struction of inhibitor by SF continues during the period of the titrations when RBC are present, we may suppose that, as virus is regenerated from complexes with inhibitor, a system prepared by adding RBC to a mixture of SF and EW progresses toward a system in which the 3 reagents have been mixed simultaneously; consequently, the inhibition titer will be lower than one might predict from a consideration of the amount of inhibitor destruction alone.

It will be seen that this interpretation of the relations among SF, vaccine, and EW inhibitor is in general accord with the explanation recently offered by Burnet[3] and by Hirst[4] for an analogous set of relations involving other viruses and normal serum as inhibitor.

*Summary.* Evidence has been presented that the mechanism of egg-white inhibition of hemagglutination of chicken red blood cells by swine influenza virus involves combination between an egg-white component and the virus, this combination obstructing the reaction of virus with red blood cells. Moreover, it has been found that the inhibitor is rapidly inactivated by highly infectious virus, with regeneration of the virus, but not by an aged formolized vaccine consisting of inactivated virus; and that the inactivation of inhibitor by virus is suppressed by convalescent (anti-influenza) swine serum. The results have been interpreted in terms of an enzymatic hypothesis of virus function in the phenomenon.

16511

## Use of Formalin-Treated Red Cells for the Study of Influenza A Virus Hemagglutinating Activity.

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Human erythrocytes, treated with formalin, the writer has found, retain their ability to react with influenza A virus in a manner quite similar to that of untreated human erythrocytes. For certain types of experiments the use of such formalized red cells possesses distinct advantages over the use of normal red cells.

To prepare formalized red cells, equal volumes of a 50% (by volume) suspension of washed red cells in saline and formalin containing sodium chloride in a concentration of 0.85%, were mixed and allowed to stand for 2 to 3 days. If the formalin concentration of the mixture was between 10 and 50%, the cells became dark brown gradually over a period of 24 hours or so. The cells also became cohesive and very resistant to hemolytic agents. The stability of the cells thus treated was marked. Samples of formalized cells stored at 4°C have retained their activity undiminished for influenza A virus for almost two years now. Furthermore, the cells have retained the microscopic appearance and shape of fresh, normal, red cells.

Concentrations of formalin of less than 10% (3.6% formaldehyde) in the mixture failed to produce browning of the red cells over 2-3 days at 4°C usually, but did render the cells cohesive. Such cells hemolyzed more readily in hypotonic solutions than did the cells prepared with higher concentrations of formalin.

Before using the formalized red cells in reactions with influenza virus the uncombined formaldehyde was removed by repeated washing with saline or water. The cells were allowed to stand in contact with each wash fluid for a period of at least several hours to permit the establishment of an equilibrium between the free formaldehyde inside and outside the cells. Generally, 6 to 10 such

washings with about 5 volumes of diluent each, sufficed to yield cells with no obvious odor of formaldehyde and which released such small quantities of formaldehyde into the final wash that testing with Schiff's aldehyde reagent produced only a faint, delayed color reaction.

For studying electrolyte-free systems[1] water has an advantage over saline as the wash fluid. Water apparently removes inorganic ions from the interior of the modified cells without hemolysis as judged by examining the wash supernatant and the washed cells for their chloride content. Tests with silver nitrate on the final water supernatant have failed to demonstrate the presence of chloride ions. Similar tests done on acid digests of 3 ml of water-washed, packed, formalized red cells also failed to demonstrate the presence of chloride ions. The same quantity of normal red cells contains on the average 0.156 milliequivalents of chloride ions,[2] an amount readily detected in control tests by the technic used.

Upon standing in contact with water for 12 hours or more the water-washed formalized cells released a yellowish brown pigment to a slight degree into the water. The pigment probably was modified hemin, since it contained iron, reacted negatively for protein with Millon's, Hopkins-Cole, and biuret reagents, the xantho-protein test, and trichloracetic acid, and in addition, was non-dialyzable. The released pigment became noticeable in the clear supernatant only when the concentration of the cells in the suspension was high.

Washed formalized erythrocytes were found to adsorb and elute influenza A virus hemagglutinating activity in a manner indistinguishable from the action of normal red cells as described by Hirst.[3] Suspensions of influenza virus also caused agglutination of the formalized cells just as they agglutinate normal red cells.[4] Therefore, formalized red cells may replace normal erythrocytes in titrational tests for viral hemagglutinating activity. However, the formalized cells could not be adapted to the Salk pattern technic[5] since it was observed that these cells agglutinate spontaneously at the bottom of the tube. The turbidometric technic as devised by Hirst[6] or as modified to use the Klett-Summerson photoelectric colorimeter,[7] has been employed successfully to titrate viral activity using formalized cells. Due to the greater optical opacity and the slightly greater sedimentation rate of the modified cells as compared with the normal cells, the recorded procedures require slight modification for optimal results.

For the quantitative studies of the adsorption or elution of influenza virus by erythrocytes, formalized cells can be used advantageously in place of normal cells. Formalized cells were found to pack more quickly and firmly than normal cells upon centrifugation so that greater accuracy was obtained in preparing suspensions. Due also to the firm packing, supernatant solutions were more completely removed without loss of the sedimented cells than under comparable conditions with normal erythrocytes. No release of intracellular constituents to any appreciable degree occurs to complicate analyses of the supernatant liquid if the time of reaction is kept moderately brief and if the cells are washed with water previously. As indicated, a stock suspension of the modified cells may be prepared and used over a period of months with assured constancy of concentration and reactivity.

However, there are several disadvantages to the use of formalized red cells. Due to their cohesiveness the packed cells could not be drawn into a pipette without some previous mixing with a liquid diluent. To prepare a suspension of known concentration, a batch of packed cells, entirely free of supernatant,

[1] Flick, J. A., Sanford, B., and Mudd, S., *J. Immunol.*, in press.

[2] Peters, John P., *Body Water, the Exchange of Fluids in Man*, Springfield, Ill., Charles C. Thomas, 1935.

[3] Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

[4] Hirst, G. K., *Science*, 1941, **94**, 22.

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

[6] Hirst, G. K., and Pickels, E. G., *J. Immunol.*, 1942, **45**, 273.

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

was mixed with a known volume of diluent and the total volume of the resulting suspension measured. The concentration of the cells present was computed from the difference in the two volume measurements. Formalized red cells possessed negligible buffering power while normal red cells are known to exert a buffering action in suspension.[8] Probably due to modification of protein amino groups among others by the formaldehyde, the constituents of the cells could no longer appreciably unite with hydrogen ions. In the absence of a buffered diluent it was found difficult to maintain the suspension of cells at a constant pH. Finally, while sedimented normal red cells can be resuspended rather easily, suspensions of formalized red cells, upon sedimenting, formed a mass of almost rubbery consistency and required vigorous stirring to resuspend the cells thoroughly. These disadvantages attending the use of formalized red cells are of minor importance and are greatly outweighed by the advantages of red cells stabilized by treatment with 10 to 50% formalin for several days.

[8] Bodansky, M., *Introduction to Physiological Chemistry*, New York, John Wiley & Sons, Inc., 1938, 4th edit.

16512 P

## Practical Method for Determination of Serum Streptomycin Level Using *"Donovania granulomatis"* as Test Organism.*

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Following the successful cultivation of *"Donovania granulomatis" in vitro* in fresh yolk medium by Dienst, Greenblatt and Chen,[1] the technique for propagating this organism has been made comparable in simplicity to those used for ordinary bacteria. With the demonstration of the high degree of susceptibility of *"Donovania granulomatis"* to streptomycin,[2,3] it was conceived that this heretofore hard-to-culture micro-organism might be used in streptomycin assay. As a result of a series of experiments the following procedures have been evolved, which we consider as being simple and accurate enough for any clinical or bacteriological laboratory to use. The principle of this method is based upon the comparison of the least amount of serum to a known quantity of streptomycin that has to be present in the culture medium in order to inhibit the growth of *"Donovania granulomatis."*

The fresh yolk medium is prepared in the following manner: Yolk is removed aseptically from fresh eggs and diluted 1:1 with sterile saline. A base medium containing 1% Bacto peptone, 0.3% Bacto tryptone, 0.3% dextrose, 0.2% sea salt, and 0.12% agar is prepared and adjusted to pH 7.3. The base medium is then sterilized and allowed to cool to 50-60°C before adding an equal portion of the diluted yolk. Four cc of this medium is placed in each of a series of 8 test tubes. The serum to be assayed is diluted with sterile distilled water to 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128. To the first tube of yolk medium is added 1 cc of the 1:1 dilution of serum, to the second tube is added 1 cc of the 1:2 dilution of serum, and so on, so that the final volume of each

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[1] Dienst, R. B., Greenblatt, R. B., and Chen, C. H., accepted for publication by the *Am. J. Syph., Gonor., and Ven. Dis.*

[2] Greenblatt, R. B., Kupperman, H. S., and Dienst, R. B., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 389.

[3] Rake, G., and Dunham, V., *Am. J. Syph., Gonor., and Ven. Dis.*, 1947, **31**, 610.