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Association of Meningococcus and *B. typhosus* Toxins with Hemoglobin *in vitro*.

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Incidental to the observations that a close association exists between tetanospasmin and hemoglobin in the blood of guinea pigs suffering from tetanus intoxication[1] a series of experiments was carried out on combination of hemoglobin with bacterial toxins *in vitro*. The results are briefly presented in this communication.

The toxins employed were filtrates from the "agar washings" of cultures of meningococcus and *B. typhosus* which were highly potent in the elicitation of the phenomenon of local skin reactivity.[2] In this work they were purified by dialysis in cellophane bags No. 600 against 0.85% NaCl solution for a period of one week.[3]

The hemoglobin preparations were made in the following manner: All the work was done under strict precautions of sterility and each step controlled for bacterial contamination on aerobic and anaerobic media. Rabbit blood obtained from the heart was defibrinated by shaking with glass beads, filtered through several layers of gauze and centrifuged in order to separate the plasma from the erythrocytes. The erythrocytes were promptly washed in 1% NaCl solution by repeated centrifugalization until the washings became biuret-negative (from 5-7 washings being required). The packed red blood cells were kept frozen for several hours at –70°C in a mixture of cellosolve and dry ice and gradually thawed out in a mixture of ice and alcohol and in the refrigerator at 4°C overnight. The cells were diluted in distilled water to a hemoglobin concentration of 50-60% as determined in the Hellige haemometer.

Filtration of the solution through a Seitz filter resulted in effective removal of cellular debris, as ascertained by examination of spreads stained by Wright's method and hanging-drop preparations. In control experiments adjusting the pH to 5.5 by the addition of N/10 HCl gave no precipitate in the filtrates.[4] The clear, dark red

1 Shwartzman, G., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 112.

2 Shwartzman, G., *Phenomenon of Local Tissue Reactivity and Its Immunological, Pathological and Clinical Significance*, Paul B. Hoeber, Inc., Medical Book Department of Harper and Brothers, New York, 1937.

3 Shwartzman, G., Morell, S., and Sobotka, H., *J. Exp. Med.*, 1937, **65**, 323.

4 Jorpes, E., *Biochem. J.*, 1932, **26**, 1488.

filtrates were dialyzed at 4°C for a period of several days in cellophane bags No. 600 against distilled water. The water was changed daily. The materials removed from the bags were dried *in vacuo* by the methods of Flosdorf and Mudd. They subsequently yielded perfect solution on addition of a diluent up to the initial volume, *i.e.*, distilled water, Sörensen phosphate buffer solution of pH 6.8, plain broth or blood serum.

In the experimental work about to be described an amount of dry hemoglobin representing a yield of 0.85 g of dissolved hemoglobin was mixed with various amounts of undiluted dialyzed toxins and also toxins diluted in different diluents or in normal rabbit serum. The phenomenon-producing potency of the final concentration of toxins per one cc ranged between 50-100 reacting units. The mixtures were dried *in vacuo* in the Flosdorf-Mudd apparatus. One or several days later the dry materials were dissolved in distilled water in amounts equal to the initial volumes of hemoglobin solutions or in phosphate buffer of pH 6.8 of the same or greater volumes. The solutions were then fractionated by the method of Parsons.[5] After 7-10 repetitions of alternate freezings at −14°C and centrifugalizations at 4°C until complete thawing, fractions widely differing in hemoglobin concentration were obtained.

The materials were assayed for toxic activity by means of the phenomenon of local skin reactivity. In these tests, the rabbits were prepared by a single intradermal injection of dialyzed meningococcus of *B. typhosus* toxin (*i.e.*, the same as used for combination with hemoglobin) and 24 hours later injected intravenously with the materials tested, one or more groups of 2 rabbits serving for each material. The results of the experiments carried out in this manner were as follows:

Rabbit, cow, guinea pig and sheep hemoglobin containing no toxin and fractionated by the method of Parsons gave no reactions in prepared rabbits.

Hemoglobin held meningococcus and *B. typhosus* toxins, the activity being associated with colored fractions and totally absent from slightly colored and colorless portions. Quantitatively, toxicity was not found in hemoglobin concentrations lower than 0.012 g per one cc. The association with the hemoglobin was firm. Elution of the toxin could not be obtained either by dilution of the toxin-hemoglobin combination in 3-fold larger amounts of the initial diluent, or by the use of plain broth, phosphate buffer mixture of

[5] Parsons, quoted in *The Respiratory Function of the Blood. Part II. Hemoglobin*, p. 68, by Joseph Barcroft, Cambridge, University Press, 1928.

pH 6.8 and normal horse serum. The same firm association of toxin with hemoglobin was obtained when toxin mixed with normal horse serum was used for the combination. Further fractionations and elutions were done under aerobic conditions. The preparations in solution appeared of bright red color of oxyhemoglobin. Occasionally, dark brown preparations of methemoglobin were encountered. They were not studied in this series of experiments. The relation of oxidation and reduction to the association described will be embodied subsequently in a publication dealing with crystallized hemoglobin preparations.

Thus, it may be concluded from the experiments cited that hemoglobin is capable of entering into association with bacterial toxins *in vitro*. The combination appears firm since elution by greater dilution in water and by dilution in other diluents, as well as normal rabbit serum, is unsuccessful.

The stable association of toxic agents *in vivo* and *in vitro* with hemoglobin suggests studies on the effects of disease-producing agents upon intimate processes of cellular physiology in which hemoglobin and similar respiratory enzymes play an important rôle.

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Regeneration of *Euplanaria Dorotocephala* with Pituitary Gland Extract.*

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The literature presents few examples of effects upon invertebrate material by vertebrate hormones.[1, 2] This preliminary paper reports the accelerated regeneration in posterior and anterior portions of *Euplanaria dorotocephala* in media of beef pituitary extracts. Wulzen[3] reports the effect of feeding of ox pituitary gland upon the growth and fission of *Planaria maculata*. In the present study there was no normal feeding, for the pituitary extracts were introduced in solution and the regenerating, transected flatworms decreased in size during the experiments.

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1 Ashbel, Rivka, *Nature* (London), 1935, **135**, 343.

2 Coldwater, K. B., *J. Exp. Zool.*, 1933, **65**, 43.

3 Wulzen, Rosalind, *J. Biol. Chem.*, 1916, **25**, 625.