

PURIFICATION FROM BLACK WIDOW SPIDER VENOM OF A PROTEIN FACTOR CAUSING THE DEPLETION OF SYNAPTIC VESICLES AT NEUROMUSCULAR JUNCTIONS

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ABSTRACT

The aqueous extract of the venom glands of black widow spiders was fractionated on a column of Sephadex G-200 and then on a column of DEAE-Sephadex A-50, pH 8.2. A protein fraction was obtained that caused a great increase in the frequency of occurrence of miniature end plate potentials at the frog neuromuscular junction, and caused swelling of the nerve terminals and depleted them of their vesicles. The fraction consists of at least four protein components that are similar in their molecular weights (about 130,000) and isoelectric points (ranging from pH 5.2 to 5.5) and are immunologically indistinguishable. It contains no sugar residues and has little or no lipolytic or proteolytic activity. The fraction is toxic to mice and is different from the fractions that act on houseflies, the crayfish stretch receptor and the cockroach heart. It seems pure enough to warrant a detailed study of its site and mode of action.

The physiological effects of the extract of the venom glands of the black widow spider, *Latrodectus mactans*, particularly the variety *tredecimguttatus*, have been studied extensively and the primary effects seem to be exerted on the nervous system (2-5, 7, 8, 12, 13, 15-18, 20-23, 30, 31, 33, 36-40, 43). Some of the active agents are proteins (18), and they seem to act in at least two basic ways: (a) to depolarize the cell bodies of some neurons and (b) to cause the release of neurotransmitters from a variety of nerve endings. For example, the extract depolarizes the cell body of the crayfish stretch receptor (S. Obara and A. Mauro, unpublished data) and induces a discharge of impulses in the axon (26). This depolarizing action of the extract may be responsible

for its ability to induce a discharge of action potentials in the abdominal ganglion of the cockroach (5, 17).

In addition to depolarizing excitable cells, the extract causes a release of transmitter from cholinergic nerve endings in brain (21, 24), sympathetic ganglia (38, 39), and *Torpedo* electric tissue (25), and from adrenergic nerve endings in the iris and other tissues (20, 22, 24). At neuromuscular junctions, the extract increases the frequency of occurrence of miniature end plate potentials, and blocks neuromuscular transmission. The neuromuscular effects have been observed at the cholinergic junctions of frogs and mammals (31, 37), at adrenergic nerve endings (vas deferens) in mammals (27), at both the inhibitory (γ -aminobutyrate)

and excitatory (glutamate) junctions in lobsters (30) and at excitatory junctions in locusts (15). The extract also induces the almost complete depletion of the synaptic vesicles at neuromuscular junctions (12, 13, 15, 27, 37). The effects on the ultrastructure are largely restricted to the nerve terminals, there being few changes in the morphology of the enveloping Schwann cells or of the underlying muscle fibers. These observations suggest that the extract contains a toxin which acts presynaptically at a variety of nerve endings. This paper describes the purification from the extract of a protein fraction which is toxic to mice and which causes the depletion of synaptic vesicles at the frog neuromuscular junction. We have also partially purified several other active principles such as those toxic to houseflies (23), that which depolarizes the crayfish stretch receptor (26), and that which accelerates the cockroach heart beat (5, 33).

MATERIALS AND METHODS

Materials

Sephadex G-200 and DEAE-Sephadex A-50 were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. Aquacide II, and Azocoll were purchased from Calbiochem, San Diego, Calif. Acrylamide, *N,N'*-methylene-bisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were obtained from Eastman Kodak Co., Rochester, N.Y. Tris (Trizma Base), sodium dodecylsulfate (SDS), riboflavin, bovine serum albumin, and β -galactosidase (*Escherichia coli*) were obtained from Sigma Chemical Co., St. Louis, Mo. Bovine ovalbumin, and bovine chymotrypsinogen were obtained from Worthington Biochemical Corp., Freehold, N.J., and Coomassie brilliant blue from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y. Ampholines (pH 4-6, pH 5-7) were obtained from LKB Produkter, Stockholm, Sweden. Ammonium persulfate, basic fuchsin, and uranyl acetate were purchased from Fisher Scientific Co., Fair Lawn, N.J. Sodium Metabisulfite was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J., and periodic acid from G. Frederick Smith Chemical Co., Columbus, Ohio. Boric acid, isopropyl alcohol, and sodium barbital were obtained from Mallinckrodt Chemical Works, St. Louis, Mo., and agarose was from Miles Laboratories, Inc., Kankakee, Ill. Antivenin (*Latrodectus mactans*), MSD (black widow spider antivenin), equine origin, was purchased from Merck, Sharp and Dohme, West Point, Pa. Epon 812 was obtained from Ladd Research Industries, Inc., Burlington, Vt., and osmium tetroxide was from Polysciences, Inc., Warrington, Pa. Lead citrate was obtained from K and K Laboratories, Plainview, N.Y.

Living adult females of *Latrodectus mactans tredecimguttatus* were purchased from Mr. B. Capparella, S. Marinella, Italy.

Methods

FRACTIONATION OF THE EXTRACT: The spiders were frozen on dry ice, and the cephalothoraxes removed and stored at -20°C . The cephalothoraxes were thawed as needed, and the venom glands with the chelicera still attached were collected in a glass homogenizer kept in crushed ice. All the following steps were performed in a cold room or with solutions chilled to temperatures between 1° and 4°C . The glands from 200 to 300 spiders were homogenized manually in 1-1.5 ml per 100 spiders of cold 0.05 M Tris-HCl buffer, pH 8.2. The homogenate was centrifuged for 15 min at 15,000 *g* in a Spinco centrifuge (Beckman Instruments, Inc. Spinco Div., Palo Alto, Calif.) The supernate was frozen at -20°C , thawed, and recentrifuged for 2 h at 100,000 *g*. The high-speed supernate was used for fractionation, and it is referred to as the whole extract.

A measured aliquot of the whole extract was applied to a 2.2×90 cm column of Sephadex G-200 equilibrated with 0.05 M Tris-HCl buffer (pH 8.2) and was eluted with the same buffer at a rate of 14 ml/h. 4.0-ml fractions were collected, and their optical densities at 280 nm were read on a Zeiss spectrophotometer. The fractions pertaining to each of the separated peaks were pooled. Peaks B, C, and E (cf. Fig. 2) were fractionated further on a 0.9×20 cm column of DEAE-Sephadex A-50 equilibrated with 0.03 M NaCl in 0.05 M Tris-HCl buffer (pH 8.2). They were eluted with a continuous gradient of NaCl that varied from 0.03 M to 0.6 M salt in the Tris buffer. Fractions of 2.5 or 3.0 ml were collected at a rate of 6 ml/h, and their optical densities were read at 280 nm. The fractions belonging to each peak were pooled and dialyzed overnight in $\frac{1}{4}$ inch tubing against 100-fold dilutions of the Tris buffer. Then they were concentrated approx. 10-fold with Aquacide and dialyzed again against 0.12 M NaCl in 0.005 M Tris-HCl buffer. The protein contents of the samples were determined by the method of Lowry et al. (32) after precipitation with trichloroacetic acid (TCA) because Tris buffer interfered with the colorimetric reaction.

ENZYME ASSAYS: Proteolytic activity was assayed according to the method of Moore et al. (35) using Azocoll as a substrate. This sensitive assay is useful in screening for proteolytic activity, since Azocoll is a general substrate which can be hydrolyzed by all known proteolytic enzymes. Phospholipase activity (A and C) was determined by the sensitive radiochemical method of Franson et al. (19).

GEL ELECTROPHORESIS AND ISOELECTRIC FOCUSING: SDS-gradient gel electrophoresis was performed as described previously (1), using gradients of polyacrylamide and sucrose. The gels were stored in the cold room overnight before use, and 1 mM ethyl-

enediaminetetraacetic acid (EDTA) was added to the buffer in the upper reservoir. The SDS gels were run at room temperature for 5–12 h at current densities of from 0.75 to 1.2 mA (cf. Fig. Legends). The gels were stained with Coomassie brilliant blue. Isoelectric focusing was performed on polyacrylamide gels, according to Scheele (41), with a running time of 10 h.

GLYCOPROTEIN TEST: The periodic acid-Schiff (PAS) staining procedure for glycoprotein (Zacharius et al. [45]) was used. Either a 5% polyacrylamide non-SDS gel, or a SDS gel with a 5–10% gradient of acrylamide, was employed. When SDS gel was used, the gel was washed with the following solutions before PAS staining: (a) 25% isopropanol, 10% acetic acid, overnight; (b) 10% acetic acid, several hours; and (c) 10% acetic acid, overnight.

CROSSED IMMUNOELECTROPHORESIS AND CROSSED IMMUNOELECTROFOCUSING: Crossed immunoelectrophoresis was done according to Clarke and Freeman (14). Electrophoresis of the whole extract or the purified fraction was performed first on 3×1 inch glass plates covered with 3 ml of 1% agarose in 0.1 M sodium barbital buffer (pH 8.6). A constant current of 10 mA per gel was applied for 2 h, giving a voltage gradient of approx. 4 V/cm across the gel. Agarose strips containing the electrophoretically separated components were cut out and transferred to 2×2 inch glass plates. Then 3.5 ml of 1% agarose solution containing a suitable amount (see Results) of black widow spider antivenin was coated on the plates around the strip. Electrophoresis in the second dimension was carried out at right angles to the first and was continued for 20 h at 4 V/cm (15 mA/plate).

For cross immunoelectrofocusing (42), 1% agarose in barbital buffer was coated on 2×2 inch glass plates. The gel in the upper portion of the plate (3.0 ml) contained antivenin while the lower portion (1.0 ml) contained no antivenin. The strip (~0.5 inches) of polyacrylamide gel containing the samples that had been separated by isoelectric focusing (cf. above) was placed upside down on top of the antivenin-free portion of the agarose plate, and electrophoresis in the second direction was performed as in crossed immunoelectrophoresis. After the run was completed, the plate was washed extensively (several days with frequent changes) in 0.9% NaCl, dried under filter paper, and then stained with 0.25% Coomassie brilliant blue.

BIOASSAYS: Toxicity studies on mice and houseflies were performed, and the mean lethal dose (LD_{50}) was determined for each fraction. The details of these procedures have been reported by Frontali and Grasso (23) for houseflies and by Frontali et al. (21) for mice. One mouse toxicity unit was defined as the micrograms of protein injected per gram of body weight that caused death in 50% of the animals. The specific activity of a given fraction was defined as the number of toxicity units per microgram of protein.

The effects of the fractions on the beat frequency of the cockroach heart were assayed on the semi-isolated heart of *Periplaneta americana* prepared according to Brown (6); the frequency of the heart-beat was recorded over 1 min periods. Assays on the crayfish stretch receptor were done according to Grasso and Paggi (26).

The neuromuscular effects of the fractions were tested at room temperature on excised cutaneous pectoris nerve-muscle preparations from frogs (31). Twitching of the preparations in Ringer's solution (2.1 mM KCl; 116 mM NaCl; 1.8 mM $CaCl_2$; and 6 mM Tris, pH 7.2) was monitored visually. End plate potentials (epp's) and miniature end plate potentials (mepp's) were recorded intracellularly from single neuromuscular junctions by standard techniques after neuromuscular transmission had been blocked with a modified Ringer's solution containing 0.5 mM $CaCl_2$ and 4.0 mM $MgCl_2$.

The most striking electrophysiological effect of the whole extract is to raise the spontaneous rate of occurrence of mepp's by several orders of magnitude (31), and we used this effect to monitor the purification of the active fraction. The relation between the mepp frequency and the quantity of extract added is highly nonlinear (31), and the increase in frequency begins abruptly after the addition of a critical amount of protein. This dose-response relationship enabled us to define in a semiquantitative way the amount of protein in the various fractions required to induce a profound increase in the mepp frequency.

Fig. 1 illustrates the results of a test with the whole extract (supernatant) of venom glands. It is obvious that the addition up to 3.3 μ g protein had little effect on the mepp frequency whereas the addition of another 1.1 μ g caused a great increase. In our experiments the threshold dose was defined as the average between the last dose that had minimal effects on mepp frequency and the dose that caused an increase to levels greater than 200 per second. In Fig. 1 the threshold dose is 3.9 μ g.

To determine the activity of a particular fraction, the fraction was diluted to a convenient protein concentration with 114 mM NaCl in 6 mM Tris (pH 7.2) and small aliquots (5–100 μ l) were added directly to the muscle chamber near the recording micropipette. The preparation was monitored for 15 min; and, if no effect was observed, another addition was made. Usually, successive additions were made so as to double the amount of protein already in the chamber. Once the frequency of mepp's had begun to rise, it could not be reversed by washing (31), so that only one active fraction could be tested on a given muscle. The doses administered are given in terms of the total number of micrograms of protein added to the chamber. We prefer these units rather than units of concentration, because we were not sure that the protein was always distributed uniformly throughout the bathing solution and because the volume of solution in the chamber was not constant but may have varied by $\pm 20\%$ from day to day. Although this

technique of application cannot be used for highly quantitative work, it was adequate for monitoring the purification.

ELECTRON MICROSCOPY: Essentially the same

procedures were used as described previously (11, 29). Muscles were fixed in the recording chamber. The bathing solution was replaced with cold fixative solution containing 2% OsO₄ in 0.12 M phosphate buffer (pH

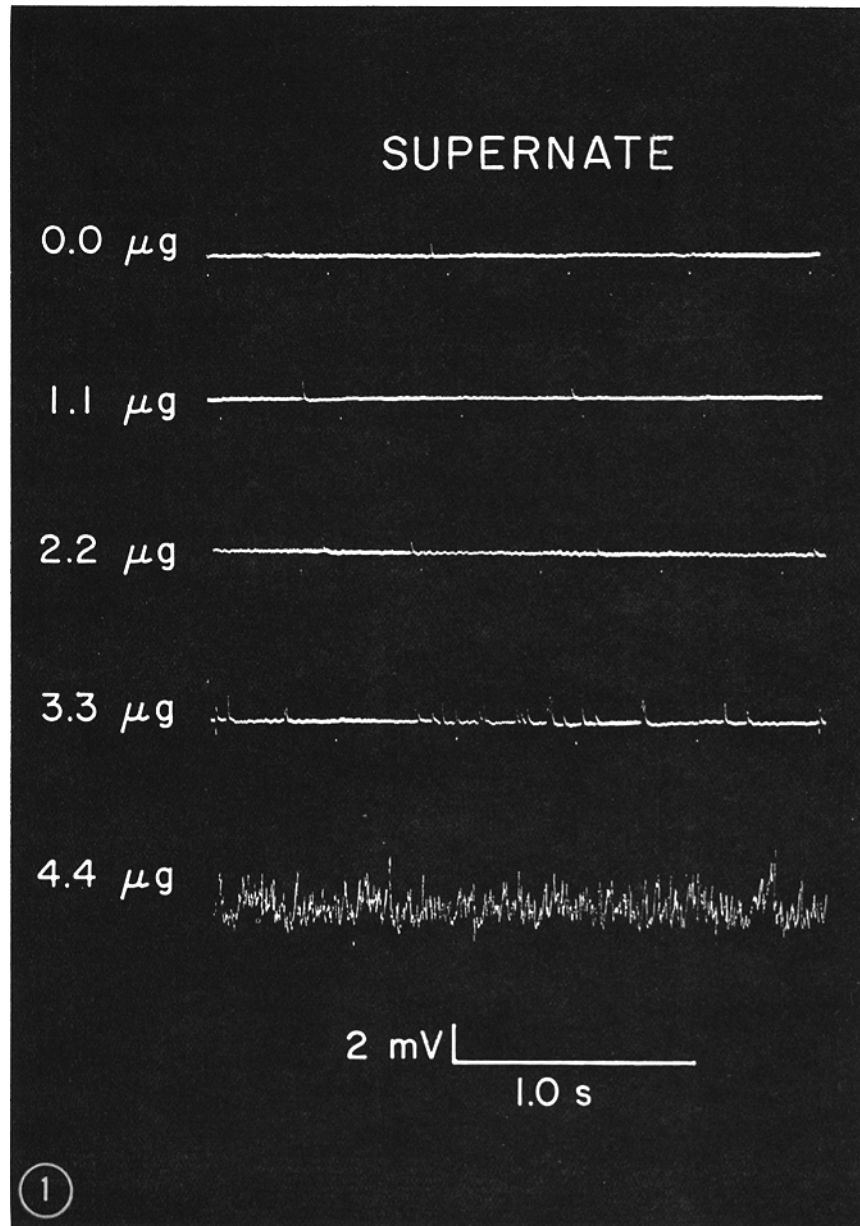


FIGURE 1 An increase in mepp frequency at frog neuromuscular junction induced by the whole extract (supernate). All the records were taken from the same neuromuscular junction, and each trace shows a sample of the mepp's recorded 15 min after the addition of the quantity of protein indicated at the left. In this experiment a great increase in frequency occurred after the addition of 4.4 μg protein. The volume of solution in the chamber was about 3.5 ml.

7.2), and the chamber was put into a refrigerator. After about 20 min the chamber was removed and under the dissecting microscope small bits of muscle containing suspected end plate regions were cut out and transferred to fresh cold fixative for a total fixation time of about 2 h. All specimens were dehydrated in alcohol and flat embedded in Epon 812. The blocks were trimmed under a dissecting microscope and the orientation of the muscle fibers was checked under a light microscope so that longitudinal sections of the nerve terminals could be cut with a diamond knife. The sections were double stained with uranyl acetate and lead citrate and were examined in either a Philips EM 200 or EM 300 electron microscope.

OBSERVATIONS ON LIVING TERMINALS: Freshly dissected cutaneous pectoris muscles of small frogs were prepared for mounting in an observation chamber. This chamber was simply constructed. A 1-cm square glass plate (approx. 0.5 mm thick) was glued to the center of a cover glass. The remaining area of the cover glass was coated with a thin layer of wax. The central transparent region of the chamber was thus thin enough to permit use of a short distance condenser. The muscle was stretched over the central glass plate and pinned to the surrounding wax. Regions of the muscles were selected in which features of the endplate could be easily recognized. To make detailed observations of the fine unmyelinated nerve terminals a Zeiss Nomarski (differential interference contrast) optical system was employed (34). A total magnification of 500 was achieved by using a $\times 40$ water immersion objective and a pair of $\times 12.5$ oculars.

RESULTS

Fractionation of the Venom and Purification of a Factor Toxic to Mice and Active on the Frog Neuromuscular Junction

When the whole extract of black widow spider venom glands was fractionated on a Sephadex G-200 column, several peaks of optical density were obtained (Fig. 2). The five peaks A-E and the low molecular weight peak were assayed for toxicity to mice and for electrophysiological activity at the frog neuromuscular junction, and only two (Fractions B and C) were active in both assays. Fractions B, C, and E were further fractionated on a DEAE-Sephadex A-50 column, with the results shown in Figs. 3 and 4. Only fractions B₅ and C₅ were active in the mouse assay and in the electrophysiological test. The fraction B₅ was over 40 times more active than fraction C₅ in the mouse assay and over 15 times more active in the neuromuscular junction assay. Since fraction B₅ was the major component of the whole extract and

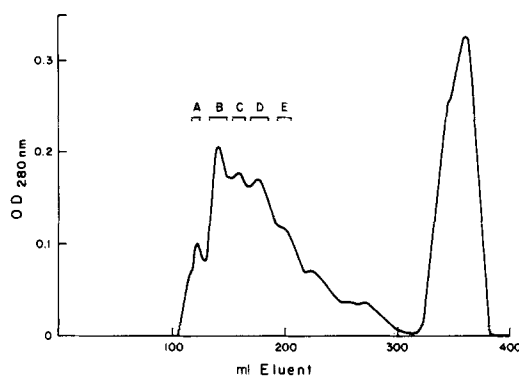


FIGURE 2 Fractionation of the whole extract on Sephadex G-200. Details are given in Materials and Methods. The fractions A, B, C, D, and E were pooled separately as shown in the figure and assayed as described. The large peak on the right represents the low molecular weight compounds.

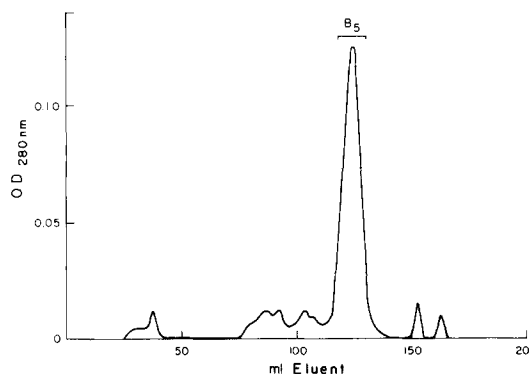


FIGURE 3 Fractionation of fraction B on a DEAE-Sephadex A-50 column. Details are given in Materials and Methods. The B₅ fraction was tested by bioassay and was used in gel electrophoresis, isoelectric focusing, and immunoelectrophoresis experiments.

was most active in the frog neuromuscular junction and in mice, most of the work was performed on it.

Fig. 5 illustrates the effect of fraction B₅ on the frequency of occurrence of mepp's at a frog neuromuscular junction. It is clear that this fraction produced the great increase in frequency that is characteristic of the whole extract but at a much lower quantity of protein. Quantitative data from one fractionation are given in Table I. In this fractionation the threshold dose for the frog neuromuscular preparation decreased successively from 1.0 μg for the whole extract to 0.4 μg for fraction B to 0.08 μg for fraction B₅, entailing a 12.5-fold purification. In the same fractionation the specific

activity of the factor lethal to mice increased successively from 6.7 to 40 to 106 toxicity U/ μ g protein, a 16-fold purification. Considering that the method for determining the threshold dose at the neuromuscular junction is not highly accurate, the purification with respect to these two biological tests seems to proceed in parallel. In three separate fractionations of fresh venom, the average threshold dose for the whole extract was 1.9 μ g of protein (range, 1.0–3.1 μ g) and the average threshold dose for the B₈ fraction was 0.2 μ g (range, 0.07–0.3 μ g). The average purification was about 10-fold (range, 9–14).

A superthreshold dose of the whole extract has a number of electrophysiological effects on neuromuscular junctions (31). The initial effects are to increase the frequency of occurrence of mepp's and to potentiate the amplitude of the epp. Fibrillation of the muscle often occurs. The mepp frequency and the amplitude of the epp reach a peak in a few minutes and then begin to decline. Ultimately the epp's vanish and the mepp frequency approaches the initial low levels and neuromuscular transmis-

sion is totally blocked. We observed that large doses of fraction B₈ produced all of these effects of the whole extract. However, when just threshold doses were used, fibrillation did not occur, the mepp frequency sometimes remained high for prolonged periods, potentiated epp's were often present after an hour, and some fibers would twitch in the low Ca- and high Mg-bathing solution.

Morphological Effects of B₈ Fraction on the Frog Neuromuscular Junctions

Another striking action of the whole extract of venom glands is to cause the depletion of synaptic vesicles at frog and mouse neuromuscular junctions (12, 29), and it was important to determine whether fraction B₈ also caused a depletion of vesicles. In these experiments the B₈ fraction was applied at five times the threshold dose, as determined on the paired muscle from the same frog, and was left on for an hour. Neuromuscular transmission was completely blocked after this

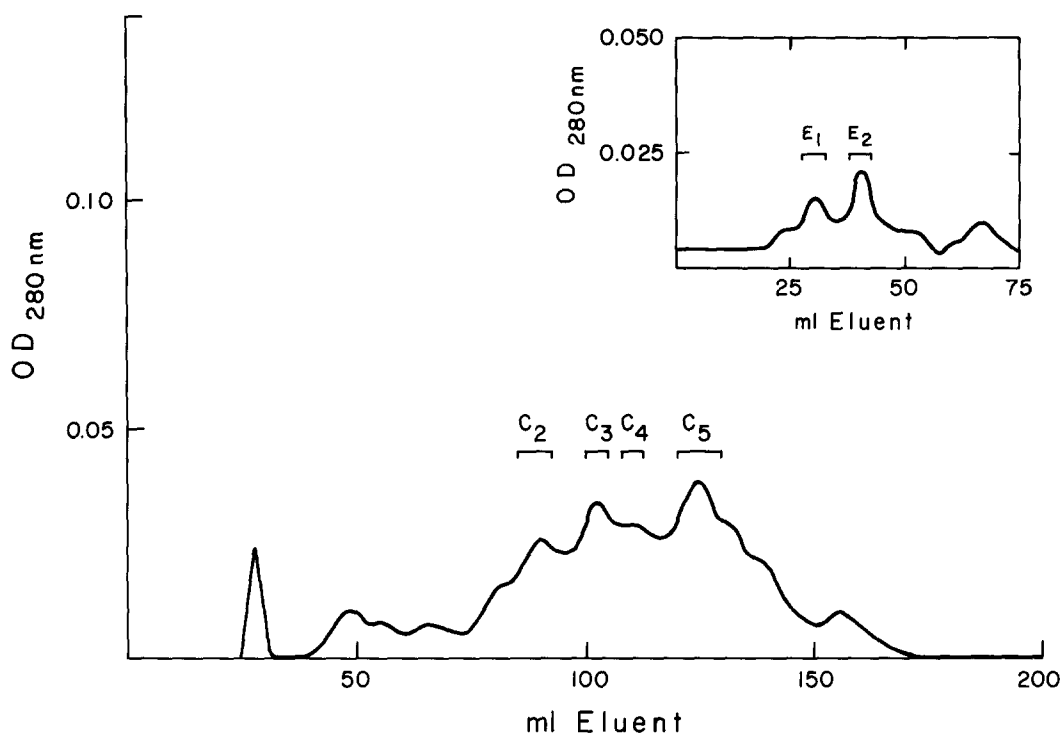


FIGURE 4 Fractionation of fractions C and E on a DEAE-Sephadex A-50 column. Details are given in Materials and Methods. The peaks C₂, C₃, C₄, C₅, and E₁ and E₂ were used for gel electrophoresis and tested by bioassays.

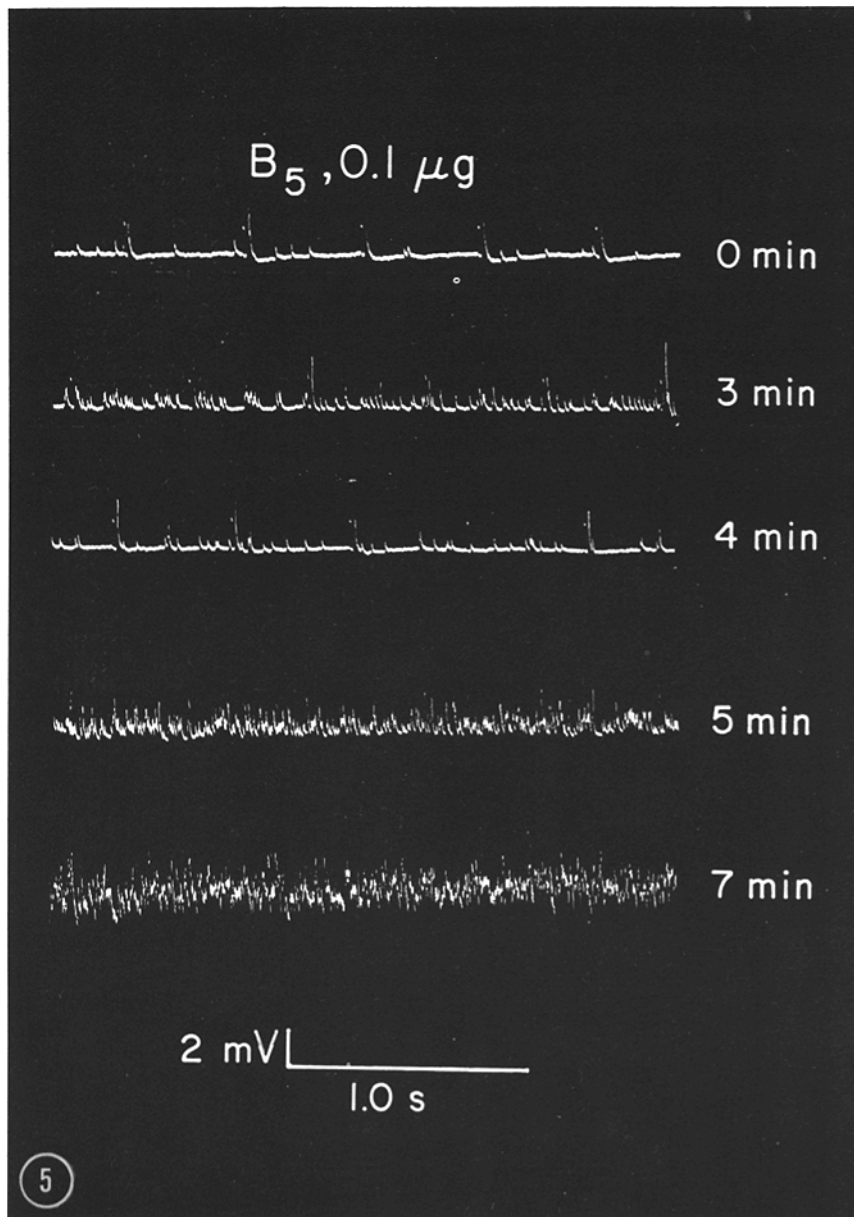


FIGURE 5 Increase in mepp frequency at a frog neuromuscular junction induced by the addition of $0.1 \mu g$ of the B_5 fraction. Each trace shows a record obtained at the indicated time following the addition of the fraction. In this experiment $0.1 \mu g$ of fraction B_5 was the smallest dose tested, and it provoked the full discharge.

time, indicating that all the terminals had been affected by the fraction. Figs. 6-8 show that the B_5 fraction caused profound depletion of the synaptic vesicles. In addition to depleting the vesicles, the B_5 fraction caused a marked swelling of the

terminal and its mitochondria as evidenced by the increase in the dimension of the terminal normal to the surface of the muscle fibers.

Clark et al. (12) previously reported that the crude extract of the venom glands produced swell-

TABLE I
Fractionation of the Whole Extract of Black Widow Spider Venom Glands

Preparation	Protein	Mouse toxicity		Threshold dose on frog neuromuscular preparation
		Toxicity units	Specific activity	
	<i>mg</i>	<i>tu</i>	<i>tu/μg</i>	<i>μg</i>
Whole extract run on Sephadex G-200	30.6	210,000	6.7	1.0
Sephadex G-200				
Peak A	0.62	<1,500	<2.5	>4
Peak B	4.00	160,000	40.0	0.4
Peak C	3.10	25,000	8.3	~1*
Peak D	2.80	<6,700	<2.4	>10†
Peak E	1.80	<1,800	<1.0	>1.6
B run on DEAE-Sephadex				
Peak B ₅	1.46	154,000	106.0	0.08
C run on DEAE-Sephadex				
Peak C ₂	0.17	<350	<2	ND
Peak C ₃	0.19	<390	<2	ND
Peak C ₄	0.20	<400	<2	ND
Peak C ₅	0.53	1,390	2.6	1.2
E run on DEAE-Sephadex				
Peak E ₁	0.09	ND	—	ND
Peak E ₂	0.11	ND	—	ND

The glands from 300 spiders were used.

ND = not determined.

* The threshold dose is approximate because C decayed rapidly, the threshold dose rising from 0.23 to 2.4 μg over the course of 4 days.

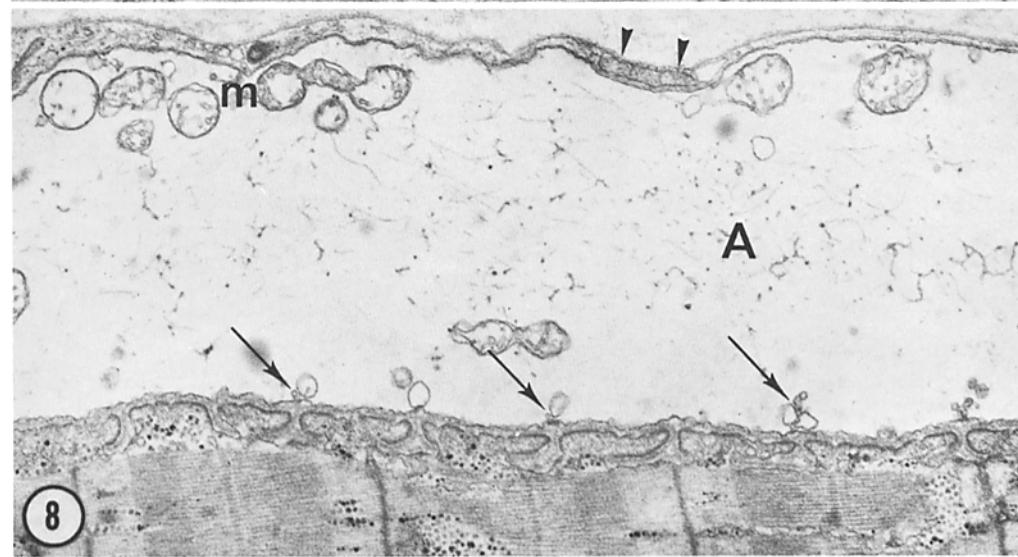
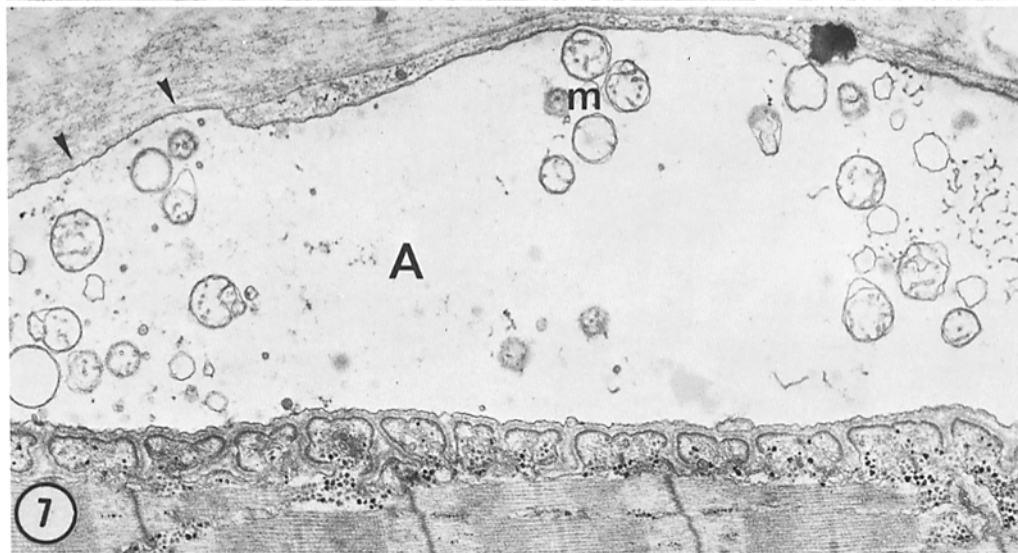
† Peak D was not tested in this particular fractionation, but in a previous one it was found to be active only after 20 μg protein had been used.

ing of the frog nerve terminal. However, the swelling induced by the B₅ fraction seemed greater, and therefore we thought that it would be possible to confirm this observation by direct observation of living nerve terminals viewed with Nomarski optics (34). Fig. 9 shows three photomicrographs of the same portion of a single nerve terminal: before (a), and 30 min (b) and 60 min (c) after, the addition of 3.2 μg of fraction B₅. There was a clear increase in the transverse dimension of the terminal. This increase, about three-fold (see the portion delineated by the arrows), corresponds roughly to the increase in the normal dimension seen in the electron micrographs. Thus, these direct observations of the nerve terminal fully corroborate the findings with the electron microscope that swelling occurs.

Gel Electrophoresis of the Separated Fractions and Isoelectric Focusing of Fraction B₅

To assess the degree of purification of the various fractions separated by the two chromatographic steps, SDS-gel electrophoresis was performed with samples of the whole extract and of each of the main fractions. The results obtained under two different conditions of electrophoresis are shown in Figs. 10 and 11.

Fig. 10 shows the results obtained with a gradient gel of 5–10% polyacrylamide. Fraction A (slot 2), which absorbed intensely at a wavelength of 280 nm (Fig. 2), shows practically no protein bands. This fraction may contain large lipid or glycolipid aggregates, which are responsible for,



most of the UV absorption, as well as a small amount of protein. Fractions B, C, D, and E (slots 3, 4, 5, and 6, respectively) each show several protein bands after electrophoresis, and the major bands in these fractions show a trend towards decreasing molecular weight, in good agreement with their order of elution from the Sephadex G-200 column. Fraction B_s was run at three different amounts of protein: 2, 5, and 20 μgs (slots 7, 8 and 9, respectively), and always gave a single band under this condition of electrophoresis. However, fraction B_s was resolved into two close bands when the conditions were varied, either by allowing greater migration of the samples through the gels or by decreasing the acrylamide concentration of the gel. One example is shown in Fig. 11 a in which a 5% polyacrylamide gel was used and B_s was allowed to migrate farther into the gel. When isoelectric focusing on polyacrylamide slab gel was performed, fraction B_s was separated into four major bands with isoelectric points distributed over a narrow range between pH 5.2 and 5.5 (Fig. 11 b).

To assess whether these protein components in fraction B_s represent similar or entirely different entities, immunoelectrophoresis techniques were employed. Fig. 12 a shows the crossed immunoelectrophoresis pattern of the whole extract. Several bands of precipitate that cross over one

another can be resolved, indicating the presence of several immunologically distinct molecules. However, fraction B_s showed only one band of precipitate when it was run into the same antivenin (Fig. 12 b). Moreover, in crossed immunoelectrophoresis, in which the four components separated by isoelectric focusing were run into an agarose gel containing the antivenin, a continuous immunoprecipitate was formed (Fig. 12 c), rather than four separate overlapping precipitates, indicating that all four components reacted with the same antibody fraction. Thus, although fraction B_s is heterogeneous regarding charge properties, no differences between the protein molecules were detected immunologically.

Chemical and Enzymatic Properties of Fraction B_s

The apparent molecular weight of the fraction B_s was determined in two ways: by its rate of migration on SDS gel (Figs. 10 and 11) and by sieve-gel chromatography (Fig. 13). On SDS gel, fraction B_s migrates at a slightly slower rate than does β-galactosidase (mol wt, ~ 130,000) and we estimated the mol wt to be somewhat greater than 130,000 by this method. The mol wt as determined by sieve-gel chromatography is 110,000 (Fig. 13). Both methods indicate that fraction B_s is a collec-

FIGURE 6 Low power electron micrograph showing a portion of a neuromuscular junction from a frog cutaneous pectoris muscle. This control muscle had been soaked for 1 h in 0.5 mM Ca and 4 mM Mg. The axonal ending is covered by a thin Schwann cell process (*Sc*), and the terminal contains numerous mitochondria, neurofilaments, elements of smooth endoplasmic reticulum, glycogen (*g*), and the normal complement of synaptic vesicles (*v*). × 18,000

FIGURE 7 Low power electron micrograph showing a portion of a neuromuscular junction from a preparation fixed 1 h after the onset of the mepp discharge induced by 3.2 μg (5 × the threshold dose) of fraction B_s added to the 3.5 ml volume of the recording chamber. The axonal ending (*A*) appears to be grossly swollen, and the cytoplasmic matrix seems less dense than normal. At the top left corner a portion of the axolemma opposite the junctional region seems to have protruded from beneath the thin Schwann cell process (arrowheads) that usually covers the terminal. The terminal contains swollen mitochondria (*m*) and some large vesicular structures but no structures resembling synaptic vesicles. × 20,000

FIGURE 8 Electron micrograph from a different neuromuscular junction from the same preparation used in Fig. 7. The swollen axonal endings (*A*) appear almost completely devoid of synaptic vesicles. The terminal contains swollen mitochondria (*m*), neurofilaments, and elements of smooth endoplasmic reticulum. A very few structures resembling synaptic vesicles and some large vesicular structures are seen closely associated with the prejunctional membrane (arrows). Arrowheads indicate a normal mitochondria within a thin Schwann cell process. × 22,000

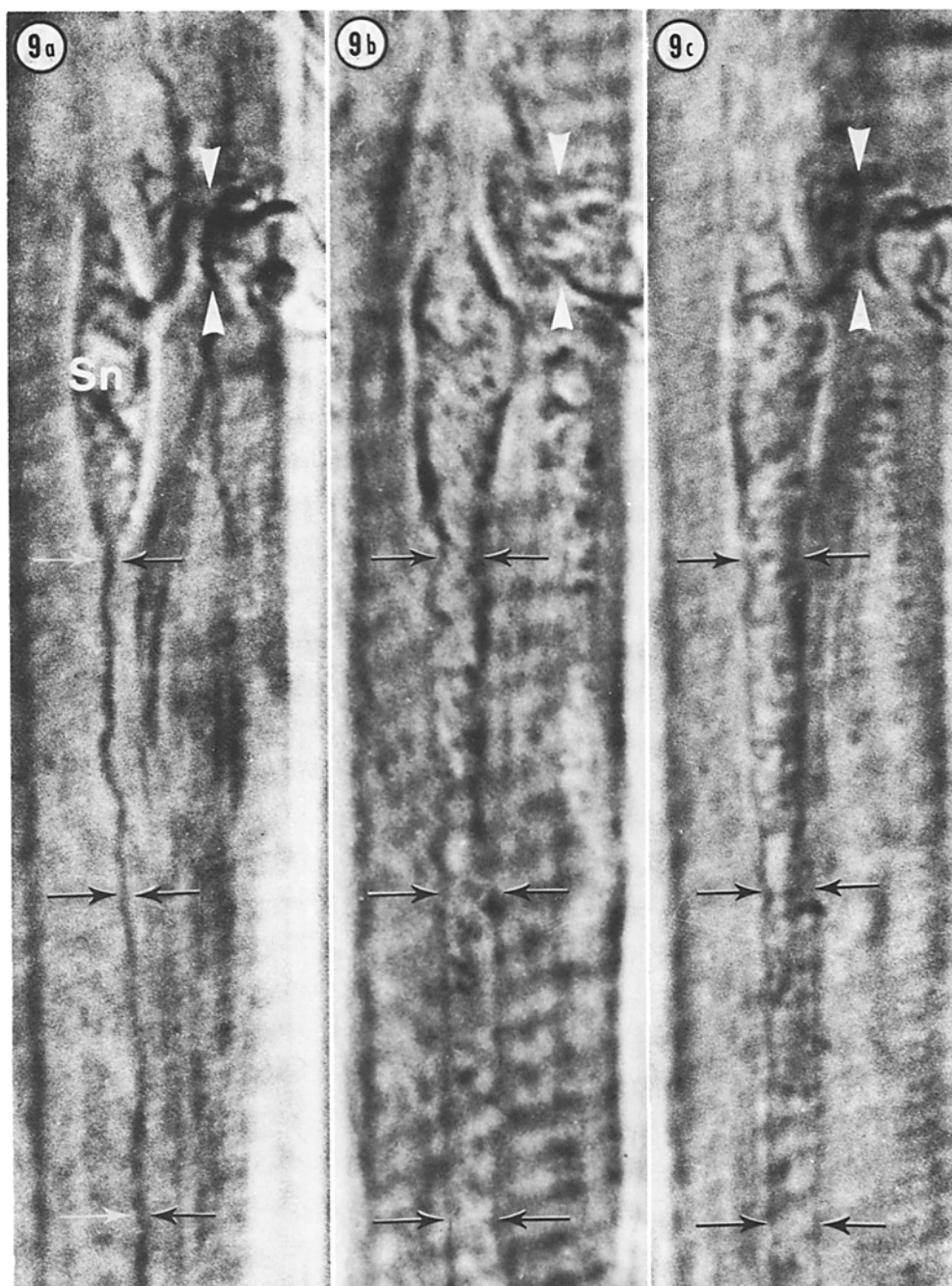


FIGURE 9 A nerve terminal in a live preparation of frog cutaneous pectoris muscle viewed with Nomarski optics. These three micrographs show the initial segment of a nerve terminal which is part of an extensive arborization. The preparation was soaking in a solution with 0.5 mM Ca and 4 mM Mg. An unmyelinated terminal branch (arrows) arises from the last myelinated segment of an axon (arrowheads) and runs along the surface of a muscle fiber. The nucleus of a Schwann Cell (*Sn*) covers the proximal portion of the terminal. The striations of the muscle fiber are not seen clearly at this focal plane. (*a*) micrograph of the untreated terminal; (*b* and *c*) micrographs of the same portion of the terminal taken 30 min and 60 min after the addition of 3.2 μ g of fraction B_s to the observation chamber. A marked increase in the transverse dimension of the terminal is evident (arrows). $\times 2,150$.

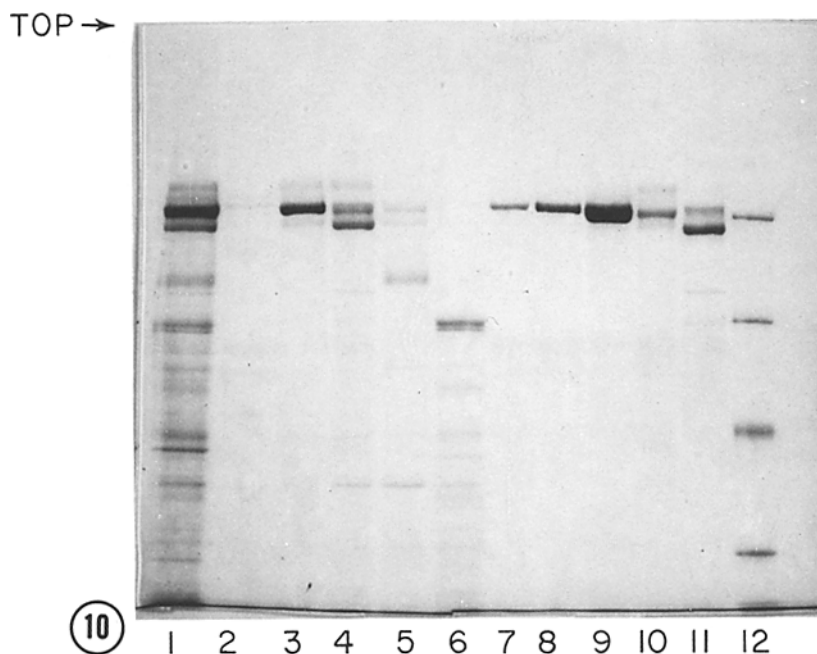


FIGURE 10 SDS-gel electrophoresis of various samples obtained after fractionation. The gel (21-cm high, 30-cm wide) contained a 5–10% gradient of polyacrylamide and a 5–13% gradient of sucrose. Electrophoresis was run at room temperature for 12 h at 20 mA. The numbered slots contained (in μg protein): (1) 50 μg whole extract; (2) 4 μg fraction A; (3) 8 μg fraction B; (4) 12 μg fraction C; (5) 6 μg fraction D; (6) 14 μg fraction E; (7–9) 2, 5, and 20 μg fraction B_5 ; (10) 8 μg fraction C_{3+4} ; (11) 9 μg fraction C_5 ; (12) standard mixture containing (from the top down) 2 μg each of: *Escherichia coli*, β -galactosidase subunit (mol wt, ~ 130,000), bovine serum albumin (mol wt, 67,000), ovalbumin (mol wt, 45,000), chymotrypsinogen A (mol wt, 25,000) and hemoglobin subunit (mol wt, 15,000) moving with the front.

tion of of large molecules, and this prompted us to look for sugar residues. With the use of the PAS staining method (45) on gels after electrophoresis, no staining could be detected in 30 μg of fraction B_5 . From the smallest amount of glycoprotein standards (ovalbumin: 45,000 mol wt, 3.5%; carbohydrate and fetuin: 40,000 mol wt, 22% carbohydrate) that gave a visible reaction when run on the same gel, we estimate that if carbohydrate is present in fraction B_5 it is less than 1% by weight. Such noncarbohydrate-containing proteins as β -galactosidase, serum albumin, chymotrypsinogen, and hemoglobin are not stained by this method.

No proteolytic activity could be detected in either the whole extract or fraction B_5 when the Azocoll assay (35) was used. For example, in an assay where 0.1 μg trypsin activity could be detected, 500 μg of the whole extract showed no activity. When the whole extract or B_5 was tested in an assay for phospholipase A and phospholipase C (19), only a questionable borderline activity

could be found. 100 μg of the whole extract, 185 μg of fraction B, and 80 μg of B_5 all gave only a very small increase over the blank, but since this increase was not stoichiometric with respect to protein concentration, and since it was neither increased by Ca^{2+} addition nor decreased by EDTA addition, it was assumed not to be due to lipase activity. If fraction B_5 has any lipase activity undetectable by this assay, it is several 100-fold less than that of an equivalent weight of purified phospholipase C. Thus, at the present time, it appears that fraction B_5 has no proteolytic activity and probably no phospholipase C or phospholipase A activity.

The fraction B_5 is rather stable when kept in Tris buffer at pH 8.2. No activity was lost during 2 months storage at -80°C . When it was stored at -20°C , either with or without 20% glycerol, erratic results were obtained, and when it was stored in solution at $0-4^\circ\text{C}$, the activity decayed with a half-life of 1–2 wk.

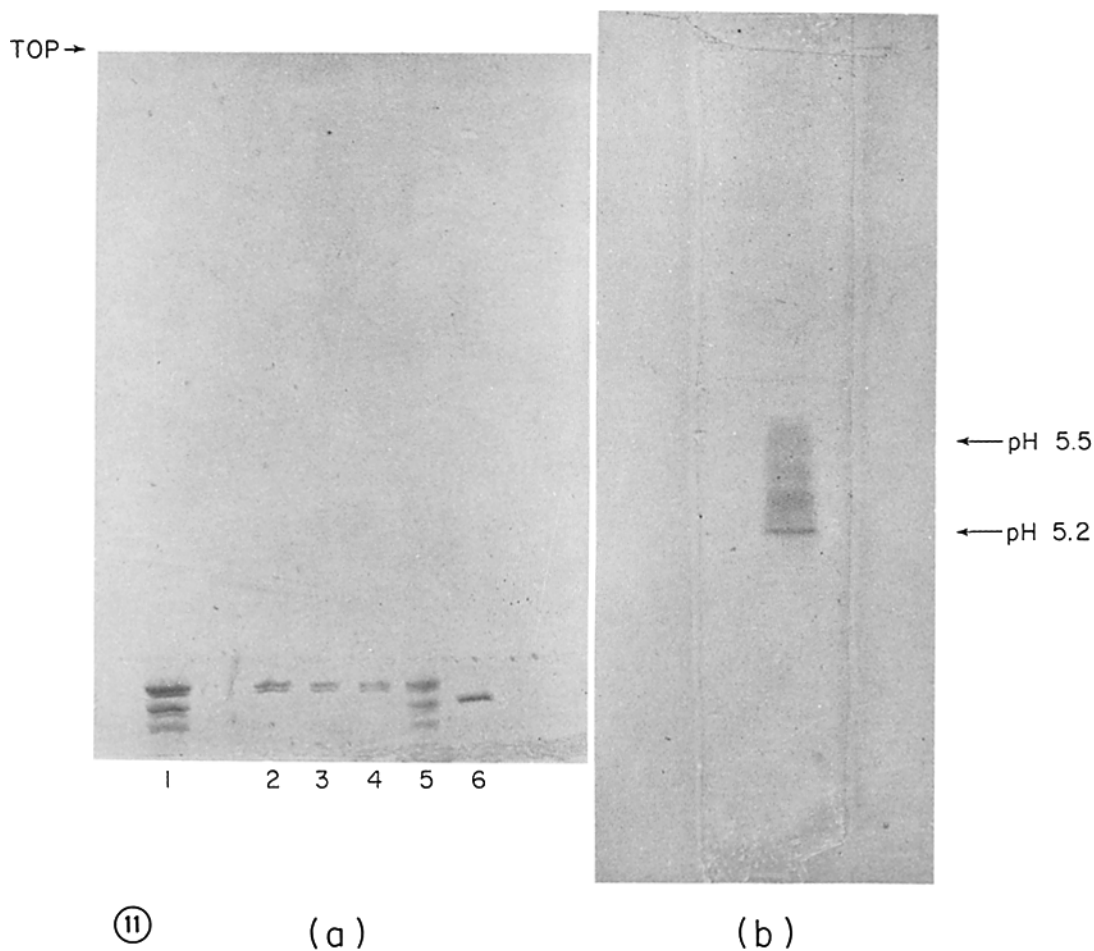


FIGURE 11 Resolution of fraction B₅ into several components. (a) SDS-gel electrophoresis with a uniform 5% polyacrylamide gel (17-cm high, 12-cm wide). A constant current of 15 mA (1.25 mA/cm) was applied for 5 h. (1) whole extract (40 μg); (2), (3), (4), fraction B₅ (10, 3, and 1 μg, respectively); (5) whole extract (15 μg); (6) β-galactosidase (2 μg). (b) Isoelectric focusing of fraction B₅ (20 μg) in a 5% polyacrylamide slab gel (14 × 12 cm) containing 1.7% each of Ampholine pH 4–6 and Ampholine pH 5–7. Constant current of 4.5 mA was applied until the voltage reached 500 V (in ~ 2 h), then constant voltage of 500 V was used. Total running time was 10 h. The measurement of pH along the gel was estimated according to Scheele (41).

Biological Properties of the Various Protein Fractions

The whole extract of the venom glands of black widow spiders has a wide spectrum of action (see introductory paragraph). Therefore, the effects of the various fractions that we obtained were qualitatively assayed, not only on mice and on the frog neuromuscular junction, but also on the crayfish stretch receptor, on the cockroach heart, and on houseflies. The results are shown in Table II. Fractions A and D had no effect on any of the preparations, and fraction B₅ affected only mice

and the frog neuromuscular junction. Only fraction E caused firing of the crayfish stretch receptor. This activity resided in the subfraction E₂. Fraction C was active on all the preparations except the stretch receptor. Subfraction C₃ was toxic to flies and was active on the cockroach heart; subfraction C₅ was toxic to mice and flies and was active on the frog neuromuscular junction. Neither of the C subfractions was pure (cf. Fig. 10), and both seemed to contain a component that migrated on SDS gel at a rate similar to that of fraction B₅.

Although fraction C was active on the frog

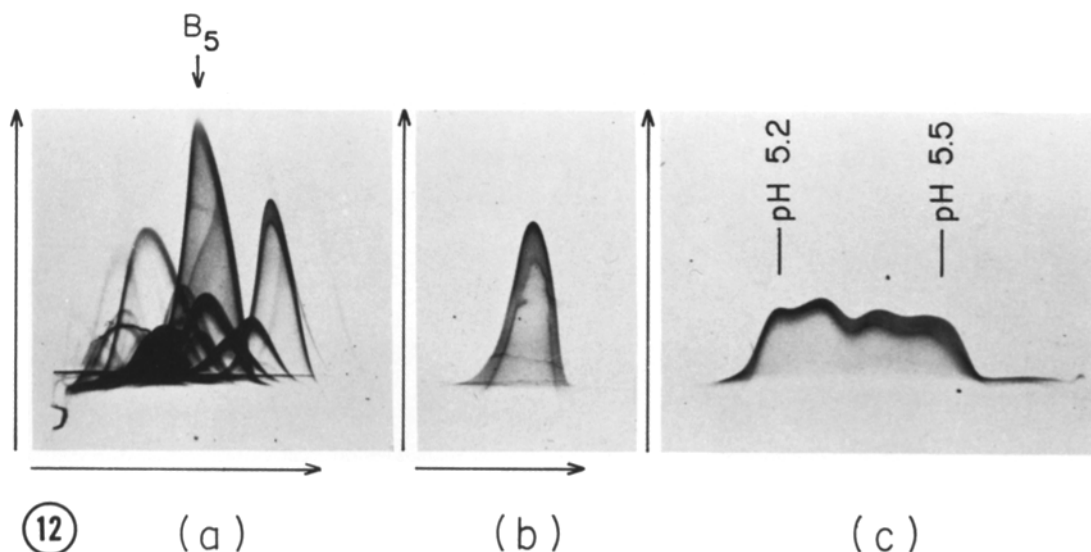


FIGURE 12. (a) Crossed immunoelectrophoresis of 5 μ l of whole extract (7.5 μ g/ μ l) performed as described in Materials and Methods. The agarose gel for the second dimension of immunoelectrophoresis contained 8% (vol/vol) antivenin. (b) Crossed immunoelectrophoresis of fraction B₅ (6 μ g); conditions were as in (a). (c) Crossed immunoelectrofocusing of fraction B₅. Isoelectric focusing was performed as described in Fig. 11b. The upper portion of the agarose gel for immunoelectrophoresis contained 10% antivenin. The arrows indicate direction of electrophoresis from negative to positive.

neuromuscular junction and was toxic to mice, its specific activity was less than that of the whole extract, and the specific activity of subfraction C₅ was less than that of fraction C. Although fractions B and B₅ were stable for up to 2 wk when kept at 1°–4°C, fractions C and C₅ lost activity in a few days when kept under the same conditions. This loss of activity in fraction C is probably not due to the presence of a contaminating protease, since we could not detect proteolytic activity in the whole extract, as mentioned above. The quick inactivation of fractions C and C₅ explains why their purification may be accompanied by a reduction in specific activity, and it accounts also for the absolute loss of toxicity units that occurred during the separation of these fractions (Table I). Of the 700 toxicity U/spider contained in the whole extract, 76% were recovered in fraction B and 12% in fraction C (total recovery 88%); after the second step of purification, all of the toxicity units contained in fraction B were recovered in fraction B₅, whereas only 5.8% of the toxicity units contained in fraction C were recovered in fraction C₅. This different resistance to inactivation shown by fractions B and C suggests that the activity of fraction C is not due entirely to contamination from

fraction B, but is probably due to a different principle.

DISCUSSION

Previous work has shown that the whole extract of the venom glands of black widow spiders increases the frequency of mepp's (12, 15, 30, 31, 37), causes the release of neurotransmitters (20, 21, 22, 24, 25) and causes a depletion of synaptic vesicles (9, 12, 13, 15, 27, 29, 37). These various effects could have been caused by different components of the extract. The present results show that at the frog neuromuscular junction a purified protein fraction, B₅, is responsible for both the increase in the frequency of mepp's and for the depletion of vesicles. We do not know whether these two phenomena result from a single action of the fraction (increasing the rate of secretion of quanta of transmitter) or whether they result from two separate actions, one on the rate of secretion of transmitter and the other on the recovery of synaptic vesicles from the axolemma, or both.

Fraction B₅ contains little, if any, sugar residues and has undetectable proteolytic or lipolytic activity. Since the fraction B₅ shows little of these enzymatic activities, it seems unlikely that its ef-

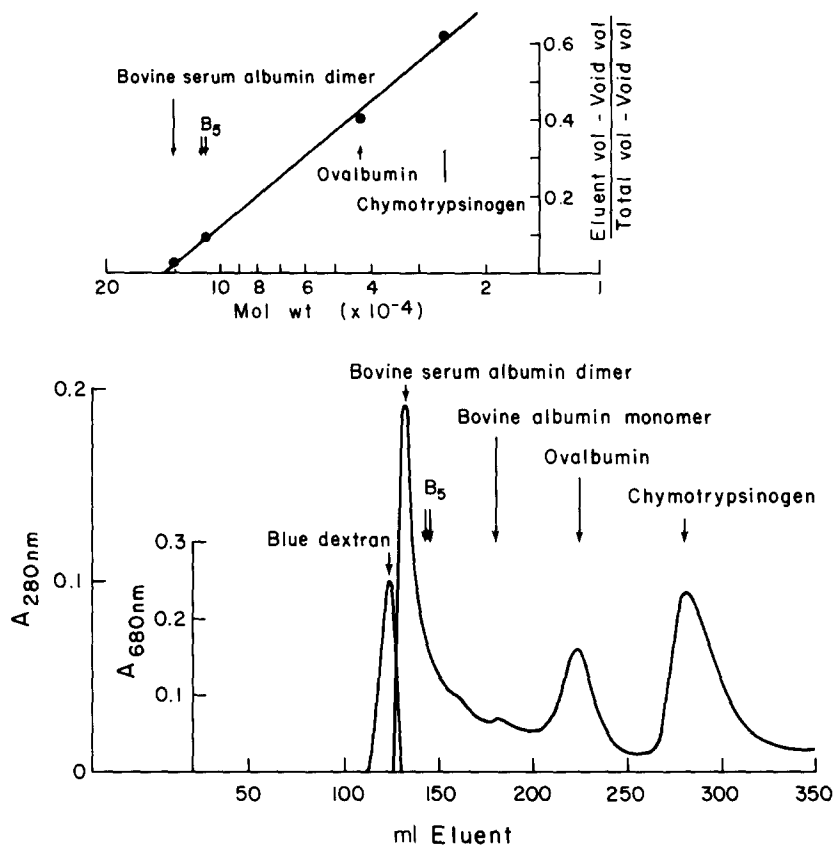


FIGURE 13 Determination of molecular weight of B_5 fraction by sieve-gel chromatography on Sephadex G-200. Blue dextran was used as a marker for the void volume and bovine serum albumin dimer, ovalbumin, and chymotrypsinogen were used as molecular weight standards. The double arrow marks the volume at which fraction B_5 were eluted. Insert gives a plot of molecular weight against the migration ratio. The double arrow marks the molecular weight of fraction B_5 .

fects on transmitter release and ultrastructure are due simply to the hydrolysis of membrane proteins or phospholipids. It is therefore probable that this fraction interacts in another relatively specific manner with the membrane of the nerve terminal and disrupts the normal chain of events involved in transmitter release.

The B_5 fraction could be separated into two components by SDS-gel electrophoresis and it could be separated into four components by isoelectric focusing. The components have similar mol wt of approx. 130,000, their isoelectric points are distributed narrowly between pH 5.2 and 5.5, and they cross-react immunologically. Thus, although fraction B_5 is not a pure protein, the components seem similar enough in their physical and immunological properties to warrant further detailed studies. Experiments are under way to test

the biological activity of each of the components of fraction B_5 .

Frontali and Grasso (23) previously fractionated the extract of black widow spider venom glands using as a first step a column of Sephadex G-100, and as a second step either column electrophoresis or ion exchange chromatography. One of their fractions, LV_3 , evidently contained fraction B_5 , although in a less purified form than in the present paper. In fact, LV_3 was highly toxic to guinea pigs (23) and to mice (44). The other two active factors, labelled LV_1 and LV_2 , which were toxic to houseflies, are probably contained in our fraction C. The electrophoresis fraction LV_4 , which was found to be active on the crayfish stretch receptor (5), clearly contained the factor presently separated as fraction E_2 . A difference between the previous work and the present experiments was

TABLE II
Qualitative Effects of Various Fractions on Different Biological Systems

	Frog Neuromuscular Junction	Mouse toxicity	Housefly toxicity	Cockroach heart beat	Crayfish stretch receptor
Whole extract	+	+	+	+	+
A	-	-	-	-	-
B	+	+	-	-	-
C	+	+	+	+	-
D	-	-	-	-	-
E	-	-	ND	ND	+
B _s	+	+	-	-	-
C ₂	-	-	-	-	ND
C ₃	-	-	+	+	ND
C ₆	+	+	+	-	ND
E ₁	-	-	ND	ND	-
E ₂	-	-	ND	ND	+

+ = Active.

- = Inactive.

ND = not determined.

the use of borate buffer previously and Tris buffer now. We found that the use of borate buffer prevented the adequate separation of fractions B and C with a Sephadex G-200 column. This effect may be due to the ability of borate to complex with sugars.

One difference between the effects of fraction B_s and of the whole extract is the degree of swelling they induce in nerve terminals and their mitochondria. The swelling induced by the fraction B_s is much more pronounced than the swelling induced by the whole extract. It is possible that this difference between the two experiments is due to differences between the actual amounts of the active fraction that were used in the experiments. We estimate that the average amount of protein added to the muscle chamber in our previous experiments with the whole extract was approx. 13 μg. Since the average purification achieved in obtaining fraction B_s was about 10-fold, we estimate that with the whole extract we used about 1.3 μg of fraction B_s. In the experiments illustrated in Figs. 7-9 we used 3.2 μg of purified B_s. Perhaps this difference in the amounts of B_s used in the two sets of experiments can explain the difference in the degrees of swelling observed.

One unexplained aspect of the venom action has been the fate of the membrane of the depleted

vesicles. The electron micrographs and the direct observations of the living preparation both show that fraction B_s induces profound swelling of the nerve terminal. The cause of the swelling is unknown; it may be due to venom-induced changes in the osmotic properties of the terminal, as witnessed by the swelling of the mitochondria. Whatever the cause, it seems likely that the swelling is accompanied by an increase in the area of the axolemma. If nerve terminals were circular in cross section, then our estimate that their diameter increased threefold would imply that the surface area also increased threefold (if their length remained constant). However, many normal nerve terminals are not circular in cross section so that some swelling could occur without an increase in surface area. Thus, the change in surface area induced by the B_s fraction is probably less than threefold. The estimates of the ratio of the area of synaptic vesicle membrane to the area of plasma membrane at the frog nerve terminal range from 1.3 (28) to 3.0 (12). The smaller estimate predicts that the area of the axolemma could increase 2.3-fold if all the synaptic vesicles were incorporated into it. Thus it is possible that most of the extra membrane needed to surround the swollen terminals is derived from vesicles that have become permanently in-

incorporated into the axolemma, as suggested previously (10).

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