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Isolation from Fresh Human Serum of a Fraction which Inhibits Certain Serological Reactions.*

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The suppressive action of fresh sera, human or of other animal species, on immune precipitation as well as on bacterial agglutination (prezone phenomenon) has been reported in numerous publications.[1-6] Moreover, it has been general practice to heat sera prior to use in flocculation tests for syphilis, although the reason for this procedure has never been adequately explained.[7]

Two years ago, Major Charles R. Rein of the United States Army (Medical Corps), in a personal communication to the authors, reported that the addition of fresh normal human serum to heated syphilitic sera causes the inhibition of formation of aggregates in various flocculation tests. He also noted that heated normal serum does not show this inhibiting effect. At his suggestion, the present authors undertook a study of this phenomenon, and now report further evidence for the existence, and methods for the isolation, of a thermolabile fraction of human serum responsible for the inhibition of certain serological reactions.

The Inhibitory Effect of Whole Serum. The inhibitory action of fresh human serum was studied in the Kahn, Kline, Mazzini, and Boerner-Jones-Lukens precipitation tests, and was demonstrated in these tests with approximately 500 fresh syphilitic sera. The inhibition of the flocculation of the specific polysaccharide of the Type III pneumococcus and its homologous rabbit antiserum was also demonstrated.

Syphilitic sera were found to give negative reactions in the fresh state, and to be positive only after heating or standing for given periods of time dependent upon the temperature. Thus, at −35°C, these sera were found to be negative, even after one year, demonstrating that the inhibitor present in the serum could be preserved at this temperature. However, raw syphilitic sera stored at 3-4°C lost their inhibitory powers, and thus gave positive serological reactions, after several weeks. Further, as the temperature of storage increased, the period of time necessary for the achievement of serological positivity decreased. Finally, the inhibitory factor in syphilitic sera was found to be destroyed in 10 to 30 minutes at 56°C. The inhibitory effect of fresh non-syphilitic sera was found to be similarly labile at these conditions of temperature and ageing.

The addition of strong electrolytes such as the chlorides, bromides, and iodides of sodium, potassium, and ammonium, to final concentrations above 2%, caused a syphilitic serum to show a positive reaction with about the same intensity as that shown by the serum inactivated by heating at 56°C for 30 minutes. This observation is undoubtedly related to the rationale for the Kline,[8] Laughlen,[9] and Briceño Rossi[10] tests, all of which employ hypertonic salt solutions. The effect of salt on the inhibitor, in contrast to the heat effect, was shown to be reversible, inasmuch as the removal of the salt by dialysis, or dilution of it to isotonicity, caused the serum to react

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2 Douris, R., Mondain, C., and Beck, J., *C. R. Soc. de biol.*, 1932, **110**, 15.

3 Heidelberger, M., *J. Exp. Med.*, 1941, **73**, 681.

4 Doladilhe, M., *Ann. de l'Inst. Pasteur*, 1934, **53**, 379.

5 Anthadse, V., *Ann. de l'Inst. Pasteur*, 1930, **45**, 203.

6 Boyd, W. C., *Fundamentals of Immunology*, 1943, Interscience Publishers, Inc., N.Y.

7 Eagle, H., *The Laboratory Diagnosis of Syphilis*, C. V. Mosby Co., St. Louis, 1937.

8 Kline, B. S., *J. Lab. Clin. Med.*, 1929, **14**, 764.

9 Laughlen, G. F., *Can. Med. Assn. J.*, 1935, **33**, 179.

10 Briceño Rossi, A. L., *Rev. de Sanidad y Asistencia social*, 1942, **7**, 333.

negatively once more.

Non-electrolytes such as sucrose, glucose, urea, and glycerol, used in concentrations as high as 5%, did not cause inactivation, reversible or irreversible, of the inhibitor in fresh serum.

The Separation of the Inhibitory Factor from Fresh Syphilitic Serum. Fifty ml of a clear syphilitic serum was enclosed in a Visking cellophane membrane (20/32, No. 213) and dialyzed with mechanical rotation at 1°C against 8 liters of a phosphate buffer of ionic strength 0.02 and pH 5.4. The buffer was prepared by adding 153 ml of 0.5 M KH_2PO_4 solution and 1.73 ml of 1 N KOH to sufficient distilled water to make 4 liters of solution.[11] After 48 hours of dialysis, the contents of the bag were centrifugalized in a brine-cooled centrifuge at 1°C at 2000 r.p.m. for 30 minutes, and the supernate (labeled "supernate A") poured off, neutralized with 1 N NaOH, and rendered isotonic with the requisite amount of 18% NaCl. The precipitate (labeled "precipitate A"), now sedimented, which had formed in the dialysis, was washed 3 times by suspension in and centrifugation with the phosphate buffer at pH 5.4. After the third washing, the precipitate was evenly distributed in 50 ml of the buffer, and 5 ml of this suspension was withdrawn into another tube. Both portions of the precipitate were then centrifugalized and the supernates discarded.

When tested in the Kline and Kahn tests, supernate A was found to be positive by itself, whereas 5 ml of supernate A combined with the sediment obtained from the 5 ml of the suspension of precipitate A, was found to be negative. It was also shown that the addition of precipitate A to a pneumococcus SSS III-specific antiserum system resulted in the inhibition of flocculation in the region of antibody excess.

The inhibitor substance was further separated from precipitate A by extraction of this latter fraction in twice its volume of a buffer of pH 6.6 prepared by dissolving 75 ml of 0.5 M KH_2PO_4 and 10.58 ml of 1N KOH in sufficient distilled water to make one liter of solution. The buffer, first cooled to 1°C, was mixed with precipitate A, and the mixture allowed to stand in a water bath at this temperature for 30 minutes. At the end of this time it was centrifugalized at 1°C, and the sedimented precipitate was washed twice with the same buffer. The precipitate was then dissolved in 1.8% NaCl to a volume equal to one-tenth of the volume of serum from which it had been prepared. This fraction, labeled "precipitate B," represented, as found in several preparations, from 0.6 to 1% of the total serum proteins, and usually contained, in addition to the inhibitory factor, a small amount of C'1 of complement.[11] Occasionally, precipitate B was found to be entirely free from C'1 activity.

In the preparation described above, syphilitic serum was used in order to demonstrate that the removal of the inhibitor causes the remaining portion of the serum to become serologically positive. However, it is emphasized that a similar procedure may be used for the separation of the inhibitor from normal (non-syphilitic) serum. Table I shows the inhibitory action of the several fractions prepared from normal serum.

Summary. From human serum (syphilitic and non-syphilitic) a fraction comprising from 0.6 to 1% of the total serum proteins, and containing an inhibitor for certain serological reactions such as the Kline and Kahn tests, has been isolated by means of chemical fractionation with phosphate buffers. This inhibitory substance as it occurs in serum, may be reversibly inactivated by hypertonic solutions of electrolytes, and irreversibly by heat.

TABLE I.
The Inhibitory Action on the Kline Test of Fresh Human Serum and Several of Its Fractions.

0.3 ml of heated syphilitic serum mixed with 0.15 ml of:*	Kline test
Heated normal serum	++++
Fresh " "	—
Precipitate A†	— or ±
" B‡	— or ±

* All fractions used in 1:1 dilution with respect to whole serum.

† Comprising about 5% of the total serum proteins.

‡ Comprising from 0.6 to 1% of the total serum proteins.

11 Ecker, E. E., Pillemer, L., and Seifter, S., *J. Immunol.*, 1943, **47**, 181.