METHOD OF ISOLATION OF TETANUS HEMOLYSIN AND A STUDY OF SOME OF ITS PROPERTIES

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A method of obtaining tetanus hemolysin by gel filtration is described. Its properties are studied.

It has been claimed [2, 4, 7-9, 12] that besides tetanus neurotoxin, another factor concerned in the pathogenesis of tetanus is a tetanus hemolysin which, according to data described by these workers, possesses hemolytic and local necrotic actions. Other workers [5, 6, 11, 13] deny that the hemolysin plays a role in this disease. The conflicting nature of the data in the literature is perhaps due to the difficulty of isolating the hemolysin in a pure form and of separating it from the neurotoxin. Hardegree [10] separated tetanus neurotoxin and hemolysin by fractionation of the toxin on Sephadex G-100 in columns measuring 45 × 4 cm (ratio of height to diameter 11 : 1). Under these conditions, however, the hemolysin fraction contained substantial amounts of neurotoxin as an impurity, possibly because the column was not tall enough. In the investigation described below, therefore, an attempt was made to isolate tetanus hemolysin by means of columns with a higher height/diameter ratio.

Filtrates of broth cultures of strains Kolle Nos. 154 and 471 (Copenhagen) of Clostridium tetani, obtained by cultivation for 7 days on casein-plant nutrient medium, were used. The specific activity was $2.3 \cdot 10^5 - 5.6 \cdot 10^5$ MLD for albino mice per milligram total nitrogen, and $1 \cdot 10^7 - 2 \cdot 10^7$ MLD/mg protein nitrogen. The specific activity of the hemolysin was 3-30 hemolytic units/mg total nitrogen. The unit of hemolytic activity was taken to be the amount of hemolysin in solution which, in the course of 1 h at 20°, caused the hemolysis of an equal volume of a 0.5% suspension of sheep's erythrocytes.

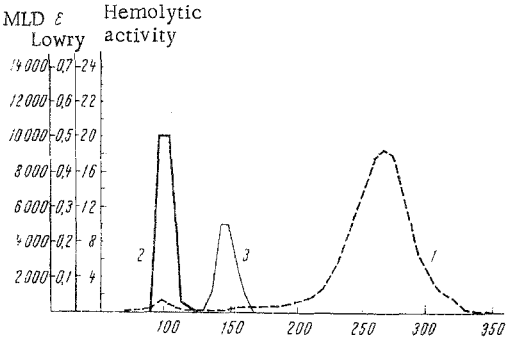

Fig. 1. Separation of tetanus neurotoxin and hemolysin on a Sephadex G-100 column measuring 1400 × 16 mm. 1) Extinction with Lowry – Folin reagent; 2) MLD for albino mice; 3) hemolytic activity (in conventional units). Abscissa: elution volumes.

The tetanus toxins were fractionated on columns with Sephadex G-100 with a working size of 1400 × 16 mm (height / diameter ratio 87.5 : 1) and 500 × 22 mm. Isotonic boro-borate buffer, pH 7.3, was used for elution. Toxins were applied to the columns in a volume of 5 ml. Fractions 6.5 ml in volume were collected at a filtration rate of 15-20 ml/h. The content of nitrogenous substances in the collected fractions was determined from extinction with Lowry – Folin reagent, toxicity (MLD) was estimated in albino mice, and hemolytic activity was measured. The molecular weight of the proteins eluted in the various fractions was calculated from the graduation curve obtained for Sephadex G-200 [3] and by Squire's formula [14]. Ultracentrifugation of the fractions, concentrated with gum arabic, was carried out on a Spinco Model L ultracentrifuge.

The biological properties of the hemolysin were assayed on 4 chinchilla rabbits weighing 2.5-3 kg, which received four subcutaneous injections, at intervals of 4 days, each of 1 ml of

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TABLE 1. Effect of Hemolysin on Erythrocyte Count and Hemoglobin Concentration in Rabbits

Group	Rabbit No.	Hemoglobin (in %)			No. of erythrocytes per mm³ (in thous.)		
		initial value	after 2nd injection	after 4th injection	initial value	after 2nd injection	after 4th injection
Experimental	60	10,5	9,3	9,0	3 560	3 050	3 090
	69	10,7	10,1	8,6	3 770	3 410	3 150
	101	12,1	12,0	10,7	4 650	4 100	3 960
	138	11,3	9,6	9,8	3 610	2 950	2 750
	Mean	11,15	10,25	9,52	3 897	3 370	3 233
Control	1	10,6	10,4	10,2	3 820	3 940	3 860
	2	11,7	12,0	11,8	4 540	4 320	4 260
	3	11,3	9,6	10,4	4 110	4 020	4 210
	4	11,4	10,9	11,5	3 260	3 510	3 630
	Mean	11,25	10,72	10,97	3 940	3 920	3 990

a solution of tetanus hemolysin with an activity of 2 conventional units. This preparation contained not less than 1 MLD of neurotoxin for albino mice in 1 ml. The control group of four rabbits received subcutaneous injections of physiological saline. Blood analyses for the hemoglobin concentration and erythrocyte count were made before the first injection of hemolysin and 3 days after the 2nd and 4th injections.

During fractionation of the tetanus toxins on columns with Sephadex G-100, two peaks were obtained with Lowry – Folin reagent. The first peak corresponded to proteins with molecular weight higher than 100,000, and the second, which was many times larger than the first, was formed by compounds of low molecular weight (Fig. 1). Nearly all the toxic activity was associated with the proteins of the first peak, while hemolytic activity was associated with compounds eluted between the first peak of high-molecular-weight proteins and the second peak of low-molecular-weight compounds.

The fractions with greatest hemolytic activity contained 10-15 hemolytic units per ml and their toxicity was 3-5 MLD for albino mice per ml. The specific activity reached 5000-8000 hemolytic units/mg nitrogen, i.e., it was more than 2000 times higher than that of the original native preparation.

Subcutaneous injection of tetanus hemolysin into rabbits in a dose of 2 hemolytic units did not produce manifestations of tetanus. Neither necrosis nor induration developed at the site of injection. Meanwhile, changes were found in the hemoglobin concentration in the blood and in the erythrocyte count (Table 1).

After injection of tetanus hemolysin into rabbits the hemoglobin concentration in the blood and the erythrocyte count fell (Table 1), and this effect was more marked after the 4th injection of hemolycin. The hemoglobin concentration was reduced on the average by 1.6% (1.4-2.1) and the erythrocyte count by 660,000 (470,000-860,000). This decrease compared with the control is statistically significant [1].

The following properties of hemolysin were studied: the effect of pH, temperature, and certain chemicals on its activity. Hemolysin exhibited specific activity within the pH range 3.0-90., with an optimum at pH 5.0. Raising the temperature from 10 to 20° increased the rate of the hemolytic reaction by three times, and the temperature coefficients for increases of temperature from 20 to 30 and from 30 to 40° were 2.2 and 1.9, respectively.

Heating at 60° destroyed the tetanus hemolysin in less than 30 min, and at 50° in less than 1 h, while after heating at 40° for 2 h about 90% of the activity was lost.

Sodium citrate and oxalate in concentrations up to 0.05%, calcium chloride in concentrations up to 0.05%, calcium chloride in concentrations up to 0.1%, and 8-hydroxyquinoline in concentrations up to $2.5 \cdot 10^{-3}$ M had no effect on the activity of tetanus hemolysin. Cysteine in a concentration of 0.033% depressed its activity by 57.5%, and mercuric chloride in concentrations of $2 \cdot 10^{-4}$ M and $2 \cdot 10^{-3}$ M depressed it by 88.5% and 100%, respectively. Iodine in dilutions of 1 : 900 and 1 : 450 depressed activity of the hemolysin by 51.3 and 100%, respectively, and glucose in concentrations of 1 and 5% by 20 and 30%, respectively.

Calculation of the molecular weight of the tetanus toxins from the rates of their elution from Sephadex gel [3, 14] gave values of 140,000 for the neurotoxin and 55,000-60,000 for the hemolysin. The sedimentation constants were 7.58 S and 3.22 S, respectively.

These results demonstrate that it is possible to obtain by gel filtration a tetanus hemolysin in a relatively pure form, suitable for the study of its properties.

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