The Isolation and Crystallization of Tetanal Toxin[1]

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A toxic protein from the filtrates of *Clostridium tetani* identical in behavior to tetanal toxin (spasmin) has been isolated and crystallized in this laboratory. The crystalline material contains between 3,500 and 4,000 flocculating units (Lf) and between 50,000,000 to 75,000,000 mouse minimal lethal doses (MLD)/mg. of nitrogen. This Lf value compares favorably with the figure of 3,300 Lf/mg. of nitrogen estimated on the basis of combining nitrogen to be the true Lf value for pure tetanal toxoid (*2*). The MLD content of the crystalline protein/mg. of nitrogen is at least 200 times greater than the purified toxin prepared by Eaton and Gronau (*1*), and also at least 50 per cent greater than the purest preparations of Pickett and co-workers (*4*).

The method employed for the purification and crystallization of the toxin involves the use of methanol under rigidly controlled conditions of pH, ionic strength, protein concentration, and temperature (*5*). The parent tetanal toxin used in this study was prepared by the method of Mueller and Miller (*3*). The major steps in the isolation and crystallization of the toxic protein are summarized in Table 1, together with assays and analyses of the various fractions. It will be noted that the activity of the crystalline toxin/mg. of nitrogen has been increased more than 500 times on an Lf basis and 800 times on an MLD basis over the parent toxin filtrates. Four crystalline preparations show a remarkable constancy in the Lf and MLD contents/mg. of nitrogen. The Lf and MLD units/mg. of nitrogen remained constant during three additional recrystallizations. The yield per liter of parent toxin filtrate is less than 1 mg. of nitrogen.

TABLE 1

GENERAL PROCEDURES

Preparation	No.	Mg. N/ml.	MLD/ml. $\times 10^6$	Lf/ml.	Kf* in min.	MLD/mg. N $\times 10^6$	Lf/mg. N	MLD/Lf $\times 10^4$	Yield Per cent of parent toxin
Parent toxin	..	3.01	0.2	20	15	0.066	6.6	1.0	..
Material insoluble in 40% MeOH at pH 5.2, μ 0.09, and temp. $-5°$ C. Precipitate dissolved in 0.15 M sodium acetate.	1	0.320	5	300	10	15.6	937	1.66	95
Precipitate 1 adjusted to 15% MeOH at pH 5.5, μ 0.03, and temp. $-4°$ C. Precipitate dissolved in 0.15 M sodium acetate.	2	0.172	4	250	10	23.2	1453	1.6	90
Precipitate 2 adjusted to 12% MeOH at pH 4.0, μ 0.075, and temp. $-4°$ C. Precipitate discarded. Supernatant employed.	3	0.054	1.8	112	10	33.3	2074	1.6	83
Supernatant 3 adjusted to 30% MeOH at pH 4.0, μ 0.02, and temp. $-6°$ C. Precipitate discarded. Supernatant employed.	4	0.034	1.5	90	10	44.1	2646	1.66	80
Supernatant 4 adjusted to 25% MeOH at pH 5.1, μ 0.02, and temp. $-8°$ C. Crystals and slight amount of amorphous material formed precipitate.	5	0.041	2.25	140	10	54.9	3415	1.6	60
Precipitate 5 recrystallized as in No. 5. Crystals formed precipitate.	6	0.022	1.5	80	10	68.1	3636	1.87	45
Precipitate 6 recrystallized three times as in No. 5. Crystals formed precipitate.	7	0.015	1.0	55	10	66.6	3666	1.81	27

* Kf determined at 20 Lf units.

Crystallization of the toxic protein occurs slowly at $-8°$ C. under the conditions shown in Table 1. The crystals disintegrate rapidly at temperatures above $-5°$ C. and dissolve in the mother liquor at temperatures above 0° C. This behavior has presented technical difficulties in microphotographing the crystals. However, a sketch of the crystals is presented in Fig. 1.[2]

The colorless crystals dissolve instantly in 0.15 M sodium acetate at pH 6.5. The solution gives positive reactions with protein reagents. Both the Molisch test and the nitroprusside test are negative.

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[2] We are indebted to Theodora Bergsland, medical artist at the Institute of Pathology, Western Reserve University, for her painstaking sketch of the crystals.

The crystals appear uniform and possess constant biological activity upon recrystallization. Their activity is destroyed by heat, acid, or alkali. This would indicate that the crystalline protein is identical with tetanal toxin.

Complete chemical, physical, and biological characterization of the crystalline toxin will have to await the accumulation of larger quantities of material. The detailed procedure for the isolation and crystallization of the toxin as well as its characterization will be presented at a later date.

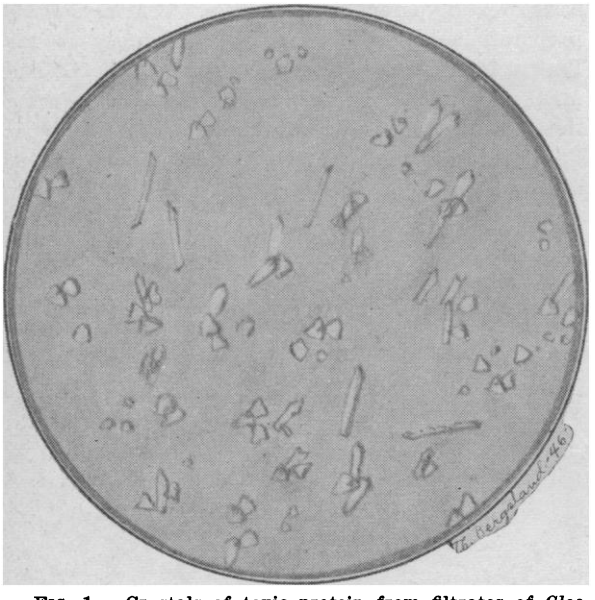

FIG. 1. Crystals of toxic protein from filtrates of *Clostridium tetani*, magnified 430 times.

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The Effect of the Prepartum Diet of the Cow on the Vitamin A Reserves of Her Newborn Offspring[1]

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The importance of the prenatal nutrition of the calf is recognized, but the problem has been given scant consideration experimentally. Analyses of livers (*1, 3, 5, 6, 7, 8, 12*) and of blood (*14, 15*) from young calves (fetuses and newborn) revealed low vitamin A reserves. The general uniformity of these results in conjunction with observations of laboratory animals has led to the belief that the gestation diet of the dam has no appreciable effect on the vitamin A content of the fetal calf liver. Recently, however, Braun and Carle (*3*) noted that the vitamin A content of the fetal calf liver, though low, was in direct relationship to the diet of the mother. Unfortunately, conclusions from many of the data are vitiated by pathological complications in the experimental subjects.

In view of the foregoing evidence, steps were taken to ascertain the effects of the plane of carotene and vitamin A intake of the dam during the latter stages of gestation on the vitamin A reserves of the normal newborn offspring.

Procedure. The experimental subjects were healthy dairy animals of the Ayrshire, Holstein, and Jersey breeds, of which the latter constituted about 50 per cent. During the immediate prepartum period, the dams were placed under three dietary regimes with respect to the carotene and vitamin A intake: standard, high carotene, and high vitamin A. All the cows were fed a basal ration consisting of good-quality alfalfa hay, sorghum silage and a concentrate mixture. Group I (the standard, or control) was restricted to this ration, but Group II (high carotene) was grazed on pasture forage in addition, and Group III (high vitamin A) was fed a vitamin A supplement.[2] The carotene intake was undetermined, but the vitamin A consumption per cow was one million U.S.P. units daily. The prepartum period of pasture grazing ranged from 14 to 90 days, and of vitamin A supplementation from 8 to 45 days.

Samples of venous blood were drawn from the calves, usually within four hours after birth and always before colostrum ingestion. The collection of blood in the early postpartum stages was necessitated by the tendency of the vitamin A concentration in the serum to decrease in the fasted newborn (*9*). Subsequently several of the calves from dams in each group were sacrificed to obtain their livers for analysis.

The general analytical procedures adopted in the determinations of carotene and vitamin A were the Kimble (*11*) for blood and a modification of the Guilbert and Hart (*6*) for liver. The major deviation in the latter case was in the use of ether extract from a sample of dry tissue (dehydrated by grinding with anhydrous sodium sulfate) for saponification.

Results. The data in Tables 1 and 2 reveal no marked differences between the vitamin A reserves of calves from cows on the basal ration and those on the high carotene but a significantly higher storage in

[1] Contribution No. 162, Department of Dairy Husbandry, and No. 304, Department of Chemistry.

[2] "Dry vitamin A" supplied by Distillation Products, Inc., Rochester, N. Y.