed in part to the smaller number of infected cells available in the DE system, the "visceral" part of the allantois being removed at the time of deembryonation. Furthermore, the volume of medium in DE preparations (10 ml.) as a rule is larger than the amount of allantoic fluid in intact chick embryos, as used for *in ovo* growth curves (5 to 8 ml.). However, if corrections are made for these differences in volumes of fluids, *i.e.* if the total yield of virus is estimated by multiplying the ID_{50} and HA titration values by the volumes of allantoic fluid or medium, it is found that *in ovo* usually far more than double the amount of virus is liberated but the loss in entodermal cells in DE preparations is certainly not more than half if one assumes that they are all equally susceptible. As will be shown (19), the differences in yield of infectious virus are more pronounced in the early part of the incubation period than later on when similar ID_{50} values may be found in both types of tests. However, the HA titers show throughout marked differences and it is likely that inactivation *in ovo* at 37°C. may explain to some extent the smaller discrepancy between the infectivity titers at the later stage of infection. On the other hand, there is some evidence that on deembryonation the vitality of the tissue is reduced and thus the rate of production of infective virus is decreased. On prolonged incubation, however, the DE preparations seem to catch up to some extent with virus production *in ovo*. Additional support for this view will be presented (19).

The question remained then to what extent certain results of DE experiments presented here and in subsequent papers (19, 29) are comparable to what occurs in the intact chick embryo. It was necessary, therefore, to obtain a closer comparison between the two technics and the combined *in ovo*–DE technic was designed for this purpose. The infectious process is permitted to proceed *in ovo* to any given stage and then deembryonation is performed and the medium is analyzed 1 to 3 hours later. In this short period after deembryonation the tissue has not as yet suffered extensively by the disruption of blood and nutrient supply and indeed the yields of hemagglutinins obtained under these conditions are virtually identical with those observed in the intact chick embryo during the same time interval, and only a slight decrease in infectious virus was noted. By use of this technic it could be shown for instance that liberation of virus from infected cells is a prolonged process not only under the conditions of DE experiments, but also in the intact chick embryo (19).

SUMMARY

The usefulness of the deembryonation technic has been analyzed as a tool in the study of various problems in the growth cycle of influenza virus in the entodermal cells of the allantoic of chick embryos.

Various improvements in the deembryonation technic have been described. The method readily permits repeated sampling of the medium at various stages after infection (cumulative growth curves) or frequent exchanges of the medium (differential growth curve). However, the yield of infectious virus or of hemagglutinins is less than that observed in the intact chick embryo. The difference observed is greater than can be accounted for by the reduction in the available host cells and is assumed, therefore, to be due in part to interruption of blood and nutrient supply to the cells. This handicap can be overcome by the combined *in ovo*–deembryonation technic, in which deembryonation is performed at any desired time after infection of the intact chick embryo, and the medium is collected and analyzed after 1 to 3 hours of further incubation.

The value of the technic is demonstrated by the fact that liberation of virus from infected cells can be detected earlier than in the intact egg. Furthermore, it continues at a nearly constant rate for many hours, thus proving to be erroneous previous inference which had been based upon *in ovo* experiments.

The technic also permits readily the addition and subsequent removal of substances that might interfere with viral propagation. As an example a study was made of the effect of the receptor-destroying enzyme of *V. cholerae* (RDE) when added to the medium of eggs infected prior to deembryonation. By carefully grading the dose of virus and using an appropriate amount of RDE, one-step growth curves were obtained indicating that those cells not directly invaded by the seed virus were subsequently protected against infection by action of the enzyme. The smaller the amount of virus the less RDE was required in order to note a protective effect. With a decrease in the period of exposure to RDE regeneration of cell receptors became increasingly more apparent in that correspondingly greater amounts of virus were produced and liberated late in the incubation periods. These results confirmed and extended those reported by Stone.

More extensive applications of these technics will be reported in subsequent papers of this series.

BIBLIOGRAPHY

1. Henle, W., *Advances Virol.*, 1953, **1**, 141.
2. Henle, W., Henle, G., and Rosenberg, E. B., *J. Exp. Med.* 1947, **86**, 423.
3. Henle, W., *J. Exp. Med.* 1949, **90**, 1.
4. Cairns, H. J. F., and Edney, M., *J. Immunol.* 1952, **68**, 155.
5. Weller, T. H., and Enders, J. F., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 124.
6. Cushing, R. T., and Morgan, H. R., *Proc. Soc. Exp. Biol. and Med.*, 1952, **79**, 497.
7. Womack, C. R., and Kass, E. H., *J. Immunol.*, 1953, **71**, 152.
8. Fulton, F., and Armitage, P., *J. Hyg.*, 1951, **49**, 247.
9. Fulton, F., and Isaacs, A., *J. Gen. Microbiol.*, 1953, **9**, 119.
10. Bernkopf, H., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, 680.

11. Bernkopf, H., *J. Immunol.*, 1950, **65,** 571.
12. Fazekas De St. Groth, S., *Nature*, 1948, **162,** 294.
13. Isaacs, A., and Edney M., *Australian J. Exp. Biol. and Med. Sc.* 1950, **28,** 635.
14. Henle, W., *Cold Spring Harbor Symp. Quant. Biol.*, 1953, **18,** 135.
15. Henle, W., *J. Exp. Med.*, 1949, **90,** 13.
16. Henle, W., and Henle, G., *J. Exp. Med.*, 1949, **90,** 23.
17. Himmelweit, F. O., personal communication.
18. Daniels, J. B., Eaton, M. D., and Perry, M. E., *J. Immunol.*, 1952, **69,** 321.
19. Henle, W., Liu, O. C., and Finter, N. B., *J. Exp. Med.*, 1954, **100,** 53.
20. Liu, O. C., and Henle, W., *J. Exp. Med.*, 1951, **94,** 269.
21. von Magnus, P., *Acta. path. et microbiol. scand.*, 1951, **28,** 278.
22. Ada, G. L., and French, E. L., *Australian J. Sc.*, 1950, **13,** 82.
23. Burnet, F. M., and Stone, J. D., *Australian J. Exp. Biol. and Med. Sc.*, 1947, **25,** 227.
24. Hoyle, L., *J. Hyg.*, 1950, **48,** 277.
25. Paucker, K., Liu, O. C., and Henle, W., data to be published.
26. Schlesinger, R. W., personal communication.
27. Stone, J. D., *Australian J. Exp. Biol. and Med. Sc.*, 1948, **26,** 49.
28. Ackerman, W. W., and Maassab, H. F., *J. Exp. Med.*, 1954, **99,** 105.
29. Liu, O. C., Paucker, K., and Henle, W., data to be published.