

# BIOCHEMICAL PROPERTIES OF THE TOXINS OF CLOSTRIDIUM NOVYI AND CLOSTRIDIUM HEMOLYTICUM

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The report of Oakley, Warrack, and Clarke (1947) on the toxins of the *Clostridium novyi* (*oedematiens*) group and of *Clostridium hemolyticum* indicates the complexity of this topic and presents the pertinent historical data. It is known that the toxins *C. novyi* and *C. hemolyticum* involve a lecithinase catalyzing the hydrolysis of lecithin to phosphorylcholine and a diglyceride. In view of the existence of such toxic enzymes as the lecithinases, it was considered possible that the hemotoxins produced by these species might be related to lysolecithin. This phospholipid is a strongly hemolytic substance produced from lecithin by lecithinase A activity and destroyed by lecithinase B hydrolysis to yield nonhemolytic end products. We have demonstrated the ability of lecithinase B to reduce the hemolytic potency of the toxins of *C. novyi*, type B, and *C. hemolyticum*, and this is taken as evidence for the presence of lysolecithin in these toxins.

## METHODS

*Cultures and media.* The strains of organisms used are listed in table 1; their authenticity had been established by one of us previously. They were maintained in the following medium containing desiccated beef heart particles: 10 g tryptone, 2.5 g proteose peptone, 2.5 g peptone, 5 g glucose, 4.2 g Na<sub>2</sub>HPO<sub>4</sub> (anhydrous), 0.54 g KH<sub>2</sub>PO<sub>4</sub> (anhydrous), 500 ml beef heart infusion, and 500 ml distilled water. Tube cultures in this medium, incubated at 37 C for 24 to 48 hours in Brewer jars, served as inocula for subsequent cultures. The following medium was used for toxin production: 30 g proteose peptone no. 2, 10 g yeast extract, 8.5 g NaCl, 0.1 g MgSO<sub>4</sub>, 4.2 g Na<sub>2</sub>HPO<sub>4</sub> (anhydrous), 0.54 g KH<sub>2</sub>PO<sub>4</sub> (anhydrous), 1 g sodium thioglycolate, 100 ml beef heart infusion, and 900 ml distilled water; 0.5 per cent glucose was added after sterilization. Cultures in 3,000-ml Erlenmeyer flasks were incubated at 37 C for 24 to 72 hours in oat jars. Growth was satisfactory with a 1 to 2 per cent inoculum; the final pH was in the range of 6.0 to 6.6. With *C. novyi*, type C, and *C. hemolyticum*, it was necessary to add about 10 g of desiccated heart particles to obtain satisfactory growth in flask cultures. The clear supernatant liquids, obtained by centrifugation after gassing of the cultures had ceased, were saturated with neutral ammonium sulfate. The coagulum which rose was removed, dissolved in a minimum quantity of distilled water, and dialyzed overnight through cellophane against cold running tap water. The nondialyzed material was centrifuged and the supernatant liquid reduced in volume to roughly one-half by vacuum distillation. The concentrated toxic solutions thus obtained were refrigerated and used as needed.

*Preparation of lecithinase B.* A crude enzyme preparation, obtained by the method of Contardi and Ercoli (1933), was dried from acetone (yield: 8 g per kg of rice bran). Enzyme activity was tested on lysolecithin obtained by the lecithinase A action of snake venom (*Crotalus adamanteus*, the Florida rattlesnake) on soybean lecithin. Commercial lecithin was purified by repeated treatments with ethanol and acetone until a light yellow product was obtained; it was stored under acetone in a refrigerator. About 25 mg of desiccated snake venom were mixed with 500 mg freshly dried lecithin in 50 ml of phosphate buffer, pH 7.0. The mixture was placed in a 45 C water bath for 24 hours and the presence of lysolecithin ascertained by hemolysis tests (positive in dilution of 1:120 with rabbit erythrocytes). In later control experiments for lysolecithin egg yolk suspension also served as a source of lecithin. The yield of lysolecithin

TABLE 1  
*Strains of Clostridium novyi and Clostridium hemolyticum studied*

MC CLUNG COLLECTION NUMBER	NAME OF ORGANISM	TYPE*	PREVIOUS CODE DESIGNATION	SOURCE
842	<i>C. novyi</i>	A	N21B	Dr. I. M. Danielson, Lederle Laboratories
41	<i>C. novyi</i>	B	B.D. 19	Dr. A. W. Turner, Australia
45	<i>C. novyi</i>	B	B.D. Rose	Dr. A. W. Turner, Australia
162	<i>B. gigas</i> ( <i>C. novyi</i> )	(B)	Demnitz 17	Dr. A. Sordelli
50	<i>C. novyi</i> (Kraneveld bacillus)	C	Strain I, bacillus osteomyelitis; bacillosa bubalorum	Dr. F. C. Kraneveld, Java
808	<i>C. hemolyticum</i>		2504	Dr. A. M. Jasmin, Montana State College

\* According to Scott, Turner, and Vawter (1934).

was greater under the latter conditions: hemolysis in a dilution of 1:12,800. To test for the activity of the lecithinase B preparation, mixtures of this enzyme and lysolecithin from both sources were made. The lecithinase B preparation reduced the hemolytic potency of the lysolecithin by 32- to 128-fold. It was thus apparent that the rice bran preparation possessed lecithinase B activity.

*Indicator techniques for toxin activity.* The determination of lecithovitellin (LV) values was performed as follows: Egg saline was prepared by removing aseptically one egg yolk (hen) and mixing with 250 ml of sterile calcium saline, pH 7.6. The latter solution consisted of 0.005 M calcium acetate in 0.85 per cent sodium chloride. To the egg-yolk-saline mixture, 20 g of kaolin or "supercel" were added and the suspension was mixed and centrifuged to clarity. The supernatant was sterilized by passage through a Seitz filter (60-mm pad), stored in a refrigerator, and used within 48 hours. The toxins were diluted in sterile

calcium saline, pH 7.6, all dilutions being made to a volume of 0.5 ml. To each dilution were added 0.5 ml of the egg saline, the tube contents were shaken gently, and the mixtures were placed in a 37 C water bath for 3 hours and then refrigerated overnight. The LV values were graded from negative to four plus; dilutions showing the same appearance as the egg saline similarly diluted were considered negative.

For the determination of hemolytic values, fresh rabbit erythrocytes were washed at least three times with saline, pH 7.0, and used in 3 per cent saline suspensions. The toxins were diluted in sterile calcium saline, pH 6.8, all dilutions being made to a volume of 0.5 ml. To each dilution were added 0.5 ml of the erythrocyte-saline suspension, and the remainder of the procedure was as described for the LV determinations. The hemolytic values were graded from negative to four plus; under no conditions were readings accepted if the control tubes showed signs of hemolysis.

For the determination of lethal values, white mice, weighing about 20 g, were used. The toxins were inoculated intraperitoneally, using 0.5 ml of the diluted material. Three mice per dilution were inoculated and the least amount of toxic preparation causing death of at least two of the three mice within 48 hours was taken as the MLD.

*Action of lecithinase B on toxins.* To determine the effect of the lecithinase B preparation of the toxins, 100 mg of the enzyme material were suspended in 4 ml of sterile calcium saline, pH 6.8, and 1 ml of toxic solution was added. The mixtures were placed in a 37 C water bath for 24 hours and then tested for LV, hemolytic, and lethality values; suitable controls, without lecithinase B, were always included.

#### RESULTS

The toxins were tested for LV, hemolytic, and lethality values; the results have been placed in table 2. Also included are the results obtained when 100 mg of lecithinase B were mixed with 5 ml of a 1:5 dilution of the toxins and incubated as indicated above. It can be seen that lecithinase B treatment of the toxins of *C. novyi*, type A, did not affect the LV and hemolytic values. On the other hand, similar treatment of the toxins of *C. novyi*, type B (including *B. gigas*), and of *C. hemolyticum* markedly reduced the hemolytic values of these toxins, without affecting the hemolysis due to the lecithinase (LV-inducing) quota of these toxins. It may be concluded that an additional hemolytic factor is involved in the "hemolysin" of these toxins, and this factor behaves as lysolecithin. The effect of lecithinase B upon lethality was tested for those toxins shown to contain a lysolecithin hemolysin. Table 2 shows that such treatment caused a decrease in lethal action of the *C. novyi*, type B, and *C. hemolyticum* but not of *B. gigas*. On this point, however, reservation is advocated because the highest lethal dose inoculated was 5 MLD. A lethal dose higher than this value could not be tested because of the problem of dilution. The potency of the lecithinase B preparation was such that it could not be diluted past the levels reported; moreover, the crude enzyme preparation itself is toxic. The

toxin of the Kraneveld bacillus (*C. novyi*, type C) was so weak in its activities that lecithinase B inhibitory action could hardly be evaluated.

A partially purified preparation of *Clostridium perfringens*, type A, was subjected to the action of lecithinase B. No reduction in hemolytic values was noted with this toxin.

The data for the concentrated toxins presented above were comparative over many trials. Prior to ammonium sulfate precipitation the culture supernatants were all tested similarly, and corresponding, though weaker, reactions were

TABLE 2  
Influence of lecithinase B on the LV, hemolytic, and lethality values of toxins\*

Number	TOXIN Organism	WITHOUT LECITHINASE B			WITH LECITHINASE B		
		LV	Hemolysis	Lethality	LV	Hemolysis	Lethality
842	<i>C. novyi</i> , type A	1:160	1:320	0.000165	1:160	1:320	Not tested
N241†	<i>C. novyi</i> , type A	0.16	0.04	0.015	0.16	0.04	Not tested
41	<i>C. novyi</i> , type B	1:160	1:5,120	0.018	1:160	1:320	0.09 (5 MLD) did not kill
45	<i>C. novyi</i> , type B	1:640	1:40,960	0.02	1:320	1:640	0.05 (2.5 MLD) did not kill
162	<i>B. gigas</i> ( <i>C. novyi</i> , type B)	1:320	1:2,560	0.20	1:320	1:640	0.4 (2 MLD) killed
50	Kraneveld bacillus ( <i>C. novyi</i> , type C)	1:8	1:32	Nonlethal	Not tested	Not tested	Not tested
808	Culture supernatant of <i>C. hemolyticum</i>	1:1,280	1:81,920	0.0625	1:640	1:1,280	0.25 (4 MLD) did not kill

\* Reported as highest dilution giving positive reaction. The lethality values are given as the least amount (ml) causing death.

† Desiccated toxin: values indicate least amount (mg) giving reaction.

generally obtained. Only one exception to this generalization was noted: in the case of *C. hemolyticum* precipitation with ammonium sulfate caused a slight decrease in the hemolytic and lethal values under the prevailing conditions; in this case the data in table 2 were obtained with original culture supernatants.

We wish to indicate here that the terminology used by Macfarlane and Knight (1941) and others with regard to some of the lecithinases is in error. The enzyme responsible for the LV reaction is correctly named "lecithinase D" and not "C." This is in accordance with the correct usage by Contardi and Ercoli (1933), the original investigators of this problem. Lecithinase C is a choline phosphatase and catalyzes the hydrolysis of lecithin to yield choline and a phosphodiglyceride.

## DISCUSSION

The data presented permit the conclusion that the lecithinase D activities of *C. novyi* and *C. hemolyticum* toxins bear no direct relation to the lethal values. The toxins lacking a lysolecithin hemolysin showed hemolytic values very close to the LV values, indicating the presence of a hemolytic lecithinase. The toxins studied, with hemolytic values in excess of LV values, include those of *C. novyi*, type B, and *C. hemolyticum*. It has been shown that the excess hemolytic values probably are due to lysolecithin, on the basis of lecithinase B inhibition of hemolysis. Moreover, some of the lethal action of the latter two groups of toxins appears attributable to the action of lysolecithin. This is not surprising since Belfanti (1925) showed the marked cytolytic action of lysolecithin in brain, capillary endothelium, and white blood cells in addition to erythrocytes.

Oakley, Warrack, and Clarke (1947) demonstrated the existence of a toxin common to *C. novyi*, type B, and *C. hemolyticum* toxins; this was called beta toxin and acted as a hemolytic, necrotizing lecithinase. In our study the occurrence of the lysolecithin hemolysin as a common toxic component is noted. The function of lysolecithin as an antigenic component can be explained on the basis of the assumption that this phospholipid occurs in nature as a lysolecithoprotein, in the same fashion as its parent substance, lecithoprotein. This assumption satisfies the serological data as well as the knowledge that the toxic component was precipitated by ammonium sulfate saturation and is not dialyzable through cellophane. Demnitz (1934), Sordelli and Ferrari (1937), Keppie (1944), Hayward and Gray (1946), as well as Oakley, Warrack, and Clarke (1947), supplied evidence to show that not all the hemolysins of *C. novyi*, type A, and *C. novyi*, type B, were identical. Moreover, there were interrelationships between the hemolysins of *B. gigas* (*C. novyi*, type B) and *C. hemolyticum*. We suggest that a common antigen of these toxins is the lysolecithin hemolysin described above.

The possibility that the rice bran preparation contained substances other than lecithinase B is appreciated. It may be admitted, however, that this enzyme was present and that certain differences were noted with some of the toxins. The differences reported appear to be significant and are not contradicted by existing knowledge. As for the hypothetical substances involved, they must be viewed as constituting such experimental risks as are common in enzyme chemistry.

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## SUMMARY

With lecithinase B, the specific hydrolytic enzyme acting on lysolecithin, as an indicator system, it was found that the toxins of *Clostridium novyi*, type B, and *Clostridium hemolyticum* contained a hemolysin showing the properties of lysolecithin. Treatment of these toxins with lecithinase B reduced lethality. The toxins of *C. novyi*, type A, and *Clostridium perfringens*, type A, do not contain such a hemolysin. The lecithinase D activities of *C. novyi* and *C. hemolyticum* toxins bear no relation to the lethal values.

## REFERENCES

- BELFANTI, S. 1925 Untersuchungen über Lysozithine. I. Ueber die Bedeutung der Lysozithine bei der Pathogenese von Intoxikationen und Infektionen. *Z. Immunitäts.*, **44**, 347-363.
- CONTARDI, A., AND ERCOLI, A. 1933 Sopra alcune scissioni enzimatiche della lisocitina e della lecitina. Nuovi isomeri delle lecitine. *Gazz. chim. ital.*, **63**, 37-46.
- DEMnitz, A. 1934 The question of the identity of *Bac. gigas* (Zeissler) with the causal organism of a bovine hemoglobinuria occurring in the region of the Andes in North and South America. *In* Medicine in its chemical aspects. I. G. Farbenindustrie AG, Leverkusen, Germany. 2104 p.
- HAYWARD, N. J., AND GRAY, J. A. B. 1946 Haemolysin tests for the rapid identification of *Cl. oedematiens* and *Cl. septicum*. *J. Path. Bact.*, **58**, 11-20.
- KEPPIE, J. 1944 A study of the antigens of *Cl. oedematiens* and *Cl. gigas* by *in-vivo* and *in-vitro* methods. Ph.D. Thesis, Cambridge University, England.
- MACFARLANE, M. G., AND KNIGHT, B. C. J. G. 1941 The biochemistry of bacterial toxins. I. The lecithinase activity of *Cl. welchii* toxins. *Biochem. J.*, **35**, 884-902.
- OAKLEY, C. L., WARRACK, G. H., AND CLARKE, P. H. 1947 The toxins of *Clostridium oedematiens* (*Cl. novyi*). *J. Gen. Microbiol.*, **1**, 91-107.
- SCOTT, J. P., TURNER, A. W., AND VAWTER, L. R. 1934 Gas edema diseases. Twelfth Internatl. Vet. Congress, **2**, 168-187.
- SORDELLI, A., AND FERRARI, J. 1937 Comunidad antigénica entre las toxinas de *B. hemolyticus*, *B. gigas*, *B. perfringens* y *B. oedematiens*. *Folia Biol.*, **74-75**, 320-324.