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THE CHEMISTRY AND PHARMACOLOGY OF A CENTRAL NERVOUS SYSTEM STIMULANT FROM THE SEA ANEMONE, *Stoichactis kenti*

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN PHARMACOLOGY

DECEMBER 1971

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ABSTRACT

A central nervous system stimulant has been isolated from the sea anemone, Stoichactis kenti. A chromatographically homogeneous fraction has been obtained from the crude extract by dialysis and gel filtration on Sephadex G-50. Attempts were made to purify the active fraction further by ion exchange column chromatography, but the specific activity of the active fraction was not increased.

The active substance was found to be water soluble, heat and acid labile and stable to alkali. It showed a positive color reaction with ninhydrin on paper (circular) chromatography, using n-butanol: acetic acid:water (4:1:5) system. Steroids, steroidal glycosides, nucleic acids, lipids and carbohydrates were found to be absent in the active fraction, when tested with specific reagents. The active fraction has a characteristic u.v. maximum at 277.5 nm. Determination of protein by Lowry's method and estimation of nitrogen by Kjeldahl's method indicated the active fraction was rich in protein. An acid and alkaline hydrolysis of the active fraction was carried out and hydrolysates were analysed both by two dimensional chromatography and Technicon auto aminoacid analyzer. The following amino acids were identified by comparing with standard aminoacids: cysteine, aspartic acid (asparagine), threonine, serine, glutamic acid (glutamine), proline, glycine, alanine, valine, cystine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine. From these results it was concluded that the active fraction was a polypeptide containing seventeen different aminoacids.

Based on behavior on Sephadex G-50, the approximate molecular weight of the active fraction was estimated to be in the range of 2,500 - 3,000.

Signs of central nervous system stimulatory activity produced by the active fraction in male mice included fighting episodes, increased motor activity and clonic convulsions. The ED50 of the active fraction based on fighting episodes was 6.4 mg/kg. The fighting episodes occurred at a frequency of 2-3 times/minute. After the administration of the active fraction intraperitoneally, the fighting episodes started within 4-6 minutes, peaked at 15 minutes and waned within about 30 minutes. The LD50 dose of the active fraction was 12.2 mg/kg. Toxic symptoms such as ataxia, catalepsy and tonic convulsions were observed before death.

Phenobarbital sodium, chlorpromazine and methocarbamol completely blocked the fighting response of the active fraction even at the ED100 dose level but did not change the LD50. The antagonism of the active fraction induced stimulant activity (as measured by fighting episodes) by these drugs suggests that this activity was probably mediated centrally.

Reserpine and tetrabenazine pretreatment markedly increased the stimulant effect of the active fraction by decreasing the ED50 of the active fraction by 50%. Such treatment increased toxicity twofold. α-methyl p-tyrosine methylester HCl (α-MPT) pretreatment did not alter the ED50, while the LD50 was significantly decreased. When α-MPT treatment was incorporated in reserpine or tetrabenazine treated

animals, the stimulatory activity of the active fraction was completely blocked even at the ED100 (9.3 mg/kg) dose level. The active fraction produced a significant decrease in brain norepinephrine content at the ED50 and the ED100 doses during the stimulation period.

Both the active fraction and reserpine produced a hyperthermic response in mice.

DL-dopa treatment restored the active fraction induced stimulant action (fighting episodes) which was abolished after combined treatment with α-MPT and reserpine and reserpine and disulfiram. DL-dopa also increased the LD50 of the active fraction. The active fraction at the ED50 dose significantly decreased brain dopamine content.

Pretreatment with p-chlorophenylalanine did not alter the ED50 and the LD50 of the active fraction. No change in brain serotonin content was observed after administration of the active fraction at the ED50 dose.

The active fraction at the ED50 dose significantly inhibited the re-uptake mechanism of norepinephrine during the stimulation period. It also elevated normetanephrine levels at the ED50 dose.

Propranolol but not phentolamine treatment completely blocked the stimulatory action of the active fraction, with no change in the LD50. Atropine treatment decreased the toxicity, with no change in the ED50. On the otherhand physostigmine blocked the stimulatory action and increased toxicity by twofold.

In conclusion, the results suggest that the active fraction causes stimulant action by releasing active norepinephrine from functional pools and inhibiting its re-uptake, thus making more norepinephrine available at adrenergic receptors.

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CHAPTER I

GENERAL INTRODUCTION

Many natural products derived from marine and terrestrial plants and animals have been found to contain significant biological activity. Investigations of these crude products have led to the synthesis of many useful drugs. The drugs that have been obtained from marine organisms were few in contrast to those obtained from terrestrial organisms. In several recent reviews concerning the potential of the sea as a source of pharmaceutical products (Schwimmer and Schwimmer, 1955; Halstead, 1957, 1968; Nigrelli, 1958, 1967; Burkholder, 1968), many interesting substances isolated from marine organisms with biological activity were described. The number of potentially useful substances from the marine environment has intensified the need for more biochemical studies on marine plants and animals. These substances have been shown to possess a variety of biological activities: antiviral, antimicrobial, tumor inhibitory, anticoagulant, neurotropic, analgesic, cardioinhibitory, phychopharmacological, and numerous other biological activities (Baslow, 1969). The scientific importance of marine biotoxins is frequently underestimated. In the past, biotoxins were investigated primarily for their toxic properties, but now it is recognized that at nontoxic doses, many of these so-called toxins can elicit significant biological activity. It follows that since these poisons can become clinically useful drugs, research is justified to determine their pharmacological and chemical properties. Another

aspect of this subject which perhaps is equally deserving of consideration relates to the exploration of new and little-known biologically active chemical structures. It was estimated that of the thousands of marine organisms known or believed to contain biotoxic substances, less than one per cent of them have been examined for their pharmacological activity (Halstead, 1965).

During the past two and one-half years there has been a cooperative effort at the University of Hawaii to collect and study biota, primarily from the Pacific basin, for pharmacological and chemical investigation. At the suggestion of Dr. Frank Tabrah, coelenterates were examined for anti-cancer activity because of the unique structural features of their organelles. Among the coelenterates collected for study under this program was a sea anemone. Dr. Cadet Hand, Bodega Bay Marine Biology Station, California, kindly examined a specimen and said it was undoubtedly Stoichactis and "most probably Stoichactis kenti". Interestingly, the non-dialysable (retentate) fraction of the alcoholic extract of this species was found to possess anticancer activity while the dialysable fraction contained a highly toxic principle. As Halstead (1965) mentioned, in natural products research, of which biotoxicology is a segment, ethnic marine biology serves a useful purpose in providing leads on the types of biological activity. Unfortunately, this knowledge of ethnic marine biology is generally not well developed. Consequently it is helpful to employ ancillary methods in the search for pharmacological activity in an unknown marine biochemical substance. Toxicity is often a useful

indicator of biological activity and, as such, provides the first approximation of pharmacological properties of that substance. Such reasoning prompted further research on this toxic dialysable compound extracted from Stoichactis kenti to determine its chemical nature and pharmacological activity.

Phylum Coelenterata or Cnidaria:

The coelenterates or cnidarians are simple metazoans having primary radial, biradial, or radio-bilateral symmetry. They are composed essentially of two epithelial layers encasing a single internal cavity - the gastrovesicular cavity, or coelenteron, which opens only at the mouth. A dominant characteristic of the group is the presence of tentacles equipped with nematocysts and for at least a part of their life span, most coelenterates are attached or sedentary. The phylum coelenterata or cnidaria is generally divided into three classes: (1) Hydrozoa, (2) Scyphozoa and (3) Anthozoa.

Halstead (1965) listed twenty species of Hydrozoa (Hydroids), thirty-two species of Scyphozoa (Jellyfishes) and seventeen species of Anthozoa (sea anemones, corals) reported to be venomous. The compounds of pharmacological interest, including toxins present in the cnidarians, have been reviewed (Welsh, 1961; Halstead, 1965; Russel, 1967).

A variety of chemical substances exhibiting biological activity in various test systems have been found in coelenterate tissue. Early attempts to isolate toxic components of coelenterates (tentacles of Actinia sp.) by Richet and Portier (1903) yielded three active extracts:

"Thallasin", "Congestir" and "Hypnotoxin". None of these was chemically identified as these substances were found not to be specific compounds but rather mixtures having deleterious biological effects. Thallasin has been shown to be a releaser of histamine in tissues, and upon intravenous injection in the cat causes the appearance of a "slow contracting substance" in the plasma. In 1923, Ackermann et al. isolated tetramethylammonium chloride or tetramine from Actina equina. Since then, several nitrogenous bases have been isolated from various coelenterates which include N-methylpyridinium hydorxide, homarine, trigonelline and γ-butyrobetaine. Large amounts of serotonin were found in the coelenteric tissues of C. parasitica, tentacles of sea anemones, Actinia equina and Anemonia sulcata yielded histamine (Mathias et al., 1957) and also epinephrine and norepinephrine were reported to be present in a sea anemone (Carlyle, 1970).

The paralyzing factor of certain sea anemones is heat labile, destroyed by trypsin and chymotrypsin, precipitated by fifty per cent acetone or ethanol, nondialyzable and present in the soluble fraction of tentacle homogenates. These characteristics suggest the protein nature of the paralyzing factor. Scheuer (1969) reported a toxin called "palytoxin" with empirical formula $C_{41}H_{73}NO_{20}$ in the extracts of the sea anemone Palythoa sp. Several high molecular weight fractions have also been isolated from the nematocysts of other cnidarians.

Picken and Skaer (1966) reviewed the chemistry of the nematocyst capsule and content. Among the substances discussed are proteins, sulfur containing amino acids, hydroxyproline, glycine, tyrosine,

arginine, proline, alanine, glutamic acid, aspartic acid, a succino-oxidase inhibitor, hexosamine, uronic acid, orthodiphenols, mineral salts, alkaline and acid phosphatases, 5-nucleotidase and cholinesterase. However, no literature was found on the pharmacology or chemistry of the Stoichactis genus which may be new to the zoological literature.

Stoichactis kenti (class Anthozoa) is a large anemone found commonly on the reefs of Tahiti. The anemone was collected from the reef of Arue Bay in Tahiti by Jack Randall and was preserved by immersion in 95% ethanol and refrigerated at $4^{o}C$.

A crude 30% ethanolic extract of this sea anemone exhibited marked central stimulating activity in mice. Attempts have been made to isolate the active principle in a pure form in order to study its chemical and pharmacological properties in greater detail. The data obtained by us is presented in two parts, one dealing with the isolation and identification of the active principle and the other its pharmacological properties.

CHAPTER II

A. EXPERIMENTAL AND RESULTS

1. EXTRACTION AND PURIFICATION

i. Solvent Extraction: To aid in the isolation of the biologically active substance, fractions at each stage of the extraction and purification procedure were bioassayed. Details of bioassay tests are presented elsewhere in the section on pharmacological methods.

Extraction was initiated by thoroughly grinding the whole anemone (about 1200 gm wet weight) in a Waring blender with 30% aqueous ethanol. The homogenate was centrifuged (30,000 x g, 30 min) and the supernatant separated from the residue which was in turn triturated and recentrifuged twice in the same extraction medium. An aqueous solution of the residue at 5X the concentration of original crude extract showed no activity whereas the supernatant at the original concentration contained all the activity. The combined extracts and washings (3.0 liters) were evaporated under reduced pressure at $50^{o}C$ to 600 ml which was equivalent to 0.5 ml per gram of original wet solids.

ii. Dialysis is a process which separates substances according to their molecular size. In this process a small volume of a solution of the mixture is placed in a semipermeable membrane and stirred in a large volume of water (usually 20 volumes to 1 volume of solution). The substances that can pass through the membrane diffuse out. An artificial kidney unit (Dow Chemical Co.) composed of $1M^2$ surface area of hollow cellulose fibers suspended in a sealed chamber with ends of

fibers leading to an inlet and outlet was used. The cut off point of this dialysis unit was approximately 6,000 molecular weight. Dialysis tubing (1" diameter, union carbide), whose cut off point was approximately 10,000 molecular weight, was also used.

Three hundred milliliters of the extract was continuously and countercurrently dialyzed in artificial kidney unit against 15.5 liters of distilled water for four hours. The retentate and dialysate were evaporated under reduced pressure at 50°C to the original volume and tested for biological activity. The retentate was found to contain all the activity while the dialysate was completely inactive. The retentate (27.4 mg/ml) was then redialyzed in dialysis tubing against 16 liters of water for 48 hours at 4°C with one change of water after 24 hours. Both retentate and dialysate were again evaporated under reduced pressure at 50°C to the original volume of 300 ml and tested for biological activity. The dialysate (3 mg/ml) which now showed the activity (same concentration as the original crude extract) was then purified by gel filtration.

iii. Gel filtration: Gel filtration is a chromatographic technique utilizing the molecular sieving properties of porous gel particles. The gel particles consist of a three-dimensional network with pore size small enough to prevent selectively large molecules from entering the pores but large enough to allow the penetration of small molecules. On a bed of such gel particles, in equilibrium with a suitable solvent, large solute molecules will pass through the interstitial space, while small solute molecules will distribute equally

inside and outside the gel granules and pass through the bed at a slower rate. The dextran gels (Sephadex) used in this process were Sephadex G-25 and G-50. Sephadex G-25 retards molecular movement up to molecular weight approximately 5,000 and Sephadex G-50 retards up to approximately 10,000 molecular weight. A column size 45 cm x 8 cm (height to diameter ratio of about 6:1) was used. The amount of dry Sephadex G-25 or G-50 to be used was determined by calculating the bed volume (G-25 1 g per 5 ml bed volume; G-50 1 g per 10 ml bed volume) and stirred in a beaker with distilled water and allowed to swell overnight. After decantation to discard the finest gel particles, the suspension was transferred into the column, mounted in an absolutely vertical position. The opening at the bottom of the column was secured during the initial application of the gel suspension, but when a layer of a few centimeters had formed, it was opened gradually as flow during the packing procedure makes the packing more even. The maintenance of a discrete band of blue dextran (M.W. > 200,000) solution as it passed through the column was the criterion of good packing. The excess eluant at the top of the bed was removed by suction and the rest of the eluant allowed to drain into the bed until the top was just free of excess water. The sample was then slowly pipetted onto the top of the bed. The surface was first washed with a small quantity of eluant then by a larger quantity of eluant to start the elution. Determination of the void volume (Vo) was done by placing a narrow zone of solution of blue dextran and eluting with the solvent, the elution volume of this substance taken as the void volume Vo.

Fractionation was monitored spectrometrically with ultraviolet light of 280 nm wavelength.

A fifty milliliters aqueous solution of the active dialysable fraction at a concentration of 16 mg/ml was centrifuged to remove traces of insoluble material and applied to a column of Sephadex G-25 previously equilibrated in chloroform saturated distilled water. The column was eluted with chloroform saturated distilled water. The void volume (Vo) was 960 ml, while the elution volume (Ve) of the active fraction was 1090 ml at the U.V. absorbtion peak. Since Ve/Vo was nearly equal to 1.0, the separation of the active fraction from impurities was not possible on this column and Sephadex G-50 was then selected. Fifty-five milliliters of the active fraction at a concentration of 16 mg/ml was applied to this column and once again eluted with chloroform saturated distilled water at a flow rate of 6.0 ml/min. The void volume (Vo) was 939 ml. Two fractions were collected at elution volumes (Ve) at 2210 ml and at 2648 ml for the two major U.V. absorbtion peaks. The distribution coefficient Ve/Vo of the first fraction was in the range 2.10-2.50, with maximum U.V. absorbance at 2.35 and of second fraction was in the range 2.75-2.90, with maximum U.V. absorbance at 2.82 (Fig. 1). Both fractions were then lyophilized and tested for biological activity. The first fraction was found to contain all the activity and the second fraction was completely devoid of activity. The first fraction from the Sephadex G-50 was again run on the same column for further purification and the active fraction was found at Ve/Vo of 2.34 (range, 2.10-2.50) with no other peaks (Fig. 2).

Figure 1. THE ELUTION PATTERN OF THE SEA ANEMONE EXTRACT ON SEPHADEX G-50.

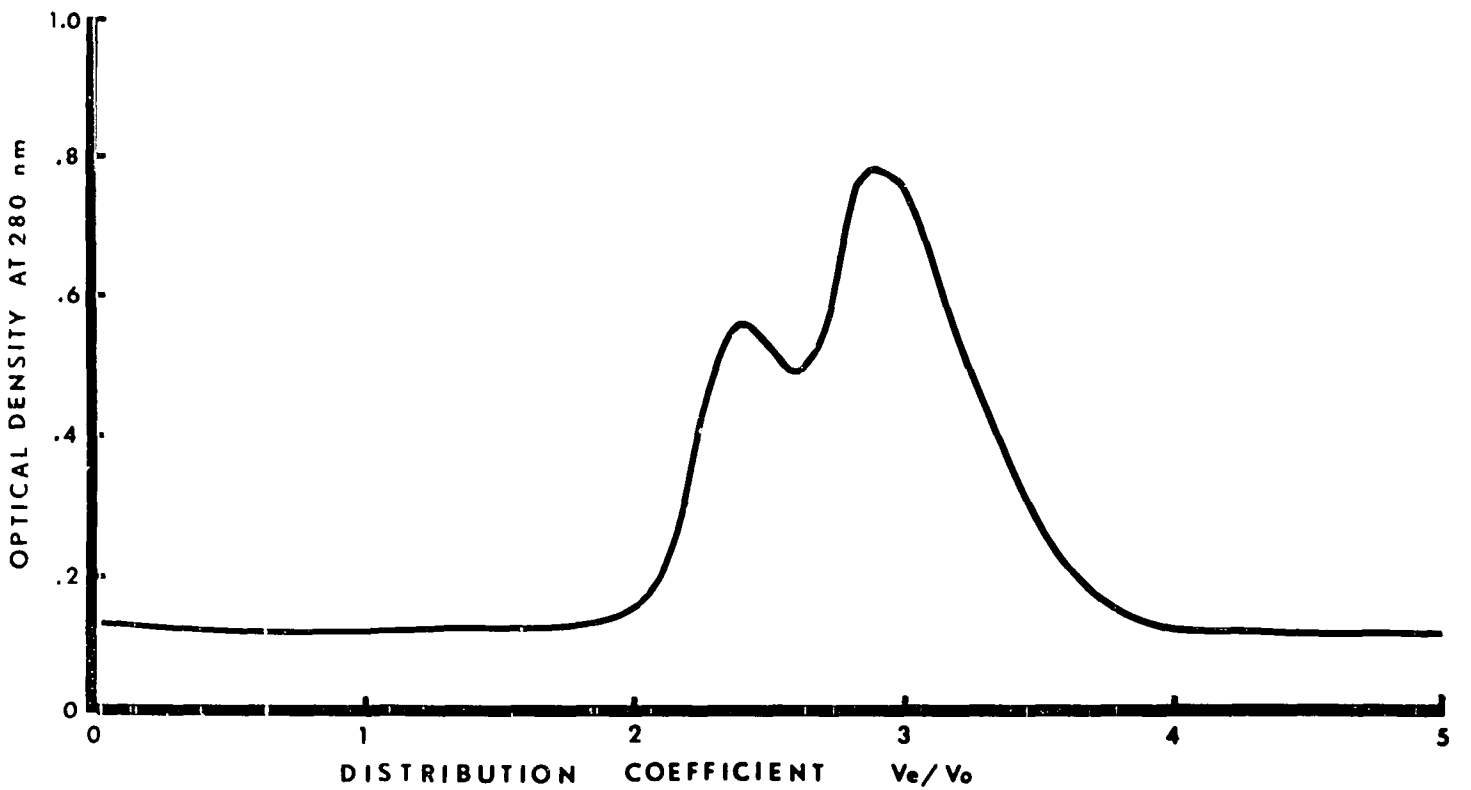
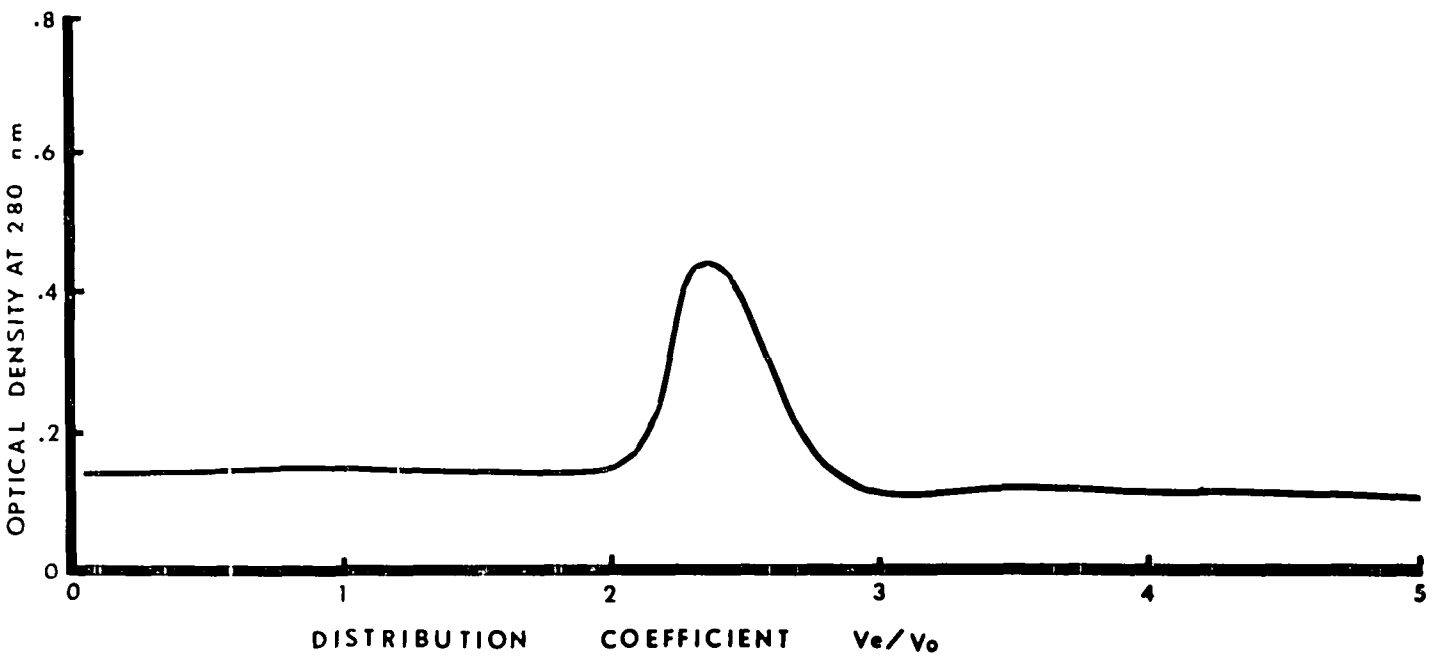

1.0
.8
.6
.4
.2
0
OPTICAL DENSITY AT 280 nm
0
1
2
3
4
5
DISTRIBUTION COEFFICIENT Ve/Vo

Figure 2. THE ELUTION PATTERN OF THE ACTIVE FRACTION ON SEPHADEX G-50.


.8
.6
.4
.2
0
OPTICAL DENSITY AT 280 nm
0
1
2
3
4
5
DISTRIBUTION COEFFICIENT Ve/Vo

This active fraction was lyophilized (1.8 gm) and tested again and showed activity at a concentration of 3.1 mg/ml, whereas the same level of activity was observed at a concentration of 250 mg/ml of the crude extract. Further purification of the active fraction was attempted using ion exchange chromatography.

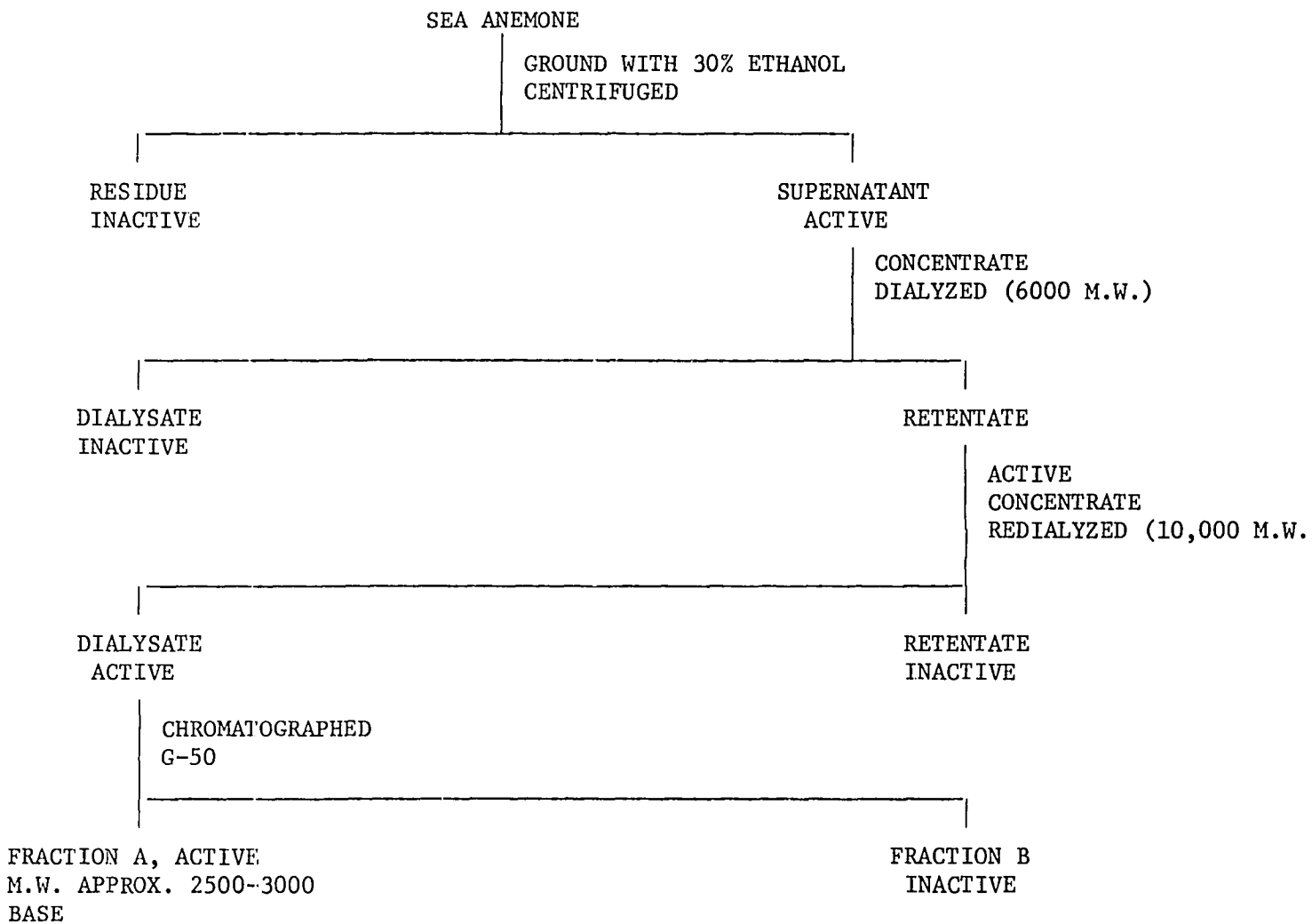
iv. Ion exchange chromatography: An ion exchange system is an insoluble material containing chemically bound charged groups and mobile counter ions. An anion exchange resin will exchange negative ions and a cation exchange resin exchanges positive ions. Diethyl-aminoethyl cellulose (DEAE, an anion exchange resin) was used for adsorption of materials with net negative charge and carboxymethyl cellulose (CM, a cation exchange resin) for adsorption of materials with a net positive charge. Tris buffer was used for the DEAE system and sodium phosphate to buffer the CM cationic exchange resin. Before commencing with the actual experiment, the ion exchange resin was allowed to reach ionic equilibrium with the starting buffer. The resin was first treated with 10X the buffer use concentration but same pH. Two bed volumes of buffer at use strength and pH were then run through the ion exchanger bed to allow the system to reach equilibrium.

One hundred milligrams of the active fraction obtained from Sephadex G-50 was extracted three times with chloroform and twice with 1-butanol saturated with water to remove lipids and pigments. The aqueous portion, after removal of the dissolved butanol under reduced pressure at 50°C, was dissolved in 1 ml of 0.01M Tris HCl buffer. This

solution was introduced into a 12 cm x 2 cm column of DEAE-cellulose and the flow rate was adjusted to 1 ml/min. The fractionation was monitored U.V. wavelength 280 nm. Three peaks were observed and each was collected separately and tested for biological activity. Only the first fraction was found to be active, whose elution volume nearly equals the void volume (1/3 bed volume).

This fraction was lyophilized (34.5 mg), dissolved in 1 ml of 0.01M sodium phosphate buffer pH 7.0 and was next introduced in CM cellulose column size of 31 cm x 1.5 cm and the flow rate was adjusted to 1 ml/min. To elute the fractions, the concentration of buffer was increased from 0.01M to 0.1M stepwise, and then increased to 0.5M whereupon the active fraction was eluted. This fraction showed activity at the same concentration as before ion exchange treatment, indicating the specific activity was not increased. The flow chart given on the following page summarizes the isolation of the active principle.

ISOLATION OF THE ACTIVE FRACTION FROM THE SEA ANEMONE, Stoichactis kenti



2. IDENTIFICATION

i. Heat Stability: Ten milligrams of the active fraction was dissolved in three milliliters of distilled water and heated for one hour at 100°C in a boiling water bath and tested. No fighting episodes were observed when the heat treated fraction was given to 10 mice at what would ordinarily be the ED50 dose (6.4 mg/kg), thus indicating that the active substance was heat labile.

ii. pH Stability: Ten milligrams of the active fraction was treated with five milliliters of 0.1N HCl or 0.1N NaOH and kept at room temperature for about twenty hours. After this treatment, the samples were evaporated to dryness under vacuum at 50°C, and each taken up in three milliliters of distilled water. The pH of each was adjusted to 7.0 and then tested for biological activity. The active fraction treated with 0.1N NaOH, at the ED50 dose elicited stimulatory activity in mice. However, after acid treatment, the activity was absent at the ED50 dose [10 animals], indicating that the active fraction was acid labile and base stable.

iii. Adsorption: Activated charcoal was added to the active fraction solution in the ratio of 1:1. The adsorbant was removed by centrifugation and the supernatant was tested for activity. The supernatant showed negligible activity at the ED50 dose indicating that the active material was adsorbed and could not be eluted with water. Therefore charcoal could not be used as a purification step.

iv. Paper Chromatography (circular): The principle of this technique is that substances to be analyzed are resolved into circular zones instead of spots. The solvent is fed from a glass capillary tube, and a drop of solvent placed at the center of the circular filter paper starts a run. The filter paper discs (Whatman papers) are specially machine stamped into five triangular sectors and such sectoring permits the analysis of five samples per disc and also achieves better equilibration on the top and bottom of the paper. Approximately one hundred microliters of the active fraction (3.1 mg/ml) was placed on the paper disc at the joint of the capillary and air dried. The disc was then placed between two glass dishes, the lower containing the developing solvent system and allowed to run for eight hours. The developed chromatogram was then air dried and sprayed with the specific reagents to detect various chemical groups in this active fraction. The solvent systems used were (1) n-butanol:acetic acid:water (6:2:2 v/v) and (2) upper phase of n-butanol:acetic acid;water (4:1:5 v/v).

v. Proteins: The developed chromatograms with either solvent systems were sprayed with 0.2 % solution of ninhydrin in acetone. After spraying, they were heated to not more than 80^{o}C. A faint violet color band appeared indicating the presence of peptide linkages.

vi. Sugars: a) Anthrone (300 mg) was dissolved in boiling glacial acetic acid (10 ml), ethanol (20 ml), phosphoric acid (3 ml) and water (1 ml) were then added to the solution. After spraying with this anthrone reagent, the chromatogram was heated for 5-6 min

at 110°C. No visible colors appeared indicating the absence of ketoses and oligosaccharides.

b) An ethanolic solution of aniline (4%) was mixed before use with an ethanolic solution of diphenylamine (4%) and concentrated phosphoric acid (5:5:1). After the chromatogram was sprayed, it was carefully heated for about 5 min. at 80°C. No visible colors appeared indicating the absence of hexoses.

c) Ten per cent of phosphoric acid was added to a 1% 1-naphthol solution. After the chromatogram was sprayed, and heated at 90°C, no visible colors appeared, also indicating the absence of sugars.

d) The chromatogram was sprayed with a saturated aqueous potassium metaperiodate solution which was allowed to react for 6 min. The chromatogram was then sprayed with a mixture of 0.1M benzidine in methanol, acetone, and 0.2N HCl (10:2:1). No characteristic colors appeared, indicating the absence of sugars with a free α-diol configuration. The paper was then sprayed with a mixture of 0.1 M ethanolic benzidine solution and 0.8 N HCl (1:1) and heated for 1 minute at 110°C. The test was negative indicating the absence of amino sugars and ketoses.

vii. Steroids: 1) The chromatogram was sprayed with a saturated solution of antimony trichloride in chloroform and heated for four minutes at 90°C. No colored spots were observed indicating the absence of steroids. 2) The chromatogram was placed on a glass plate, wetted with a solution of sulfuric acid (20%) in acetic anhydride, and covered with another glass plate. No characteristic colors

appeared indicating the absence of Δ^5-unsaturated 3-sterols.

viii. Steroid glycosides: The chromatogram was sprayed with a solution of m-dinitrobenzene (0.2%) in absolute ethanol and after the alcohol was evaporated it was sprayed again with aqueous KOH (2.5N) and heated at 70-100°C for 10 minutes. No characteristic colors were seen indicating the absence of steroid glycosides.

ix. Anthrone Method for Sugars: The anthrone reagent was made by dissolving two hundred milligrams of anthrone in one hundred milliliters of concentrated sulfuric acid. Two milliliters of this reagent were added to one ml of sample (3 mg/ml) concentration. The test tube was kept in ice and was shaken to mix. It was then placed in boiling water bath for sixteen minutes, and cooled rapidly. The intensity of the color was read at 625 nm. Absorbance was the same as the blank indicating the absence of sugars in the active fraction.

x. Acid Hydrolysis: The most common method for total hydrolysis of proteins or peptides is heating with an excess of 6N HCl at 105°C for a period of twenty-four hours in evacuated sealed tubes. Partial losses of some amino acids occur during the course of hydrolysis and the content of amino acids in the hydrolysate does not completely reflect the composition of the original protein or peptide. Among the amino acids which are liable to decompose during acid hydrolysis are tryptophan and cysteine. To some extent tyrosine, and very low amounts serine and threonine are also decomposed. The active fraction was subjected to acid hydrolysis for qualitative study rather than quantitative study. All the precautions necessary for quantitative estimation were not observed. The active fraction (3 mg)

was transferred to an ampule (4 ml size) with one milliliter of 6N HCl and then the ampule was flame sealed. The ampule was placed in a thermostatically controlled oven set at 108°C for about twenty-four hours. After hydrolysis was completed, the excess hydrochloric acid was removed by evaporating in a petri dish over a boiling water bath to dryness. Water was added to the residue and the solution evaporated to dryness again. This procedure was repeated twice. Finally, the residue was dissolved in one milliliter of distilled water for further analysis of individual amino acids by paper chromatography and amino acid analyzer.

The acid hydrolysate (100 µl) was spotted and chromatograms were developed using the solvent system, upper phase of n-butanol: acetic acid:water (4:1:5 v/v). After spraying with 0.2% ninhydrin w/v in acetone and heated for ten minutes at a temperature not exceeding 80°C, approximately seven to eight intense violet bands appeared indicating the presence of a number of different amino acids.

xi. Alkaline Hydrolysis: As tryptophan is destroyed by acid, the estimation of this amino acid by chromatography requires the use of alkaline conditions. Two milligrams of active fraction was boiled with ten milliliters of 15% barium hydroxide in an oil bath at 125°C under reflux for 18-20 hours. The barium was removed with a slight excess of 1N sulfuric acid. The pH was adjusted to about 7.0. The filtrate was lyophilized and taken up in 1 ml of 10% isopropyl alcohol. The alkaline hydrolysate (100 µl) was spotted and developed using the same solvent system as acid hydrolysate. p-Dimethylaminobenzaldehyde (1 g) was dissolved in acetone (8 ml), water (1 ml) and

conc. HCl (1 ml). After spraying with this specific reagent for tryptophan, no characteristic color appeared, indicating the absence of tryptophan in the active fraction.

xii. Two Dimensional Chromatography: The principle of this technique is the successive development of the chromatogram with two different solvents (e.g., a basic and an acidic solvent), whose advancing fronts are at right angles to each other. About one hundred microliters of acid hydrolysate and unhydrolysed sample were spotted by means of a capillary on a line two inches distant and parallel from one edge of the filter paper sheet (18" x 22"). After the spots were dry, the papers were folded sharply along a line one inch from the edge. The sample was, therefore, one inch from the fold. A glass solvent trough was used for developing these chromatograms. A glass rod which serves for supporting the paper was situated along side the trough. As a reference standard, about one hundred microliters of known amino acid mixture was spotted as described before on another sheet of paper. The chromatogram papers were now transferred to the chromatographic cabinet and mounted on separate glass rods. The edges of the papers in the trough were secured by another glass rod. The chromatograms were run in the first dimension using the solvent system n-butanol:acetic acid:water (4:1:5 v/v) for about sixteen hours. The chromatograms were dried in a hood at room temperature and then developed in the second dimension with the solvent system phenol:water (4:1 w/v) with ammonia (0.5%) kept separately in a petri dish at the bottom of the tank for about twenty hours.

The chromatograms were then dried in a hood at room temperature to complete dryness and sprayed with ninhydrin reagent. These were allowed to develop color overnight in a dark room. The standard amino acid mixture chromatogram was compared to the amino acid map obtained by Smith et al. (1955) and all amino acids were identified by carefully measuring their relative distances from the origin. The active fraction acid hydrolysate was then compared to the standard amino acid chromatogram and the following amino acids were identified: (1) sulfur amino acids (cystine or cysteine), (2) aspartic acid or asparagine, (3) glutamic acid or glutamine, (4) threonine, (5) serine, (6) glycine, (7) histidine, (8) arginine, (9) leucine, (10) isoleucine, (11) tyrosine and (12) alanine. Two other spots obtained could not be identified.

The chromatogram spotted with unhydrolysed sample revealed only one weak colored spot upon spraying with ninhydrin, confirming the homogeneity of the active fraction.

Acid hydrolysate samples were then analyzed using a Technicon aminoacid autoanalyzer. The amino acids were separated by ion exchange chromatography and then reacted with ninhydrin to give colors that were measured colorimetrically. The following amino acids were identified from the standard amino acid chart: (1) cysteine, (2) aspartic acid (asparagine), (3) threonine, (4) serine, (5) glutamic acid (glutamine), (6) proline, (7) glycine, (8) alanine, (9) valine, (10) cystine, (11) isoleucine, (12) leucine, (13) tyrosine, (14) phenylalanine, (15) lysine, (16) histidine and (17) arginine.

In the presence of the amides, asparagine and glutamine, which have elution times close to threonine and serine, it was necessary to make a separate four hour run on a basic hydrolysate sample in order to determine serine and threonine as well as the amides. The peaks of aspartic acid, threonine, serine and glutamic acid were well separated in this short run. However, it was not possible to separate glutamine and asparagine peaks from their respective acids. Based on the behavior of the active fraction on the anion and cation exchange columns (no retardation on anion exchange and strong retardation on cation exchange resins), it is probable that the amide forms of aspartic and glutamic acids are present in the sample.

xiii. Chromatography of acid hydrolysate for detection of individual amino acids. Glycine: The chromatogram was sprayed with a 0.2% solution of phthalic aldehyde in acetone and heated at 100°C for 2 min. It was then dipped in a 1% solution of potassium hydroxide in 95% ethanol and heated for a further 10 min. at 100°C. A characteristic green color revealed the presence of glycine in the active fraction.

Tyrosine and Histidine:

(Pauly's Reagent) Sulfanilic acid (3 g) was dissolved in water (200 ml), concentrated hydrochloric acid (6 ml) and n-butyl alcohol (14 ml). Before spraying the chromatogram, 20 ml of the latter solution was mixed with sodium nitrite (0.3 g). After 5-10 minutes drying in the air, the chromatogram was sprayed with 10% sodium carbonate solution. Red and pale orange spots appeared indicating the presence of histidine and tyrosine respectively.

Sulfur containing amino acids: The chromatogram was first dipped in a solution containing 0.5 g of sodium cyanide in 2 ml of water diluted to a volume of 25 ml with methanol. This was dried and then dipped into nitroprusside solution prepared by dissolving 250 mg of sodium nitroprusside in 1 ml of dilute sulfuric acid and then adding 2 ml of 28% ammonium hydroxide and methanol to a volume of 25 ml. A deep red color was observed indicating the presence of cystine or cysteine or both.

Proline: The chromatogram was sprayed with 0.2% isatin in acetone and then heated for 10 minutes in an oven at $70^{o}C$. A blue color indicatng proline, dark purplish blue indicating aspartic and glutamic acids, light brown indicating serine, threonine and tyrosine and red color indicating valine were all observed.

Arginine: (Sakaguchi Reaction). The chromatogram was first sprayed with a 16% solution of urea mixed with a 0.2% solution of 1-napththol in ethanol (5:1) and after drying at a temperature below $40^{o}C$, it was sprayed with a solution prepared by dissolving bromine (3.3 ml) in 5% NaOH (500 ml). A pink spot was observed indicating the presence of guanidine derivatives like arginine in the active fraction.

xiv. Estimation of protein by Lowry's method: This method is a sensitive colorimetric method to determine the protein content of a solution.

Reagent A: 2% sodium carbonate + 0.02% sodium potassium tartarate

Reagent B: 1 ml of 0.5% copper sulfate + 49 ml of reagent A.

Reagent C: Folin-Ciocalteu (1927) phenol reagent (stock solution diluted 1:1 ml water)

To 0.5 ml of solution containing one hundred micrograms of sample, 0.5 ml of 1N sodium hydroxide and 5.0 ml of reagent B were added. This was allowed to stand ten minutes at room temperature. 0.5 ml of reagent C was added very rapidly and mixed within a second or two. After thirty minutes, the sample was read spectrophotometrically at 740 nm. The absorbance (O.D.) at 740 nm for the active fraction (100 µg) was compared to the absorbance obtained by bovine serum albumin sample (100 µg) after subjecting to Lowry's method. This is used to determine proteins only and the intensity of the color produced varies from one protein to another. In our experiment, the sample gave similar intensity as that of bovine serum albumin. This is taken only as an indication of the peptide nature of the active principle.

xv. Determination of nitrogen by Kjeldahl method: The accurate determination of the total protein nitrogen is a good indication of the state of purity of the protein sample. The percentage of nitrogen for a pure protein should be in the range of 13.5 or above. The standard microanalytical procedure of Kjeldahl is the most widely used for the determination of nitrogen.

Reagents: 1. Concentrated sulfuric acid (nitrogen free).

2. Catalyst mixture, prepared by grinding to a fine powder 80 g. of K_2SO_4, 20 g of $CuSO_4$, 5 H_2O and 0.34 g of sodium selenate.
3. Boric acid (AR), 4% solution
4. 1N HCl
5. 15N NaOH
6. Indicator, prepared by mixing equal volumes of bromocresol green and methyl red.

25 mg. of the active fraction was weighed into a long-neck 100 ml Kjeldahl flask. Concentrated sulfuric acid (5 ml) was added carefully and the mixture heated on a digestion rack until charring was just apparent. This was then cooled and 1 g. of catalyst mixture was added. Heating was resumed and digested for about four hours. The cooled contents were diluted with 300 ml of distilled water. The distillation apparatus was flushed with steam for a few minutes. The delivery tube was then immersed beneath the surface of a 4% boric acid solution with added indicator contained in a 300 ml flask. The contents of the Kjeldahl flask were washed quantitatively into the distillation chamber and 60 ml of 15N NaOH was added. About 250 ml of distillate was collected. The ammonia trapped in the boric acid solution was then titrated with standard acid to a neutral gray end-point. The blank was also titrated with standard acid. Material which is all polypeptide or protein will generally have a nitrogen content of 13.5-16%, as determined by microanalytical method. The percentage of nitrogen of the active fraction determined by this method (macro) was 12.7%. Since the method used here was not microanalytical, the nitrogen percentage was well within the limits for a pure protein sample, confirming that the active fraction was rich in protein or polypeptide content.

xvi. Ultraviolet absorption spectrum. To determine the U.V. wave length of maximum absorption for the active fraction, 1 mg/ml active fraction solution was scanned through U.V. spectrum (Beckman DU-2 spectrophotometer). Active fraction (0.9 ml) exhibited maximum

absorption at 277.5 nm (Fig. 3). To detect the presence of nucleic acids, which have a high absorption maximum at 260 nm in contrast to proteins, which possess a weak maximum near 280 nm, the absorption ratio was measured. The ratio of absorption at these wavelengths for the active fraction was 1.42.

Using Kalckar (1947) formula to determine the amount of protein in mg per ml,

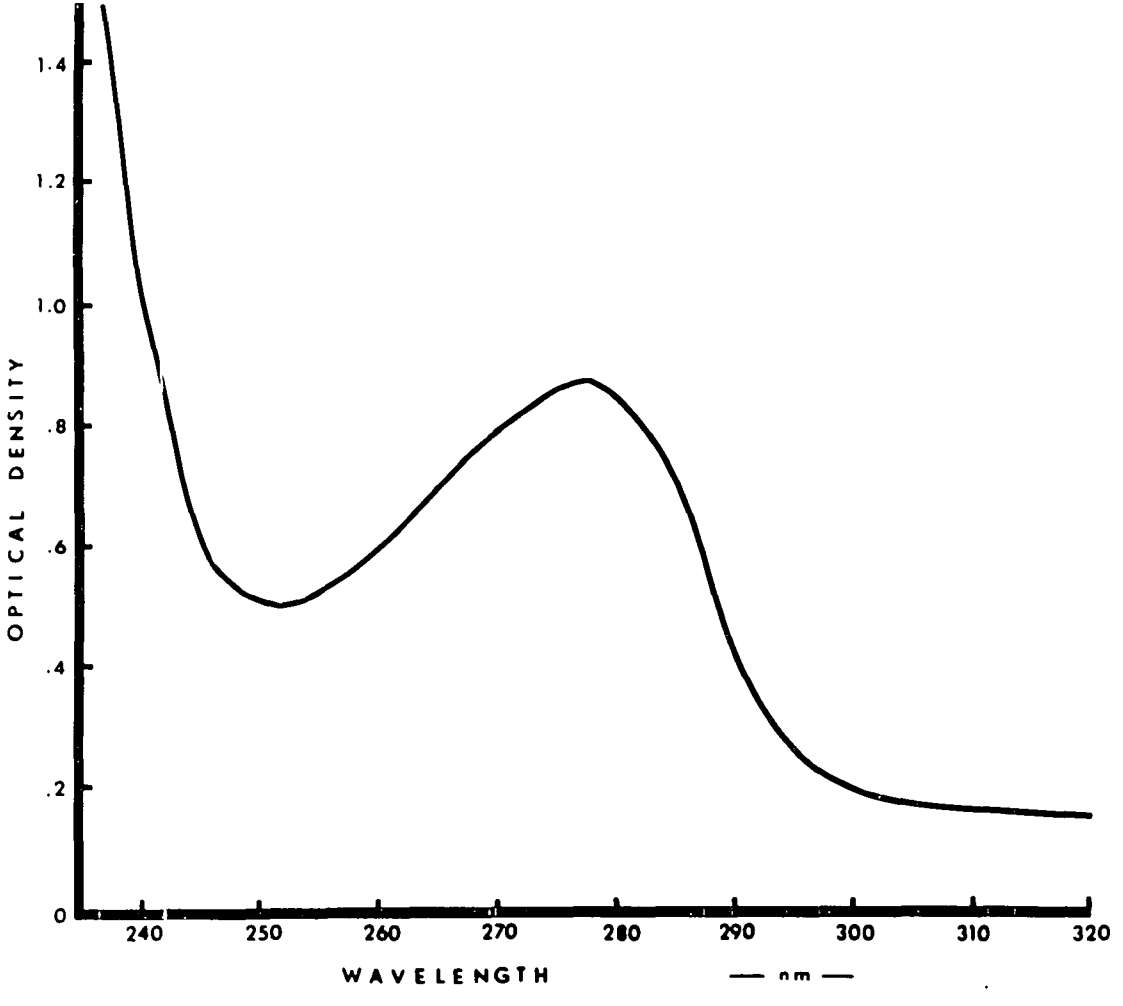
$$\text{Protein in mg per ml} = 1.45\ E\ 280 - 0.74\ E\ 260$$

$$E\ 280 = 0.85$$

$$E\ 260 = 0.60$$

Substituting these values in the above equation, the amount of protein in mg per ml = 0.90.

Figure 3. THE ULTRAVIOLET ABSORPTION SPECTRUM OF THE ACTIVE FRACTION IN WATER.


OPTICAL DENSITY
1.4
1.2
1.0
.8
.6
.4
.2
0
240
250
260
270
280
290
300
310
320
WAVELENGTH
— nm —

B. DISCUSSION

A central nervous system stimulant has been isolated from the sea anemone, Stoichactis kenti. The active substance was found to be water soluble, heat and acid labile and stable to alkali. A chromatographically homogeneous fraction has been obtained from the crude extract by dialysis and gel filtration on Sephadex G-50.

Steroids, steroidal glycosides, nucleic acids, lipids and carbohydrates were found to be absent in the active fraction, when tested with specific reagents. It showed a positive color reaction with ninhydrin. After hydrolysis of a sample and spraying with ninhydrin after chromatography, several colored spots appeared. This indicated that the substance was a polypeptide.

The active fraction has a characteristic U.V. maximum at 277.5 nm. The ratio of U.V. absorbance at 260 and 280 nm was 1.42. A 100 microgram solution of this active fraction gave the same absorbance at 740 nm, Lowry's method, as that of a 100 microgram solution of bovine serum albumin. Protein determination of a 1 mg/ml solution of the active fraction by measuring the absorbance at 280 nm and 260 nm and applying Kalckar's formula indicates the fraction to be rich in protein or polypeptide. The total nitrogen value for the sample by Kjeldahl's method was found to be 12.7%, which was in fairly good agreement with the suggested 13.5% value for a protein.

From these observations we concluded that the active fraction was a polypeptide containing aromatic aminoacids. On two dimensional

paper chromatography, using n-butanol:acetic acid:water and phenol: water systems and spraying with ninhydrin only one spot was observed. This was taken as a criterion of purity and further chemical work was carried out on this basis of purity.

An acid and alkaline hydrolysis of the active fraction were carried out and hydrolysates were analysed both by two dimensional paper chromatography and Technicon auto aminoacid analyzer. The following aminoacids were identified by comparing with standard aminoacids: Cysteine, aspartic acid (asparagine), threonine, serine, glutamicacid (glutamine), proline, glycine, alanine, valine, cystine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine. Specific confirmatory tests have been performed for specific aminoacids like, arginine, histidine, etc. Alkaline hydrolysate was examined for the presence of tryptophan and found to be absent.

During the isolation of the active principle, the activity was found to be retained by the artificial kidney (cut off point 6000 M.W.) and was in the dialysable fraction when a dialysis tubing was employed (cut off point 10,000 M.W.). On gel filtration using Sephadex G-25, (exclusion limit 5000) the active fraction was excluded from the column. However, the active principle was retarded by a column of Sephadex G-50 and distribution coefficient (Ve/Vo) for the active fraction was found to be 2.3. This suggested a molecular weight for the active fraction might be in the range of 2500-3000. An approximate calculation of the molecular weight of the active principle on the basis of one aminoacid residue each, indicates the molecular

weight to be 2345. The apparent inconsistency of the behavior on dialysis and gel filtration on Sephadex G-50 can possibly be explained as due to the formation of micelles as the concentration in the dialysis bag is fairly high, while on the column the substance gets diluted and thereby the necessary critical micelle concentration is not attained. In dialysis, diffusion rate control under practical nonequilibrium conditions could also be an explanation for the retention in the artificial kidney. On ion exchange column chromatography, the active fraction was retarded by the cation exchanger suggesting the basic nature of the active principle. An examination of the aminoacid composition reveals the presence of basic aminoacids in the active principle and the possibility of glutamic acid and aspartic acid being present in their amide forms cannot be ruled out.

From these results we conclude that the chemical nature of the central nervous system stimulant isolated from the Sea anemone Stoichactis kenti is a polypeptide containing 17 different aminoacids.

CHAPTER III

THE PHARMACOLOGY OF THE CENTRAL STIMULANT FRACTION

A. INTRODUCTION

In the natural products program designed to screen several marine organisms for anticancer activity, the sea anemone, Stoichactis kenti was one of the specimens gathered for study. For testing the biological activity, a non-lethal dose had to be determined, for which the crude extract (0.1 ml) of this sea anemone was administered by intraperitoneal injection into male white mice. Soon after the administration, symptoms observed in mice included increased activity and tremors. After ten minutes there was ataxia, decreased muscle tone, flaccid paralysis, anoxic convulsions and death apparently resulting from respiratory failure. At much lower doses, mice exhibited stimulant behavior with frequent fighting episodes followed by tonic convulsions and recovered after a period of thirty minutes.

The actions of drugs on the central nervous system can be studied in a variety of ways. Biochemical techniques can be used to monitor chemical changes in the brain when drugs are administered, or can follow neurophysiological changes and thirdly, can use the techniques from experimental psychology to investigate the effects of drugs on behavior. In this research, investigations have been largely concerned with study of neurotransmitter changes in the brain and the correlation of the changes induced with changes in behavior.

In recent years, several investigations have been undertaken specifically aimed at relating the brain function to metabolism and

disposition of neurohumors in the brain. The following compounds are present in the brain and are suspected of being involved in synaptic transmission: (1) acetylcholine, (2) norepinephrine, (3) dopamine, (4) serotonin (5-hydroxytryptamine) and (5) γ-aminobutyric acid. A great number of other amino acids including aspartic acid, glutamic acid and glycine and their derivatives have been investigated as possible central transmitters. Similarly, a wide variety of chemically heterogeneous compounds have been isolated from the central nervous system and investigated for possible neurohumoral transmitter functions. Among these are polypeptides, such as substance P and bradykinin, long chain unsaturated hydroxy acids, (prostaglandins) and other substances, such as histamine, and cyclic AMP (Goodman and Gilman, 1970).

There has been a gradual accumulation of evidence suggesting a causal link between the effects of drugs on the psyche and on central nervous system catecholamine metabolism. The discovery that reserpine and many other centrally acting drugs like amphetamine can affect the storage and metabolism of catecholamines in the brain has stimulated great interest in the actions of drugs on the brain catecholamines.

Hess (1954) put forth an attractive hypothesis, the central theme of which concerns the functional integration of the autonomic nervous system with the rest of the brain. He proposed that a subcortical system coordinates autonomic, somatic and psychic functions and consists of separate antagonistic divisions, the ergotropic and trophotropic. The ergotropic division integrates sympathetic with

somatomotor activities and is associated with arousal, increased sympathetic activity, enhanced skeletal muscle activity and activated psychic state. In contrast, the trophotropic division integrates parasympathetic with somatomotor activities and produces drowsiness and sleep, decreased skeletal muscle activity and lowered responsiveness to external stimuli. In this scheme the ergotropic division is an adrenergic system with norepinephrine serving as its neurohormone, Brodie et al., 1959) and is related to the reticular activating system (Rothballer, 1957).

By histochemical fluorescence method and biochemical determinations it has been demonstrated that norepinephrine, dopamine and 5-hydroxytryptamine occur in the central nervous system and the amines are stored in specific neurons (Carlsson, Falck and Hillarp 1962, Dahlström and Fuxe 1964, Fuxe 1964). The cell bodies of these neurons are located in the lower brain stem, i.e., the medulla oblongata, pons and mesencephalon, while their terminals are found in almost all parts of the brain and spinal cord. Norepinephrine and 5-hydroxytryptamine terminals are particularly abundant in certain areas of the hypothalamus, limbic system and neocortex, whereas dopamine containing terminals are found in the caudate nucleus and adjacent striatal areas. These monoamines fulfill some of the criteria for the identification of central nervous system transmitters. First, the neurons in the central nervous system contain high concentrations of norepinephrine, dopamine or 5-hydroxytryptamine in their nerve terminals with much lower concentrations in other parts of the nerve cell. The fine

terminal branches have the varicosities, characteristic of peripheral adrenergic neurons and make synaptic contact with neuronal cell bodies or dendrites. Morphologic evidence therefore is very suggestive for a transmitter function of the monoamines in the central nervous system. (Anden, Carlsson and Häggendal, 1969). Second, the enzymes necessary for the synthesis and inactivation of the amines are present in the central nervous system and their concentration is directly proportional to the distribution of these monoamine containing neurons (Anden and Corrodi, 1966). Third, the monoamine containing neurons can be manipulated pharmacologically by drugs whose actions are fairly established and upon application, the monoamines can produce the same constellation of effects as the physiological transmitters. Furthermore, the monoamine neurons of the central nervous system resemble peripheral neurons in possessing specific uptake mechanisms for external monoamines.

The ubiquitous distribution of monoamine terminals suggest that these substances may be involved as both excitatory and inhibitory transmitters in a wide variety of neuronal pathways in the central nervous system (Iverson, 1967). Drugs influencing the monoamine neurotransmission have such widespread effects as on the central regulation of the autonomic nervous system (spinal cord, lower brain stem), on motor function (spinal cord, neostriatum), on the endocrine system (hypothalamus), as well as on the integrative mechanisms associated with conditioned avoidance response and with mood (probably limbic system and cerebral cortex) (Anden et al., 1969). Microelectrophoretic studies have indicated that many nerve cells exist

in the brain which respond to noradrenaline (Bloom et al., 1963, Bradley and Wolstencroft, 1965). Gunne and Reis (1963) indicated that repeated electrical stimulation of the amygdaloid nucleus under conditions which will result in the ultimate production of a defense reaction or rage behavior is associated with a marked reduction in brain norepinephrine. Fuxe (1965), has also shown histologically that stimulation of the amygdala produces a decrease of brain substance presumed to be catecholamine. This effect appeared principally in the thalamus, to lesser extent in the medulla oblongata, and not at all in the midbrain. Mayert and Levi (1964) also reported a fall in brain norepinephrine level with no change in brain serotonin or acetylcholine in shock treated rats.

To investigate further the physiological role of brain monoamines, various percursors, 3,4 dihydroxyphenylalanine (DOPA), 3,4 dihydroxyphenylserine (DOPS) and 5-hydroxytryptophan (5-HTP) have been used as tools (Carlsson, 1964). In contrast to the monoamines, these precursors are able to penetrate the blood brain barrier and undergo decarboxylation to their respective amines, dopamine, norepinephrine and 5-hydroxytryptamine. 5-Hydroxytryptophan causes tremors, convulsions and hyperextension of limbs, suggestive that 5-hydroxytryptamine neurons participate in the control of motor functions. It was unable to antagonize the akinesia caused by reserpine. DOPA stimulates spontaneous motility and may in suitable dosage restore reserpinized animals almost to normal (Carlsson et al., 1957; Carlsson, 1959). DOPS, not being a physiological precursor of noradrenaline,

was decarboxylated very slowly by the decarboxylase and produced little effect. However, in the absence of monoamine oxidase, the central effects were excitatory, similar to those of DOPA. The data on monoamine precursors available, thus, suggest that dopamine, noradrenaline and 5-hydroxytryptamine are largely excitatory transmitters in the brain.

The role of catecholamine in affective disorders is derived from pharmacological studies. These studies have shown consistently the relationship between drug effects on catecholamine metabolism, especially norepinephrine, and affective or behavioral states (Schildkraut, 1965). The drugs that cause depletion and inactivation of central norepinephrine such as reserpine and tetrabenazine produce sedation or depression, whereas drugs that increase or inhibit the inactivation of brain norepinephrine such as amphetamine, iproniazid, tranylcypromine and imipramine are associated with behavioral stimulation or excitement and generally exert antidepressant effects.

The mechanisms by which some CNS stimulants alter or influence central monoamine concentration and/or metabolism has been defined to some extent and are discussed in the following sections.

Amphetamine and analogues: In laboratory animals the major effect of amphetamine and amphetamine-like drugs is characteristically an increase in spontaneous motor activity. This is a short-lived psychic stimulation mediated by the release of active norepinephrine (Iverson, 1964) or both norepinephrine and dopamine (Taylor and Snyder, 1970) from nerve cells and by the blockade of subsequent inactivation of

released norepinephrine by the cellular reuptake mechanism. This hypothesis is supported by the finding that large doses of d-amphetamine can cause a lowering in the levels of noradrenaline in the brain (Moore, 1963) and can cause the release of radioactive noradrenaline from brain stores previously labelled by intraventricular injection of H^3-noradrenaline (Glowinski and Axelrod, 1966). Studies with α-methyl tyrosine, a tyrosine hydroxylase inhibitor, have suggested that amphetamine is an indirectly acting sympathomimetic amine whose central action requires an uninterrupted synthesis of norepinephrine (Weismann et al., 1966; Hanson, 1967). In animals pretreated with reserpine the stimulant action elicited by amphetamine is potentiated. As the catecholamine stores although are markedly reduced from the storage granule, the mechanism for and release by amphetamine is unimpaired since the synthesis is not inhibited (Glowinski, Iverson and Axelrod, 1966). There is considerable evidence that amphetamine can also act as a competitive inhibitor of monoamine oxidase and as a partial agonist (Glowinski, Axelrod and Iverson, 1966).

It is observed clinically that the period of acute amphetamine stimulation, particularly after large doses, is often followed by a "rebound period" of profound mental depression and fatigue. This period of depression following amphetamine stimulation may reflect a temporary depletion of norepinephrine stores available for continued release (Schildkraut, 1965). Although both reserpine and amphetamine reduce the level of norepinephrine in the brain, they appear to release the amine in a different manner. Amphetamine action results in

the formation of more normetanephrine while that of reserpine causes the formation of more deaminated metabolites. The difference in the pharmacological actions of these two drugs on the central nervous system may be related to the fact that in the case of amphetamine, norepinephrine is released in a physiologically active form, whereas with reserpine only the inactive metabolites are released (Glowinski and Axelrod, 1965). The suggested mode of action of amphetamine thus includes (a) release of active catecholamines onto central adrenergic receptors, (b)inhibition of transmitter re-uptake (c) inhibition of monoamine oxidase and (d) participation as a partial agonist. The net result of these actions would lead to a temporary increase of norepinephrine at the adrenergic receptor. Among the amphetamine analogues, methamphetamine and phenmetrazine elicited behavioral excitation, whose action was inhibited by pretreatment with α-methyl tyrosine but not by reserpine. This implies that the effect of these drugs is dependent on the continuous synthesis of catecholamine (Weissman et al., 1966; Fuxe and Ungerstedt, 1970). The second group of stimulants, (amphetamine analogues) consisting of pipradol, benzphetamine, methylphenidate and NCA (7-benzyl-1-ethyl-1,4-dihydro-4 oxo-1,8-naphthyridine-3 carboxylic acid, Aceto et al., 1967), action is inhibited by reserpine, but not by α-methyl tyrosine. Scheel-Krüger (1971) reported that both groups of these stimulants interact with dopamine and norepinephrine metabolism.

Tricyclic antidepressant drugs: Most widely used drugs for the treatment of depression are the tricyclic antidepressants. The tertiary amine tricyclic antidepressants include imipramine, chlorimipramine and amitryptyline, while the secondary amines are represented by desmethylimipramine, nortryptyline and protryptyline. The ability to inhibit the transmitter uptake mechanism in the neuronal membrane is a characteristic action of the tricyclic drugs. Since transmitter uptake is one of the main mechanisms responsible for the termination of norepinephrine effect, the tricyclic drugs are believed to act by prolonging the sojourn of the neurohormone in the region of receptor sites (Sigg et al., 1963). Studies using brain slices indicated that desmethylimipramine may also act on the intraneuronal concentrating mechanism of storage vesicles (Stienberg, 1970). Recently it has also been demonstrated that the amine uptake mechanism for 5-hydroxytryptamine in serotonin neurons could be blocked by tertiary amines of tricyclic antidepressants but not by secondary amines (Carlsson et al., 1969; Ross et al., 1969). In the blockade of norepinephrine uptake, secondary amines are more active than tertiary amines and the efficacy of secondary amines in enhancing psychomotor activity is much greater than tertiary amines, suggesting the predominant role of norepinephrine. Regarding elevation of mood in depression, tertiary amines appear to be superior, suggesting the involvement of 5-hydroxytryptamine neurons (Carlsson et al., 1969).

Monoamine oxidate inhibitors: These drugs inhibit monoamine oxidase, an enzyme responsible for the metabolism of norepinephrine, dopamine and 5-hydroxytryptamine. Inhibition of this enzyme causes increased locomotor activity accompanied by concomitant increase in the intraneuronal levels of catecholamines and serotonin. Since the behavioral change is better related to the rise in brain norepinephrine than to the elevation of serotonin, the stimulation has been postulated to result from the spillover of free norepinephrine onto receptor sites (Spector et al., 1960, 1963). The drugs that elicit stimulation by this mechanism of action include nialamide, phenelzine, tranylcypromine and pargyline.

The mechanism of action by which some stimulants cause neurophysiological changes has been defined to some extent. These drugs stimulate the central nervous system by either blockade of inhibition or enhancement of synaptic excitation. Strychnine is the prototype of drugs that selectively block postsynaptic inhibition and picrotoxin has a blocking effect on presynaptic inhibition. Pentylenetetrazol does not block either presynaptic or postsynaptic inhibition. The mechanism of stimulant action caused by pentylenetetrazol is not yet clear. Lewin and Esplin (1961) have suggested that it stimulates excitatory and inhibitory neurons and that the convulsive manifestations are due to excitation of cerebral structure relatively unopposed by inhibition.

The stimulatory symptoms observed with the active fraction obtained from the sea anemone were very similar to those of amphetamine,

a central nervous system stimulant. Since amphetamine action is related to central catecholamine metabolism, it was supposed that this active fraction also could alter central monoamines to elicit the stimulatory action. Therefore, the major aim of this investigation was to study the alterations in the concentration and/or metabolism of monoamine in the brain and to correlate the behavior changes with these effects. Drugs which interfere with the synthesis, storage and metabolism of monoamines and their interactions with the active fraction were also studied to investigate the site of action of this active fraction. A part of this investigation also included a comparison of the central effect of this fraction with that of known central nervous system stimulant.

B. METHODS

Male (Swiss Webster albino) mice weighing approximately 20 g. each were used. All animals were housed under normal laboratory conditions. Food and water supply was similar for all animals.

Drug interactions were examined by determining LD50 and ED50 values in mice. The LD50 was defined as the dose that killed 50% of the animals after twenty-four hours and the ED50 was defined as the dose causing fighting episode, increased motor activity and clonic convulsions in 50% of the animals. LD50 and ED50 values with confidence limits were calculated according to the method of Litchfield and Wilcoxon (Litchfield and Wilcoxon, 1948).

All drugs were administered intraperitoneally unless otherwise stated. Each treated group and a parallel control group of animals (injected with corresponding volumes of physiological saline) consisted of ten animals.

The following drugs and dosage were employed. Reserpine, 2.5 mg/kg (Serpasil^{R}, Ciba Pharmaceutical Co.), tetrabenazine methanesulfonate, 64 mg/kg (Hoffman-La Roche), DL p-chlorophenylalanine, 300 mg/kg (Nutritional Biochemicals), phenobarbital sodium, 100 mg/kg (Merck & Co.), chlorpromazine, 10 mg/kg (Smith Kline & French Labs), methocarbamol, 400 mg/kg (Robins Co.), DL-α-methyl-p-tyrosine methyl ester HCl, 250 mg/kg (α-MPT, Regis Chemical Co.), DL-Dopa, 100 mg/kg (Cal Biochem), disulfiram, 400 mg/kg (Ayerst Labs), propranolol, 10 mg/kg (Inderal^{R}, Ayerst Labs), phentolamine mesylate, 10 mg/kg subcutaneously (Regitine^{R}, Ciba Pharmaceutical Co.), tetrodotoxin,

10 μg/kg (Sankyo Chemicals), atropine sulfate, 25 mg/kg (City Chemical Co.), physostigmine, 0.8 mg/kg (Merck & Co.), ℓ-H^3-norepinephrine, 0.1 μm/2 ml of medium, sp. activity 2.13 c/mM (The Radiochemical Center, Amersham) and tranylcypromine, 1.25 x 10^{-5}M (Smith Kline & French Labs).

Pretreatment time schedules: The following is a list of the time schedules employed when the mice were pretreated with various drugs. Reserpine and Tetrabenazine given 24 hours and 1 hour respectively before the active fraction. α-MPT given three times, the first twenty four hours, the second eighteen hours and the third four hours prior to the active fraction. DL-dopa and disulfiram were administered one hour and three hours respectively before the active fraction. p-Chlorophenylalanine was given three days consecutively prior to the active fraction. Propranolol, tetrodotoxin, atropine sulfate, phentolamine and physostigmine were given thirty minutes, fifteen minutes, thirty minutes, one hour and five minutes respectively before the administration of the active fraction. Phenobarbital sodium, chlorpromazine and methocarbamol were administered ten minutes, fifteen minutes and five minutes before the active fraction.

Body temperature determination: Rectal temperature measurement of mice was measured using a thermocouple. The probe was inserted into the rectum to a constant depth of about 2 cm. Each test was made on five groups, consisting of ten animals per group. One group was treated with reserpine, the second group with the active fraction, 6.4 mg/kg, and the third received the active fraction after being

pretreated with reserpine two hours earlier. The remaining two groups receiving saline alone and saline after reserpine pretreatment served as controls. Rectal temperatures were taken for every fifteen minutes for a period of two hours. The animals were kept two hours before the experiment in a room at a temperature of about 25°C.

Estimation of brain norepinephrine: Doses of the active fraction 1.0, 3.2, 6.4 and 9.3 mg/kg were administered intraperitoneally to mice. For each dose, five determinations were made to calculate the standard error and P value. The brains of five animals were pooled for each determination. The control group received saline solution intraperitoneally. After five, fifteen and thirty minutes the mice were decapitated and the brains were dissected out and frozen immediately in dry ice. The time intervals were selected since the latent period for the onset of stimulation was about five minutes, and the duration of action was about twenty minutes. By thirty minutes the stimulation period was over.

Brain norepinephrine was chemically assayed by a procedure which was a modification of that described by Anton and Sayre (1962). About two grams of brain tissue (brains pooled from five mice) were homogenized in 10 ml of 0.4N perchloric acid (Mallinckrodt) in a glass tissue homogenizer submerged in an ice bath. The homogenate was transferred to a 50 ml polyethylene centrifuge tube and spun at 30,000 x g in cold room for ten minutes. The clear supernatant was adjusted to 25 ml with 0.4N perchloric acid and transferred to a 50 ml beaker containing 400 mg aluminum oxide (Woelm Neutral Activity

Grade I), 200 mg of EDTA (Fisher) and 10 mg of sodium metabisulfite (B&A). Under constant and rapid stirring the mixture was brought to and maintained at pH 8.6 with 10N and 1N sodium hydroxide. The stirring was continued for five minutes. After the stirring was stopped, the aluminum oxide quickly settled and the supernatant was pipetted and discarded. The precipitated aluminum oxide was washed with 10 ml of distilled water (4 or more times) and transferred into a small glass column with distilled water. After rinsing the column with a small quantity of distilled water (<10 ml), norepinephrine was eluted with 3 ml of 0.05N perchloric acid.

A 0.2 ml aliquot of the perchloric acid eluate was taken in a test tube and 0.1 ml of buffer (0.05M phosphate, pH 7.0) was added and the contents thoroughly mixed. To this, 0.02 ml of a freshly prepared 0.25% solution of potassium ferricyanide (Fisher) was added, mixed thoroughly and allowed to stand for exactly one minute. Then 0.2 ml of fresh alkaline ascorbate was added and mixed immediately. Within 15 to 30 seconds, 0.5 ml of distilled water was added, mixed thoroughly and the sample transferred to a cuvette. This was read in Aminco-Bowman spectrophotofluorometer within five minutes.

The same procedure was followed for the blanks except the ferricyanide was omitted.

Instrument performance (sensitivity and wave lengths) was initially checked with a reference solution of quinine sulfate (0.1 mg/ml in 0.1N H_2SO_4). Standard solutions of norepinephrine (Nutritional Biochemicals) were prepared in four concentrations 50, 100, 200, and

400 ng/ml and 0.2 ml of each norepinephrine solution was reacted as described above and read in spectrophotofluorometer at an excitation wavelength of 396 nm and an emission wavelength of 523 nm. The plot of percentage transmission against the concentration was linear and was used to calculate the norepinephrine content of the sample.

The aluminum oxide was prepared in a hood as follows: 100 gm. of aluminum oxide were taken in a 1000 ml beaker containing 500 ml of 2N HCl. This was covered with a watch glass and heated at 90°C to 100°C for 4-5 minutes with continuous and rapid stirring by means of a magnetic stirrer. The beaker was removed from the heater and the heavier particles of aluminum oxide were allowed to settle for one and a half minute. The yellow supernatant fluid was discarded along with the finer particles of aluminum oxide. The precipitate was washed twice with fresh 250 ml portions of 2N HCl at 70°C for 10 minutes. The supernatant was discarded with the finer aluminum particles each time. Then the precipitate was stirred with 500 ml of 2N HCl for 10 minutes. After decanting the HCl, the precipitate was washed repeatedly with fresh 200 ml portions of distilled water until a pH of 3.4 was reached. Each time the finer particles were decanted. Finally, the aluminum oxide was transferred to a glass plate, heated at 120°C for one hour, at 200°C for two hours and then stored in an incubator at 37°C to keep the powder dry.

Alkaline ascorbate was made by completely dissolving 10 mg of ascorbic acid (Nutritional Biochemicals) in 0.1 ml of distilled water and then adding 5.0 ml of 10N NaOH (Mallinckrodt).

Estimation of dopamine in the brain: The method of Anton and Sayre (1964) was used. Brains from treated and control mice were dissected out and frozen as described for the norepinephrine determinations. The reagents and the extraction procedure employed for dopamine were similar to those described for norepinephrine.

To a 0.2 ml portion of the 0.05N perchloric acid eluate from the aluminum oxide column, 0.01 ml of ethanol (70% v/v) and 0.10 ml of the buffer (0.5M KH_2PO_4, pH 7.0) were added. To this, 0.02 ml of the periodate solution (0.5% $NaIO_4$) and then in one minute 0.10 ml of alkaline sulfite solution were added. The following were added as rapidly as possible with mixing in the order presented: 0.28 ml water, 0.10 ml citrate buffer, and 0.17 ml phosphoric acid (3M). This sample was then read in the Aminco-Bowman spectrophotofluorometer at excitation wavelength of 392 nm and emission wavelength of 327 nm. The dopamine content was estimated from the linear transmission-concentration plot of 100,200,400 and 600 ng/ml concentrations of a standard solution (Nutritional Biochemicals).

Alkaline Sulfite solution was prepared by dissolving 125 mg anhydrous sodium sulfite (Baker) in 0.5 ml distilled water to which 4.5 ml of 5N NaOH (Mallinckrodt) was added.

Citrate buffer, 0.5M, pH 4.0 was made by adjusting a solution of 1.0M citric acid (Baker) to pH 4.0 with sodium hydroxide and diluting with water to 0.5M.

Estimation of brain serotonin: Brain serotonin was measured by the method of Udenfriend (1955). Brains from mice treated with the active fraction (6.4 mg/kg) and from control mice were dissected out and frozen as in the previous methods. About 2.5 gm of brain tissue (brains pooled from 6 mice) were homogenized in 5.0 ml 0.1N HCl. The homogenate was centrifuged at 30,000 x g in cold room for ten minutes and 3 ml aliquot of the homogenate was transferred to a 60 ml glass stoppered tube and adjusted to pH 10.0 by the addition of anhydrous sodium carbonate (B&A). After addition of 5 ml of borate buffer (pH 10.0), the solution was diluted with water to 15 ml and to this 5 g of sodium chloride and 15 ml of n-butanol were added. The tube was then shaken for ten minutes, subjected to centrifugation and the fluid decanted from the solid material into a glass stoppered centrifuge tube. After further centrifugation the aqueous layer was removed by pipette and the butanol phase washed by shaking with an equal volume of borate buffer and centrifuged. Ten ml of the butanol phase was then transferred to another tube containing 20 ml of heptane and 1.5 ml of 0.1N HCl. After shaking and last centrifugation, the supernatant solvent was removed, and the aqueous phase containing the extracted serotonin (5-hydroxytryptamine) assayed on the Aminco-Bowman spectrophotofluorometer at excitation wavelength of 290 nm and emission wavelength of 560 nm, against suitable blanks treated in the same manner. Serotonin content was read from a standard curve prepared from four concentrations, 100, 200, 400 and 600 ng/ml of standard solutions of serotonin (Mann Research Laboratories). Fluorescence was

determined after addition of 0.3 ml of concentrated HCl to 1.0 ml of serotonin containing aqueous phase.

In the preparation of the borate buffer, to 94.2 gm of boric acid (B&A) in three liters of water were added 165 ml of 10N NaOH (Mallinckrodt). The buffer solution was then saturated with purified n-butanol (Matheson Coleman & Bell) and sodium chloride (Mallinckrodt) by addition of these substances in excess and shaking. The final pH was brought to approximately 10.0.

Reagent grade n-butanol and n-heptane (Mallinckrodt, A.R.) were purified by shaking first with an equal volume of 0.1N NaOH, then with an equal volume of 0.1N HCl (Baker), and finally twice with distilled water.

Uptake Studies: Three groups of mice, each consisting of five animals, were treated with 6.4 mg/kg of the active fraction. At intervals of five, fifteen and thirty minutes the treated and the saline control mice were decapitated and the brains were rapidly removed and kept in beakers containing ice-cold Krebs-Henseleit medium ($4^{o}C$). The cerebral hemispheres were separated from the rest of the brain and the white matter underneath the gray was carefully removed with a spatula. Thin slices of cerebral cortex were made on a tissue chopper and about 30 mg of the cerebral slices were then transferred to 15 ml tubes containing 2 ml of the ice cold medium and tranylcypromine (1.25×10^{-5}M). This mixture was agitated and incubated at $37^{o}C$ under 95% 0_2 -5% $C0_2$ gas mixture in a shaker for five minutes, after which ℓ-H^3-norepinephrine (0.1 μM) was added and the incubation continued

for thirty minutes. At the end of the incubation period the content of each beaker was poured over Whatman No. 542 filter paper discs moistened with ice-cold 0.9% saline in a Buchner funnel and connected to a vacuum. The tissues were washed with ten milliliters of ice-cold saline and the disc containing tissue was partially dried under vacuum. Each tissue piece was then weighed and approximately 25 mg of tissue and 50 µℓ of the medium from each sample was also taken into separate vials. Half an ml of Soluene (Packard) was added to each vial and kept overnight in a dark room. After the tissues were completely solubilized, fifteen milliliters of scintillation fluid were added and radioactivity measured in a Packard liquid scintillation counter.

Preparation of Krebs-Henseleit bicarbonate medium: The composition of the medium was 119.2 mM NaCl, 4.78 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 7 H_2O and 25 mM $NaHCo_3$. This total mixture was mixed for ten minutes with gas mixture of 95% O_2 and 5% CO_2. Finally to this medium 11 mM glucose, 0.2 mg/ml ascorbic acid and 0.05 mg/ml EDTA were added.

Scintillation fluid was made by dissolving 5 g 2,5-diphenyloxazole (PPO) and 50 mg of 1,4 bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (POPOP) in one liter of toluene.

Estimation of brain normetanephrine: Brain normetanephrine was measured by a method of Leonard and Tonge, 1969, which was a modification of that described by Anton & Sayre (1966). Brains from mice treated with the active fraction at a dose of 6.4 mg/kg and control mice were dissected out and frozen as described for the estimation of norepinephrine. About 1.5 gm of tissue (brains pooled from 4 mice) were

homogenized in 6 ml of 1.0N HCl. The homogenate was centrifuged at 30,000 x g for thirty minutes in cold room and the clear supernatant transferred to a 15 ml beaker containing 2 ml of 1M Tris buffer (Sigma). The pH was adjusted to 6.8-7.2 before the addition of 2 ml of borate buffer (pH 10.0). Fourteen grams of K_2HPO_4 (Mallinckrodt) and 8 ml of n-butanol were added and the mixture was shaken for ten minutes, centrifuged and the organic layer removed. The aqueous phase was shaken again for ten minutes with 4 ml n-butanol and after centrifugation, the butanol extracts were pooled and shaken for an additional ten minutes with 4 ml of 0.1N HCl and 18 ml n-heptane. The butanol-heptane phase was discarded and the acid phase was mixed with 9 gm of K_2HPO_4 and 1 ml of borate buffer. The resultant saturated solution was shaken for ten minutes with 15 ml of ether (Mallinckrodt). The ether layer was removed and shaken with 1.5 ml of 0.1N HCl. Aliquots of the acid phase were then used for fluorophor formation.

To a 0.2 ml aliquot of the acid extract the following ingredients were added with thorough mixing: 0.20 ml of distilled water, 0.10 ml of EDTA and 0.05 ml of citric acid-sodium acetate buffer (0.80M, pH 5.0). To this, 0.05 ml of the periodate solution (20 mg/ml) was added and allowed to stand for exactly four minutes. Quickly 0.10 ml of the sulfite solution (100 mg/ml) and 0.30 ml of alkaline ascorbate reagent was added and this was then read in an Aminco-Bowman spectrophotofluorometer. Each sample was read at excitation wavelength of 396 nm and emission wavelength of 500 nm against suitable blanks and

the normetanephrine content estimated from a standard curve produced from a standard solution of DL normetanephrine (Winthrop) at four concentrations 50, 100, 200 and 400 ng/ml.

Citric acid-sodium acetate buffer, 0.8M, pH 5.0, was made by adjusting a 2.0M citric acid solution (Baker) to pH 5.0 with a 2.0M solution of sodium acetate (Matheson, Coleman & Bell) and then diluting with distilled water to 0.8M.

The borate buffer, 1.0M, pH 10.0 was made by dissolving 30.92 gm of boric acid (B&A) in 450 ml of distilled water and adjusting to pH 10.0 with solid and 5.0N NaOH. This solution was then saturated with sodium chloride and n-butanol, readjusted to pH 10.0 and made up to 500 ml with distilled water and filtered through two sheets of Whatman No. 1 paper.

Alkaline ascorbate consisted of 10 mg of ascorbic acid dissolved in 0.1 ml of distilled water, and 10.0 ml of 10N NaOH added and thoroughly mixed.

Statistics: Standard errors of the mean were calculated for all the values in the experiments and delineated by vertical bars on the graphs and parentheses in the tables. Students' *t* test was used to evaluate the level of significance between experimental and control groups (Dunn, O. J., 1967).

C. RESULTS

Behavioral effects: Signs of central nervous system stimulant activity were observed five minutes after administration of the active fraction at doses ranging from 1 to 9 mg/kg. The signs included increased motor activity and fighting episodes (Fig. 4). This stimulatory activity peaked at about fifteen minutes and all animals recovered by thirty minutes. At higher doses (range 11 to 15 mg/kg) the onset of stimulant action was about 2-3 minutes, followed by severe tonic convulsions and death by about fifteen minutes.

ED50 and LD50: The fighting episode induced by the active fraction occurred at a frequency of 2-3 times/minute and was used as an objective measure of the stimulant activity of the drug. The increase in motor activity produced by the drug generally paralleled the fighting episode. However, no attempt was made to quantitatively measure the increase in motor activity. The ED50 of the active fraction based on the fighting episode (See Fig. 4) was found to be 6.4 mg/kg. After the administration of the active fraction at the ED50, the fighting episodes which occurred started within 4-6 minutes, peaked, and waned in about 20 minutes. When the LD50 (12.2 mg/kg) of the active fraction was injected, toxic symptoms such as ataxia, catalepsy and tonic convulsions were observed before death.

Effect of phenobarbital, chlorpromazine and methocarbamol: Phenobarbital sodium (100 mg/kg), chlorpromazine (10 mg/kg) and methocarbomal (400 mg/kg) completely blocked the fighting episode of the active fraction even at the ED100 (9.3 mg/kg) dose level. On the otherhand the

Figure 4. THE FIGHTING EPISODE IN THE MOUSE AFTER INTRAPERITONEAL ADMINISTRATION OF THE ACTIVE FRACTION AT THE ED50 DOSE.

LD50 dose of the active fraction was not significantly increased (Table 1) and the animals which died still showed convulsions. Phenobarbital sodium, chlorpromazine and methocarbamol at the same doses did not modify either the normal spontaneous behavior or produce death in control animals (given physiological saline instead of the active fraction).

Effect of reserpine, tetrabenazine and α-MPT: Reserpine pretreatment (24 hours) (Table 2) decreased the ED50 of the active fraction by 50% and accelerated the onset of the stimulant action. On the other hand, such treatment increased toxicity twofold. Tetrabenazine methanesulfonate administered one hour previously, similarly affected the response to the active fraction as tetrabenazine decreased the ED50 of the active fraction by approximately 50%, while increasing its toxicity significantly (Table 2). α-MPT, 250 mg/kg given three times, the first twenty four hours, the second eighteen hours and the third four hours prior to the active fraction (Table 2), did not affect the ED50 significantly. The LD50 of the active fraction, on the other hand, was significantly decreased (Table 2). When similar α-MPT treatment was incorporated in the reserpine and tetrabenazine treated animals, the fighting episodes, clonic convulsions and increased motor activity of the active fraction were completely blocked even at the ED100 (9.3 mg/kg) dose level (Table 2). The effects on the toxicity was not statistically significant when LD50's are compared but tended to be enhanced. Reserpine and tetrabenazine as well as combinations of these agents with α-MPT produced sedation and decreased motor

Table 1

THE EFFECT OF PHENOBARBITAL, CHLORPROMAZINE AND METHOCARBAMOL ON THE ED50 AND THE LD50 OF THE ACTIVE FRACTION

*Drug	Active fraction	
	ED50 mg/kg	LD50 mg/kg
None (Control)	6.4 (7.6 - 5.4)	12.2 (13.6 - 10.9)
PHENOBARBITAL SODIUM, 100 mg/kg	**	12.7 (14.1 - 10.5)
CHLORPROMAZINE, 10 mg/kg	**	13.4 (14.5 - 11.0)
METHOCARBAMOL, 400 mg/kg	**	12.4 (13.7 - 11.1)

ED50 and LD50 were calculated by the method of Litchfield and Wilcoxon (1949).

*Active fraction administered after pretreatment.

**Even at ED100 of active fraction, stimulant activity could not be observed.

All drugs were administered intraperitoneally.

Table 2

THE EFFECT OF AGENTS WHICH PRODUCE CATECHOLAMINE DEPLETION ON THE ED50 AND THE LD50 OF THE ACTIVE FRACTION

*Drug	Active fraction	
	ED50 mg/kg	LD50 mg/kg
None (control)	6.4 (7.6-5.4)	12.2 (13.6-10.9)
Reserpine, 2.5 mg/kg	3.7 (5.2-2.6)	7.5 (9.0- 6.2)
Tetrabenazine, 64 mg/kg	3.4 (5.3-2.1)	8.0 (9.6- 6.6)
α-MPT, 250 mg/kg	5.7 (6.5-4.9)	8.2 (10.1- 6.7)
Reserpine + α-MPT	**	5.3 (6.8- 4.1)
Tetrabenazine + α-MPT	**	5.1 (7.1- 3.7)

ED50 and LD50 were calculated by the method of Litchfield and Wilcoxon (1949).

*Active fraction administered after pretreatment.

**Even at ED100 of active fraction, stimulant activity could not be observed.

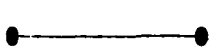
All drugs were administered intraperitoneally.

activity but did not produce death in control animals given physiological saline. α-MPT alone did not produce any gross behavioral changes.

Effect on the body temperature: As shown in Figure 5, the active fraction had significant effect on the mouse body temperature thirty minutes after administration. Reserpine (2.5 mg/kg., 2 hr pretreatment) alone increased the body temperature maximally 0.9°C at thirty minutes. The active fraction in the reserpine pretreated animals increased this hyperthermic response as the body temperature increased significantly by 2.1°C with peak effect occurring within fifteen minutes (Table 3). No change in body temperature was observed in control group of animals

Effect of DL-dopa and disulfiram: DL-dopa pretreatment (1hr) decreased the ED50 of the active fraction by 44 percent without affecting the LD50 significantly (Table 4). DL-Dopa at the same dose did not cause any stimulation in control animals given saline. As previously indicated, the active fraction failed to cause any stimulatory action after combined pretreatment with α-MPT and reserpine (Table 2). However, DL-dopa administration in such treated animals restored the excitatory action of the active fraction to the control level. Such treatment also decreased LD50 of the active fraction; the latter was increased beyond twofold (Table 4). α-MPT and disulfiram administered three hours previously, did not significantly increase the ED50 or the LD50 of the active fraction (Table 4). Such action of α-MPT and disulfiram was antagonized by the DL-dopa pretreatment. The active

Figure 5. THE EFFECT OF THE ACTIVE FRACTION AND RESERPINE ON THE MOUSE BODY TEMPERATURE.



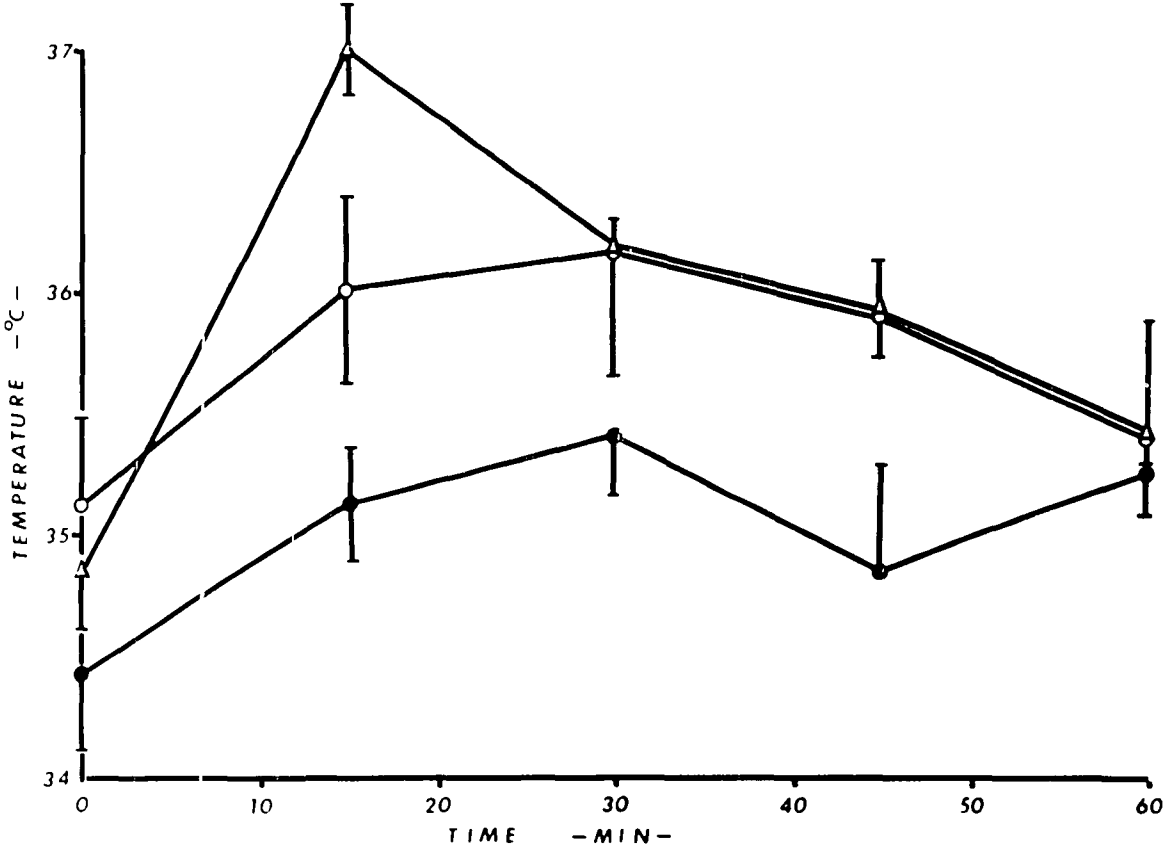

37
36
35
34
TEMPERATURE -°C-
0
10
20
30
40
50
60
TIME -MIN-

Table 3

THE EFFECT OF THE ACTIVE FRACTION AND RESERPINE ON THE BODY TEMPERATURE (Mouse)

DRUG	DOSE (I.P.)	TIME (Min)	TEMP. ^{o}C[b]	INCREASE IN TEMP. ^{o}C
ACTIVE FRACTION	6.4	0	34.4 ± .33	-
		15	35.1 ± .24	0.7
		30	35.4 ± .23	1.0[c]
RESERPINE	2.5	0	35.1 ± .33	-
		15	36.0 ± .38	0.9
		30	36.1 ± .54	1.0
RESERPINE + ACTIVE FRACTION	6.4[a]	0	34.9 ± .29	-
		15	37.0 ± .20	2.1[d]
		30	36.1 ± .18	1.2[e]

[a] Active fraction was administered after reserpine treatment (2.5 mg/kg)

[b] Expressed as the Mean ± S.E.

[c] $P < 0.05$ [d] $P < 0.001$ [e] $P < 0.01$

Table 4

THE EFFECT OF DOPAMINE ON THE ED50 AND THE LD50 OF THE ACTIVE FRACTION

*Drug	Active fraction	
	ED50 mg/kg	LD50 mg/kg
None (control)	6.4 (7.6-5.4)	12.2 (13.6-10.9)
DL-Dopa, 100 mg/kg	3.6 (5.3-2.4)	13.6 (14.7-12.6)
Reserpine + α-MPT + DL-Dopa	5.5 (7.7-3.9)	11.5 (14.2- 9.2)
α-MPT + Disulfiram, 400 mg/kg	9.0 (11.2-7.3)	13.7 (14.9-12.6)
Reserpine + Disulfiram	**	9.7 (11.4- 8.2)
α-MPT + Disulfiram + DL-Dopa	5.7 (7.1-4.6)	12.8 (14.4-11.3)
Reserpine + Disulfiram + DL-Dopa	9.9 (11.2-8.6)	14.2 (15.7-12.8)

ED50 and LD50 were calculated by the method of Litchfield and Wilcoxon (1949).

*Active fraction administered after pretreatment.

**No stimulant activity was observed even after treatment with ED100 of active fraction.

All drugs were administered intraperitoneally.

fraction was devoid of any stimulatory action in animals treated with both reserpine and disulfiram, but the excitatory activity was restored by DL-dopa and the ED50 of the active fraction was increased by 55 percent. Also DL-dopa treatment increased its LD50 by 30 per cent over the animals treated with reserpine and disulfiram. There were no deaths in saline control groups after combined treatments as described in Table 4.

Effect of p-chlorophenylalanine: P-chlorophenylalanine, an inhibitor of tryptophan hydroxylase, was administered three days consecutively with a dose of 300 mg/kg. This treatment had no significant effect on the ED50 or the LD50 of the active fraction. P-chlorophenylalanine at the same dose did not modify the behavior or produce death in saline control animal.

Effect of propranolol, phentolamine, physostigmine, atropine and tetrodotoxin: In Table 5 is presented the effects of propranolol, phentolamine, physostigmine, atropine and tetrodotoxin on the active fraction the ED50 and the LD50. Propranolol, a β-adrenergic receptor blocking agent was administered before the active fraction. The stimulant action of the active fraction was completely blocked even at the ED100 level, but the LD50 did not change significantly. On the other hand, α-adrenergic receptor blockade by phentolamine, administered subcutaneously one hour before, did not alter both the ED50 and LD50 of the active fraction. Intraperitoneal administration of atropine sulfate and tetrodotoxin thirty and fifteen minutes pretreatment, respectively, had very little effect on the ED50 of the

Table 5

THE EFFECT OF PROPRANOLOL, PHENTOLAMINE, ATROPINE, TETRODOTOXIN AND PHYSOSTIGMINE ON THE ED50 AND THE LD50 OF THE ACTIVE FRACTION

*Drug	Active fraction	
	ED50 mg/kg	LD50 mg/kg
None (control) I.P.	6.4 (7.6-5.4)	12.2 (13.6-10.9)
Propranolol, 10 mg/kg I.P.	**	11.5 (12.5-10.5)
Phentolamine, 10 mg/kg S.C.	5.6 (8.4-3.7)	11.1 (12.6- 9.7)
Atropine, 25 mg/kg I.P.	5.4 (6.9-4.2)	***
Tetrodotoxin, 10 µg/kg I.P.	5.0 (6.4-3.9)	***
Physostigmine, 0.8 mg/kg I.P.	**	6.0 (7.3- 4.9)

ED50 and LD50 were calculated by the method of Litchfield and Wilcoxon (1949).

*Active fraction administered after pretreatment.

**No stimulant activity was observed even after treatment with ED100 of active fraction.

***No mortality was observed even after treatment with LD100 of active fraction.

I.P. = Intraperitoneally

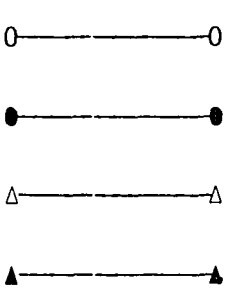
S.C. = Subcutaneously

active fraction. However, after these drugs the LD50 dose of the active fraction was not determinable since no deaths occurred at usual LD50 nor at the LD100. Cholinesterase inhibition with physostigmine five minutes prior to the active fraction treatment abolished the stimulant action even at the ED100 (9.3 mg/kg) dose; toxicity was also increased on the basis of the 50 percent reduction in the LD50, over the control. These drug treatments (Table 5) did not change the behavior or produce death in saline controls.

Effect of the active fraction on brain norepinephrine content: Figure 6 illustrates the effect of the active fraction upon the brain norepinephrine content after five, fifteen and thirty minute intervals. At the ED50 dose the active fraction decreased brain norepinephrine content at the three time intervals. The percent decrease observed was 13.1, 22.2 and 34.2 at five, fifteen and thirty minutes, respectively. The P values were $<.01$, $<.001$, and $<.001$ for the three time intervals, respectively. The ED100 dose (9.3 mg/kg) likewise significantly decreased brain norepinephrine levels at all three time intervals ($p < .001$; Table 6). Even at the ED20 dose (3.2 mg/kg) the percent decrease of 13.8 at fifteen minutes was significant ($p < .01$), whereas at the five and thirty minute intervals the decrease was not significant. A non-effective dose (1.6 mg/kg) did not alter the brain norepinephrine content at all time intervals tested.

Effect of the active fraction on brain dopamine content: In Figure 7 is illusted the effects of the active fraction on the brain dopamine content at the five, fifteen and thirty minute time intervals. The

Figure 6. THE EFFECT OF THE ACTIVE FRACTION ON NOREPINEPHRINE CONTENT IN THE MOUSE BRAIN



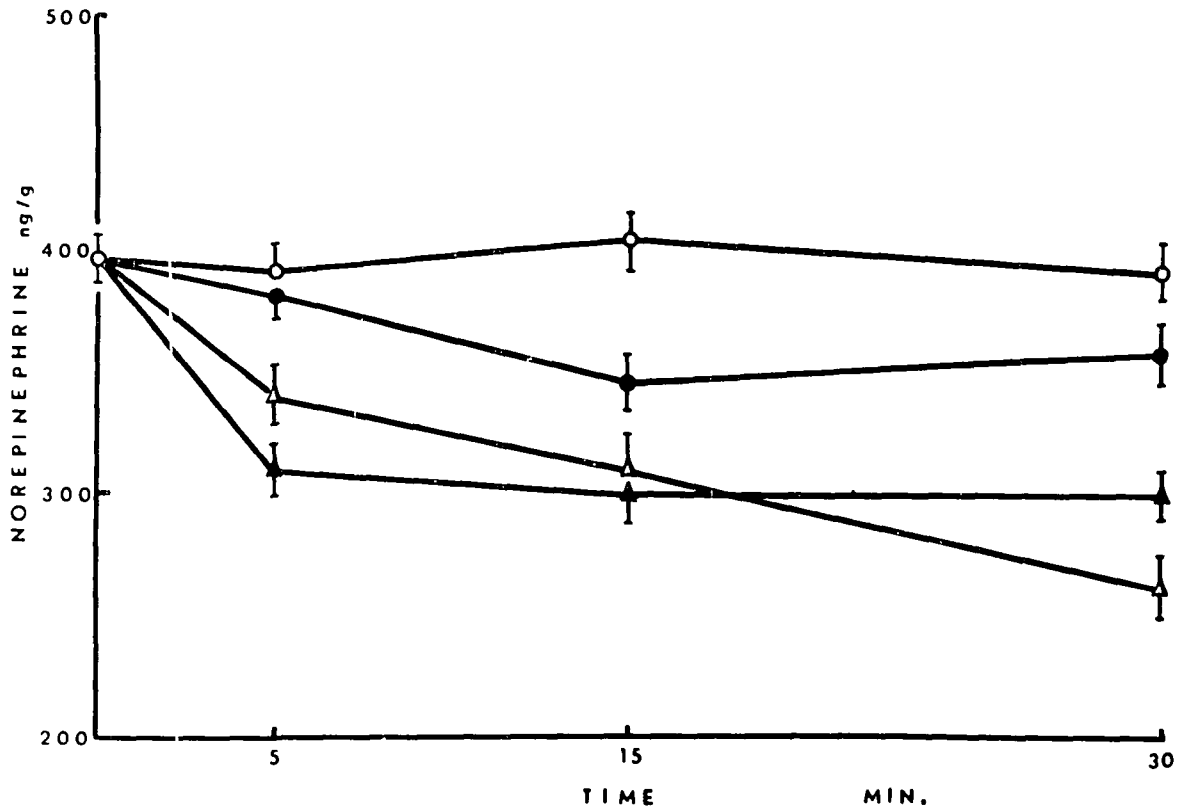

500
400
300
200
NOREPINEPHRINE ng/g
5
15
30
TIME
MIN.

Table 6

THE EFFECT OF THE ACTIVE FRACTION ON NOREPINEPHRINE CONTENT IN THE MOUSE BRAIN

DOSE[a]	TIME (min)	N	NOREPINEPHRINE[b]	% DECREASE[c]
CONTROL	0	7	397 ± 11.6	-
1.6	5	5	393 ± 4.3	n.s.
	15	5	406 ± 5.7	n.s.
	30	5	395 ± 6.2	n.s.
3.2	5	5	381 ± 8.1	n.s.
	15	5	342 ± 6.2	13.8[d]
	30	5	360 ± 15.3	9.3
6.4	5	5	345 ± 8.9	13.1[d]
	15	5	309 ± 7.0	22.2[e]
	30	5	261 ± 16.4	34.2[e]
9.3	5	5	311 ± 12.0	21.8[e]
	15	5	298 ± 14.0	25.0[e]
	30	5	296 ± 10.6	25.4[e]

[a] mg/kg of the active fraction, i.p.

[b] ng/g brain expressed as the Mean ± S.E.

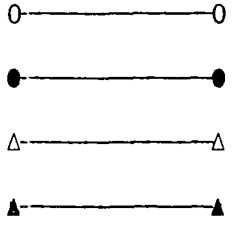
[c] Percentage decrease from control

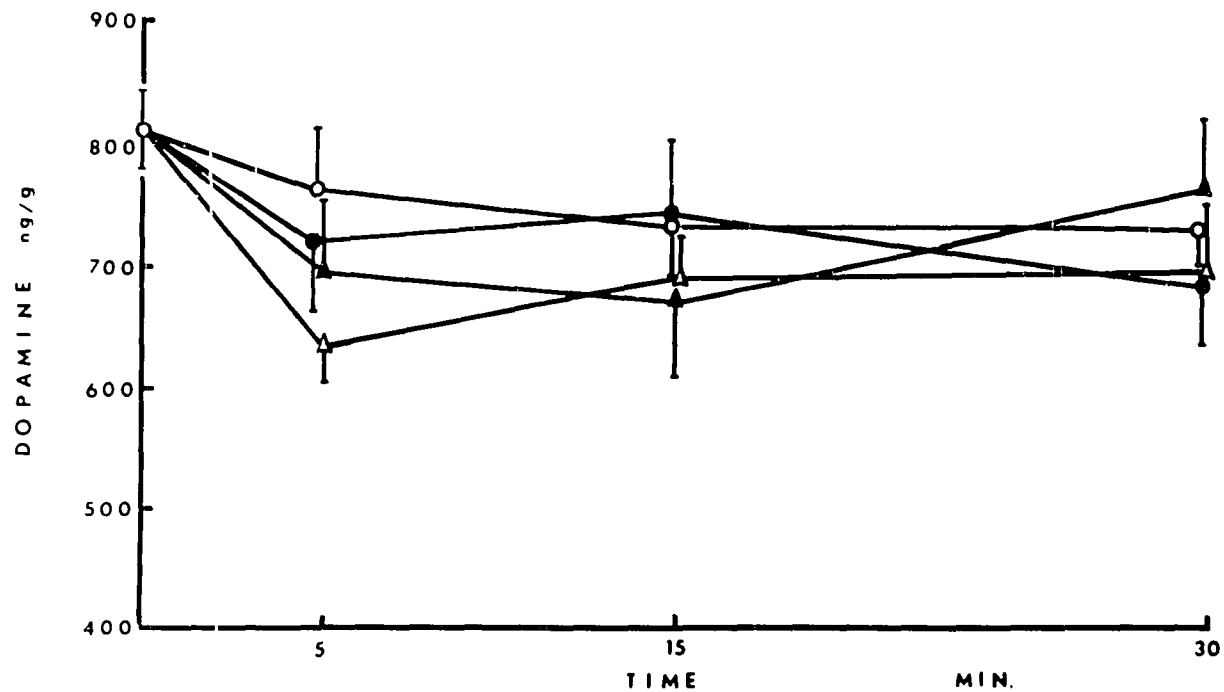
n.s. not significant

[d] P <0.01

[e] P <0.001

Figure 7. THE EFFECT OF THE ACTIVE FRACTION ON DOPAMINE CONTENT IN THE MOUSE BRAIN.




900
800
700
600
500
400
DOPAMINE ng/g
5
15
30
TIME
MIN.

active fraction at the ED50 dose caused a decrease of 21.5, 15.3 and 14.7 percent at the five, fifteen and thirty minutes intervals respectively, with the decrease being significant only at the five and fifteen minute time periods (Table 7). At the ED100 dose (9.3 mg/kg) the percent decrease in brain dopamine was 13.6, 16.2 at the same times, but these differences were not significant. At the ED20 dose (3.2 mg/kg) and at the non-effective dose (1.6 mg/kg) although a decrease in brain dopamine was observed the change was not statistically significant (Table 7).

Effects of the active fraction on brain Serotonin content: The effects of the active fraction on the brain Serotonin content after five, fifteen and thirty minute time intervals are shown in Figure 8. The brain serotonin content of the animals treated with the ED50 dose of the active fraction did not differ from the saline control (Table 8).

Effect of the active fraction on the uptake of ℓ-H^3-norepinephrine by brain slices (cerebral cortex) in vivo: Figure 9 represents the effect of the active fraction on the uptake of ℓ-H^3-norepinephrine by brain slices after five, fifteen and thirty minute intervals. The ED50 dose significantly inhibited by 22.8 percent the uptake at the fifteen minute interval (Table 9). There was no significant effect on the uptake after the five minute period or at thirty minute interval.

Effect of the active fraction brain normetanephrine content: In Figure 10 is illustrated the effect of the active fraction on the content of brain normetanephrine, one of the main metabolites of endogenous norepinephrine. At the ED50 dose, the active fraction significantly elevated the brain normetanephrine content at five, fifteen

Table 7

THE EFFECT OF THE ACTIVE FRACTION ON DOPAMINE CONTENT IN THE MOUSE BRAIN

DOSE[a]	TIME (min)	N	DOPAMINE[b]	% DECREASE[c]
CONTROL	0	6	807 ± 33.3	-
1.6	5	5	765 ± 45.6	n.s.
	15	5	731 ± 42.2	n.s.
	30	5	729 ± 32.7	9.7
3.2	5	5	711 ± 68.6	12.0
	15	5	737 ± 63.8	8.7
	30	5	682 ± 65.3	15.6
6.4	5	5	634 ± 30.6	21.5[d]
	15	5	684 ± 40.6	15.3[e]
	30	5	688 ± 54.8	14.7
9.3	5	5	697 ± 60.3	13.6
	15	5	677 ± 69.5	16.2
	30	5	765 ± 49.4	n.s.

[a] mg/kg of the active fraction, i.p.

[b] ng/g brain expressed as the Mean ± S.E.

[c] Percentage decrease from control

n.s. not significant

[d] $P < 0.001$

[e] $P < 0.05$

Figure 8. THE EFFECT OF THE ACTIVE FRACTION ON SEROTONIN (5-HYDROXY TRYPTAMINE) CONTENT IN THE MOUSE BRAIN.

O———————O ACTIVE FRACTION at 6.4 mg/kg i.p.

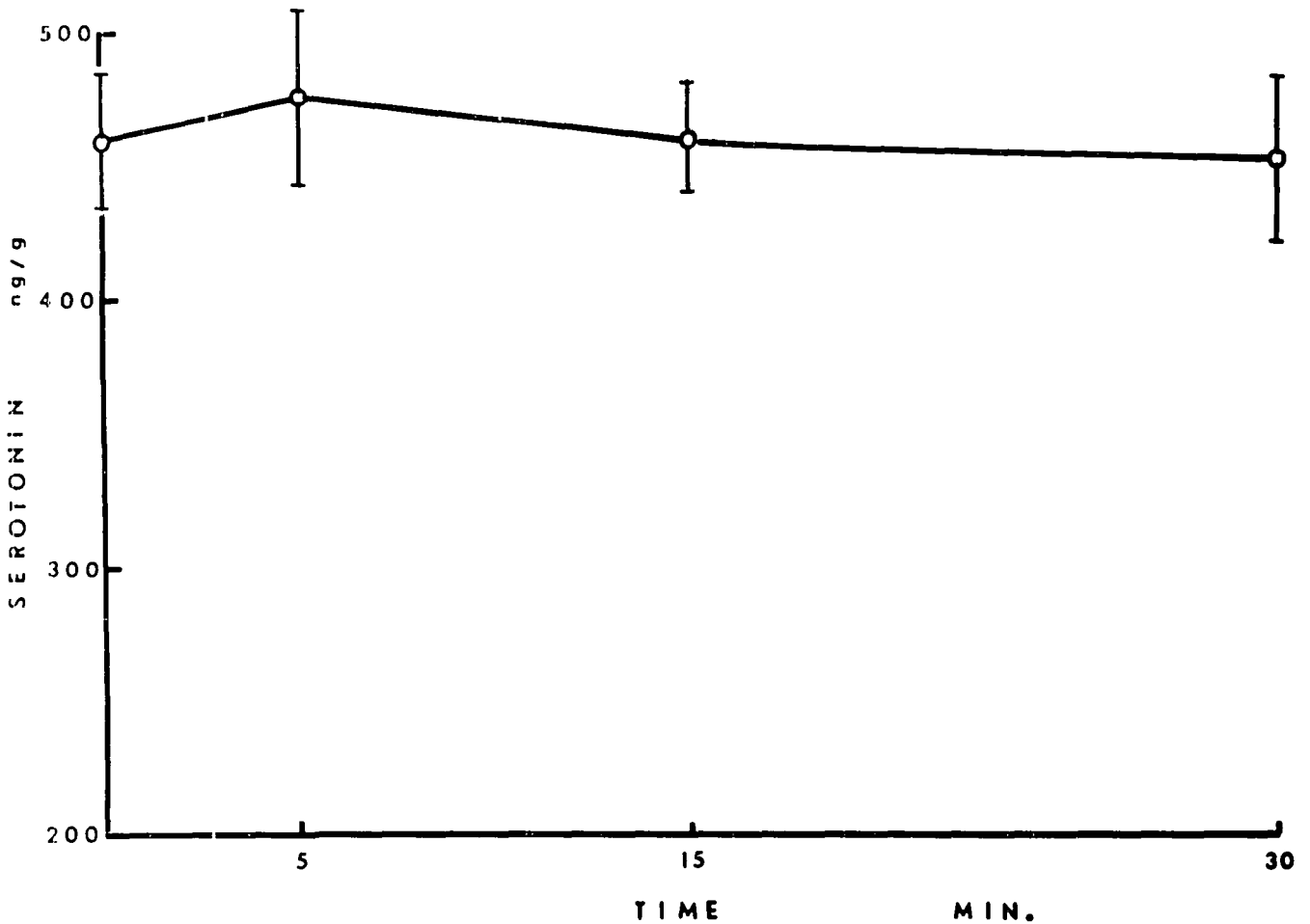

500
400
300
200
SEROTONİN ng/g
5
15
30
TIME MIN.

Table 8

THE EFFECT OF THE ACTIVE FRACTION ON SEROTONIN (5-HYDROXYTRYPTAMINE) CONTENT IN THE MOUSE BRAIN

DOSE[a]	TIME (min)	N	5-HYDROXYTRYPTAMINE[b]	% DECREASE[c]
CONTROL	0	7	461 ± 23.9	-
6.4	5	5	478 ± 30.9	-
	15	5	460 ± 20.6	n.s.
	30	5	450 ± 29.4	n.s.

[a] mg/kg of the active fraction, i.p.

[b] ng/g brain expressed as the Mean ± S.E.

[c] Percentage decrease from control

n.s. not significant

Figure 9. THE EFFECT OF THE ACTIVE FRACTION ON THE UPTAKE OF ℓ-H^3-NOREPINEPHRINE INTO THE MOUSE BRAIN SLICES (CEREBRAL CORTEX) IN VIVO.

O———————O ACTIVE FRACTION at 6.4 mg/kg i.p.

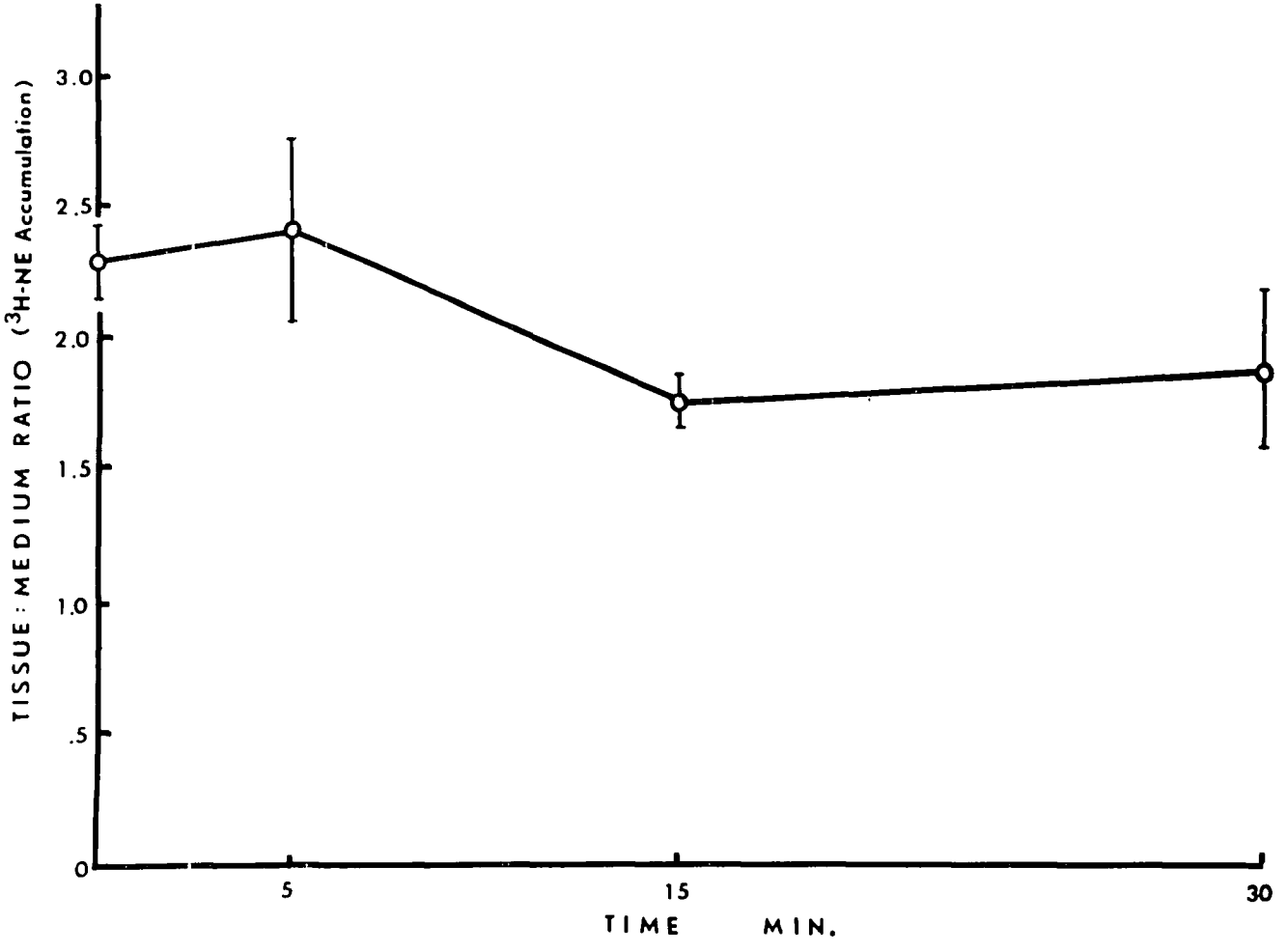

TISSUE : MEDIUM RATIO (^{3}H-NE Accumulation)
3.0
2.5
2.0
1.5
1.0
.5
0
5
15
30
TIME MIN.

Table 9

THE EFFECT OF THE ACTIVE FRACTION ON THE UPTAKE OF ℓ-H^3-NOREPINEPHRINE INTO THE MOUSE BRAIN SLICES (CEREBRAL CORTEX) IN VIVO

DOSE[a]	TIME (min)	N	ℓ-H^3-NE ng/30/g brain[b] min slice	T/M[c]	% INHIBITION[d]
CONTROL	0	5	37.6 ± 2.3	2.28 ± 0.14	-
6.4	5	5	40.9 ± 3.4	2.40 ± 0.35	n.s.
	15	7	27.7 ± 1.5	1.76 ± 0.10	22.8[e]
	30	5	31.8 ± 3.2	1.88 ± 0.29	n.s.

[a] mg/kg of the active fraction, i.p.

[b] Expressed as the Mean ± S.E.

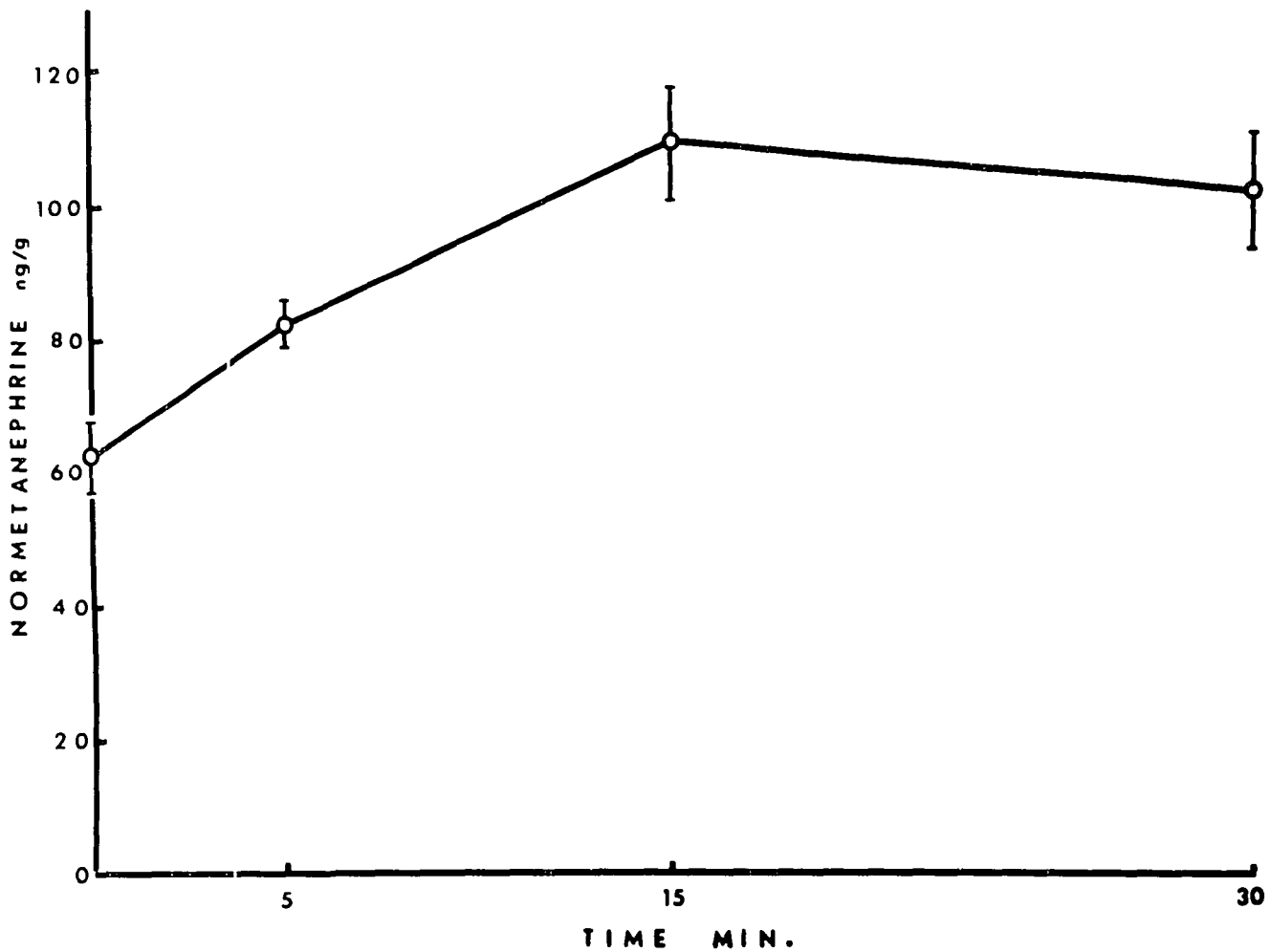
[c] Tissue-Medium ratio of ℓ-H^3-NE

[d] Percentage inhibition of uptake from Control

[e] P <0.02

Figure 10. THE EFFECT OF THE ACTIVE FRACTION ON NORMETANEPHRINE CONTENT IN THE MOUSE BRAIN.

0——————0 ACTIVE FRACTION at 6.4 mg/kg i.p.


120
100
80
60
40
20
0
NORMETANEPHRINE ng/g
5
15
30
TIME MIN.

and thirty minute time intervals. The percentage increase was 32.2, 77.1 and 64.5 at the respective time periods (Table 10).

Table 10

THE EFFECT OF THE ACTIVE FRACTION ON NORMETANEPHRINE CONTENT IN THE MOUSE BRAIN

DOSE[a]	TIME (min)	N	NORMETANEPHRINE[b]	% INCREASE[c]
CONTROL	0	5	63 ± 5.3	-
6.4	5	5	83 ± 3.5	32.2[d]
	15	5	111 ± 8.9	77.1[e]
	30	5	103 ± 8.4	64.5[e]

[a] mg/kg of the active fraction, i.p.

[b] ng/g brain expressed as the Mean ± S.E.

[c] Percentage increase from control

[d] $P < 0.05$

[e] $P < 0.001$

D. DISCUSSION

The behavioral effects of many drugs are thought to involve the catecholamines, norepinephrine or dopamine, in the central nervous system. Investigators have had only limited success in ascertaining whether particular effects were attributable to interactions with norepinephrine or dopamine or both (Glowinski, Axelrod & Iverson, 1966; Wolf et al., 1969). It was reported that the behavioral excitation elicited by central nervous system stimulant amphetamine and its analogues is associated with an effect on both brain dopamine and norepinephrine (Jonas & Scheel-Krüger, 1969). One mechanism by which the most widely used stimulants of the central nervous system might act is through the release of stored catecholamines (Trendelenburg et al., 1962). The results of present study show that the stimulant action of the active fraction was related to central catecholamine metabolism.

The antagonism of the active fraction stimulant activity by centrally depressant drugs phenobarbital sodium, chlorpromazine, and methocarbamol suggests that the excitatory behavior produced by the active fraction was probably mediated centrally.

The use of monoamine depleting agents such as reserpine, tetrabenazine and α-MPT has been of value in attempts to clarify the mechanism of action of other drugs which evoke excitation. In this regard reserpine pretreatment markedly increased the stimulant effect of the active fraction. Similar effect was observed with pretreatment of tetrabenazine methanesulfonate, which selectively depletes central

catecholamines (Chen et al., 1968). Rech (1964) reported that pretreatment of mice with reserpine (5 mg/kg) greatly increased the CNS stimulant effect of d-amphetamine in mice. He suggested that increase in CNS excitability might be attributed to the supersensitivity of the post-synaptic receptor site developed by the depletion of endogenous norepinephrine. While reserpine produces a marked decrease in brain norepinephrine levels by action on storage granules (Carlsson, 1966), it does not impair norepinephrine synthesis (Glowinski, Iverson & Axelrod, 1966) and the brain is partially capable of accumulating the amine within 24 hr of reserpine administration. Consequently, norepinephrine may have been available for release by the active fraction even after reserpine. Also reserpine pretreatment may sensitize the residual norepinephrine stores to the releasing action of the active fraction. This is supported by the hypothesis that brain catecholamines are stored in at least two different neuronal pools aside from the readily releasable pool. The reserpine-resistant pool of the catecholamines in contrast to the reserpine sensitive pool is closely associated and related to the pool consisting of newly synthesized catecholamines (Carlsson et al., 1966; Rech et al., 1968).

α-MPT causes depletion of brain norepinephrine and dopamine by blocking their synthesis at the rate limiting step of tyrosine hydroxylation (Carlsson and Lindqvist, 1962). This treatment did not significantly change the ED50 of the active fraction. When norepinephrine synthesis is inhibited the intraneuronal storage mechanism for the neurohumoral transmitter remains intact even though the granules are

depleted (Spector et al., 1965; Corrodi et al., 1966). From our results, it was assumed that the previously formed tissue catecholamine would be still available for release to cause the stimulatory action. The stimulant action of amphetamine is effectively blocked by α-MPT, suggesting that it requires an uninterrupted synthesis of norepinephrine for its action (Hanson, 1967). The absence of stimulant action produced by the active fraction after combined treatment with α-MPT and reserpine or tetrabenazine might suggest that the presence of a certain amount of tissue catecholamines is required for activity. Previous work indicates that about 90 percent depletion of brain catecholamines is observed in mice treated with both reserpine (2.0 mg/kg) and α-MPT (250 mg/kg), whereas reserpine or α-MPT alone causes only 63 percent depletion (Menon et al., 1967).

From the biochemical studies, it was observed that the active fraction produced a significant decrease in brain norepinephrine at the ED50 and the ED100 doses. A significant decrease in brain norepinephrine content was observed at the ED20 (3.2 mg/kg) dose only at the fifteen minute interval. No decrease in brain norepinephrine was observed with a dose of the active fraction which was subthreshold in terms of the fighting response. The decrease in norepinephrine content by the active fraction might be explained on the basis of either a reduction in the net synthesis or excessive release of norepinephrine from the nerve endings. Since there was no decrease in the norepinephrine content in mice treated with a non-effective dose, it was evident that stimulatory effect was related to central catecholamines. d-Amphetamine produces a 34 percent depletion in brain

norepinephrine at the peak time of stimulation (Taylor and Snyder, 1971).

Both the active fraction and reserpine produced a hyperthermic response in mice. The reserpine induced hyperthermic effect in mice has been explained by the release of catecholamines, since the response was inhibited by adrenergic blocker, propranolol (Mantegazza et al., 1970). Amphetamine induced hyperthermia was strongly antagonized by a β-adrenergic blocking agent, propranolol (10 mg/kg) and kö 592 (Gessa et al., 1969). The active fraction induced hyperthermia may thus be related to a similar mechanism.

After marked decrease of tissue catecholamines by combined treatment with α-MPT and reserpine, the active fraction failed to cause central excitatory action. However, DL-dopa restored the stimulant action. The central stimulant action of d-amphetamine given to a cat in a very large dose (10 mg/kg) is reported to be almost completely blocked by pretreatment with reserpine and α-MPT. However, the activity of d-amphetamine is restored by a small dose of DL-dopa (Carlsson, 1970). Since inhibition of tyrosine hydroxylase by α-MPT also reduced the rate of synthesis of dopamine, the avilability of this catecholamine may also be required for some of the central actions of the active fraction. To elucidate further the role of dopamine in the active fraction, disulfiram, an effective inhibitor of dopamine β-hydroxylase was used. The increase of stimulant action of the active fraction by DL-dopa in animals after combined treatment with α-MPT and disulfiram and restoration after combined treatment

with reserpine and disulfiram indicate the importance of dopamine for this stimulant action. In reserpine (10 mg/kg) pretreated mice amphetamine produced a pronounced increase in motility, which was blocked by α-MPT (200 mg/kg) but not at all by FLA-63 (50 mg/kg), a dopamine β-hydroxylase inhibitor. In mice with their monoamine stores intact, α-MPT blocked the stimulant action of amphetamine. FLA-63 also had some inhibitory effect, though less complete. These data indicate that dopamine plays a dominant role for the central stimulant action in mice (Carlsson, 1970). It is thus tempting to speculate that in the present experiments the excess of dopamine can act as a transmitter agent and thus compensate for the loss of norepinephrine.

From the estimations of dopamine content in the brain after treatment with the active fraction at various doses, it was observed that the active fraction significantly decreased dopamine content at the ED50 dose. The decrease was not significant at the ED100 (9.3 mg/kg) and the ED20 (3.2 mg/kg) doses. These results suggest that the role of dopamine in this stimulant action at best is minor. If the assumption is correct that the decrease in catecholamine levels in the brain was due to a reduction in the net synthesis of these amines, the ED50 dose of the active fraction should have been increased after α-MPT pretreatment. However after α-MPT pretreatment the ED50 was not significantly altered indicating that the active fraction was not affecting the catecholamine synthesis. Since the brain dopamine content was not increased, it also suggest that the

active fraction was not lowering norepinephrine content by inhibiting dopamine β-oxidase enzyme, which converts dopamine to norepinephrine. Taylor and Snyder (1970) reported no changes in brain dopamine content after amphetamine treatment.

The possible role of brain serotonin in the central excitatory action of the active fraction may be eliminated since pretreatment with p-chlorophenylalanine, a tryptophan hydroxylase inhibitor, did not change the ED50 of the active fraction. In the biochemical estimation studies, it was observed that the active fraction at the ED50 dose did not alter the serotonin content of the brain over that of the control. These results also suggest that the active fraction was not decreasing the brain catecholamine levels by direct stimulation of monoamine oxidase. If the active fraction acts by direct stimulation of this monoamine oxidizing enzyme, brain serotonin content should also be decreased. However, there is considerable evidence that amphetamine acts as a competitive inhibitor of monoamine oxidase (Glowinski, Axelrod & Iverson, 1966). The striking increase in O-methylated metabolites and profound decrease in deaminated metabolites observed after amphetamine treatment (15 mg/kg, i.p.) in all the structures of the brain suggest that amphetamine acts as an inhibitor of MAO (Blaschko et al., 1962).

Another possible mechanism of reducing brain catecholamine levels is by inhibition of the re-uptake mechanism. In the uptake studies, the active fraction at the ED50 dose significantly inhibited the norepinephrine re-uptake process at the fifteen minute time interval.

While no inhibition was observed at five minutes and at thirty minute time interval. This might suggest that the active fraction was acting by release of norepinephrine and inhibiting its re-uptake during the period of stimulation by the nerve terminal. By inhibition of the re-uptake mechanism the active fraction makes more norepinephrine available at the receptors level and thus can enhance its action. Amphetamine also inhibits re-uptake mechanism in the cerebral cortex, a noradrenergic region (Taylor & Snyder, 1971). Inhibition of norepinephrine uptake alone probably cannot account for the fighting episodes and increased motor activity, since desmethylimipramine, a more potent inhibitor of norepinephrine re-uptake, does not by itself enhance locomotor activity (Taylor & Snyder, 1971). The increased motor activity might be the consequence of a combination of norepinephrine release and inhibition of its re-uptake.

The active fraction at the ED50 dose significantly elevated normetanephrine levels at all time intervals examined. These results suggest that the reduction of norepinephrine level was a consequence of increased release of the amine from the neuron and a decreased re-uptake into the neuron.

The absence of stimulant action of the active fraction by treatment with propranolol but not phentolamine indicates the exclusive role of β-adrenoceptors in this action. Amphetamine in doses ranging from 2.5 to 10 mg/kg increased spontaneous motility and a straight log dose effect relationship could be obtained. In mice pretreated with propranolol (10 mg/kg) this effect was reduced and the dose-response

curve was displaced and flattened (Mantegazza et al., 1970). However in rats, propranolol (6.0 mg/kg) enhances the frequency of amphetamine induced rearing during the first five minutes after administration. This enhancement does not exist for higher doses (Cahn and Herold, 1970). Phentolamine inhibits the rearing activity induced by amphetamine in rats. These results indicate that amphetamine induced exploratory behavior (rearing) in rats involves an α-adrenergic mechanism, suppressed by phentolamine and enhanced by propranolol. It has been shown previously that certain neurons excited by norepinephrine can be blocked by β-adrenergic blocking agent, dichloroisoproterenol (Yamamoto, 1967) and that activation of α-adrenoceptors in the central nervous system of mice induces sedation (Delbarre & Schmidt, 1971). The reduction in brain norepinephrine caused by amphetamine may be the result of its action on adrenoceptors within the brain, and thereby reducing the further synthesis of the catecholamines by a feed back mechanism (Smith, 1962). However this mechanism was unlikely for the action of the active fraction, since it was unable to cause stimulation in mice pretreated with both α-MPT and reserpine, which markedly decreases catecholamines in the brain.

It was suggested that there are two interacting systems in the brain: an inhibitory cholinergic system which exerts its actions normally by inhibiting an activating system which, as the name implies normally activate behavior, the activating system assumed to be adrenergic (Carlton, 1963). The interactions of cholinergic and anticholinergic drugs could be explained in light of this hypothesis.

The role of endogenous norepinephrine in the toxicity has been investigated by several workers. The drugs that have been reported to protect against this toxicity might be exerting their protection by blocking the effects or the release of endogenous norepinephrine. Conversely, procedures which increase stores of norepinephrine available for release or prevent its reduction should enhance the toxicity (Moore, 1964). The changes in the LD50 of the active fraction after interactions with several drugs, that alter the catecholamine metabolism, can be explained by the above hypothesis.

Reserpine, tetrabenazine, α-MPT and their combined treatments significantly reduced the LD50 of the active fraction indicating the relationship of low levels of endogenous norepinephrine to the increased toxicity. However, significant reduction in the toxicity was observed after pretreatment with DL-dopa in animals treated with either reserpine and α-MPT or reserpine and disulfiram. Adrenergic blockers like propranolol and phentolamine did not alter the LD50 over the control. Also chlorpromazine, an adrenolytic agent, insignificantly increased the LD50 of the active fraction. Both α- and β-adrenergic blockers (phenoxybenzamine 10 mg/kg, tolazoline 30 mg/kg, MJ-1999 30 mg/kg, and propranolol 10 mg/kg) antagonize the effects of aggregation on the toxicity of amphetamine which suggest that endogenous catecholamines might be involved in this phenomenon (Weiss et al., 1961). The increase in the toxicity observed after physostigmine and no toxicity after atropine cannot be explained by above hypothesis. There is a possibility that cholinergic mechanism

might be involved in this toxic action.

In conclusion, the results suggest that the active fraction acts like amphetamine in causing stimulant action by releasing active norepinephrine from functional pools and inhibiting its re-uptake, thus making more norepinephrine available at adrenergic receptors.

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