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SEMI ANNUAL PROGRESS REPORT

A STUDY OF "IMMEDIATE" SENSITIZATION BY ADSORPTION OF ANTIGENS AND ANTIBODIES *IN VITRO*

INTERIM TECHNICAL REPORT

ON

"Studies on the Mode of Action of Sea Urchin Toxin. Enzymatic and Immunological Behavior"

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Prepared by

George A. Feigen

Associate Professor of Physiology
Stanford University, School of Medicine
Stanford, California

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Previous studies (1, 2) from this laboratory have shown that most of the toxic potency found in the crude pedicellarial venom of *Tripneustes gratilla* is concentrated in the fraction precipitating in the presence of 65 per cent saturated ammonium sulfate. During the course of pharmacological experiments (2) it was observed that the LD_{50} in mice was somewhat lower than the median effective dose required for the direct stimulation of the guinea pig ileum. A plausible reason for this amplification in toxicity is that certain constituents of the active fractions of toxin might have a proteolytic action which could result in the formation of active plasma kinins of the sort described for the action of snake venom (3) and for the catalysis of serum proteins by kallikrein (4).

In the present study we show that the interaction of crude sea urchin toxin, or its purified fractions, with certain substrates in the plasma produces dialyzable, heat stable substances that have the capacity to initiate contractile reactions of the guinea pig ileum and the rat uterus. A preliminary analysis of the interaction suggests that the toxin behaves kinetically as an enzyme and that its proteolytic action is selective on certain components of the globulin fraction of mammalian plasma. The enzymatic activities of the selected toxin preparations have a temperature optimum at 26°C. Precipitating antibodies produced to the formalinized toxoids can quantitatively neutralize the lethal effects as well as the *in vitro* pharmacological actions.

MATERIALS

Toxin Preparations

Two general types of toxin preparation were used in these experiments. The first type, described in our previous publications, was obtained by fractional precipitation with ammonium sulfate. The physical and pharmacological properties of these fractions may be obtained by reference to the code on p. 162 of the preceding report (2). The second type was prepared by gel filtration on Sephadex G-200 (Pharmacia). The starting material was the total pedicellarial yield of 65 specimens which were processed according to the general scheme exhibited in Figure 1. Percolation through Sephadex resolved the crude material into three independent fractions exhibited by the peaks in Figure 2. The yields, lethal toxicities, and optical properties of the several preparations are given in Table I.

Bovine Serum Proteins

Whole bovine serum was obtained from the Bakte Scientific Laboratories, Berkeley, California. Before use, 100 ml of serum was dialyzed against 18 liters of 1% NaCl for 48 hours at 4°C. Bovine γ-globulin and bovine serum albumin were obtained from Armour Pharmaceutical Co. Each preparation was dissolved in 25 ml of 1% NaCl solution. The former was repeatedly precipitated in the presence of 1/3-saturated ammonium sulfate and the latter in 2/3-saturated salt at pH 7.8. After the third precipitation each preparation was taken up in 1% NaCl and dialyzed against 30 liters of NaCl solution for 72 hours at 4°C. The dialyzed proteins were centrifuged at 4°C and the supernatants stored at -20°C.

Human Serum Proteins

Two general kinds of human serum protein fractions were used in these experiments. The preliminary work was done on preparations obtained by salt fractionation, whereas the latter work was performed on commercially available Cohn fractions (obtained by alcohol precipitation) some of which were furnished to us as unknowns by the Hyland Laboratories.

The globulins obtained by salt precipitation were prepared according to a method modified from Kendall (5). In the present case, 1300 ml of human plasma was clotted by the addition of 20 mg Ca Cl_2 per 100 ml of fluid. After filtration 1000 ml of serum was treated with saturated ammonium sulfate (SAS) and the fractions precipitating at 33 and 50 per cent saturation were removed. In each case the precipitate was taken up in 1% NaCl and dialyzed against distilled water so as to provide for a water-soluble and a water-insoluble subfraction. Each subfraction was then repeatedly precipitated at 33 or 50 per cent SAS as required and dialyzed against distilled water, as before. After six precipitations each fraction was taken up in 60 ml of 1% NaCl, exhaustively dialyzed at 4°C, and then frozen. Fractions 1 and 2 were obtained in the presence of 1/3-saturated ammonium sulfate, and Fractions 3 and 4 were obtained in the presence of 1/2-saturation. At each precipitation subsequent treatment produced one water-soluble and one water-insoluble subfraction. Thus fractions 1 and 3 were water-soluble, while 2 and 4 were water-insoluble.

The Cohn fractions of human serum, supplied by the Hyland Laboratories as knowns, varied in their content of α- and β-globulins. According to Penell (6) Fraction III-0 was expected to contain 5 per cent α-globulin and 84 per cent β-globulin, whereas Fraction IV should have had 89 per cent of α- and 10 per cent of β-globulins. Subsequently, they supplied fractions III_1, IV_4, $IV_{5\ \&\ 6}$, and II + III as unknowns. Electrophoretically pure α_2-M globulin was obtained from Dr. H. H. Fudenberg, University of California Medical Center, San Francisco.

Toxoid

The subcutaneous inoculation of small quantities of active toxin into rabbits resulted in extensive necrosis of the site; hence, in order to produce a suitable antitoxin for use in the present experiments, it was necessary to immunize the animals with a formaldehyde-inactivated material. The crude toxin and the various fractions were toxoided by treating 1-ml portions of various preparations, made up to a concentration of 2 mg/ml, with an equal volume of 0.02% aqueous formaldehyde. The immunizing antigens were prepared by emulsifying one part of the toxoid with 2 parts of Freund's complete adjuvant.

Stock Solutions

All of the toxin and substrate preparations required for enzymological studies were kept as frozen stock solutions in quantities sufficient for a given day's work so that the activity of the materials would not be affected by repeated thawing and refreezing. Stock solutions of sea urchin toxin were made by dissolving 60 mg of the lyophilized powder in 30 ml of 0.15 M phosphate buffer at pH 7. These batches were dialyzed against 6 liters of 1% NaCl for 24 hours and stored in 5-ml bottles at -20°C. The substrate stocks were first extensively dialyzed against 1% NaCl, analyzed for TCA-precipitable N, adjusted to a protein concentration of 5 mg/ml, distributed into 5-ml serum bottles and stored at -20°C.

METHODS

Enzymological.

The stock solutions of toxin were thawed and then suitably diluted with Tyrode's immediately before the experiment. The final reaction volumes were 5 ml, and all experiments were made in duplicate. All the reaction mixtures, as well as toxin-free and substrate-free controls were maintained in a thermostatically controlled Dubnoff metabolic shaker for the time and temperature required by the experimental protocol.

At the end of the prescribed incubation period the samples were "inactivated" by being placed in a 56°C water bath for 30 minutes. After inactivation a 2.5-ml aliquot of each sample was transferred to a cellophane bag. The bag was placed in a 25 ml container and 2.5-ml of Tyrode's was added to the vessel. The vials were placed on a rocking device and the active product permitted to dialyze for 24 hr at 4°C. The dialyzate as well as the remainder of the original undialyzed sample were frozen at -20°C before bioassay.

Pharmacological and Chemical Estimations.

Bioassays were made on the isolated guinea pig's ileum, as described in our previous report (2), or on the sensitized rat's uterus. In the latter case virgin female rats weighing about 150 grams were injected subcutaneously with stilbestrol (10 µg/100 g) 20 hours before the experiment. The animals were killed by a blow to the base of the skull, the uteri removed, and single horns were suspended in a 4 ml bath filled with oxygenated de Jalon's solution containing 1.66 µg/ml of atropine. The isometric or isotonic contractions of the uterine horns were recorded through the electro-optical system previously described or by means of the usual kymographic technique (2). The responses were referred to a calibration curve and are expressed as equivalent bradykinin concentrations. Chemical estimations for histamine (7) or nitrogen (8) were made according to previously published methods.

Immunological.

Antitoxin: Antisera were produced to the various preparations by inoculating rabbits with two one-milliliter portions of the emulsified antigen given by deep subcutaneous injection in the nuchal region. Three weeks after the inoculation, test bleedings were made from the marginal ear vein for the detection of precipitating antibodies; subsequently, the rabbits were bled by cardiac puncture. The blood was permitted to clot, the serum harvested, and titrated. All of the sera had precipitating antibodies and, in specific instances, were shown to fix complement. The γ-globulin fraction, obtained by repeated precipitation in the presence of 1/3-saturated ammonium sulfate, was also found capable of neutralizing toxin *in vitro* and *in vivo*, as judged by lethal toxicity tests and assays on isolated tissues.

Immunochemical Determinations: Immunoelectrophoresis and immunodiffusion (Ouchterlony) tests were carried out on substrate and toxin preparations as microdeterminations customarily used in this laboratory. Quantitative precipitin and complement fixation tests were carried out on the antisera according to Campbell *et al.* (9).

RESULTS

Preliminary Characterization.

The object of the initial studies was to determine whether a physiologically active material could be produced from the interaction of whole serum, or certain of its well defined components, with active toxin and to determine the pharmacological behavior of the product so that the course of the reaction could be followed reliably.

Dialyzed bovine serum, purified bovine γ-globulin, and bovine serum albumin were incubated for 15 minutes at 37°C with a constant concentration of the most active preparation of sea urchin toxin, [SUT(64-2) 65 *as*], according to the general procedure

described in "Methods". From the results given in Table IIA it is apparent that only the whole serum produced a measurable quantity of dialyzable pharmacologically active material at the concentration, reaction time, and temperature of the experiment. Similar studies made on the globulin fractions prepared from human serum showed that the globulin precipitated at the higher salt concentration had significantly greater activity than that obtained at 1/3-saturation. Furthermore, as shown in Table IIB, the water-insoluble fractions were approximately three times as effective as the water-soluble preparations.

Although the method of separation used does not produce materials of recognized purity, it has the advantage of yielding large quantities of stable substrates of defined potency which could be used to study some of the common enzymological parameters of action of various toxin preparations. Additionally, since the bulk of human γ_2-globulin is precipitable at approximately 1/3-saturated ammonium sulfate, and is water-soluble, the present results are in accord with the findings in the case of bovine serum fractions to the effect that the γ-globulin is not an effective substrate in the reaction.

The dialyzable product elicited a slow contraction of the guinea pig's ileum and a rapid oxytocic response in the uterine horn of the stilbestrol-primed rat. The amount of material required to produce a slow contraction appeared to depend upon the time of incubation with toxin, but the response to a constant dose decreased with repeated exposure, although the preparation remained sensitive to histamine.

A study was made of the effectiveness of several pharmacological blocking agents with respect to the active principle. Separate portions of guinea pigs' ilea were titrated with histamine, acetylcholine, serotonin, and bradykinin, and the concentration, of antagonist required to block a 50% response to the agonist was determined. Thus, doses of unknown material required to produce a 50% response were tested on tissues in the presence of appropriate concentrations of pyribenzamine, atropine, phenylbutazone, and d-bromolysergic acid. The results presented in Table III show that whereas pyribenzamine and atropine have little effect against the dialyzable product, d-bromolysergic acid, and phenylbutazone, respectively, produce effective blockade.

The product of interest evidently possessed the general properties of a plasma kinin; consequently, it was necessary to determine to what extent maneuvers designed to affect enzyme inhibitors and kininases would influence the net output of the reaction-product in our work. For this purpose we compared the natural kinin outputs of acid-treated and untreated bovine serum in response to (a) 50% dilution in saline, and (b) in response to the incubation with sea urchin toxin.

By comparing the appropriate protocols in Table IV it is evident that acid-treated serum as well as the untreated sample produced physiologically active materials in their respective dialyzates. However, for comparable reaction times the untreated serum yielded less material than the acid-treated counterpart. In both cases the presence of sea urchin toxin tended to decrease the amount of material available at the end of the experimental period but that effect was significantly lower in the untreated than in the acid-treated samples.

The possibility that the crude toxin might have natural kininolytic activity was tested by incubating samples of synthetic bradykinin with crude toxin. Table V shows that bradykinin, in quantities sufficient to produce a 50 to 60 per cent response in the isolated ileum, was completely inactivated in 30-180 minutes by the concentrations of toxin used in the experiments reported in Table IV.

The results of the preliminary studies gave plausible grounds for inferring that the production of physiologically active materials resulted from an enzymatic attack of one or more substances in the toxin upon a substrate in the plasma. Confirmatory evidence was sought by determining whether such variables as toxin concentration, substrate concentration, and temperature would influence the net kinetics of the system in accordance with this hypothesis.

Toxin Concentration.

The effect of toxin concentration on the amount of product formed in 15' at 37°C was studied by incubating 0.308 mg/ml aliquots of pseudoglobulin fraction 4 with five concentrations of [SUT(64-1)65 *as*] which ranged from 0.029 to 0.098 mg N/ml at pH 7.5. The results of the bioassays made on the dialyzed products given in Table VI show a monotonic nonlinear increase of product formation with increasing concentration of toxin. The departure from linearity was not unexpected since the prior work had shown that the toxin also had the property of inactivating or degrading the product. If the variation of product formed at constant time can be taken as gross index of the variation of velocity with toxin concentration, an estimate of the potency of the enzyme(s) can be got by determining the concentration of toxin required for the half-maximal reaction. Taking 10.5×10^{-7} moles/liter as the maximum equivalent histamine concentration yielded under the conditions of the present experiment, the present data can be normalized by means of the logistic transformation of von Krogh (10), and the toxin concentration giving the half reaction obtained by interpolation. A plot of the transformed data given in Figure 3 shows that the half-maximum reaction in the present case is given when the enzyme concentration is 0.185 mg N/ml.

Substrate Concentration.

The relationship between substrate concentration and reaction velocity was studied by determining the time-courses of product formation at 37°C for each of five substrate concentrations, ranging from 0.155 to 0.308 mg/ml of pseudoglobulin, Fr. 4, in the presence of 0.093 mg N/ml of [SUT(64-1)65 *as*]. The concentration of product yielded at the various time intervals for the entire experimental array is given in Table VII. A preliminary arithmetic plot of these results (Fig. 4) gives a family of sigmoidal curves characterized by progressively increasing maxima and diminishing lag periods in response to increasing substrate concentration.

The initial velocity constants for these curves were evaluated from the modified form of the first-order law as applied to growth data by Lotka (11) and Brody (12), and to sensitization kinetics by Feigen and Nielsen (13). In the present case the working expression can be written

$$1 - P/P_{max} = e^{-k(t-t^4)}$$

in which P_{max} is the maximum product concentration for a given category of substrate concentration, P is the product concentration formed at time t, and t^4 is the time at which the extrapolated curve crosses the time axis. The values of P_{max} were obtained graphically by successive approximations.

Plots of $1 - P/P_{max}$ against $t - t^4$ on semi-logarithmic coordinates were linear for each of the time courses, showing that the data could be grossly described by first order kinetics. The first order velocity constants were obtained from the slopes of the lines given in Figure 5 and tested by direct and reciprocal plots for conformance to the Michaelis-Menten relationship. The direct plot showed an upward concavity

indicating that the reaction might have been complicated by a modifier. Accordingly the data were tested for linearity by determining the goodness of fit to expressions of a higher order. Figure 6 shows that the data are adequately linearized by a plot of the first order velocity constants against the square of the substrate concentration.

Temperature Optimum.

Since the preliminary characterization experiments had shown that the toxin preparation had the capacity not only to form the product but to destroy it as well, the possibility existed that the temperature coefficient and the concentration-dependence of the two reactions could be such as to confound the estimation of the temperature optimum if only one toxin-substrate system were studied at various temperatures. Accordingly, the temperature optimum was studied by measuring the net production of active material from the interaction for an array of six toxin concentrations at each of the four temperatures. The substrate was 0.20 mg/ml (Cohn Fraction IV_4 in all cases but the toxin ranged from 0.04 to 0.40 mg/ml for each of the four temperatures: 19, 26, 27, and 41°C.

The results, given in Table VIII, show that although the initial reaction velocity appears to rise with an increase of temperature up to 37°C there occurs a decline in activity at the higher toxin concentration at 37°C and 41°C. A convenient way of demonstrating the temperature optimum is to exhibit the variation of the maximum net output of active product from each array of concentrations as a function of temperature. On this basis the maximum output as seen from Figure 7 occurs at 26°C. It is apparent from Table VIII that little destruction takes place at the lower temperatures since the dependence of product formation on toxin concentration tends to increase with temperature. At 37°C the dependence is even steeper but is overtaken by the velocity of product degradation, while at 41°C both activities appear to be inhibited.

Substrate Specificity.

A preliminary resolution of substrate effectiveness was made by comparing the concentrations of product yielded by the attack of toxin (SUT[64-2] 65 *as*) upon Cohn fractions selected for their differing content of α- and β-globulins. Fraction III-0 was expected to contain about 84% β-globulins (68% β_1, 16% β_2) and about 5% mixed α-globulins, whereas Fraction IV-1 according to Pennell, (*op. cit.*) normally contains 89% mixed α-globulins and 10% mixed β-globulins.

Comparative experiments were first made to determine the substrate potencies of III-0, IV-1, and normal beef serum at two toxin concentrations: 0.20 and 0.40 mg/ml [SUT(64-2) 65 *as*]. Tests were made on guinea pig ileum and rat uterus. In general, more bradykinin-like activity was elaborated by Fraction III-0 than by IV-1 with respect to the guinea pig ileum, although it was about the same for the rat uterus. Both substrates, as seen in Table IX, were considerably more effective than diluted beef serum, which was present in approximately a 20-fold greater concentration than the Cohn fractions.

It was possible that the difference between the two assays might have been a reflection of the attack of toxin on different substrates, one furnishing material effective on guinea pig gut and the other producing substances active only on the rat uterus. The effect of toxin concentration on kinin production by the attack of SUT 65 *as* on III-0 was studied with respect to the activities of the product on guinea pig ileum and rat uterus. The results displayed in Figure 8 show that the dependence of product formation upon toxin concentration is much steeper in the guinea pig assay than in the rat uterus test. The observed results suggest that the two major molecular species in Fraction III-0 furnish qualitatively different products when attacked by an ensemble of enzymes in the toxin preparation and that the difference in amounts of material yielded is a reflection of the absolute amounts of the various substrates present.

A comparison was next made between III-0 and IV-1 at such substrate and enzyme concentrations at which the respective reactions would be maximal in order to determine whether there would be differences in output of "guinea pig kinin". Table X shows that these conditions were achieved and that the mean output of active product, as assayed on the guinea pig ileum, was about four times greater in the case of III-0 than of IV-1.

Finally, the two impure substrate preparations were tested in such a way that in one case the α_2-globulin, and in the other the β-globulin, would be constant. The concentrations of the fractions were adjusted with respect to the percentage composition of α- and β-globulins on the basis that the α-globulin content of Fraction III-0 was 5 per cent, and the β-globulin content of Fraction IV-1 was 10 per cent. The data in Table XI show that when the β-globulin content was constant there was no difference in the guinea pig assay but a considerable drop in the rat test, which appeared to be associated with the 12-fold reduction in the α-globulin concentration. In the experiments in which α- was constant the total protein concentration inevitably was two-thirds of that used in the β-constant determination because both fractions had to be adjusted to the α-globulin content of III-0, which was 5 per cent. The data for that experiment show a dramatic gain in the rat uterus assay achieved by raising the concentration of III-0. The fact that both assay values in this case failed to come up to those observed for IV-1 in the former set may be attributed to the presence of 33 per cent greater α-content in the former system. On the other hand, the influence of the β-globulins on the guinea pig assay is not very pronounced: an 8-fold change producing no difference and an increase of 140-fold in the concentration of β-globulin producing only a 2-fold increase of product formed.

The validity of these results was tested by determinations on unknowns prepared through the courtesy of Mr. Kingdon Lou of the Hyland Laboratories who furnished materials differing in their content of α-, β-, and γ-globulins. The reactions were carried out for 10 minutes at two substrate and three toxin concentrations at 30°C. The results were submitted to Mr. Lou, who then identified the samples as being II + III (β, γ), IV_4 (α, β), III (α, β, γ) IV_{5+6} (α).

Inspection of Table XII shows that the β-rich substrates furnished no measurable product in tests on the rat uterus whereas the α-rich materials yielded active substances which varied quantitatively according to the substrate concentration used in the reaction. Although an increase of toxin augmented the product formed at the lower substrate concentration a further increase of toxin at the higher substrate concentration tended to degrade the product. The same general order of effectiveness was observed in the bioassays on the guinea pig ileum as on the rat uterus except that the yield was roughly one order of magnitude lower in effectiveness in the latter case.

In order to obtain a quantitative confirmation of the difference between the α- and β-globulins, Fractions IV_4 and IV_{5+6} were tested at lower substrate concentrations in the expectation that by reducing the reaction velocity the differences between the two substrates would become more critical. Since the α-globulin content of Fraction IV_4 is 40 per cent whereas that of Fraction IV_{5+6} is 85 per cent, the data exhibited in Figure 9 evidently support the hypothesis concerning the specificity of the α-globulins as substrates in the reaction.

Conclusive proof of this argument would require positive tests with electrophoretically purified α_1- or α_2-globulins and negative tests with comparable preparations of β_1- and β_2-globulins. Unfortunately, the only preparation available in pure form was α_2-globulin. As seen in the immunoelectrophoresis pattern (Figure 10) the reaction between the preparation and the α_2-antibody reveals a single antigenic determinant. The results

of comparative tests in which α_2-globulin and Fraction IV_{5+6} were studied at four protein concentrations are shown graphically in Figure 11. Although Figure 11 shows a slightly steeper curve of product formation in the case of Fraction IV_{5+6} it be remembered that the toxin was by no means pure and, therefore, the possibility cannot be discounted that the small amount of additional material formed from Fraction IV_{5+6} might have come about from an attack of a different enzyme in the toxin preparation upon molecules unrelated to α_2-globulin, which evidently are present in the Cohn fraction. Alternatively, if α_2-globulins possess kininolytic properties, the purification of the substrate would have resulted in the concentration of the kinin-destroying factor as well as of the substrate.

Relative Enzymatic Potencies of Sephadex Fractions.

The availability of purified α_2-M globulin and the three toxin fractions which were separated on Sephadex G-200, permitted us to determine whether the lethal activity of the toxin was correlated with its proteolytic action in respect of α_2-globulin. Accordingly, samples of α_2-globulin, made up to 0.10 mg protein/ml, were incubated for 10 minutes with 1.87 mg N/ml aliquots of the various Sephadex fractions mentioned in Table I. For comparative purposes [SUT(64-2) 65 *as*] was tested in the same way. The reactions were stopped by heating the samples at 56°C and the products bioassayed on the guinea pig's ileum.

The results displayed in Figure 12 show that the greatest yield of active product, estimated as bradykinin, was given by the fraction precipitated in 2/3-saturated ammonium sulfate. As far as the Sephadex preparations were concerned the greatest net yield was given by Fraction III, 1.4×10^{-5} mg/ml (as bradykinin), and the least - 4×10^{-6} mg/ml - by Fraction I. In view of the fact that the lethal activity of the Sephadex preparations was concentrated in Fraction II (Table I) it appears evident that there does not exist a simple relationship between lethality and enzymologic potency with respect to this substrate.

Immunological Experiments.

Initial attempts to produce antibodies in rabbits against active toxin were made by inoculating 5 mg quantities of sea urchin toxin preparations intramuscularly. The injection sites ulcerated rapidly and the animals died shortly afterward from the toxic effects of the injected materials. Attempts were next made to produce a toxoid by treating the toxin with formaldehyde. Control assays in mice showed that treatment of (SUT 64-2) with 0.01% formaldehyde reduced the lethal toxicity from 777 LD_{50}/mg to zero within the limits of the test. Test inoculations of 1 mg quantities incorporated into 1 ml of Freund's complete adjuvant were well tolerated and the rabbits produced positive antisera within two weeks. Tests for immunogenicity and specificity were next made by injecting a group of 6 rabbits with the crude [SUT(64-2)] and its various daughter preparations, which were obtained by fractional precipitation with ammonium sulfate.

Serological Characterization

The results exhibited in Table XIII show that all of the antisera reacted specifically not only with the immunizing antigen but with all of the sea urchin antigens. Species and tissue specificity were lacking as is evident from the positive reactions of the antisera of rabbits 16, 17, and 18, made to pedicellarial proteins of *T. gratilla* with antigens derived from the tests of *S. purpuratus*.

In an effort to visualize the antigenic determinants the immunoelectrophoresis experiments were made with the crude preparation SUT 66 and with the various ammonium sulfate fractions. Electrophoresis of the crude preparation SUT 66 (Figure 13) brought out two antigenic determinants, one to the right and one to the left of the central well. Antisera directed specifically against 65 *as* (10) and against the crude material (16) gave single lines in the region to the right, while the specific antiserum to 100 *as* showed a single line lying to the left of the origin (12). There was no visible reaction between anti-33 *as* and the antigens in SUT 66. Electrophoresis of the individual fractions gave positive reactions. When the individual fractions were electrophoresed and tested with anti-crude SUT, only the SUT 65 *as* fraction showed a reaction with anti-crude SUT (22); this again was characterized by a single line to the right of the central well.

The residue of these tests is that there are at least two demonstrable antigenic determinants in the crude preparation and that the substance migrating as an α-globulin toward the anode is the more immunogenic. This antigen is found to be concentrated in the fraction precipitating in the presence of 2/3-saturated ammonium sulfate.

Complement Fixation and Quantitative Precipitation.

Complement fixation tests performed on the toxin-antitoxin system showed that the reaction between SUT 61 and anti-SUT(64-2) (rabbits 11 and 14) fixed complement at an antigen concentration of 0.0025 mg/ml when tested with the antiserum with a limiting dilution of 1:8. The results are displayed in Table XIV.

All of the antisera except that directed against [SUT(64-2)65 *as*] were pooled and the γ-globulin fraction obtained by precipitating 125 ml of serum with 2 volumes of saturated ammonium sulfate. The final precipitate was taken up in 1% NaCl, dialyzed and concentrated to 1/5 the original volume of serum by pressure filtration through a cellulose-acetate thimble. The final γ-globulin concentration was 17.6 mg/ml as determined by nitrogen analysis.

The antigen was geometrically diluted from an initial concentration of 0.057 mg N and 1 ml aliquots of the various dilutions treated with 0.2 ml quantities of antibody containing 1.6 mg/ml of γ-globulin. Figure 14 shows a normal precipitation curve from which it can be calculated that 0.16 mg of antibody was precipitated at optimal proportions hence that the antibody content of the γ-globulin against SUT 64 was 10 per cent of the total.

Functional Characteristics of Antibody

Protection and Neutralization in vivo: The LD_{50} of SUT(64-2)65 *as* determined from a dose mortality assay (Figure 15) was equivalent to 2×10^{-3} mg of the dried preparation. A Ramon titration carried out for the flocculation reactions between [SUT(64-2)65 *as*] and its specific antiserum (16) gave an optimum ratio of 0.2 ml antitoxin for LD_{50} *i.e.* 2×10^{-3} mg of antigen. Protection tests were carried out by injecting mice intravenously with 0.25 mg aliquots of undiluted antiserum or γ-globulin solution containing 0.072 mg protein. Table XV(A) shows that 0.072 mg γ-globulin protected mice against 2 LD_{50}, and that the protective power of 0.2 ml of antiserum (16) was complete against 1 LD_{50}, partial at 2 LD_{50}, and inadequate at 5 and 10 LD_{50}.

Neutralization experiments were made by incubating 2 LD_{50} quantities of toxin for 10 minutes with 0.2 ml portions of γ-globulin solution containing a total of 0.18, 0.072, and 0.036 mg of protein, respectively. The results given in Table XV(B) suggest that *in vitro* neutralization is a rapid process and that the critical region in the neutralization of 2 LD_{50} occurs between 0.072 and 0.036 mg of γ-globulin.

Neutralization of SUT Effect on Guinea Pig Ileum: Appropriate mixtures were set up for incubation so that at a 10-fold dilution in a 4 ml muscle bath the test dose of toxin would be 2.06×10^{-3} mg/ml and the concentration of γ-globulin would vary from 0 to 2.43 mg/ml. Table XVI shows that 2.25 mg/ml antibody blocked the response to 1 MED by 80 per cent and that 2.43 mg/ml blocked the response completely.

DISCUSSION

The present studies have produced convincing evidence that the reaction between sea urchin toxin and plasma proteins results from an enzymatic attack of toxin molecules upon specific substrates to yield dialyzable products which have the capacity to stimulate smooth muscle.

The active constitutents of sea urchin toxin are non-dialyzable heat-labile, immunogenic proteins which react with either α_2- or with β-globulins to form materials active on the rat uterus or guinea pig ileum, respectively. In addition to these proteolytic effects the toxin preparations were also found capable of destroying the product and of inactivating synthetic bradykinin. To add to the complexity of the system the plasma protein fractions used as substrates were shown to be capable of forming active materials and of inactivating the products in the *absence* of toxin.

The problems encountered in the present studies were the usual ones found in the initial experiments with kallikrein systems in which both the kininogenic and kininolytic properties, possessed by both the crude "enzymes" as well as the crude "substrates", tended to confound the expected relationship between the reactants and the amount of kinin formed.

Although the velocity of the reaction in the present experiments was found to be influenced by the concentration of toxin, the concentration of plasma protein, and by the ambient temperature, the multiplicity of substances present in the early toxin preparations as well as in the plasma protein fractions, made it impossible to demonstrate that the system obeyed simple classical enzyme kinetics. Taking first the case of the enzyme, we should expect the dependence of reaction velocity upon enzyme concentration to be linear. The dependence observed experimentally, although linear at low concentrations of toxin, appeared to decelerate as the toxin concentration was increased. An outcome of this kind is not unexpected in view of the demonstrable kininolytic behavior of the toxin, and it is probable that at higher toxin concentrations the increased rate of product destruction might have become significant.

Considering next the influence of substrate concentration on the reaction velocity we see that the dependence of the first order velocity constants upon the concentration of pseudoglobulin does not give the usual hyperbolic relationship; in fact, the reciprocal plot is parabolic in character. The function was linearized only when the first order velocity constants were plotted against the square of substrate concentration. According to Frieden (14) such a complexity can arise if the substrate contains "modifiers", *i.e.*, if the activity of the enzyme is markedly affected by compounds which are not substrates for the reaction of interest. In the case of a parabolic plot it is assumed that substrate activation may account for the departure from the usual linear reciprocal function. It is not possible at the present time to isolate the specific step in the reaction which might have been affected to produce this "modification" because the experiments in question were carried out with what, on hindsight, evidently were impure reagents: the Kendall pseudoglobulins and the toxin fraction precipitated in the presence of 2/3-saturated ammonium sulfate.

The substrate preparation used evidently was capable of forming kinins spontaneously. That finding is in accord with prior reports of the existence of inactive kallikreins in these plasma protein fractions. The precursors are easily activated and are capable of attacking α_2-globulins to form plasma kinins. In connection with the present experiments it is significant that the kinin-forming system can be rapidly generated at neutral pH by procedures as simple as dilution (15) or by exposure to wettable surfaces. In view of the fact that the bioassay does not distinguish physiologically between those kinins and the dialyzable products formed from the attack of toxin upon the substrate, it is not possible to assess whether, for example, the toxin preparations could have served to activate the plasma kallikreins at the same time that they were attacking α_2-globulins.

The substrate fractions also contained kininases and, although these were presumed to be inactivated with acid, it is useful to recall that treatment with acid not only inactivates the kininases but also promotes the activation of an enzyme system which spontaneously forms kinins.

An additional factor, unknown at the time of the experiments, was that more than one substrate within the crude fraction could have been involved in the reaction. It is entirely possible, as shown by Jacobsen, (16) that the plasma kallikrein could have acted on at least two substrates, and it is evident from our studies that both α_2- and β-globulins are capable of being attacked by sea urchin toxin. Whether or not these substrates are attacked by the same or by different molecular species in the toxin is not yet established. Finally, the toxin is capable of destroying not only the natural product formed but also of degrading synthetic bradykinin. Since bradykinin evidently is one of the established reaction products in the plasma kallikrein system it is not unlikely that the net output of natural material from the reaction system must have reflected a certain degree of bradykinin destruction.

Preparations of sea urchin toxin appeared to be highly immunogenic in rabbits, and the antibodies produced reacted normally according to the usual immunochemical and serological criteria. Although the antibody blocked all of the functional activities of the toxin:lethal toxicity, effect on smooth muscle, and enzymatic properties, it is not clear whether the antigenic determinant of the toxin molecules was exclusively involved in this effect since the antibodies to the toxoids of *T. gratilla* were shown to cross-react with the non-"toxic" proteins of the tests of *S. purpuratus*.

The fact that purified immune γ-globulin blocks the enzymatic and pharmacological actions of SUT without being itself digested is a significant corroboration of the high degree of substrate specificity of the system. Since precipitation is unimpaired it is plausible to infer that neither the identity of the antibody combining site nor the bonds linking the two halves of the F_{AB} region of the γ-globulin molecule are targets in the enzymatic reaction.

The relationship of the lethal toxicity to the enzymatic potency *in vitro* is obviously central to the understanding of the mode of action of the toxin. Is the animal killed because the venom reacts with plasma proteins to form toxic products or is that mechanism relatively less important than the direct action of sea urchin toxin on such cells as are contained in the lungs, heart, gut, or blood? Unfortunately, no simple answer can be provided this question on the basis of the present experiments. There are several reasons for this, and among them are: (1) the validity of the *in vitro* test, (2) the involvement of several substrates, and (3) the possibility that more than one product can be formed from a given substrate by different enzymes.

Although many features of the present system predispose the authors to consider the results in terms of plasma kinin formation it must be emphasized that this is done only for the convenience that such an analogy can furnish. Actually, short of isolation and

determination of structure, a plasma kinin is judged on the basis of five activities, as set forth by Lewis (17). These are: smooth muscle stimulation, vasodilatation, capillary permeability, leukocyte migration, and the production of pain. Thus far we have studied only the first of these criteria.

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TABLE 1

Properties of Sephadex Fractions of Crude Sea Urchin Toxin 1965

Toxin	Weight g	OD_{278} 1 mg/ml (dry)	Mg Total N per 1 mg (dry)	LD_{50} per mg N	MED* (GP Ileum) mg N/ml	S_{20}
Fr. I	0.106	0.398	0.028	62.5	1.38×10^{-3}	6.5
Fr. II	0.072	0.275	0.039	18,816.0	1.22×10^{-4}	4.7
Fr. III	0.036	1.247	0.112	15.1	2.0×10^{-1}	2.5

* MED is the median effective dose of toxin, obtained by interpolation of linearized dose-response curves, as the final concentration of material giving a 50 per cent contraction.

TABLE 2

Effect of Various Substrates on the Formation of Kinin in the Presence of [SUT(64-2) 65 *as*] at 37°

Preparation	Substrate Conc. (mg Protein/ml)	SUT Conc. (mg N/ml)	Histamine Equivalent moles/liter
A. Bovine Serum Proteins			
Whole bovine serum	0.4900	0.0863	2.56×10^{-7}
Bovine γ-globulin	0.3082	"	0.0
Bovine serum albumin	"	"	0.0
B. Human Serum Proteins			
SAS/3 Kendall Fraction 1	0.3082	0.0863	1.72×10^{-7}
" " " 2	"	"	4.60×10^{-7}
SAS/2 " " 3	"	"	4.34×10^{-7}
" " " 4	"	"	14.20×10^{-7}

TABLE 3

Comparative Blockade of Dialyzable Kinin and Known Drugs with Various Antagonists

Conc. of Test Compound	Blocking Agent Type	mg/ml	Initial Reaction (% of maximum)	Reaction after Blocking Agent	% Block
Dialyzable Kinin 1.21 mg/ml	Atropine	0.714	29	26.60	8
	PBZ*	0.114	35	26.00	25.00
	BOL**	0.086	29	7.00	73.00
		"	21	7.50	64.50
			35	18.00	50.00
	ΦBZ***	1.00	30	0	100.00
Bradykinin	PBZ	0.114	50	50.00	0.00
5.71×10^{-6} mg/ml	BOL (148)	0.086	42	0.00	100.00
Serotonin	PBZ	0.114	48	52.00	0.00
6×10^{-7} moles/liter	BOL (148)	0.0857	46	0.00	100.00

* PBZ Pyribenzame

** BOL(148) D-bromolysergic acid

*** ΦBZ Phenylbutazone

.TABLE 4

Effect of Toxin [SUT(64)] On the Net Production of Dialyzable Material From Normal and Acid-Treated Bovine Serum

Test System*	Incubation Time (minutes)	Dose Tested (ml)	% Reaction	% Digested
		A. Untreated Serum		
[SUT(64)] + 1% NaCl	120	0.50	0.00	
Bovine Serum + 1% NaCl	"	0.20	4.00	
		0.30	12.00	
		0.40	15.00	
		0.50	16.00	
[SUT(64)] + Bovine Serum	"	0.20	0.00	
		0.30	2.00	84.00
		0.40	3.00	86.00
[SUT(64)] + 1% NaCl	200	0.50	1.00	
Bovine Serum + 1% NaCl	"	0.50	39.00	
[SUT(64)] + Bovine Serum	"	0.50	17.22	55.00
[SUT(64)] + 1% NaCl	300	0.50	0.00	
		4.00	0.00	
Bovine Serum + 1% NaCl	"	0.50	0.00	
		4.00	35.00	
[SUT(64)] + Bovine Serum	"	0.50	0.00	
		4.00	20.33	44.00
		B. Acid-Treated Serum		
[SUT(64)] + 1% NaCl	30	0.40	0.00	
Bovine Serum + 1% NaCl	"	0.10	56.00	
		0.20	69.00	
		0.40	93.33	
Bovine Serum + [SUT(64)] +	"	0.10	9.00	65.00
		0.20	29.00	63.00
[SUT(64)] + 1% NaCl	300	0.50	0.00	
Bovine Serum + 1% NaCl	"	0.20	37.00	
		0.30	60.00	
		0.50	72.00	
Bovine Serum + [SUT(64)] +	"	0.20	15.00	59.00
		0.30	25.00	59.00
		0.50	36.00	62.00

* The reaction volume of each test system before dialysis was 10 ml and consisted of 5 ml aliquots of each of the reactants. The stock toxin concentration was 0.187 mg N/ml. Acid-treated serum was incubated at pH 2 for 30 min; then the pH was adjusted to 7.5 for testing. Test doses were added directly to the muscle bath to bring the final test volume to 4 ml in all cases.

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TABLE 5

Destruction of Bradykinin by Sea Urchin Toxin 64 at pH 7.5

Test System	Incubation Time (minutes)	Dose Tested* (ml)	% Reaction	% Inactivated
SUT	30	0.10	0.00	
BK†	"	0.10	52.86	
SUT + BK	"	0.10	0.00	100
		0.20	0.00	
SUT	180	0.30	0.00	
		0.40	0.00	
BK	"	0.30	62.22	
		0.40	93.33	
SUT + BK	"	0.30	0.00	100
		0.40	0.00	

* The stock concentration of [SUT(64)] was 0.187 mg N/ml and the stock concentration of synthetic bradykinin was 1 µg/ml

† Bradykinin

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TABLE 6

Effect of Toxin Concentration [SUT(64-2) 65 *as*] on the Formation of Dialyzable Product From Pseudoglobulin Fraction 4

Incubation Temp.	Incubation Time	Substrate (Fr. 4) (mg Protein/ml)	Toxin (mg N/ml)	Histamine Equivalent* (moles/liter) x 10^{-7}
37°C	15'	0.3082	0.0288	4.50
"	"	"	0.0431	8.00
"	"	"	0.0575	9.02
"	"	"	0.0865	10.20
"	"	"	0.0983	10.50

* Average values for three experiments

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TABLE 7

Effect of Substrate Fr. 4 (Kendall) Concentration on Formation of Dialyzable Kinin in the Presence of Sea Urchin Toxin [SUT(64-2)65 *as*] *

Substrate Conc. [S] mg protein/ml	1/[S]	S^2	Incubation Time (min)	Relative Histamine Equivalent(moles/ liter x 10^{-7})	First Order Velocity Constant k (min^{-1})
0.1547	6.46	0.0239	5	1.20	
			15	2.20	
			22	3.23	0.031
			30	4.50	
			45	6.48	
0.1858	5.38	0.0345	5	1.20	
			15	3.30	
			22	4.90	0.035
			30	6.15	
			45	9.00	
0.1981	5.05	0.0392	5	2.52	
			15	5.08	
			22	7.30	0.048
			30	10.50	
0.2170	4.61	0.0471	5	2.85	
			15	8.43	
			22	12.40	0.061
			30	17.50	
			45	18.30	
0.3082	3.24	0.0950	5	3.45	
			15	13.10	
			22	16.15	0.114
			30	17.10	
			45	17.95	

* Toxin concentration 0.093 mg N/ml

TABLE 8

Effect of the Temperature on the Reaction Between SUT(65) Sephadex I and Cohn's Fraction IV_4 (0.200 mg Protein/ml)

Temperature °C	Reaction Time (min)	SUT Concentration (mg/ml)	Bradykinin Equivalent (mg/ml)	% Maximum Kinin Activity
19	10	0.04	1.47×10^{-5}	83.3
		0.08	2.30	
		0.16	4.30	
		0.24	5.15	
		0.32	6.90	
		0.40	7.50	
26	10	0.04	1.17×10^{-5}	100.0
		0.08	2.23	
		0.16	5.68	
		0.24	6.56	
		0.32	8.00	
		0.40	9.00	
37	10	0.04	2.10×10^{-5}	77.8
		0.08	5.60	
		0.16	7.06	
		0.24	6.50	
		0.32	5.61	
		0.40	5.33	
41	10	0.04	1.05×10^{-5}	54.4
		0.08	2.17	
		0.16	2.50	
		0.24	3.21	
		0.32	4.83	
		0.40	4.69	

TABLE 9

Comparative Effectiveness of Cohn Fractions and Bovine Serum as Substrates for [SUT(64-2) 65 *as*] According to Various Bioassays

Substrate*	Substrate Conc. mg protein/ml	ENZYME SUT(64-2)65 *as* (mg/ml)	GUINEA PIG ILEUM Bradykinin Equivalent (mg/ml)	GUINEA PIG ILEUM Histamine Equivalent (M/L)	RAT UTERUS Bradykinin Equivalent (mg/ml)
III-0	0.40	0.40	1.05×10^{-4}	5.0×10^{-7}	1.11×10^{-5}
III-0	0.40	0.20	7.30×10^{-5}	4.0×10^{-7}	7.8×10^{-6}
IV-1	0.40	0.40	6.35×10^{-5}	2.8×10^{-7}	1.14×10^{-5}
IV-1	0.40	0.20	5.33×10^{-5}	2.50×10^{-7}	1.00×10^{-5}
Beef Serum	[1:10]	0.40	2.83×10^{-5}	1.73×10^{-7}	4.83×10^{-6}
		0.20	9.3×10^{-6}	5.78×10^{-8}	0.00

Incubation Period: 20 minutes

Temperature: 37°C

Inactivated: 56°C for 30 minutes

* Fraction III-0 was high in β-globulins and Fraction IV-1 was high in α-globulins. For approximate composition see text.

TABLE 10

Comparison of Substrate Specificities of Cohn Fractions III-0 and IV-1

SUT(64-2)65 *as* (mg N/ml)	Preparation	Concentration (mg Protein/ml)	Incubation Time (min)	Bradykinin Equivalent ($mg/ml \times 10^{-5}$)
1.27×10^{-2}	III-0	0.200	15	7.20
			30	7.80
			60	7.70
			150	7.50
			300	7.50
			600	8.66
			1200	8.25
1.27×10^{-2}	IV-1	0.200	15	1.20
			30	1.82
			60	1.90
			150	1.80
			300	1.75
			600	1.85
			1200	1.69

Temperature of Incubation 37°C
Inactivation at 56°C for 30 minutes

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TABLE 11

Comparison of the Specificities of Fractions III-0 and IV-1 as Substrates Tested at Constant α-Globulin and β-Globulin Content

Experimental	Preparation	Concentration (mg/ml) Substrate	Enzyme*	BK Equivalent G.P. Ileum (mg/ml)	BK Equivalent Rat Uterus (mg/ml)
β-globulin constant	IV-1	β = 0.04 α = 0.36	2.96×10^{-2}	1.0×10^{-4}	2.73×10^{-5}
	III-0	β = 0.04 α = 0.003		1.1×10^{-4}	0.00
α-globulin constant	IV-1	β = 0.0024 α = 0.021	2.96×10^{-2}	6.04×10^{-5}	2.13×10^{-6}
	III-0	β = 0.336 α = 0.021		1.15×10^{-4}	1.02×10^{-5}

* [SUT(64-2)65 *as*]

TABLE 12

Effect of Various Cohn's Fractions as Substrates in the Formation of Kinin in the Presence of Sea Urchin Toxin [SUT(64-2) 65 *as*] at 30°C

Sample Code (Unknown)	Cohn Fraction (disclosed)	Enzyme Conc. SUT(64-2)65 *as* (mg/ml)	Substrate Conc. (mg Protein/ml)	Incubation Time (min)	Bradykinin Equivalent (mg/ml) GP Ileum	Rat Uterus
1	II & III	0.080	0.200	10	0.00	0.00
2	IV_4		"	"	5.67×10^{-5}	6.05×10^{-6}
3	III_1		"	"	1.00×10^{-5}	0.00
4	$IV_{5\ \&\ 6}$		"	"	6.62×10^{-5}	5.30×10^{-6}
1	II & III	0.160	0.200	10	1.00×10^{-5}	0.00
2	IV_4		"	"	9.0×10^{-5}	7.30×10^{-6}
3	III_1		"	"	1.87×10^{-5}	0.00
4	$IV_{5\ \&\ 6}$		"	"	7.25×10^{-5}	8.35×10^{-6}
1	II & III	0.160	0.400	10	2.13×10^{-5}	0.00
2	IV_4		"	"	10.80×10^{-5}	1.78×10^{-5}
3	III_1		"	"	2.02×10^{-5}	0.00
4	$IV_{5\ \&\ 6}$		"	"	12.5×10^{-5}	1.50×10^{-5}
1	II & III	0.280	0.400	10	2.09×10^{-5}	0.00
2	IV_4		"	"	9.52×10^{-5}	1.07×10^{-5}
3	III_1		"	"	1.98×10^{-5}	0.00
4	$IV_{5\ \&\ 6}$		"	"	10.00×10^{-5}	8.5×10^{-6}

Temperature of Incubation, 37°C.

Incubation Time, 10 min.

Inactivation Temperature, 56°C for 30 min.

Globulin composition of samples:

1, $\beta + \gamma$; 2, $\alpha + \beta$; 3, α, β, γ; 4, α.

TABLE 13

Serological Reactions of Rabbit Antisera to SUT Preparations*

Rabbit #	Antisera Against	Test Antigens 1964-2 Crude	1964-2 33 *as*	1964-2 65 *as*	1964-2 100 *as*	1966 Crude	1966** *S. purpuratus*
10	64-2 33 *as*	+	+				
11	64 Crude	++	++	++	++	++	
14	64 Crude	++	++	++	+	++	
16	64-2 65 *as*	++	+	+	+		++
17	64-2 100 *as*	++	+	++	++		++
18	64 Crude	++	++	+++	+++		++
Pool: antiovalbumin		-	-	-	-		-
Pool: normal serum		-	-	-	-		-

* Ring Tests

** The *S. purpuratus* antigen was made by extracting the entire tests of the eviscerated specimens with sea water. The proteins had been precipitated in saturated ammonium sulfate, dialysed, and then freeze-dried. All antigens were tested at 0.25 and at 1.0 mg/ml.

TABLE 14

Complement Fixation by SUT 61) and Anti-SUT (64)

SUT (61) (mg/ml x 10^{-2})	Anti-SUT (64) Dilution 1	1:2	1:4	1:8	1:16
1.0	-	-	-	-	-
0.5	+	+	+	-	-
0.25	+	+	+	+	-
0.125	+	±	-	-	-
0.0625	-	-	-	-	-
0.0313	-	-	-	-	-

+ = fixation, no lysis

- = no fixation, lysis

TABLE 15

Effect of Immune Sera On Lethal Toxicity of Sea Urchin Toxin

A. Protection Tests†

Primed With		Challenged with		Protected
Pooled γ-globulin	0.25 ml (0.072 mg)	[SUT(64-2) 65 *as*]	2 LD_{50}	4/4
		"	1 LD_{50}	4/4
Antiserum (16)	0.2 ml	[SUT(64-2) 65 *as*]	10 LD_{50}	0
		"	5 LD_{50}	0
		"	2 LD_{50}	2/3
		"	1 LD_{50}	4/4

† Mice received antibody *iv* 2 hours before challenge

B. Neutralization Tests*

Toxin [SUT(64-2) 65 *as*]	Antitoxin (γ-Globulin)	Protection
2 LD_{50}	0.180	4/4
"	0.072	4/4
"	0.036	2/3

* Injected immediately after mixing

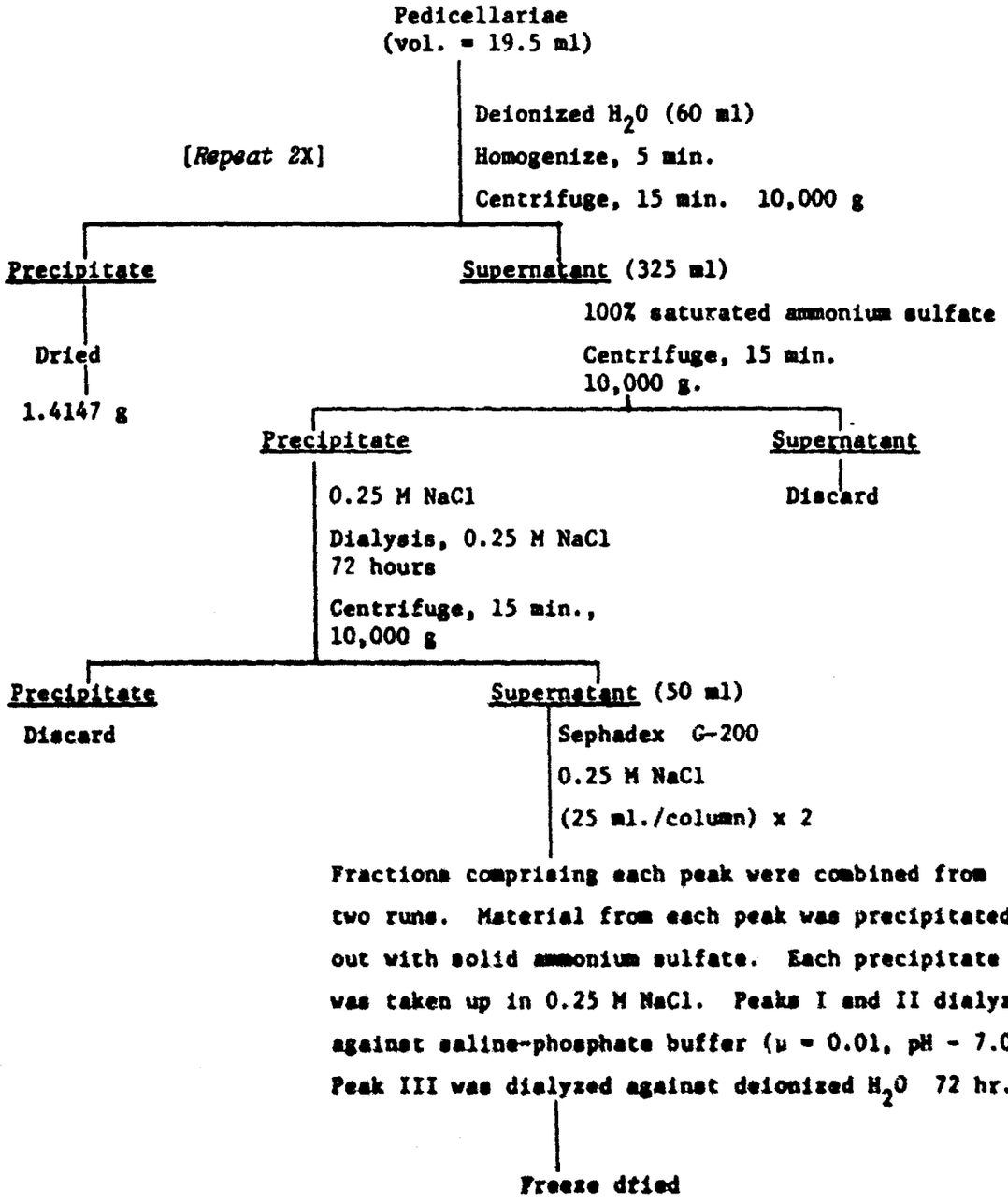
TABLE 16

Neutralization of SUT(64-2) 65 *as* By Specific Antiserum, as Judged By Direct Assay on Guinea Pig's Ileum

SUT(64-2)-65 *as* (mg/ml)	Anti-SUT(64-2)-65 *as* (mg/ml)	Per Cent of Maximum Response	Per Cent Neutralized
2.06×10^{-3}	0.0	50.0	0.0
2.06×10^{-3}	2.25×10^{-2}	9.0	82.0
2.06×10^{-3}	2.43×10^{-2}	0.0	100.0

FIGURE 1

Sephadex Fractionation of Crude Sea Urchin Toxin



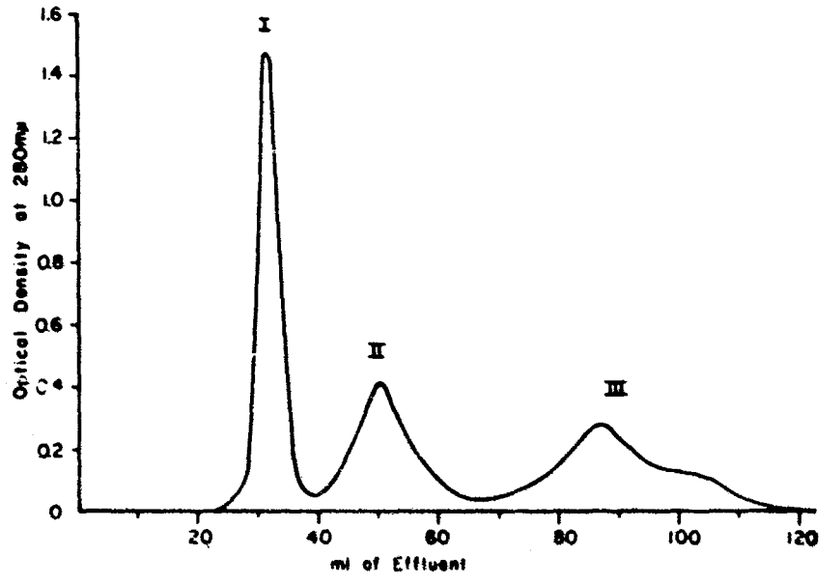


FIGURE 2 Elution diagram of SUT 65 in phosphate-buffered 0.25 M NaCl at pH 7. Optical density at 280 mμ.

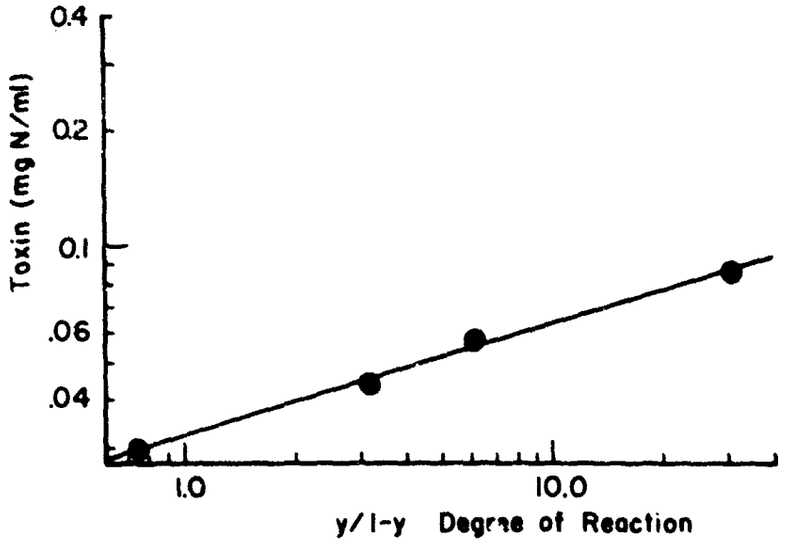


FIGURE 3 Effect of toxin concentration on the formation of dialyzable product. The reaction system consisted of 0.308 mg/ml of pseudoglobulin (Fr. 4) and various concentrations of [SUT(64-2)65 *as*]. The ratio of the per cent of total reaction, y, to the remainder, $1 - y$, is plotted as a function of toxin concentration. The dose of toxin necessary to produce a 50% response occurs at the coordinate when $y/1-y = 1$.

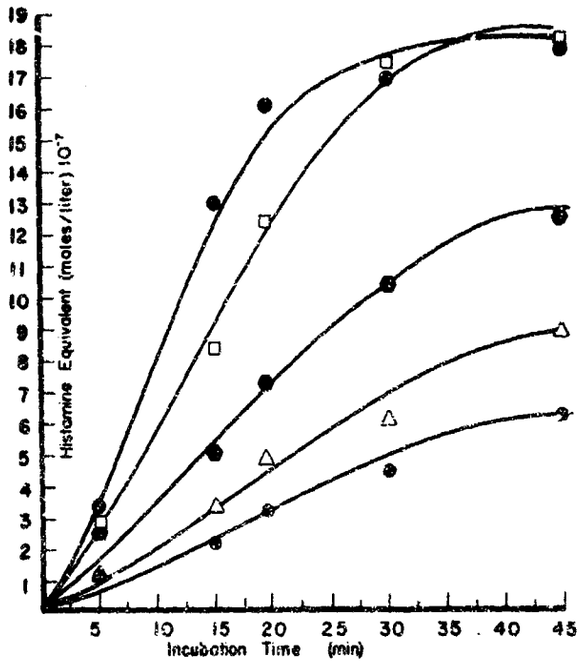


FIGURE 4 Effect of substrate concentration on the time-courses of reactions between pseudoglobulin Fr. 4 and [SUT(64-2)65 *as*] 7.5 x 10^{-3} mg/ml.

Concentration of Fr. 4

○ —— ○ 0.308 mg/ml;

□ —— □ 0.217 mg/ml;

⬡ —— ⬡ 0.198 mg/ml;

△ —— △ 0.186 mg/ml;

⊗ —— ⊗ 0.135 mg/ml

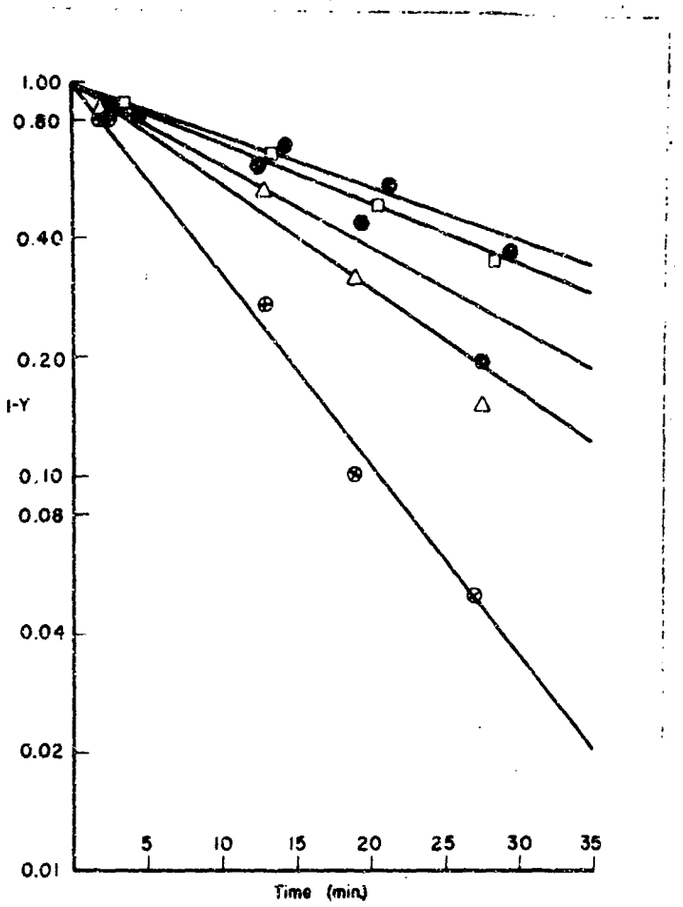


FIGURE 5 First-order plots of data presented in Figure 4, normalized according to the procedure described in the text.

Concentration of Fr. 4

0.308 mg/ml;

0.217 mg/ml;

0.198 mg/ml;

0.186 mg/ml;

0.155 mg/ml

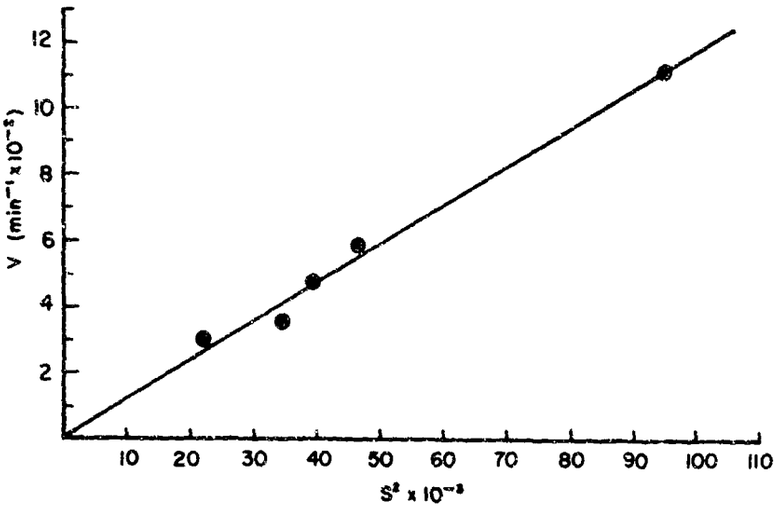


FIGURE 6 The variation of first order velocity constant *(V)*, obtained from the slopes of the lines in Figure 5, with the square of substrate concentration, (S^2). (*cf.* Figure 4).

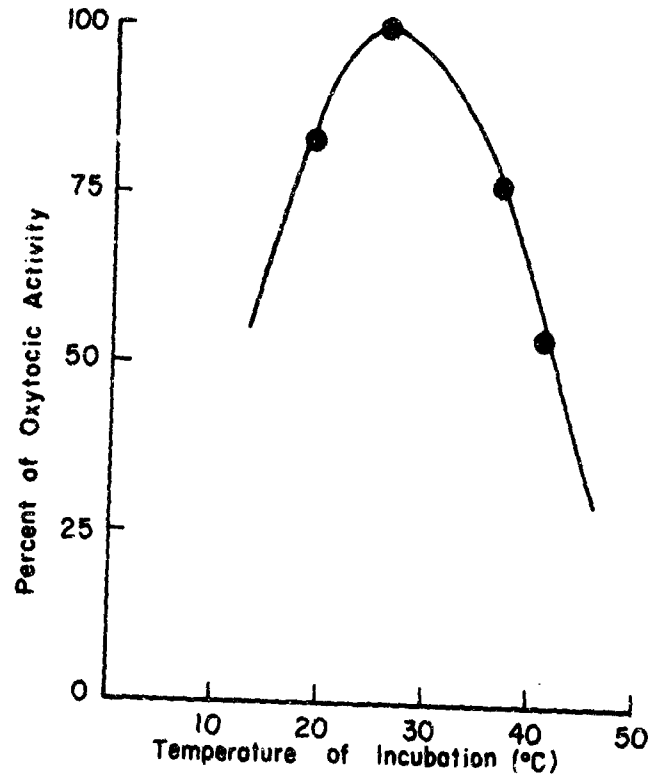


FIGURE 7 Temperature optimum for the production of maximum oxytocic activity from the interaction of SUT(65) Sephadex Fr. I and Cohn Fraction IV_4. See data in Table 8.

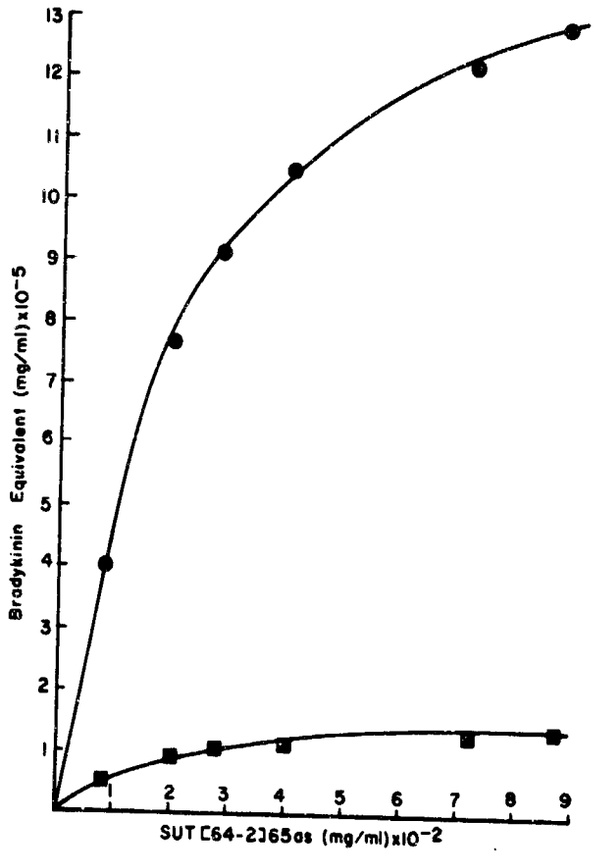


FIGURE 8 Comparative sensitivity of guinea pig (● —— ●) and rat (■ —— ■) tissues to the product formed from Cohn Fraction III-0 and various concentrations of [SUT(64-2)-65 *as*]. The reactions were carried out at 37° for 20 min, and then inactivated at 56° for 30 min before testing. The material was assayed on the guinea pig's ileum in Tyrode's at 37° and on the rat's uterus in de Jalon's solution at 30°.

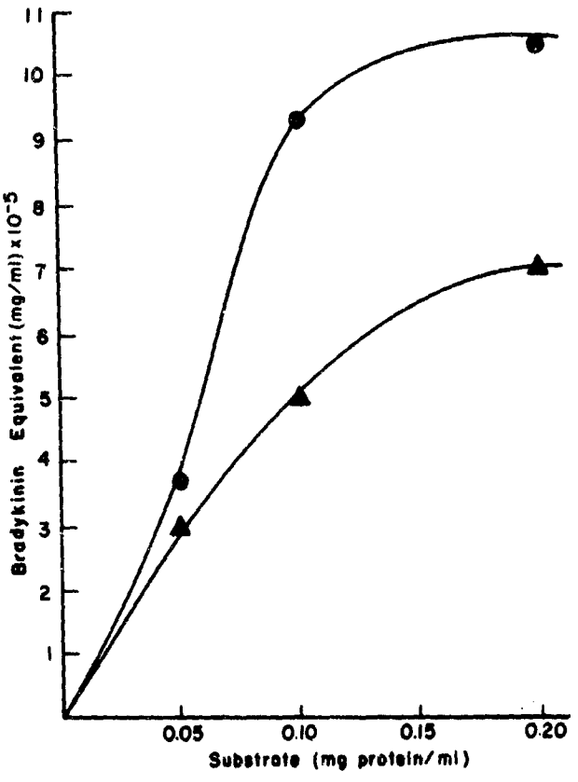


FIGURE 9 Relative potencies of Cohn Fractions IV_4 (▲——▲) and IV_{5+6} (○——○) as substrates for SUT(65) Sephadex Fr. I (9.2×10^{-3} mg N/ml).

FIGURE 10 Immunoelectrophoresis of α_2-macroglobulin (S_{20} = 17.7) (8.3 mg/ml) in central well against specific rabbit anti-α_2-macroglobulin serum in the bottom trough, and specific rabbit anti-α_1-lipoprotein serum in the top trough. Electrophoresis was carried out at 25 milliamps for 2.5 hours following which antiserum was added and then allowed to diffuse for 24 hours.

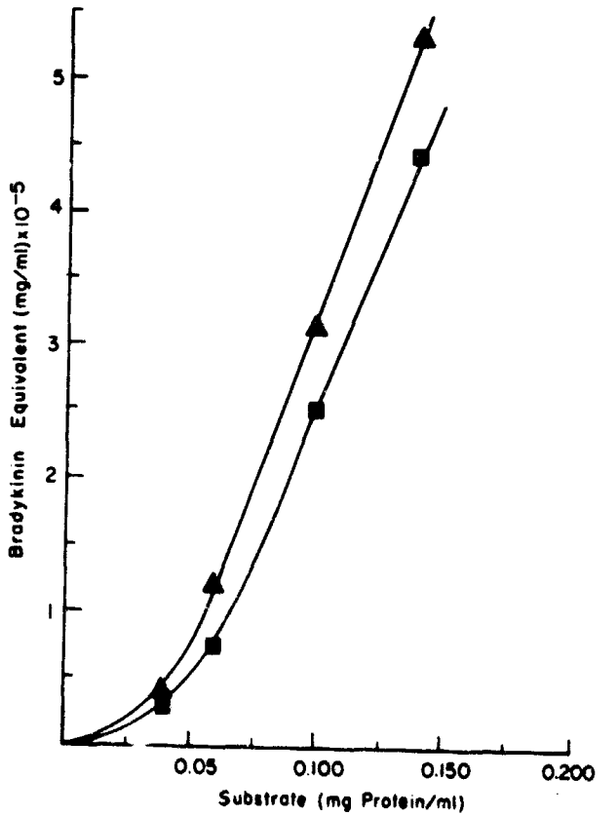


FIGURE 11 Comparative potencies of Cohn Fr. $IV_{5\ \&\ 6}$ (▲ —— ▲) and pure α_2-M globulin (■ —— ■) as substrates for 1.87×10^{-2} mg N/ml of [SUT(64-2)65 *as*].

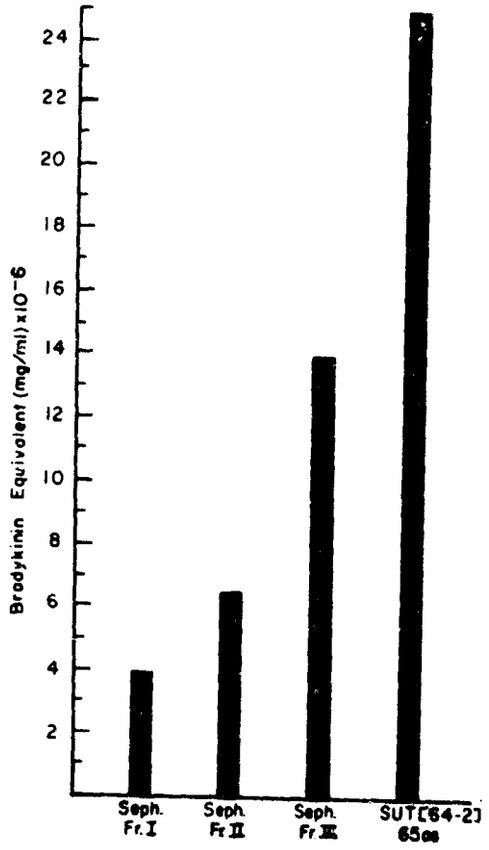


FIGURE 12 Relative potencies of various toxin preparations as enzymes in the reaction with α_2-M globulin. The substrate concentration was 0.10 mg protein/ml in all cases and the toxin preparations contained 1.87×10^{-2} mg N/ml.

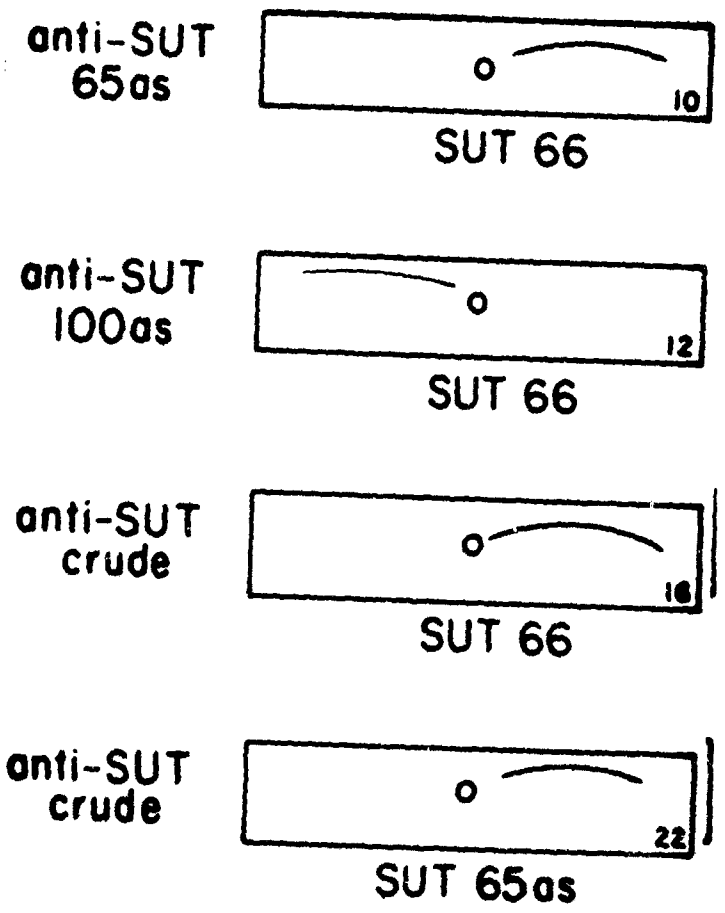


FIGURE 13 Immunoelectrophoretograms of various toxin preparations against selected anti-SUT rabbit immune sera. Immunoelectrophoresis was carried out under the conditions described in Figure 10. Anode is to the right.

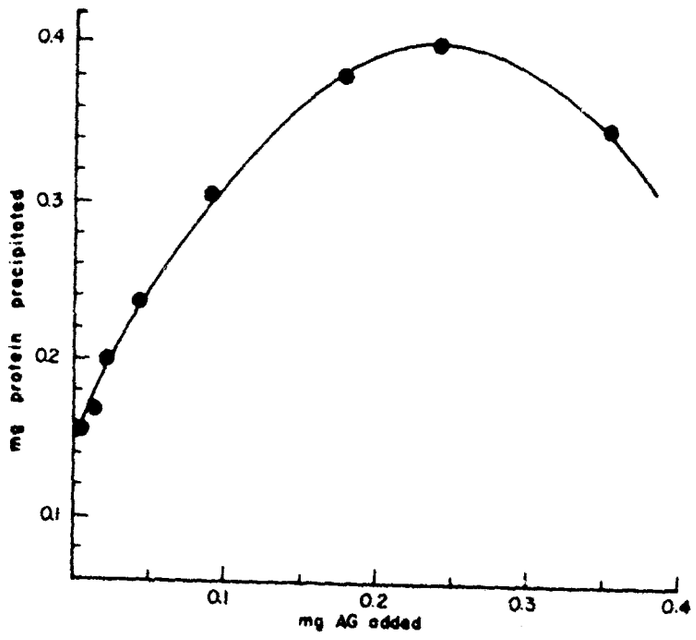


FIGURE 14 Quantitative precipitin curve of immune γ-globulin to SUT (64). Maximal precipitation occurs when the antigen is present at 0.24 mg/ml. Under these circumstances 0.162 mg of antibody is precipitated.

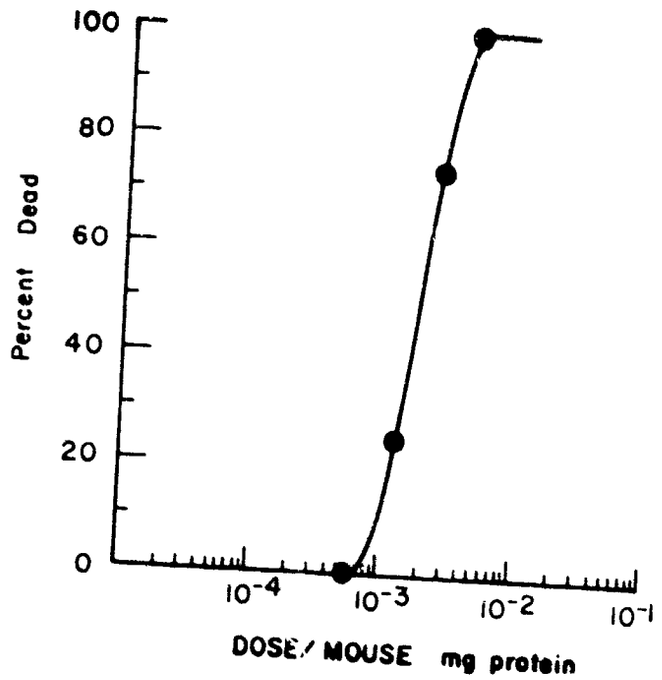


FIGURE 15 Dose-mortality curve for [SUT(64-2)-65 *as*]. Determinations were made on 4 mice per dose. The LD_{50} in mice is 1.8×10^{-3} mg/20 g.

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13. ABSTRACT

The interaction of several sea urchin toxin preparations with certain substrates in mammalian serum produces dialyzable, heat-stable substances which cause contractions of the guinea pig's ileum and rat's uterus. Evidence is presented to show that the toxin acts kinetically as an enzyme and that one of the substrates is electrophoretically pure human α_2-macroglobulin. Crude toxin preparations were also shown to be kininolytic with respect to the dialyzable reaction-product as well as to synthetic bradykinin. Rabbit antibodies to formalinized toxoids quantitatively precipitated active toxin, fixed complement, and protected mice against lethal intravenous doses in the usual quantitative protection and neutralization tests. Immunoelectrophoretic analysis brought out the existence of two distinct antigenic determinants, and additional serological tests showed that antibodies directed against the pedicellarial toxins of *T. gratilla* cross-reacted with test proteins of *S. purpuratus*.

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