HOKKAIDO UNIVERSITY

Title	PURIFICATION AND CRYSTALLIZATION OF CLOSTRIDIUM BOTULINUM TYPE C TOXIN
Author(s)	SYUTO, Bunei; KUBO, Shuichiro
Citation	Japanese Journal of Veterinary Research, 20(1-2), 19-30
Issue Date	1972-06
DOI	https://doi.org/10.14943/jjvr.20.1-2.19
Doc URL	https://hdl.handle.net/2115/1989
Type	departmental bulletin paper
File Information	KJ00003418341.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Jap. J. vet. Res., **20**, 19~30 (1972)

PURIFICATION AND CRYSTALLIZATION OF *CLOSTRIDIUM BOTULINUM* TYPE C TOXIN

Bunei SYUTO and Shuichiro KUBO

Department of Biochemistry
Faculty of Veterinary Medicine
Hokkaido University, Sapporo, Japan

(Received for publication, March 24, 1972)

A procedure for purification and crystallization of *Clostridium botulinum* type C toxin is described. The toxin was precipitated from the culture by addition of zinc chloride and eluted by dibasic sodium phosphate solution from the precipitate. The toxin was precipitated with ammonium sulfate to 34% saturation at 4°C and the toxin solution dissolved in acetate-phosphate buffer was chromatographed by the connected columns of Sephadex G-75—DEAE cellulose—QAE Sephadex A-50 at pH 7.0. The eluate had a specific toxicity of 3.8×10^7 MLD/1.0 $E_{277.5m\mu}$.

The toxin was crystallized from the solution dialysed against the saturated ammonium sulfate solution by ultrafiltration. The crystalline toxin had a specific toxicity of 3.0×10^7 MLD/1.0 $E_{277.5m\mu}$ and a faint hemagglutination activity. The crystalline toxin contained at least two components in acrylamide gel electrophoresis and the agar gel immunodiffusion test, respectively.

INTRODUCTION

Clostridium botulinum produces a toxin that is the most potent poison known, and it is classified into six types, A, B, C, D, E and F, by the immunological character of toxin produced. Among them, type C is mainly encountered in botulism in domestic animals and fowls.

Clostridium botulinum type C is further classified serologically into two types, C_α and C_β. In Japan, botulism in minks caused by the type C toxin have been recorded by KARASHIMADA et al. (1962, 1965), who reported that the toxicity of type C toxin to mink is stronger than types A, B and E toxin.

The physiological mechanism of botulism is still obscure. It is necessary first to isolate and purify the toxin for clarifying the relation between the physiological activity and the molecular structure of toxin. Working along these lines, type A toxin was isolated and crystallized by LAMANNA et al. (1946), ABRAMS et al. (1946) and DUFF et al. (1957), respectively. Type E toxin was crystallized by SHIOKAWA (1962). The other toxins have not yet been obtained in crystalline form, and relatively little information has been reported on the purification of type C toxin.

In this paper, the procedures for the purification and crystallizaion of type

C toxin are reported and the purity of the crystalline toxin is discussed.

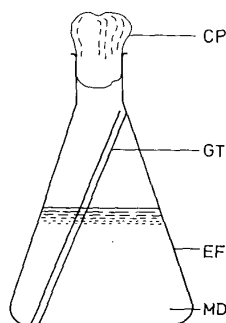
MATERIALS AND METHODS

Strain *Clostridium botulinum* type C strain Stockholm supplied kindly by Dr. G. SAKAGUCHI*, National Institute of Health, Tokyo, was used throughout in this investigation.

Media The medium for the enrichment of the organisms was composed of 0.5 g of cooked meat medium (Difco) in 10 ml of a solution containing 1% meat extract (Kyokuto), 1% peptone (Kyokuto), 0.5% glucose, 0.5% sodium chloride and 0.05% sodium thioglycolate. The medium was adjusted to pH 7.6, and autoclaved at 120°C for 20 min. Glucose was autoclaved separately as a 50% solution and added aseptically.

The medium for the prodution of the toxin was composed of 4% peptone (Kyokuto), 2% polypeptone (Daigo), 2% yeast extract (Oriental), 2% corn steep liquor (self made), 1% glucose, 0.5% sodium chloride and 0.05% sodium thioglycolate. The medium was adjusted to pH 7.9 and was autoclaved at 114°C for 15 min.

FIGURE. 1 *Incubation bottle*



EF : 5,000 ml Erlenmeyer flask
CP : cotton plug
GT : glass tube 1×30 cm
MD : medium

Toxin production When the inoculum was required for the production of the toxin, 0.5 ml of the stock culture was inoculated into the medium for enrichment, and incubated at 33°C for 18~22 hr. After incubation, 0.7 ml of the inoculum was transferred to 3,500 ml of the medium for the production of the toxin in 5,000 ml flask through a 1×30 cm glass tube stood against the inside wall of the flask as shown in figure 1, and incubated at 33°C. The organisms were allowed to spread gradually from the bottom to all over the medium. The glass tube was removed from the culture at 3 days after inoculation, and the incubation was continued for 2 or 3 more days.

Toxicity titration The toxin was diluted in 0.1 M phosphate buffer at pH 6.9. White mice weighing about 20 g were injected intravenously with 0.1 ml aliquots of the dilution of the toxin, and their survival times after injection were recorded. There were six mice per dilution. The MLD was calculated by the method of BOROFF & URSLA (1966).

Hemagglutinin test The hemagglutinating activity of toxic samples was titrated by means of semiquantitative method. The toxic sample was diluted by the method of twofold dilution with 0.1 M NaCl-0.05 M acetate-phosphate buffer, pH 7.0. The titration was performed by adding 0.5 ml of a 0.5% suspension of washed mouse erthrocytes in the same buffer to 0.5 ml of serially diluted samples. The mixtures were incubated at 30°C for 15 min and were allowed to stand overnight at 4°C. The highest dilution at which hemagglutination took place was recorded, and the minimum dose of hemagglutinin required

---

* Present address: Department of Veterinary Medicine, Faculty of Agriculture, University of Osaka Prefecture

for macroscopically appreciable hemagglutination in this method was represented as the minimum hemagglutinating dose (MHAD).

Column chromatography All chromatography was carried out at room temperature. The buffer used for chromatography was prepared by adding 8.7 ml of CH_3COOH to 1,000 ml of the solution containing 125 g of $Na_2HPO_4 \cdot 12H_2O$, followed by dilution to 10 litres. The pH of the buffer, as necessary, was adjusted to 7.0 with CH_3COOH or NaOH.

Sephadex G-75 (Pharmacia) swollen in the buffer was packed in an acryl column, 6.5×42 cm, and was washed with 4 litres of the buffer at the rate of 2.5 ml per min.

DEAE cellulose (Brown) in 0.5N NaOH was packed under slight pressure in an acryl column, 1.8×90 cm, and equilibrated with the buffer without washing with distilled water.

QAE Sephadex A-50 (Pharmacia), which was activated previously with 0.5 M acetic acid-ammonium phosphate buffer and washed with the acetate-phosphate buffer, was packed in an acryl column, 2.5×40 cm, followed by equilibration with the same buffer at the rate of 1.5 ml per min. When these three columns were used for chromatography, they were connected by vinyl tubing as in Sephadex G-75, DEAE cellulose and QAE Sephadex A-50 order.

The columns of DEAE cellulose, 1×70 cm, and QAE Sephadex A-50, 2×40 cm, for rechromatography were prepared by the procedure mentioned above.

Purity assays of toxin Acrylamide gel electrophoresis was carried out by the modified method of DAVIS (1964). For gel preparations of each concentration on acrylamide, the following procedures were used. Stock solutions, A (30% acrylamide-0.8% bisacrylamide and B (TEMED-Tris-HCl buffer, pH 8.9), were stored at 4°C. For 7.5% gel, 2 ml of solution A and 1 ml of solution B were mixed with 1 ml of H_2O and 4 ml of fresh 0.14% ammonium persulfate (w/v). The mixture was degassed and polymerized by distributing in the glass tube, 0.5×7 cm, at room temperature. For other gel preparations of lower concentration on acrylamide, the volume of solution A only was reduced and the volume of H_2O was correspondingly increased, but the contents of solution B and ammonium persulfate in the mixtures were not changed in all cases. Electrophoresis was performed without the use of large pore gel for the concentration of protein and was run at a constant current 5 mA/tube for 60~90 min at room temperature. The chamber buffer used for electrophoresis was 0.05 M or 0.005 M tris-glycine buffer, pH 8.4. Gels were stained with 1% Amino Black 10 B in 7% acetic acid and destained electrophoretically.

Immunological assays were done by the agar gel diffusion technique in 1% Special agar B (Wako) gel with veronal buffer at pH 8.6 or with 0.85% saline. Antitoxin of type C (horse serum) was supplied by the Ministry of Supply Station of Porten, England.

Protein determination The protein concentration in the effluent on the chromatography was estimated as absorbancy at 280 mμ with a Uvicord II, (LKB) and/or at 277.5 mμ with a Hitachi Parkin-Elmer 139 UV-Spectrophotometer. The protein content was calculated by multiplying by a factor of 6.25. The nitrogen content was determined by the micro-Kjeldahl method.

Determination of concentration of Cl^- The concentration of Cl^- in the effluent was determined by titration Cl^- with a standard $AgNO_3$ solution and an indicator, K_2CrO_4 solution.

RESULTS

Precipitation with zinc chloride

The toxicity of the culture reached the maximum, $0.8 \sim 1.3 \times 10^5$ mouse intravenous MLD per ml, and the pH dropped to 5.6 from 7.9 after 6 days of incubation at 33°C. The culture was filtrated with Hyflo-super-cel (Johns- Manville Products) under vacuo, and 1/35 volume of 50% (w/v) solution of $ZnCl_2$ was added slowly to the filtrate with stirring and adjusting the pH to 6.4 with N NaOH. The resulting precipitate was collected immediately by a Kubota Successive Centrifuge at 10^4 rpm and a flow rate of 300 ml per min at room temperature. The large mass of precipitate obtained was broken down into small pieces and suspended in a 0.6 M solution of $Na_2HPO_4 \cdot 12H_2O$ to 1/15 the volume of the original culture at 30°C. The suspension was stirred at room temperature for two hours until the zinc reacted with phosphate to produce insoluble compounds, and then left to stand overnight at 4°C. The insoluble compounds and excess phosphate settled down at the bottom of the vessel in this operation, and clear toxic supernatant could be obtained by decantation. This concentrated toxic solution having the potency of $12 \sim 23 \times 10^5$ MDL per ml will be referred to as the S-1. The fractions of the S-1 which contained virtually 100% of total toxic activity in original culture were pooled and stored at −20°C. Toxic activity was not affected by storage for at least for four weeks.

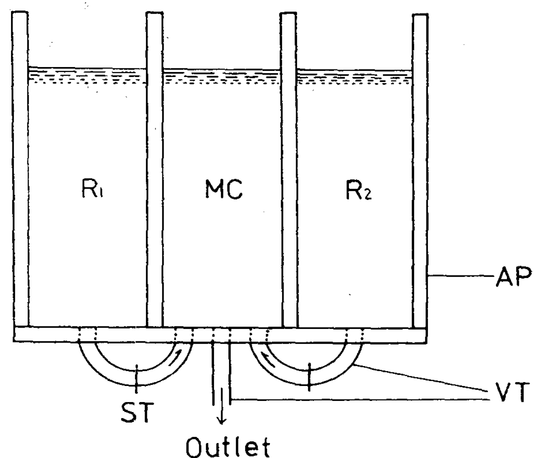
Ammonium sulfate precipitation

One half the volume of saturated $(NH_4)_2SO_4$ solution was added slowly to the S-1 with gentle stirring at 4°C. The mixture was then allowed to stand overnight at 4°C. As much supernatant as possible was siphoned off, titrated for toxicity, and discarded. The precipitate was centrifuged at 10^4 rpm for 15 min at 2°C and dissolved in the buffer to 1/300 the volume of the original culture. The undissolved materials were removed by centrifugation at 1.2×10^4 rpm for 15 min at room temperature. The supernatant having the potency of $240 \sim 390 \times 10^5$ MDL per ml was designated as S-2. The yields of toxicity in this stage were practically 100% of original toxicity in most cases, but were less than 75% in a few cases. A possible reason for this lower recovery is not clear. This fraction was prepared freshly at each time before chromatography, because the toxic activity of this fraction dropped during the storage. No hemagglutinin activity could be removed in this stage.

Column chromatography

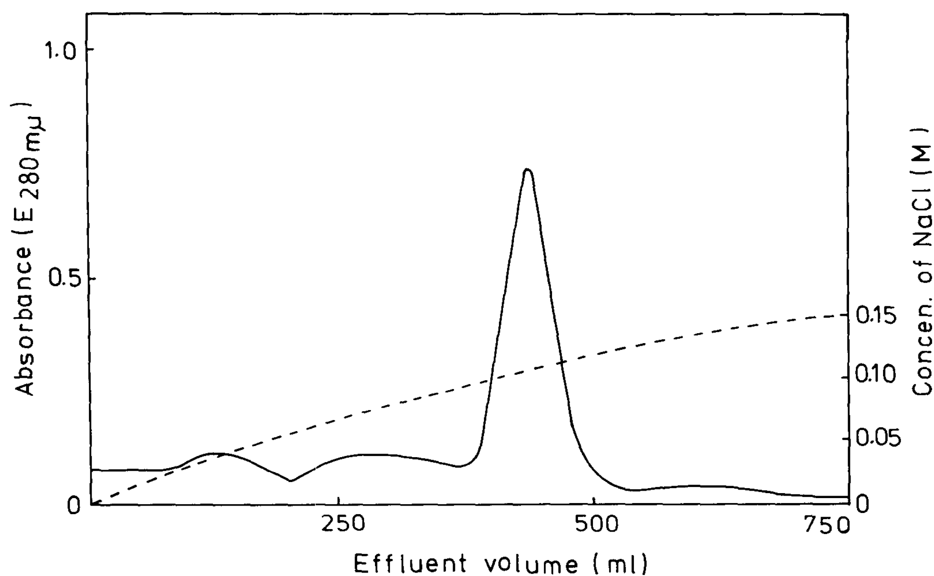
S-2, 60–80 ml, was applied on a column of Sephadex G-75 connected to DEAE cellulose column, and was eluted with the buffer, pH 7.0 and $\mu = 0.12$, at a rate of 2.5 ml per min. Since most of toxin passed through on DEAE cellulose but

FIGURE. 2 *Mixer*



MC : mixing chamber, acetate-phosphate buffer pH 7.0, μ=0.12
R_1 : reservoir, 0.3 M NaCl-acetate-phosphate buffer pH 7.0
R_2 : reservoir, acetate-phosphate buffer pH 7.0 μ=0.12
ST : stopper VT : vinyl tube
AP : acryl plate 0.5 cm (width)
Size of one chamber : 11 cm (inner height) × 4 cm (inner width) × 9 cm (inner depth)

FIGURE. 3 *Chromatography of the S-2 on QAE Sephadex A-50*



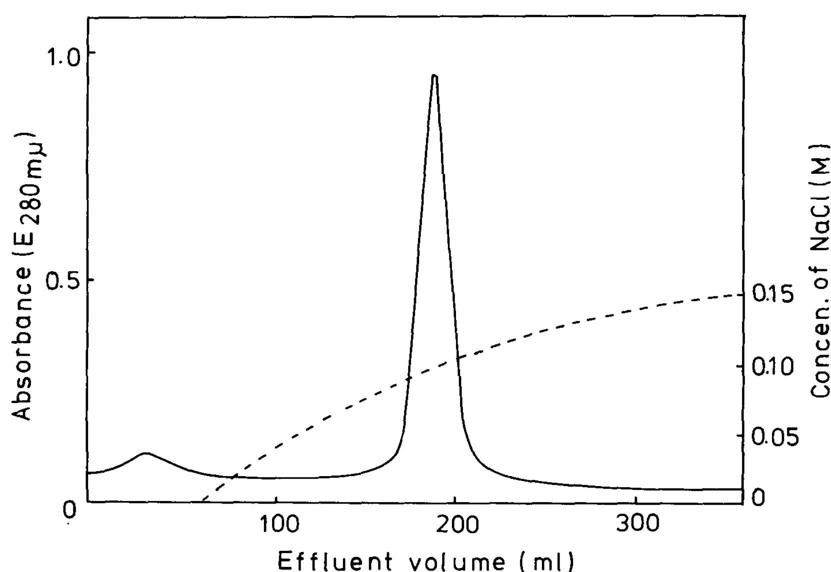
Experimental details are given in the text ; 10 ml fractions were collected.
Solide line : absorbance at 280 mμ
Broken line : NaCl concentration

not on QAE Sephadex A-50 under these conditions of pH and ionic strength, when the absorbance of the effluent of DEAE cellulose column at 280 mμ began to be detected on the monitor, the column of QAE Sephadex A-50 was connected to the column of DEAE cellulose with vinyl tubing in order to absorb the toxin. After removing the column of Sephadex G-75, the two anion exchange columns were further eluted by the buffer of an additional one volume of cellulose column at a rate of 1.5 ml per min. The column of QAE Sephadex A-50 was separated from the DEAE cellulose column and then developed by means of exponential salt gradient with the buffer and 0.3 M NaCl-buffer employing the mixer as shown in figure 2. The total volume of the eluent for the gradient elution was 900 ml, and the flow rate was 45 ml per hr. Figure 3 shows the elution pattern of chromatography. Three peaks, two minor and one major, emerged on eluting the column. The major peak was highly toxic and slightly hemagglutinating. The recovery of total toxic activity of this fraction was above 90% of the S-2. Most of hemagglutinating activity of the S-2 was removed in this connected-columns chromatography. The chromatographed toxic fraction will be referred to as S-3, and has a specific toxicity of 3×10^7 MLD/1.0 $E_{277.5m\mu}$.

Rechromatography on QAE Sephadex A-50

S-3, 120~140 ml, was dialysed against 15 volumes of the buffer for 16 hr with three changes at 4°C. The insoluble meterials, if the occurred, were removed by

FIGURE. 4 *Chromatography of the S-3 on QAE Sephadex A-50*



Experimental details are given in the text; 8 ml fractions were collected.

centrifugation at 1.2×10^4 rpm for 15 min at room temperature. The clear supernatant was loaded on the columns of DEAE cellulose-QAE Sephadex A-50 at a rate of 1 ml per min. The columns were washed with the buffer of two volumes of the columns at the same rate. The column of QAE Sephadex A-50 was separated from the DEAE cellulose column and was eluted by means of exponential salt gradient with the buffer and 0.3 M NaCl buffer, and the total volume of the eluent for the gradient elution was 600 ml. Figure 4 shows only one peak emerging on eluting the column. The apex of the peak was highly toxic and weakly hemagglutinating. The recoveries of the toxic activity of this fraction were 65~75% of S-3 and 50~60% of the starting material, respectively. The specific activity of the toxic fraction was 3.8×10^7 MLD/1.0 $E_{277.5m\mu}$. This fraction was designated as S-4.

Crystallization of toxin

S-4 was concentrated by dialysing against saturated ammonium sulfate solution at 4°C for 6~7 hr. The resulting precipitate was collected by centrifuging at 1.2×10^4 rpm and 2°C for 30 min, and was ressolved in 10 ml of the buffer. The insoluble precipitate was removed by centrifugation. The clear supernatant obtained was further concentrated by ultrafiltration with a collodion bag until the crystals of toxin appeared in the solution. If the toxin did not crystallize out of the solution after concentration to 1/3~1/4 volume, the collodion bag was immersed in the saturated ammonium sulfate solution until the mother solution at the bottom of the bag became slightly white and turbid, and was gently shaken. Repeating this procedure, the crystallization of toxin often occurred. The low concentration of toxin protein, however, led to amorphous precipitate instead of crystals. The concentration of protein required for crystallization was approximately 2%.

When the mother solution showed a silk like turbidity of toxin crystals, the solution was diluted by adding approximately 4 volumes of 1 M $(NH_4)_2SO_4$-acetate phosphate buffer, pH 7.0, and reconcentrated to the same volume again. The suspension of toxin crystals in the bag was allowed to stand for 3 or 4 days at 4°C, if necessary, with additional concentration. The toxin crystals were stored in 1 M $(NH_4)_2SO_4$-acetate phosphate buffer at 4°C. The crystals obtained was needle-like as shown in figure 5. The specific activity of the crystalline toxin was 3×10^7 MLD/1.0 $E_{277.5m\mu}$. A trace of hemagglutinating activity was detected on the both of mother solution and crystalline toxin.

The specific toxicity and recovery of toxic activity in each step of purification is summarized in table 1. The recovery of total toxic activity in final step (in crystalline form) was 20~25%.

TABLE 1 *Summary of purification*

PURIFICATION STEP	SPECIFIC TOXICITY	RECOVERY OF ACTIVITIES FROM			
		Preceding step		Starting material	
		Toxicity	HA activity	Toxicity	HA activity
	MLD/1.0 $E_{277.5m\mu}$	%	%	%	%
Culture filtrate	2.3×10^3			100	100
S—1	2.6×10^5	100	100	100	100
S—2	2.8×10^6	90~100	90~100	95	95
S—3	3.0×10^7	90	0.4~1.0	90	0.7
S—4	3.8×10^7	65~ 75	3.4	50~60	Trace
Crystal	3.0×10^7	40	0.1	20~25	Trace

Purity assays of toxin

Acrylamide gel electrophoresis Crystalline toxin revealed a single band in the acrylamide gel electrophoresis on 7.5% gel at pH 8.4 as shown in figure 6. However, two bands were observed on the gels of lower concentration than 5.5% gel.

Agar gel immunodiffusion test The agar gel immunodiffusion test with S-4 showed a single precipitin line in both gels which were prepared with a veronal buffer, pH 8.6 and with 0.8% NaCl solution. The crystalline toxin, however, showed two precipitin lines, one sharp and the other dispersed, in the gel with veronal buffer, as shown in figure 7.

DISCUSSION

Clostridium botulinum type C toxin was isolated and crystallized by a relatively simplified method. The concentration procedure with zinc chloride was effectively adapted for the first step of the large scale purification. But the repeat of the same procedure for the concentration of toxin resulted in a low recovery of toxic activity. Most of constituents acting as protective substances for the toxin in the culture were removed in the first stage, so that the toxic protein might easily be denatured with zinc chloride added in the second stage.

Sufficient precipitation of toxin protein in the preparation of S-2 was obtained at 1/3 saturation with the saturated ammonium sulfate solution. Though an increases rate of saturation resulted in a large amount of the precipitate, the recovery of total toxic activity was somewhat reduced at a higher saturation than at a 1/3 saturation.

The lower yields of toxicity were brought out by the chromatography of the S-2 on the two connected columns of DEAE cellulose-QAE Sephadex A-50 without the use of the Sephadex G-75 column. The two-column chromatography required a longer period for operation and resulted in a somewhat lower recovery than three-column chromatography since the S-2 had to be dialysed against the buffer overnight at 4°C instead of gel filtration. Most of hemagglutinin was removed by chromatography from the toxic fractions of S-2 and S-3 respectively, but it could not be completely separated at this pH. The toxic eluate obtained by chromatography at a higher pH, 7.8 or 7.9, with acetate phosphate buffer had negative hemagglutinin activity, but after concentrating the eluate it again showed a positive reaction of hemagglutinin. It seems that the hemagglutinin was not removed completely under these chromatographic conditions.

Crystalline type C toxin obtained by this procedure was highly toxic and weakly hemagglutinating. The ratio of one MHAD to 5×10^5 MLD of crystalline type C toxin was 0.5~1.0, while that of crystalline type A toxin (LAMANNA, 1948)

was calculated as 160~500. In one case, the maximal potency of the S-4 was 6.3×10^7 MLD/1.0 $E_{277.5m\mu}$ and that of the crystalline toxin was 4×10^7 MLD/1.0 $E_{277.5m\mu}$. The crystalline toxin usually had a lower toxicity than that of the S-4, and it may require a longer period for crystallization. Though the hemagglutinin content of the S-3 was higher than that of the S-4, the toxin could be crystallized from the S-3 by applying the crystallization procedure described above. The crystalline form of type C toxin was a needle shape like that of type A (LAMANNA et al., 1946) and of type E (SHIOKAWA, 1962).

Gel electrophoresis of the crystalline toxin revealed no detectable impurity on 7.5% gel, while two bands were observed on a lower gel concentration than 5.5% gel. It seems that the gel concentration of 7.5% was too high to separate these components.

The agar gel immunodiffusion test with the S-2 showed four or five precipitin lines, but with the S-4 showed a single precipitin line. The crystalline toxin showed also a single precipitin line agar gel with 0.85% NaCl solution, but two precipitin lines in agar gel with an alkaline veronal buffer, pH 8.6. Crystalline toxin of type C might be dissociated with toxin and hemagglutinin as the observation on type A toxin (WAGMAN & BETAMAN, 1951, 1953, WAGMAN, 1954, DasGUPTA et al., 1966).

REFERENCES

1) ABRAMS, A., KEGELES, G. & HOTTLE, G. A. (1946): *J. Biol. Chem.*, **164**, 63
2) BOROFF, D. A. & URSULA, F. (1966): *J. Bact.*, **92**, 1580
3) DasGUPTA, S. R., BOROFF, D. A. & ROTHSTEIN, E. (1966): *Biochem. biophys. Res. Commun.*, **22**, 750
4) DAVIS, B. J. (1964): *Ann. New York Acad. Sci., Art.* **2**, 404
5) DUFF, J. T., WRIGHT, G. G., KLERER, J., MOOR, D. E. & BIBLER, R. (1957): *J. Bact.*, **73**, 42
6) KARASHIMADA, T., ANDO, Y., IIDA, H. & SAKAGUCHI, G. (1962): *Jap. J. Bact.*, **17**, 90
7) KARASHIMADA, T., ONO, T., ANDO, Y., SAKAGUCHI, G., SAKAGUCHI, S. & KAGOTA, K. (1965): *Rep. Hokkaido Inst. publ. Hlth*, **15**, 17
8) LAMANNA, C., McELROY, O. E. & EKLUND, H. W. (1946): *Science*, **103**, 613
9) LAMANNA, C. (1948): *Pro. Soc. exp. Biol. Med.*, **69**, 332
10) SHIOKAWA, H. (1962): *Nissin Igaku*, **49**, 211
11) WAGMAN, J. & BETAMAN, J. B. (1951): *Arch. Biochem. Biophys.*, **31**, 424
12) WAGMAN, J. & BETAMAN, J. B. (1953): *Ibid.*, **45**, 375
13) WAGMAN, J. (1954): *Ibid.*, **50**, 104

EXPLANATION OF PLATE

Fig. 5 Crystals of *Clostridium botulinum* type C toxin

Fig. 6 Electrophoresis of crystalline type C toxin on acrylamide gel at pH 8.4

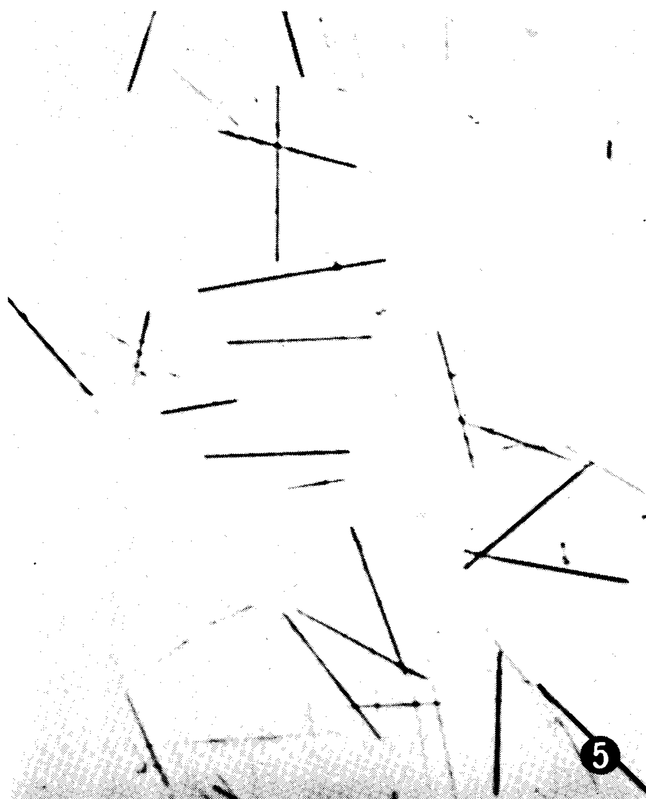
1	7.5 % gel	2	6.5 % gel
3	5.5 % gel	4	4.5 % gel
5	4.0 % gel		

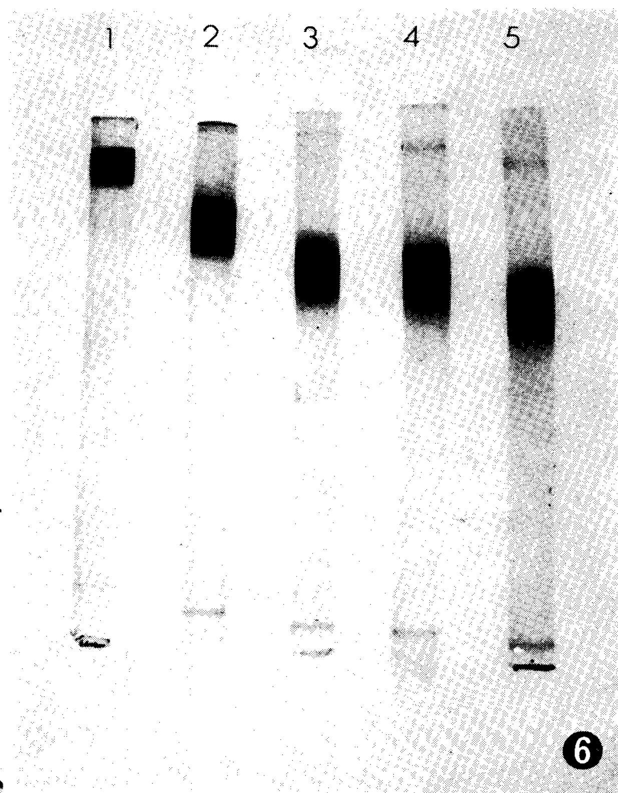
Fig. 7 Agar gel immunodiffusion test with type C toxin

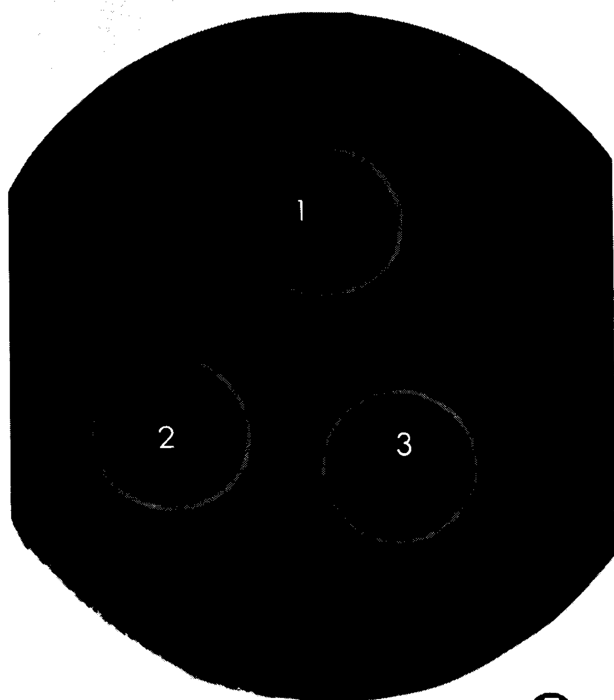
a veronal buffer at pH 8.6

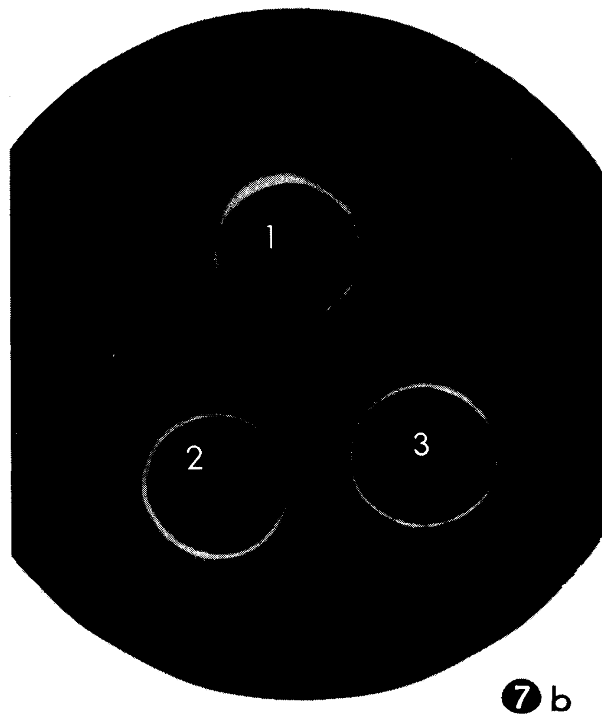
b 0.85 % NaCl

1	antitoxin	2	S-4
3	crystalline toxin		


5


1
2
3
4
5
6


1
2
3
7a


1
2
3
7b