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

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I. SUMMARY PROGRESS REPORT
1966-1969

II. ANNUAL INTERIM PROGRESS REPORT

"Physical-chemical, Immunological, and Enzymatic Properties of Purified Fractions of Sea Urchin Toxin"

By

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PREAMBLE

The mission of this laboratory since 1959 has been to present a unified and consistent account of the problem of *in vitro* anaphylaxis under such conditions as would assure control over (1) the nature and quantity of antibody bound to the tissue and, (2) over the nature and purity of the antigen used for challenge. We have approached this domain by determining the relationship of the physical-chemical, enzymatic, and immunological properties of the materials we have isolated to changes produced in the electrophysiological, pharmacological, and anaphylactic responses of organs and tissues.

During the current contract period, i.e. 1966-1969 we have broadened the sophistication of our approach by the use of antigens having functional properties over and above simple antigenicity. These antigens are purified bacterial toxins and animal venoms. The materials selected for study were chosen because they were suitable as biochemical probes for various levels of attack on tissues, because they could simultaneously function as antigens, and because they represented significant models of clinical disorders in which natural toxicity could be compounded by immediate sensitivity.

Within each category, as the problems have required, we have undertaken ancillary investigations to answer details about specific processes, about reactants which were used as biochemical probes, and about methods for quantitative estimation of reaction products.

At the present time we are concerned with three antigenic toxins which produce qualitatively distinct functional effects, at different molecular levels, and in different anatomical regions. These are streptolysin, tetanus, and sea urchin toxin.

Streptolysin was selected because it can simultaneously produce toxic and anaphylactic reactions in the heart, which are easily resolvable by pharmacological agents.

Tetanus toxin was studied because it contains at least two molecular species, each of which has a high degree of selectivity for a restricted functional receptor, one in the central inhibitory system and the other in the peripheral neuromuscular apparatus.

The third, sea urchin toxin, was chosen because it represents a relatively simple prototype of a more complex antigenic animal venom which contains all of the cardinal properties of more sophisticated animal venom complexes.

Full details on the status of work done on the three toxins are available in the reprints, manuscripts, and reports submitted with this application. The principal results of these investigations are summarized below.

SUMMARY OF PROGRESS 1966 - 1969

A. *STREPTOLYSIN O*

1. Direct Cardiotoxicity

The purpose of these studies was to determine the site and mode of action of streptolysin O by an analysis of the electrophysiological, pharmacological, and mechanical changes occurring in the isolated whole heart and separate cardiac tissues of the guinea pig pursuant to their challenge with streptolysin O.

The response of the perfused guinea pig heart to a large dose of purified streptolysin O consists of two distinct phases. A rapid, but transient, depression in rate and amplitude of contraction is superimposed on a gradual, irreversible, decline in ventricular function. At ventricular standstill, the atria beat normally, as do electrically driven ventricle strips prepared from the stricken heart. Spontaneously beating, isolated, atria respond to the toxin with a completely reversible, dose-dependent, reduction in rate and amplitude of contraction; this response shows exponential tachyphylaxis to repeated doses of toxin. The principal and characteristic electrophysiological finding in these atria is an acceleration in the rate of repolarization of the intracellular potential.

These transient atrial changes, as well as the reversible phase of the response of the whole heart, are blocked by atropine and potentiated by eserine. Acetylcholine can be detected in perfusates obtained from atria challenged with streptolysin O. On the other hand, isolated ventricle strips, prepared from normal hearts, are functionally and electrophysiologically insensitive to the toxin. It is concluded from these findings that, (i), the reversible phase of the response of the isolated heart is atrial in origin and can be accounted for by the release of acetylcholine from that tissue and that, (ii), the irreversible ventricular decline results not from damage to the contractile mechanism of the ventricle, but from a toxin-induced defect in one or more components of the conduction system.

The observed toxic action is limited to the reduced streptolysin O. The toxicity can be eliminated by (a) oxidation, (b) heat inactivation, (c) neutralization with specific antiserum, and (d) treatment with cholesterol.

2. Anaphylactic Components in the Cardiotoxicity of Streptolysin O

The main thesis underlying the experimental studies which form the basis of this study is that the cardiotoxic action of streptolysin O may be aggravated by the presence of specific antibodies in the heart. The previous work had established the nature and site of the defect in the normal heart insulted with active toxin. This showed that the early, reversible, physiological changes can be quantitatively accounted for by the release of acetylcholine, whereas the later irreversible changes, occurred because of a defect in the atrio-ventricular conduction system.

Based on the fact that the toxin liberates acetylcholine, *sui generis*, and that the anaphylactic reaction liberations histamine (1, 2) it was expected that challenge of sensitized hearts by active toxin should result in the production of both materials. In the experiments which were made it was established that the usual manifestations of cardiac anaphylaxis -- increased rate, amplitude, and histamine release -- obtain in actively and passively sensitized tissues when they are challenged with the *inactive* (oxidized) form of the toxin. On the other hand, when tissues sensitized in this way are challenged with the *active* (reduced) form of streptolysin O there is evidence that the response is biphasic; *i.e.* there appears first a reduction in force and rate on "normal" atria, which is then followed by the typical histamine-like response observed with the inactive toxin and other non-toxic protein antigens. By appropriate use of atropine and pyribenzamine it is shown that either, or both, of the responses could be selectively abolished.

B. *TETANUS*

1. Action

The lethal action of crystalline tetanus toxin results from a blockade between the specific interneurones of the spinal inhibitory pathway and the terminal motoneurones supplying skeletal muscles. This blockade leads to general extensor rigidity and, particularly, to the paralysis of the respiratory muscles. Work from this laboratory by Feigen, *et al.* has established that, in addition to this central action, crude tetanus toxin has a peripheral effect demonstrable as the increased rate of the random discharge of miniature end-plate potentials as recorded intracellularly from the intercostal muscles of the isolated hemithorax of the mouse.

The miniature end-plate potential (MEPP) is generated by a quantum of acetylcholine acting at the post-junctional connections of the skeletal muscle fiber. Therefore, the implication of our findings was that a factor (or factors) present in the crude tetanus toxin was capable of releasing acetylcholine from the presynaptic nerve terminals supplying the muscle. An analysis of this peripheral effect by Parsons, Hofmann, and Feigen, (3), showed that the increased MEPP frequency resulted from the depolarization of these presynaptic terminals and that the toxin tended to reduce the quantum content of the stimulus-evoked end-plate potentials.

2. Isolation

The centrally active material, tetanospasmin, is known to have a high degree of affinity for a crude suspension of brain known as, "Protagon", which contains a mixture of cerebrosides and gangliosides. Earlier work (4) showed that much of the lethal toxicity could be removed from crude tetanus toxin, without affecting its peripheral activity, by adsorbing the responsible molecular species on Protagon. The resulting electrophysiologically active, but tetanospasmin-poor, material was termed the "non-spasmogenic preparation", and the putative molecular species responsible for the peripheral effect was called the non-spasmogenic principle, NSP.

Since adsorption with Protagon does not destroy the central paralytic action of the responsible molecular species but merely removes these molecules from solution, it was reasonable to infer that the central and peripheral effects were due to separate components of the tetanus toxin and not to a single substance that originally possessed both properties. The aim of the present study was to test this hypothesis by showing that the molecular species responsible for each of the effects were indpendent and could be concentrated independently of each other. It had to be established that preparations having a high lethal potency had minimal peripheral action and, conversely, that materials concentrated for their peripheral effect had a low level of lethal toxicity.

Accordingly, two different kinds of fractionation procedures were employed. The lethal toxicity of the preparation was increased by purifying the tetanospasmin by repeated precipitation with methanol at low temperatures, according to the method of Pillemer *et al.* (5). The electrophysiological potency of the starting material was enriched by combining ammonium sulfate fractionation, to isolate the peripherally active component, with adsorption on cerebroside-ganglioside complex, to reduce the residual lethal activity of this fraction.

Our studies have shown that these two major isolation schemes, and their subsequent modifications, produced independent enrichment of the two biological activities and that the materials isolated by the respective fractionation procedures were no only antigenically and immunoelectrophoretically independent of each other, but also that they differed in respect to their ultracentrifugal and chromatographic behavior.

3. Characterization of the Non-spasmogenic Principle

The non-spasmogenic principle had been isolated in small quantities as a single antigen, and sufficient material was available to carry out determinations of molecular weight and a tentative amino acid analysis. This year we concentrated our efforts at improving the yield so that sufficient material could be available for physical-chemical and biochemical characterization. Ultracentrifugal analyses of the best fraction available last year showed the lethal fraction to have a Svedberg coefficient of 7 whereas the most potent MEPP-fraction was associated with 4*S* particles. This year, further preparative chromatographic separations were made on the MEPP-rich starting material (Fraction IV). Chromatography on Sephadex G-200 gave 3 major peaks. The yields of repeated runs were separately pooled on the basis of elution diagrams and each pool was concentrated by pressure-dialysis. No appreciable MEPP activity was found in the first 2 peaks; it appeared to be concentrated in Peak III, which had no lethal activity. This peak had only one antigenic determinant and gave a Svedberg coefficient of 2; but all of the lethal toxicity of the parent was quantitatively recovered in Peaks I and II which contained 5*S* and 4*S* molecules, respectively. The MEPP material accounted for only 7% of the total protein of the starting material.

C. *SEA URCHIN TOXIN*

1. Collection, Processing, and Early Studies of Purification

Specimens of *Tripneustes gratilla* (Linnaeus) were obtained from the flats and slopes of a series of coral reefs in Kaneohe Bay and Waikiki, Oahu, Hawaii.

The heads of globiferous pedicellariae were removed from the animals immediately after each collecting trip. All animals were thoroughly cleaned with a strong sea water spray, the wash water passed through two successive stainless steel sieves with mesh openings of 1.95 mm (13 meshes/in), respectively, and the contents of the latter sieve transferred to a large volume of filtered sea water. The pedicellariae and debris were allowed to settle by gravity until detritus and other particulate matter were the only materials remaining suspended, and the latter were removed by decantation. This procedure was repeated several times until the rinse water was free of all suspended matter. Finally, the isolated heads were transferred to tared polyethylene bottles, the excess sea water removed after a period of settling, and the contents frozen and stored at -20°C.

The frozen mass of heads was homogenized with 4 vol of filtered sea water for 5 min in a Waring Blender, and the resulting homogenate centrifuged at 10,000 g for 15 min. After the supernatant had been removed the sediment was twice resuspended in cold sea water, homogenized, and centrifuged as above. The supernatants from the three extractions were combined and stored at 5°C, while the residue was dried to constant weight. The pooled supernatant was then dialyzed against distilled water for 18 hr at 5°C, lyophilized, and the dried material stored at -20°C for subsequent analysis.

The extracts were next fractionated by being brought sequentially to 0.33, 0.65, and 1.00 saturation with ammonium sulfate. The precipitates formed at each step were dissolved in water, dialyzed exhaustively against distilled water and then dried by lyophilization.

One of the active principles was shown to be a pH-stable, heat-labile protein, which was precipitated from the crude extract in the presence of 65 per cent saturated ammonium sulfate. The most active protein fraction showed a single 2.6*S* peak in the analytical ultracentrifuge. Preliminary studies on the physiological behavior of the active toxin suggested that the crude material had a rather broad range of action: it lysed the red cells of certain mammals and fish, it produced a precipitate drop in systemic arterial pressure of the anesthetized rabbit, and it elicited contractions of the isolated guinea pig intestine.

2. The Release of Histamine and Other Agents from Tissues

Direct tests with crude non-dialyzable preparations of sea urchin toxin as well as with the fractions prepared by ammonium sulfate precipitation induced prolonged contractions of the isolated guinea pig's ileum in proportion to the dose used. The response was not blocked with atropine, only partially blocked with *Pyribenzamine*, blocked with various degrees with D-bromolysergic acid and *Mellaril*, and completely blocked with phenylbutazone.

The dose-response curve, obtained by pooling the data over a range of concentrations, obeyed the usual logistic function, and this could be employed reliably to assess changes in potency resulting from various physical and chemical treatments. Gentle heating of toxin reduced the magnitude and the slope of the dose-response curve, showing that the incubation of toxin for a constant time was inversely dependent on its concentration; carbon treatment decreased the median effective dose but increased the heterogeneity of the response, and treatment with ammonium sulfate concentrated most of the activity of the starting material in the fraction precipitating in the presence of 65 per cent saturated salt solution. The toxin was shown to release dialysable pharmacologically active agents from ileal, pulmonary, and cardiac tissues of the guinea pig as well as from colonic and pulmonary preparations of the rat.

Preliminary pharmacological tests excluded acetylcholine but suggested that histamine, as well as several other substances, could be released by the reaction. Subsequent specific chemical analyses confirmed the presence of histamine.

The release of histamine by the isolated tissues was shown to be quantitatively dependent upon the toxin concentration and the temperature. Although none of the fractions was as powerful as the parent material, the preparation obtained in the presence of 65 per cent saturated ammonium sulfate was the most potent histamine releaser among the fractions, being only slightly less active than the starting material.

3. Enzymatic Action on Plasma Substrates

During the couse of the pharmacological experiments reported above it was observed that the LD_{50} in mice appeared to be lower than the median effective dose required for the direct stimulation of isolated tissues. A plausible reason for this amplification of toxicity appeared to be that the toxin might have a proteolytic activity which could result in the formation of plasma kinins from an attack on plasma proteins. Similar processes were known for the action of snake venom and for the enzymatic catalysis of serum proteins by kallikrein, known to be liberated in anaphylaxis.

The crude pedicellarial toxin of *Tripneustes gratilla* obtained from 1,000 specimens in 1966 and 2,000 specimens during 1967 was variously fractionated, and the fractions further purified by chromatography on Sephadex. The enzymological properties of these materials were studied with respect to toxin concentration, substrate concentration, and temperature. The kinetics of the reaction system with respect to whole plasma and to its pseudoglobulin fractions are of a complex order owing to (a) "natural" kinin formation of the substrate, (b) to the inactivation of the reaction product (and of synthetic bradykinin) by the toxin, and (c) to the existence of more than one enzyme in the "purified" material. Plots of first order velocity constants against the square of substrate concentration were linear suggesting the existence of substrate "modifiers". The dependence of reaction velocity on enzyme concentration was not of the classical type. The temperature optimum of the system was 26°C.

A study of the reactions of various Cohn fractions with more purified enzymes suggested that the substrate furnishing the kinin activity on guinea pig tissues was α_2-globulin and conclusive proof was obtained in studies with immunoelectrophoretically pure human α_2-macroglobulin. Parallel assays on guinea pig and rat tissues suggested that the substrate responsible for the oxytocic effect in the rat, on the other hand, is a β-globulin. Rabbit adjuvant, gave normal quantitative precipitin curves with "active" antigens (or toxoids) and they fixed complement. Immunoelectrophoresis showed 2 major antigens, the more immunogenic being concentrated in the 2/3 SAS fraction while the other was present among the 1/3 SAS materials. Biologically, the antisera showed quantitative antitoxic action in the usual protection and neutralization tests in whole animals as well as on isolated tissues. Toxin-induced inhibition of active Na transport in the toad bladder was also neutralized by the antitoxin.

4. Analysis of the Enzymatic Behavior of Purified Components

Operations during 1968-1969, reported in detail in the attached PROGRESS REPORT, were directed to the further purification and characterization of the proteolytic enzymes, to the elucidation of the point of attack of the enzymes on the natural (and synthetic) substrates, and to the isolation and the pharmacological characterization of the products formed in the reaction.

a. *Production, Isolation, and Pharmacological Properties of Reaction Products.*

Studies preliminary to the production of a large scale batch of active material showed that (a) the effective operating temperature range was 27°-30°C as at higher temperatures the destruction of the natural product, as well as that of synthetic bradykinin, was too great to insure an effective net yield; (b) the formation of active kinin from the attack of SUT on heat-treated plasma (HTP) was associated with a loss of TCA-precipitable nitrogen from the reaction mixture; (c) the inactivation of synthetic bradykinin by SUT could be partially blocked by the kininase inhibitor, Trasylol.

Several large batches were prepared by treating heated acidified human plasma, or α_2-globulin, with SUT or trypsin. The peptides produced by this digestion were recovered by precipitating the reaction mixture with ethanol, extracting the ethanolic residue with acidified *n*-butanol, and precipitating the butanolic extract with ether. Selected samples were purified by filtration on Sephadex G-25 and then chromatographed on paper, along with synthetic bradykinin. Paper chromatography gave 5 distinct spots, 3 of them having kinin activity. The products isolated on paper were tested for activity by the usual pharmacological procedures described in previous reports. The active product formed by the attack of trypsin on HTP was almost entirely bradykinin-like while that formed by SUT showed that another active peptide was present along with bradykinin.

b. *Studies of Competitive Reaction Kinetics Affecting Yield of Product.*

The rate of kinin formation, substrate digestion, and degradation of bradykinin were studied at 27°, 32°, and 37°C, at various toxin and substrate concentrations in order to evaluate the kinetics of formation of bradykinin-like material from the digestion of HTP. Since 27°C appeared to be near the optimal temperature for bradykinin formation, two adaptations of the Prado extraction procedure, as well as gel filtration on Sephadex G-25, were used to isolate dialyzable products from the digestion reaction at 27°C.

c. *Physical-Chemical, Immunological, and Enzymatic Properties of Purified Toxin Fractions.*

Active fractions were obtained by gel filtration on Sephadex or by chromatography on hydroxylapatite. The immunological relationships of the fractions prepared by the two methods were studied, their ultracentrifugal and analytical properties were determined, and their catalytic specificities were determined.

The substrate systems used for detection of proteolytic activity were azocasein, TAME, and ATEE. Initial studies showed that although the crude toxin attacked azocasein and ATEE, it had very little effect on TAME, suggesting that the major proteolytic action was chymotrypsin-like.

Further studies were then made with the purified fractions. None of these attacked TAME and only 1 Sephadex and 2 hydroxylapatite fractions attacked ATEE. The chymotrypsin activity appeared to be concentrated in Fraction II of the hydroxylapatite preparations: the K_m value was 1.33×10^{-2} in contrast to that of 4.1×10^{-3} for the crude starting material.

Although the greatest lethal potencies were found in Fr. II of HOA and Fr. II of Sephadex the latter was devoid of chymotrypsin specificity. We conclude from these results that the lethal toxicity is not clearly correlated with proteolytic action of this kind, which leads to the formation of bradykinin. Indeed, the intravenous injection of large doses of synthetic bradykinin fails to kill mice. It is unlikely to be due to hemolysis since that property is present quite potently in some of the non-lethal preparations.

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FUTURE PLANS

1. Introduction

Man's traditional concern with animal venoms and bacterial toxins has undergone a profound change over the past century from a practical study of measures to insure his own survival, to the use of venoms as highly specific physiological and biochemical probes. In certain instances animal venoms have played a significant role in the elucidation of critical steps in blood coagulation (1), and in the analysis of certain mechanisms of immediate hypersensitivity such as the discovery of slow-reacting substance by Kellaway and Trethewie (2), and the mode of formation of bradykinin by Rocha e Silva *et al.* (3).

In spite of their wide-spread use as specific reagents information about toxins and animal venoms remains largely fractional and a general pattern of design of venom complexes has emerged only in the case of the terrestrial snake. Although toxins and venoms of the same order of potency as those of snakes have been known to occur in other *phyla*, particularly among primitve marine forms, there has not appeared any systematic effort to study their ontogenetic development, phylogenetic evolution, or ecological significance from the biochemical point of view.

Most snake venoms contain ensembles of enzymes (as well as activators and inhibitors) of a sort which has led some students to conclude that their function subserved a digestive purpose. They have been found to contain phospholipase A, proteases, L-amino acid oxidase, cholinesterase, nucleases, phosphodiesterase, and monoesterase hyaluronidase digestion enzymes. Their action in very specific ways on certain cells such as erythrocytes, mast cells and platelets makes them highly useful as agents for the study of the process of release of vasoactive agents.

In an attempt to simplify the problem our aim has been to provide a detailed description of the chemistry and mode of action of a relatively simple venom-complex of a primitive marine echinoderm, the sea urchin *Tripneustes gratilla* (Linnaeus). The species of interest has a wide distribution in the littoral of the Indo-Pacific area. It has globiferous pedicellariae which contain a venom that is highly lethal when injected intravenously into the mammalian circulation, and stings received by humans can cause serious local effects which may be aggravated by generalized hypersensitivity in persons repeatedly exposed.

2. Rationale

In many respects, the actions we have discovered so far have shown us that sea urchin toxin may be only qualitatively, but not functionally, different from the venoms of higher forms. Like snake venoms, the crude poison of the sea urchin has a multiplicity of effects. It contains proteases and phospholipases as well as much simpler, dialyzable, substances; and, like snake venoms, this echinoderm toxin has hemolytic, histamine-releasing, and neurotoxic properties.

In neither case is the significance of these peripheral effects completely understood but it is generally conceded that neither hemolysis nor histamine release are the major causes of the lethal action, although the hypotension and increased peripheral vascular permeability can enhance the toxic actions of the other venom components. Although many snake venoms contain active neurotoxins which appear to be chiefly responsible for the lethal effects in mammals but, as we have seen, there seems to be no simple correspondence between the neurotoxic and the enzymatic activities.

Since most of the cardinal aspects of toxicity associated with the action of the more complex venoms are reproduced by the less sophisticated venom of the sea urchin we have at hand an extremely useful model for working out the relative importance of the various components to the characteristic envenomation syndrome since the numbers and enzyme specificities of these components are much more restricted in the sea urchin than in the snake.

The preparation and properties of crude sea urchin toxin and of certain fractions are described in the PROGRESS REPORT. The crude material contains a variety of enzymes which produce hemolysis and release vasoactive agents when applied to tissues. The nature of the release products has been described by Feigen *et al.* (4). One of the principal components that is responsible for the major portion of the intravenous toxicity is a trypsin-like material which produces vasoactive peptides when it attacks α_2-globulins. It appears in the second fraction eluted from Sephadex G-200 in phosphate buffered saline (pH 7) and has a sedimentation constant of 4.7. This material is antigenic and corresponds to the principal antigenic determinant of the parent material. Its direct action on the tissue is quantitatively neutralized by antibody made to the formalinized toxoid.

During the past 3 years we have shown that the pedicellarial venom is an ensemble of about 6-8 antigenically distinct protein components. Potent (lethal) preparations could be produced by fractional precipitation with ammonium sulfate, by gel filtration on Sephadex, and by chromatography on hydroxylapatite. A survey of the esteratic properties of the 3 Sephadex fractions and 6 Hydroxylapatite (HOA) preparations was made by determining whether they could break down azocasein and hydrolyze the synthetic substrates TAME and ATEE. None of the preparations attacked TAME and only 3 - Sephadex I and HOA I and II - attacked ATEE. The K_m values were Sephadex I, 2×10^{-3} *M*; HOA I, 3.04×10^{-3} *M*; and HOA II, 1.33×10^{-2} *M*.

Several large batches of reaction product were prepared by treating heated, acidified plasma with crude SUT. The peptides were recovered by extracting the dried ethanolic residues with acidified *n*-butanol and precipitating the butanolic extract with ether. Selected samples were further purified by gel filtration on Sephadex G-25 and then chromatographed on paper. Paper chromatography gave 5 distinct spots, 3 of them having kinin activity, and 1 of them corresponding to synthetic bradykinin. Neither the hemolytic activity nor the proteolytic action were clearly correlated with the lethal toxicity.

3. Outline of Research Plan

The aims of the present research are:

a. To isolate and characterize the protein constituents of bacterial toxins, and of sea urchin toxin.

b. To determine the point and method of attack of the various constituents.

c. To employ the various immunogenic toxin components in the analysis of an experimental model in which toxicity or envenomation is compounded by anaphylaxis.

d. To determine the immunologic kinships and differences that exist among these components and digestive enzymes.

(i) Within the species, and

(ii) Between homologous components in different species.

The contemplated studies will be made according to the following general plan:

I. Analysis of the Mode of Action of Purified Components of a Primitive Animal Venom

A. Isolation and characterization of protein constituents
1. Extraction and purification
2. Physical-chemical characterization

B. Pharmacological Properties
1. Lethal toxicity
2. Detection and estimation of pharmacological agents

C. Physiological Measurements
1. Mechanical response
2. Electrophysiological properties

D. Immunological Factors
1. Immunization
2. Isolation and characterization of antibodies
3. Immunochemical determinations
4. Anaphylaxis

D. Biochemical Studies
1. Detection and characterization of enzymes in known isolates
2. Hematological problems

II. Anaphylactic Components in the Cardiac Reaction to Active Bacterial Toxins and Animal Venoms

A. Direct action of toxins on the normal heart.

B. Studies on the preparation and modes of biochemical action of toxin.

C. Effect of antigens on kinetics and characteristics of various antibodies formed.

D. Action of toxins on sensitized hearts.

E. Action of antibodies on heart-fixed toxins.

4. Applications of Research

These studies can furnish information on:

a. The general problem of envenomation mechanisms

b. Evolutionary significance of venoms in relation to enzymology

c. Anaphylaxis

a. Envenomation:

The first point has been discussed at length in the Introduction.

b. Evolutionary Significance of Venoms in Relation to Enzymology

The study of venoms and toxin has been profitable in the wealth of information directly applicable to studies of enzyme action, the process of neuronal conduction and impulse transmission, blood coagulation, and the process of red cell lysis. The discovery, for example, of the widespread occurrence of bradykinin structures in the toxins of molluscs, wasps, hornets, and certain amphibians is compelling evidence for the eventual significance of the occurrence of this vasoactive substance as a byproduct of various primitive defense mechanisms, such as anaphylaxis, in man. This is further supported by the findings that kinins found in marine toxins often occur in association with other physiological mediators such as histamine, acetylcholine, and serotonin which have an almost universal distribution in the animal and plant kingdom.

Studies of active peptides produced by lower forms and those produced in man by the action of his own enzymes or by the enzymes in venom complexes of lower animals, is very often revealing of the means by which neurohormones or digestive hormones, such as gastrin, may be formed in the mammal.

The applications to the specific problem of anaphylaxis are inescapable.

c. *Mechanism of anaphylaxis*

As a class, proteins of marine animals are known to be potent allergens for mammals. Indeed, the recognition of anaphylaxis as a distinct immunopathologic entity came about, historically, through the efforts of Portier and Richet (5) to produce protective antisera against the toxins of *actinae*. For many years attention was given the principal physiological signal of the anaphylactic reaction: the specific release of histamine.

With the development of more critical pharmacological techniques for assay and of highly sensitive chemical methods evidence has been mounting in this and other laboratories for many years that many humoral agents besides histamine are produced in anaphylaxis. One of the central questions in this field has been the source of the cell and the mode of humoral release.

Although crude venoms and generalized anaphylactic reactions of isolated tissues often liberate the same array of humoral substances from affected tissues it is not known to what extent the two processes contain common elements in the reaction sequence, nor is it known, in many instances whether the mediators are liberated from the same population of cells.

Recent work in immunopharmacology has pointed to the high degree of reagent selectivity in the release of humoral agents. Evidence from Austen's laboratory (6) has established that the interaction of antigens with physicochemically distinct immunoglobulins results in the rapid output of distinctly different substances through the selective attack on different classes of cells and, conversely, that several entirely independent immunological mechanisms can lead to the release of the same mediators from a single type of cell (the rat's peritoneal mast cell), some of these being complement-independent, while others being dependent on various factors in the complement system.

Thus, it was found that while mast cells were involved in the liberation of histamine and serotonin but that only the polymorphonuclear leukocytes liberated slow-reacting substance. The selection of the cell type was determined by the class of antibody, SRS-A release being mediated by hyperimmune (IgA) antibody while the release of histamine and serotonin was associated with other antibodies migrating with the IgG group.

If the character of the antibody -- its electrophoretic class, for example -- determines the selection of the target cell it is at once evident that the character of the anaphylactic response can be controlled by the sensitizing antigen and the route and method of immunization.

It is clear that studies of the selectivity of the anaphylactic release of humoral agents can be materially improved with the availability of highly selective agents which can function both as enzymatic probes as well as antigens. Protein toxins are excellent examples of such "double agents". If they are selected for a highly specific mode of action it becomes possible to probe for either the "toxic" or the anaphylactic effect, independently or (simultaneously) from a given tissue -- or cell type -- by the use of the same pair of reagents.

The power of this technique can be illustrated by the ramifications in which it already has been applied and is submitted as a manuscript (see Appendix A in Supporting Data). The bacterial toxin, Streptlysin O, elaborates acetylcholine from unsensitized tissues. Tissues sensitized with anti-streptlysin O liberate only histamine when tested with inactive toxin, but liberate both histamine and acetylcholine when tested with active toxin. Each release can be independently exhibited by blocking the other, or both can be blocked by the simultaneous presence of pyribenzamine and atropine.

We see at once that the two agents cannot be produced by different cells, that acetylcholine is not produced as a typical feature of anaphylactic reaction in this case, that histamine is not produced by the active (toxic) group but arises as the result of the combination of the non-toxic moiety with the antibody and that cytotropic antibodies do not protect ACh-releasing cells against the action of the catalytic center of the molecule. Since the toxin is active only in the reduced form it is also evident that it cannot draw sustenance for its reduction from the by-products of the anaphylactic reaction which occurs when the system is challenged with oxidized (inactive) toxin.

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DETAILED EXPERIMENTAL RESEARCH PLANS

The program contemplated during the next period is a continuation of our general attack on the problem of *in vitro* anaphylaxis. The emphasis during the next contract period will be focussed on two principal aspects of our general interest. I. The analysis of the mode of action of purified components of a simple but representative animal venom -- sea urchin toxin -- and, II. A study of the anaphylactic components in the tissue (cardiac) reaction to active bacterial toxins and animal venoms.

PART I. An Analysis of The Mode of Action of Purified Components of a Primitive Animal Venom.

A. *CHEMICAL*

1. Extraction and Purification of Sea Urchin Toxin.

All animals are thoroughly cleaned with a strong sea water spray, the wash water passed through two successive stainless steel sieves with different mesh openings, and the contents of the second sieve transferred to a large volume of filtered sea water. The pedicellariae and debris are allowed to settle by gravity until detritus and other particulate matter remain suspended. This material is removed by decantation. This procedure is repeated several times until the rinse water is free of all suspended matter. Finally, the isolated heads are transferred to tared polyethylene bottles, the excess sea water removed after a period of settling, and the contents frozen and stored at -20°C.

The frozen material is homogenized with 4 volumes of distilled water for 5 min in a Waring Blendor and the homogenate is centrifuged at 10,000 g. The supernate is removed and the sediment is re-extracted in this way two additional times.

The proteins in the extracts are then brought down in the presence of saturated ammonium sulfate. The precipitate is separated by centrifugation, dialyzed against 1/60 *M* phosphate buffer at pH 7.2, and concentrated by pervaporation to a concentration of about 0.5 mg N/ml. Sufficient NaCl is then added to bring the final salt concentration to 0.3 *M*. The crude toxin solutions are then distributed in 50 ml batches into plastic bottles and stored in the deep freeze. These preparations are referred to as the *Starting Material*.

The next step in the purification is accomplished by gel filtration on Sephadex, followed by separation on hydroxylapatite gel. The Sephadex treatment provides a ready means of collecting all of the material having a high lethal potency into Fraction II. The starting material is applied to a Sephadex G-200 column in the cold room and continuosly eluted with 0.25 *M* NaCl. The first fraction is discarded and the second fraction concentrated by pressure dialysis and pooled with similar material obtained in repeated runs.

Further purification is made on hydroxylapatite. Fraction II of Sephadex (or other fractions as necessary) is applied to an hydroxylapatite column equilibrated with phosphate buffer at an ionic strength of about 0.001 μ. The column is developed by successive elutions of phosphate buffer of increasing ionic strength. Materials obtained on repeated chromatography are pooled as to the identity of the fraction, concentrated by pressure dialysis.

Further purification steps will be undertaken depending on the outcome of the various tests made to characterize the materials. Thus, depending on the results obtained by immunoelectrophoresis and Ouchterlony immunodiffusion, it will be determined whether subsequent purifications should be made by electrophoretic or electrochromatographic methods; or by techniques involving the use of charged absorbents such as carboxymethylcellulose, DEAE Sephadex, or Amberlite IRC 50. The method of choice for the identification of enzymes and toxins from snake venoms, for example, has been electrophoresis on starch gel, according to the method of Smithies (1).

Starch gel electrophoresis provides for resolution of components in small quantities, but as very small amounts are required for the various functional tests, active fractions can be identified immunologically and plans for large scale production of a given fraction -- by chromatography, for example, can be decided upon from the results obtained by electrophoresis and immunodiffusion.

2. Physical-Chemical Characterization

a) Protein: The protein content of toxin preparations obtained in preliminary experiments is determined by the biuret reaction as standardized by Gornall *et al.* (2). A crystalline bovine serum albumin (Armour and Company) is used as a reference protein. Estimation of protein nitrogen contents of toxin preparations obtained in later experiments is based upon tungstate precipitates by the micro-modification of the Kjeldahl method of Lanni *et al.* (3) and Feigen *et al.* (4).

b) Light Absorption: Absorbance determinations of toxin preparations are routinely made at a wavelength of 278 mμ in a Beckman DU spectrophotometer, while toxin solutions were analyzed in a B and L Spectronic 505 spectrophotometer in a wavelength range of 200 mμ to 650 mμ.

c) Ultracentrifugation: The sedimentation behavior of toxin solutions is determined on a model E analytical ultracentrifuge (Spinco). All measurements are made at 20°C in a centrifugal field of 201,400 g.

Crude sea urchin toxin, SUT(67), is subjected to electrophoresis on polyacrylamide gel by the procedure of Ornstein and Davis. Toxin samples were prepared in an acrylamide reagent solution (3% acrylamide, pH = 6.7) and this solution photopolymerized in small

diameter glass tubes (0.635 cm ID x 7.62 cm), each containing recently polymerized "spacer" and "separator" gels (3% acrylamide, pH = 6.7 and 7.5% acrylamide, pH = 8.9, respectively). The total quantity of protein does not exceed 200 μg/gel.

As these are required, pending the outcome of separation procedures, materials of interest will be further characterized for solubility profiles according to the usual phase-rule solubility tests, for intrinsic viscosity by extrapolating specific viscosities to infinite dilution, and osmotic pressure. Molecular weights obtained by these methods will be compared to those calculated from the results obtained from equilibrium, ultracentrifugation, and amino acid analyses which will be run to obtain a value for the partial molar volume. Further details of the application of these techniques to the determination of molecular weight of tetanus toxin have been published by Tomita and Feigen (5).

B. *PHARMACOLOGICAL*

1. Lethal Toxicity

The lethal toxicity is determined by the dose-mortality functions in groups of mice injected intravenously with the toxin preparation. The LD_{50} values are derived from these data by the von Krogh transformation described in our previous work (6), and the specific biological potency is expressed as the ratio LD_{50}/mg N.

2. Detection and Estimation of Pharmacological Agents

It is intended to detect and to estimate several vasoactive agents under appropriate circumstances and as the experimental design requires. These are: histamine, serotonin, acetylcholine, bradykinin, SRS-A.

In selected cases, where methods are available, histamine and serotonin will be estimated chemically. Once the distribution and conditions for identification (or exclusion by blocking agents) of the vasoactive agent are available, quantitative studies will be made by bioassay, particularly if the number of samples is excessive.

a) Preparations of Tissues for Bioassay

Isolated tissues for both the direct pharmacological work and for the production of physiologically active materials is obtained from male guinea pigs, ranging in weight between 300 and 500 g, and from female rats

weighing between 150 and 200 g. All animals are first injected with sodium heparin (1 mg per 100 g) and killed 15 min later by a blow to the base of the skull.

i) *Guinea Pig Ileum:* Ileal tissue is obtained from guinea pigs by gently freeing it from the peritoneum and delivery it into a beaker of oxygenated Tyrode's solution at 37°C. When the entire length of gut has been transferred, it is tied off at the oral and cecal ends, severed, and stored for 1 hr at 5°C in oxygenated solution. It is then gently warmed to 37°C after which the luminal contents were thoroughly flushed out. One-third of the distal portion of the gut is cut into 2-cm portions, each of which was secured to a glass L-rod and mounted in a water-jacketed 4 ml muscle bath in such a way that the free end of the gut strip could be connected to the lever arm of a Statham (G7A) strain gauge. The electro-mechanical signals generated by the "isometric" contractions were transduced and recorded through the electro-optical system of an oscillograph.

ii) *Rat Colon and Duodenum:* These tissues are obtained from rats and treated in essentially the same manner as described for the guinea pig intestine with the exception that de Jalon's solution at 21°C is substituted for Tyrode's at 37°C.

iii) *Uterus:* Uterine tissue is obtained from rats primed with estradiol 24 hours before the test. The preparation is maintained in de Jalon's medium.

iv) *Stomach:* Preparations of the fundus are used for the bioassay of serotonin.

v) *Cardiac Tissues:*

a. *Whole Hearts:* All cardiac tissues are prepared according to a common general procedure. Each animal is primed with an intraperitoneal dose (5 mg/kg) of sodium heparin, and killed 10 minutes later by a blow to the base of the skull. The thoracic cage is opened and a cannula inserted into the ascending aorta. The cannulated heart is then excised, connected to the standpipe of a perfusion apparatus*, and perfused at 37°C with oxygenated Chenoweth's solution at a pressure head of 40 cm H_2O. After being thoroughly equilibrated and trimmed of extraneous tissue, such hearts are either left in place for studies involving the intact whole organ, or removed and appropriately dissected for experiments on the separate cardiac tissues.

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* Anderson Heart Perfusion Apparatus

b. *Separated Cardiac Tissues:* In experiments dealing with the mechanical and electrical responses of isolated atrial pairs, the heart is removed from the perfusion system and the atria dissected as a single tissue from the ventricles. For the studies requiring individual left or right atria, the atrial pair is surgically separated so that the sino-atrial node remains intact in the right atrium. Certain experiments require the preparation of isolated ventricle strips. Such tissues are obtained from hearts treated in the foregoing manner, and prepared as described by Feigen, *et al.* (7).

b) Challenge

Cardiac tissues are challenged either by adding toxin to the perfusate or by adding the material to the surviving fragments in a bath equilibrated with O_2/CO_2 mixture. The challenging agent is present in a predetermined concentration. For studies of kinetics of release it is convenient to employ superfused atria in which the rate of drip, hence the volume of the fraction is constant at prescribed intervals.

The perfusates are saved for estimations of vasoactive agents. Various enzyme inhibitors may be added depending on the experimental requirements to preserve the agent at interest.

c) Assays:

i) *Chemical*

a. *Histamine:* The chemical estimation of histamine is based on a combined procedure of McIntire *et al.* (8), for its separation, and that of Shore, *et al.* (9) for its fluorometric estimation as the estimation as the condensate of *o*-phthaldialdehyde (OPT). Since very little protein is usually present in the samples, the precipitation step is omitted. In practice, 2 ml aliquots of the perfusates or of the histamine standards are treated with 0.6 g of a salt mixture (1 g $Na_3PO_4 . H_2O$ + 6.25 g Na_2SO_4) and 5 ml of *n*-butanol. The mixture is shaken for 10 min, and the phases separated by a short centrifugation at room temperature.

A 5 ml portion of the supernatant organic phase is passed through a 100 mg pad of cotton-acid succinate. The pad is next washed with ethanol and then with distilled water, after which the histamine is eluted from the pad with two successive 1 ml portions of 0.1 N HCl. The histamine hydrochloride is rendered alkaline with 0.3 ml of 2 N NaOH and treated with 0.1 per cent (w/v) solution of OPT in absolute methanol. The reaction is stopped with 0.2 ml of 3 N HCl and the intensity of the fluorescence was read on an Aminco-Bowman spectrophotofluorometer or a Turner fluorometer. In the former instrument the activating wavelength used was 365 mμ, and the emission is measured at 440 mμ.

b. *Serotonin:* In the absence of catecholamines serotonin is extracted from the butanolic solution after the histamine has been removed by the CAS column. Five ml of the butanolic solution is extracted with 12 volumes of acidified heptane and 1 ml of 0.1 N HCl. The aqueous layer is further acidified with 1/3 volume of 12 N HCl and estimated on the fluorometer. In the presence of a significant amount of adrenaline or noradrenaline the combined histamine-serotonin method cannot be used because the alkali used in the extraction of histamine converts these compounds into the corresponding adrenolutives which fluoresce in the same region as serotonin.

To prevent this conversion from taking place the extraction is made with acidified butanol containing NaCl. The extraction is made with acidified heptane and the fluorescence of the aqueous layer is read directly after further acidification with 12 N HCl.

ii) *Bioassays*

a. *Histamine and SRS-A:* Assays for histamine and SRS-A are conducted on the atropinized guinea pig ileum. The assay for SRS-A, in the sample is conducted on the same preparation which has been additionally blocked with an antihistamine. Serotonin can be blocked by *d*-bromolysergic acid.

b. *Acetylcholine:* Nanogram quantities of ACh can be estimated on eserinized leech muscle.

c. *Serotonin:* 5-hydroxytrytamine is estimated on the fundus of the rat's stomach.

d. *Bradykinin:* This polypeptide contracts rat's uterus as does eledoisin but it is distinguished from the latter in its relaxing effect on the rat duodenum.

iii) *Reduction of Observations*

The responses to the direct effects of toxin and to the materials from venom-treated tissues are quantitated on the isolated tissues. Each tissue is first standardized at 37°C by recording its reactions to a range of concentrations of histamine, serotonin, or bradykinin as required in the experiment. Immediately following each response the bath is drained and the tissue washed three times. A period of 4 min is allowed for equilibration between each challenge and the testing or callibration procedure, which is repeated until a consistent response is obtained. When the tissue has been thus calibrated with the standard compound, and the overall maximum contraction has been recorded, it is challenged with a suitable dose of toxin or with several dilutions of bath fluid containing material released from the tissue-toxin reaction mixture. At the end of the assay, each test strip is re-calibrated with the standard compound.

The dose-response curves are sigmoidal, and the potency of the unknowns are found by interpolation from the standard curve. For more critical work, particularly that which involves the comparison of inhibitors, the sigmoidal functions are linearized by the logistic transformation of von Krogh as previously mentioned. In that case the magnitudes of all reactions are converted into fractions of the maximal response and fitted to the relationship:

$$X = K[y/(1 - y)]^{1/n},$$

in which X is the concentration of the compound giving the relative response, y, and K is a constant equal to the dose giving a 50 per cent response. A plot of log X against log $y/(1 - y)$ is linear with a slope of $1/n$. The value of K can be either calculated from the numerical results or found by interpolation from the plot. Unless otherwise stated the bioassays are expressed as equivalent histamine concentrations in moles per liter.

C. *PHYSIOLOGICAL MEASUREMENTS*

1. Mechanical Response of Cardiac Tissues

The mechanical reactions of intact perfused hearts to challenge consists of changes in the amplitude, rate, and rhythm of beat. These reactions are recorded on an Offner Dynograph Type 506 by means of a transducer attached through a thread to the apex of the ventricles.

The mechanical activity of spontaneously beating, isolated atria is studied on tissues mounted vertically in water-jacketed muscle baths having a volume of 3.5 ml. The recording system consists of a Statham G-7A transducer, G-18 control box, and the Offner Dynograph. These preparations were maintained at 30°C in order to increase the amplitude of contraction, decrease the rate, and prolong the period of stability. At this temperature the rate is 53%, and contraction 210%, of that at 37°C, and preparations are stable for at least 20 hours.

2. Electrophysiological

a) Atria and Ventricles (Action Potentials)

Simultaneous recordings of the electrophysiological and mechanical activity of atrial and ventricular tissues are made on horizontally mounted tissues according to methods described previously (10).

Intracellular resting and action potentials, measured with standard glass microelectrodes, and mechanical activity, monitored by a moveable-anode transducer, are displayed on a twin-beam oscilloscope and photographed by an oscilloscope camera. Atrial preparations are stimulated with a biphasic 1.0 msec pulse, 10 volts above threshold, at a rate approximately 10 beats per minute above the natural rate. Ventricle strips are similarly driven at a rate of 180 beats per minute. All preparations are equilibrated with O_2/CO_2 and maintained at 37°C.

b) Intercostal Muscles (MEPPs)

Miniature end-plate potentials are recorded from the mouse hemithorax preparations for (i) study of mode of action of toxin, (ii) bioassay of NSP fraction of tetanus toxin. The preparations are mounted in a perspex clamp and then placed in a muscle bath, and maintained with Liley's solution at 37°C. Intracellular potentials are measured with KCl-filled microelectrodes connected by an Ag-AgCl bridge to the input of a cathode-follower circuit. The potentials are displayed on one beam of a Tektronix 502 oscilloscope and photographed with a Grass oscilloscope camera. Potentials are recorded from at least twelve cells before and after the introduction of a test solution.

The results are acceptable only if the frequency remains stable, the amplitude at least 250 μV, and the resting membrane potential is in excess of -60mV. The average resting frequency is 5.35 ± 0.29 sec^{-1} at 37°C (11). The observations are reduced by plotting the mean percentile increments of test to control frequency obtained in at least 5 experiments against the final concentration of toxin present in the bath.

The range of response is limited to a 50% increase above control frequency and the preparations are quantitatively bioassayed by the concentration required to produce one-half the limiting response. The midpoint of the reaction curve, the $MEPP_{50}$, is used as an index, analogous to the LD_{50} to evaluate the electrophysiological potency of the toxin.

3. Active Transport

There is good evidence for the view that a factor in the neurotoxic effect of certain snake venoms (12) is due to a defect in active transport of the neurone. Since sea urchin toxin evidently has a primary action at the cell membrane, the influence of various preparations upon the movement of electrolytes will be investigated in greater detail by classical studies of active transport through the frog skin and the toad bladder.

The classical Ussing apparatus is used for this purpose. This consists of two lucite chambers and 4 Ringer-Agar electrodes which are used to measure a) the transmembrane potential, and b) the short circuit current. The biological membrane is clamped between the chambers and oxygenated nutrient solution is added to the two sides. The Ringer-Agar bridges are connected to calomel electrodes and then to a potentiometer circuit.

The frog skin and toad bladder have the property of actively transporting Na^+ into the animal. Since the conditions of electrical neutrality require that the net transfer of cations and anions be equal, there is no net flow of electric current across the skin, i.e., the transported Na^+ is accompanied by an equivalent amount of Cl^-. If the bathing solutions are joined by an external conductor (short-circuit), the skin becomes a source of electric current due to the active transport of Na^+, i.e., there is a net Na^+ flux across the membrane.

To measure the ionic current (Na^+ transport) a second pair of Ringer-Agar bridges are placed at the outer ends of the chambers. The second set of bridges is connected in series with Ag-AgCl electrodes and a voltage supply consisting of a battery and a voltage divider. A microammeter completes the short-circuit and measures the current drawn through it. By adjusting the potential across the membrane to zero and using identical bathing solutions on both sides, ions that are not subject to active transport pass through the membrane at the same rate in both directions and do not give rise to a net transport of electricity; ions (Na^+) that are subject to active transport more predominately in one direction in the absence of an electrochemical gradient resulting in a net transport of electricity across the membrane. The current running through the short-circuit when the transmembrane potential is adjusted to zero is a resultant of the net transport of Na^+ across the membrane and is measured by the microammeter.

The effect of toxin preparations on the movement of ions and water molecules across the frog skin or toad bladder can be determined by using radioactive isotopes. For example, Na^{22} can be added to the mucosal side and by sampling the serosal side the rate of sodium movement across the membrane can be measured.

Previous work in this laboratory (13) has shown that some of the effects of toxin can be neutralized by specific antisera.

D. *IMMUNOLOGICAL*

The purpose of the immunological studies is 1) to provide reagents for the detection of active fractions; 2) to determine the relationship between the protective power *in vivo* to the effect of enzyme action *in vitro* and, 3) to study the process of anaphylaxis to the whole toxin and to its separate fractions.

1. Immunization

Antisera will be produced by the immunization of rabbits and guinea pigs. The antigen is first toxoided in the presence of 0.01% formaldehyde. All antigens are incorporated into complete Freunds adjuvant (CFA) and injected into multiple sites in areas around the neck and upper back of the recipient. At fourteen and twenty-eight days following this initial injection, the animals are given "booster" injections of 5 mg of the various antigens, administered intradermally. At regular intervals, the animals are bled by cardiac puncture;

the serum separated, the γ-globulin fraction obtained, and the antibody titer of these preparations determined by quantitative precipitation. The serum is dialyzed for 3 days with 3 changes of 1% NaCl. The γ-globulin fraction is dialyzed 3 days with 3 changes of borate-buffered saline, pH 8.45, $\mu = 0.1$.

A certain proportion of the guinea pigs will be saved for studies of active sensitization.

2. Isolation and Characterization of Antibodies

The whole antiserum and the γ-globulin fractions are variously characterized by their immunoelectrophoretic, ultracentrifugal, and chromatographic properties and the various electrophoretic classes of antibodies prepared according to the chromatographic techniques.

a) Preparation of Electrophoretically Distinct Antibodies

For studies of anaphylaxis it is important to have at hand antibodies which are of a restricted electrophoretic type. Anaphylactic antibodies (i.e. homocytotropic) in the guinea pig are γ_1- and heteroaptotropic; antibodies of the rabbit, which sensitize guinea pig tissues, are γ_2-. Electrophoretically distinct antibodies are prepared as follows.

i) Rabbit γ-globulin: Rabbit antibodies are separated into γ_1 and γ_2 by the method of Onoue *et al.* (14). An initial fractionation is carried out by gel filtration on Sephadex G-200 column. The buffer used to equilibrate the column and to elute the protein is a Tris buffer of 0.02 *M* pH 8.0, containing 0.5 *M* NaCl. A crude globulin fraction, obtained by one third saturation of serum with $(NH_4)_2SO_4$, is dialyzed against the Tris buffer before application to the column. Fractionation is accomplished on DEAE-cellulose. The cellulose is washed and equilibrated with the starting buffer of 0.067 *M* Tris-phosphate, pH 8.5. The crude globulin fraction, or those from Sephadex fractionation, is dialyzed against the starting buffer. The pH and salt gradient was produced by a cone-sphere arrangement reported by Fahey *et al.* (15). The final buffer is 0.5 *M* Tris-phosphate pH 4.0

This fractionation yields antibodies of three different protein types identified as the γ-type (6 - 7*S*), the β_2-type (16*S*) and the β_1-type, (probably 9*S* and 6*S*). The γ-type contains γ-globulins of a wide range of electrophoretic mobility. The γ-type can be fractionated on DEAE cellulose into two distinct fractions which differ in their electrophoretic mobility and are referred to as γ_1 and γ_2.

ii) Guinea Pig γ-globulin: Guinea pig antibodies are separated by the method of Askonas, *et al.* (16). A crude globulin fraction is

dialyzed against 0.02 *M* sodium-phosphate buffer (pH 7.5), and the immunoglobulins are fractionated by stepwise elution from DEAE-cellulose column. (a) 0.02 *M* sodium phosphate pH 7.5 (elutes approx. 80% of γ_2-globulins), (b) 0.05 *M* buffer, pH 7.5 (a small amount of γ_2-globulin present, plus some other proteins), (3) 0.1 *M* buffer, pH 6.5 elutes γ_1-globulins.

iii) *Immunochemical determinations:* Immunoelectrophoresis and immunodiffusion (Ouchterlony) tests are carried out on substrate and toxin preparations as microdeterminations customarily used in this laboratory. Quantitative precipitin and complement fixation tests are made on the antisera according to Campbell *et al.* (17).

The functional characteristics of the various antibodies will be tested with respect to protection, complement-fixation, neutralization, and passive sensitization against active and inactive toxins.

iv) *Anaphylaxis:* The existence in sea urchin toxin of a variety of enzymatic components having, in one sense, a common immunogenetic provenance makes this system particularly useful to the elucidation of the anaphylactic component in reaction to a toxin in an individual who has had previous exposure.

The clinical significance of this problem is inescapable if one considers the number of deaths caused by anaphylaxis to bee stings which contain a toxin of a comparatively low order of potency.

The phenomenon of cardiac anaphylaxis is particularly suited for the resolution of these problems because:

a) cardiac tissues can selectively release a variety of humoral substances;

b) it lends itself to a variety of mechanical; electrophysiological, and biochemical measurements, permitting correlation between chemical and functional events;

c) cardiac anaphylaxis is an explosive process, and its intensity is equisitely related to the immunological input, to direct and passive sensitization *in vitro*;

d) it has primary relevance to clinical conditions such as, for example, rheumatic heart disease.

E. *BIOCHEMICAL STUDIES*

1. Detection and Characterization of Enzymes in Venom Complexes

The intent of this study is to identify the components which have high lethal potency as judged from the preceding preparative and pharmacological study and to make a detailed study of the substrate specificity and kinetic behavior of the highly purified material.

For this purpose the various isolates obtained by the several methods of purification will have to be screened by a battery of rapid and disjunctive tests of enzymatic behavior. In order to restrict the range of inquiry, a useful guide is to consider the possible biological significance of venoms, particularly those of the snake.

ARGUMENT

Unlike other animals serpents cannot mix the secretion products of their digestive glands with the food by chewing or tearing. Instead, they use the highly specialized injection apparatus to introduce digestive agents into the prey. In the course of phylogenetic development the length of the maxilla has become shortened and the position of the fangs has shifted toward the front of the mouth, and they thus developed into weapons of defense and attack.

The presence in these venoms of proteases, peptidases, phosphatases, esterases, and lecithinases is in accord with these assumptions. All of these agents are found in the digestive apparatus of other animals with the possible exception of a specific kind of L-amino acid oxidase and hyaluroridase. Some enzymes are purely digestive, others are purely toxic, and a number of enzymes has been found to display both activities.

Formidable barriers to diffusion of these poisons is raised by the hyaluronic acid polymers that constitute the intercellular connective tissue of the skin. These are overcome by the presence of hyaluronidase which reduces the viscosity of the polymerized hyaluronic acid. The tissues of the prey usually contain substances that inactivate hyaluronidase - antinvasin - which is overcome by the proimvasin found in the typical snake venom.

The effectiveness of hyaluronidase is supported by the action of phospholipase which attacks the lipids of endothelial cell membranes and leads to the formation of lysolecithins which increase the permeability of the cell membranes. Powerful proteinases dissolve the blood vessels leading to the extravasation of red cells and serum into tissues giving rise to a widespread hemorrhagic edema. Absorption of the other toxic principles occurs from the deading edge of the edema and the venom is absorbed by the lymphatics, which become markedly swollen and hemorrhagic.

There are also present other, not directly toxic materials, in venom complexes. One of these is L-amino oxidase, whose activity is not neutralized by antivenoms. It is commonly observed that envenomated animal carcasses putrefy at a greater than normal rate, even though venoms themselves fail to show high proteolytic action, although they can activate other proteases. The substance of observations is that the L-amino oxidase (ophioamino oxidase) functions as an activator not only of venom proteases, but of many proteolytic enzymes.

From this discussion the principle emerges clearly and for this reason in addition to the proteases and phospholipases it is necessary to study the effects on blood clotting, curariform action, to detect coagulases, cholinesterases, and other constituents.

The following battery of rapid methods will be used for the preliminary screening of enzyme activity and in specific instances, warranted by the degree of toxicity, more detailed kinetic studies of a particular system will be undertaken.

It is assumed here that the material is presented as an electropherogram on starch gel. Samples are selected from the unstained block on the basis of indications given by a starch gel layer which has been stained with Amido black 10B. Starch blocks are cut into 10 mm-segments according to the method of Master and Rao (18).

For testing, the segment at interest is put into the barrel of a syringe and the fluid expressed. Usually 0.4 ml is obtained in this way, sufficient for enzyme detection and a toxicity determination.

Proteolytic Activity: Aliquots of the expressed fluid are placed on uncovered strips of photographic film, placed in a covered petri dish and incubated overnight. Active preparations dissolve the gelatin. Alternatively, the digestion of azocasein can be used as a quantitative method by determining the free dye in perchloric acid supernates.

Clotting or Anticoagulant Activity: Samples are tested by the addition of 0.1 ml portions by Quick's prothrombin time method in the presence of recalcified human plasma.

Phospholipase A: Filter paper discs are soaked in the press juice and applied to a lecithin substrate incorporated into an Agar gel in a petri dish. The lecithin is prepared by adding 2.5 ml of 0.2% lecithin and 2.5 ml packed sheep cells to 45 ml of 1% Agar in physiological saline. Each plate contains 10 ml of this mixture. The reaction is incubated overnight at 37°C. Enzyme activity is revealed by hemolysis around the test discs.

Nucleases: A 3% RNA solution is applied to a starch block after electrophoresis. The system is incubated overnight at 37°C, and then treated with 0.5 *M* HCl. Nucleases are detected by the appearance of a clear zone.

Phosphodiesterase: The presence of this activity is revealed by treating the gel with 0.1% *p*-nitrophenyl-UMP. The system is positive for diesterase if an intense yellow zone appears after incubation for 10 min at 25°C.

Monoesterase: Monoesterase activity is detected by incubating 0.4 ml of press fluid with 0.5% Na-*p*-nitrophenylphosphate. Positive reactions are shown by the appearance of yellow *p*-nitrophenol after 2 hr of incubation.

5'-Nucleotidase: Incubation of 0.4 ml of sample with adenosine monophosphate will result in the formation of inorganic phosphate, which is detected by the classical method of Fiske and SubbaRow.

L-Amino Acid Oxidase: Eluted materials are added in 0.2 ml portions to 0.5 ml of 3 mg/ml L-histidine HCl. After a 24-hour incubation aliquots of the reaction mixture are applied to 24 cm filter paper circles. Chromatography is carried on in the presence of butanol-HA-H_2O (4:1:5). The reaction is developed by a spray of diazolised sulfanilic acid. The appearance of an additional band above that of histidine indicates a positive test for L-amino oxidase.

Cholinesterase: Acetylcholinesterase is estimated by determining residual ACh in heated reaction mixture by suitable pharmacological tests on guinea pig ileum and eserinized leech muscle.

2. Hematological Problems

a. Quantitative hemolysis

One ml quantities of various geometric dilutions of heated and unheated toxin are tested in the presence of 8 ml of 0.4% sheep erythrocytes. The samples are centrifuged, the supernatants are analyzed spectrophotometrically for hemoglobulin. The H_{50} value is obtained from a dose-hemolysis curve. Variants of this method can be applied to detect lysolecithin by carrying out the reaction in the presence of egg yolk substrates.

b. Clotting

Russell's viper venom is a powerful plasma coagulant in the presence of Ca^{++} and it requires such lipids as are present in commercial lecithin, cephalin, or platelets. The active lipid which enhances viper venom appears to be associated with phosphatidylethanolamine. Venom thus activated can produce clotting in plasmas deficient in Factor 8.

Russell's viper venom has both thrombin - and thromoplastin-like actions, the latter can be detected in the cone stage prothrombin test by substituting it for thromboplastin.

These tests will be applied in a quantitative way to fractions of sea urchin toxin having phospholipase activity.

In the succeeding discussion we illustrate the relevance of the study of animal venoms to the analysis of the immunopharmacological reactions, using cardiac anaphylaxis as a model.

PART II. Anaphylactic Components in the Cardiac Reaction to Active Bacterial Toxins and Animal Venoms

Background

The advances in prophylactic immunization, chemotherapy, and the application of new modes of palliative management, although increasing the recovery rate from normally serious bacterial invasions, have brought along with them the increased probability of secondary immunopathology. Although the clearest case of the process of secondary immunological damage is exemplified by the sequelae of reinfection by hemolytic streptococci, evidence is accumulating that this may be true in other diseases involving the elaboration of highly potent and antigenic bacterial toxins. The increased survival rate in tetanus owing to the prevention of spasms by curariform and other agents appears to have revealed targets other than the nervous system which were heretofore unknown, and there is now an increasing awareness that patients who survive the acute respiratory paralysis may die from either the direct or indirect effects of the toxin on the cardiovascular system. Similarly, immunogenic animal venoms may put the victim in double jeopardy.

For the past 30 years many students (19, 20, 21, 22) of the problem have reported the presence of circulating antibodies in the sera of patients with acute rheumatic fever and rheumatic heart disease, which had the capacity of reacting with constituents of cardiac tissues; later, it was demonstrated that bound γ-globulin was present at the sites of damage in the heart. It appears from the subsequent work of Kaplan *et al.* (23) that the deposited γ-globulin suggests an active pathological condition brought on by an antecedent streptococcal infection which caused this accumulation. A direct test showed that rabbits produced antibodies to cell walls of group A streptococci which then had the capacity to bind heart muscle.

The presence of heart-bound antibody and the well established cardiotoxic effect of the streptolysin have lead to the present study of the relationship between the cardiotoxic and immunologic properties of streptolysin O (24,25). Our experimental model very faithfully fulfills the conditions found in the clinical condition, and therefore it probably reflects the process of primary insult of the sensitized heart by active toxin.

The primary thesis underlying our work is that the direct action of the toxin on the heart may be exaggerated by an additional reaction between the toxin and the specific, anaphylactic, antibody bound to the cardiac tissues. We have presented elsewhere (24), a detailed analysis of the cardiotoxic properties of streptolysin O, and we have shown also, that the direct reaction between the toxin and the heart can occur simultaneously with the anaphylactic reaction between the toxic and the tissue-bound antibody, each of the reactions being signalled by the release of two distinct pharmacological agents.

In the following sections we present experimental designs for the analysis of factors contributing to the immediate toxicity and immediate anaphylaxis of tetanus toxin and sea urchin toxin, in addition to more extensive studies of streptolysin. As stated elsewhere in this proposal, these three substances have highly selective modes of toxic attack; they are all highly immunogenic and, at least in two cases, they apear capable of being fixed to cardiac tissues. Since the receptor group in each instance is highly specific an excellent tool is at hand for the resolution of some general problems in immunopharmacology by comparing results obtained in studies of direct and reversed passive anaphylaxis.

Experimental

In experiments with streptolysin we have established that the toxin liberates acetylcholine when it is acting directly on a normal heart, and that the anaphylactic reaction to the inactive toxin liberates histamine. These results showed that the usual manifestations of cardiac anaphylaxis -- increased rate, amplitude, and histamine release -- obtain in actively and passively sensitized tissues when they are challenged with the *inactive* (oxidized) form of the toxin. On the other hand, when tissues sensitized in this way are challenged with the *active* (reduced) form of streptolysin O there is evidence that the response is biphasic; i.e. there appears first a reduction in force and rate of contraction, characteristic of the effect of acetylcholine seen on "normal" atria, which is then followed by the typical histamine-like response observed with the inactive toxin and other non-toxic protein antigens. By appropriate use of atropine and pyribenzamine it is shown that either, or both, of the responses can be selectively abolished.

Several questions naturally arise from this demonstration both in connection with the action of the streptococcal toxin and to the action of antigenic animal venoms. Some of these are:

1. *To what extent is the aggravation of toxicity - as judged by the pharmacological reaction - a general problem of specifically acting toxins?*

2. *Is the pharmacological agent released from the same or different sites in the direct and anaphylactic reactions?*

3. *Is sensitization mediated by a different kind of antibody from that operating in a "protective" way?*

4. *Is the pharmacological agent released determined by specific antibodies acting on the same or different sites?*

5. *Is the severity of the combined reaction in actively immunized animals dependent on the character of the antigen (or its route of administration) in eliciting anaphylactic or protective antibodies?*

6. *Are toxins fixed to different types of cells as revealed by the nature of the pharmacological agent released on direct exposure?*

7. *Can antibodies be sufficiently selective in eliciting different responses from cell bound toxin, when anaphylactic and non anaphylactic immunoglobulins are used to evoke reactions from a test domain.*

8. *Can two toxins, having different direct actions, be made to compete for anaphylactic site on the cell?*

In order to approach an answer to these questions it is first necessary to establish the following general conditions:

1. *What substances are released by the heart in anaphylaxis, in terms of the antigens, antibodies, and substrates involved?*

2. *What substances are released by the direct action of toxins in their active groups, i.e. what is the mode of action of the toxin?*

3. *What sorts of antibodies are elicited by immunization with the toxins at interest?*

4. *Are there differences in the selectivity of fixation among toxins as determined by challenge? (fixation of inactive toxin?)*

A. DIRECT ACTION OF TOXINS ON THE NORMAL HEART

1. Purpose: To establish a base line of information on how the differences in functional specificity of these toxins are reflected in their:
   - a. Point of attack
   - b. Electrophysiological response and mechanical response
   - c. Pharmacological output in guinea pig and rat hearts

2. Method:

a. Site of Attack: The responses of whole hearts to insult with active toxins are compared with those elicited from the separated cardiac tissues.

i) the whole heart response is examined with respect to rate, amplitude, block, and coronary flow.

ii) the responses of the separate tissues are studied electrophysiologically. Records of intracellular potentials are taken from the separated atria and ventricles to determine whether:

a) the toxin causes a defect in transmission of the signal - presence of pacemaker potentials, absence of action potentials elsewhere (failure of propagated response).

b) the toxin affects the permeability of the cell- as evidenced by a shift in resting potential.

c) it affects the apparatus responsible for depolarization and/or repolarization.

From this battery of tests it will be possible to assess whether difficulties are localized in the atria, ventricles, or the conduction system and will give an indication as to the nautre of the defect, i.e. whether it is directed at the contractile system, or whether it stems principally from the abnormalities of impulse generation or transmission. The electrophysiological response will be used as a preliminary guide to indicate whether there is any significant, or selective effect on ion transport, as judged by the quantitative analysis of variations in resting potential, depolarization and repolarization velocities, as well as the height of the reversed (overshoot) potential. As necessary, the effects of toxins on nodal tissue on Purkinje fibers will be determined.

b. Pharmacological Output: samples of perfusate and bath fluid occurring during the reaction of the whole or separated tissues will be examined for: Histamine, serotonin, SRS-A, Acetylcholine, and kinins (or reagents forming them, such as kallikrein).

The significance of the release of vasoactive substances to the functional changes observed in the heart or its isolated tissues will be studied in terms of the following variables:

i) The effect of the type and concentration of toxin on the assortment of vasoactive substances released. This will determine whether there is a preferential release for one or several of the materials and will indicate in a preliminary way, whether the functionally distinct toxin "probes" attack selected cells, or affect particular metabolic pathways.

ii) The Effect of the vasoactive effluent on normal cardiac preparations from normal hearts. Those tests carried out with effluents, heated so as to inactive residual toxin, will give presumptive evidence in most cases (except ACh, which is rapidly destroyed) as to whether the intensity or the character or functional changes are generally related to the output of the most abundant agent released.

iii) The effect of selected blocking agents on the cardiac reaction to toxin. On the basis of the preliminary screening tests (b) the experiments in (a) will be repeated in the presence of:

specific pharmacological blocking agents: Pyribenzamine, atropine, d-bromolysergic acid, dietyl carbamazine,

specific receptors for toxic groups: cholesterol (SLO), cerebroside-ganglioside complexes (tetanospasmin), soy-bean trypsin inhibitor, "Trasylol" (SUT).

Direct effects of vasoactive agents. Although the effects of the common vasoactive agents are known for specific test tissues selected for their reactivity as test objects they are not so well established in a comparative way on a given mammalian heart. The fact that histamine produces positive inotropic and chronotropic reactions in the isolated guinea pig heart was established relatively recently by Feigen *et al.* (10) and by Erspamer in 1960 (26). The direct cardiovascular actions of SRS-A, for example, are not well-documented and the actions of serotonin are contradictory. In the case of histamine, for example, it is not unlikely that the inotropic and chronotropic effects are not primary, but may occur because of the secondardy stimulation of the output of noradrenaline. This fact also complicates the estimation of serotonin. The mediator-dependent release of other mediators is not uncommon, such sequential obligatory releases obviously would complicate the interpretation of the selectivity of a toxic or anaphylactic process, and it will be necessary to examine the effluents of hearts treated with each mediator in turn, for the release of other mediators.

B. STUDIES ON THE PREPARATION AND MODES OF BIOCHEMICAL ACTION OF TOXIN

1. Purification and Characterization of Toxins

Separation of sea urchin toxin on Sephadex and hydroxylapatite at a graded ionic strength, elution at various pH values, conserve activity. In both cases, the various fractions will be characterized physically, chemically, and immunologically. Details are presented under A. CHEMICAL.

2. Enzymological Studies

Since the development of sea urchin toxin is the particular contribution of this laboratory the studies under this heading will be limited to the analysis of the point of attack of the various components which have been obtained as single antigenic determinants.

The various preparations obtained which have single antigenic determinants will be tested for their general and specific mode of attack:

a. General: This will include the preliminary assessment of the activity of the material to determine if it functions as an activator or inhibitor in reaction with various gross fractions of plasma, in comparison to the standard SUT-Cohn Fr. IV, as well as the SUT-bradykinin system. This will include the use of inhibitors such as Trasylol, and ε-amino caproic acid; various SH and S-S reacting agents such as cysteine thioglycollate iodoacetate; esterase inhibitors such as DFP.

b. Specific: When the general type of activity has been established, and depending on the results obtained in the pilot runs as to the nature of the attack, further tests will be made with the appriate synthetic substrates. If it is an esterase as determined by DFP the methyl esters, benzoyl-L-arginine, and p-trisylarginine will be used. If it is a peptidase, the appropriate substrates would be n-acetylphenylalamine ethyl ester, nacetyl-tryptophan ethyl ester.

C. EFFECT OF ANTIGENS ON KINETICS AND CHARACTERISTICS OF VARIOUS ANTIBODIES FORMED.

These necessary studies will be carried out as described under "Isolation and Characterization of Antibodies" to determine the specificities, anaphylactic and mast cell specific protective properties, of antibodies formed as a function of the type of antigen and method of immunization (site, adjuvant, frequency) to determine if particular differences in the types of antibodies formed are responsible for possible differences in reactions when the actively sensitized organs are used.

D. ACTION OF TOXINS ON SENSITIZED HEARTS

The experimental design consists of the challenge of actively and passively sensitized hearts with inactive and active toxins in direct and reversed sequence on the character of the response, with specific reference to the type of antibody responsible for the anaphylaxis and the nature of the vasoactive output. Additionally the question is put as to how nearly it might approach the clinical model. The plan is displayed in the accompanying protocol.

This protocol will be replicated for each toxin on the cardiac preparation most appropriate for the determination of the functional and pharmacological results.

The surrogate of the clinical model is the actively sensitized heart, because the antibody load and type is not under control. The effect of type of antibody is revealed by comparison between γ_1 and γ_2 in the passive sensitization experiments. *In vivo*, as compared to *in vitro*, transfer is to control for possible complement or substrate attachments during fixation.

Tests for independence of the toxic from the anaphylactic effects is secured by reversal of order of challenge with active or inactive form of the antigen. Tests of protective action of antibody, for example homocytotropic γ_2 of the guinea pig, would be revealed by the failure of response to active toxin when that agent is used in initial challenge.

Independence of the two processes, either in terms of cell types sensitized or biochemical mechanisms will be evident from the fact that the active toxin would release its typical profile, when presented at first or second challenge, in the latter instance, the release of histamine might be significantly reduced.

In selected cases, as conditions warrant, further investigations will be made to determine whether the release of the particular vasoactive agent can be interfered with. Thus the release of SRS-A for example, which takes place through a different biochemical pathway could be blocked specifically by diethylcarbazazine, without interfering with the release of histamine or,

Effects of Method of Sensitization, Functional Activity of Antigen, and Sensitization of Antibody on Physiological and Pharmacological Responses of Cardiac Preparation

Sensitization			Challenge		Response							
Mode of	Route	Antibody Type	+ active toxin - inactive toxin		Physiological			Pharmacological				
			Initial	Rechallenge	Rate	Amplitude	Block	Histamine	Ach.	SRS	BK	Serotonin
Active			+	-								
			-	+								
	In vivo	γ_1	+	-								
			-	+								
		γ_2	-	+								
			+	-								
	In vitro	γ_1	+	-								
			-	+								
		γ_2	+	-								
			-	+								

possibly, with the release of toxin-activated SRS-A. In this connection, if histamine and SRS-A are released by different biochemical measurements, the question naturally arises whether the energetics of sensitization and release are grossly different. Experiments to reveal these factors will be made by a temperature analysis using the SRS-A/histamine ratio as an index.

E. ACTION OF ANTIBODIES ON HEART FIXED TOXINS

The clinical model for this condition would involve any situation in which the diseased recipient receives remedial passive immunization with antibodies containing a specificity for the heart-fixed toxin, it is also basically a model for the rejection reaction.

In the present instance, since the various toxins such as SLO and tetanus, at least have known modes of attachment, the following questions can be examined with selected types of antibody.

The hearts are sensitized by *in vitro* perfusion or *in vivo* administration of the inactive toxin.

a. Effect of anaphylactic vs. non-anaphylactic antibody. If only the anaphylactic antibody elicits a reaction in spite of the different modes of attachment, it is likely that the effect is due to the attachment of antibody to susceptible cells.

If a reaction is elicited by non-anaphylactic antibody, is the character of the reaction, as displayed in the array of pharmacological agents, different for each type of antigen. Can the anaphylactic antibody, applied subsequently, produce an additional response, that is the same for all antigens?

b. Site of Action

i) Competition between the cell and the specific receptor for:

a) Toxic action
b) Anaphylactic action

by means of cholesterol in the case of SLO, and cerebroside-ganglioside in the case of tetanus.

ii) Challenge of hearts with antibody after they have been discharged with active toxin: If the results are positive, and independent of antigen provided that the tests are made with non sensitizing antibodies this would suggest that the toxin, is in fact, also attached to anaphylactically competent cells either of the SRS-A or histamine releasing types. Further experiments with DFP and diethylcarbamazine would be disjunctive.

c. **Energetics**

A study of the energetics by methods previously described of the sensitization of various toxins in comparison to the energetics of binding to specific adsorbents would provide additional evidence of the similarity or difference in sites of attachment.

d. Requirement for Complement Components

The effect of functional complement complexes, added during the reaction, would be relevant to the analysis of the cell type affected and would be analogous to the situation seen when rat mast cells are treated with rabbit-anti rat serum globulins because the latter also has property of non-specific attachment.

e. Fixation

In selected instances hearts treated with active and inactive toxins will be examined after the application of specific fluorescent antibodies, or by appropriate immunoautoradiographic techniques.

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

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ANNUAL INTERIM PROGRESS REPORT

"Physical-chemical, Immunological, and Enzymatic Properties of Purified Fractions of Sea Urchin Toxin"

By

George A. Feigen, Roger A. Pfeffer, and Lahlou Hadji

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ANNUAL INTERIM PROGRESS REPORT

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STUDIES ON THE MODE OF ACTION OF SEA URCHIN TOXIN. III. *ANALYSIS OF THE ENZYMATIC BEHAVIOR OF PURIFIED COMPONENTS.*

Production, Isolation, and Pharmacological Properties of Kinins Formed by the Attack of Sea Urchin Toxin on Human Plasma.

Further Studies of Kinin Formation Reaction Kinetics and Isolation of Products.

Physical-chemical, Immunological, and Enzymatic Properties of Purified Toxin Fractions.

A. The Production, Isolation and Pharmacological Properties of Kinins Formed by the Attack of Sea Urchin Toxin on Human Plasma.

A. The Production, Isolation, and Pharmacological Properties of Kinins Formed by the Attack of Sea Urchin Toxin on Human Plasma

Previous studies of the enzymatic action of sea urchin toxin (SUT) on human serum fractions indicate that the human α_2-globulins can serve as substrates for the release of a dialyzable, heat-stable, and pharmacologically active kinin similar to bradykinin (1). However, studies of the production of this kinin were confounded by its rapid destruction at higher temperatures and enzyme concentrations, and the small amount of substrate available. In order to study some of the kinetics of kinin formation related to enzyme concentration and temperature, before committing the meager supply of α_2-globulin, an adaptation of the method of Prado *et al.* (2) was used which employed heat-treated plasma (HTP) as a substrate. Heating the plasma decreased the kininase activity, but the outside dialysate from the HTP substrate still had a low level of kinin activity. Subsequent experiments in the present study showed that there was no decrease in this spontaneous activity in acid-treated, heat-treated plasma (HTPA).

The pharmacological activity of the kinins produced by the attack of SUT and trypsin were compared by bioassay on the guinea pig's heart and ileum, and the rat's heart, duodenum, and uterus. The blocking agents atropine, pyribenzamine, and bromolysergic acid were used as in previous work (3) to indicate which kinins were produced by each system. The analysis and comparison of the dialyzates and precipitates from the SUT-HTP and trypsin-HTP digestion indicates that the liberation of pharmacologically active substances follows the same kinetic path in both cases, but that more than one kinin is liberated by the action of SUT, while bradykinin is the principal kinin liberated by the action of trypsin.

MATERIALS

Toxin.

The toxin was prepared from the pedicellariae of 2,000 *Tripneustes gratilla* sea urchins collected from the Waikiki area of Hawaii. The pedicellariae were removed from the sea urchins by washing with sea water as has been previously described (4).

The total yield of pedicellariae, 782 g, was divided into 12 batches and ground for 10 minutes in a Waring blendor containing 175 g of ice and 200 ml of a 1/60 M phosphate buffer containing 8.8 g/L $NaH_2PO_4 \cdot H_2O$ and 12.9 g/L Na_2HPO_4. The suspension was centrifuged at

4500 RPM for 1.5 hours and the supernatant was made up to saturation with ammonium sulfate (SAS). The precipitate was filtered through Whatman number 1 paper and resuspended in SAS. The suspension was centrifuged, the pellet dissolved in 1/60 M phosphate buffer, and filtered through Whatman number 52 paper. At this point similarly treated solutions from the second and third extractions of the ground pedicellariae were pooled and then divided into 4 samples which were dialyzed against water for 2 hours and then against 15 L of 0.12 M phosphate buffer (containing 39.6 g of $NaH_2PO_4 \cdot H_2O$ and 86.4 g of $Na_2HPO_4 \cdot 7H_2O$ made to pH 7.2 with 15 ml of 5 N NaOH) for 24 hours. After dialysis the bags were removed and pervaporated at 4°C to 1/3 of their original volume. The pervaporates were pooled and dialyzed against the 0.12 M phosphate buffer for 2 hours. The dialysate was made up to 0.3 M with NaCl and stored in the deep freeze in plastic bottles. The crude toxin, referred to as SUT-67, contained 0.521 mg of TCA precipitable N per ml.

The purity of the SUT-67 toxin was checked by means of ascending Sephadex fractionation against (μ = 0.01) ionic strength phosphate buffered saline at pH 8.0. The three components that were found were collected in several steps and then concentrated by pressure dialysis against the same buffer. A second fractionation of the three components showed only one peak in each case.

The identity of the three Sephadex components was determined by immunoelectrophoresis at 25 volts with one per cent barbital buffered agar, pH 8.3, at 0.05 μ ionic strength. Anti-toxin to the SUT-67 and the three Sephadex fractions were produced by rabbits injected with one mg of 0.01 per cent formaldehyde-treated toxin in Freund's complete adjuvant. The purity of the three Sephadex components was also determined by means of immunodiffusion against the anti-serum to the toxoids of the three Sephadex components and the crude SUT-67.

Substrate.

Human blood plasma (Type A Rh + no. R9575 and A Rh - no. H7304) obtained from the Stanford Blood Bank was heated at 56-58°C for 3 hours. A copious residue formed which was discarded after centrifugation. The supernatant was dialyzed for 24 hours against 18 Liters of a constant flow of 1 per cent NaCl solution in a 6 liter cylinder. The heat-treated plasma (HTP) was then stored in plastic bottles in the freezer. The pH of this HTP was 7.84, and nitrogen analysis indicated that there was 9.4 mg total N per ml.

Enzyme.

Twice-crystallized trypsin, obtained from Nutritional Biochemical Co. (Number 9680) was used to digest the HTP in the control experiment.

Substrate Control.

As a control of the effect of the HTP on the tissues used for bioassay, 20 ml of HTP was brought to pH 2 with 6 N HCl and incubated for 30 minutes at 37°C. The acid-treated HTP (HTP-A) was then neutralized with 5 N NaOH.

Reagents.

All of the salts used to prepare the physiological solutions were either of reagent or analytical grade and were dissolved in water which had been redistilled over alkaline permanganate in an all-glass still. Preparation of the Tyrode's, Chenoweth's, and de Jalon's solutions, as well as the pharmacological reagents used in this study have been previously described (3). For the precipitation of the peptides formed by the digestion of HTP ether (Mallinckrodt analytical reagent) and twice distilled n-butanol (Baker analytical reagent) were used.

Experimental Animals.

The guinea pigs and the white Wistar rats used in these experiments for biological assays were supplied by Martin Farms of Palo Alto, California.

METHODS

Optimal Temperature and Enzyme-Substrate Ratios.

Four milliliters of HTP substrate was added to a geometrically diluted series of 7 blood tubes containing 1.0 ml of either SUT-67 (starting at 0.521 mg ppt. N/ml) or trypsin (starting at 2.0 mg/ml). The tubes were mounted in a shaker at either 30 or 37°C. After 10 minutes of incubation, the tubes were removed and placed in a water bath at 56°C for 30 minutes. After this heat treatment, 4 ml of the mixture was placed in dialysis tubing and dialyzed against 4 ml of 1 per cent NaCl at 4°C on a shaker. Control tubes of SUT-67, trypsin, and HTP were diluted with 1 ml of Tyrode's and treated in the same manner. All experiments were made in duplicate, and both the dialyzed and the non-dialyzed material was checked for pharmacological activity.

HTP Control.

In order to determine whether the HTP substrate itself was contributing to the pharmacological activity found in the previous experiment, acid-treated HTP was compared with HTP on the guinea pig gut.

Three samples were used containing either 5.0 ml of HTP or HTP-A substrate + 5 ml of Tyrode's, 10 ml of Tyrode's, or 5 ml of either HTP or HTP-A + 5 ml of Tyrode's.. Two 10-cm pieces of guinea pig ileum selected at random, were placed in both sample 2 and 3 and the samples were incubated for 30 minutes at 37°C. Four milliliter aliquots from each of the tubes were dialysed overnight against 4 ml of 1 per cent NaCl on a shaker at 4°C, and the dialyzate was examined for kinins by bioassay. Two micrograms per ml of Semicarbazide was added to retard the destruction of any histamine formed. The experiment was performed in duplicate.

Isolation of Peptides from Digestion of HTP.

The digestion mixture consisted of 70 ml of SUT-67 and 30 ml of glucose-free Tyrode's; this was added to 250 ml of HTP at 30°C for 10 minutes. The peptides produced by this digestion were recovered as follows: at the end of the incubation period the mixture was poured into 1000 ml of boiling ethanol and heated at 80°C for 30 minutes. The copious protein precipitate was removed by filtration and centrifugation of the filtrate. The supernatant was evaporated to dryness on a rotary evaporator in a water bath at 50°C for 18 hours. The residue was taken up in 25 ml of distilled water and centrifuged. The lipid layer and the pellet were saved for a second extraction and the supernatant was saturated with 8.9 g of NaCl. This solution was brought to pH 1.5 with 4 N HCl, and an equal volume of n-butanol added. The butanol-water mixture was thoroughly mixed and then separated by centrifugation. The butanol fraction was pooled with the second butanol fraction, added to 400 ml of anhydrous peroxide-free ether, and stored overnight on ice. The precipitate that formed was collected by centrifugation and dried over anhydrous $CaCl_2$ in vacuum. Trypsin-HTP digests were treated in the same way. There were 0.13019 g recovered from the SUT-67 digest and 0.07048 g from the trypsin digest.

Chemical Assays.

Nitrogen estimations were made by the Kjeldahl-Nessler micromethod as modified by Feigen *et al.* (5) on either the soluble material or on 5 per cent tri-chloracetic acid precipitates. Chemical estimations for histamine were based on a combined procedure of McIntire *et al.* (6) for its separation, and on Shore *et al.* (7) for its fluorometric estimation as previously described (3).

Pharmacological Assays.

Bioassays of kinin activity were made on the guinea pig ileum, the rat uterus, the guinea pig heart, and the rat heart and were compared

to known standards of histamine, acetylcholine, serotonin, and bradykinin both before and after blocking with sufficient pyribenzamine, atropine, and bromolysergic acid to give a 100% block of the MED of agonist.

Guinea pig ileum was tested at 37°C in Tyrode's using isotonic contractions recorded on a kymograph according to previously published methods (3). Standard reaction to histamine, bradykinin, ACh, or serotonin were recorded between each unknown determination.

The rat uterus assay followed previously described methods (3) except that the virgin female rats were injected with 10 micrograms of "Progynon" in olive oil 24 hours before use. After sacrifice of the rats, the uterine horns were removed, tied off, and mounted in 4 ml baths at 30°C. The isotonic contractions were recorded on a kymograph. De Jalon's solution was used to prepare and bath the tissue and the tissue was gassed with pure oxygen. Standards of bradykinin were used between every unknown. The duodenal strips were taken from the same rats used for uterus studies and were prepared in the same way.

Guinea pig hearts were perfused *in situ* by means of a cannula which was inserted into the ascending aorta as previously described (3) and then mounted on the Anderson perfusion apparatus. The tests were performed at 37°C in Chenoweth's solution. Rat hearts were tested in the same manner.

RESULTS

The Effect of SUT-67 Concentration and Temperature on the Digestion of HTP.

The outside dialysate resulting from the action of a varying concentration of SUT-67 on a constant amount of HTP substrate had kinin-like effect on the guinea pig ileum, causing it to contract. The amount of kinin formed varied directly with the amount of enzyme added at both 30 and 37°C incubation temperatures, as can be seen in Figure 1. The total amount of dialysable kinin-like material formed by the action of 0.104 mg ppt N/ml of SUT-67 on 4 ml of HTP was 0.8 micrograms BKE at 37°C and 3.1 micrograms BKE at 30°C. Since SUT-66 has been previously (1) shown to be capable of digesting bradykinin, as well as the α_2-globulin, it seems likely that the apparent increased formation of BKE at 30°C, compared to 37°C, is due to the more rapid destruction of the kinin product at the higher temperature. For the 10 minute incubation period used in these experiments, 30°C would seem to be better for the isolation of kinins.

Both the enzyme and the substrate controls also liberated a kinin-like material, and these values are shown as intercepts on the y axis, (Fig. 1). The amount of kinin liberated at 30°C was more than 3 times the amount liberated at 37°C by the HTP substrate. This indicates that the kinin was formed by enzymatic action, since a simple mechanical factor,

such as dilution, would normally produce more kinin at the higher temperature. The greater spontaneous formation of the kinin at the lower temperature is probably due to the more rapid enzymatic breakdown of the kinin from an enzyme system in the HTP substrate. Therefore, there must be at least two enzyme systems capable of forming and breaking down kinins in the SUT-67 HTP digestion mixture: one from the SUT-67 and one from the HTP substrate.

When the amount of SUT-67 enzyme was below 0.1 mg ppt N/ml in the digestion mixture, the amounts of kinin formed were not significantly different from the controls. This indicates that the enzyme/substrate ratio controls the level of enzymatic activity, and that when substrate exists in excess, there is little or no enzymatic activity. This may be due to an inhibition of the enzyme-substrate interaction by the spontaneous formation of the kinin by the HTP substrate. For subsequent experiments the SUT-67 level was kept at 0.104 mg ppt N per ml.

The Effect of Trypsin Concentration on Digestion of HTP.

A comparison of the amounts of dialyzable kinin formed by varying concentrations of SUT-67 or trypsin acting on a constant amount of HTP is shown in Figure 1. The results show that changes in enzyme concentration have the same effect on the amounts of kinin formed in both systems. This indicates that kinetically SUT and trypsin have a similar action on the HTP substrate. At the highest enzyme concentration tested, containing 0.29 mg ppt N/ml, the yield of bradykinin equivalents was 0.96 micrograms per 4 ml of HTP at 37°C. This value is only slightly higher than the 0.08 micrograms found for the SUT-67 digest. As in the SUT-67 HTP experiment, both the dialysates from the HTP substrate and the enzyme control had a pharmacological activity. The substrate control liberated 0.025 micrograms BKE, similar to the 0.03 micrograms found in the SUT-67 HTP substrate control.

Dialyzable Products Formed by the Action of SUT-67 on HTP.

Pharmacological assays of the dialysable products formed by the action of SUT-67 on HTP indicated that more than one kinin might be involved since the substrate, HTP, gave a reaction that could have been due either to the presence of a histamine-like kinin in the HTP or to the release of histamine by the gut from the action of HTP. The chemical assay of the pooled dialysates indicated that histamine was present, and this was confirmed by the 45% block of kinin activity by pyribenzamine. These results, shown in Table I, indicate that SUT-67 acting on HTP produces more than one kinin. Although the external HTP dialyzate and the HTP both showed no chemical histamine and were not blocked by PBZ, it was still possible that HTP itself acted as a histamine releaser on the guinea pig ileum.

The Action of HTP and HTP-A Dialyzates on the Guinea Pig Ileum.

Because the HTP substrate that had been extensively redialyzed and still caused contraction of guinea pig ileum, it was necessary to compare the HTP with HTP treated with acid (HTP-A) to determine whether the contraction was caused by kinins resulting from dilution during dialysis, an antibody to guinea pig in human plasma, or a non-specific histamine releaser.

The results in Table II indicate that a dialyzable kinin, capable of causing guinea pig gut contraction, is liberated from substrate plus Tyrode's, gut tissue plus Tyrode's, and substrate plus gut tissue when either HTP or HTP-A were sed as the substrate. This reaction was not blocked by a dose of PBZ sufficient to block a histamine control that was ten times the concentration of the unknown kinin. Since the chemical histamine assay also indicated that little or no histamine was present or released, the results show that histamine is not a factor in the reaction of the substrate with the gut. Furthermore, acid treatment does not decrease or prevent the release of kinin-like material from the substrate.

Dialyzable Products Formed by the Action of Trypsin on HTP.

The dialysable products formed in the digestion of HTP that had kinin-like activity appeared to be similar to bradykinin in their action on the guinea pig gut in terms of the delay before the onset of contraction. These contractions were not blocked by a dose of PBZ sufficient to block a ten-fold concentrated dose of histamine, but a chemical assay to confirm or deny the presence of histamine was not made. These results are shown in Table I.

Isolation of Kinins from the Action of SUT on HTP

The ether-precipitable peptides from the SUT-HTP digest were analyzed for kinin-like activity using guinea pig gut and rat uterus bioassays. The digestion of HTP by SUT-67 produced a total of 0.13 g of ether-precipitable material which was bioassayed on the guinea pig ileum and the rat uterus. The results in Table III show that of the 0.13 mg dry weight of the ether precipitate there was only 1.3 to 2.47 micrograms of kinin-like material. The ratio of bradykinin equivalents for guinea pig ileum over rat uterus was 6.8, somewhat higher than the 1.6 ratio found by Prado *et al.* (2) for the digestion of HTP by trypsin, as can be seen in Table IV.

Pharmacological Activity of Precipitable Kinins from the HTP-SUT Digest

The results of pharmacological tests of the SUT-HTP ether precipitate dissolved in one per cent saline on the guinea pig ileum and

the rat uterus are shown in Table V. The results of the ileum work, recorded isometrically from a transducer, do not shed any light upon the nature of the kinin, but the results from the rat uterus indicate that the precipitate is a mixture of a bradykinin-like material that is partially blocked by BOL-148 and one or more kinins which can be partially blocked by atropine. The blocking studies indicate that small amounts of kinins with pharmacological activity similar to histamine, serotonin, and acetylcholine may be present. Most of the activity of the SUT-HTP precipitate, however, must be caused by some other kinin, because ATR and BOL blockade do not prevent the pharmacological activity and the ether precipitate is active on rat uterus, which is not sensitive to histamine. The action of the precipitable material on the guinea pig and rat hearts in Table VI also shows that more than just bradykinin-like kinins must be present, as there is a decrease in both flow rate and frequency for SUT-HTP treated guinea pig hearts, while hearts treated with bradykinin show a flow increase and no frequency change. The rat heart substantiates this view, as there is a 75% decrease in amplitude of beat with SUT-HTP compared to an 8 - 15% increase with the bradykinin standards.

The rat duodenum results show that a bradykinin-like material is present in the SUT-HTP digest since the rat duodenum relaxes when the solutions made from the ether precipitate is added to the bath. These results, shown in Table III, indicate that only 0.0037 micrograms of BKE is present per mg of SUT-HTP digest by dry weight.

Pharmacological Activity of Precipitable Kinins from the HTP-Trypsin Digestion

The pharmacological activity of the HTP-Trypsin ether precipitable material on the guinea pig ileum and rat uterus is shown in Table VII. The results are consistent with the idea of a single bradykinin-like material being formed from the action of Trypsin on HTP. Neither ATR nor PBZ has a blocking effect on the action of the precipitate on the guinea pig ileum, and the level of blocking with BOL is consistent with that for known bradykinin standards. Similarly, ATR has almost no effect on the action of the Trypsin-HTP precipitate on the rat uterus, and the level of blocking by BOL is similar to that of the known bradykinin standard.

The action of the ether-precipitable material on the rate of flow, the amplitude and the frequency in the guinea pig heart was the same as for bradykinin, as can be seen in Table VI, while in the rat heart the unknown and the bradykinin standard differed in that in the digest the amplitude of beat was decreased while the bradykinin standard increased the amplitude.

The HTP-Trypsin digest had a relaxing effect upon the rat duodenum similar to the action of bradykinin. It is therefore probable that the trypsin-HTP ppt. contain only one pharmacologically active product which is similar to bradykinin.

DISCUSSION

A comparison of the total yields of pharmacologically active material from the outside dialysates and the ether-precipitable material obtained from the SUT-HTP digests shows that there was as much kinin-like material present in the outside dialysate from the 4.0 ml of HTP substrate (0.8 μg) as there was in the ether precipitable material from the digestion of 250 ml of HTP (1.3 - 2.4 μg). The outside dialysate and the ether precipitate from the trypsin digest of the HTP shows the same trend: there was 0.96 μg from the 4.0 ml HTP in the outside dialysate compared to only 3.6 μg from the 50 ml HTP used in the ether precipitate experiment. These results indicate that a great deal of kinin-like material is lost in one or more of the steps leading to the ether precipitate. It is unfortunate that a step by step bioassay for kinin-like material in the various phases of the extraction was not made or that a study of the efficiency of the recovery of bradykinin was not done.

The results of the optimal substrate and enzyme ratios at 30 and 37°C compared to the results of the trypsin digestions of the HTP substrate, seen in Figure 1 indicates that SUT-67, like trypsin, is a proteolytic enzyme which digests proteins to form peptides, some of which are pharmacologically active. At equivalent temperatures, substrate volumes, and incubation periods approximately equal amounts of pharmacologically active material are formed by the action of SUT-67 or Trypsin on the HTP. Since there was twice as much SUT as there was trypsin, in terms of precipitable N, this would indicate that SUT is about half as efficient as the twice crystallized trypsin.

Chemical and biological assays of the external dialysate obtained from the digestion of HTP by SUT-67 showed that there were at least two different kinins formed; one that was similar to bradykinin in its action on the guinea pig ileum, as shown by its resistance to blocking by atropine or bromolysergic acid, and a histamine-like kinin which was blocked in part by pyribenzamine. The possibility that the histamine-like kinin was spontaneously released by the HTP was eliminated by the results of the HTP-A experiment shown in Table II. The outside dialysate from the trypsin-HTP digestion appeared to contain only bradykinin, as it was not affected by the blocking agents, atropine, pyribenzamine, or bromolysergic acid. Chemical assay showed that there was histamine in the SUT-HTP digest.

A study of the pharmacologically active materials in the SUT-HTP ether precipitate indicates that a bradykinin-like material is present in low yield that causes the rat uterus to contract even when blocked by atropine or bromolysergic acid. Since the rat uterus is not sensitive to histamine, this material was eliminated from consideration. The rat duodenum assay also indicates that bradykinin-like material is present since the SUT-ether precipitable material causes the duodenum to relax, eliminating

kinins similar to eledoisin, as well as acetylcholine and serotonin, all of which cause the duodenum to contract. The results of the study of the flow, the amplitude and the rate changes in guinea pig and rat hearts indicates that there must be another kinin besides bradykinin present in the SUT ether precipitate, since these results are diametrically opposed to those obtained on hearts exposed to bradykinin standards.

The ether precipitates resulting from the digestion of the HTP by trypsin indicate that only a bradykinin-like material is present since the rat uterus, guinea pig ileum, rat duodenum, and guinea pig heart all gave results which were consistent with the bradykinin standards. These results agree well with those of the outside dialysate, which also indicated that only one kinin was formed as a result of the action of trypsin on HTP.

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TABLES

I A Comparison of the Pharmacological Activity of the Outside Dialysates From the SUT-67 and Trypsin Digest of the Heat-Treated Human Plasma Using Guinea Pig Ileum

II A Comparison of the Pharmacological Activity of the Outside Dialysates From Acid-Treated Human Plasma and Heat-Treated Human Plasma Reacted With Guinea Pig Ileum

III Yields of Ether-Precipitable, Bradykinin-like Material From the Digestion of Heat-Treated Human Plasma by SUT-67

IV Yields of Ether-Precipitable, Bradykinin-like Material from the Digestion of Heat-Treated Human Plasma by Trypsin

V A Comparison of the Degree of Blocking of the SUT-67 HTP Precipitates with Various M.E.D. of Antagonists

VI A Comparison of the Effects of Bradykinin and SUT and Trypsin Digests on the Guinea Pig and Rat Hearts

VII A Comparison of the Effects of Blocking Agents on the Ether Precipitable Material from the Trypsin-HTP Digest and on Kinin Standards

TABLE I

A Comparison of the Pharmacological Activity of the Outside Dialysates From the SUT-67 and Trypsin Digest of the Heat-Treated Human Plasma Using Guinea Pig Ileum

Outside Dialysate From	Bradykinin Equivalents μg	Chemical Histamine M/L	Per Cent Block by 0.1 μg/ml PBZ
Pooled SUT-HTP	6.1×10^{-2}	1.1×10^{-7}	45
HTP	3.5×10^{-2}	0	0
SUT-67	1.5×10^{-2}	3.0×10^{-8}	-
Trypsin-HTP	2.4×10^{-1}	-	0
Non-dialyzed HTP	6.2×10^{-2}	0	0

TABLE II

A Comparison of the Pharmacological Activity of the Outside Dialysates From Acid-Treated Human Plasma and Heat-Treated Human Plasma Reacted with Guinea Pig Ileum

Outside Dialysate From	Histamine M/L	μg/ml Bradykinin (0.1 μg/ml PBZ)	Chemical Histamine M/L
HTPA + Tyrodes	5.4×10^{-7}	1.4×10^{-2}	8.5×10^{-8}
Ileum + Tyrodes	6.4×10^{-7}	1.1×10^{-2}	0
HTPA + Ileum	5.9×10^{-7}	1.1×10^{-2}	0
HTP + Tyrodes	4.9×10^{-7}	1.1×10^{-2}	0
Ileum + Tyrodes	5.4×10^{-7}	1.1×10^{-2}	0
HTP + Ileum	7.0×10^{-7}	1.2×10^{-2}	4.5×10^{-8}

TABLE III

Yields of Ether-Precipitable, Bradykinin-like Material From the Digestion of Heat-treated Human Plasma By SUT-67

Test Tissue	Bradykinin per mg dry wt. (μg)	Total Bradykinin (μg)	Total Dry wt. of Precipitate (μg)
Guinea Pig Ileum	0.019	2.47	130,190
Rat Uterus	0.010	1.30	130,190
Rat Duodenum	0.004	0.28	130,190

TABLE IV

Yields of Ether-Precipitable, Bradykinin-Like Material From the Digestion of Heat-Treated Human Plasma by Trypsin

Test Tissue	µg Bradykinin per mg dry wt.	Total µg Bradykinin	Total µg dry wt. of Ether Precipitate
Guinea Pig Ileum	0.052	3.67	70,480
Rat Uterus	0.0077	0.54	70,480
Rat Duodenum	0.0037	0.28	70,480
Ileum/Uterus Ratio	6.8		

TABLE V

A Comparison of the Degree of Blocking of the SUT-67 HTP Precipitates With Various M.E.D. of Antagonists

Test Tissue	Test Material		Per Cent Block by: ATR(1)	PBZ(2)	BOL(3)
Guinea Pig Ileum	SUT-HTP	1.	74	85	
		2.	78	85	
		3.	82	100	
	Bradykinin		71	46	
			84	89	
	Acetylcholine		100		
	Histamine			100	
	Serotonin				100
Rat Uterus	SUT-HTP	1.	19		54
		2.	0		53
		3.	20		64
	Bradykinin		0		19
	Acetylcholine		100		
	Serotonin				100

1. ATR = 0.83 μg/ml Atropine
2. PBZ = 0.1 μg/ml Pyribenzamine
3. BOL = 8.3 μg/ml D-Bromolysergic acid

TABLE VI

A Comparison of the Effects of Bradykinin and SUT and Trypsin Digests on the Guinea Pig and Rat Hearts

Test Substance	Response	Guinea Pig	Rat
Trypsin-HTP	Flow	90% increase	46% decrease
	Amplitude	8% decrease	14% decrease
	Rate	no change	no change
SUT-HTP	Flow	71% decrease	87% decrease
	Amplitude	40% decrease	75% decrease
	Rate	50% decrease	25% decrease
2 μg Bradykinin	Flow	30% increase	20% decrease
	Amplitude	25% decrease	12% increase
	Rate	no change	no change
10 μg Bradykinin	Flow	56% increase	12% decrease
	Amplitude	15% decrease	9% increase
	Rate	no change	no change

TABLE VII

A Comparison of the Effects of Blocking Agents on the Ether Precipitable Material From the Trypsin-HTP Digest and On Kinin Standards

Test Tissue	Test Material		Per Cent Block by ATR (1)	PBZ(2)	BOL(3)
Guinea Pig Ileum	Trypsin-HTP	1.	33	0	24
		2.	24	74	25
		3.	0	0	34
		4.*	0	0	0
	Bradykinin	1.	46	0	0
		2.	48	0	24
		3.	28	0	38
		4.*	28	0	31
	Histamine			100	
	Acetylcholine		100		
	Serotonin				100
Rat Uterus	Trypsin-HTP*		4		44
	Bradykinin*		0		60
	Acetylcholine*		100		
	Serotonin*				100

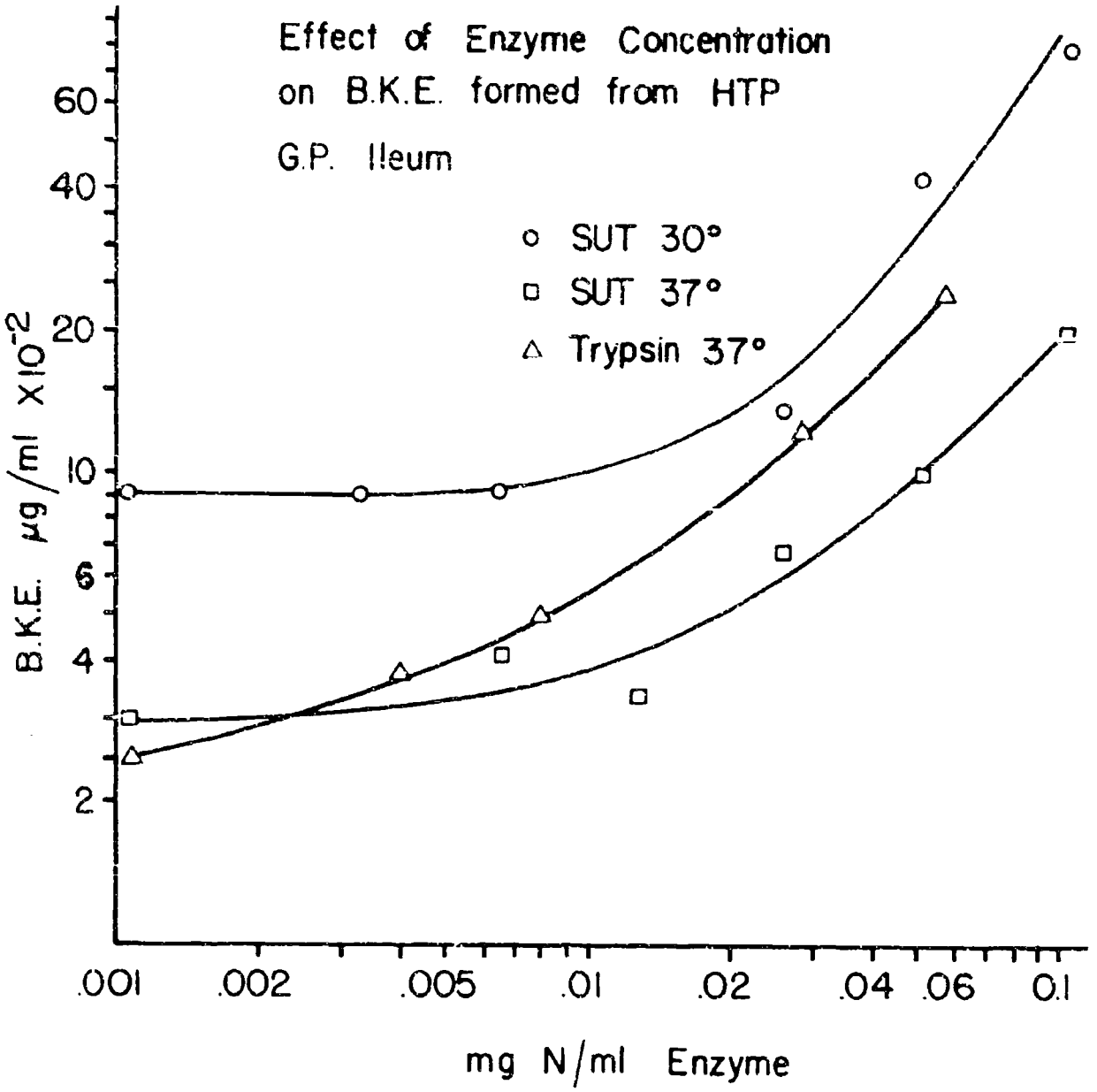
(1) 0.83 μg/ml Atropine

(2) 0.10 μg/ml Pyribenzamine

(3) 8.3 μg/ml D-Bromolysergic Acid

*Experiments done with one blocking agent per gut strip

Fig. 1


Effect of Enzyme Concentration
on B.K.E. formed from HTP
G.P. Ileum
SUT 30°
SUT 37°
Trypsin 37°
B.K.E. μg/ml X10⁻²
60
40
20
10
6
4
2
.001
.002
.005
.01
.02
.04
.06
0.1
mg N/ml Enzyme

B. Further Studies of Kinin Formation Reaction Kinetics and Isolation of Products

D. Reaction Kinetics and Identification of Products

INTRODUCTION

In the preceding section it was shown that a kinin, with pharmacological properties similar to those of bradykinin, was formed by the action of SUT-67 on heat-treated human plasma (HTP). The enzymatic action of the SUT-67 toxin was found to be greater at 30° than at 37°C. Isolation of the bradykinin-like peptides from the crude digest was made by the method of Prado *et al.*, (1) but the amount of bradykinin recovered was small and there was a large amount of inactive material associated with it.

In the present study the rate of bradykinin formation, substrate digestion, and degradation of bradykinin were studied at 27°, 32°, and 37°C, in order to evaluate the kinetics of formation of bradykinin-like material from the digestion of HTP. Since 27°C appeared to be near the optimal temperature for bradykinin formation, two adaptations of the Prado extraction procedure, as well as gel filtration on Sephadex G-25, were used to isolate dialyzable products from the digestion reaction at 27°C in order to determine the most efficient way to recover bradykinin from the crude digest.

Further purification of the Sephadex fraction by paper chromatography showed that there was a peptide present with the same mobility as the bradykinin standards used for comparison. This peptide was eluted from the paper and shown to have bradykinin-like activity on the rat uterus.

MATERIALS

Materials used in these experiments that have not been previously described were: Sephadex G-25, Whatman #1 filter paper for chromatography, Glacial acetic acid, ninhydrin, and acetone.

METHODS

The reaction mixtures consisted, in each case, of 4 ml of HTP substrate and 1 ml of SUT-67. Samples were incubated for varying lengths of time on a shaker at 27°, 32°, or 37°C. In the time study the SUT-67 concentration was kept constant at 0.26 mg ppt N/ml. In the study of the effect of concentration the SUT-67 was geometrically diluted, starting with a concentration of 0.52 mg ppt N/ml. Controls from which either substrate or enzyme were omitted were similarly treated.

Isolation and Identification of Digestion Products

Since the first experiments indicated that one hour's incubation at 27°C gave the best yields of bradykinin 240 ml of HTP was digested by 60 ml of SUT-67 containing 0.26 mg ppt N/ml. After the reaction was stopped by heating the mixture to 60°C for 30 minutes, the reaction mixture was distributed in 50 ml aliquots into dialysis bags and dialyzed against 1,500 ml of distilled water for 24 hours in the cold room. A sample of the external dialysate was taken for analysis, and the remainder was refrigerated. This procedure was repeated and the 1st and 2nd external dialysates were pooled, evaporated to 110 ml, and then frozen. The third external dialysate and the reaction mixture were discarded. The equivalent bradykinin content in the reaction mixture was determined by bioassay on the rat uterus.

For comparative purposes aliquots of the external dialysate were purified in two ways: i.e. 25 ml by solvent extraction and precipitation according to the Prado technique, as described in the Section A of this report, and 5 ml by ascending column chromatography on Sephadex G-25. In the latter case, the contents of all tubes containing active materials were pooled, evaporated to dryness *in vacuo*, and taken up in 1.0 ml of water for further fractionation and bioassay. Five 10 µL aliquots of the material obtained from the column were repetitively spotted on Whatman #1 filter paper along with a bradykinin standard similarly eluted from a separate G-25 column. The solvent system employed was butanol-glacial acetic acid - water (4:1:1). The paper was dried for 4 days and one half was developed in 0.2% ninhydrin in acetone. The suspected bradykinin areas in the non-developed portion were cut out, eluted with 1 ml of 1% NaCl, and bioassayed.

Destruction of Synthetic Bradykinin by SUT-67

The kininolytic power of SUT-67 was measured by its ability to destroy synthetic bradykinin at 27°, 32°, and 37°C. The experimental conditions were identical with those used for the digestion of HTP by toxin. Thus, 4-ml quantities of substrate solution containing 0.2 µg of bradykinin were incubated with 1 ml (0.52 mg N) of SUT-67 at the temperatures specified for a period of 10, 20, 40, and 60 minutes. All samples were dialyzed and the dialysates bioassayed for bradykinin on the rat's uterus.

Protein Digestion by SUT-67

Additional evidence for the proteolytic activity of SUT-67 was sought by determining the relationship between the formation of bradykinin and the digestion of proteins in the substrate solution. The latter was estimated as the increase in non-precipitable N as measured on the supernates remaining after precipitation with 10% trichloroacetic acid. The supernate N was measured according to the method of Feigen, *et al.*, (2).

RESULTS

The Effect of Temperature on the Time-Course of Kinin Formation

The time-course of bradykinin formation from the digestion of HTP by SUT-67 is summarized in Table I. It is clear that the amount of bradykinin formed is directly related to the length of time of incubation at 27° and 32°C. At 37°C, however, the amount of BKE formed reaches a maximum at 10 minutes and then declines, indicative of the activation of the kininase. The results show that SUT-67 forms more BKE at 27°C (1.2×10^{-2} μg/hour), than at 32°C (1.1×10^{-2} μg/hour), or at 37°C, (8.8×10^{-3} μg/hour).

It is evident from these results that the competitive enzyme systems involved in kinin formation and destruction are highly temperature-dependent. In both cases the lowest temperature produces the greatest rate of net product output. As the temperature is increased to 32°C, the net output declines with time. At the highest temperature the separation in the activity between the kininogenic and the kininolytic enzymes is most apparent. This is particularly well illustrated in assays on the rat's uterus (shown in Fig. 1): there is a great burst of kinin formation between 0 and 10 minutes followed by a rapid fall so that the net production of kinin after an hour's incubation at 37°C falls below that obtained at 32° and 27°C.

Although the guinea pig assay (Fig. 2) follows the same general pattern as the rat's uterus, the actual yields of active material are, on the whole, higher for the respective temperatures and it is significant to note that even the controls (0 time) had a significant level of activity, showing that some of the kinin activity did not result from an interaction between the HTP and SUT-67. Since the guinea pig ileum is sensitive to other agents beside bradykinin, it is likely that the difference in results may have reflected the elaboration of other active materials in the reaction. The chemical demonstration that significant amounts of histamine were present at 0 time and varied phasically during the course of the reaction does not entirely explain the difference between the two assays.

Digestion of Bradykinin

Confirmatory evidence that the increased kininase activity could account for the reduction of the net output of active material was sought by direct tests on the inactivation of synthetic bradykinin at 27°, 32°, and 37°C. These results are shown in Table II. They confirm that the toxin has a rapid destructive effect on this peptide with an apparent optimum at 32°C. Since more than one material evidently is produced in the attack of SUT on HTP, these results only partially account for the time-courses in the preceding experiment.

Digestion of Protein by SUT-67

Tables I and III show that the increase in the level of NPN formed with the time of digestion, and with toxin concentration, in general parallels the changes seen in the production of active kinin estimated on the rat's uterus. The close correlation between kinin formation and protein destruction is exhibited in Fig. 1 for the 27°C experiment which contains values taken from both the time-course and the enzyme-dependence experiments. As the temperature of reaction is increased, the relationship departs from linearity because of the evident degradation of the kinin produced at the higher temperatures.

Isolation of Kinins Formed in the large-scale Reaction at 27°C

A large-scale preparation was made under the conditions found in the foregoing pilot studies to insure the maximal net production of kinin. A solution of heat-treated plasma containing about 2.5 grams of protein N was treated with 60 ml of crude SUT-67 made up to a concentration of 0.26 mg protein N/ml. The course of the reaction was monitored by taking samples for bioassay at various times during the 60-minute incubation period. The results of these assays, exhibited in Table IV confirm the pilot results about the speed of the reaction. Thus, at 1 minute the concentration of active material was already 50 per cent of that achieved at the end of 60 minutes. The reaction was stopped by heating the system to 60°C for 30 minutes and dialyzing it three times against 1500 ml of water. Aliquots of each dialysate were bioassayed and the first two dialysates (which contained activity) (Table V) were pooled and concentrated in a rotary evaporator to 110 ml. According to the bioassay carried out on the concentrate the total recovery of dialyzable nitrogenous material was 16.8 mg N which contained 6.2 μg of bradykinin-equivalent material. The final recovery of BKE fell somewhat short of that predicted by the control assay at 60 minutes. Taking into account the reaction volume, 300 ml, and the concentration of BKE, 5.5 μg/ml as obtained by bioassay, we should have obtained 24 μg as the total yield. The total yield actually obtained was equivalent to 16.5 μg of bradykinin. After the dialyzates had been concentrated, additional losses occurred such that the final yield was 6.2 μg of BKE. Since there was only 6.2 μg of BKE recovered for 16.83 mg protein N, a ratio of 0.37 μg BKE/mg protein N, it is evident that there is a great deal of pharmacologically inert material present in the dialysate.

An estimate of the comparative efficiencies of the pilot and the large scale reactions can be made by comparing the BKE yields with respect to the protein N inputs in the two cases. On the basis that 0.6 μg BKE was yielded for 41.4 mg of protein N in the first case, and 16.5 μg BKE was obtained from 25412 mg protein N in the second instance, the yield ratio of the large scale reaction, 6.49×10^{-3} μg/mg N, was only 45 per cent of the figure 14.4×10^{-3} μg/mg N obtained in the pilot experiment.

The isolation of active kinin from the dialysate was made by two methods, for comparative purposes. In the first instance a 25 ml aliquot of the pooled external dialysate was treated by the solvent extraction method of Prado *et al.*, (1) and in the second case a 5 ml aliquot was fractionated directly on a Sephadex G-25 column. The effluent profile at 260 mμ for the separation is shown in Fig. 4. It is significant to note that most of the BKE activity is associated with the region of minimal UV absorption, i.e. between tubes 108 and 125. The data in Table VI show that of the 1.4 μg BKE subjected to the solvent extraction, 0.75 μg, or 54 per cent was recovered, whereas 100 per cent recovery was obtained by the column method.

Identification of Bradykinin by Paper Chromatography

Samples of active materials obtained by the purification of the external dialysate on Sephadex were further analyzed by descending paper chromatography and compared to the results obtained with synthetic bradykinin. The untreated dialysate contained at least 10 different peptides or amino acids but after chromatography only 7 ninhydrin-positive components remained. The bradykinin standard gave 2 spots, only one of which had pharmacological activity. The unknown had 3 active areas, one of them matching identically the more mobile spot of bradykinin.

These results confirm our prior inference that although bradykinin was formed in the reaction there were also present other active bradykinin-like materials which could account for the difference between the two assay methods to monitor the purification.

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2. Feigen, G. A., J. M. Campbell, G. B. Sutherland, and G. Markus, "Simultaneous Estimation of Serum Proteins and Oxypolygelatin by Phenol:Nitrogen Ratio," J. App. Physiol. 7:154-160, 1954.

TABLES

I Effect of Temperature on the Time-Course of HTP Digestion by SUT-67

II Temperature and The Rate of Digestion of Bradykinin by SUT-67

III The Effect of SUT-67 Concentration and Temperature on the Digestion of HTP

IV The Time-Course of Kinin Formation During the Digestion of 240 ml of HTP by 60 ml of 0.26 mg Protein N/ml SUT-67 at 27°C

V Recovery of Dialyzable Kinins From the Digestion of 240 ml of HTP By 60 ml of 0.26 mg ppt N/ml SUT-67 at 27°C

VI A Comparison of the Yields of Dialyzable Kinins Between The Prado and Sephadex G-25 Extraction Procedures

TABLE I

Effect of Temperature on the Time-Course of HTP Digestion by SUT-67

ASSAY			Time in Minutes 10	20	40	60
Rat uterus	BKE*	27°	5.4	8.4	10.00	12.0
		32°	4.8		9.0	11.0
		37°	14.00	10.00	9.3	8.8
GP Ileum	BKE*	27°	11.00	14.00	16.00	17.00
		32°	9.2	10.00	12.00	13.00
		37°	10.00	9.2	7.6	9.2
GP Ileum	Hist.**	27°	1.00	2.1	2.9	3.1
		32°	1.8	2.2	3.0	3.3
		37°	4.6	4.1	2.4	3.7
Chemical	Hist.***	27°	4.1		3.1	3.0
		32°	3.6	5.9	4.2	5.5
		37°	3.7	4.5	3.2	4.1
mg/ml Protein N digested	****	27°	5.7	6.9	8.8	9.2
		32°	7.5	9.0	11.0	12.2
		37°	10.00	10.00	8.6	9.8

* BKE in μg/ml x 10^{-2}

** Hist. in moles/L x 10^{-6}

*** Hist. in moles/L x 10^{7}

**** mg/ml N digested x 10^{-2}

TABLE II

Temperature and The Rate of Digestion of Bradykinin by SUT-67

	Temp.	Time in Minutes			
		10	20	40	60
μg/ml BKE digested	27°	3.40	3.58	3.81	4.00
x 10^{-2}	32°	3.00	3.53	4.00	
	37°	2.80	3.32	3.68	3.83
Per cent BKE digestion	27°	85.00	89.00	95.00	100
	32°	75.00	88.40	100	
	37°	70.00	83.00	92.00	98.3

TABLE III

The Effect of SUT-67 Concentration and Temperature on the Digestion of HTP

		SUT-67 Concentration in mg ppt N/ml				
		6.5×10^{-3}	1.3×10^{-2}	2.6×10^{-2}	5.2×10^{-2}	$7{,}04 \times 10^{-1}$
GP Ileum	27°	9.1		12.5	17.00	20.00
BKE formed *	32°	14.0	16.00	14.00	20.00	20.00
(SUT-67)	37°	3.1	8.0	13.00	11.00	13.00
mg ppt N	27°	2.5	2.5	5.3	10.00	15.00
digested **	32°	2.4	3.6	5.7	11.40	13.50
(SUT-67)	37°	2.3	3.2	4.5	7.9	19.00

* BKE $\times 10^{-2}$ µg/ml

** mg ppt N/ml $\times 10^{-2}$

TABLE IV

The Time-Course of Kinin Formation During the Digestion of 240 ml of HTP By 60 ml of 0.26 mg Protein N/ml SUT-67 at 27°C

	Time in Minutes				
	1	10	20	40	60
BKE μg/ml x 10^{-2} *	2.7	3.4	4.4	4.3	5.5

* Bioassayed on the Rat's uterus

TABLE V

Recovery of Dialyzable Kinins From the Digestion of 240 ml of HTP By 60 ml of 0.26 mg ppt N/ml SUT-67 at 27°C

Sample	vol.	BKE µg/ml	Total BKE µg BKE	Total mg N/ml	Total mg N Recovered
HTP-SUT-67 Digest	300	5.5×10^{-2}	16.5	10.34	2542
1st external dialysate	1500	3.1×10^{-3}	4.7	0.0107	16.05
2nd external dialysate	1500	1.0×10^{-3}	1.5	-	-
3rd external dialysate	1500	0	0	-	-
Pooled concentrated 1st & 2nd external dialysate	110	5.6×10^{-2}	6.2	0.153	16.83

TABLE VI

A Comparison of the Yields of Dialyzable Kinins Between The Prado and Sephadex G-25 Extraction Procedures

Method		Input			Recovery			
	Sample volume (ml)	Total mg N	Total µg BKE	µg/BKE / mg N	Total mg N	Total µg BKE	µg BKE / mg N	% BKE Recoverd
Prado	25	3.83	1.4	0.36	0.56	0.75	1.33	54
Sephadex G-25	5	0.77	0.28	0.36	0.12	0.28	2.39	100

FIGURES

1. Time Course of BKE Formation from HTP at 27, 32, and 37°C as Assayed on the Rat's Uterus
2. Time Course of BKE Formation from HTP at 27, 32, and 37°C as Assayed on the Guinea Pig Ileum
3. Correlation Between Kinin Formation and Plasma Protein Digestion [SUT-67 + HTP; 27°C]
4. Elution Profile of Dialyzable Material from the Hydrolysis of HTP by SUT-67 on Sephadex G-25

Fig. 1

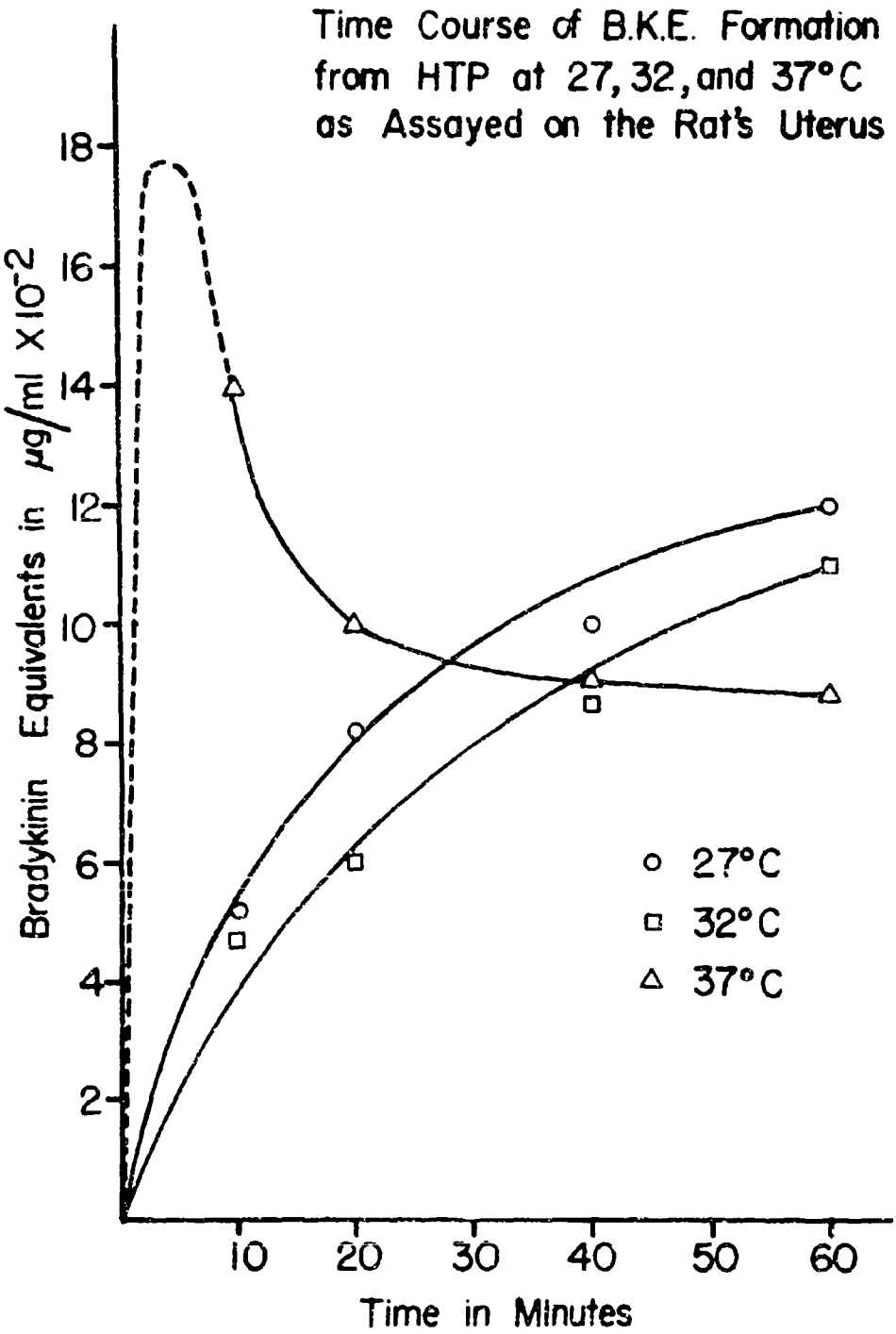

Time Course of B.K.E. Formation from HTP at 27, 32, and 37°C as Assayed on the Rat's Uterus
Bradykinin Equivalents in μg/ml X 10⁻²
18
16
14
12
10
8
6
4
2
10
20
30
40
50
60
Time in Minutes
27°C
32°C
37°C

Fig. 2

Time Course of B.K.E. Formation from HTP at 27, 32, and 37°C as Assayed on the Guinea Pig Ileum

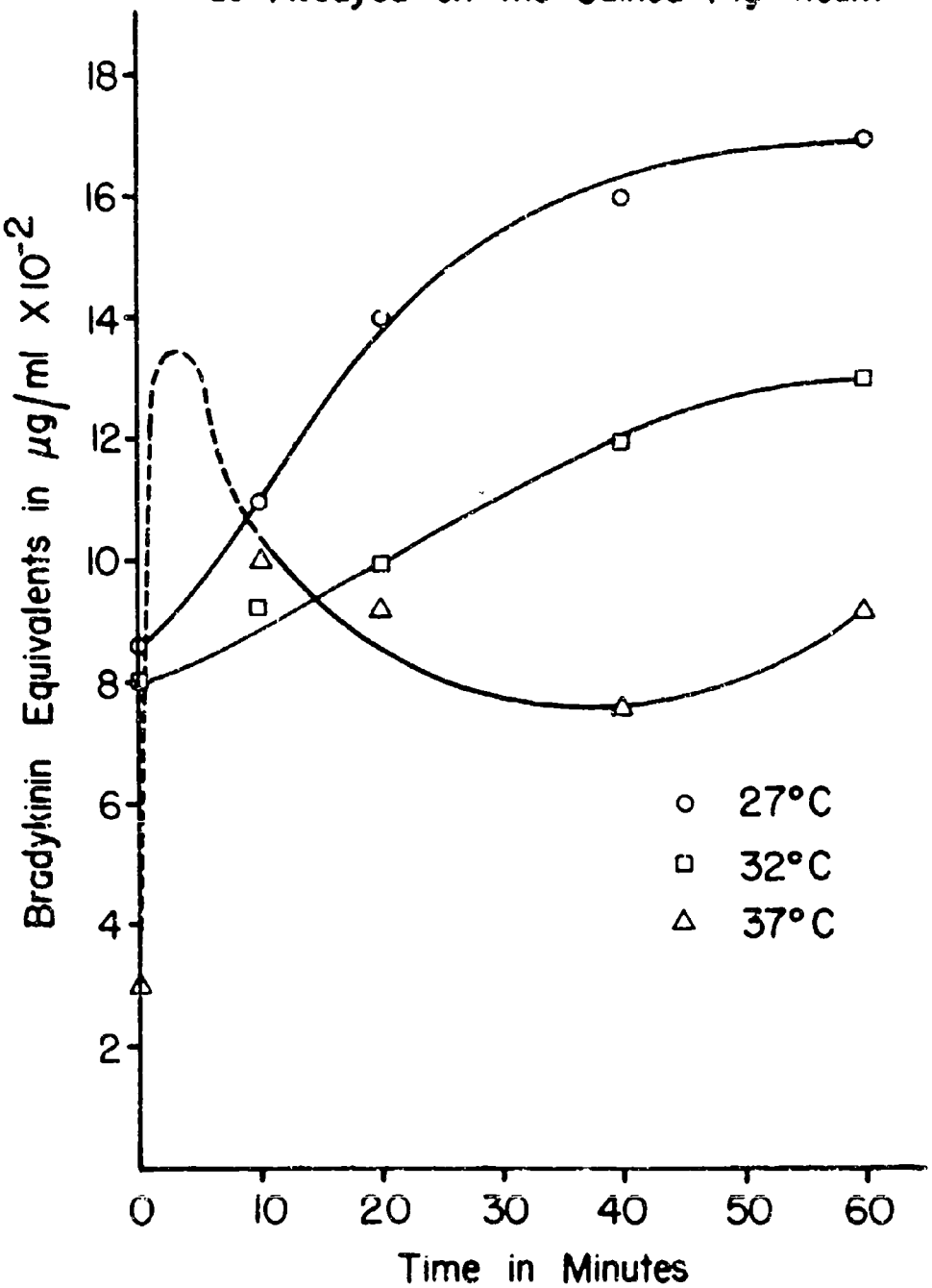


Fig. 3

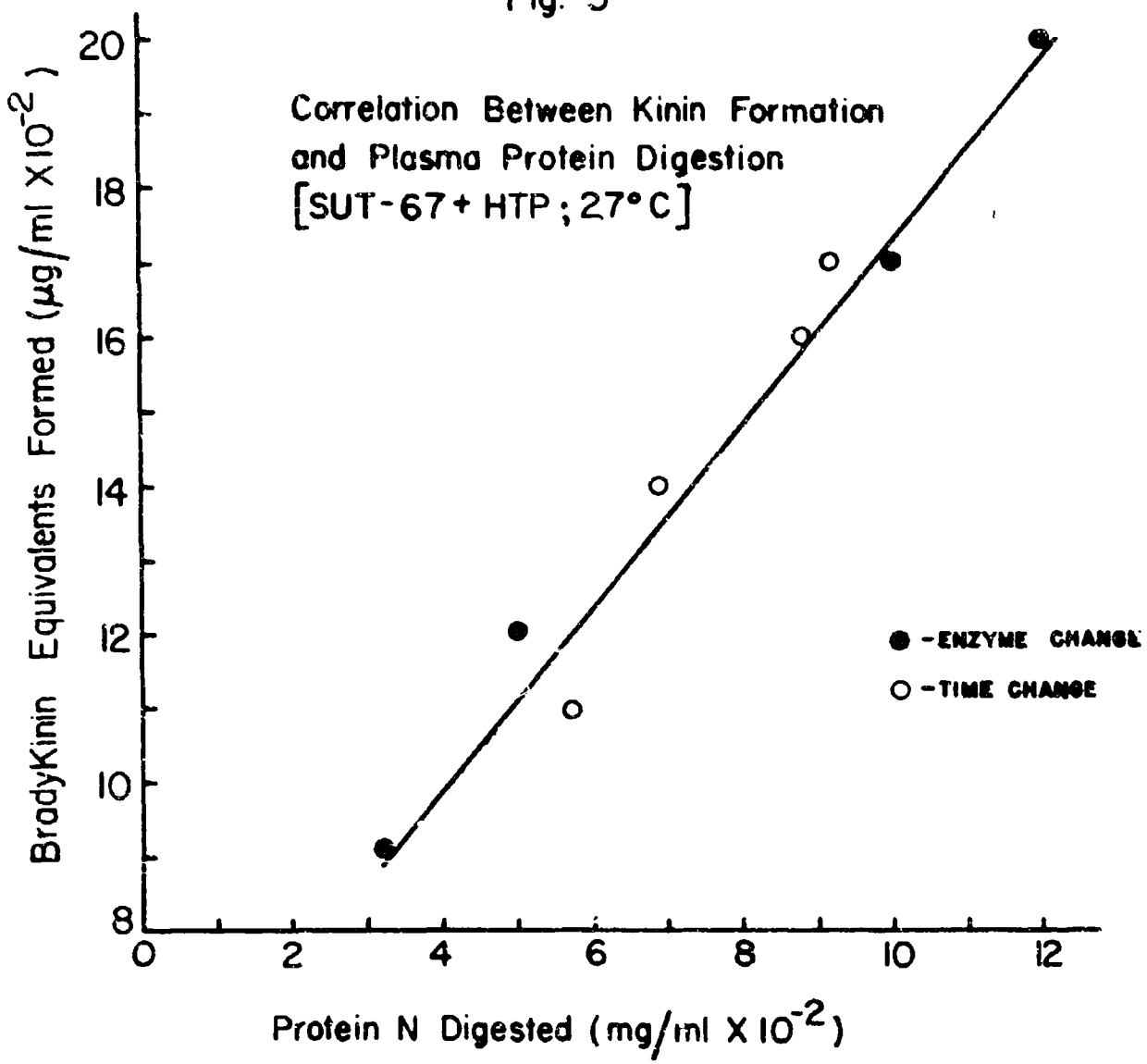
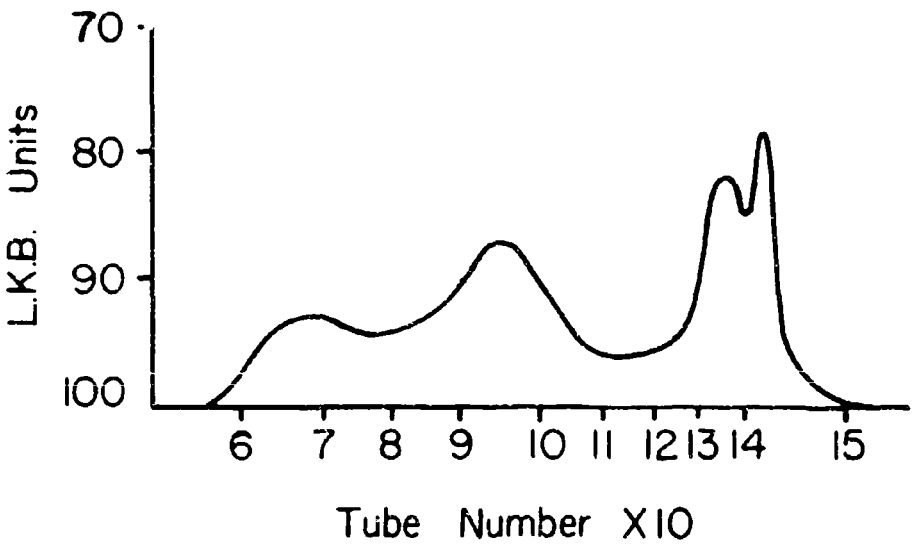

Correlation Between Kinin Formation
and Plasma Protein Digestion
[SUT-67 + HTP ; 27° C]
BradyKinin Equivalents Formed (μg/ml X10^-2)
20
18
16
14
12
10
8
0
2
4
6
8
10
12
Protein N Digested (mg/ml X 10^-2)
● -ENZYME CHANGE
○ -TIME CHANGE

Fig. 4

Elution Profile of Dialyzable Material from the Hydrolysis of HTP by SUT-67 on Sephadex G-25



C. Physical-chemical, Immunological, and Enzymatic Properties of Purified Toxin Fractions

C. Physical-Chemical, Immunological, and Enzymatic Properties of Purified Toxin Fractions.

INTRODUCTION

Studies described in the present section were directed to the further purification and characterization of the proteolytic enzyme, and to the elucidation of the point of attack of the enzymes on the natural (and synthetic) substrates.

MATERIALS AND METHODS

Three thousand specimens of *tripneustes gratilla*, L., were collected and processed in Honolulu in 1968. Extraction of the pedicellaria obtained from 2000 specimens gave 0.259 grams of crude pedicellarial toxin. This harvest yielded a starting material of far greater potency than heretofore had been the case. The LD_{50} was 9.8×10^{-5} mg N/20 g mouse, or 10^4 LD_{50}/mg N. Our previous most active crude preparation had 10^3 LD_{50}/mg N and the most active fraction obtained by purification on Sephadex G-200 had 18×10^3 LD_{50}/mg N.

The shelf-stability of the crude preparation was improved by storing it in the deep-freeze, dissolved in 0.3 *M* NaCl. Whereas the lyophilized material had a 1/2-life of about 36 months, the frozen preparation, retested on separate occasions over a 300-day period, showed essentially no loss in potency if the random variation of the assay method is taken into account. These results are exhibited in Table I.

TABLE I

Effect of Storage Time on the Potency of Crude Pedicellarial Toxin (SUT-1967)

Time of LD_{50} Determination (days)	LD_{50} in mg ppt Nitrogen	LD_{50}/mg ppt N
0	1.29×10^{-4}	7.75×10^3
77	1.15×10^{-4}	8.60×10^3
105	1.75×10^{-4}	5.71×10^3
139	1.49×10^{-4}	6.71×10^3
167	1.30×10^{-4}	7.75×10^3
194	1.63×10^{-4}	6.15×10^3
230	1.30×10^{-4}	7.75×10^3
261	1.70×10^{-4}	5.88×10^3
292	1.49×10^{-4}	6.71×10^3

Mean LD_{50}/mg N = 7.00 ± 0.98 ($\times 10^3$)

RESULTS

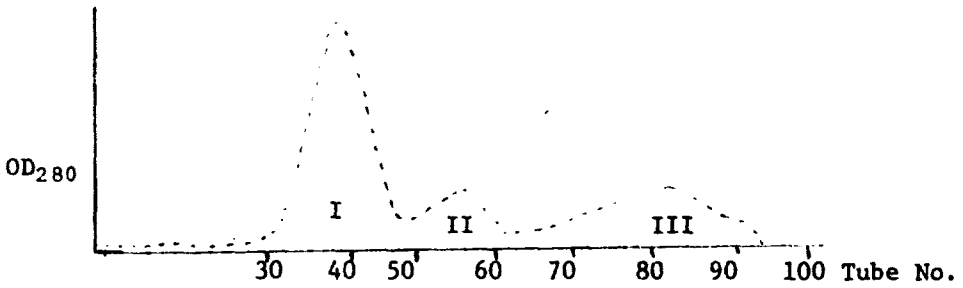
Enzymes

The fact that the most active Sephadex fraction (II) appeared to have more than one specificity suggested that the components of SUT might not be separable by molecular weight alone. In order to get a greater degree of resolution and a high capacity we next employed hydroxylapatite gel and carried out comparative studies of the activities of fractions produced by Sephadex and Hydroxylapatite.

1. Sephadex Fractionation of SUT-67

The fractionation of SUT-67 was carried out in the cold (4°C) using a Sephadex Gel G-200 equilibrated with phosphate buffered saline 0.01 μ; pH 7.0. Three components appeared:

FIG. 1. Fractionation of SUT-67 on Sephadex G-200



The toxicity of the parent toxin SUT-67 (7.7×10^3 LD_{50}/mg ppt. N) was distributed as follows among the three fractions (as seen in Table II):

TABLE II

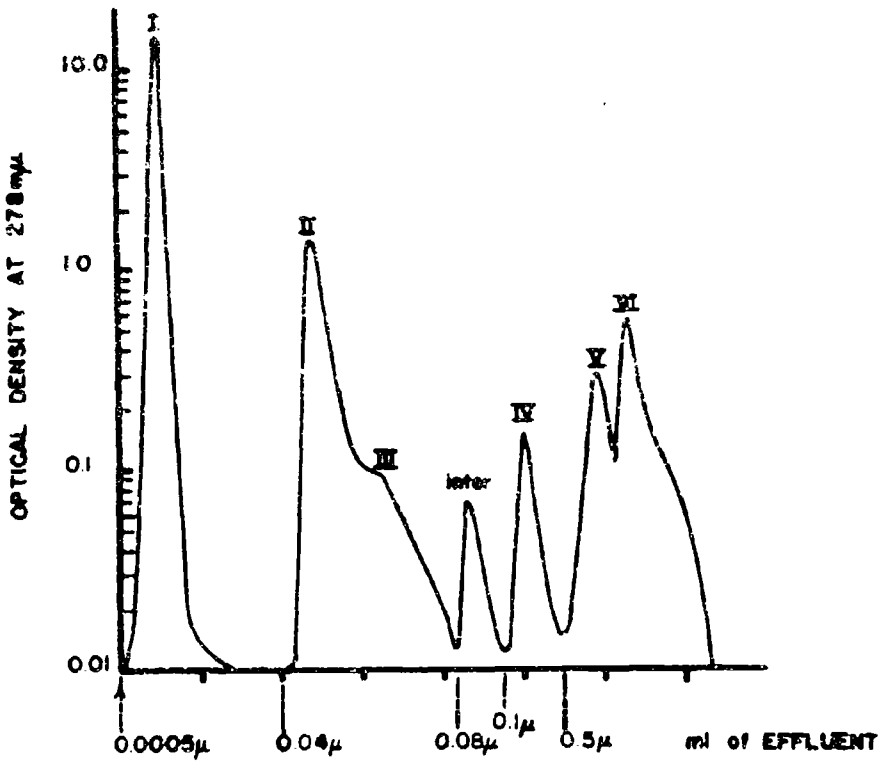
Distribution of Toxicity in SUT-67 and Its Sephadex Fractions

Material	LD_{50}/mg precipitable N
SUT-67	7.75×10^3
Fr. I	3.5×10^3
Fr. II	2.0×10^4
Fr. III	1.4×10^3

Each fraction, after elution from the column, was concentrated by pressure dialysis against phosphate buffered saline (μ= 0.01) pH 7.0 and rechromatographed. A single component was obtained for each fraction.

Fig 2

ADSORPTION CHROMATOGRAPHY of CRUDE SEA UNCINA TOXIN with HYDROXYLAPATITE (STEPWISE ELUTION with PHOSPHATE BUFFER pH 6.8 of INCREASING IONIC STRENGTH (μ))



Immunological Analysis of SUT-67 and Its Sephadex Fractions.

Antibodies were produced in the rabbit against the parent toxin and Fr. I, II, and III, by subcutaneous injections of the formalin-treated materials in complete Freund's adjuvant on opposite sides of the neck. Two weeks later the rabbits were given a booster dose intradermally of toxoided material in complete Freund's. The rise in antibody titer could be followed by ring testing. Only late secondary antibodies showed any strong affinity for the antigens, as revealed by immunoelectrophoresis and quantitative precipitation analysis.

Quantitative Precipitation: Precipitation did not occur in AG excess even though a visual OP could be observed in certain cases, but was absent after storage at 4°C for 48 hours.

Immunoelectrophoresis: Immunoelectrophoresis in 1% barbital agar (0.05 μ) revealed 5 components for SUT-67; one cathodic and four anodic. Fr. I shares 3 components, the cathodic one being unique to it. Fr. II is the purest fraction, sharing 1 major component (anodic) with SUT-67. Fr. III shares the other major anodic component with SUT-67 and also possesses 5 additional separate and *unique* antigenic anodic components that were probably exposed during the fractionation procedure and that produced precipitating antibodies only during the late secondary response.

2. Hydroxylapatite Fractionation of SUT-67

Preparation of Hydroxylapatite Columns: Twenty grams of hydroxylapatite (Bio-Gel HTP from Bio-Rad Laboratories) was washed with 200 ml of phosphate buffer pH 6.8 (0.001 μ) and then equilibrated with 500 ml of the same buffer. In this fashion the calcium phosphate can be stored at 4°C until ready to be used.

Fractionation: Three grams of the adsorbent was poured into a small Pharmacia column (2.5 x 30 cm) and washed for 3 hours with phosphate buffer pH 6.8 (0.0005 μ), before use.

During fractionation, the eluting buffer was maintained 20 cm above the surface of the calcium phosphate, thus allowing a fast elution rate of about 20 drops per minute. The small column prevented dilution of the fractions and allowed sharper peak resolutions. The procedure is summarized in Table III and an elution diagram of the chromatographic separation of SUT-67 on hydroxylapatite is presented in Figure 2.

TABLE III

Summary of the Elution Procedure for the Fractionation of SUT-67 On Hydroxylapatite

Phosphate Buffer pH 6.8 (μ)	Fraction(s) Eluted	Temp. of Elution °C
0.0005	I	4
0.04	II	"
0.04	III	"
0.08	Intermediate	"
0.1	IV	"
0.5	V	Room temp. (24°C)
	VI	" " "

Recovery: After fractionation, the preparations were dialyzed against phosphate buffer pH 7.0 (0.01 μ) and then concentrated by pressure dialysis (negative pressure: 15 inches of Hg). Nitrogen determinations were carried out for pre- and post-dialysis fractions. The per cent recovery of total and precipitable N is as follows:

TABLE IV

Per Cent Recovery of Nitrogen in the Fractionation of SUT-67 on Hydroxylapatite

Fraction	% Recovery Total N	% Recovery of ppt. N
I	60	complete
II	77.5	"
III	62.0	"
Intermediate	72.0	"
IV	69.0	"
V	81.0	"
VI	50.0	"

Resolution of Sephadex Fractions on Hydroxylapatite

This study shows that both Sephadex Fraction I and Sephadex Fraction III can be further resolved on hydroxylapatite by elution at increasing ionic strength. The total number of fractions obtained by HOA was at least 6 as compared to the 3 given by Sephadex G-200. Rechromatography of Sephadex fractions on Sephadex and hydroxlyapatite is presented in Figures 3 - 6.

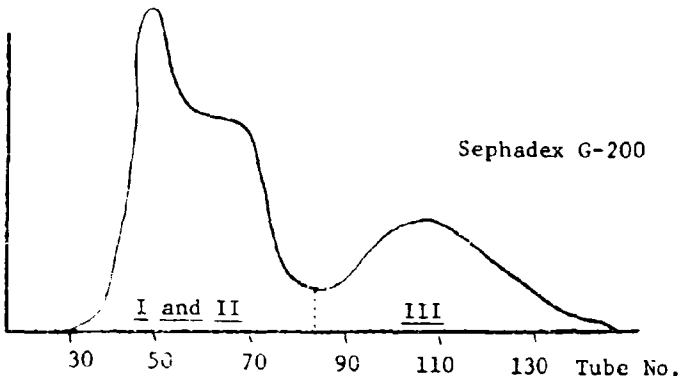
Fig. 3. Fractionation of Pooled Sephadex Peaks I and II on Sephadex G-200.

Fig. 4. Elution Diagram of SUT-67 on Sephadex G-200.

Fig. 5. Fractionation of Sephadex Fraction III on Hydroxylapatite.

Fig. 6. Fractionation of Sephadex Fr. I on Hydroxylapatite.

FIG. 3



I and II are pooled together, concentrated, and rechromatographed.

FIG. 4

Refractionation of I and II
Each Sephadex Fraction is chromatographed [on HOA] below:

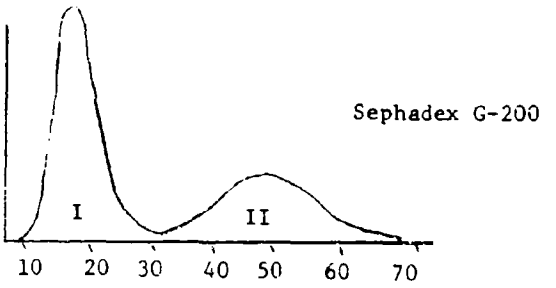


FIG. 5

Sephadex Fr. III

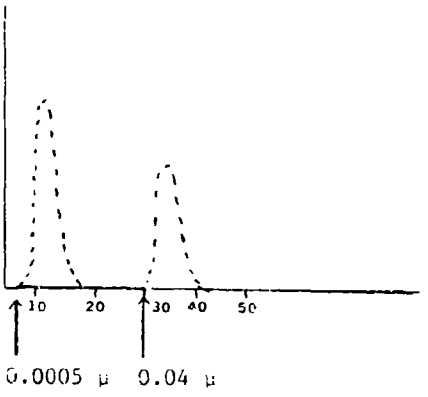
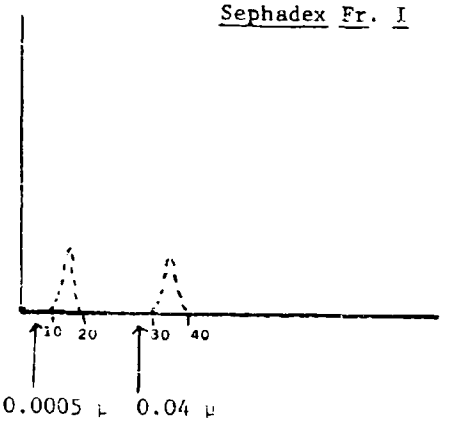


FIG. 6

Sephadex Fr. I



Substrates

The availability of a variety of physically distinct fractions now permitted a more sophisticated evaluation of the point of attack of certain of the proteolytic enzymes. Studies with azocasein, TAME, and ATEE showed that the crude preparation attacked azocasein and ATEE, but had only a slight effect on TAME suggesting that the major proteolytic activity was chymotrypsin-like.

The purified fractions attacked the synthetic substrates according to classical Michaelis kinetics: the substrate curves were rectangular hyperbolas with respect to substrate concentration and linear with respect to enzyme concentrations, permitting us to calculate meaningful K_m and V_{max} values. These general results are exhibited in Tables V, VI, VII, VIII, and IX, and are shown in Figures 7 - 12.

SUMMARY

A summary of the physical-chemical, enzymological and pharmacological tests is given in Table X for the Sephadex and hydroxylapatite fractions.

The greatest lethal potencies are present in Fr. II of the hydroxylapatite series and Fr. II of the Sephadex set. The molecular species producing this potency is in the range 5.7 - 6.4*S*. In the Sephadex series this has no trypsin or chymotrypsin-like potency while in the OH-A set it is associated with the highest K_m value for ATEE. Hemolysis was produced by all of the preparations tested.

We conclude from these results that the lethal toxicity is not clearly correlated to the proteolytic activity, *i.e.* to the formation of bradykinin; indeed, the injection of large doses of synthetic bradykinin fails to kill mice. It is significant that although specific antibody to fraction (Sephadex) I completely blocked its lethal toxicity, it had no effect on its ability to hydrolyze ATEE. The lethal toxicity is unlikely to be due to hemolysis since that is present quite potently in some of the non-lethal preparations.

TABLES

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TABLE V

Digestion of the Artificial Substrates TAME (Tosyl Arginine Methyl Ester) And ATEE (N-acetyl-l-tyrosine ethylester) by Trypsin*, Chymotrypsin** and SUT-67

Enzyme	Concentration	Substrate	Substrate Conc.	Rate of Digestion (in µl of 0.1 N NaOH/hr)
Trypsin	0.0075***	TAME	0.01 *M*	10,814
SUT-67	0.0102†	TAME	0.01 *M*	75
Chymotrypsin	0.0063***	ATEE	0.014 *M*	8,043
SUT-67	0.0127†	ATEE	0.014 *M*	490

* Trypsin: 2X crystalline (Nutritional Biochemical Corporation)

** Chymotrypsin: 3X crystalline (Nutritional Biochemical Corporation)

*** milligrams dry weight

† mg N/ml

TABLE VI

Digestion of the Artificial Substrates ATEE (N-Acetyl-l-tyrosine ethylester) And TAME (Tosyl Arginine methyl ester) by Sephadex G-200 Fractions I, II, And III From SUT-67

Enzyme	mg ppt N/ ml	Substrate	Substrate Conc. M/L	Inhibitor	LD_{50} mg ppt N/ mouse	Rate of Digestion (in µl of 0.1 N NaOH/hr)
SUT-67 Seph.						
Fr. I	0.0057	ATEE	0.014	None	2.85×10^{-4}	336
Fr. I	0.0009	TAME	0.01	None	2.85×10^{-4}	32
Fr. I	0.0057	ATEE	0.014	0.1 ml Anti-Fr.I*	0	336
Fr. II	0.0065	ATEE	0.014	None	5.0×10^{-5}	0
Fr. II	0.0010	TAME	0.01	None	5.0×10^{-5}	0
Fr. III	0.0066	ATEE	0.014	None	7.1×10^{-4}	0
Fr. III	0.0011	TAME	0.01	None	7.1×10^{-4}	0

* Rabbit antibody

TABLE VII

Digestion of Azocasein in 0.1 *M* Borate Buffer pH 8.4 by Sephadex G-200 Fraction I, II, and III From SUT-67 And Ammonium Sulfate Fractions, 33% 65%, and 100% From SUT-64-2

Enzyme	mg ppt N/ml	Substrate (1.3%)	Δ OD_{390}
None	0	Azocasein	0
SUT-67	0.1767	"	0.316
SUT-67	0.1767	None	0
SUT-67 Seph.			
Fr. I	0.0312	Azocasein	0.164
Fr. II	0.0356	"	0
Fr. III	0.0363	"	0
SUT-64-2			
AS 33%	0.0404	Azocasein	0.158
AS 65%	0.0420	"	0.221
AS 100%	0.0670	"	0.373

TABLE VIII

Digestion of Bradykinin, Bradykinin in the Presence of 500 units of Trasylol, And Heat-Treated Human Plasma by Sephadex G-200 Fractions I, II, and III From SUT-67

Enzyme	mg ppt N/ml	Substrate	Inhibitor	BKE After Digestion µg/ml
SUT-67	0.0530	Bradykinin 0.04 µg/ml	none	0
Seph. Fr. I	0.0093	"	"	0
Seph. Fr. II	0.0107	"	"	2.7×10^{-2}
Seph. Fr. III	0.0109	"	"	3.9×10^{-2}
0	0	"	"	3.1×10^{-2}
SUT-67	0.0530	Bradykinin 0.04 µg/ml	Trasylol 500 units	0
Seph. Fr. I	0.0093	"	"	0
Seph. Fr. II	0.0107	"	"	3.9×10^{-2}
Seph. Fr. III	0.0109	"	"	2.5×10^{-2}
0	0	"	"	3.9×10^{-2}
SUT-67	0.0530	Heat-Treated Plasma 10.34 mg ppt N/ml	none	3.3×10^{-2}
Seph. Fr. I	0.0093	"	"	2.8×10^{-2}
Seph. Fr. II	0.0107	"	"	1.9×10^{-2}
Seph. Fr. III	0.0109	"	"	0
0	0	"	"	0

TABLE IX

Digestion of 1.3% Azocasein in 0.1 *M* Borate Buffer pH 8.4 by C Hydroxylapatite Fractions I-II, III, and IV From SUT-67

Enzyme	Substrate (1.3%)	mg ppt N/ml	mg ppt N LD_{50}	Δ OD_{390} units/hr
None	Azocasein	0	0	0
Fr. I and II	"	0.0166	0	48
Fr. III	"	0.0126	1.5×10^{-4}	19
Fr. IV	"	0.003	5.6×10^{-4}	53

TABLE X

Physical-chemical, Enzymological, and Biological Properties Of SUT Fractions

Preparation		N/mg		LD_{50}/mg		Enzymology			Hemolysis
Type	No.	Total TCA P/T %	S_{20}	LD_{50} / mg Total N	LD_{50} / mg/ppt N	Azocasein U/mg N*	TAME K_m	ATEE K_m	H_{50} (mg N)
Sephadex	Crude	67.6	20-60-2.1	5.24×10^3	7.75×10^3	1.2	0	4.07×10^{-3} *M*	2.0×10^{-5}
"	I	40.4	20 and 6.4	1.85×10^3	3.5×10^3	3.50	0	1.97×10^{-3} *M*	3.0×10^{-4}
"	II	62.0	5.68	1.24×10^4	2.0×10^4	0	0	0	1.4×10^{-5}
"	III	51.5	2.0	7.3×10^2	1.4×10^3	0	0	0	3.0×10^{-5}
Hydroxyl-apatite	Crude	67.6		5.24×10^3	7.75×10^3		0	4.07×10^{-3} *M*	2.0×10^{-5}
"	I	53.1	2.2	0	0		0	3.04×10^{-3} *M*	2.9×10^{-5}
"	II	66.1	6.4	1.0×10^4	1.5×10^4		0	1.33×10^{-2} *M*	1.2×10^{-6}
"	III	91	6.4	6.0×10^3	6.6×10^3		0	0	9.8×10^{-6}
"	VII	100		2.2×10^3	2.2×10^3		0	0	
"	IV	100		5.5×10^3	5.5×10^3		0	0	1.0×10^{-5}
"	V	100		0	0		0	0	4.6×10^{-5}
"	VI	100		0	0		0	0	7.3×10^{-5}

* 1 unit = 1 OD/hr in the presence of 1.3% Azocasein

FIGURES

7. Time Course of Hydrolysis of ATEE and TAME by SUT-67

8. Effect of pH on Hydrolysis of ATEE by Sephadex Fr. I (SUT-67)

9. Effect of Substrate Concentration on Velocity of Destruction of ATEE by SUT Sephadex I

10. The Effect of Enzyme Concentration on the Hydrolysis of 0.014 M ATEE by Sephadex Fr. I (SUT-67) at 30°C

11. Comparison of Enzymatic Hydrolysis of 0.014 M ATEE by 0.0104 mg N/ml SUT-67 and 0.007 mg N/ml Sephadex Fr. I

12. Effect of Various Toxin Fractions on the Destruction of ATEE at 30°C

Fig. 7

Time Course of Hydrolysis of ATEE and TAME by SUT-67

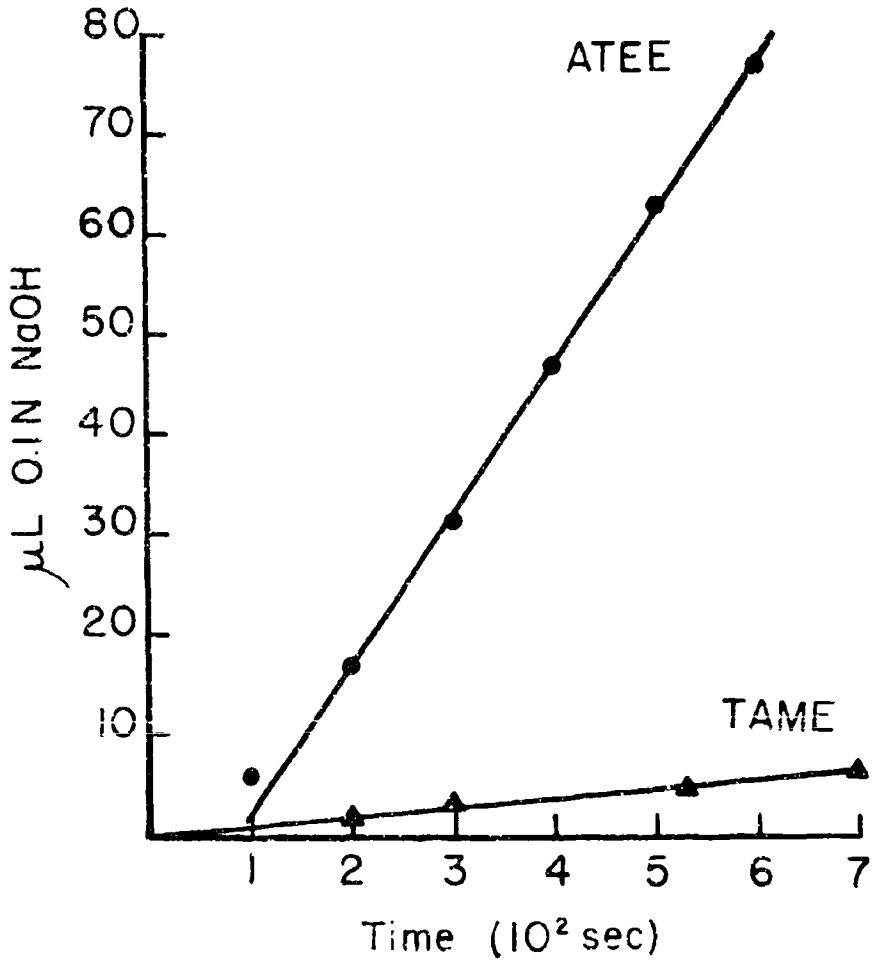


Fig. 8

Effect of pH on Hydrolysis of ATEE by Sephadex Fr. I (SUT-67)

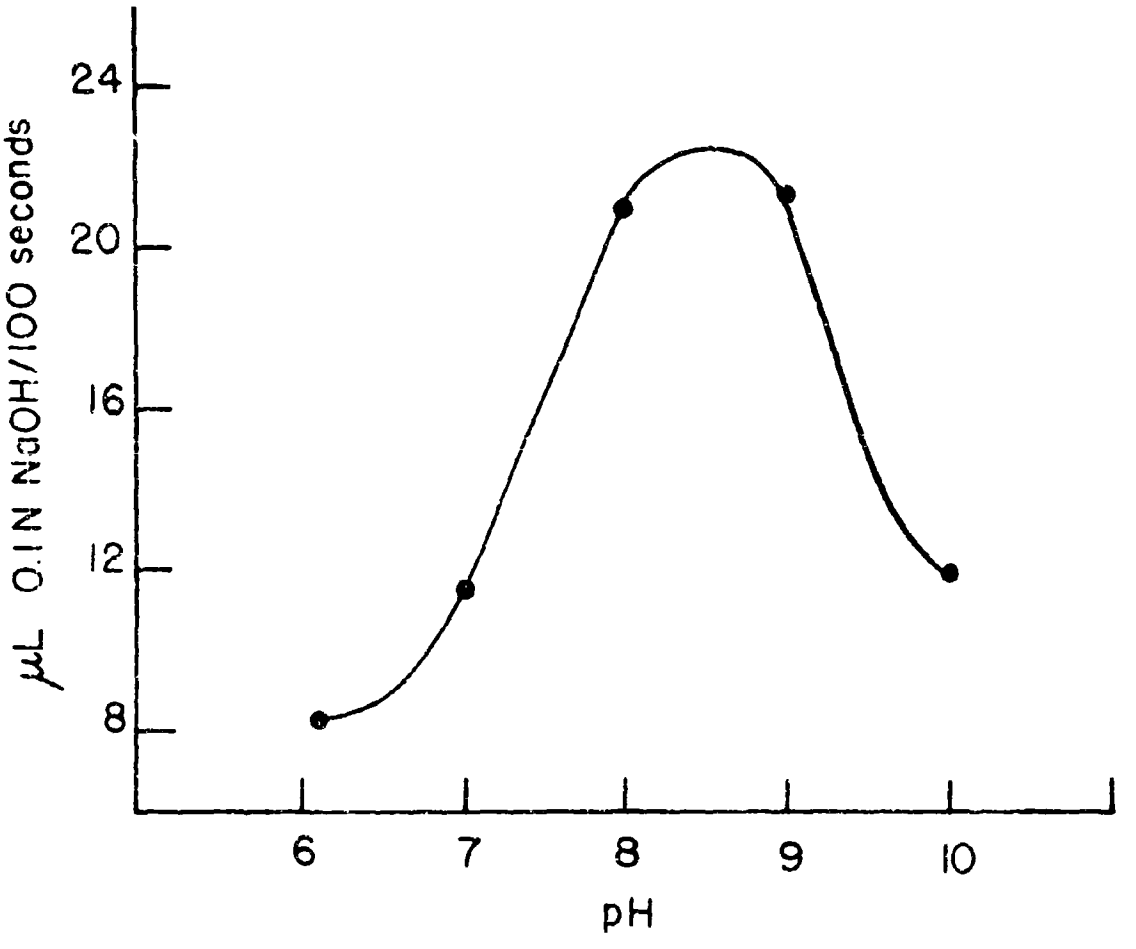


Fig. 9

Effect of Substrate Concentration
On Velocity of Destruction of ATEE
By SUT Sephadex I

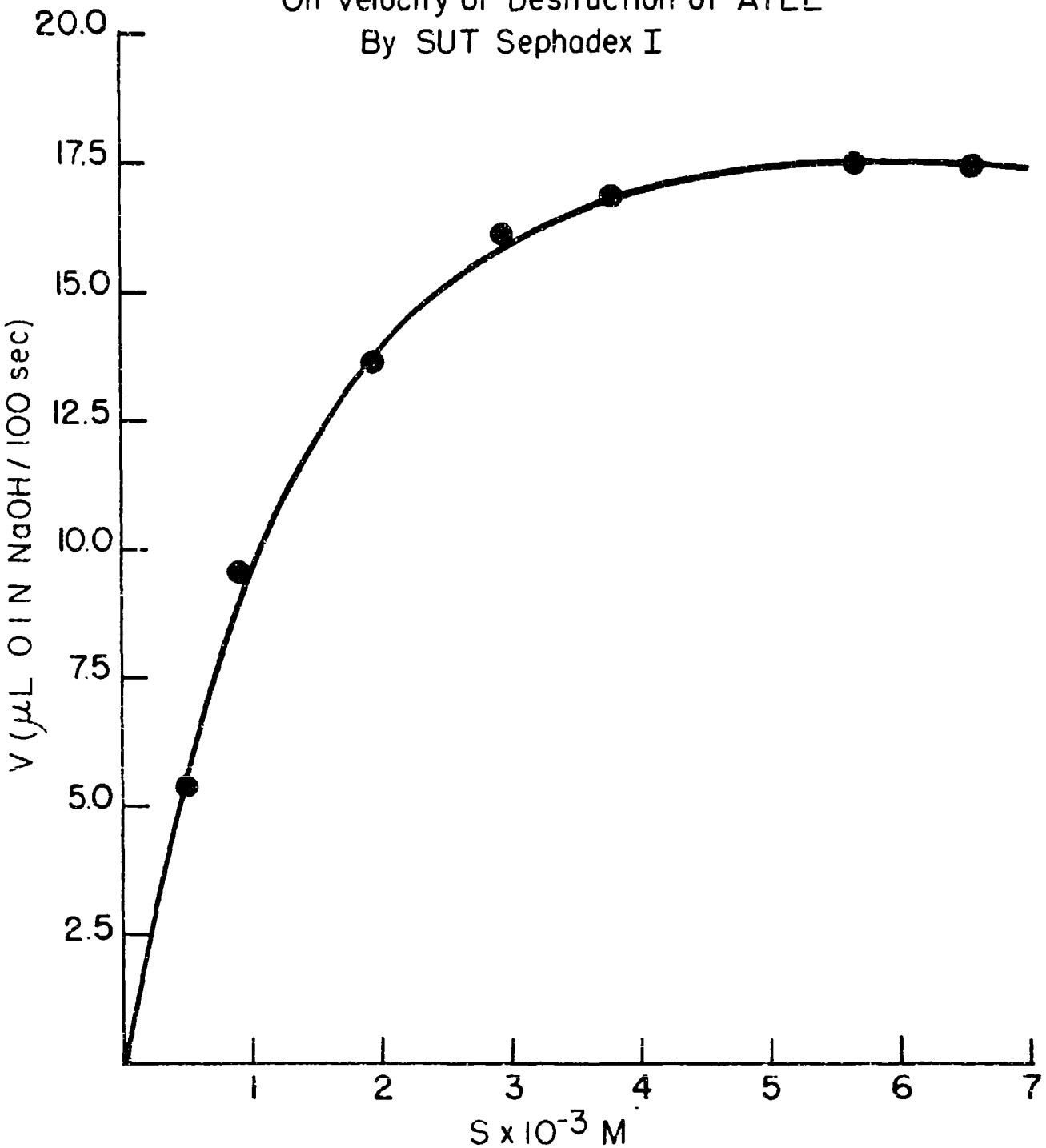


Fig. 10

The Effect of Enzyme Concentration on the Hydrolysis of 0.014 M ATEE by Sephadex Fr. I (SUT-67) at 30°C

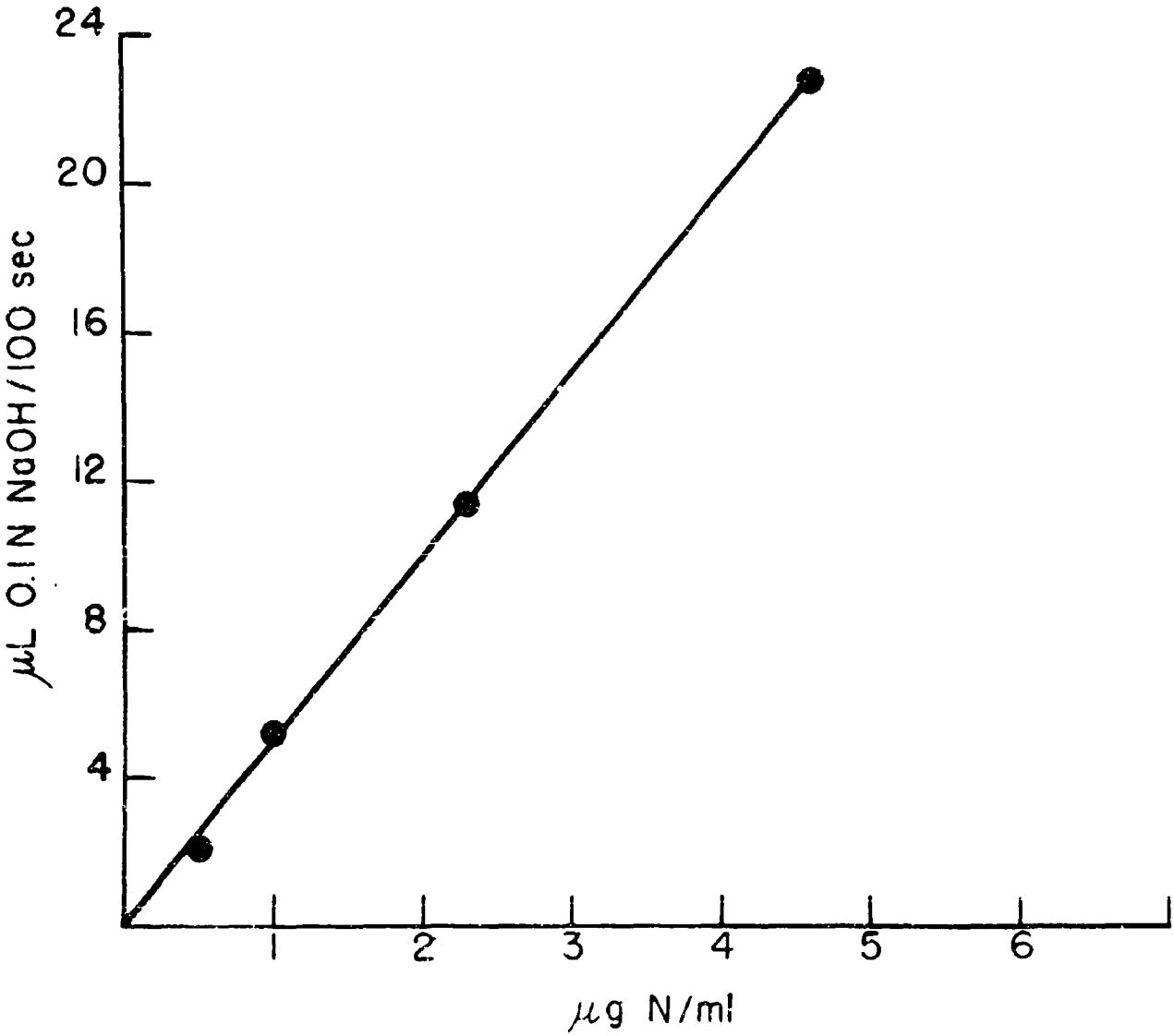


Fig. 11

Comparison of Enzymatic Hydrolysis of 0.014 M ATEE by 0.0104 mg N/ml SUT-67 (●) and 0.007 mg N/ml Sephadex Fr. I (■)

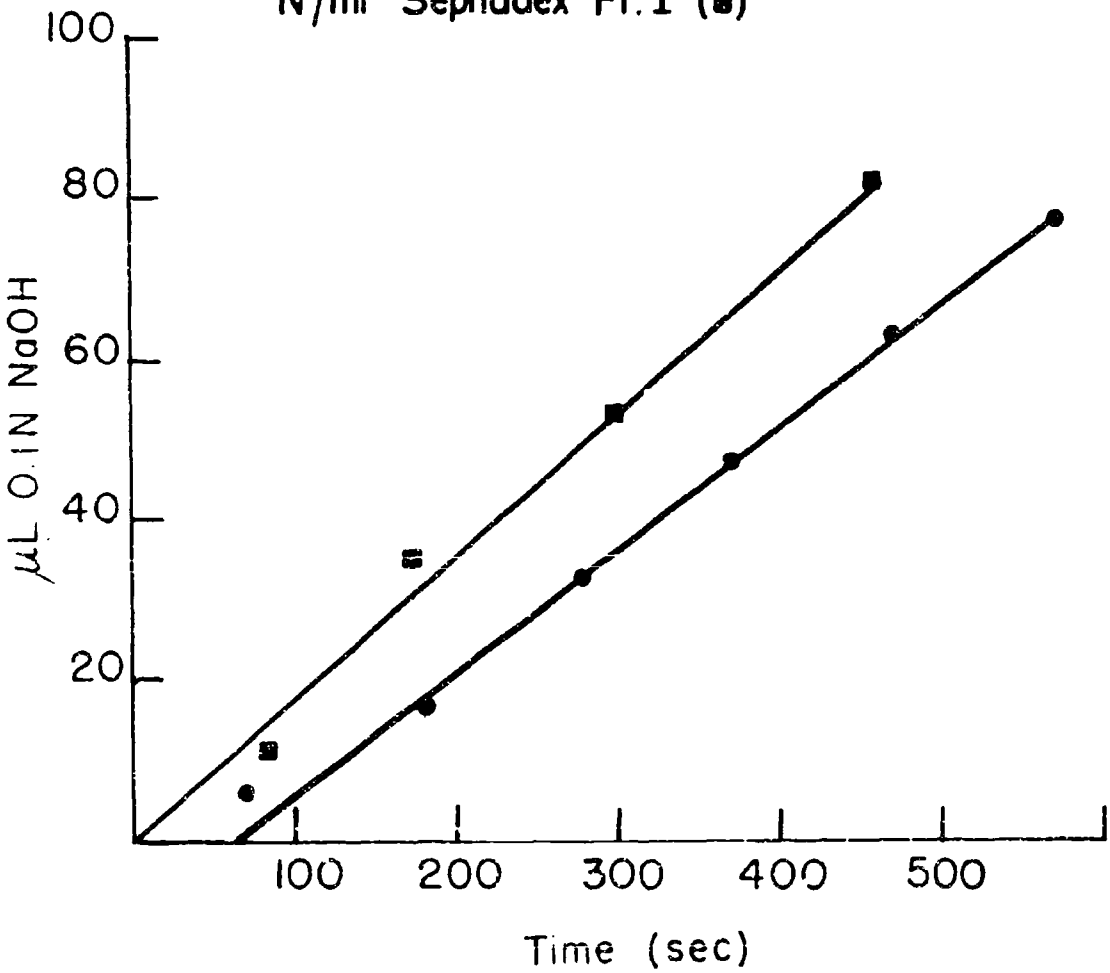
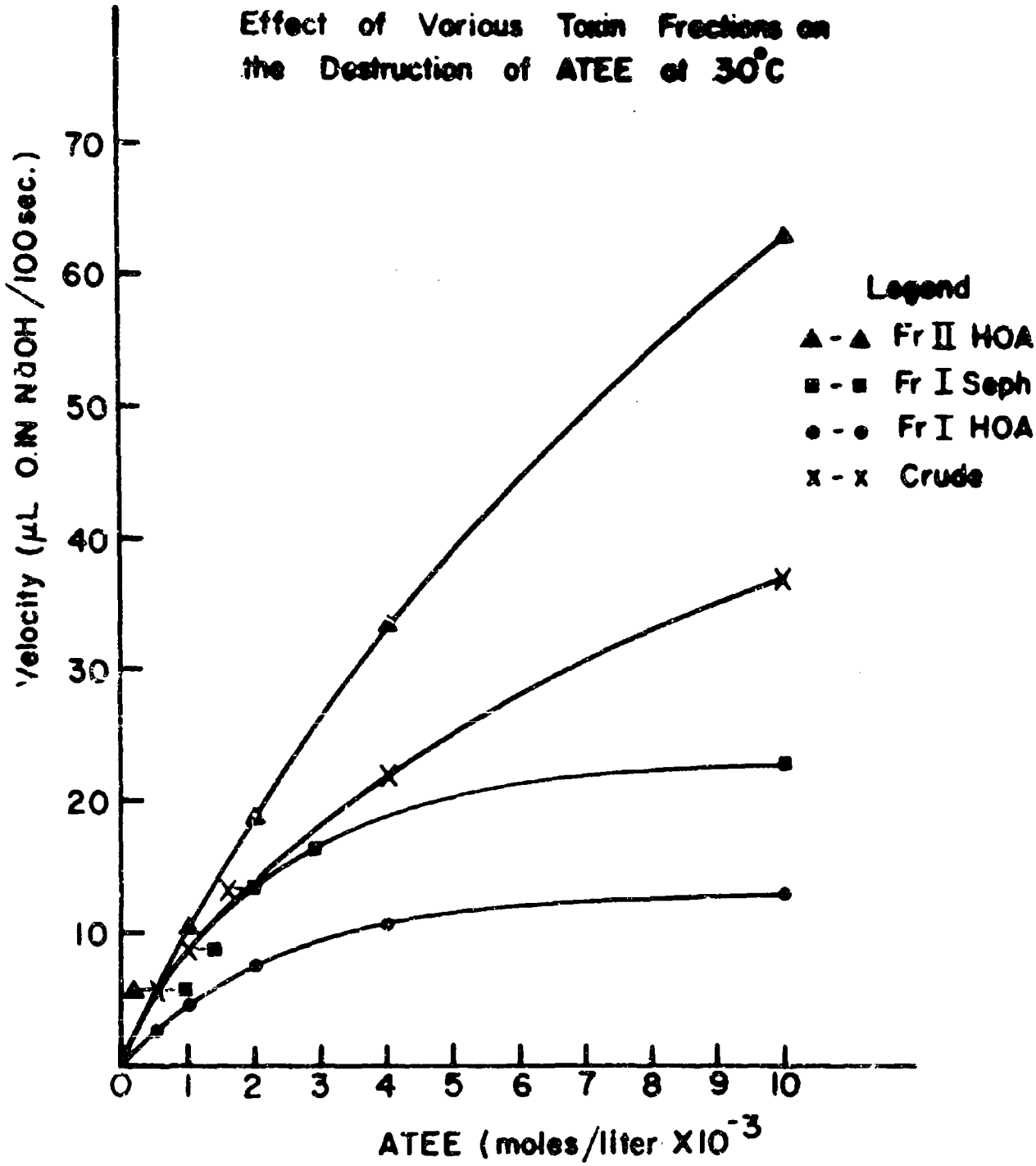


Fig. 12


Effect of Various Toxin Fractions on the Destruction of ATEE at 30°C
Velocity (μL 0.1N NaOH/100 sec.)
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
ATEE (moles/liter X10^-3
Legend
▲-▲ Fr II HOA
■-■ Fr I Seph
●-● Fr I HOA
x-x Crude

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Security Classification

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13. ABSTRACT: The mission of this lab has been to present a unified & consistent account of the problem of in vitro anaphylaxis under such conditions as would assure control over 1. the nature & quantity of antibody bound to the tissue and, 2. over the nature & purity of the antigen used for challenge.

During the current contract period we have broadened our approach by the use of antigens having functional properties over & above simple antigenicity. The materials selected for study were chosen because they were suitable as biochemical probes for various levels of attack on tissues & because they could simultaneously function as antigens. They thus represented significant models of clinical disorders in which natural toxicity could be compounded by immediate hypersensitivity.

The preparation and properties of one of these agents, sea urchin toxin, and of certain purified fractions are described in the Progress Report. The crude material contains a variety of enzymes which produce hemolysis and release vasoactive agents when applied to tissues.

The pedicellarial venom is an ensemble of about 6-8 antigenically distinct protein components. Potent (lethal) preparations can be produced by fractional precipitation with ammonium sulfate, by gel filtration on Sephadex, and by chromatography on hydroxyl apatite. A survey of the esteratic properties of the 3 Sephadex fractions and 6 Hydroxylapatite (HOA) preparations was made by determining whether they could break down azocasein and hydrolyze the synthetic substrates TAME and ATEE. None of the preparations attacked TAME and only 3- Sephadex I and HOA I and II - attacked ATEE. The K_m values were Sephadex I, 2×10^{-3} M; HOA I, 3.04×10^{-3} M; and HOA II, 1.3×10^{-2} M.

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14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT

Several large batches of reaction product were prepared by treating heated, acidified plasma with crude SUT. The peptides were recovered by extracting the dried ethanolic residues with acidified n-butanol and precipitating the butanolic extract with ether. Selected samples were further purified by gel filtration on Sephadex G-25 and then chromatographed on paper. Paper chromatography gave 5 distinct spots, 3 of them having kinin activity, and 1 of them corresponding to synthetic bradykinin. Neither the hemolytic activity nor the proteolytic action were clearly correlated with the lethal toxicity.

1. *In Vitro* Anaphylaxis
2. Sea Urchin Toxin
3. Venoms
4. Enzymes
5. Marine Animal Toxins
6. Cardiac Anaphylaxis
7. Kinin formation
8. Action on Synthetic Substrates

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