SOME ALTERATIONS IN CHICKEN ERYTHROCYTES WHICH FOLLOW TREATMENT WITH INFLUENZA AND NEWCASTLE DISEASE VIRUS

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A great deal has been learned about viral hemagglutination since the report by Hirst in 1941 that influenza viruses agglutinate chicken red blood cells. Of special interest have been those observations that suggested that this reaction may serve as a model for the study of viral infections.

In 1942 Hirst reported that PR8 and Lee viruses were eluted from the red blood cells at different rates and that erythrocytes that had adsorbed and fully dissociated from these viruses were no longer capable of adsorbing a detectable amount of fresh homologous or heterologous virus or of agglutinating in their presence. He postulated that this alteration of the erythrocytes was analogous to an enzyme-substrate reaction and suggested that hemagglutination might be an *in vitro* counterpart to natural infection. This suggestion received support from his subsequent work (Hirst, 1943), which indicated that the cells of the respiratory tract resembled red blood cells in their capacity for adsorbing and subsequently dissociating from influenza viruses. Burnet and Bull (1943) in the course of comparative observations on the behavior of freshly isolated influenza strains commented on an apparent parallelism between the susceptibility of erythrocytes from various species to agglutination and the susceptibility of those species to infection. Burnet and his co-workers (1945, 1946) also investigated the agglutinability of red blood cells that had once adsorbed and dissociated from hemagglutinating viruses. Their results, unlike those of Hirst (1942), indicated that there were varying degrees of refractoriness to agglutination depending upon the virus used initially and those used later to test the modified cells. Burnet (1945*a*,*b*,*c*) presented a linear arrangement of these agents—mumps, Newcastle disease, most influenza A strains, influenza B, and swine influenza—and stated that cells treated with any one of them were subsequently refractory to agglutination by the homologous virus and those viruses preceding it in the series, but were still susceptible to agglutination by those following it.

Ziegler and Horsfall (1944) noted certain analogies between interference and the resistance of modified erythrocytes to further agglutination. In their studies of interference in the chick embryo they found that, although PR8 virus causes interference with infection by Lee virus, Lee did not interfere with subsequent infection by large amounts of PR8. This lack of reciprocal interference in the presence of certain viruses is unlike the results reported by Hirst (1942)

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with PR8- and Lee-treated red blood cells, but might be consistent with the observations of Burnet (1945*a*,*b*,*c*; Burnet *et al.*, 1946), although he did not report the effect of PR8 in his series. A review of the literature on interference such as is presented by Ziegler and Horsfall (1944) indicates that viruses widely different in antigenicity may cause interference and that this may or may not be reciprocal depending upon the particular agents studied. This has been further documented by the more recent work of Duffy (1944) showing reciprocal interference in the chick embryo with St. Louis encephalitis and Western equine encephalitis viruses, and by that of Lennette and Koprowski (1946) in tissue culture in which yellow fever and West Nile viruses are shown to interfere with infection by PR8 virus, although PR8 does not interfere with infection by the former viruses. The importance of the conditions under which these experiments are performed is shown by a report of Henle and Henle (1945) in which, after using inactivated virus as a first inoculum and an unspecified concentration of active virus as the second, they presented evidence, unlike Ziegler and Horsfall (1944), for reciprocal interference in the chick embryo between PR8 and Lee.

The foregoing findings have many implications for the student of infectious disease. Consequently, it was felt that a reinvestigation with more quantitative techniques of the alterations that take place in erythrocytes following contact with hemagglutinating viruses might reconcile the different results reported by various workers. It was hoped that the results obtained might offer further support for the use of the hemagglutination phenomenon as a tool in the study of the pathogenesis of viral infections. Therefore, the duration and the specificity of the *in vitro* changes in chicken red blood cells following treatment with the influenza viruses, PR8 and Lee, and Newcastle disease virus (NDV) were investigated, and an attempt was made to learn whether similar changes might occur *in vivo* in infected chick embryos. In the present communication it will be shown that the alterations produced in erythrocytes are long-lasting, that the degree of the refractory state for adsorption and agglutination of altered cells varies depending upon the virus employed, and that similar changes do not necessarily occur in infected chick embryos.

MATERIALS AND METHODS

Viruses. The PR8 strain of influenza A virus, the Lee strain of influenza B virus, and a strain of Newcastle disease virus originally recovered by Dr. Beaudette of Rutgers University were used in this study. For convenience they will henceforth be termed PR8, Lee, and NDV, respectively. Pools of allantoic fluids from 12- and 13-day-old chick embryos that had been infected via the allantoic sac were employed as the source of the virus. Between tests these pools were stored at either 4 C or −72 C.

Cells. Chicken red blood cells were collected in an excess of 2 per cent sodium citrate and washed several times with saline; the desired concentration was prepared from the sediment obtained after centrifugation at 1,500 rpm for 10 minutes.

"Treated" cells were prepared by mixing a suspension of cells with undiluted allantoic fluid. The mixtures were permitted to remain at 37 C and room temperature for varying periods of time before the cells were separated and washed with saline. Washings were repeated many times with intervening periods at 37 C to permit complete elution of absorbed virus. Between procedures the cells were stored at 4 C. Such cells were prepared with PR8, Lee, and NDV, and with normal allantoic fluid and saline. Since several different concentrations of cells were used, more exact details of preparation will be reserved for the experimental section. However, whenever treated cells are referred to in this communication, it is meant to indicate that the cells have been subjected to a procedure such as has just been outlined.

Saline. An 0.85 per cent solution of sodium chloride containing 0.01 M phosphate buffer at pH 7.2 was used throughout.

Sera. Specific immune sera were prepared for each of the viruses by injecting rabbits intravenously with 10 ml of a pool of infected allantoic fluid. In preparing NDV and Lee antisera, the rabbits were given a second such injection after 6 weeks—approximately 2 weeks after the last injection serum was collected.

Hemagglutination tests. The hemagglutination titers of the viruses were obtained by making serial dilutions with saline in a volume of 0.4 ml, adding an equal quantity of a 1.5 per cent red blood cell suspension, and observing the pattern of the sediment after 60 minutes. For PR8 and Lee viruses the reaction was permitted to proceed at room temperature and for NDV usually at 4 C, as previously described (Florman, 1947). In some of the early experiments tests with NDV were conducted at room temperature and read after 30 minutes. The highest dilution of virus giving 2+ agglutination was considered the end point and constituted one hemagglutinating unit of virus. This method was used with fresh red blood cells and with those that had previously been treated with viruses when information was sought regarding their degree of agglutinability.

Infectivity titrations. The quantity of active virus present in an infected allantoic fluid was determined by titration in chick embryos. Tenfold dilutions were made in 10 per cent normal horse serum broth, and inoculations were carried out by the intra-allantoic route. After 24 hours' incubation of the inoculated embryos the allantoic fluids were collected and tested for hemagglutination. The highest dilution of the inoculum that had produced infection in 50 per cent of the embryos was considered to contain one embryo infective dose (1 E.I.D.).

Antibody titrations. Because tests with NDV are best carried out in the cold and since uniformity was desired, all antibody titrations were done at 4 C by a method previously described for NDV (Florman, 1947). The sera were inactivated at 56 C for 30 minutes, and serial twofold dilutions were prepared in a volume of 0.2 ml. To each there were added 0.2 ml of a virus dilution containing 32 hemagglutinating units. The mixture was shaken and incubated in a 37 C water bath for 60 minutes. After chilling, 0.4 ml of a cold 1.5 per cent chicken cell suspension were added, and the tubes were reshaken and left at 4 C for 60 minutes. At the end of this period the pattern of the sedimented

cells was observed. The lowest dilution of serum permitting 2+ agglutination was considered the end point and constituted one unit of hemagglutination-inhibiting antibody.

EXPERIMENTAL RESULTS

Alterations in agglutinability of virus-treated red blood cells in the presence of fresh virus. In general the published findings of earlier workers were confirmed (Hirst, 1942; Burnet, 1945*a*,*b*,*c*). Chicken red blood cells treated with NDV, Lee, and PR8 showed a modified agglutinability in the presence of fresh homologous or heterologous virus. Cells treated with any one of the three viruses were subsequently inagglutinable in the presence even of large amounts of NDV. However, the degree of alteration of the cells with respect to agglutination with PR8 or Lee was not uniform. As will be shown below, the manner in which the cells were treated and tested, especially with PR8 and Lee, made a difference in the result obtained.

Persistence of alterations in agglutinability of treated red blood cells. Before any elaborate experiments could be undertaken with altered cells, it was necessary to determine how long after treatment they remained modified. Aliquots of a 3 per cent suspension of freshly collected chicken red blood cells were treated with either NDV, PR8, or Lee by mixing with an equal amount of undiluted, infected allantoic fluid containing the desired virus and by incubating the mixtures at 26 C for a total of 48 hours. After several washings a 1.5 per cent suspension was prepared from each batch of treated cells and a saline control. Then 0.4 ml of these cells were added to 0.4 ml of serial dilutions of fresh preparations of each of the three viruses and hemagglutination end points were determined. At the time the first tests were performed 3 days had elapsed since the cells were treated. Similar tests were subsequently carried out on the tenth and seventeenth days after treatment. In the interim the cells were stored at 4 C as 3 per cent suspensions. In this experiment all the hemagglutination tests were done at room temperature and read after 1 hour except those with NDV, which were read at the end of 30 minutes.

As is shown in table 1, all cells treated in the manner described were inagglutinable by NDV for at least as long as 10 days. Except for those treated with NDV, they were also inagglutinable in the presence of Lee virus for at least as long as 17 days. In the presence of PR8, however, although the NDV- and Lee-treated cells showed diminished agglutinability for as long as 17 days, only the PR8-treated cells were inagglutinable. The appearance with NDV-treated cells of partial agglutinability in the presence of Lee virus on the seventeenth day could not be explained. With other preparations of NDV-treated cells complete loss of agglutinability in the presence of Lee was rarely observed. From this experiment it may be concluded that cells treated with any of these three viruses show altered agglutinability for at least as long as 17 days, and that no essential difference is detectable between results obtained with cells tested on the third and on the tenth days after treatment. In another similar experiment in which only NDV- and Lee-treated cells were studied, such alterations were observed to persist for as long as 21 days.

Effect of variations in treatment of red blood cells on their agglutinability in the presence of fresh virus. Many difficulties were encountered in obtaining uniform results when treated cells were tested for agglutinability in the presence of fresh virus. It appeared that even though cells were consistently inagglutinable in the presence of large amounts of NDV, the manner of treatment markedly affected the results obtained with PR8 and Lee. Although no attempt was made to investigate this subject completely, two experiments will be presented to illustrate this point.

In one experiment chicken erythrocytes were prepared as a 3 per cent suspension, mixed with equal parts of undiluted, infected allantoic fluid, incubated for

TABLE 1

Persistence of diminished agglutinability by viruses of red blood cells treated with viruses

CHICKEN RBC* TREATED WITH:	VIRUS-INFECTED ALLANTOIC FLUID	HEMAGGLUTINATION TITER† NO. OF DAYS FOLLOWING TREATMENT OF RBC		
		3	10	17
Saline	NDV	1,280	640	
NDV	"	10	10	
Lee	"	10	10	
PR8	"	10	10	
Saline	Lee	1,280	1,280	1,280
NDV	"	10	10	160
Lee	"	10	10	10
PR8	"	10	10	10
Saline	PR8	1,280	640	320
NDV	"	320	160	160
Lee	"	320	160	40
PR8	"	10	10	10

* Three per cent suspension of RBC added to undiluted allantoic fluid and held at 26 C for 48 hours.

† Reciprocal of dilution giving titration end point.

a total of 17 hours at 37 C, and washed several times before use. These cells will be referred to as "saturated cells." In the second experiment chicken red blood cells were prepared as a 20 per cent suspension, mixed with equal parts of virus, incubated for a total of 6 hours at 37 C, washed several times, and finally stabilized by the addition of a small amount of normal allantoic fluid before use. These cells will be referred to as "unsaturated cells."

Because of the fact that PR8 virus is eluted relatively slowly from red blood cells, it was often difficult to obtain stable suspensions of PR8-treated cells that would not agglutinate spontaneously in saline. Since stable preparations are essential for all tests of comparative agglutinability, very long incubation periods and many washings were usually required. Fortunately it was observed that by the addition of small amounts (e.g., 12 per cent) of normal allantoic fluid, which contains an hemagglutination inhibitor, as was indicated above, the period of preparation could be greatly shortened.

From the tenth to the twelfth days after the preparation of the saturated and unsaturated cells was started, hemagglutination tests were conducted. Dilutions of NDV, Lee, and PR8 viruses were made in saline so that each dilution contained a known number of hemagglutinating units. In the first experiment these ranged from 32 to 256 units and in the second from 1 to 512 units. To 0.4 ml of each virus dilution there were added 0.4 ml of a 1.5 per cent suspension of virus-treated and control chicken cells. Tests in which NDV was used as the agglutinating agent were performed at 4 C; the others were done at room temperature. At the end of 1 hour the degree of agglutination in each tube was noted and compared.

The results are shown in table 2. It is seen that both the saturated and the unsaturated cells were equally inagglutinable in the presence of NDV. However, the unsaturated cells were apparently less altered, especially by NDV and PR8.

TABLE 2

Agglutinability by viruses of red blood cells variously treated with viruses

CHICKEN RBC TREATED WITH	TREATMENT	RESULTS* OF HEMAGGLUTINATION TESTS WITH INDICATED NUMBER OF HEMAGGLUTINATING UNITS OF VARIOUS VIRUSES																										
		NDV										Lee										PR8							
		512	256	128	64	32	16	8	4	2	1	512	256	128	64	32	16	8	4	2	1	256	128	64	32	16	8	4	2	1
Saline	Saturated 3%		4	4	4	4							4	4	4	4						4	4	4	4					
NDV	suspension of		0	0	0	0							3	3	2	1						4	4	4	4					
Lee	RBC treated		0	0	0	0							0	0	0	0						4	4	4	3					
PR8	at 37 C for 17		0	0	0	0							0	0	0	0						3	2	0	0					
NAF†	hr		4	—	—	4							4	—	—	4						4	—	—	—					
Saline	Unsaturated	4	4	4	4	4	4	4	4	3	2	4	4	4	4	4	4	4	4	4	3	4	4	4	4	3	3	3	2	2
NDV	20% suspen-	0	0	0	0	0	0	0	0	0	0	4	4	4	4	4	3	2	2	2	2	4	4	4	4	3	3	2	2	±
Lee	sion of RBC	0	0	0	0	0	0	0	0	0	0	±	±	0	0	0	0	0	0	0	0	4	4	4	4	3	2	1	0	0
PR8	treated at 37 C	0	0	0	0	0	0	0	0	0	0	4	4	4	3	2	0	0	0	0	0	4	4	4	3	3	3	2	±	±
NAF	for 16 hr	4	4	4	4	4	4	4	3	3	2	4	4	4	4	4	4	4	4	4	3	4	4	4	4	4	4	3	2	1

* 4 = complete agglutination; 3, 2, and 1 = partial agglutination (2 is considered end point); 0 = no agglutination.
† NAF = normal allantoic fluid.

In the presence of Lee and PR8 these cells show less loss of agglutinability than did the saturated cells, although in both experiments the differences from the controls were in the same direction. The differences between the reactivity of Lee-treated cells with Lee virus were minimal. In neither instance could definite agglutinability be demonstrated even in the presence of 256 hemagglutinating units of virus.

If the first experiment is considered alone, it would appear that PR8 virus was most effective in modifying the agglutinability of chicken red blood cells, followed closely by Lee and then by NDV. However, if the second experiment is considered alone, the order is slightly changed. Lee is the most effective in altering the reactivity of the cells, followed by PR8 and then NDV. If these results are considered in terms of exhausting the receptors of the erythrocytes for these viruses, as has been suggested, they offer evidence to support Burnet (1945*b*) that the receptor for NDV is most readily removed. The receptor for

Lee would seem to be more quickly exhausted than that for PR8, although when prolonged treatment is permitted, that for PR8 virus appears to be slightly more effectively removed.

Table 2 also shows the advantage of testing treated cells against more than one dilution of virus. In the first experiment quite different results would have been obtained if a single dilution of 256 or of 64 units of PR8 had been used, and similarly in the second experiment, if just 64 or 16 units of Lee had been employed.

Neither of these experiments shows the complete reciprocal loss of agglutinability in the presence of PR8 and Lee by PR8- and Lee-treated cells reported by Hirst (1942). However, the results of tests with saturated cells and 64 or fewer hemagglutinating units in the first experiment do approach this and suggest that the method of treatment be considered in all its details in any discussion of alteration of agglutinability of treated erythrocytes.

Alterations in adsorptive capacity of treated red blood cells. From his studies with PR8 and Lee, Hirst (1942) reported that changes in agglutinability of treated cells are paralleled by changes in their capacity to adsorb fresh virus. A number of experiments were, therefore, conducted with NDV, PR8, and Lee viruses in order to learn whether changes could be detected in the adsorptive capacity of treated cells comparable to the modifications in their agglutinability described previously.

Appropriate mixtures of red cells and viruses were prepared. After a designated time the amount of virus adsorbed by the cells as reflected in a decrease in the hemagglutinating titer of the supernatant was determined. In a few experiments the amount of virus that could be subsequently eluted from the sedimented cells was also measured.

Experiments were carried out with the same saturated and unsaturated chicken red cells that were used in the agglutination experiments presented in table 2. However, the former cells were 12 days old at the time of the test and the latter only 2 and 3 days old. Since it was not necessary to have completely stabilized cells, the unsaturated cells were merely suspended in saline. Adsorption with NDV was permitted to take place for 5 to 10 minutes at 4 C, with Lee for 10 minutes at room temperature, and with PR8 for 40 minutes at room temperature. These intervals were selected after preliminary experiments with untreated red cells had shown them to be satisfactory for each of the viruses. After the sediment was washed, virus was permitted to elute into fresh saline at 37 C. In the experiment with saturated cells the detection of eluted virus was not begun until after the cells and virus had been together at room temperature for 1 hour; in the experiment with unsaturated cells this was begun immediately after the previously indicated intervals. In the former experiment the amount of virus adsorbed by a 3 per cent cell suspension was studied, and in the latter the amount adsorbed by a 10 per cent suspension was determined.

The results are summarized in table 3. The first experiment was with saturated cells. It was found that none of the treated cells adsorbed any NDV that could be detected either by reduction in titer of the supernatant or by elution

from the sedimented cells. Although none of the virus-treated cells reduced the titer of the Lee supernatant, the NDV- and Lee-treated cells apparently adsorbed some of this virus for it was subsequently eluted from them. The greater sensitivity of elution as an index of adsorption capacity is even better illustrated with these cells and PR8. In no instance with virus-treated cells was the titer of the supernatant significantly reduced, yet all three of the types of virus-treated cells later showed elution of some virus. The fact that a 50 per cent reduction in virus concentration must take place before it is reflected by a single tube change in the former test no doubt accounts for the foregoing findings. The results of the second experiment in which more concentrated

TABLE 3

Adsorption of viruses by red blood cells variously treated with viruses

TREATED WITH	CHICKEN RBC TREATMENT	CONCENTRATION USED FOR ADSORPTION	HEMAGGLUTINATION TITERS* OF VIRUS REMAINING IN THE SUPERNATANT AND ELUTED FROM THE RBC SEDIMENT FOLLOWING ADSORPTION OF INDICATED VIRUSES					
			NDV		Lee		PR8	
			Supernatant	Eluate from RBC	Supernatant	Eluate from RBC	Supernatant	Eluate from RBC
		per cent						
(Control—no RBC)	Saturated 3% suspension of RBC treated at 37 C for 17 hr	3	640	—	1,280	—	320	—
Saline			160	64	320	16	<20	512
NDV			640	<4	2,560	8	160	16
Lee			1,280	<4	2,560	4	640	32
PR8			640	<4	1,280	<4	160	32
(Control—no RBC)	Unsaturated 20% suspension of RBC treated at 37 C for 6 hr	10	128	—	1,024	—	384	—
Saline			48	16	32	>128	<4	38
NDV			128	<2	128	96	32	54
Lee			128	<2	1,024	2	128	18
PR8			128	<2	1,024	4	128	13
NAF			48	16	32	>128	<4	35

* Reciprocal of dilution giving titration end point.

although unsaturated cells were used differed from the first chiefly in quantitative aspects. In those instances in which virus was adsorbed, it was usually reflected by both a drop in titer of the supernatant and a greater elution of virus from the sedimented cells. This might have been because a more concentrated cell suspension was used, and hence more cells were available for adsorbing virus, or because the less completely treated cells were less modified, or because of a combination of both factors. It would seem that the most reliable results would be obtained if saturated cells and more concentrated cell suspensions were used.

A comparison of the results presented in tables 2 and 3 in general confirms Hirst's impression that there are parallel changes in the agglutinability and absorptive capacity of treated cells. This correlation is especially good when

NDV is used as the test agent, somewhat less so when PR8 is the virus, and only fair in the presence of Lee. Even though PR8-treated cells were agglutinated by 32 units of Lee in the second experiment, the eluate gave the only evidence that there had been any adsorption of virus. Conversely, the Lee-treated cells that were inagglutinable by 256 units of Lee apparently did adsorb some virus, as could subsequently be detected by elution. These findings indicate once again that the alterations produced in red blood cells are to a degree specific for the virus used. Those induced by NDV and PR8 are most apparent when NDV is used as the test agent, less so if Lee is the virus to be adsorbed, and least in the presence of PR8. In addition, in arranging these three viruses in order of ascending degrees of effectiveness in modifying the adsorptive capacity of chicken erythrocytes the order is also NDV, Lee, and PR8 although the difference between PR8 and Lee is very slight.

Attempt to correlate alterations in treated red blood cells with those in infected chick embryos. The superficial resemblance of the induction of refractory red blood cells to interference has been noted by others (Ziegler and Horsfall, 1944; Stone, 1947). It was hoped that the present investigation might offer support for the use of hemagglutination as a model for the study of viral infections. The results of experiments with virus-treated red blood cells such as those that have been presented above suggested a direct approach to this question. In all of the experiments described erythrocytes treated with PR8 were subsequently unable to adsorb or to be agglutinated by NDV, whereas cells treated with NDV were still able to adsorb and to be agglutinated by PR8 almost as well as were the controls. Therefore, if hemagglutination were a satisfactory model of virus infection, changes similar to these might be expected to occur in chick embryos following infection with these viruses. Chick embryos infected by PR8 should be resistant to infection by NDV, whereas embryos infected by NDV should still be susceptible to PR8.

Before this possibility could be examined, a method had to be devised that would permit the accurate detection of relatively small amounts of one of these viruses in the presence of the other. After a number of trials with allantoic fluids containing a mixture of two viruses, the following procedure was found to be sufficiently sensitive for this purpose: Serial dilutions of a fluid were made in triplicate in 0.2-ml quantities. There were then added to each tube of one series 0.2 ml of a 1:20 to 1:100 dilution of immune rabbit serum containing 32 antibody units against one of the viruses, to each tube of the second series the same amount of rabbit antiserum against the second virus, and to the third series a dilution of normal rabbit serum equal to that of the more concentrated of the other two sera. It was, however, necessary to select sera that, when diluted as just indicated, would show no nonspecific inhibition or chicken red blood cell agglutinins. The subsequent procedures were similar to those employed in titrating antisera. The mixtures were shaken, incubated at 37 C for 60 minutes, and chilled, after which 0.4 ml of a cold 1.5 per cent chicken red blood cell suspension were added. The end point in the presence of each of these sera was the highest dilution of a fluid giving a 2+ agglutination. As controls, allantoic

fluids that contained only one of the two viruses were similarly examined. With each fluid the titers obtained in the presence of normal rabbit serum were compared with those obtained in the presence of each of the two antisera. Reduction of the hemagglutination titer by an immune serum is a measure of the amount of homologous virus present. However, this reduction may be obscured and made to appear less if heterologous virus is also present in the mixture. Therefore, the degree of reduction in titer of a control fluid that contained only

TABLE 4

Serologic analysis of mixtures of allantoic fluids containing two viruses

MIXTURE OF INFECTED ALLANTOIC FLUID		RABBIT SERUM*	RESULTS† OF HEMAGGLUTINATION-INHIBITION TESTS FINAL DILUTION OF ALLANTOIC FLUID MIXTURES										INHIBITION END POINT
PR8	Lee		4	8	16	32	64	128	256	512	1,024	2,048	
ml	*ml*												
1.0	0.0	Normal	4	4	4	4	4	4	3	2	0	0	512
		Anti-PR8	4	2	0	0	0	0	0	0	0	0	8
		Anti-Lee	4	4	4	4	4	4	2	0	0	0	256
0.0	1.0	Normal	4	4	4	4	4	4	3	2	0	0	512
		Anti-PR8	4	4	4	4	4	4	3	1	0	0	256
		Anti-Lee	3	0	0	0	0	0	0	0	0	0	4
0.5	0.5	Normal	4	4	4	4	4	4	3	2	1	0	512
		Anti-PR8	4	4	4	4	4	3	0	0	0	0	128
		Anti-Lee	4	4	4	4	4	3	0	0	0	0	128
0.95	0.05	Normal	4	4	4	4	4	4	3	3	1	0	512
		Anti-PR8	4	4	4	2	0	0	0	0	0	0	32
		Anti-Lee	4	4	4	4	4	4	2	0	0	0	256
0.05	0.95	Normal	4	4	4	4	4	4	3	2	0	0	512
		Anti-PR8	4	4	4	4	4	4	3	0	0	0	256
		Anti-Lee	3	3	3	0	0	0	0	0	0	0	16

* Constant amount of serum was used, final dilutions ranged from 1:20 to 1:100, corresponding to approximately 32 hemagglutination-inhibition units.

† 4 = complete agglutination; 3, 2, and 1 = partial agglutination (2 is considered end point); 0 = no agglutination.

one virus was first determined and then compared with the degree of reduction demonstrable with the same sera and a fluid suspected of being a mixture. If in an immune serum the decrease in titer of the unknown was less than that exhibited with the control fluid, heterologous virus was assumed to be present. With this method it was possible with considerable accuracy to determine the composition of mixtures of any two of the group of PR8, Lee, and NDV, as is done, for example, in table 4 with PR8 and Lee. It was also possible to undertake the study of the changes in susceptibility of chick embryos that follow infection.

Several experiments were performed in which 11-day-old chick embryos were given two intra-allantoic inoculations 24 hours apart. These were given through a small shell hole that had been made directly over the embryo. Reinoculations were done through a paraffin seal over the site of the first inoculation. The volume of the first inoculum was 0.1 ml and of the second 0.5 ml. The inocula

TABLE 5

Serologic analysis of representative allantoic fluids from chick embryos inoculated with two viruses

INOCULA (INTRA-ALLANTOIC)					RABBIT SERUM*	HEMAGGLUTINATION INHIBITION END POINTS†	VIRUS DEMONSTRATED	
First		Interval	Second					
0.1 ml	E.I.D.		0.5 ml	E.I.D.			PR8	NDV
		hours						
NDV.....	$10^{5.0}$	24	Broth	—	Normal	512	0	+
					Anti-PR8	512		
					Anti-NDV	16		
NDV.....	$10^{5.0}$	24	PR8	$10^{5.7}$	Normal	256	0	+
					Anti-PR8	256		
					Anti-NDV	8		
PR8.....	$10^{4.0}$	24	Broth	—	Normal	512	+	0
					Anti-PR8	8		
					Anti-NDV	256		
PR8.....	$10^{4.0}$	24	NDV	$10^{6.7}$	Normal	512	+	0
					Anti-PR8	16		
					Anti-NDV	256		
Broth.....	—	24	NDV	$10^{6.7}$	Normal	512	0	+
					Anti-PR8	512		
					Anti-NDV	16		
Broth.....	—	24	PR8	$10^{5.7}$	Normal	1,024	+	0
					Anti-PR8	16		
					Anti-NDV	512		

* Constant amount of serum was used, final dilution ranged from 1:20 to 1:100, corresponding to approximately 32 hemagglutination-inhibition units.

† Reciprocal of dilution giving titration end point.

contained large amounts of virus in order to simulate the conditions under which the red blood cells were treated. They contained approximately 10,000 and 500,000 E.I.D., respectively, of PR8, and 100,000 and 5,000,000 E.I.D., respectively, of NDV. Twenty-four hours after the second inoculation the eggs were removed from the incubator to the refrigerator and chilled before the allantoic fluids were collected. These fluids were analyzed for virus content by the method described above.

The results presented in table 5 are representative of those obtained in numerous double infection experiments. The susceptibility of the embryo was so altered by whichever virus first infected it that the secondary introduction of very large amounts of heterologous virus failed to induce infection. Unlike what occurred *in vitro* with red cells, there was no evidence that the PR8 virus was able to infect cells that had been previously infected by NDV. Indeed, a total of 11 embryos were given a preliminary inoculation of 100,000 E.I.D. of NDV, followed after 24 hours by 500,000 E.I.D. of PR8. When the allantoic fluids were removed 24 hours later, NDV was the only virus that could be detected in any of them. Yet the controls indicated that this amount of PR8 produced maximal infection in normal chick embryos.

Ziegler and Horsfall (1944), by using very large inocula comparable to those in the present experiments, were able to show that, although infection of chick embryos by PR8 uniformly interfered with subsequent infection by Lee, a primary infection by Lee was not always able to prevent later infection by large amounts of PR8. Such results would seem to be consistent with what one might have predicted from agglutination experiments with treated erythrocytes such as are given in table 2. Consequently, the failure to show a similar correlation between the results with PR8 and NDV in the foregoing experiments appears significant. It indicates that different mechanisms are probably involved in inducing a refractory state in red blood cells and in modifying cells of chick embryos so that a second infection is blocked. It also emphasizes an inadequacy of the hemagglutination phenomenon as a model of virus infections.

DISCUSSION

The nature of the characteristic changes that are produced in erythrocytes by hemagglutinating viruses is still not known. It has been suggested that they result from "exhaustion of the receptors" of the cells (Hirst, 1942). Indeed, Burnet *et al.* (1946) have presented evidence that a lecithinase, if permitted to act for varying periods of time, may modify the reactivity of red blood cells in a linear fashion somewhat similar to the hemagglutinating viruses. From this it was implied, as by Hirst (1942) previously, that the exhaustion may be enzymatic in nature. Bovarnick and de Burgh (1947) have recently presented evidence that the receptors of erythrocytes for the several hemagglutinating viruses may be different. They prepared a lipid extract from sheep red blood cells that prevented agglutination of human and chicken erythrocytes by mumps virus but not by PR8, whereas a similar extract from human red blood cells prevented agglutination of these cells by both mumps and PR8. Although the evidence is not yet complete, it is very suggestive that in hemagglutination there is a reaction between a component in or on the red blood cell surface and the virus that results in a permanent modification of the cell surface. This modification appears to be specific for the hemagglutinating virus.

The changes in the reactivity of virus-treated red blood cells are so striking that it was natural to inquire whether similar alterations might occur in tissue cells after infection, even though the many differences between these two types

of cells and in the procedures are quite obvious. A simple experiment with which to seek an answer to this question was suggested by the observation that cells treated with PR8 were subsequently unable to adsorb or to be agglutinated by NDV, whereas cells treated with NDV subsequently absorbed and were agglutinated by PR8 virus to essentially the same degree as were controls. Therefore, chick embryos were infected first with PR8 or NDV and subsequently challenged with large amounts of the heterologous virus. Unlike the virus-treated red blood cells, they manifested no selective blocking of the second virus. Whichever virus was introduced first prevented infection by the second.

The foregoing findings are consistent with the results of experiments by Henle and Henle (1947) that also indicate a difference between the capacity of a virus to render red blood cells refractory to agglutination and its capacity to block infection. These authors treated influenza viruses with ultraviolet light for varying periods of time and were able to demonstrate that the property of blocking infection could be destroyed much more quickly than the property of rendering red blood cells refractory to agglutination.

SUMMARY

Alterations characteristic of the virus employed take place in chicken red blood cells following treatment with Newcastle disease virus (NDV) and the two influenza viruses, PR8 and Lee. These changes are reflected in parallel losses in the adsorptive capacity and agglutinability of treated cells for fresh homologous and heterologous virus. These modifications in red blood cells may persist for at least as long as 21 days. The extent of the alterations is directly proportional to the intensity of viral treatment. When a 3 per cent suspension of chicken erythrocytes is treated for 17 hours at 37 C with NDV, Lee, or PR8 viruses, the most marked changes are produced by PR8, slightly less striking ones follow treatment with Lee, and the least modifications follow treatment with NDV. If the cells are less thoroughly treated, the order is Lee, PR8, and NDV. Further evidence is presented that the hemagglutination phenomenon is an inadequate model for the study of viral infections. The modifications in erythrocytes that follow treatment with NDV are not correlated with changes that follow infection of chick embryos by this virus. Although red blood cells that have been treated with NDV are still capable of adsorbing and being agglutinated by PR8, chick embryos that have been infected by NDV are after 24 hours no longer susceptible to infection with as much as 500,000 embryo infective doses of PR8.

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