row. spleen and liver.[20,21] The anemia of Hodgkin's disease has been ascribed to erythrophagocytosis (in lymph nodes, liver, lungs and bone marrow),[22] and the severe anemia of primary splenic panhematopenia has been attributed to excessive phagocytic function of the spleen.[23] Throughout the literature, macrophages have been the chief phagocytic cell reported. Only in patients with severe pernicious anemia,[9] and a severe "secondary anemia" (probably of a leukemia)[13] have granulocytes been the predominating phagocytic cell type.

In animals, toxic or infectious states have led to anemic conditions associated with erythrophagocytosis.[24] Most prominent of these is the bartonellosis of rats,[25] similar to Oroya fever in man. It is of interest that this condition is reported to be improved by treatment with PGA.[26] Other studies in man and rodents indicate an increased phagocytic function of granulocytes in anemia (including pernicious anemia), the increase in phagocytosis being roughly proportional to the severity of the anemia.[27]

The clinical and experimental data suggest, therefore, that erythrophagocytosis may be associated with a PGA-deficient state. Further investigation is required to ascertain if such a relationship exists.

Summary. Blood cell cultures grown in the presence of PGA-antagonists exhibited marked erythrophagocytosis by granulocytes when compared with cultures containing no antagonist or containing antagonist and PGA. It is suggested that PGA-deficiency might account for erythrophagocytosis observed in certain instances of anemia in humans and animals.

20 *Report of First Expedition to South America,* 1915, Cambridge, Harvard University Press, pp. 50-51.

21 Ash, J. E., and Spitz, S., *Pathology of Tropical Diseases,* W. B. Saunders Co., Philadelphia, 1945, pp. 25-26.

22 Marschal, G., Mahoudeau, D., Loc'h Le, and Brun, C., *Bull. et mem. Soc. d'hop de Paris,* 1941, **57**, 15.

23 Doan, C. A., and Wright, C., *Blood,* 1946, **1**, 10.

24 Jaffe, R. H., in Downey, H., *Handbook of Hematology,* Paul B. Hoeber, Inc., New York, 1938, p. 1017.

25 Weinman, D., *J. Infect. Dis.,* 1938, **63**, 1.

26 Smith, H. M., and Emery, F. E., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 178.

27 (a) Berry, L. J., Leyendecker, R. M., and Spies, T. D., Morphologic Hematology, Special Issue No. 1 of *Blood,* Grune and Stratton, New York, 1947, p. 98; (b) Berry, L. J., and Haller, E. C., *ibid.,* p. 108; (c) Berry, L. J., and Haller, E. C., *ibid.,* p. 117.

16494

Electrokinetic Change in Human Erythrocytes During Adsorption and Elution of PR8 Influenza Virus.*

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Hirst[1,2] and McClelland and Hare[3] found that PR8 strain of influenza virus could be adsorbed by chicken erythrocytes with their accompanying agglutination. On standing it was also found that the virus was gradually eluted from the cells and that the cells could no longer adsorb subsequent additions of that

* Aided by grants from the National Foundation for Infantile Paralysis, Inc., and from the Commission on Influenza, Army Epidemiological Board, Office of Surgeon General, U. S. Army.

1 Hirst, G. K., *Science,* 1941, **94**, 22.

2 Hirst, G. K., *J. Exp. Med.,* 1942, **76**, 195.

3 McClelland, L., and Hare, R., *Can. Pub. Health J.,* 1941, **32**, 530.

virus. The agglutination phenomenon was rapidly developed into a quantitative test[4-6] for influenza viruses and became an invaluable tool in the elucidation of their properties and in their purification.[7-9] Little is known concerning the physical or chemical mechanism of the agglutination, however, and the present work is the initial aspect of such an investigation.

Since no detectable change in the electrokinetic characteristics of red blood cells in the presence of egg albumen, gelatin, casein, hemoglobin and fibrinogen has been observed, it has been concluded that the cell retains its surface properties even in the presence of large amounts of proteins.[10] The present paper, however, reports observations that the electrokinetic potential of human erythrocytes is greatly decreased by minute additions of influenza virus, that this decrease accompanies the adsorption and elution phenomena, and that the reaction is dependent upon the concentration of added virus.

Materials and methods. Preparation of virus. The PR8 strain of Type A influenza virus maintained by egg passage was employed. Twenty-five dozen 10-day embryonated hen's eggs were inoculated by the allantoic route with 0.1 ml of infectious allantoic fluid diluted to 10^{-3} with 0.9% NaCl. The inoculum before dilution had an agglutination titer of 1-5120 by a pattern test. The eggs were sealed with paraffin and were incubated at 36°C for 48 hours. The allantoic fluids were harvested by pouring through a funnel lightly plugged with glass wool. The resulting 1900 ml of infectious allantoic fluid had an agglutination titer of 1-5120. Adult chicken red blood cells were added to make a 2% suspension, and adsorption was allowed to take place for 2 hours at 4°C. The cells were centrifuged out of the suspension and were resuspended in 80 ml M/15 sodium potassium phosphate buffer, pH 7.4,[11] at 37°C for 90 minutes for elution to take place. The cells were removed and washed by centrifugation with 50 ml buffer at 37°C. The supernates were combined and clarified at 3000 r.p.m. for 20 minutes. The virus was sedimented from this solution in a high speed angle centrifuge for 90 minutes at 14,400 r.p.m. at 4°C. The resulting pellets were dissolved in 900 ml buffer at 4°C. Fresh chicken erythrocytes were added to make a 2% suspension and an identical cycle of adsorption, elution and differential centrifugation was repeated. The total yield was 12.0 ml of virus preparation† having a titer of 1-160,000 and containing 2.8 mg of material per ml when dried in air for 3 hours at 110°C and corrected for the salt content. The concentrated virus material when coated on collodion particles in M/15 sodium potassium phosphate buffer, pH 7.4 had an electrophoretic mobility of 0.67 μ/sec./volt./ cm at a concentration of 0.14% at 22.5 ± 1.5°C. Electron micrographs showed little evidence of extraneous material or particles of smaller size, the purification procedure having been designed to reduce the amount of normal, acidic component.

Titration of virus. The titrations were carried out by the procedure of Salk,[6] employing a suspension of 0.25% chicken red blood cells, with the exception of the end point taken. The modification used served to decrease the great differences in reading end points inherent in the 2-fold dilution procedure. The readings

[4] Hirst, G. H., and Pickels, E. G., *J. Immunol.*, 1942, **45**, 273.

[5] Miller, G., and Stanley, W. M., *J. Exp. Med.*, 1944, **79**, 185.

[6] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

[7] Lauffer, M. A., and Stanley, W. M., *J. Exp. Med.*, 1944, **80**, 531.

[8] Sharp, D. G., Taylor, A. R., McLean, I. W., Beard, D., Beard, J. W., Feller, A. E., and Dingle, J. H., *J. Immunol.*, 1944, **48**, 129.

[9] Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.

[10] Abramson, H. A., Moyer, L. S., and Gorin, M. H., *Electrophoresis of Proteins*, Rheinhold Pub. Corp., N. Y., 1942, 307.

[11] Clark, W. M., *The Determination of Hydrogen Ions*, The Williams and Wilkins Co., Baltimore, 1928, 212.

† Hereinafter the term, virus, refers to this concentrated material containing the PR8 strain of influenza virus, Type A, and the quantitative values refer to the dry weight of the preparation.

TABLE I.

Relation Between Mobility of Erythrocytes and Hemagglutination Titer with Different Concentrations of Virus.

16.9 μg/ml			3.11 μg/ml			0.933 μg/ml			0.350 μg/ml			0.311 μg/ml		
Min.	Mobility	Titer	Min.	Mobility	Titer	Min.	Mobility	Titer	Min.	Mobility	Titer	Min.	Mobility	Titer
							Controls.							
—33		960	—26		160	—21		64	—19	1.28		—22		10
—10	1.34		—10	1.33		— 8	1.38		— 9		12	—17	1.37	
235	1.28		1391	1.28		15	1.35		51	1.30		19	1.34	
375	1.30					155	1.31		235	1.30		379	1.31	
1486	1.30					388	1.35		1473	1.35		1445	1.38	
						1719	1.39							
							Virus.							
5	0.96		14		160	10	1.37		20	1.25		12		16
8		480	18	1.25		16		32	29		0	40		2
30		160	36		35	34		8	69		0	45	1.21	
50	0.68		88		10	35	1.37		126	1.19		91	1.28	
60		240	98	1.01		62		8	149		0	104		0
100	0.61		170		20	75	1.19		195	1.17		176		0
184		800	193	0.69		82		8	225		0	184	1.28	
207	0.52		235		50	99		8	313	1.05		244	1.21	
265		1280	267	0.65		134		8	712		0	260		0
314	0.57		291		70	173	1.18		734	0.96		348	1.19	
340		960	364		70	179		8	1380		0	353		0
870	0.49		369	0.56		230	1.03		1390	0.86		389	1.12	
895		1280	385		160	234		8	1583		2	743	1.14	
1422		1120	1361		240	251		16	1656	0.78		763		0
1505	0.50		1431	0.58		282		16	1725		4	1389		0
1681	0.50					364	0.91		1749	0.66		1467	0.99	
						365		16	2785		16	1775	0.60	
						1435		64	2893	0.52		1807		6
						1494	0.66					2873		24
						1688		128						
						1693	0.63							

of +, ±, or O were read as in Salk's method. However, ± instead of + was taken as the end point. Where + proved to be the observed end point followed by O, ± was interpolated halfway between the two values. All titrations were run in duplicate and when the duplicates differed, as they occasionally did, their values were averaged. The dilutions for the titrations were made in the same stock M/15 sodium potassium phosphate buffer, pH 7.4, as were those for the electrophoretic experiments.

Microelectrophoretic measurements. The electrophoretic mobility was measured by the method of Moyer.[12] Human type O red blood cells rather than chicken red blood cells were used because of their smaller size and lower sedimentation rate. They were prepared by washing 3 times with M/15 sodium potassium phosphate buffer, pH 7.4 and were diluted to 10% suspensions in the buffer immediately after their preparation. They were stored at 4°C. The erythrocytes were never used after 7 days although they maintained their original electrokinetic properties at least until 10 days after shedding. In one experiment in the presence of 5.6 μg of virus suspension per ml there was no significant electrophoretic difference between type O and type AB cells. Therefore, type O cells from a single donor were used throughout. Agglutination of the erythrocytes into groups of 2-6 in the presence of the virus could regularly be seen in the microscope during the measurements. These groups did not seem to differ in mobility from single cells and were also used in measuring mobility. Equal numbers of 12-15 measurements in each direction were made in the microelectrophoresis cell at the 0.21 level. These were averaged. The erythrocytes were suspended at a concentration of 0.25% in buffer. The measurements were conducted at 22.5° ± 1.5°C. The instrument was rigorously cleaned with dichromate cleaning solution and distilled water before the introduction of each sample.

Experimental. Two 250 ml flasks were used to contain the control and the virus-red blood cell suspensions for the duration of a single experiment. That one serving as the control flask contained 100 ml of a 0.25% suspension of human type O erythrocytes in buffer. The second flask contained 150 ml of purified virus preparation in the selected dilution with the same stock buffer for the particular series of observations. Just prior to the addition of the erythrocytes to the virus suspension, a 3.75 ml sample was withdrawn for subsequent titration. The same volume of the 10% stock suspension of red blood cells was then added to the virus suspension and the time was noted. The time of admixture of the reagents is considered to be zero time for comparison of the different runs at the various concentrations. Measurements of agglutinating activity of the virus and of electrophoretic mobility of the red cells which served as controls, and which were made before zero time are tabulated in negative time increments, Table I. At convenient intervals after the admixture of reagents, microelectrophoretic measurements were made on the virus and control samples. These measurements usually required 10-15 minutes. The average time between the first and last readings was selected as the time of the measurement from zero time.

Concurrent with the microelectrophoretic measurements 3.0 ml samples of the virus-erythrocyte suspensions were periodically withdrawn for titration. These were immediately centrifuged at 2,500 r.p.m. for 10 minutes to remove the erythrocytes. The supernates were carefully withdrawn from the red cell precipitates, and the time was noted. The supernates were stoppered and placed in the refrigerator at 4°C for subsequent titration. Upon completion of a run, usually within 1800 minutes from zero time, all the supernates and the original virus suspension were titered together under the same conditions with one pool of chicken erythrocytes.

Results. The relation between the quantity of virus found in the supernates during the adsorption and elution processes and the electrophoretic mobility of the erythrocytes at varying time intervals is shown in Table I and Figs. 1, 2, 3. The concentrations of the virus material were 16.9, 3.11, 0.933, 0.350 and 0.311 μg per ml respectively. The titers are expressed as dilutions of the stock virus sus-

[12] Moyer, L., *J. Bact.*, 1936, **31**, 531.

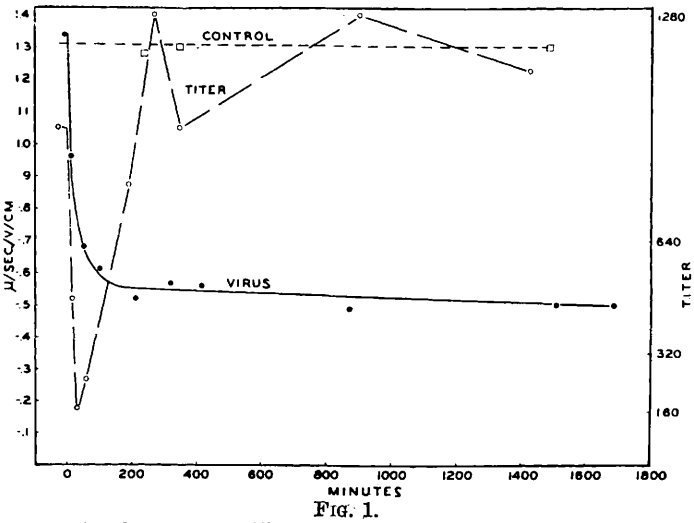

FIG. 1.

Relation between mobility of 0.25% erythrocytes and adsorption-elution in the presence of excess virus, 16.9 μg/ml.

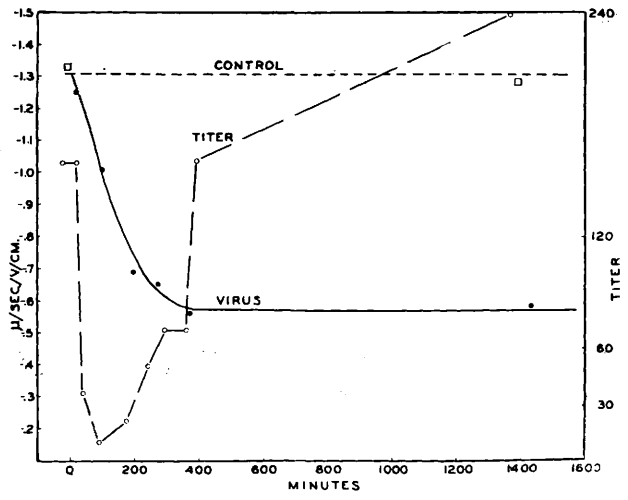

FIG. 2.

Relation between mobility of 0.25% erythrocytes and adsorption-elution in the presence of the optimal concentration of virus, 3.11 μg/ml.

pension containing 2.8 mg/ml.

At the higher virus concentrations, 16.9 μg/ml (Fig. 1, Table I), there is an initial sharp drop in titer as adsorption of virus takes place on the cells. This is followed by rapid elution. Corresponding to the adsorption of the virus by the erythrocytes there is a sharp drop in the electrophoretic mobility of the cells from the normal value of 1.32 to about 0.55 μ/sec/v/cm. When the virus is eluted from the cells, as indicated by the rise in titer of the supernate, there is no corresponding rise in the electrophoretic mobility. It remains at the lower value.

As the virus concentration is gradually decreased down to 0.311 μg/ml, (Figs. 2, 3, Table I), the sharp initial drop in titer persists, but the elution is delayed. The negative slope of electrophoretic mobility also decreases. When a concentration of 0.350 μg/ml is reached (Fig. 3), the virus remains adsorbed to the erythrocytes for as long as 1400 minutes

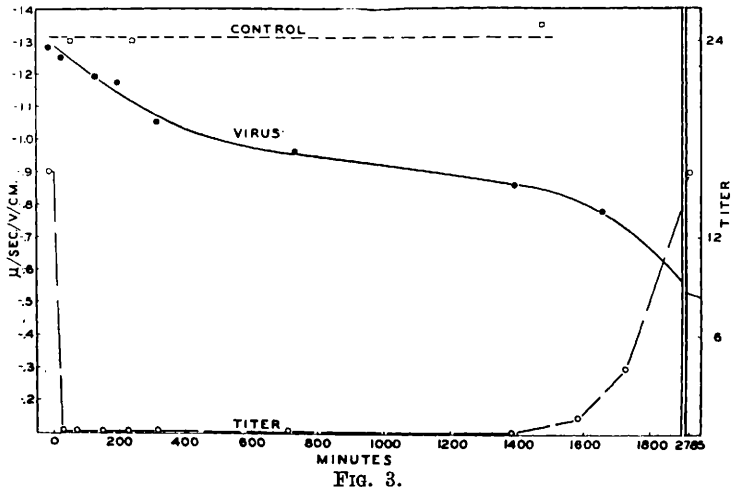

FIG. 3.

Relation between mobility of 0.25% erythrocytes and adsorption-elution in the presence of insufficient virus, 0.350 μg/ml.

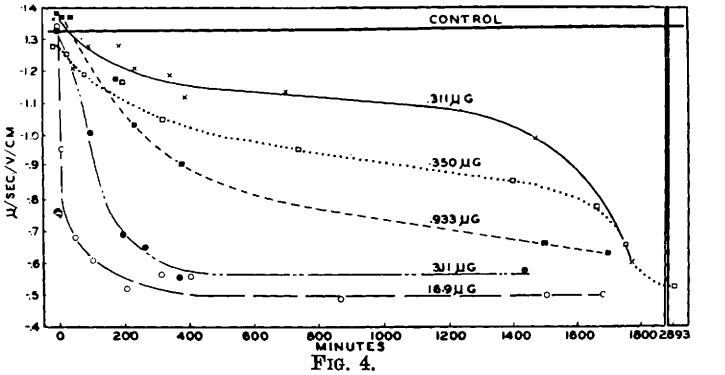

FIG. 4.

Comparison of mobility of 0.25% erythrocytes in the presence of various concentrations of virus illustrating how the mobility pattern changes with the concentration of virus.

before elution is detected, and at this time the electrophoretic mobility also begins to fall to the same low value reached by those cells treated with the higher concentrations of virus. It may be seen, therefore, that the shapes of both the electrophoretic mobility curves and the adsorption-elution curves are not only interdependent but also are concentration dependent. The dependence of adsorption and elution upon virus concentration at constant concentration of erythrocytes is illustrated in Fig. 5. For greater clarity the curve for 0.350 μg virus/ml has been deleted as it falls so close to the curve at 0.311 μg virus/ml. The dependence of electrophoretic mobility upon virus concentration is illustrated in Fig. 4. The heavy line representing the controls, no virus added, is a composite of 19 points taken concurrently with the curves made in the presence of the various concentrations of virus. It was drawn by the method of least squares. The standard deviation, σ, is 0.035.

Discussion. The changing shape of the electrophoretic mobility curves as the virus concentration is increased, (Fig. 4), leads to the concept that there might be an optimal ratio of virus to cells at which all the receptors of the cells are acted upon at the same time. At this concentration it might be expected that for a short interval little or no virus would be found in the supernate. It would rapidly be adsorbed on, and then eluted from, the cells. From the adsorption-elution titrations, Fig. 5, it appears that the optimal virus concentra-

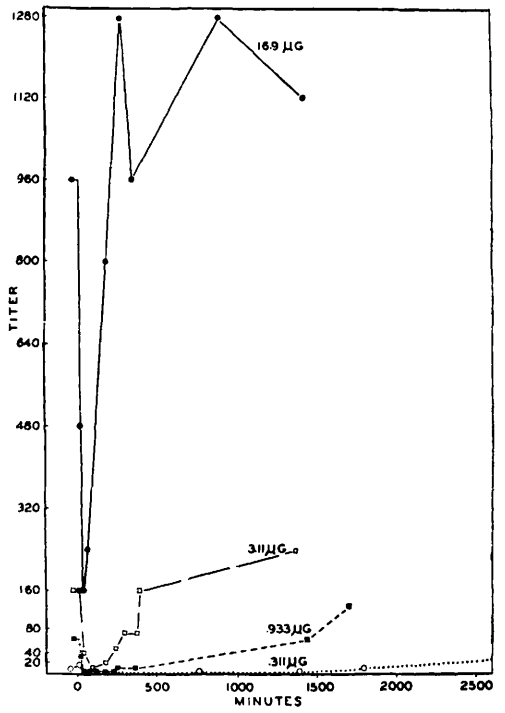

FIG. 5.

Comparison of the supernatant virus titers illustrating the changing pattern of adsorption-elution for various concentrations of virus in contact with 0.25% erythrocytes.

tion in the presence of 0.25% cells would be very close to 3.11 μg virus/ml. At a concentration of 16.9 μg/ml all the virus was not removed from the supernate while at 0.933 μg/ml it remained attached to the cells for over 200 minutes. Using the values, 3.11 μg virus/ml, 40 mμ as the radius of the anhydrous virus,[7] 1.27 as its density,[7] and 5.54×10^6 as the number of erythrocytes per cu mm of whole blood by actual count, it may be calculated that at the point of simultaneous saturation of the cell receptors by the virus, there are 298 PR8 particles to one erythrocyte. If one considers the erythrocyte as a sphere with a surface area of $120\mu^2$,[13] and the virus as a disk with a radius of 40 mμ, it is evident that 23,900 PR8 particles would be required to cover the erythrocyte with just a single layer. According to this calculation at the optimal concentration only 1/80 of the surface area of the cell is actually covered by the virus, a value inconsistent with the possibility of the cell's assuming the charge of a complete layer of the adsorbed virus. To support this evidence it is found that after elution of the virus from the cells, the cells continue to have the same lowered mobility. This mobility is lower than that of the virus itself and indicates that it is the mobility of the altered cells.

The changing shapes of the electrophoretic mobility curves with time for various virus concentrations (Fig. 4) may be interpreted to mean that at low virus concentrations the virus is adsorbed indiscriminately by the individual cells in chance amounts. When virus particles elute they are immediately adsorbed by other receptors on the same or on adjacent erythrocytes, gradually reducing the electrokinetic potential of all the cells in the suspension. As the ratio of virus particles to unreacted receptors on the cells increases, the rate at which the electrophoretic mobility falls off rapidly increases. The same low value of 0.5-0.6 μ/sec/v/cm is eventually reached as was attained 1500 minutes before by those cells in contact with higher virus concentrations.

That the decrease in electrophoretic mobility of the erythrocytes takes place while they are in intimate contact with the virus is indicated in Figs. 1, 2, and 3. Particularly in Fig. 3 where the virus and cells are in contact for a long time, it may be seen that some kind of reaction expressed by the decrease in mobility is in progress, and that this reaction is not complete until elution is complete. These results clearly indicate an ionogenic change in the character of the erythrocyte which is exhibited by its altered electrophoretic mobility. The nature of the ionogenic change, whether it is caused by loss of a charged substance from the cell, change of the spatial configuration of charged groupings of the cell, or adsorption by the cell of charged elements of the solvent after the cell's treatment by virus, is still obscure. Further experiments are in progress to determine a more exact interpretation of the phenomenon.

Aggregation of the virus after high speed centrifugation and disaggregation after ad-

[13] Best, C. H., and Taylor, N. B., *The Physiological Basis of Medical Practice*, The Williams and Wilkins Co., Baltimore, 1943, 11.

sorption and elution by the erythrocytes are clearly indicated in Fig. 5. It may be seen that for each concentration of virus in the adsorption-elution curves, the titer before adsorption is considerably lower than after elution. That packing of the virus during sedimentation causes aggregation is borne out by the electron micrographs which were made after centrifugation and before adsorption and elution by the erythrocytes. The micrographs showed most of the virus particles in a state of aggregation.

Conclusions. The electrophoretic mobility of human type O erythrocytes was found to decrease from 1.32 to 0.5-0.6 μ/sec./v./cm in the presence of 0.311 to 16.9 μg/ml of a highly purified preparation of the PR8 strain of influenza virus.

Adsorption and elution of the virus, and agglutination of the erythrocytes was found to be concurrent with the decrease in electrophoretic mobility of the erythrocytes.

The time of intimate contact of virus with cells and the pattern of decrease of electrophoretic mobility are not only interdependent but also dependent upon the concentration of added virus.

At the optimal ratio of virus to erythrocytes, as determined by the mobility and titration curves, it has been calculated that only 1/80 of the erythrocytic surface is covered by virus particles and that this value corresponds to about 298 PR8 virus particles to one erythrocyte.

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Relation of Induced Sulfonamide Resistance to Biochemical Properties in Alpha Streptococci.

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A decrease in the production of hydrogen peroxide accompanying an induced increase in resistance to sulfonamide bacteriostasis was first observed in a strain of pneumococcus by MacLeod.[1] Since some streptococci produce hydrogen peroxide, it seemed desirable to determine whether or not these organisms would produce less peroxide when they were made resistant to sulfonamide, whether other biochemical changes would be produced, and what relation these changes might have to the mechanism of resistance.

Loss of peroxide producing ability by resistant organisms. Five strains of greening streptococci originally obtained from saliva were tested for susceptibility to sulfathiazole by the serial dilution method, and for hydrogen peroxide production by the method of Main and Shinn[2] with slight modifications. They were then made resistant by transferring at intervals of 3 to 5 days in broth containing one-half the inhibiting concentration. All organisms were transferred 15 times. Sulfathiazole susceptibility and hydrogen peroxide production were then determined again. The results of the determination are shown in Table I. The sulfathiazole values represent the average of 3 or more determinations, none of which varied more than one tube from the value given. The peroxide production is expressed in values obtained from 3 or more determinations, none of which varied more than 5 μg/ml from the value given. Ability to produce peroxide was lost by all strains after they became resistant.

Comparison of other biochemical properties. Other biochemical changes were also investigated. Dehydrogenase activity was

[1] MacLeod, Colin M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 215.

[2] Main, Edna R., and Shinn, Laurance, E., *J. Biol. Chem.*, 1939, **128**, 417.