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STUDIES OF BIOCHEMICAL AND TOXIC PROPERTIES OF SUBSTANCES OF BIOLOGICAL ORIGIN

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FINAL REPORT

1969-1970

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TABLE OF CONTENTS

TABLE OF ORGANIZATION

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INTRODUCTION

Our studies of the biochemical, immunochemical, and toxic properties of substances of biological origin fall into two general categories: (1) studies of the mode of action of bacterial and animal toxins on the isolated tissues of normal animals and, (2) studies of these systems as complicated by the presence of specific antibodies on target cells, which have the effect of producing anaphylaxis concomitantly with the toxic reaction.

This laboratory has had a long tradition in studies of *in vitro* anaphylaxis, particularly those having to do with the physical-chemical variables which determine the degree of sensitization and the magnitude of the response of shocked tissues and organs. These studies, perforce, have required the determination of physical-chemical enzymatic, and immunological and pharmacological reactions during anaphylaxis.

During the past year we have been engaged in the "double insult" problem. In this case the antigen has an unique, characteristic, and identifiable action in addition to its immunological specificity, and can therefore function either as a biochemical probe in the system of interest or as a passive antigen depending upon its degree of activation. The latter can be controlled to come at a preselected point in time in the reaction sequence. The probes of choice have been protein toxins, both of microbial and zoological origin, particularly of the lower marine orders.

The bulk of the progress in the analysis of the double insult problem has been achieved in studies of cardiac, particularly atrial, anaphylaxis to the active and inactive forms of streptolysin O (SLO) as the toxin used was highly purified, containing little or no contaminating DPNase. The physiological analysis of the mode of action of the active toxin upon the isolated heart (1) and the concomitant release of acetylcholine and histamine by the sensitized heart (2) in response to challenge with the active agent had shown that the toxic and anaphylactic moieties of the SLO molecule are independently manipulated and that they activate quite distinct but highly specific mechanisms in the heart. The question which naturally arises concerns the conditions that determine the quantities and proportions of anaphylactic to protective antibodies that may be raised in response to immunization with SLO. Human neutralizing antibody is protective but human γ-globulin is not cytophilic for guinea pig tissues. Active immunization of guinea pigs with SLO always leads to anaphylaxis and the passive transfer of rabbit antibodies - known to protect hemolytic systems - likewise produces anaphylaxis on passive transfer.

In order to generalize the problem several other kinds of antigenic toxins were studied. Two of these were echinoderm toxins -- highly antigenic to mammals and known to have a direct toxic action on the heart in their active state. These were the toxins of the sea urchin, *Tripneustes gratilla* and the starfish, *Acanthaster planci*. The third toxin, tetanus, has no direct effect on the heart, but is a classical antigenic material to which

antibodies are widely distributed in the population, owing to large scale prophylactic immunization. All of these toxins in their active and inactive forms produced anaphylaxis in the passively sensitized heart. Since recent reports have suggested that many of the cases of acute human anaphylaxis, regardless of the antigen appear to involve the conduction system and musculature of the heart, the degree of generalization appears to be justified (3).

For these reasons, a large section of the present report (Section I) deals with the isolation, physical properties and enzymatic behavior of these toxins, and in particular with the release of plasma kinins.

The activation of the bradykininogen system appears to play a significant role in the course of anaphylaxis to penicillin, and may involve an antibody response somewhat different from that evoked by the classical protein antigens. Evidence for this is presented in the atypical coronary response seen in experimental cardiac anaphylaxis to penicillin conjugates of rabbit serum albumin in Section II. This section, appropriately, contains an account of the variations in bradykininogen levels occurring as a consequence of acute penicillin anaphylaxis in man.

REFERENCES

1. Reitz, B. A., Prager, D. J., and Feigen, G. A., An Analysis of the toxic actions of purified streptolysin O on the isolated heart and separate cardiac tissues of the guinea pig, J. Exp. Med., 128:1401, 1968.

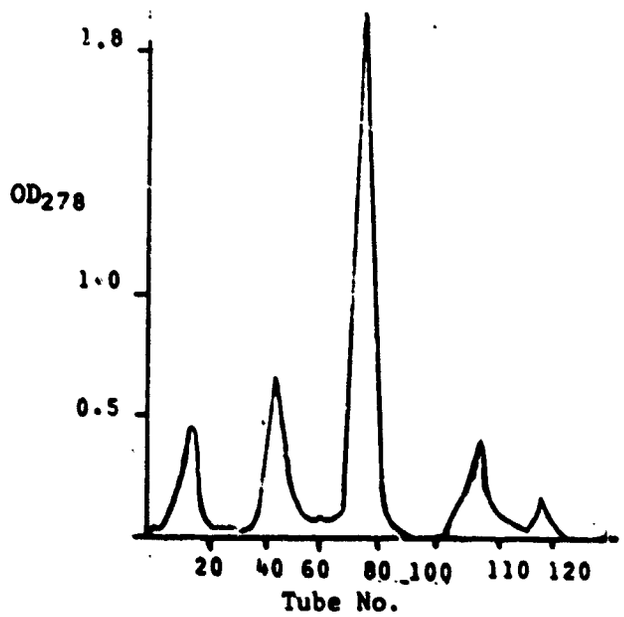
2. Feigen, G. A., and Prager, D. J., Experimental Cardiac Anaphylaxis: Physiologic, Pharmacologic and Biochemical Aspects of Immune Reactions in the Isolated Heart, Am. J. Cardiol., 24:474, 1969.

3. Booth, B. H., and Patterson, R., Electrocardiographic changes During Human Anaphylaxis, J.A.M.A., 211:627, 1970.

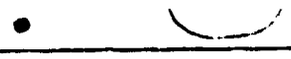
I. Preparation, Properties, and Functional Behavior of Protein Toxins

A. Tetanus

Repeated chromatographic runs were made to accumulate stocks of various fractions of partially purified tetanus toxin so that enough material would be available for physical chemical and biological characterization. The parent material was received from the Wellcome Laboratories as a lyophilized powder obtained as a fraction precipitating between 70 - 100% SAS from acid-treated crude bacterial toxin. Chromatography of 100 mg of TD698B on hydroxylapatite yielded the fractions represented by the effluogram given below:



The biological, physical, and immunological properties are available in the table below:

OH-A Fractions	LD_{50} mg ppt N	$MEPP_{50}$ mg ppt N	S_{20}	Immunoelectrophoresis - +
Crude	5.71×10^{-3}	5.5×10^{-7} 7.0×10^{-6}	3.90	
I	7.47×10^{-5}	None		
II	Atoxic	5.8×10^{-5}	2.2	
III	Atoxic	None		
IV	Atoxic	3.7×10^{-9}		
V	Atoxic	None	4.48	
VI	Atoxic	Not Tested	4.86	

This method of fractionation represents a significant improvement over the previous separation on Sephadex as described by Tomita and Feigen (1). Preparation IV represented the most powerful NSP fraction and it corresponded immunoelectrophoretically to Sephadex Fr. II of the Sephadex series from which the non-spasmogenic fraction was isolated originally. Antisera raised to toxoids made from the starting material sensitized the heart for *in vivo* and *in vitro* passive cardiac anaphylaxis.

REFERENCES

1. Tomita, J. T., and Feigen, G. A., Serological identification and physical-chemical properties of the non-spasmogenic principle (NSP) in tetanus toxin, Immunochemistry, 6: 421, 1969.

B. Streptolysin O

Earlier work had shown that the cardiac tissues of sensitized guinea pigs would react anaphylactially when tested with toxoided or oxidized streptolysin O but that both the toxic and anaphylactic reactions could be exhibited when sensitized tissues were challenged with active (reduced) SLO (cf. Phase 2). These studies were amplified during the present contract period to include the following variables: antibody concentration, activation of toxin, specific and non-specific blocking agents: atropine, immune human globulin, and cholesterol; effect of temperature.

Hearts of normal guinea pigs were sensitized by perfusion with various concentrations of rabbit anti-SLO γ-globulin. The concentrations ranged from 0.12 to 18.8 μg of anti-SLO/ml. Atria were dissected from the sensitized heart as a single tissue and arranged in 32°C muscle baths for recording of mechanical response. The test sequence was arranged as follows:

Phase	A	B	C
1	Inactive SLO	Active SLO	Active SLO + Atropine
2	Active SLO	Active SLO	Active SLO
3	Histamine	Histamine	Histamine

Phase 1: Challenge with inactive toxin (A) produced only an increase in amplitude, which varied in magnitude and time of onset with increasing antibody concentration. On the other hand, challenge with active toxin (B) produced first a decrease in amplitude followed by an increase, the decrease being higher, and the increase being lower, as the antibody concentration was increased. The principal difference owing to atropine (C) was that the initial toxic response (decreased amplitude) was greatly inhibited and the subsequent anaphylactic rise was lowered (Fig. 1).

Phase 2: Tissues initially challenged with inactive toxin (A), now gave a profound toxic response to active SLO without much anaphylactic reaction, suggesting that no tachyphylaxis of the cholinergic apparatus had taken place although the tissue had been desensitized to the initial anaphylactic challenge (Fig. 1). This finding gives strong support to the idea that the toxic and anaphylactic moieties of the SLO molecule are independent.

Phase 3: The sensitivity to histamine was greatest in group A and least in group C (Fig. 1). The toxic depressor response, always appeared before the anaphylactic response. An inquiry was made to determine whether the phase difference resulted from a phase difference in the attack of SLO molecules on target cells or whether it could be accounted for exclusively on pharmacological grounds, i.e. that the dose-response characteristics of the tissue to histamine and acetylcholine determined the phasic characteristics. Experiments with solutions containing various proportions of authentic histamine and ACh showed that the tissues responded first to ACh and then to histamine (Fig. 2), but that the extent of the ACh depression depended inversely on the histamine concentration, suggesting - for the overall reaction - that the character of the overt anaphylactic response was the independent variable and that - even though it was manifested later in time - could determine the extent of the toxic reaction.

Antigen: The anaphylactic dose-response curves of systems sensitized with varying concentrations of anti-SLO and challenged with a constant concentration of antigen were typically sigmoidal. Challenge with the activated antigen, however, produced a leftward shift in the dose-response function. The increase in sensitivity is reflected in a shift of the 50% sensitizing dose of antibody from about 5.0 µg/ml to less than 2.5. This result suggests that activation brings out additional antigenic moieties in the SLO molecule since the range of reactivity of a constant amount of antigen is now increased.

Antibody: Preliminary experiments showed that passive sensitization with equipotent concentrations of specifically precipitable anti-SLO gave greater anaphylactic and lower toxic responses when sensitization was accomplished with the γ-globulin fraction than when it was done with whole serum. A study now in progress on six hydroxylapatite fractions obtained by chromatographing the 30-50% saturated ammonium sulfate cut shows these fractions to vary in their anaphylactogenic power. These results will be reported shortly.

An examination of the electrical properties of single atrial cells exposed to mixtures of histamine and acetylcholine were carried out at Oxford by Dr. Bramah N. Singh and Dr. George A. Feigen. Acetylcholine alone increased the resting potential and the action potential, shortened the repolarization time, and decreased the mechanical response. Histamine alone had only a slight effect on the resting potential; the action potential was slightly decreased, as was the depolarization rate. The repolarization time was prolonged and the contraction was greatly increased. In the presence of a constant dose of ACh, the resting potential action potential, and depolarization rate were not greatly affected by the addition of moderate amounts of histamine, but both the repolarization time and the contraction returned to control values. High concentrations of histamine precipitated fibrillation in the presence of ACh. A test with that (5.5×10^{-6} *M*) concentration of histamine alone greatly increased the repolarization time and the amplitude of contraction (Experiment 2, Figure 3).

Taken together, the release of ACh and histamine during anaphylaxis to active SLO seems to account for the obvious changes occurring during the episode. Since the atrium contains no measurable histaminase activity but does have powerful cholinesterases, it is evident that the effect of ACh will be shorter-lived than that of histamine. Hence, the degree of anaphylactic histamine release might be expected to swamp out the acetylcholine effect. At a given concentration of toxin the acetylcholine released will be constant and independent of antibody concentration; under the same conditions of challenge the histamine (anaphylactic)effect will be determined by the antibody concentration. Hence one might predict that the greater the anaphylactic reaction the lesser will be the toxic (cholinergic) response. Such a negative correlation is exhibited in Fig. 4. Evidently the presence of atropine changes the character of the relation between the anaphylactic and toxic reactions: the toxic response evoked by SLO is much lower for a given anaphylactic reaction when atropine is present.

Heating: Heating the atria at 45°C to inactivate the anaphylactic reaction did not prevent the toxic reaction from taking place (Fig. 6). Cholesterol blocked the toxic but not the anaphylactic reactions of sensitized atria.

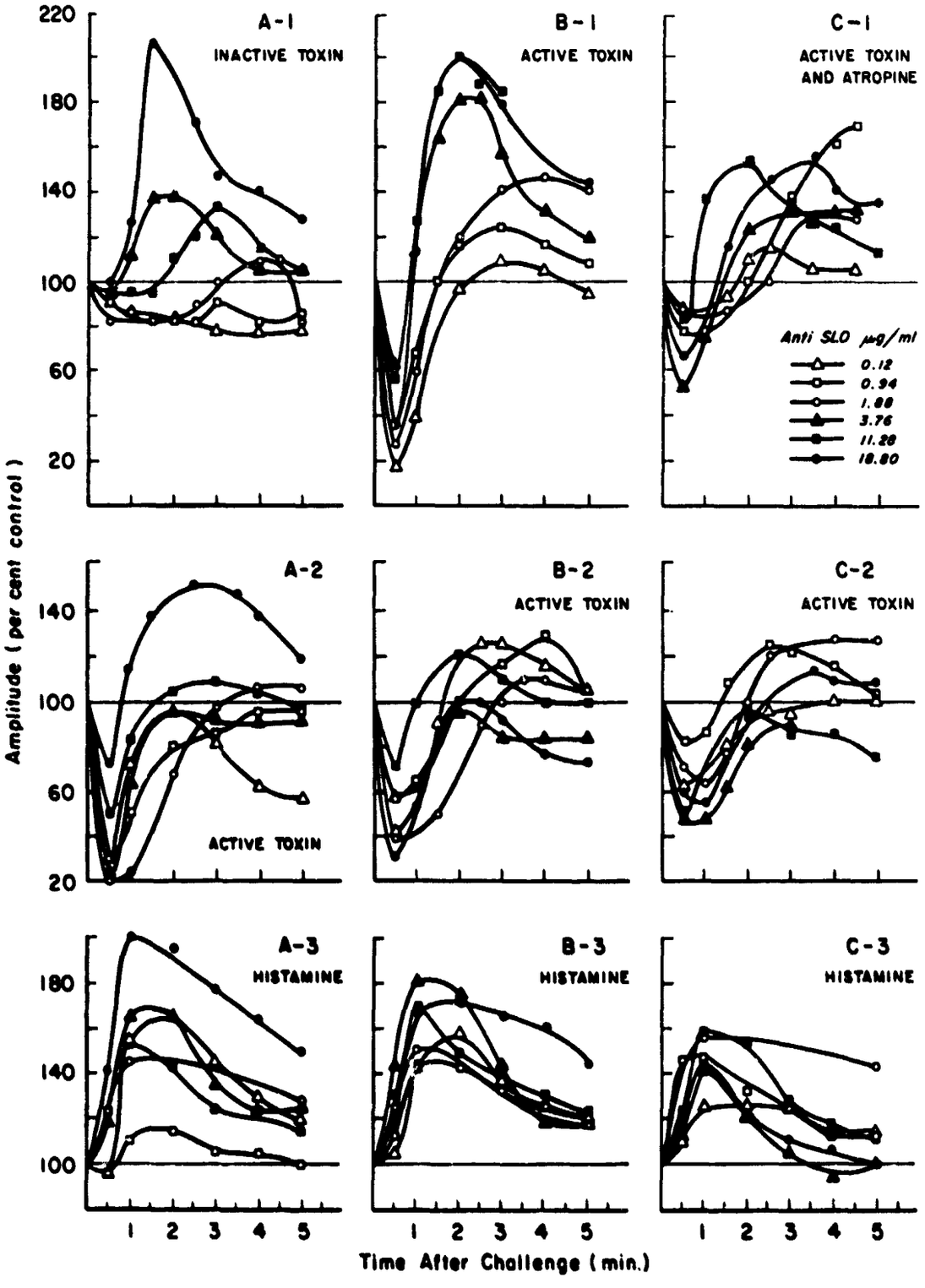


Figure 1. Effects of Antibody Concentration on Toxic and Anaphylactic Reactions to Streptolysin O.

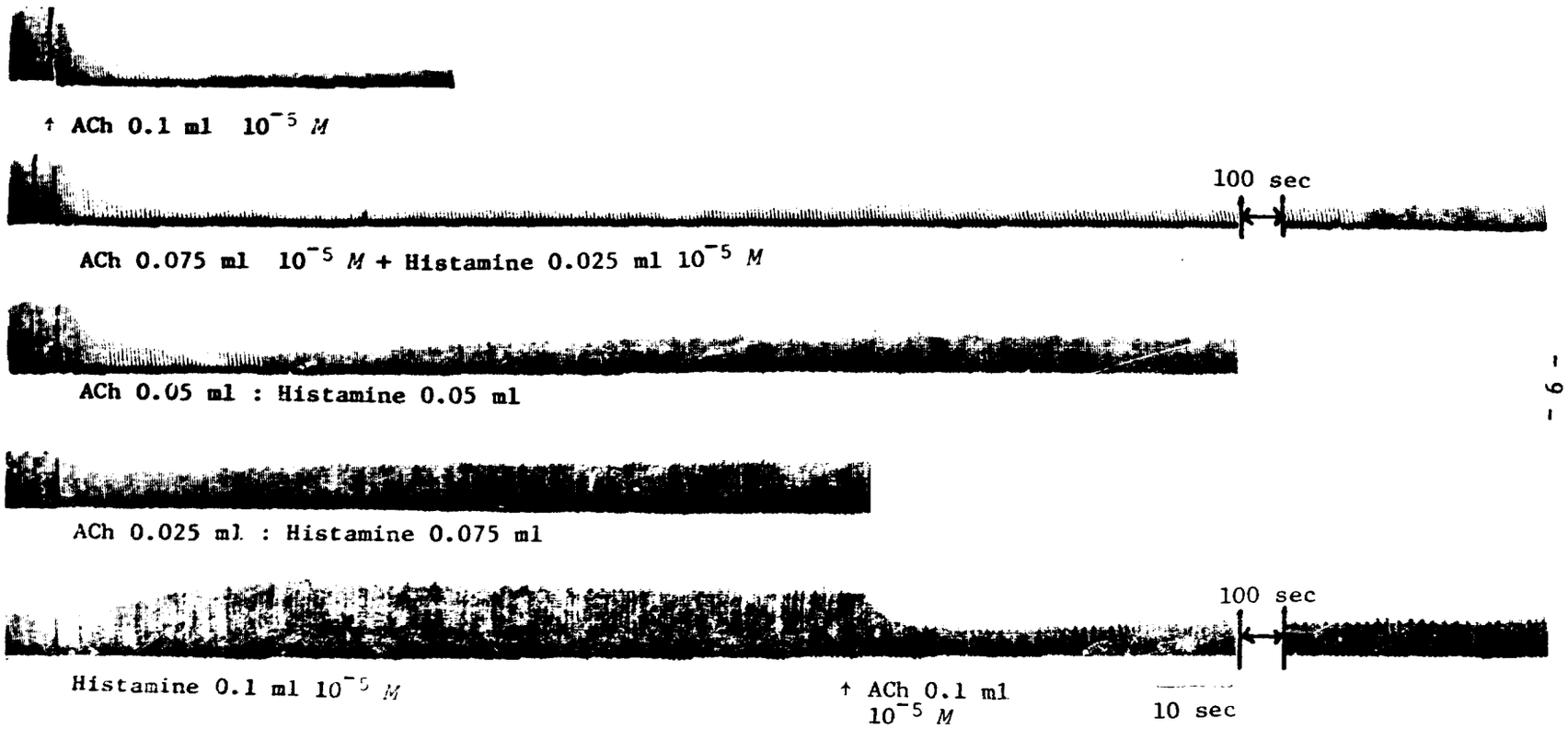


Figure 2. Inotropic and Chronotropic Reactions of Guinea Pig Atria to Mixtures of Acetylcholine and Histamine

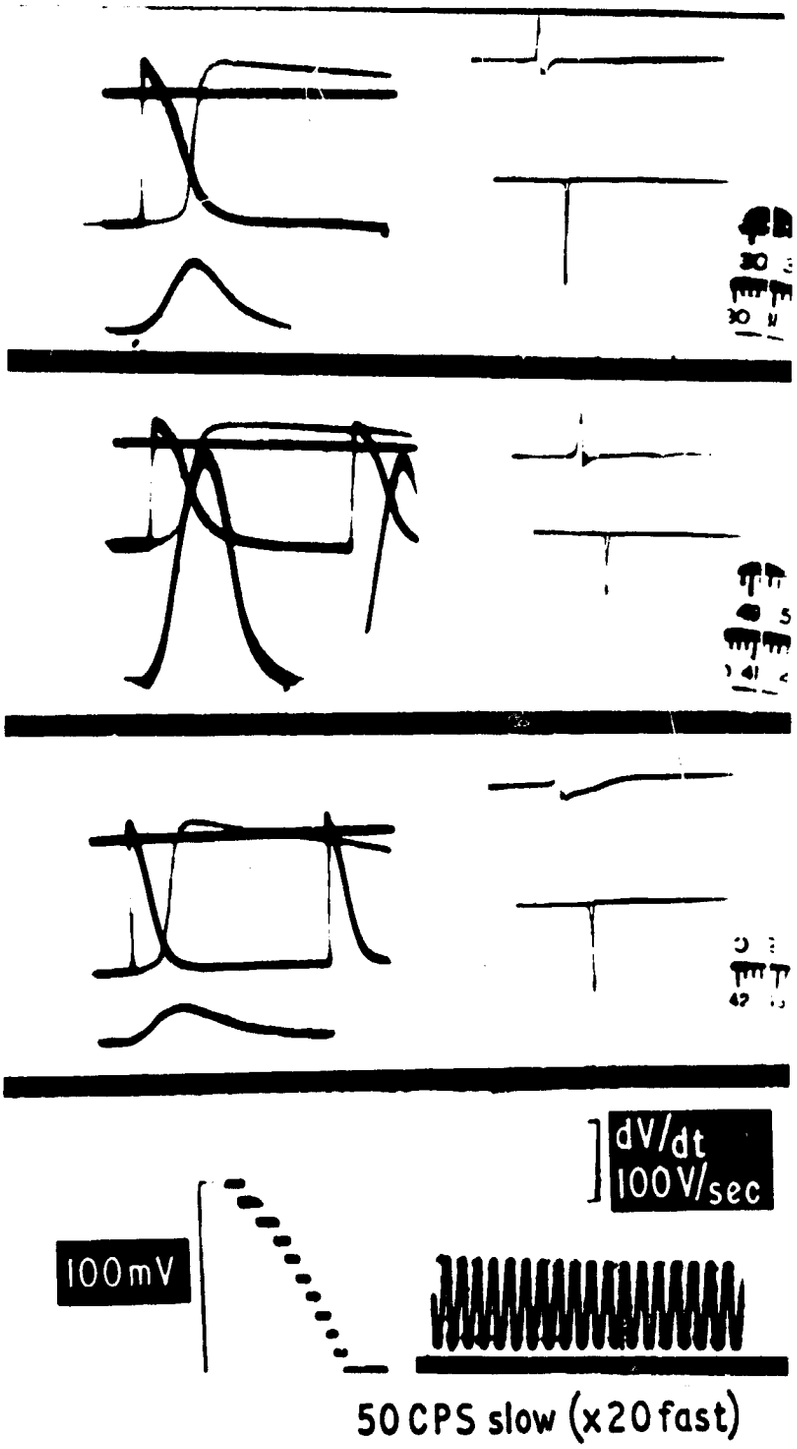


Figure 3. Intracellular potential changes induced in guinea pig atria treated with mixtures of histamine and acetylcholine. In each frame the upper complex is the superimposed slow and fast potential record; the lower trace is the contraction.

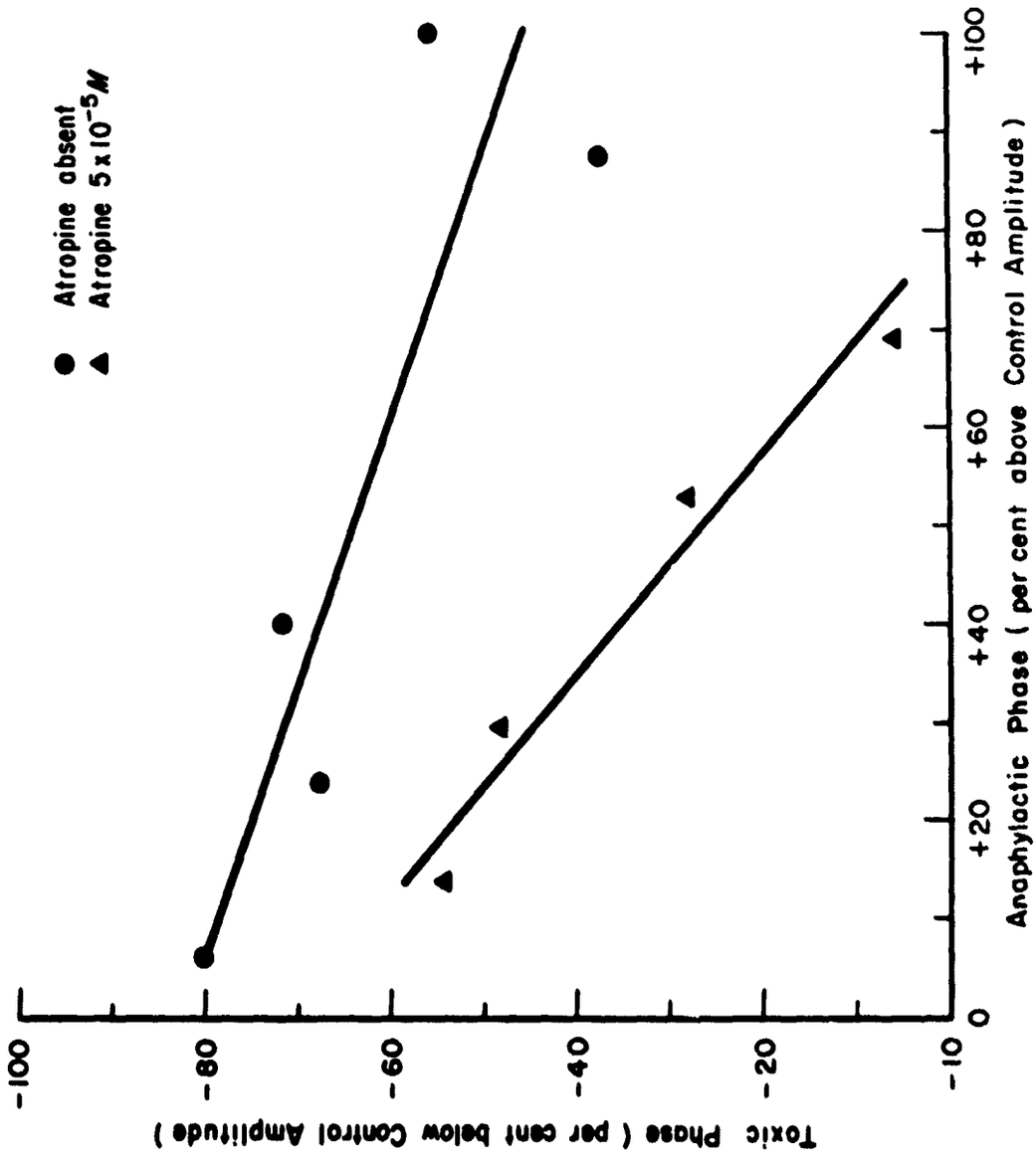


Figure 4 Correlation between Toxic and Anaphylactic Responses of Sensitized Atria Challenged with Active Streptolysin O.

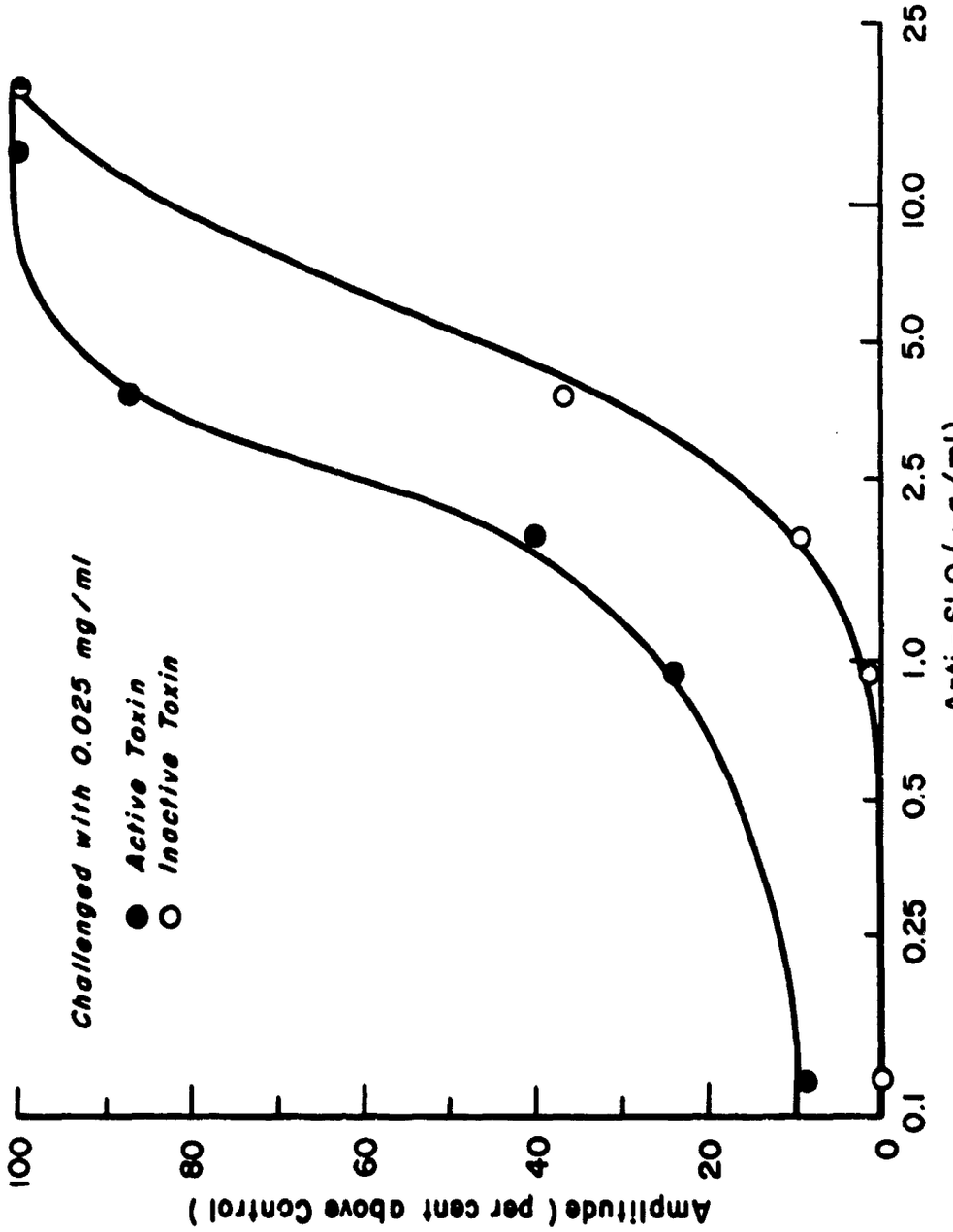


Figure 5. Effect of Priming Dose of Antibody (*in vitro*) on the Anaphylactic Reaction to Active and Inactive Streptolysin O.

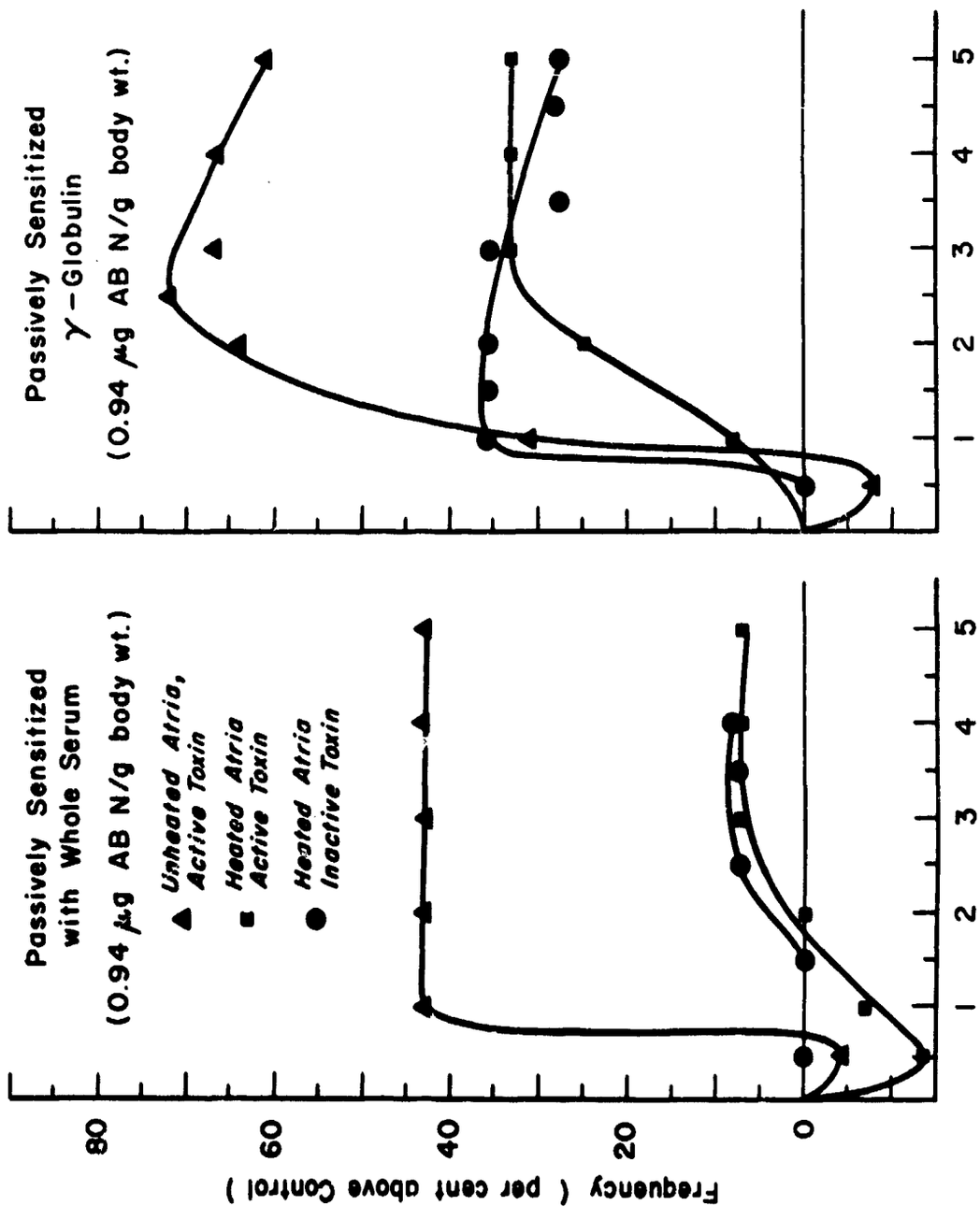


Figure 6. Chronotropic Response of Sensitized Atria to Challenge With Streptolysin O.

C. Sea Urchin Toxin

The sea urchin *Tripneustes gratilla* (Linnaeus) has a wide distribution in the littoral of the Indo-Pacific area. It has a globiferous pedicellariae which contain a venom that is lethal when injected intravenously into mammals. The venom is antigenic and stings received by humans can cause both serious local effects as well as generalized hypersensitivity in persons repeatedly exposed.

An earlier study under the present grant (1) had shown that a dialyzable product, having the properties of a plasma kinin, could be produced by incubating heated, acidified serum, (or certain of its globulin fractions), with various preparations of sea urchin toxin. From an array of studies in which the plasma protein and the toxin concentrations were systematically varied, it was deduced that the toxin behaved kinetically as an enzyme, and that the principal substrate was an α_2-globulin. The toxin also had the capacity of destroying the product. In an effort to clarify that problem it was necessary to establish the general identity of the principal pharmacologically active product in the reaction and to determine the points of attack of the purified toxin preparations.

In the present study we show that by controlling the temperature and time of digestion it is possible to separate active peptides from the reaction mixture either by solvent extraction or direct dialysis which can be further purified by gel filtration and resolved by paper chromatography into two groups of pharmacologically active components, one of which is identical physically (Fig. 1) and physiologically (Fig. 2) with synthetic bradykinin.

The next phase deals with the separation of active components from the ensemble of proteins by chromatography on hydroxylapatite and Sephadex, the determination of the point of attack, and a study of their reaction kinetics as enzymes.

The protein constituents of the pedicellarial toxin of the sea urchin (SUT) *Tripneustes gratilla* (L) were separated by chromatography on Sephadex G-200 or hydroxylapatite (Table I) and characterized by analytical ultracentrifugation. Toxicity was associated with molecular distributions in the range 5.7 - 6.4 *S*. There was a linear correlation between the digestion of plasma proteins by SUT and the yield of kinin. Comparative studies on the digestion of azocasein showed that all of the proteolytic activity in the Sephadex series was concentrated in Fraction I; it was greatest in Fraction I of the hydroxylapatite (HOA) set, but was present in moderate potency in three other HOA fractions.

Tests on the Sephadex fractions showed a correlation between the kinin-forming and the bradykinin-destroying potencies of the preparations, (Table I). None of the preparations tested hydrolyzed TAME but four of them hydrolyzed ATEE, and studies of pH optima, substrate variation, and enzyme variation (Fig. 3) showed conformance to classical chymotrypsin activity. From an analysis of Lineweaver-Burk data the enzymatic power of Sephadex Fraction I was increased almost 3-fold and that of Fraction II of the hydroxylapatite set was concentrated 6.5 times by purification (Fig. 4).

The present results show that the lethal toxicity does not depend on the formation of bradykinin from the heated plasma. Table II shows that the most toxic preparation of the Sephadex series (Fr. II) has no measurable esterolytic or proteolytic action, and, additionally, tests in this laboratory have shown that mice can survive intravenous doses of bradykinin at least up to 0.1 mg/20 g.

Many of the marine toxins are hemolytic. Sea urchin toxin hemolyzes rabbit cells directly but will lyse sheep cells only in the presence of lecithin. Although all of the SUT preparations tested hemolyzed rabbit cells, there appeared to be no clear relationship between hemolysis and lethal toxicity. Death from an effective intravenous dose of any of the SUT preparations occurs within a few seconds. The terminal signs in mice include respiratory stridor and convulsions, and blood samples taken from the heart or great vessels immediately post-mortem show no signs of hemolysis.

The components of SUT were cardiotoxic and antigenic, and they evoked anaphylaxis in isolated hearts. The same antisera protected mice against death in neutralization tests but did not protect against the esterolysis of ATEE.

REFERENCES

1. Feigen, G. A., Sanz, E., Tomita, J. T., and Alender, C. B., Studies on the mode of action of sea urchin toxin. II. Enzymatic and immunological behavior, Toxicon, 6:17, 1968.

TABLE I

Effect of Toxin Concentration and Reaction Time on the Relation of Protein Digestion to Kinin Formation at 27°C

Type of Experiment	Enzyme (1.0 ml) SUT-67 (mg N/ml)	Substrate (4.0 ml) HTP (mg N ml)	Time Min.	NPN Formed (mg N/ml)	Kinin Formed µg BKE/ml x 10^{-2}
Variable Enzyme	0.104	10.34	10	0.120	20.0
	0.052	"	"	0.100	17.0
	0.026	"	"	0.053	12.4
	0.013	"	"	0.033	9.1
Variable Time	0.052	"	10	0.057	11.0
	"	"	20	0.069	14.0
	"	"	40	0.088	16.0
	"	"	60	0.092	17.0

TABLE II

Comparison of the Kininolytic Properties of Sea Urchin Toxin Preparations at 32°C

Toxin Preparation		Kininolysis		Kininogenesis	
Type	Concentration mg N/ml*	Substrate Present: Bradykinin µg/ml	Bradykinin Found After Digestion µg/ml	Bradykinin Destroyed %	Substrate Present: HTP mg N/ml*	Net BK Formed** µg/ml
SUT-67 (Crude)	0.0530	4×10^{-2}	0	100	8.27	3.3×10^{-2}
Seph. Fr. I	0.0093	"	0	100	"	2.8×10^{-2}
Seph. Fr. II	0.0107	"	2.7×10^{-2}	32.5	"	1.9×10^{-2}
Seph. Fr. III	0.0109	"	3.9×10^{-2}	0	"	0
0	0	"	3.9×10^{-2}	0	"	0

* Concentration expressed as mg of TCA-precipitable N

** Equivalent bradykinin concentration per milliliter as found by bioassay against bradykinin standards corrected for dilution

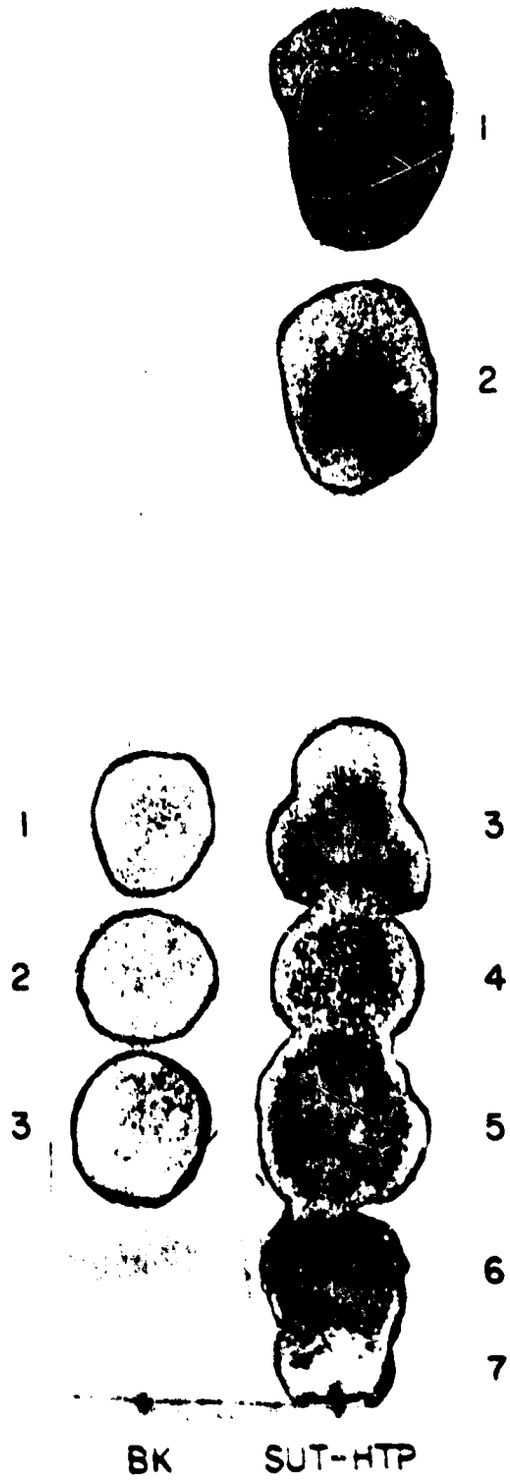
TABLE III

Summary of Functional Activities of Various Sea Urchin Toxin Preparations

Type	SPECIFIC TOXICITY	ENZYMOLOGY				Hemolysis
	LD_{50} per mg protein N	Azocasein (OD_{390}/mg N hr^{-1})	ATEE K_m	ATEE V_{max}*	ATEE V_{max}/mg N	H_{50} (mg N)** Rabbit Cells
Crude Toxin						
SUT-67	7.75×10^3	3.58	5.5×10^{-3}	55.5	213.4	2.0×10^{-5}
Sephadex Fractions						
Fr. I	3.5×10^3	10.51	1.92×10^{-3}	26.67	592.6	3.0×10^{-4}
Fr. II	2.0×10^4	0	no activity	-	no activity	1.4×10^{-5}
Fr. III	1.4×10^3	0	"	-	"	3.0×10^{-5}
Hydroxylapatite Fractions						
HO-A Fr. I	0	20.31	3.57×10^{-3}	20.0	188.6	2.9×10^{-5}
HO-A Fr. II	1.5×10^4	4.27	1.46×10^{-2}	154.0	1412.8	1.2×10^{-6}
HO-A Fr. III	6.6×10^3	0.20	no activity	-	no activity	9.8×10^{-6}
HO-A Intermediate	2.2×10^3	4.38	"	-	"	
HO-A Fr. IV	5.5×10^3	0.93	"	-	"	1.0×10^{-5}
HO-A Fr. V	0	3.94	"	-	"	4.6×10^{-5}
HO-A Fr. VI	0	0.53	"	-	"	7.3×10^{-5}

* µL 0.1 NaOH/100 sec

** mg protein N to produce 50% lysis



BK - Synthetic Bradykinin

SUT-HTP - Active Materials from Plasma Digestion

Figure 1. Resolution of Peptides by Paper Chromatography

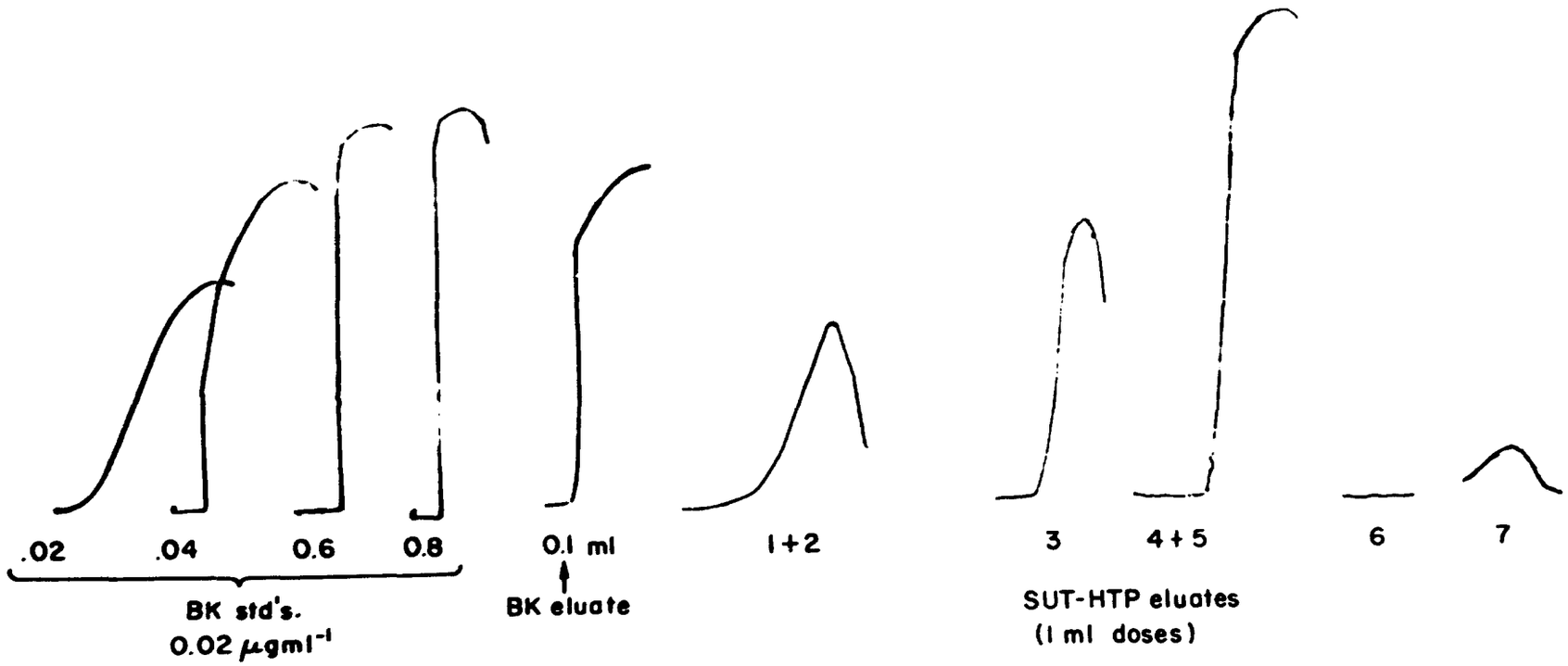


Figure 2. Bioassay of Peptides Eluted From Chromatogram in Fig. 1.
(Guinea Pig Ileum 37°C)

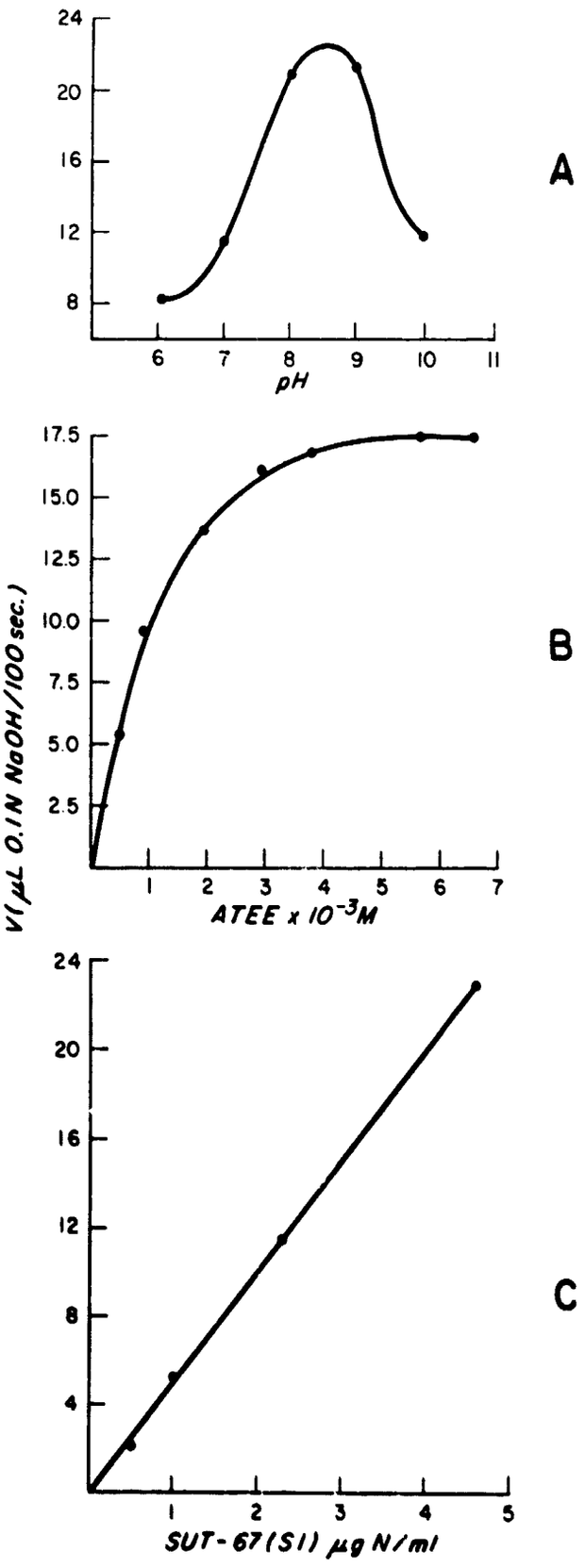


Figure 3. The Velocity of Substrate Digestion of Purified SUT as Influenced By pH, Substrate Concentration, and Toxin Concentration.

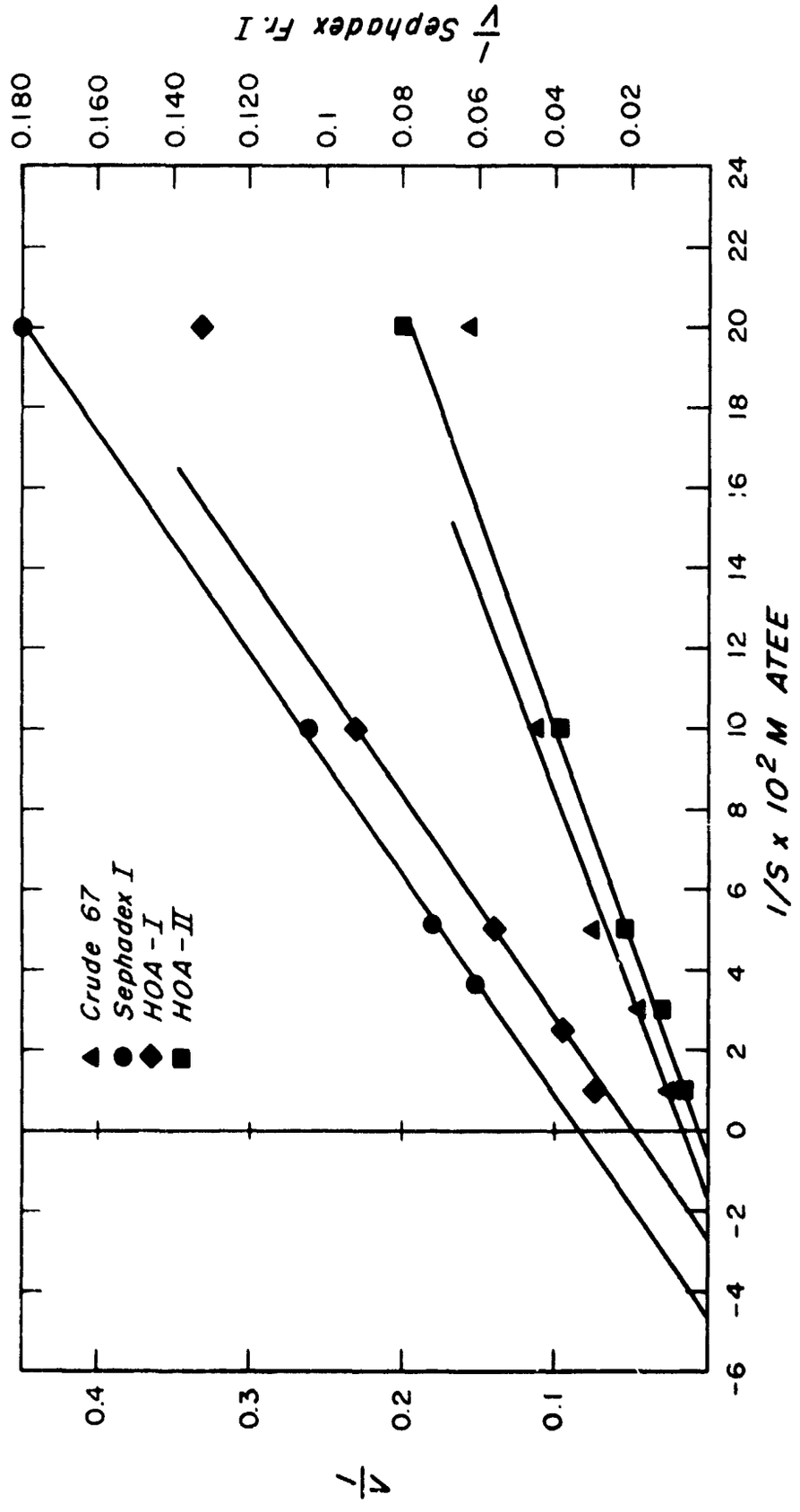


Figure 4. Lineweaver-Burk Plots for Various SUT Preparations.

D. Crown of Thorns Toxin

The uncontrollable proliferation of the starfish, *Acanthaster planci* (Linnaeus) in the Indo-Pacific area has posed a severe environmental problem owing to its depredation on the Great Barrier Reef as well as in the American Trust Territories. It has the potential, also, of offering a severe problem - as do all marine toxins - to the personnel whose economic or scientific activities take them to this area, as suggested by the report of Barnes and Endean (1) on the sequelae of puncture wounds sustained by humans.

Preliminary investigations on the proteins extracted from the spines, as well as the washings taken from the ventral surface, of several animals received in this laboratory from the South Pacific showed that the extracts were lethal to mice on intravenous injection, and that they exerted toxic reactions on various isolated organs such as the uterus, gut, and heart. The extracts were immunogenic, and the passive transfer of antibodies from rabbits to guinea pig tissues sensitized the latter anaphylactically so that the system had a valuable potential for the continuation of our studies on the double insult problem.

Preparation and Physical Properties of Starfish Toxin:

Two general types of extract were prepared. In the first case the extract was limited to the spines and in the second case both the spine and the sea water washings (taken from the ventral surface) were used as starting materials. Two 100-gram samples of spines were homogenized in a Waring blendor in 200 ml of 1/60 *M* phosphate buffer at pH 7.2 to which had been added 175 g of ice. The proteins were brought down by saturating the solution with solid ammonium sulfate. The precipitate was redissolved in 250 ml of the 1/60 *M* phosphate buffer. The precipitation step was repeated, the material was redissolved, dialyzed against 15 L of continuously flowing 0.12 *M* phosphate buffer, and pervaporated to 228 ml. The final extract was a dark red brown solution at 0.1 *M* PO_4.

The crude extract was further fractionated by chromatography on Sephadex G-200 or hydroxylapatite. Three fractions were obtained in the first case and six in the second.

The absorption spectrum of the crude toxin, given in Fig. 1, shows absorption maxima at 280 and 424 mμ.

Analytical and Toxic Properties:

Crude Extract: The comparative recovery of protein from the spine extract (prep. IX-42) and the spine + surface preparations (prep. IX-86) given in Table I demonstrates first that five times as much protein was obtained in the second case and that for an approximately constant ratio of precipitable to total N the specific toxicity was quadrupled.

Sephadex Fractions: Fig. 2 compares the separation of the spine + surface material with the separation of a mixed chromatogram in which the sample was enriched with "surface" extract alone. It is evident from the result of this experiment that the minor peak is due to the surface toxin and that the major peak represents the spine extract. The comparative toxicities of the various sephadex fractions is exhibited in Table II. The results show Fr. I, representing the spine extract, to be virtually atoxic and the surface material to be approximately 3 times as lethal as the starting material.

Pharmacological Properties of the Toxin:

Pharmacological tests for toxicity of the starfish toxin preparations were made on guinea pig ileum, rat uterus, and guinea pig heart by appropriately challenging these preparations with untreated, heated, and formalinized samples of the toxin.

Active Toxins: Active toxins caused a biphasic contraction of ileal strips. A second challenge was without effect although the tissue still reacted to the usual test agents. Perfusion of the guinea pig heart with active toxin in concentrations of 0.01 - 0.03 mg protein N/ml rapidly and irreversibly decreased the coronary (Fig. 3) perfusion rate, the reduction being associated with ventricular (Fig. 4) but not atrial standstill.

Heated Toxin: Toxin preparations heated to 60°C for 1 hour produced only a slight reduction in the perfusion rate and contraction amplitude of the isolated heart. This preparation caused the guinea pig ileum to contract but there was a rapid tachyphylaxis.

Formalinized Toxin: Toxin preparations treated with 0.01% formaldehyde increased the perfusion rate but decreased the amplitude of the normal heart. The pattern of the ileal reaction was similar to that seen in the case of the treated toxin.

Immunological Behavior:

Methods:

Immunization. Antisera to crude *A. planci* toxin IX-86 was produced in rabbits by injection into multiple sites around the neck of heat-treated toxin (3.39 mg ppt N) in complete Freund's adjuvant. At eleven days a booster injection containing 0.57 mg ppt N of heat-treated toxin in complete Freund's was administered and the rabbit bled by cardiac puncture one week later. The serum was separated, the γ-globulin fraction obtained, and the antibody titer determined by quantitative precipitation.

Immunoelectrophoresis. Immunoelectrophoresis was carried out on the toxin and its various fractions to determine the complexity and number of antigenic components.

Anaphylaxis. Studies of *in vitro* anaphylaxis in guinea pigs sensitized by passive transfer were made in response to challenge by active toxin and its sephadex fractions or by toxoided material. The experimental design consisted essentially in the challenge of the passively sensitized heart with the various antigens and the study of the effect on amplitude and frequency response of the heart and the nature of the output of the vasoactive agents. Reverse passive anaphylaxis was also studied in which normal heart and ileum were challenged with various dilutions of toxins and after an appropriate period of time re-challenged with antibody.

Results:

Immunoelectrophoresis. The crude toxin showed four main antigenic determinants. Three of the determinants appear in Sephadex Fr. I while all four appear in Fr. II. Fraction III contains two of the determinants in common with the crude toxin and Fr. II, and one in common with Fr. I. One of the determinants in the crude fraction belongs solely to Fr. III.

Anaphylaxis. Guinea pigs were passively sensitized with 1.0 ml of dialyzed rabbit anti-*A. planci* (containing 0.2960 mg ppt AbN per ml of serum) the night before use. Cardiac and ileal tissue were excised and treated as described previously. The sensitized hearts were challenged with various doses of treated and non-treated toxin and its Sephadex fractions.

Challenge of sensitized heart with the toxin resulted in anaphylactic responses in all cases tested. At the higher doses of toxin the measurable activity of the heart ceased. Recovery of normal rhythm of heart beat at decreased amplitude ensued after challenge with lower doses. All the perfusates of specifically shocked hearts contained histamine. The time-course of histamine release was an explosive process, which reached a peak at approximately 60 seconds following challenge. As seen in Figure 5, the active Sephadex fraction released over 5 times as much histamine at the peak than did the formalinized preparation.

Hemolytic Properties. The hemolytic potential of the starfish toxin was studied on the erythrocytes of the rabbit, sheep, mouse, and guinea pig. The titrations were carried out by incubating 1-ml portions of 2% RBC suspensions with 1-ml aliquots of toxin for 15 minutes at 37°C. At the end of the incubation period each tube was treated with 5 ml of ice cold 1% NaCl, centrifuged for 10 min at 2500 rpm, and the supernates read at 540 mμ in a Klett colorimeter. The per cent hemolysis was determined with reference to a standard and the hemolytic titer, expressed by H_{50}, (the concentration of toxin producing 50% lysis) is entered in Table IV.

The hemolytic potencies (Table IV) obtained on sheep cells were, in general, somewhat lower than those obtained with sheep erythrocytes. The specific hemolytic activity was greatest in the case of the crude spine extract than in that of the "surface" toxin; among the fractions, the hemolytic activity was evidently concentrated in Sephadex Fraction III.

REFERENCES

1. Barnes, J., and Endean, R., A Dangerous Starfish, *Acanthaster planci* (Linnaeus), The Medical Journal of Australia, 1: 592, 1964.

TABLE I

Characteristics of *A. planci* Spine Toxin Preparations

	Preparation IX-42	Preparation IX-86
Weight of Spines (g)	100	98
Total mg N Extracted	41.86	198.4
Mg ppt N Extracted	29.38	119.9
Ratio of Precipitable to Total N	0.702	0.605
Protein (mg)	256.3	1240.0
LD_{50}/mg ppt N	88.5	375

TABLE II

Characteristics of Spine Toxin Fractions* Separated by Gel Filtration On Sephadex G-200

	Crude	Seph. Fr. I	Seph. Fr. II	Seph. Fr. III
Mg tot N	4.405	2.944	0.7066	0.2100
Mg ppt N	2.665	1.744	0.3314	0.0853
LD_{50}/mg ppt N	375	28	301	1290
P/T	0.605	0.5925	0.4690	0.406

* Determined on preparation IX-86

TABLE III

Amino Acid Analysis of Spine Toxin
(Sephadex Fraction I IX-42)

Amino Acid	Nos. of residues
Lysine	14.9
Histidine	4.8
Arginine	7.9
Aspartic Acid	21.1
Threonine	14.0
Serine	11.9
Glutamic Acid	20.1
Phenylalanine	8.2
Proline	10.1
Glycine	14.1
Alanine	12.2
Valine	17.6
Methionine	6.0
Isoleucine	8.4
Leucine	13.8
Tyrosine	8.4

TABLE IV

Hemolytic Potencies of *A. planci* Toxin Preparations

Preparation IX-86	Rabbit H_{50}/ mg ppt N	Sheep H_{50}/ mg ppt N
Crude	113	106
Seph. Fr. I	18	12
Seph. Fr. II	20	-
Seph. Fr. III	77	31
Surface Extract	69	70

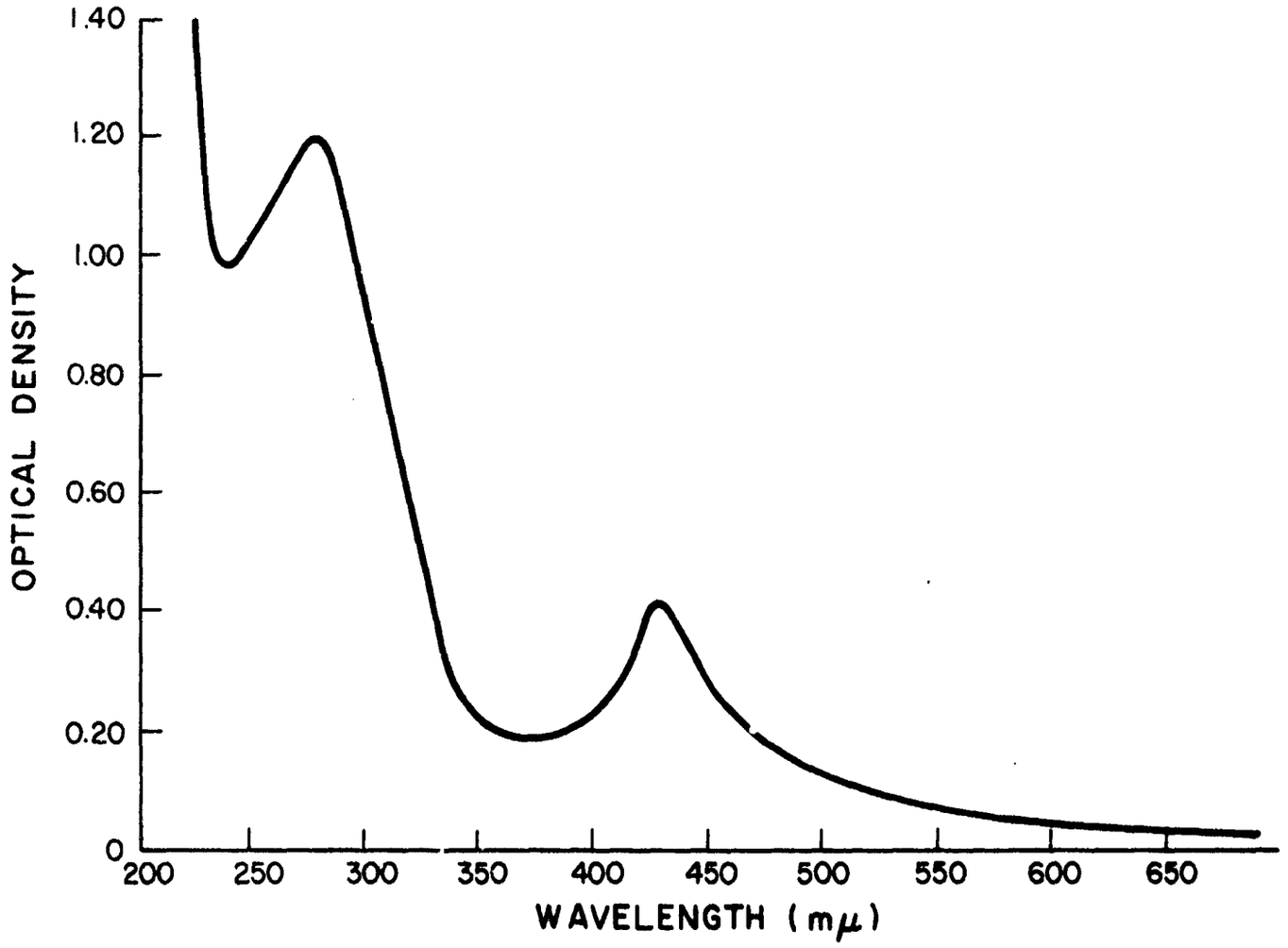


Figure 1. Absorption spectrum of *A. planoi* Spine Toxin.

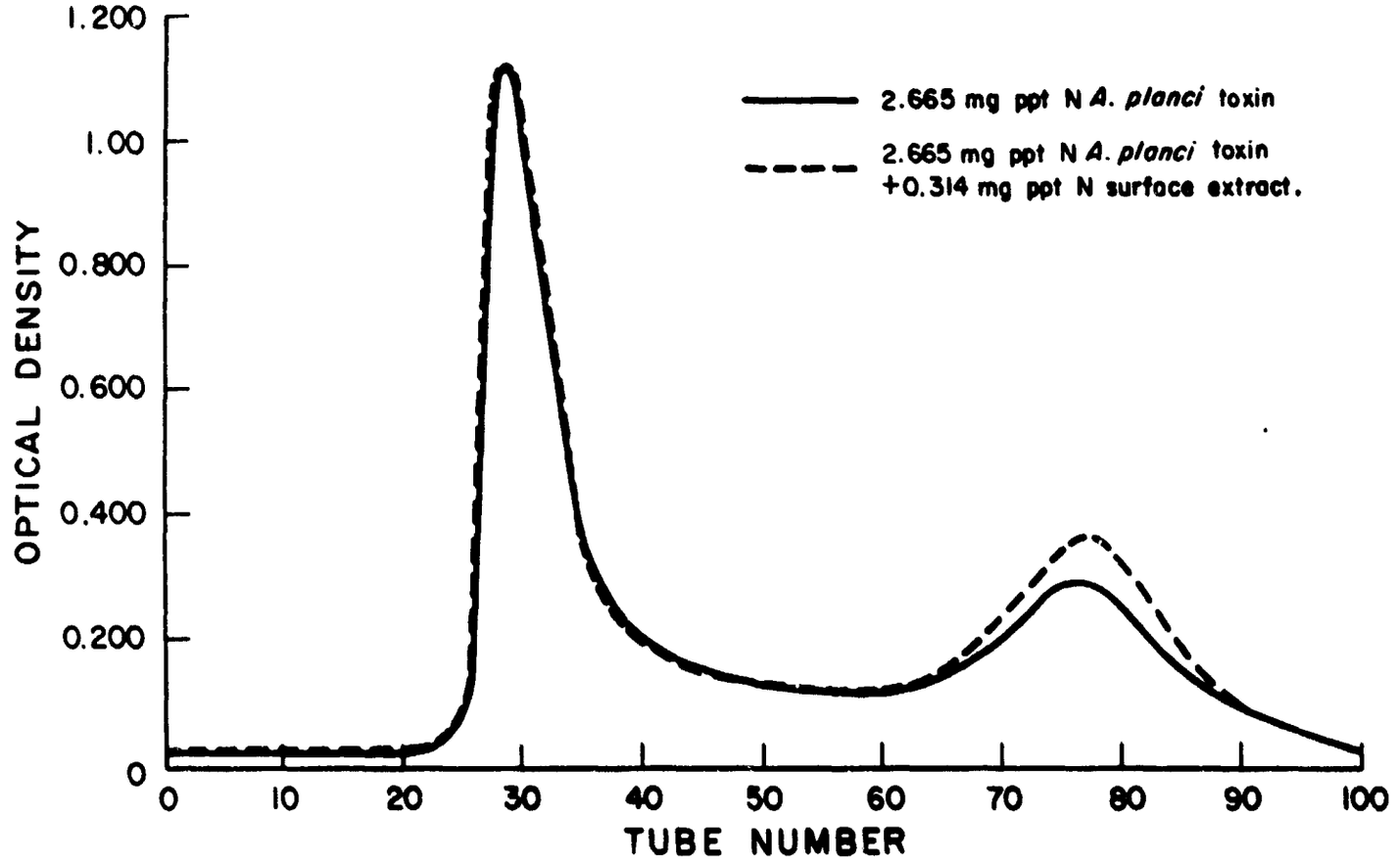


Figure 2. Fractionation of *A. planci* Spine Toxin on Sephadex G-200 (Prep. IX-86).

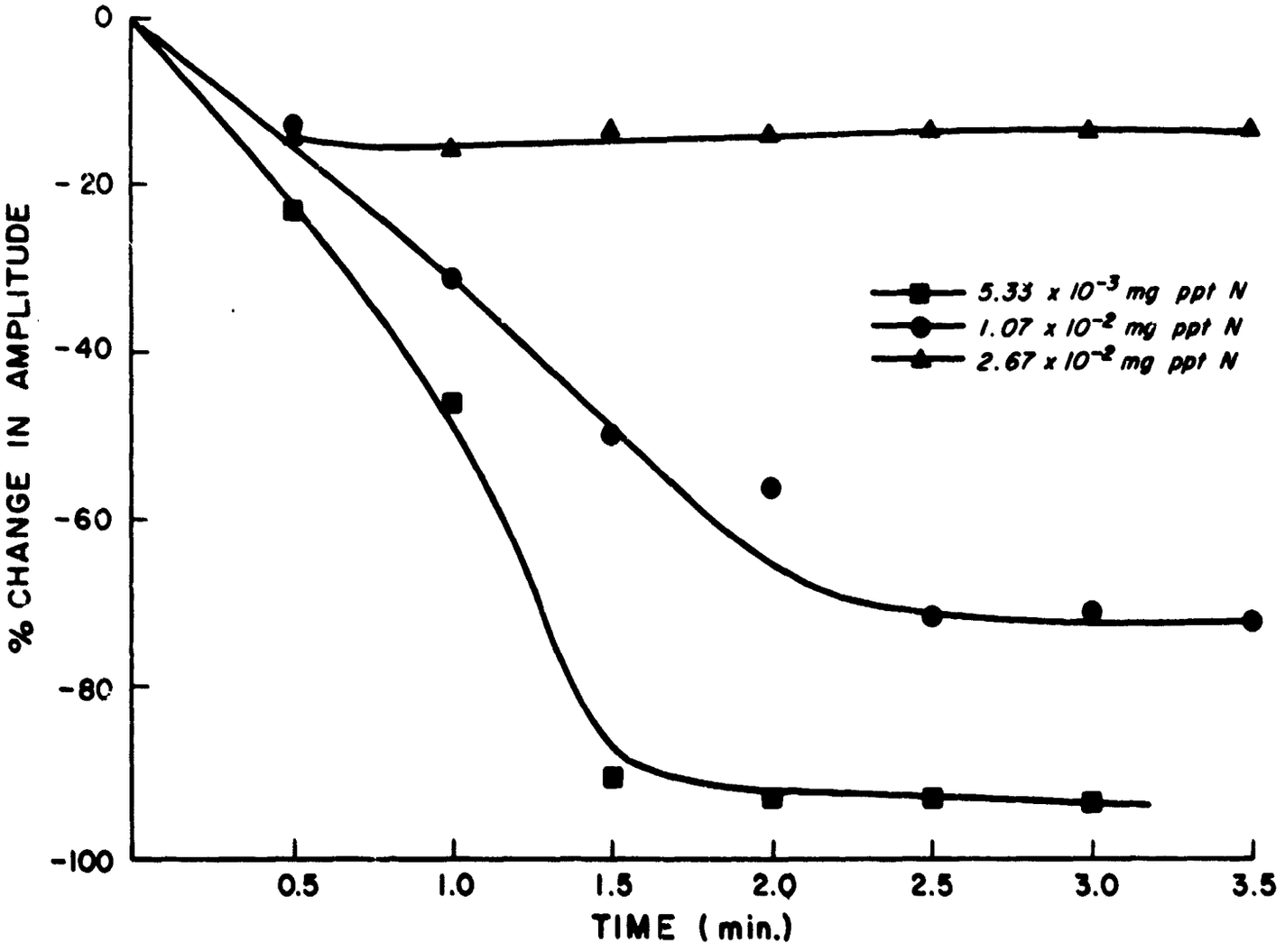


Figure 3. Per Cent Change in Amplitude and Contraction of Guinea Pig Heart (*in vivo*) Challenged with *A. planoi* Toxin (Crude).

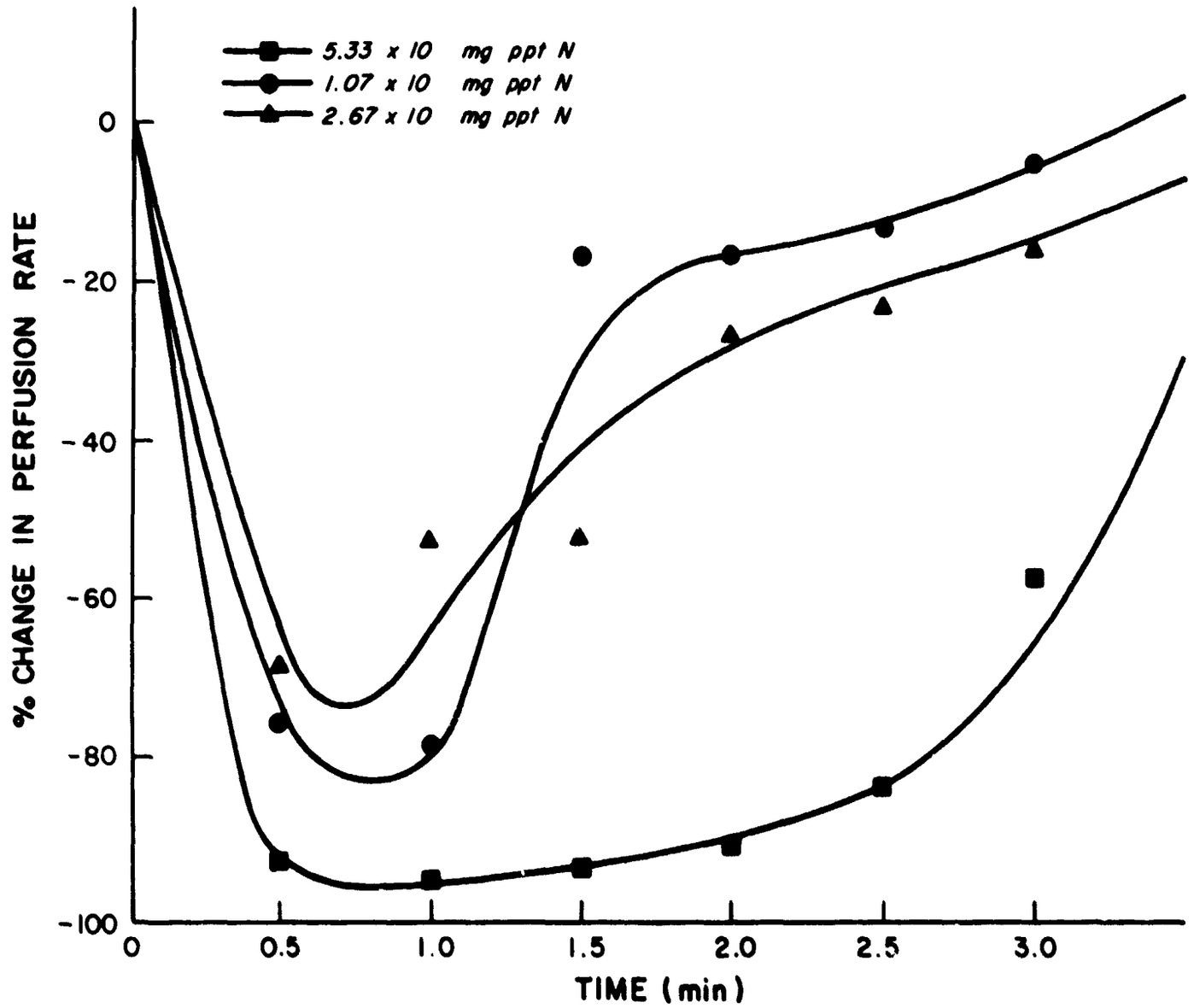


Figure 4. Per Cent Change in Perfusion Rate of Guinea Pig Heart *(in vitro)* Challenged with *A. planoi* Toxin (crude).

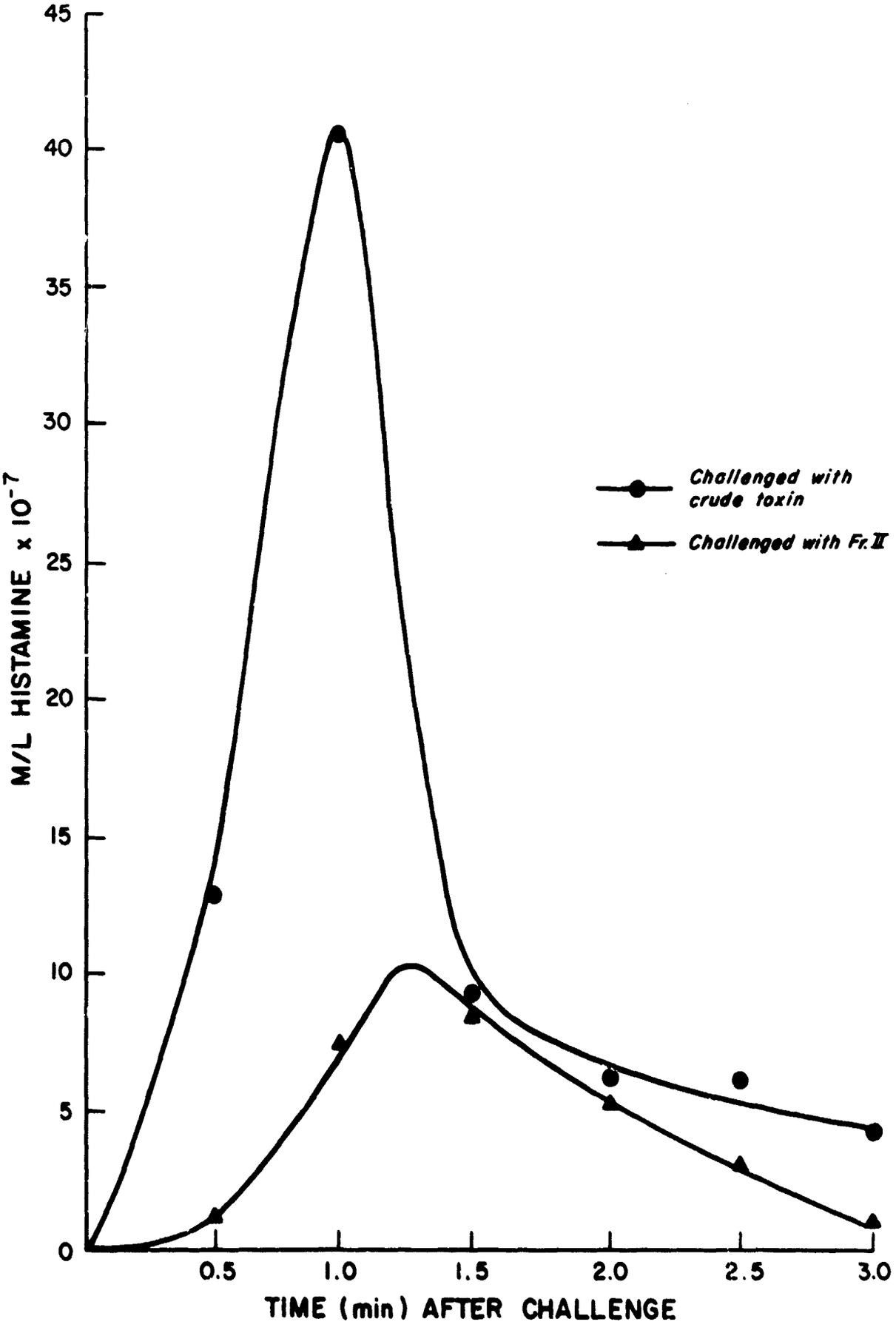


Figure 5. Time-Course of Histamine Release in Sensitized Hearts.

II. Studies on Anaphylaxis to Penicillin

Acute reactions to penicillin in man can proceed to cardiovascular collapse and cardiac standstill. Whether or not the heart itself is a primary target organ in the clinical syndrome is unsettled: that it can be a primary target organ in the guinea pig is borne out by the studies of passive transfer cited in our earlier progress report (July 1969).

In that report it was noted that sensitizing antibodies prepared against immunogens containing a penicilloyl hapten had a functional effect that distinguished them from rabbit antibodies prepared against the usual protein haptens. The functional difference involved an increase rather than a decrease in coronary flow rate which is part of the usual sequelae of cardiac anaphylaxis, suggesting that the active material released when the sensitized heart was challenged with penicillin haptens could not consist entirely of histamine.

In the present report we exhibit certain features of anaphylaxis to penicillin: A) the demonstration of the influence of the thermolabile antibody on coronary flow and histamine release; and B) the reduction of the kininogen level in human anaphylaxis.

A. Passive Anaphylaxis to Heated and Unheated Rabbit Antibodies to Penicillin

The process of immediate cardiac anaphylaxis is characterized by an increase in the rate and force of heart beat, A-V block, and the measurable release of histamine. In general the coronary flow is reduced and this reduction is also observed when the organ is tested with synthetic histamine in control experiments. In a previous report (July 1969) we have described instances of passive sensitization in which the coronary flow was increased rather than decreased. These occurred during experiments in which the hearts were passively sensitized with rabbit antibodies against a conjugated antigen consisting of penicilloyl-coupled rabbit serum albumin.

The present pilot experiments were made to determine whether these abberant effects were owed to the presence of an antibody sufficiently different in its functional properties from the usual γ_2-produced in rabbit in response to a protein antigen. One of the possibilities which might account for the specific case posed by the penicillin coupled antigen - is that it might have evoked the formation of heat labile antibodies of the reaginic type.

The aim of these studies was to determine the effects of heated and unheated penicillin antisera on the coronary flow and whether these changes were associated with alterations of the precipitation behavior and the release of histamine.

MATERIALS AND METHODS

Rabbit anti-[Pen-RSA] prepared as described in a previous report (July 1969) was heated to 56°C for five hours.

Guinea pig hearts were sensitized *in vitro* with 2 ml of unheated antiserum, and *in vivo* with unheated and heated antibody which contained 1.18 mg of Ab/ml. The hearts were challenged with 1.0 mg of Pen-RSA in the presence of semicarbazide (2 γ/ml). Mechanical responses were recorded by the usual methods and the histamine was assayed both chemically and biologically.

RESULTS

Quantitative Precipitin Titration:

Comparative titrations made with heated and unheated antisera are shown in Fig. 1. Although there is a 3% reduction in the precipitating antibody at optimal proportion after incubation at 56°C, the striking change is seen in the narrowing of the precipitation range suggesting an increase in the homogeneity of the system owing to the elimination of the heat labile antibody.

Anaphylactic Reactions:

The character of the coronary flow is exhibited for the several experiments in the appropriate group of curves in Fig. 2. The phasic reactions in flow are expressed as minima and maxima in Table I along with the corresponding histamine outputs.

1. Unheated Serum:

a. *In Vitro Sensitization*. Hearts sensitized *in vitro* reacted with an increased coronary flow which averaged about 40% above control. Histamine was detected only in one instance. (Table IA).

b. *In Vivo Sensitization*. Hearts prepared from animals which received 2.4 mg of Rabbit Anti-[Pen-RSA] reacted to the antigen with a distinct biphasic character of the coronary flow. There was first a decrease amounting (in rough average) to a 36% reduction followed by a 50% increase above control. Histamine was released in all cases, the average being 6.8×10^{-6} moles/g dry heart/min, (Table IB).

2. Heated Serum:

a. *In Vivo Sensitization*. The average reduction in the first phase and the average increase in the second were both about 1/3 lower than in the corresponding test with unheated serum, and the mean histamine output was reduced by 57% (Table IC).

CONCLUSIONS

Tentatively we conclude that the reduction in coronary flow in the first phase apparently is associated with the release of histamine. It is also evident that the inactivation of the thermolabile fraction is associated with the release of histamine. Since the character of the precipitin curve changes only slightly in terms of the maximal point but drastically in its range of heterogeneity one may conclude that the number of degrees of freedom for interaction are thereby reduced, thus a higher proportion of histamine releasing cells may be occupied by globulins having no functional capacity with respect to antigen in the case of the heated serum as compared to the unheated preparation.

Finally, the mode of sensitization appears to be a significant determinant of the character of the reaction. If the histamine release determines the phasic response of the coronary flow, it is evident that the intraperitoneal administration of the antibody involves additional factors in the final sensitization that either inhibit histamine release or influence its preferential fixation to cells which elaborate coronary dilators rather than histamine.

TABLE I

Anaphylactic Reactions in Guinea Pig Hearts Sensitized with Unheated and Heated Rabbit Anti-[Pen-RSA]

Sensitization	Tissue	Δ Coronary Flow with Respect to control = 0 Min.	Max.	Histamine Release Moles H/g dry Heart/min	Mean Change Due To Treatment
A. Unheated Serum [*in vitro*]	1	- 4	+ 46	$<1.48 \times 10^{-7}$	
1.18 mg Ab/ml - 1 ml	2	- 6	+ 47	$<1.48 \times 10^{-7}$	
cycled 4X	3	- 4	+ 32	1.48×10^{-7}	
	Mean	- 4.6	+ 41		
B. Unheated Serum [*in vivo*]	1	-39	+ 30	7.89×10^{-6}	
2 x 1 x 1.18 mg Ab ml	2	-57	- 4	5.29×10^{-6}	
Rabbit Anti [Pen-RSA]* for 30 hours	3	-12	+124	6.76×10^{-6}	
	Mean	-36	+ 50	6.76×10^{-6}	
C. Heated Serum [*in vivo*]	1	-28	+ 32	2.80×10^{-6}	
2 ml x 1.04 mg Ab	2	- 4	+ 62	3.35×10^{-6}	
Rabbit Anti [Pen-RSA]* for 30 hours ip	3	-39	- 3	2.56×10^{-6}	
	Mean	-24	+ 33	2.89×10^{-6}	[B-C] -57.3%

* Anti-[Pen-RSA] Rabbit Antiserum raised against penicilloyl-rabbit serum albumin

Heated Serum incubated at 56°C for 5 hours

B. Bradykininogen Levels in Man Subsequent to Acute Penicillin Anaphylaxis

A white male patient, aged 53, admitted for chronic alcoholism, developed an upper respiratory infection which, on further examination, was diagnosed as pneumonia. Penicillin therapy was recommended and although he had received "numerous doses of penicillin in the past" the patient was reported to be non-allergic to this antibiotic. He received 2 million units intravenously of potassium penicillin G and was noted to be wheezy 5 minutes after the injection. A few seconds later he complained of profound weakness. His respiratory frequency dropped to 2 - 5/minute and shortly afterward the heart beat was absent. The heart beat was restored after a sharp blow to the chest and after appropriate intravenous medication the blood pressure rose from <70 mm Hg to 140/80 and eventually was maintained at 120/80. Shortly after the acute episode the patient's face had a puffy appearance and the tongue and epiglottis, which had appeared normal at intubation, were markedly swollen. The patient was given erythromycin, appropriately sedated, and by the next morning evidently had recovered. Blood samples were received periodically in this laboratory for the estimation of bradykininogen levels in the plasma. The preshock sample had been permitted to clot and therefore could not be used for estimating the patient's kininogen level. For this purpose "normal" control plasmas were obtained from various members of this laboratory.

According to the time-course the patient's kininogen levels, shown in Table II, (estimated as bradykinin formed by the treatment of heated plasma with trypsin), reached their lowest point in the first sample obtained on the day following the acute episode and they returned, apparently, to a stable level, i.e. consistent with control normal samples, on the fifteenth day following shock.

Antibodies to penicillin were detected in tests with penicilloyl polylysine and penicilloyl-Rabbit γ-globulin in the preshock serum. None was detectable on the first post-shock day, but positive reactions were observed on the 11th and 13th day after the episode. No serological reactions appeared after the periods noted.

TABLE II

Variation of Bradykininogen Levels Following Acute Anaphylaxis to Penicillin In Man

(Trypsin method of Diniz and Carvalho, N.Y. Acad. Sci. 104:77, 1963)

Sample #	Date	Test dose of 1/20 dilution (ml)	Reaction (mm)	Total BKE Found in Sample	BKE/ml Bath	γBK/ml Plasma *	Ring Test † Pen-poly lysine	Ring Test †† Pen-R γ-glob.
0	Pre-shock**	0.5	24	0.06	0.015	0.6	±	±
	Shock 3/4/70							
1	Post-shock 3/5	0.5	22	0.054	0.013	0.52	0	0
2	Post-shock 3/13	0.6	28.5	0.083	0.021	0.7	++	++
3	Post-shock 3/17	0.6	23	0.057	0.014	0.466	±	+
4	Post-shock 3/17	0.6	25	0.08	0.02	0.666	±	0
5	Post-shock 3/19 (glass) ⁴	0.25	26	0.083	0.021	1.68	0	0
6	Post-shock 3/23 (glass)	0.6	33.5	0.20	0.05	1.67	0	0
7	Post-shock 3/26	0.3	29	0.10	0.025	1.666	0	0
8	Post-shock 3/30	0.3	11	0.063	0.016	1.333	0	0
9	Post-shock 6/23	0.2	20	0.065	0.163	1.63	good	Faint
Normal Control								
GAF	6/10/70	0.3	11	0.063	0.016	1.065		
LBS	6/10/70	0.3	16	0.08	0.02	1.332		
MC	6/10/70	0.5	26	0.12	0.03	2.00		

* Bradykinin found by bioassay x dose in bath x dilution of original plasma sample

** Serum sample

† Penicilloyl polylysine-[Cilligen] Sigma 6×10^{-7} M

†† Pen Rγ-Globulin (Prep. 52-81) 0.5 mg/ml

⁴ Stored in glass bottles by clinic

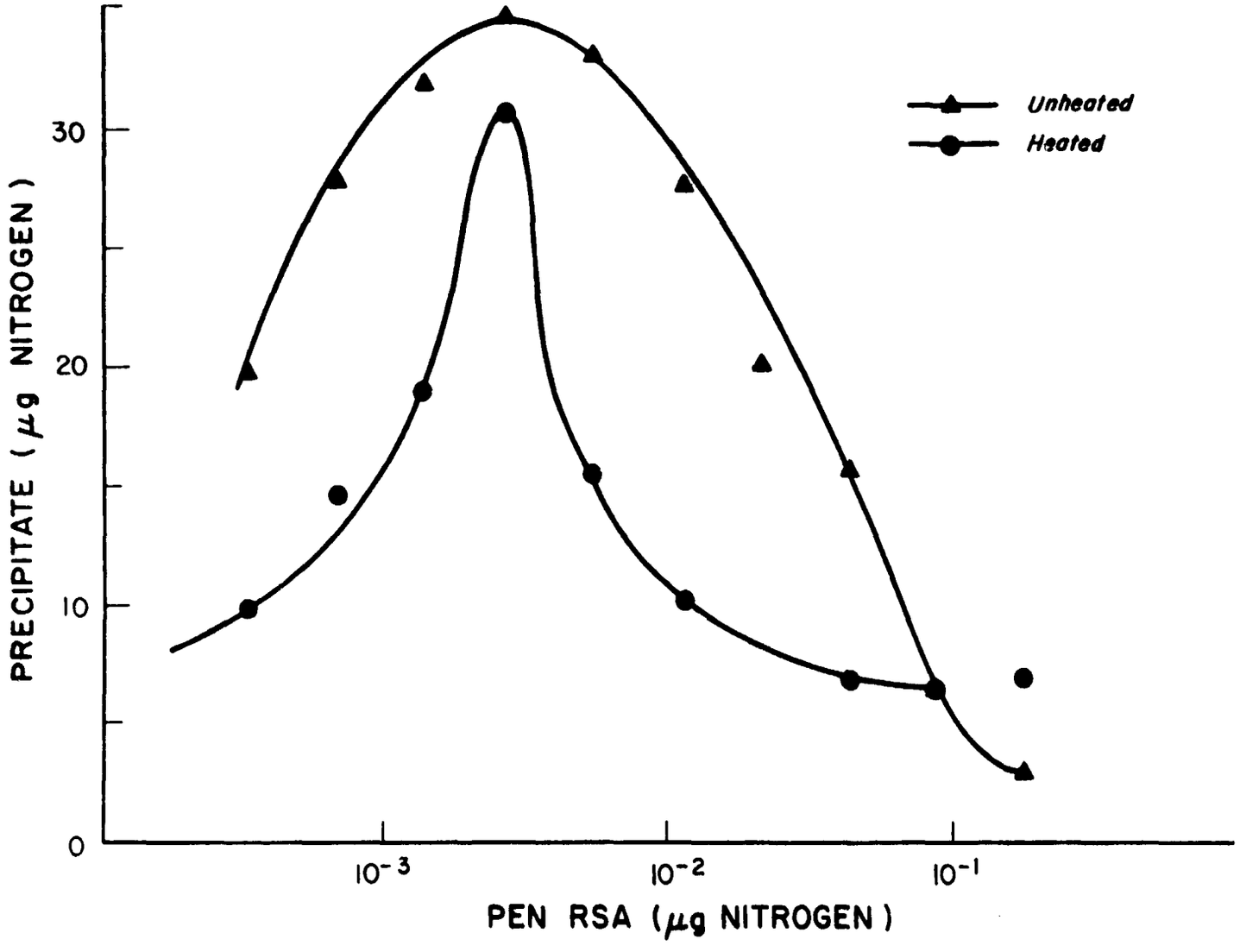
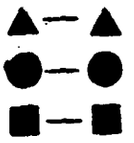


Figure 1. Titration of Rabbit Serum Anti Pen-RSA

Figure 2. Effect of Heating Antibody and Mode of Passive Transfer on Coronary Responses of Penicillin Sensitive Hearts.

A. Hearts passively sensitized *in vitro* with 1.18 mg unheated Anti-[Pen-RSA].

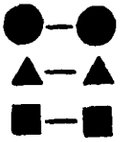


Heart #1
Heart #2
Heart #3

B. Hearts passively sensitized *in vivo* with 2.36 mg of unheated Anti-[Pen-RSA].

Heart #1
Heart #2
Heart #3

C. Hearts passively sensitized *in vivo* with 2.0 mg heated Anti-[Pen-RSA].



Heart #1
Heart #2
Heart #3

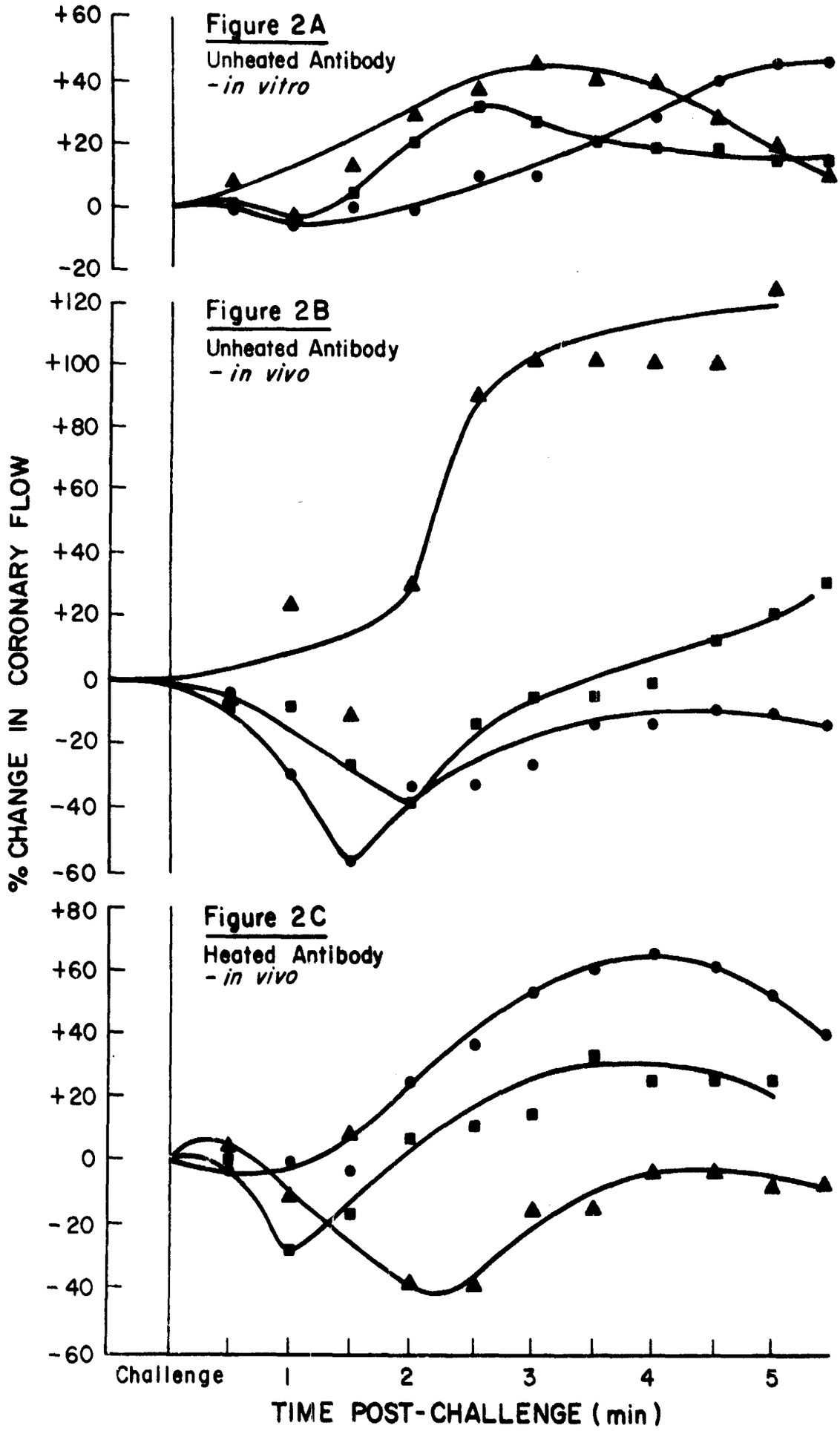


Figure 2A
Unheated Antibody
- in vitro
Figure 2B
Unheated Antibody
- in vivo
Figure 2C
Heated Antibody
- in vivo
+60
+40
+20
0
-20
+120
+100
+80
+60
+40
+20
0
-20
-40
-60
+80
+60
+40
+20
0
-20
-40
-60
% CHANGE IN CORONARY FLOW
Challenge
1
2
3
4
5
TIME POST-CHALLENGE (min)

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13. ABSTRACT

The present studies of the biochemical, immunochemical, and toxic properties of substances of biological origin fall into two general categories: (1) studies of the mode of action of bacterial and animal toxins on the isolated tissues of normal animals and (2) studies of these systems as complicated by the presence of specific antibodies on target cells, which have the effect of producing anaphylaxis concomitantly with the toxic reaction.

Before proceeding with studies of the mode of action and immunology of the several antigens it was necessary to purify and characterize them. Accordingly, the results of purification studies are presented for tetanus toxin, sea urchin toxin, and starfish (Crown-of-Thorns) starfish.

The bulk of the progress in the analysis of the double insult problem (2) has been achieved in studies of cardiac, particularly atrial, anaphylaxis to the active and inactive forms of streptolysin O (SLO) as the toxin used was highly purified, containing little or no contaminating DPNase. The physiological analysis of the mode of action of the active toxin upon the isolated heart (a) and the concomitant release of acetylcholine and histamine by the sensitized heart (b) in response to challenge with the active agent had shown that the toxic and anaphylactic moieties of the SLO molecule are independently manipulated and that they activate quite distinct but quite specific mechanisms in the heart.

In order to generalize the problem several other kinds of antigenic toxins were studied. Two of these were echinoderm toxins -- highly antigenic to mammals and known to have a direct toxic action on the heart in their active state. These were the toxins of the

sea urchin, *Tripneustes gratilla* and the starfish, *Acanthaster planci*. The third toxin, tetanus, has no direct effect on the heart, but is a classical antigenic material to which antibodies are widely distributed in the population, owing to large scale prophylactic immunization. All of these toxins in their active and inactive forms produced anaphylaxis in the passively sensitized heart. The activation of the bradykininogen system appears to play a significant role in the course of anaphylaxis to penicillin, and may involve an antibody response somewhat different from that evoked by the classical protein antigens. Evidence for this is presented in the atypical coronary response seen in experimental cardiac anaphylaxis to penicillin conjugates of rabbit serum albumin in Section II. This section, appropriately, contains an account of the variations in bradykininogen levels occurring as a consequence of acute penicillin anaphylaxis in man.

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Security Classification

KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Anaphylaxis to toxins						
Bacterial						
Streptolysin						
Tetanus						
Animal Toxins						
Invertebrate Toxins						
Echinoderm Toxins						
Crown of Thorns Starfish						
Sea Urchin						
Plasma Kinins						
Kinetics of formation and destruction levels in Human Anaphylaxis to Penicillin						