

after planting  $10^{-5}$  mg bacilli while 0.1% horse serum albumen supports growth after planting  $10^{-7}$  mg bacilli.

*Summary.* Crystalline horse serum albumen was shown to be strongly growth-promoting for tubercle bacilli grown in synthetic media. Horse serum globulins were without effect or inhibited growth.

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#### Association of Tetanospasmin with Hemoglobin in Acute Stages of Tetanus Intoxication of Guinea Pigs.

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The object of this communication is to report on a new observation that tetanus toxin (tetanospasmin) may be found intimately associated with the hemoglobin of guinea pigs during the paralytic stages of the disease. A part of an extensive series of experiments on this problem is briefly summarized, as follows:

Guinea pigs weighing each 350 g were injected subcutaneously in the abdominal region with tetanus toxin\* in doses ranging between 20 to 40 M.L.D. and bled from the heart during the stages of complete paralysis and spasmodic contractions (*i.e.*, 24-48 hours following the subcutaneous injection). The pooled blood was defibrinated by shaking with glass beads; filtered through several layers of gauze, and centrifuged at room temperature for one-half hour. The clear, dark red supernatant plasma and the sedimented red blood cells were placed in separate containers.

Washing of red blood cells in 1% NaCl solution was repeated until the supernatant fluid became biuret-negative (usually 4 washings are required). The washed cells were diluted 1:5 in distilled water. When fairly complete laking and some crystallization occurred about one hour later, the solution was centrifuged for one-half hour also at room temperature. This procedure resulted in separation of a dense dark red sediment containing masses of hemoglobin crystals; a superimposed loose whitish precipitate consisting of cellular debris and some hemoglobin crystals; and finally, a clear dark red supernatant fluid.

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\* Tetanus toxin in powder form obtained from Eli Lilly Research Laboratories (Lilly 27994, 400,000 M.L.D. per g), through the courtesy of Dr. H. M. Powell.

The serum was fractionated with the aid of the somewhat modified method of Parsons,<sup>1</sup> originally recommended for crystallization of hemoglobins and in these experiments consisting of repeated alternate freezing to  $-14^{\circ}\text{C}$  and centrifugalization of the serum at  $4^{\circ}\text{C}$  until complete thawing. The procedure enabled the separation of colorless, faintly colored, medium colored, and dark red fractions. Small amounts of crystals and whitish debris were also obtained.

Following determinations of the concentration of hemoglobin by the Sahli method modified by Wintrobe (Hellige haemometer), all the materials were promptly injected into guinea pigs weighing each 350 g. The volume injected into each guinea pig ranged between 1 cc-10 cc and never exceeded 10 cc. One cc was given subcutaneously over the abdomen, the remaining amount was introduced subcutaneously over both thighs in equally divided doses. Examinations for symptoms of generalized and local tetanus were made daily. Test guinea pigs recorded as having shown tetanus intoxication died following generalized paralysis and spasms. On post-mortem examination no other pathological findings were observed except localized inflammation at the site of injection without pus formation, and some congestion of lungs and adrenals. The results of the tests were consistently as follows:

The blood defibrinated by means of glass beads elicited tetanus.

The washed red blood cells, the cellular debris and packed hemoglobin crystals suspended in a small amount of 0.85% NaCl solution failed to produce the symptoms.

Laked red blood cells tested before centrifugalization, and the clear dark red supernatant fluid obtained after centrifugalization of the laked cells, both possessed a considerable amount of tetanus toxin, which in sufficient concentration produced acute paralysis and death within 48 hours following the subcutaneous injection.

The plasma containing a certain amount of hemoglobin also had the tetanospasmin. Fractionation proved, however, that the major portion of the toxin was associated with the dark red portion; a small amount capable of producing only local tetanus being present in the medium-colored fraction, and none at all found in the colorless and slightly colored parts.

Quantitative studies emphasize the remarkable fact that under the above experimental conditions the tetanospasmin concentration of the blood is approximately directly proportional to the hemoglobin concentration. With doses 20-40 M.L.D. of the toxin injected sub-

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<sup>1</sup> Parsons, quoted in *The Respiratory Function of the Blood... Part II. Hemoglobin*, p. 68, by Joseph Barcroft, Cambridge, University Press, 1928.

cutaneously per guinea pig, weighing 350 g, each M.L.D. recovered from the blood of the intoxicated guinea pigs corresponded to 0.035 g-0.007 g of hemoglobin in the preparations and fractions tested, in volumes ranging between 1 cc-10 cc. The effects of these total amounts of hemoglobin were the same, irrespective of the volumes used.

The active blood preparations may be considered predominantly oxyhemoglobin. Occasional batches of methemoglobin obtained after prolonged storage and reduced hemoglobin were not studied in this series.

No symptoms were elicited in test-guinea pigs with blood fractions of control guinea pigs receiving no injections and of those injected with tetanus toxin heated at 80°C for ½ hour prior to the injection. Also, blood fractions of guinea pigs which received an old inactive toxin gave no tetanus.

In this and the following paper the term "hemoglobin" refers to complex substances obtained in solution following laking of washed erythrocytes and removal of cellular debris. Hemoglobin is defined, therefore, by the biological properties, its chemical relationships being disregarded for the moment. The term "association" is merely descriptive and indicates a combination which may either occur directly with the hemoglobin or through the intermediary of some unidentified constituent with which the hemoglobin is associated.

All the data considered together, especially observations on the toxic activity of hemoglobin obtained by means of lysis of washed red blood cells, indicate clearly that under the experimental conditions described, a close association exists between hemoglobin and tetanospasmin in the blood recovered from guinea pigs during the acute stages of the disease.

Investigations on association of certain viruses with hemoglobin are under progress.