STUDIES ON ANGIOTENSIN CONVERTING ENZYME

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ABSTRACT

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By

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Since angiotensin I converting enzyme acts as a dipeptidyl carboxypeptidase on the decapeptide angiotensin I by liberating histidyl-leucine and the hypertensive octapeptide angiotensin II, we sought a relatively inexpensive peptide substrate analog to routinely assay and characterize this enzyme. An enriched preparation of human angiotensin converting enzyme was isolated from the middle portion of three fractions obtained by gel filtration of serum over Sephadex G-200 in 0.1 M tris-hydrochloric acid buffer at pH 8.0, with subsequent dialysis and lyophilization. Commercially available porcine angiotensin converting enzyme was included for comparison. In addition to converting angiotensin I to angiotensin II, both preparations catalyzed the hydrolysis of the peptides L-phenylalanyl-glycyl-glycine, L-phenylalanyl-glycyl-L-phenylalanyl-glycine, L-phenylalanyl-glycyl-glycyl-L-phenylalanine, glycyl-L-histidyl-glycine and hippuryl-glycyl-glycine. This was demonstrated by incubating various concentrations of converting enzyme for one to three hours with the substrate analog in 0.05 M phosphate buffer at pH 7.25 containing 0.1 M sodium chloride. The dipeptides liberated were identified by thin-layer chromatography and quantitively determined using colorimetric and fluorometric techniques.

Two methods were developed for the direct chemical assay of human plasma angiotensin converting enzyme. They were based on the spectrophotometric and spectrofluorometric determination of histidyl-glycine, which was a product of the hydrolysis of the substrate analog glycyl-L-histidyl-glycine. o-Phthalaldehyde reacted with the imidazole moiety of histidine to produce a fluorophore and a chromophore under acid and alkaline conditions respectively; the activity of the converting enzyme was proportional to the fluorophore and chromophore concentration.

Glycyl-L-histidyl-glycine was hydrolyzed at a rate about twice that of the natural substrate angiotensin I, with a K_m of 6.0×10^{-4} M as compared to 4.5×10^{-5} M for angiotensin I. The hydrolysis of angiotensin I and glycyl-L-histidyl-glycine were both inhibited by EDTA, pyrophosphate, p-chloromercuribenzoate, 8-hydroxyquinoline, *Bothrops Jararaca* venom extract and two new natural inhibitors, human urinary factor and plasma inhibiting factor.

The reaction of o-phthalaldehyde and β-mercaptoethanol with amino acids, peptides, and proteins yielded a fluorescent product. Neither the mechanism of the reaction nor the structure of the fluorophore had heretofore been characterized. For the amino acid derivatives, this was elucidated by the synthesis of possible intermediates and characterization of a solid reaction product by absorption and resonance spectroscopy. Evidence was found that the hemimercaptal was produced with one aldehyde group of o-phthalaldehyde and that a Schiff base with the amino acid was formed at the other aldehyde group. The reaction was further extended to peptides and proteins and characterized. The fluorescence of the protein-o-phthalaldehyde-β-mercaptoethanol adduct was found to be proportional to protein concentration and molecular weights of the proteins investigated. The reaction with proteins showed essentially the same characteristics as the reaction with amino acids. Energy transfer was observed from the trytophan residues in bovine sorum albumin to the o-phthalaldehyde-β-mercaptoethanol adduct. The fluorescence from the o-phthalaldehyde-β-mercaptoethanol reaction was used for the estimation of the molecular weight of four purified proteins, for the quantitative determination of proteins, peptides, and amino acids. It can also be used for the assay of proteases and peptidases, since cleavage of peptide bonds results in a net increase in the number of free amino groups and thus an increase in derivable fluorescence.

STUDIES ON ANGIOTENSIN CONVERTING ENZYME

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James John Summary

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To my fellow men:

> "A hundred times every day I remind myself that my inner and outer life depend on the labors of other men, living and dead, and that I must exert myself in order to give in the same measure that I have received."
>
> --- A. Einstein

To the Department of Army, Office of the Surgeon General:

I sincerely appreciate the selection and total personal financial support of this endeavor.

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Biochemical research is done in a laboratory adorned with equipment and men of intellect. I appreciatively acknowledge that MSU has responded in full measure to the following exhortation:

> "Take interest, I implore you, in those sacred dwellings which one designates by the expressive term: laboratories. Demand that they be multiplied, that they be adorned. These are the temples of the future. . . temples of well-being and happiness. There it is that humanity grows greater, stronger, better."
>
> --- L. Pasteur

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To Dad and Janie:

"To one fixed trust my spirit clings."

--- J. Whittier

To the Profession of Chemistry:

"The chemists are a strange class of mortals, impelled by an almost insane impulse to seek their pleasure among smoke and vapor, soot and flame, poisons and poverty. Yet, among all these evils, I seem to live so sweetly that may I die if I should change places with a Persian king!"

--- J. Becker
17th Century Chemist

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LIST OF ABBREVIATIONS

Acid Citrate-Dextrose	ACD
Benzyloxycarbonyl	Z
Bovine Serum Albumin	BSA
t-Butyloxycarbonyl	BOC
p-Chloromercuribenzoate	PCMB
o-Dihydroxymethylbenzene	DMB
Diisopropylfluorophosphate	DFP
Dithiothreitol	DTT
Distilled and Deionized	DDI
Ethylenediaminetetraacetate	EDTA
Glutathione	GSH
Human Urinary Factor	HUF
o-Hydroxymethylbenzaldehyde	HMB
β-Mercaptoethanol	βME
o-Phthalaldehyde	OPT
Plasma Inhibiting Factor	PIF

I. INTRODUCTION

Hypertension is regarded as a physiological state of elevated blood pressure and, in humans, may increase with age or certain diseases. Essential hypertension in man is of ill defined etiology, whereas renal hypertension may be explained by a series of biochemical or molecular events. These have been described as comprising the renin-angiotensin system.

The operation of the renin-angiotensin biochemical apparatus can be initiated when reduced renal perfusion pressure develops. The resulting dimished renal blood flow and arterial pressure stimulates the renal cortex to secrete into the blood stream the proteolytic enzyme called renin. A plasma glycoprotein of the α-2 globulin fraction has been identified as the substrate (also called angiotensinogen) that undergoes limited proteolysis by renin, liberating from the N-terminal end the biologically inactive decapeptide angiotensin I.

The second phase of the renin-angiotensin system comes into play when angiotensin I encounters plasma or tissue converting enzyme, a dipeptidyl carboxypeptidase that catalyzes the hydrolysis of angiotensin I into the vasoactive octapeptide angiotensin II and the C-terminal derived dipeptide histidyl-leucine. Angiotensin

II has a prime role in the regulation of blood pressure by its ability to effect arteriole constriction and to stimulate the production of aldosterone by the adrenal glands. Aldosterone causes sodium retention by the kidney and increases blood volume with the concomitant elevation in pressure within the renal circulation.

Examination of past investigations into the renin-angiotensin system indicates that the second phase involving the reaction catalyzed by angiotensin converting enzyme to produce the vasopressive product has been least thoroughly researched. Boucher *et al.* (60) put it this way: "Although converting enzyme is a cornerstone of the renin-angiotensin system, little is known about its physiological significance and importance." Since angiotensin converting enzyme occupies a central position in the sequence of hydrolytic reactions to produce angiotensin II, examination of its enzymic properties may provide additional clues to the mechanism of renovascular hypertension and aldosteronism. This research effort, therefore, was directed toward the isolation and biochemical characterization of this enzyme in order to develop a more detailed knowledge of its physical and chemical properties as a protein; to examine the structural requirements for its catalytic activity and control; and to provide information about its role at the cellular level. Implied in the latter was the development of new methods of direct chemical assay in order to implement these studies. Thus experiments were undertaken to structurally modify substrates and the enzyme, to introduce new natural inhibitors and substrate analogs, and to study hormonal influences on the hydrolysis of substrates.

Throughout this research endeavor, we have sought for a relatively inexpensive substrate analog to routinely assay and characterize this enzyme in order that medical and clinical laboratories could incorporate the assay for the evaluation of human hypertensive diseases. To this end, new substrate analogs for the assay of human angiotensin I converting enzyme, using spectrophotometric and spectrofluorometric techniques, were investigated in detail.

During the development of a fluorescence technique for the assay of converting enzyme, a sensitive method for quantitation of proteins and peptides was explored. Using purified proteins and o-phthalaldehyde, a rapid method for the confirmation of the molecular weights of four purified proteins was investigated.

A thorough study was undertaken to elucidate the mechanism of the reaction of o-phthalaldehyde and β-mercaptoethanol with amino acids, to characterize its reaction with peptides and proteins, and to determine the chemical structure of the fluorophore that is produced.

II. LITERATURE REVIEW

Thirty-six years after Tigersted and Bergman (1) found that kidney cortical extracts contained renin (a substance which increased arterial blood pressure), Goldblatt *et al*. (2) in 1934 demonstrated that experimental hypertension could be sustained in the dog by renal artery stenosis. Six years later Braun-Menendez *et al*. (3) and Page and Helmer (4) independently found that renin liberated a heat stable and dialyzable vasoconstrictor, angiotensin (hypertensin, angiotonin), from a plasma protein. The plasma protein, angiotensinogen, was subsequently shown to be a glycoprotein (5), and occurred with the alpha-2 globulin fraction of blood plasma (6,7). Skeggs *et al*. (8) incubated angiotensinogen with trypsin, and subsequently isolated a tetradecapeptide, which also served as a polypeptide renin substrate. Renin by now was identified as a proteolytic enzyme, present in the renal cortex and cleaved the leucyl-leucyl bond of angiotensinogen and liberated the physiologically much less active decapeptide angiotensin I (9-11). Angiotensin I was subsequently found to be hydrolyzed by a plasma and/or tissue converting enzyme at the phenylalanyl-histidyl bond with the simultaneous production of the vasoactive octapeptide angiotensin II and the dipeptide histidyl-leucine (12). The amino acid sequence of angiotensin I and II isolated from horse blood (13), but not bovine (14,15), has been shown to be identical to human angiotensin (16,17). To recapitulate, the chemistry involved is shown in Scheme I.

Skeggs et al. isolated three major (A, C_1, C_2) and two minor (B_1, B_2) forms of the angiotensinogen glycoprotein (18). The three major forms after purification were found to have the same amino acid content and molecular weight of 58,000, but their carbohydrate moieties differed in the amount of sialic acid, glucosamine or hexose contents. Any other function of these five forms of angiotensinogen is unknown since they all yield angiotensin I at approximately the same rates (10).

Angiotensin I converting enzyme was first partially purified from horse plasma in 1956 by Skeggs and coworkers (19) and they suggested that the enzyme was a metalloprotein. Recently Dorer et al. (20) from the same laboratory again partially purified the same enzyme using different methodology but could not unequivocally establish the metal requirement of the native enzyme. Bahkle (21) partially purified a particulate enzyme from dog lung and found it to be qualitatively similar to horse plasma converting enzyme, a finding which was recently reaffirmed (22). Oparil et al. (23) reported lung angiotensin converting enzyme was an exopeptidase capable of hydrolyzing the decapeptide, but not the nonapeptide. Partially purified preparations of plasma

SCHEME I.--The Renin-Angiotensin System and Role of Angiotensin Converting Enzyme

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1 8 10 14

Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-Glycoprotein

Angiotensinogen ↓ Renin

1 8 9 10

Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe-His-Leu + Leu-Val-Tyr-Ser-Glycoprotein

Angiotensin I (weakly active) ↓ Converting Enzyme

1 8 9 10

Asp-Arg-Val-Tyr-Ileu-His-Pro-Phe + His-Leu

Angiotensin II (active) ↓ Angiotensinases

Inactive Peptides

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angiotensin converting enzyme was reported to inactivate bradykinin and hydrolyze various peptides (24). Thus a definitive characterization of converting enzyme using direct chemical methods was needed to aid in the understanding of its cellular role in the mechanism of hypertension, since it had been found in heart, liver, aorta, ileum (25) as well as blood and lung (19,26).

The vasopressor response to injections of angiotensin I or II is of short duration since many tissue extracts of the body contain hydrolytic (peptidase) enzymes which destroy the active angiotensin II (27). Bumpus et al. (28) and Regoli et al. (29) characterized the activities of these enzymes in rat plasma and found that the major component was the aminopeptidase angiotensinase A (60%) and an endopeptidase that cleaved angiotensin II into two tetrapeptides (30). The action of plasma angiotensinases on human angiotensin I as substrate has not been studied in detail, since angiotensin II has been used primarily for degradative studies. Skeggs et al. (31) reported that the trypsin derived tetradecapeptide substrate but not angiotensinogen was hydrolyzed by angiotensinases. Ng and Vane (26) reported, however, that a 105,000 x g supernatant of dog lung homogenate contained "destroying enzymes" which inactivate both angiotensin I and II.

For many years the assay of the components of the renin-angiotensin system solely depended upon the pressor response of angiotensin II in animals (32-40). Of late radioimmunoassay techniques (41-49,58,59), though sensitive are exceedingly involved, expensive and lengthy. Simpler, sensitive and more direct methods which utilize gas-liquid chromatography (50), fluorometry (51-54), and spectrophotometry (20,24,55) have been introduced for the assay of some isolated components of the renin-angiotensin system. Direct chemical methods were introduced in order to assay for the converting enzyme during the purification process and its biochemical characterization.

Roth (77) empirically developed a sensitive fluorometric method for the detection of amino acids using o-phthalaldehyde (OPT) and β-mercaptoethanol (βME). Although some basic parameters of the reaction were advanced, no reaction mechanism or further application to peptides and proteins was given.

OPT, a weakly fluorescent aromatic dicarbonyl compound, reacts in strong acid or alkali with many amino compounds to produce nonfluorescent and fluorescent products. For example, glycine can be determined colorimetrically with OPT (78-84), but usually sensitive fluorometric methods were used for the determination of an imidazole moiety (54,89,90), polyamines (87-90), and glutathione (91,92). In contrast, OPT in the presence of βME, produces highly fluorescent compounds with amino acids under relatively mild alkaline conditions. Variations of Roth's procedure for amino acids were employed in several analytical procedures, and the ease, sensitivity, and rapidity of the method encouraged further investigation and extrapolation to peptides and proteins.

III. EXPERIMENTAL PROCEDURE

A. MATERIALS AND EQUIPMENT

1. Materials. Unless otherwise stated, all commercial chemicals were analytical reagent grade and used without further purification.

a. Substrates. Glycyl-L-histidyl-glycine, L-phenylalanyl-glycyl-glycyl-L-phenylalanine, L-phenylalanyl-glycyl-L-phenylalanyl-glycine and L-phenylalanyl-glycyl-glycine were purchased from Sigma Chemical Corporation; angiotensin I was purchased from Schwarz-Mann and hippuryl-glycyl-glycine from Nutritional Biochemicals. Only the L isomers of peptides and amino acids were used in this study.

b. Proteins. Ovalbumin, aldolase and chymotrypsinogen A were procured from Pharmacia Fine Chemicals; Versatol A, a lyophilized human serum residue, was purchased from General Diagnostics; bovine serum albumin and porcine angiotensin converting enzyme was supplied by Pentex Corporation.

c. Chemicals and Biologicals. o-Phthalaldehyde, β-mercaptoethanol, aldosterone, *Bothrops Jararaca* pit viper venom, L-histidyl-leucine, L-histidyl-glycine and chromatographically pure amino acids were ordered from Sigma Chemical Corporation. Phenol Reagent, for the total protein determination, was purchased from Fisher Scientific Co.

2. Major Equipment Items

a. Spectrophotometers. Absorption spectra were recorded with a Beckman DB spectrophotometer and a Sargent SRL recorder or obtained manually using a Beckman DU spectrophotometer. Some absorbance measurements were accomplished with a Coleman Jr. II spectrophotometer.

b. Fluorometers. Fluorescence was measured with a Coleman Model 12 C filter fluorometer and an Aminco-Bowman Spectrophotofluorometer.

c. Spectrometers. Beckman IR-5 and Unicam SP 1000 Infrared spectrometers were used for infrared analysis, and the Varian T-60 NMR was used for recording the resonance spectra.

d. Chromatography Columns. Gel filtration of human serum proteins was accomplished using 2.5 x 100 cm columns, connected to a 3-way stopcock and Mariotte flask, supplied by Pharmacia Fine Chemicals.

B. ANALYTICAL METHODS

1. Protein Determinations and Analyses. All water used throughout this study was distilled and deionized (DDI) water. All solution concentrations indicated in % were prepared by weight to volume (w/v) unless otherwise noted.

a. Total Protein Determination by the Folin-Ciocalteau-Lowry Procedure (62,63). For the determination, the following reagents were prepared: 1 volume of Phenol Reagent was diluted with 2 volumes of DDI water; anhydrous sodium carbonate (2 g), potassium sodium tartrate tetrahydrate (2 g) and copper sulfate pentahydrate (1 g) were separately diluted to a deciliter (dl) with water. For the protein control, lyophilized human serum (Versatol A, Lot 2275049) was diluted with water to yield a concentration of 4.5 g/dl. After 20 minutes, the solution was quantitatively transferred to a volumetric flask and diluted to 200 ml with 0.9% saline. The final concentration of proteins was 1.13 mg/ml. To prepare the Folin working solution, 0.5 ml of the 1% copper sulfate and 0.5 ml of 2% sodium potassium tartrate was added to 49 ml of 2% sodium carbonate. The solution was used immediately.

For the protein determination, 0.20 ml of sample, standard and water designated as "test," "standard" and "blank" was added to 4.0 ml of Folin working solution. The tubes were mixed, allowed to stand 10 minutes, and then 0.4 ml of phenol reagent was added to each. The absorbances of the solutions were measured at 580 nm between 30 and 60 minutes after the addition of the phenol.

b. Total Protein Determination by Direct Spectrophotometry According to Warburg and Christian (64). Protein fractions eluting from the chromatography columns were monitored at 280 nm on a Beckman DU spectrophotometer, using 1.0 cm quartz cells and a slit width of 0.5 mm.

c. Electrophoresis of Proteins in Polyacrylamide Gels (67). The separating gel was prepared by dissolving 700 mg of acrylamide and 21 mg of bisacrylamide in 10 ml of 0.3 M tris, pH 8.8, and adding 0.060 ml of 5% ammonium persulfate and 0.020 ml of N,N,N',N'-tetramethylenediamine. After mixing, it was poured into glass tubes, carefully covered with a little water, and allowed to set 20 to 30 minutes.

A multicompartmental disc electrophoresis apparatus employed is described elsewhere (68a & 68b). Briefly, each tube had its own upper buffer reservoir and current supply which allowed for independent removal of a gel tube without disturbing the others. The lower common buffer tank held 1400 ml, the same total volume of the eight upper reservoirs. The electrodes were composed of 20-guage platinum wire. Current was supplied by a Spinco Duostat Model RD constant current regulated DC power supply.

Following polymerization, 0.025 ml of protein sample (10 mg/ml in 20% sucrose containing bromphenol blue as the tracking front) was carefully layered on the gel, and the tube inserted into the empty upper buffer reservoir. The 0.05 M borate buffer, pH 9.2, was gently poured into each upper tank and into the lower buffer reservoir. As an alternate method for sample application, the tubes containing the gel were inserted into the empty upper buffer reservoirs, then filled with buffer, and using a pasteur pipet, the sample was layered onto the gel surface. A 3 mA current was applied to each tube until the bromphenol blue tracking front migrated to the bottom of the gel tube (ca 35-45 minutes).

The gels were carefully extruded by inserting between the gel and the glass tube wall a 22-guage needle attached to a hypodermic syringe filled with water to provide lubrication. The gels were electrolytically destained for 2 hours. The destaining unit, powered by a 12 volt, 1 1/2 amp battery charger, contained in a discarded battery case, used 7% acetic acid as the electrolyte and two stainless steel plates as electrodes.

Polyacrylamide gel electrophoretic analysis was done on DEAE subfractionated plasma and serum protein preparations as described in Part C. Following electrophoresis, the gels were cross-sectioned with a razor blade every 5 mm in order to locate angiotensin converting enzyme activity. Two bands were slightly stained as a result of the addition of the tracking dye bromphenol blue. This provided a convenient spatial correlation between gels, since converting enzyme activity could not be detected within a single gel section. Ten gels were sectioned at a time and the slices soaked overnight in 2 ml of 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride, and the substrate (angiotensin I, 38.5 nmoles; Gly-His-Gly, 373 nmoles) at 4°. The mixtures were incubated for 2 hours at 37° and assayed fluorometrically.

d. Protein Analysis by Fluorometry (116). Usually 10.0 ml of a buffered o-phthalaldehyde (OPT) containing β-mercaptoethanol (βME), prepared by diluting 2.5 ml of OPT (10 mg OPT diluted to 10 ml with β-mercaptoethanol in methanol) to a deciliter with 0.05 M borate buffer, pH 9.5, was mixed with a 0.20 ml aliquot of 1.0 mg/ml protein solution. After standing at room temperature from 10-60 minutes, fluorescence was measured in a fluorometer. Protein standards were run simultaneously and the protein concentration obtained from the standard curve. Spectrofluorometric scans were measured under conditions described in the next paragraph.

2. Amino Acid and Peptide Determination and/or Analysis

a. Spectrophotofluorometric Analysis.* Fluorescence of solutions containing proteins, peptides, or amino acids with fluorescence generating reagent was measured with several instruments. For routine use the Coleman fluorometer (excitation 365 nm; emission greater than 495 nm) was sufficient. These readings were checked for continuity on Aminco-Bowman spectrophotofluorometer at various intervals (excitation at 340 nm and emission at 455 nm for solutions using βME; excitation at 365 nm and emission at 495 nm for solutions without βME). Corrected fluorescence and partial quantum

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*I wish to thank Dr. J. F. Holland for his aid and expertise in obtaining and interpreting fluorescence spectra.

efficiency scans were obtained using a unique computer centered fluorometer that was capable of simultaneous absorbance-fluorescence measurements (101). The corrected fluorescence curves were acquired in the form of quanta fluoresced per unit wavelength and were automatically corrected for the intensity of the excitation beam and absorption in the sample cell (102). The resulting quantum efficiency curve produced a quantity which was linearly related to quantum efficiency at each point along the wavelength axis of the excitation scan.

In a typical procedure for the quantitation of amino acids and peptides, usually 10 ml of buffered OPT-βME, prepared as described in section B.1.d., was mixed with an aliquot of amino acid or peptide that contained 50 to 100 nmoles. After standing at room temperature from 10 to 60 minutes, the fluorescence was measured in either the Coleman or Aminco-Bowman fluorometer.

b. Analysis of Peptides and Amino Acids by Thin-Layer Chromatography (65). Filter paper strips, saturated with migrating solution composed of n-butanol, acetic acid, and water (4:1:1) were placed along four sides of a glass covered Brinkman chromatography chamber. Solution was added to a depth of 8 mm, and the closed system allowed to equilibrate overnight at 24° before the silica gel G plates (Analtech) or silica gel F_{254} plates (Merck) were migrated to a height of 10 to 15 cm.

For visual detection of the separated amino acids or peptides, the plates were dried at 110° for 10 minutes and sprayed with a modified ninhydrin reagent (66) prepared in the following manner:

Solution I: To 50 ml of 0.2% anhydrous ethanolic ninhydrin solution, was added 10 ml of glacial acetic acid and 2 ml of 2,4,6-collidine. Solution II: To 1 g of copper nitrate trihydrate was added 100 ml of anhydrous ethanol. Just before use as a spray, 50 parts of solution I were mixed with 3 parts of solution II.

The plate was sprayed and held over a hot plate until even color development occurred; then the spots were observed by transmitted light and outlined with pencil.

The enzymatic lytic products of Gly-His-Gly were separated using the migrating solution composed of acetone, water, acetic and formic acids (70:20:8:2) as utilized by Detterbeck and Lillevik (115).

c. *Visible and Ultraviolet Absorption Spectroscopy*. The electronic absorption spectra of amino acids, peptides and proteins in the visible and ultraviolet were recorded at 24° with a Beckman DB spectrophotometer and Sargent SRL recorder. Quantitative absorbance measurements and some spectral scans were obtained manually using either the Beckman DU or Coleman Jr. II spectrophotometers. Ultraviolet absorbance measurements were made in aqueous solutions using quartz cuvets of 1.0 cm light path except where noted.

d. Infrared Spectroscopy.* Infrared spectra of compounds were obtained with a Beckman IR-5 or a Unicam SP 1000 infrared spectrometers. The solid compounds were scanned in Nujol mulls.

e. Nuclear Magnetic Resonance Spectroscopy.* NMR spectra were obtained with a Varian T-60 NMR spectrometer. The OPT reaction preparations were dissolved in D_6-acetone, OPT in carbon tetrachloride, glycine in D_2O and β-mercaptoethanol was run neat.

C. PREPARATIVE METHODS:

1. Desalting of Human Plasma on Sephadex G-25. About 2 g of Sephadex G-25 were swelled overnight in 0.9% saline and poured into a 10 ml pipet having a glass wool plug covered with glass beads at the lower end. With a Pasteur pipet, 1.0 ml of fresh human venous plasma was applied directly to the drained surface of the bed and eluted with saline. The 0.6 ml effluent fractions were monitored on the Beckman DU spectrophotometer at 280 nm and by the turbidity produced when a drop was layered on a 20% trichloroacetic acid solution.

2. Preparation of Human Serum Angiotensin Converting Enzyme by Gel Filtration with Sephadex G-200 (69). About 17 g of

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*I wish to thank Ms. Elizabeth I. Pupko for her skilled technical assistance in obtaining NMR spectra and Professor A. Timnick for help in obtaining IR spectra.

Sephadex G-200 was heated on a boiling water bath for 5 hours in 650 ml of 0.1 M tris-hydrochloric acid buffer, pH 8.0, containing 0.2 M sodium chloride. The swollen gel was allowed to cool to room temperature and a slurry was carefully poured down the 2.5 x 100 cm column wall. The flow was immediately started after filling the column, and the gel was washed with 1500 ml of buffer in order to thoroughly equilibrate the gel. In most experiments, upward flow elution was used in order to decrease the tendency of the bed to pack due to gravity. The column preparation was modified in later experiments when it was reported (114) that improved flow rates could be achieved by using an internal support of siliconized glass beads 6 mm in diameter.

Using a three-way valve, 2.0 ml of freshly drawn human serum was applied and immediately followed with 2 ml of 10% sucrose in buffer to ensure sharp sample application. The flow rate was adjusted to 15 ml per hour and 5 ml fractions were collected. The entire operation was conducted at 24°. Proteins in the effluent were monitored at 280 nm on a Beckman DU spectrophotometer.

3. Purification of Human Serum Angiotensin Converting Enzyme by DEAE-Sephadex Ion Exchange Chromatography (70). The DEAE-Sephadex A-50 was allowed to swell in a large excess of 0.02 M phosphate buffer, pH 6.8, containing 0.075 M sodium chloride, for 24 hours. Before use, the ion exchanger was shaken and was allowed to settle for 7 minutes, and the supernatant

buffer discarded. The process of removing fine particles was repeated five times.

About 40 mg of enriched human converting enzyme preparation from the middle cut of three fractions obtained by gel filtration of serum over Sephadex G-200 was dissolved in 0.5 ml of 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride and centrifuged for five minutes. The clear supernatant was applied to a 1 x 32 cm column containing *ca.* 1.5 g of the ion exchanger and eluted with a stepwise ionic strength gradient of sodium chloride (0.075, 0.15, 0.20, and 0.30 M sodium chloride in 0.02 M phosphate buffer, pH 6.8). The 2.5 ml effluent fractions were monitored on the Beckman DU spectrophotometer at 280 nm.

4. *Human Blood Plasma Protein Fractions by Cohn's Ethanol Procedure* (76). Blood plasma is obtained after removing blood cells by centrifugation. Fractionation is accomplished with ethanol by controlling such variables as pH, alcohol, ionic strength, protein concentration and temperature. The protein pastes* used in this study were designated as Fraction I, Fraction II + III, Fraction IV-1, Fraction IV-4 and Fraction V.

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*The Cohn fractions were kindly provided by Dr. K. B. McCall, Assistant Director, Human Plasma Products Section, Division of Laboratories, Michigan Department of Health, Lansing, Michigan.

5. Partial Purification of Human Urinary Factor (HUF) and Plasma Inhibiting Factor (PIF). Since angiotensin converting enzyme had low demonstrable activity in human serum (104), blood and urine were examined for possible angiotensin converting enzyme inhibitors. Freshly voided human urine was dialyzed overnight against an equal volume of water and the dialysate was acidified with glacial acetic acid to pH 3.5. A method for isolating amino acids and peptides was then applied (115). A chromatography column (0.6 x 15 cm) was filled with moist cation exchange resin (Dowex 50W-X8, 100-200 mesh, H^+ form, 300 mg/2 ml of urine dialysate), and the acidified dialysate was poured gently onto the column. The acidic effluent was discarded, and the resin was eluted with 4 ml of 2 N triethylamine in 20% aqueous acetone. The eluate was evaporated overnight *in vacuo* over concentrated sulfuric acid. The residue (7 mg/2 ml of urine) was redissolved in 4.0 ml of water. The plasma inhibiting factor was prepared similarly.

Further purification of HUF was accomplished by adding to a column of Dowex 1-X8 (OH^- form) 0.2 ml of HUF, the aqueous residue obtained by Dowex 50W-X8 fractionation, and eluting with water. The wash was collected and lyophilized.

Extraction of an active component was attempted using either chloroform/methanol (3:2), benzene or petroleum ether. The Dowex 50W-X8 purified HUF and PIF preparations were subjected to acid hydrolysis by mixing equal volumes of PIF or

HUF solution with concentrated hydrochloric acid, sealing them in a capillary tube, and heating at 90° for _ca_. 24 hours.

6. _Extraction of Bothrops Jararaca Pit Viper Venom_ (110). _B. Jararaca_ venom (5 mg) was extracted by the addition of 1.0 ml of absolute ethanol, triturated to a fine suspension, and allowed to stand at 37° for 48 hours, and centrifuged to remove turbidity. The crude extract was preincubated with 1 mg of the angiotensin converting enzyme preparation for 15 minutes, incubated with the substrate for 90 minutes, and assayed according to the microfluoremetric procedure (Section D.2.b.).

7. _Preparation of Canine Adrenal Gland Extract_ (117). Freshly excised adrenal glands* (2 g) from a mongrel dog were extracted with 99% acetone overnight with stirring at 4°. Further extraction was done similarly with 80% aqueous acetone and the two extracts combined. The acetone solution was filtered and concentrated overnight _in vacuo_, the remaining residue extracted twice with petroleum ether, and the aqueous mixture incubated at 37° to remove traces of petroleum ether. Aliquots were incorporated directly into the assay procedure.

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*We are indebted to C. C. Chou, Professor of Physiology and Medicine, for performing the adrenalectomy.

8. Synthesis of Intermediates Involved in the Fluorometric Assays

a. Reaction Product of o-Phthalaldehyde and Glycine. One gram (0.15 moles) of OPT was dissolved in 3 ml of methanol and added to 0.55 g (0.15 moles) of glycine in 4 ml of 50% aqueous methanol. After 4 minutes the solution turned a deep green and the product crystallized upon slow evaporation of solvent at 40°. The colored product melted between 105 and 110° and migrated only as one component upon silica gel thin-layer chromatography, when either methanol/water (1:1) or butanol/acetic/water (4:1:1) was used as a migrating solution. The TLC plates showed no new component when sprayed with ninhydrin. Ultraviolet, infrared, and nuclear magnetic resonance spectra were obtained on the solid preparation.

b. Reaction Product of OPT, Glycine and β-Mercaptoethanol. Synthesis was effected as described immediately above, except that 0.5 ml of pure β-mercaptoethanol was incorporated into the OPT solution, and upon its addition to glycine the solution turned a deep red. Slow evaporation of the solvent at 40° yielded a red crystalline product that melted in the range of 105 to 110° and gave one spot upon TLC when methanol/water (1:1) was the migrating solution. However, when butanol/acetic acid/water (4:1:1) was used, four colored products plus one ninhydrin positive component appeared. When a 10 μl aliquot of the reaction mixture was taken before evaporation of the methanol, and diluted to 10.0 ml with 0.05 M borate buffer at pH 9.5, an intense blue fluorescence was seen. Ultraviolet, infrared and nuclear magnetic resonance spectra were obtained on the solid preparation.

c. Synthesis of o-Hydroxymethylbenzaldehyde (HMB).

(1) OPT-Ethylene Glycol Monoacetal Synthesis. One gram (0.07 moles of OPT) and 2 g (0.08 moles) of aluminum trichloride were mixed with 10 ml of ethylene glycol and the mixture was warmed for *ca.* 5 minutes to dissolve the components. The resulting dark-orange solution was extracted with 30 ml of benzene, and the extract was washed successively with 1% sodium hydroxide and 2% sodium carbonate. The washed benzene extract was dried over calcium sulfate and then distilled free of benzene to yield an orange syrupy residue of the acetal. IR spectra of the product indicated that steric hindrance apparently prevented ethylene glycol addition to both aldehyde groups of OPT.

(2) Borohydride Reduction. One ml of the acetal mixture was dissolved in 5 ml of n-propyl alcohol, and to this was added 0.3 g of sodium borohydride, and the mixture gently refluxed for 10 minutes. Gentle heating was continued until bubbling ceased and the colored solution cleared. Ten ml of water was added and the mixture allowed to stand overnight at 37°. When concentrated sulfuric acid was added until pH 3 resulted, a viscous layer of HMB separated from

solution. These same properties of the product were described by Rodd (103). The pale yellow liquid was washed with 2% sodium carbonate and dried over calcium sulfate. The structure of HMB and OPT ethylene glycol monoacetal was verified by IR spectroscopy, reaction with phenylhydrazine and a positive Tollen's test.

d. Synthesis of o-Dihydroxymethylbenzene (DMB) or o-Xylylene Glycol. One gram of OPT was dissolved in 5 ml of n-propanol and to this was added 0.3 g of sodium borohydride, and the mixture was gently refluxed for 10 minutes. Sodium borohydride reduction was effected as previously described with the acetal. After addition of 10 ml of water, the mixture was concentrated by distillation to *ca*. 8 ml, whereupon DMB separated as a white solid, (MP 60-63°; Rodd (103) reported 64°). The structure was verified by IR spectroscopy.

9. Synthesis of Modified Substrates for Angiotensin Converting Enzyme (118).

a. N-Acetyl Glycyl-Histidyl-Glycine Synthesis. To a 1.0 ml reaction vial containing 2.0 mg Gly-His-Gly dissolved in 50 µl of 0.05 M phosphate buffer, pH 7.25, was added 50 µl of saturated sodium acetate. The solution was cooled to 0° and 4 µl of ice-cold acetic anhydride was added in 1 µl aliquots every 15 minutes. A reaction was evidenced by immediate clearing of the solution. The resulting mixture was then diluted to 3.6 ml with 0.05 M phosphate buffer, pH 7.25, thus yielding a 2 mM solution of N-acetyl-Gly-His-Gly. Thin-layer chromatography of the preparation, using acetone/water/acetic acid/formic acid (70:20:8:2) as the migrating solvent, showed one ninhydrin negative component. The N-acetyl-Gly-His-Gly was visualized by placing the TLC plate in a closed chamber containing an iodine vapor saturated atmosphere (R_f, 0.58).

b. Glycyl-Histidyl-Glycine Ethyl Ester Synthesis. Gly-His-Gly (1.0 mg) was suspended in 50 µl of absolute ethanol containing 0.1 M hydrochloric acid and allowed to stand at 4° for five days. Thin-layer chromatography of the reaction mixture, using acetone/water/acetic acid/formic acid (70:20:8:2) as the migrating solution, demonstrated one primary ninhydrin positive component, the ethyl ester of Gly-His-Gly (R_f, 0.40) and a trace of Gly-His-Gly (R_f, 0.16).

c. Angiotensin I Ethyl Ester Synthesis. The ethyl ester of angiotensin I was prepared as described immediately above, except 0.2 mg were dissolved in 20 µl of the acidic ethanol solution. Thin-layer chromatography of the mixture, using acetone/water/acetic acid/formic acid as the migrating solution, showed one spot (R_f, 0.84) corresponding to ethyl ester of angiotensin I.

D. ASSAY METHODS FOR DETERMINATION OF ENZYMATIC ACTIVITY.

1. Current Methodology for Detecting Peptide Bond Cleavage

a. *Ninhydrin Assay of Angiotensin Converting Enzyme* (71,72). A 4 M lithium acetate buffer was prepared as follows: 24 g of anhydrous lithium hydroxide was added to 60 ml of water and stirred until about half was dissolved; cautiously and in small portions were added 64 ml of glacial acetic acid. Warming and vigorous bubbling occurred. The solution was diluted almost to 250 ml with water, and the pH measured after a 1:3 dilution with water. If the pH was not 5.2 ∓ 0.05, it was adjusted to this value with 1 M lithium hydroxide or glacial acetic acid. After cooling to 20°, the solution was diluted to 250 ml.

(1) Ninhydrin reagent. To 1.0 g of ninhydrin (Pierce Chemical Co.) was added 37.5 ml of dimethylsulfoxide and 12.5 ml of 4 M lithium acetate buffer. The reagent was used immediately, since the cherry red solution faded to a light yellow when exposed to air. Potency was maintained for 1-2 weeks if stored at 4° in small brown bottles filled to the brim.

(2) Standards. A stock standard solution of 50 mM glycyl-glycine was prepared by diluting 660.6 mg Gly-Gly to a deciliter with water. To prepare a dilute 100 nmoles/ml Gly-Gly standard, 1.0 ml of the 50 mM stock standard was diluted to 500 ml with water. This standard was further diluted with water to give working standards of suitable concentration.

(3) Substrates. To prepare a 0.05 M sodium phosphate buffer, 3.55 g of anhydrous dibasic sodium phosphate and 3.0 g sodium chloride was dissolved in ca. 250 ml of water and titrated to pH 7.25 with 0.1 M hydrochloric acid and diluted to 500 ml with water. To prepare 1.0 mM (1000 nmoles/ml substrates) 8.6 mg of Phe-Gly-Gly-Phe, 8.6 mg. of Phe-Gly-Phe-Gly and 5.9 mg of Hippuryl-Gly-Gly were diluted to 20 ml and 7.0 mg of Phe-Gly-Gly were diluted to 25 ml with the 0.05 M phosphate buffer.

(4) Angiotensin Converting Enzyme. Enriched preparations by Sephadex G-200 fractionation of human serum ("Fraction II"), or a subfraction ("Fraction III") of Fraction II obtained by column chromatography with DEAE-Sephadex A-50 and commercially available porcine plasma converting enzyme were used for assay.

(5) Procedure. To 0.5 ml of substrate was added 0.5 ml of enzyme preparation and the solution was incubated for 3 hours at 37°, using 0.5 ml of substrate and 0.5 ml of phosphate buffer as the reagent blank. All tubes were placed in a boiling water bath for 5 minutes to terminate the enzymatic reaction and centrifuged if turbid. A 0.5 ml aliquot was removed from the Hippuryl-Gly-Gly reaction mixture and 0.1 ml aliquot was taken from the Phe-Gly-Gly, Phe-Gly-Gly-Phe, Phe-Gly-Phe-Gly incubated mixtures as well as the corresponding blanks, and each was diluted to 1.0 ml with water. To each was added 0.5 ml of ninhydrin reagent.

Likewise, 0.5 ml of ninhydrin was added to 1.0 ml of working standard and 1.0 ml of water for the ninhydrin blank. The tubes were mixed on a vortex mixer for 30 seconds and covered with aluminum caps. The tubes were placed in vigorously boiling water for exactly 15 minutes, then cooled for 10 minutes in tap water. To each was added 2.5 ml of water, mixed on a vortex mixer and centrifuged if necessary. The Coleman Jr. II spectrophotometer was set at 580 nm and the absorbance read versus water in 1/2 in. cuvets.

b. Fluorometric Assay of Angiotensin Converting Enzyme: Determination of Histidyl-Leucine with o-Phthalaldehyde Reagent, Macro-Method. (54).

(1) Reagents. For the diluent, a 0.05 M sodium phosphate buffer, pH 7.25, containing 0.1 M sodium chloride was prepared. The substrate, angiotensin I (1 mg or 770 nmoles/ml) was then diluted to 1.0 ml with phosphate buffer. For the standards, a stock histidyl-leucine solution was prepared by dissolving 1.0 mg or 3720 nmoles/ml in water. Aliquots were removed to give working standards of suitable concentration. In addition 2 N sodium hydroxide, 6 N hydrochloric acid, and 10 mg/ml of o-phthalaldehyde in methanol were other reagents required for the determination. Enriched preparations of converting enzyme from Sephadex G-200 fractionation of human serum ("Fraction II"), from the subfractionation of Fraction II on DEAE-Sephadex A-50 ("Fraction

III"), and commercially available porcine plasma converting enzyme were used for assay.

(2) Procedure. The assay procedure was initiated by incubating 0.1 ml of the enzyme preparation with 0.1 ml of angiotensin I at 37°. Meanwhile, a substrate and enzyme blank were prepared by adding 0.05 ml of each to 7.55 ml of phosphate buffer. At the end of the one to three hour incubation period, 0.1 ml of the incubation mixture was added to 7.5 ml of buffer. Into all tubes was pipeted 1.0 ml of sodium hydroxide and 0.25 ml of o-phthalaldehyde. The tubes were mixed on a vortex mixer and allowed to stand for four minutes. Then 0.5 ml of 6 N hydrochloric acid was added to each. For standards, 2.5, 5.0, 10.0 and 20.0 µl of stock His-Leu was pipeted into 7.6 ml of buffer, and sodium hydroxide, OPT, and hydrochloric acid added as above. A reagent blank without His-Leu was also prepared. The fluorescence was measured either in the Aminco-Bowman spectrophotofluorometer or the Coleman Model 12 C filter fluorometer.

2. Methodology Developed in This Study

a. Fluorometric Assay of Angiotensin Converting Enzyme Using Substrate Analog Glycyl-Histidyl-Glycine and the o-Phthalaldehyde Reagent to Determine Histidyl-Glycine, Macro-Method. Angiotensin converting enzyme was serially diluted with 0.05 M phosphate buffer at pH 7.25, containing 0.1 M sodium chloride, to yield 3.25, 1.12, and 0.56 mg protein per 0.5 ml. For the substrate 2.7 mg of Gly-His-Gly were dissolved in 10.0 ml of the same phosphate buffer to yield a 1.0 mM solution. Aqueous His-Leu (1 mg/ml) was used as a stock standard.

For the hydrolysis of substrate as a function of time, 0.5 ml of 1.0 mM Gly-His-Gly was incubated with 0.5 ml of enzyme (3.25 mg) at 37°. A 0.1 ml aliquot of the reaction mixture was removed at 0, 15, 30, 45, 75, 90, 105, and 120 minutes after initiation of the reaction and quickly frozen in a methanol-dry ice bath.

For the catalyzed hydrolysis of substrate as a function of enzyme concentration, 0.50 ml of substrate was incubated at 37° for 2 hours with 0.50 ml of enzyme containing 3.25, 1.12, and 0.56 mg of protein. Zero time controls were frozen as above. To each of the 0.10 ml aliquots was added 7.50 ml of 0.05 M phosphate buffer, 1.0 ml 2 N sodium hydroxide, 0.25 ml OPT (10 mg/ml in methanol). After 5 minutes, 0.50 ml 6 N hydrochloric acid was added and the fluorescence measured in the Aminco-Bowman spectrophotofluorometer. For standards, 2.5, 5.0, 10.0, 15.0, and 20.0 μl of stock His-Leu was added to a 0.60 ml buffer, followed by sodium hydroxide OPT, and then hydrochloric acid as above.

b. Spectrophotofluorometric and Spectrophotometric Assay of Angiotensin Converting Enzyme Using Substrate Analog Glycyl-Histidyl-Glycine and the o-Phthalaldehyde Reagent to Determine Histidyl-Glycine, Micro-Methods.

(1) Fluorometric Assay. Partially purified converting enzyme preparation (0.05 - 1.0 mg) was dissolved in 0.10 ml of 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride, and incubated at 37^{o} for 90 minutes with 0.10 ml of 1.0 mM Gly-His-Gly or angiotensin I (1.0 mg/ml). The enzymatic reaction was initiated with the addition of Gly-His-Gly or angiotensin I substrate solution and terminated with the addition of 0.1 ml of 2 N sodium hydroxide. The fluorogenic reaction was initiated by the addition of 0.05 ml of OPT (10 mg/ml in methanol). This solution was allowed to stand for 5 minutes; then 0.1 ml of 6 N hydrochhloric acid was added, and the final volume brought to 1.0 ml (or 2.5 ml if less sensitivity was desired). The addition of acid precipitated the proteins which were removed by centrifugation at *ca.* 2000 rpm for 5 minutes in an International Model H clinical centrifuge. After 10 to 30 minutes following the addition of hydrochloric acid, the fluorescence was measured in the Aminco-Bowman spectrophotofluorometer.

(2) Spectrophotometric Assay. The procedure is identical to the one described immediately above, except the aborbance of the solution was measured at 420 nm, in 1.0 cm cuvets using a Beckman DU spectrophotometer, after the addition of sodium hydroxide. Addition of hydrochloric acid was omitted and the final volume was brought to 1.0 ml with water. The absorbance was best measured 20 to 30 minutes after the addition of the OPT reagent.

c. Fluorometric Assay of Angiotensin Converting Enzyme: Determination of Lytic Dipeptides from Substrate Analogs with the o-Phthalaldehyde Reagent in the Presence of β-Mercaptoethanol. In a typical assay, 0.5 ml of 1.0 mM Phe-Gly-Phe-Gly was mixed with 0.5 ml of converting enzyme solution and incubated for 3 hours at 37°. For blanks, 0.05 M borate buffer, pH 9.5, and enzyme preparation were combined as above and boiled for 5 minutes. A 0.5 ml aliquot of both digest and blank was added to 9.5 ml of borate buffered OPT. Borate buffered OPT was prepared by diluting 2.5 ml of OPT (10 mg OPT diluted to 10 ml with 5 μl/ml of β-mercaptoethanol in methanol) to a deciliter with 0.05 M borate buffer, pH 9.5. The fluorescence was measured between 5 and 25 minutes after the addition of the borate buffered OPT reagent.

d. Spectrophotometric Assay for Angiotensin Converting Enzyme in Human Plasma Using Substrate Analog Glycyl-Histidyl-Glycine or Angiotensin I and the o-Phthalaldehyde Reagent to Measure the Lytic Dipeptides, Micro-Method. The substrates, 1.0 mM Gly-His-Gly and 0.77 mM angiotensin I, were dissolved in 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride. To 0.10 ml of acid citrate-dextrose (ACD) plasma and 0.10 ml of substrate was added 0.10 ml of phosphate buffer and the mixture incubated at 37° for 10 minutes. The reaction was terminated by the addition of 0.20 ml of 6N sulfuric acid and 0.20 ml of 10% sodium tungstate. The precipitated proteins were removed by centrifugation at 2000 rpm for 5 minutes in an International Model H clinical centrifuge. To 0.40 ml of the clear supernatant was added 0.10 ml of 2 N sodium hydroxide followed by 0.050 ml of OPT (10 mg/ml in methanol). After 10 minutes, the absorbance of the solution was measured in 1.0 cm quartz cuvets at 420 nm with a Beckman DU spectrophotometer. The absorbance of a protein blank (or blank containing the inhibitor in enzyme inhibitor studies) was subtracted and the net absorbance compared to a His-Gly (Gly-His-Gly as substrate) or His-Leu (angiotensin I as substrate) standard curve to ascertain the amount of dipeptide liberated during the incubation period.

3. Converting Enzyme Activity in Cohn Human Plasma Protein Fractions. Each of the Cohn human plasma fractions (Fraction I, Fraction II + III, Fraction IV-1, Fraction IV-4 and Fraction V), previously lyophilized from ethanol pastes, were dissolved in 0.9% saline and dialyzed overnight against water at 4°. The dialyzed fractions were shell frozen and lyophilized. These preparations were dissolved in 0.05 M sodium phosphate buffer, pH 7.25, containing 0.1 M sodium chloride, centrifuged, and the supernatant was serially diluted to give concentrations from 0.5 to 2.5 mg/ml. One ml of each was incubated for 3 hours at 37° with 1.0 ml of 1.0 mM Hippuryl-Gly-Gly; a 0.20 ml aliquot from the incubation mixture was removed and the liberated Gly-Gly determined by the ninhydrin procedure.

A fluorometric assay was also performed on each of the Cohn fractions using the substrate analog Gly-His-Gly. To 0.10 ml (1.0 mg) of the dialyzed and lyophilized Cohn fraction was added 0.10 ml of substrate and 0.30 ml of phosphate buffer. The mixture was incubated at 37°. for 60 minutes and assayed by the microspectrophotofluorometric procedure.

To demonstrate inhibition by inorganic pyrophosphate, 8-hydroxyquinoline, and diisopropylfluorophosphate on angiotensin coverting enzyme detected in the Cohn fractions, 0.10 ml of 10 mM pyrophosphate, 1.0 μl of 200 ng/ml diisopropylfluorophosphate, and 50 μl of 20 mM 8-hydroxyquinoline were incorporated into the above described 0.50 ml incubation mixtures.

E. REACTION MECHANISMS

1. Factors Influencing Angiotensin Converting Enzyme Activity on Substrate Analogs

a. Effect of Chelating Agents. Porcine plasma converting enzyme was dissolved in 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride, and serially diluted with the same buffer to protein concentrations of 1.5, 0.75 and 0.375 mg/ml. To 5.0 ml of the 1.5 mg/ml enzyme preparation was added 2.0 mg of disodium ethylenediaminetetraacetate (EDTA) and left overnight at 4°. The protein concentration was serially diluted as above.

b. Influence of Anions. Chloride-free phosphate buffer was prepared by dissolving 7.1 g of anhydrous dibasic sodium phosphate in double deionized water, titrating to pH 7.25 with phosphoric acid, and diluting to a liter with water. Both porcine plasma and human converting enzyme (Fraction II) preparations were dissolved in chloride-free buffer. Substrates were prepared as described in section D.1.a. (Ninhydrin Assay); however, sodium chloride was deleted from the substrate preparations.

Equal volumes of substrate and enzyme were combined, incubated for 3 hours at 37°, and the liberated products quantitated by the ninhydrin assay.

c. Influence of Inhibitors.

(1) Inhibition Studies Using Fraction II. Gly-His-Gly (100 nmoles) was incubated for 90 minutes with 0.50 mg of converting enzyme preparation (Fraction II from Sephadex G-200 preparation) and the indicated amount of inhibitor (Table IV) in total volume of 0.30 ml. The liberated His-Gly was determined spectrophotometrically as detailed in section D.2.b.

(2) Inhibition Studies Using Fraction II and ACD Plasma. Gly-His-Gly (100 nmoles) was incubated for 90 minutes with 0.50 mg of converting enzyme preparation (Fraction II from Sephadex G-200 preparation) and the indicated amount of compound or biological extract (Table VI) in total

volume of 0.30 ml. The liberated His-Gly was determined spectrophotometrically as detailed in section D.2.b. Similarly, 0.10 ml of ACD plasma was incubated for 10 minutes with the indicated amount of compound or biological extract, and the liberated His-Gly determined spectrophotometrically as detailed in section D.2.d.

In all inhibition studies, the concentration of the compound or biological extract was made equimolar in every tube in order to correct for any effect that the compounds or extracts may have had on the assay procedure.

d. Effect of Esterified and N-terminal Modified Substrates. Angiotensin I (77.0 nmoles), ethyl ester of angiotensin I (77.0 nmoles), Gly-His-Gly (100 nmoles), ethyl ester of Gly-His-Gly (100 nmoles) and n-acetyl Gly-His-Gly (100 nmoles) were incubated for 90 minutes with 0.50 mg of angiotensin converting enzyme preparation in 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride and a final volume of 0.30 ml. Similarly, 0.10 ml of ACD plasma was substituted for the Fraction II preparation and incubated for 10 minutes with the substrates. The liberated His-Gly and His-Leu were determined spectrophotometrically as described in section D.2.d.

2. Small Peptide Hydrolysis by Human ACD Plasma

ACD plasma (0.10 ml) was incubated for 10 minutes at 37^{o} with 0.10 ml of 1.0 mM peptide (Table V) in a total volume of 0.30 ml. The enzymatic reaction was terminated and the proteins precipitated by the addition of 0.20 ml of 0.6 N sulfuric acid and 0.20 ml of 10% sodium tungstate. After centrifugation, 2.0 ml of buffered OPT, prepared as described in section B.1.d., was added to 0.40 ml of the protein free supernatant. After 10 minutes the fluorescence was measured in the Aminco-Bowman spectrophotofluorometer with the excitation monochromator set at 340 nm and the emission monochromator set at 455 nm. The fluorescence of an unincubated blank, containing all components of the reaction mixture, was measured simultaneously and the resulting value subtracted from the fluorescence value obtained from the incubated sample.

Peptide hydrolysis, catalyzed by a porcine plasma angiotensin converting enzyme preparation, was measured in an identical manner, except 0.10 ml of a 5.0 mg/ml solution of the enzyme was substituted for the plasma and incubated for 140 minutes. The buffered OPT reagent (2.0 ml) was added directly to the incubation mixture and the fluorescence measured. Appropriate blanks were prepared as above.

3. Studies on the Mechanism of the Fluorogenic Reaction of o-Phthalaldehyde and β-Mercaptoethanol with Amino Acids, Peptides, and Proteins

a. Relative Fluorescence Produced from the Reaction of OPT, HMB, and DMB Plus βME with Asparagine.

Buffered HMB-βME

reagent was made by addition of 5 µl of HMB (10 mg/ml in methanol) to 0.70 ml of βME solution (5 µl/ml in 50% methanol) and diluting to 10.0 ml with 0.05 M borate buffer, pH 9.5. The buffered DMB-βME reagent was composed of 10.0 mg of DMB preparation and 0.10 ml of βME diluted to 10.0 ml with the same borate buffer.

Similarly buffered solutions of OPT, HMB, and DMB were prepared without βME. To produce the fluorescent derivatives, 0.20 ml of a 2.0 mM solution of asparagine was diluted to 10.0 ml with each of the aforementioned reagents and the developed fluorescence was measured fluorometrically.

b. *Effect of Oxygen on Fluorescence.* A 0.20 ml aliquot of 2.0 mM glycine was diluted to 10.0 ml with buffered OPT-βME reagent and the resulting fluorescence was measured. With another identical sample, oxygen was bubbled through the solution for 10 minutes, and then its fluorescence was measured and compared with the unoxygenated sample. A similar sample without βME was prepared and compressed nitrogen bubbled through the solution for 10 minutes and the fluorescence noted.

c. *The Influence of Amino and Carboxyl Group Proximity on Fluorescence.* Aqueous solutions of 2.0 mM glycine, β-alanine, γ-aminobutyric and ε-aminocaproic acids, as well as the ethyl ester of glycine, were prepared, and from each a 0.20 ml aliquot was taken and diluted to 10.0 ml with buffered OPT-βME reagent. The resulting fluorescence from each was measured.

d. Titration of the Fluorescent Derivative with p-Mercuribenzoate. To 10.0 ml of buffered OPT reagent containing 200 μmoles of βME was added 0.20 ml of 2.0 mM glycine, and this solution was titrated with 20.0 mM p-mercuribenzoate. The fluorescence was noted after each 0.10 ml addition.

e. The Effect of Reducing Agents on the Production of Fluorescence. Sodium borohydride, sodium bisulfite, and dithiothreitol were substituted for βME in the preparation of the buffered OPT reagent and reacted with glycine as before. The intensity of fluorescence resulting from each reagent was measured.

f. Fluorescence as a Function of Protein Concentration and Molecular Weight. Solutions of bovine serum albumin (1.95 mg/ml), aldolase (0.55 mg/ml), chymotrypsinogen A (2.85 mg/ml) and ovalbumin (2.50 mg/ml) were prepared in 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride, which produced protein concentrations of 28.9, 3.50, 114.0 and 55.7 nmoles/ml respectively. Aliquots of each protein solution (0.050 or 0.025 ml) were diluted to 10.0 ml with buffered OPT-βME reagent and the resulting fluorescence was measured.

g. The Effect of pH on the Fluorescence of the Protein Derivatives. Four buffered OPT-βME reagent solutions were prepared as follows. OPT (0.050 ml, 10 mg/ml in methanol) plus 0.50 ml of βME (5.0 μl/ml in methanol) were diluted to a final volume of 20.0 ml with either 0.05 M borate buffer, pH 11.0; 0.05 M borate buffer, pH 9.5; 0.05 M phosphate buffer, pH 7.25; 0.05 M acetate buffer, pH 5.2. For testing the effect of each on fluorescence, 0.10 ml of bovine serum albumin (2.5 mg/ml) was diluted to 10.0 ml with the previously described buffered OPT-βME reagents at their specified pH. To observe the fluorescence of the bovine serum albumin adduct as a function of pH after the fluorophore had been formed at pH 9.5, bovine serum albumin was reacted with buffered OPT-βME reagent and, after 10 minutes, the solution was titrated to the desired pH.

h. Binding of OPT to Bovine Serum Albumin. A constant amount of albumin (0.10 ml of a 15 nmoles/ml solution) was reacted with increasing aliquots of OPT solution (74.5 nmoles per ml of 0.05 M borate buffer of pH 9.5) ranging from 0.05 to 2.0 ml and making the final volume to 10.0 ml with borate buffer containing 1450 nmoles/ml of βME. Fluorescence was measured after 10 minutes.

i. Binding of βME to OPT-Bovine Serum Albumin. Aliquots ranging from 0.65 to 0.20 ml of βME (5 μl/ml in methanol) were reacted with constant amounts of OPT and albumin (0.10

ml of 10.0 mg/ml and 0.10 ml of 15.0 nmoles/ml respectively) and diluted to 10.0 ml with 0.05 M borate buffer, pH 9.5. After 10 minutes the fluorescence was measured.

IV. RESULTS

A. ISOLATION, PURIFICATION AND ASSAY OF ANGIOTENSIN CONVERTING ENZYME PREPARATIONS

1. Angiotensin Converting Enzyme Preparations and Their Assay

a. *Angiotensin Converting Enzyme Activity in Desalted Human Plasma.* In order to increase the sensitivity of the ninhydrin procedure for converting enzyme assay, the background of ninhydrin positive substances was partially reduced by removal of lower molecular weight compounds from the plasma proteins using Sephadex G-25. A typical elution curve is seen in Figure 1. The fractions containing the proteins, as detected by trichloroacetic acid turbidity and absorbance at 280 nm, were incubated with Hippuryl-Gly-Gly for 14 hours and assayed by the ninhydrin procedure. No converting enzyme activity could be demonstrated in the protein fraction.

b. *Human Serum Angiotensin Converting Enzyme by Gel Filtration.* A typical protein elution pattern from Sephadex G-200 column is shown in Figure 2. Three peaks successively appeared, the fractions corresponding to the second peak ("Fraction II"), containing most of the activity, were pooled, dialyzed overnight against water and lyophilized.

Figure 1.--Elution pattern obtained by chromatography of human plasma on a column of Sephadex G-25 in 0.9% sodium chloride. One ml of plasma was applied to a 2 g bed of the gel and eluted with 0.9% sodium chloride. Peak A contained plasma proteins and the arrow indicates the absence of turbidity with 20% trichloroacetic acid in the effluent fraction. Peak B indicates non-proteinaceous substances absorbing appreciably at 280 nm.

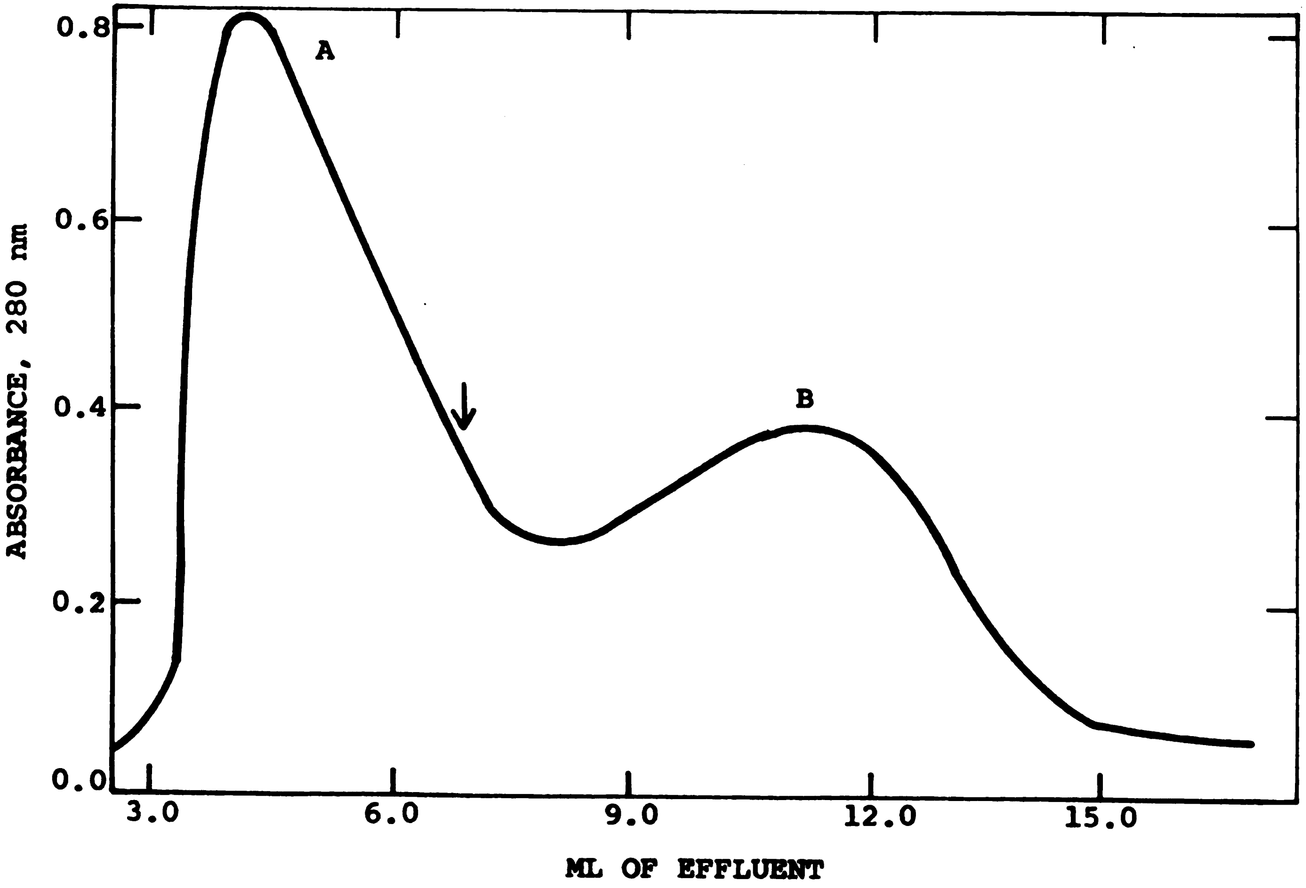
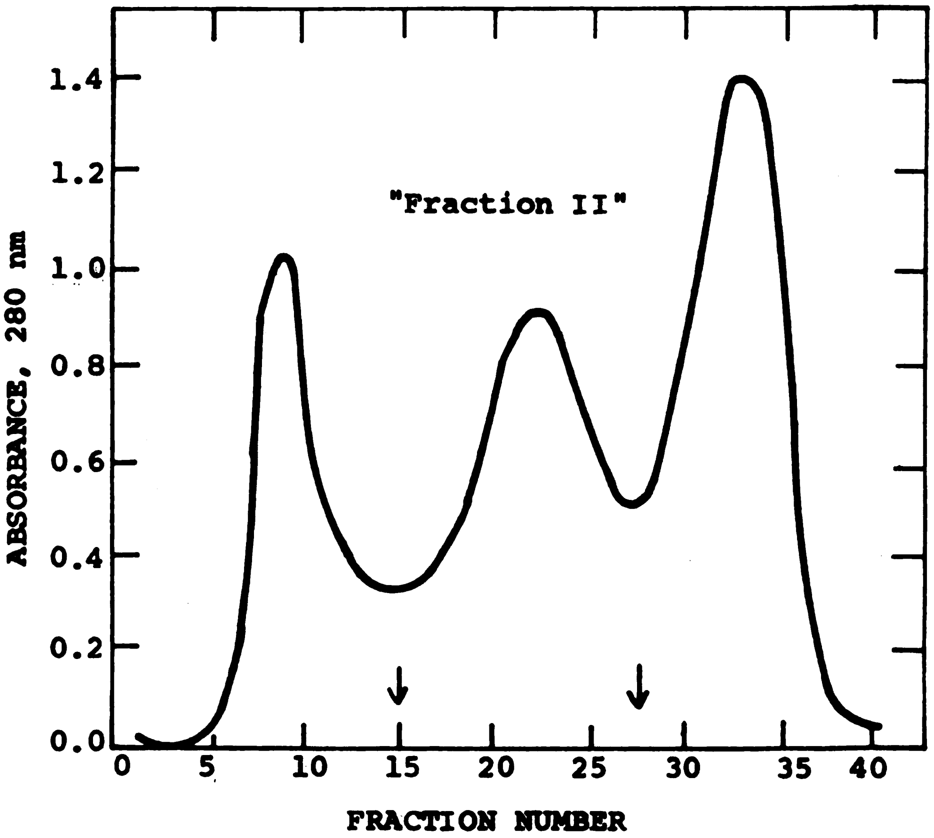

ABSORBANCE, 280 nm
0.8
0.6
0.4
0.2
0.0
A
B
3.0
6.0
9.0
12.0
15.0
ML OF EFFLUENT

Figure 2.--Gel filtration of human serum on Sephadex G-200. Elution pattern was obtained by chromatography of human serum on 2.5 x 100 cm column of Sephadex G-200 (17 g) in 0.1 M tris-hydrochloric acid buffer, pH 8.0, containing 0.2 M sodium chloride. The middle peak, Fraction II, contained angiotensin converting enzyme. The fractions between the arrows were pooled, dialyzed and lyophilized.


ABSORBANCE, 280 nm
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
FRACTION NUMBER
0
5
10
15
20
25
30
35
40
"Fraction II"

c. Purification by DEAE-Sephadex Ion Exchange Chromatography. The lyophilized preparation, Fraction II from Sephadex G-200, was applied on a DEAE-Sephadex A-50 column and eluted using a stepwise ionic strength gradient. The effluent plot is shown in Figure 3, indicating four major protein fractions. The peak that was eluted with 0.2 M sodium chloride contained converting enzyme which hydrolyzed angiotensin I, Hippuryl-Gly-Gly, Phe-Gly-Gly, Phe-Gly-Gly-Phe, Phe-Gly-Phe-Gly and Gly-His-Gly. The natural substrate, angiotensin I, was used in the assay of the lyophilized Fraction III preparation and the amount of His-Leu enzymatically released was quantitated fluorometrically. As seen in Figure 4, as little as 4.0 nmoles of His-Leu could be readily detected. Greater sensitivity (100-1000 pmoles/ml) could be easily attained using the microspectrophotofluorometric procedure.

Diagramatic representations of polyacrylamide gel electrophoresis patterns of Fraction II, the four DEAE-Sephadex fractions, and porcine plasma preparation (Figure 5) indicate that additional purification techniques are necessary if purer preparations are desired.

Table I and Scheme II outline the partial purification of human angiotensin converting enzyme from 2 ml of serum, using angiotensin I as substrate to monitor the progress of purification by fluorometric assay. About a 12.5 fold purification was achieved after gel filtration and

Figure 3.--Effluent diagram of Fraction II (Middle Fraction Sephadex G-200), human serum. Forty mg of protein were dissolved in 0.5 ml of 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride, and chromatographed on DEAE-Sephadex A-50 using a stepwise ionic strength gradient of sodium chloride in 0.02 M phosphate buffer, pH 6.8. 1 x 35 cm column, 25° C.

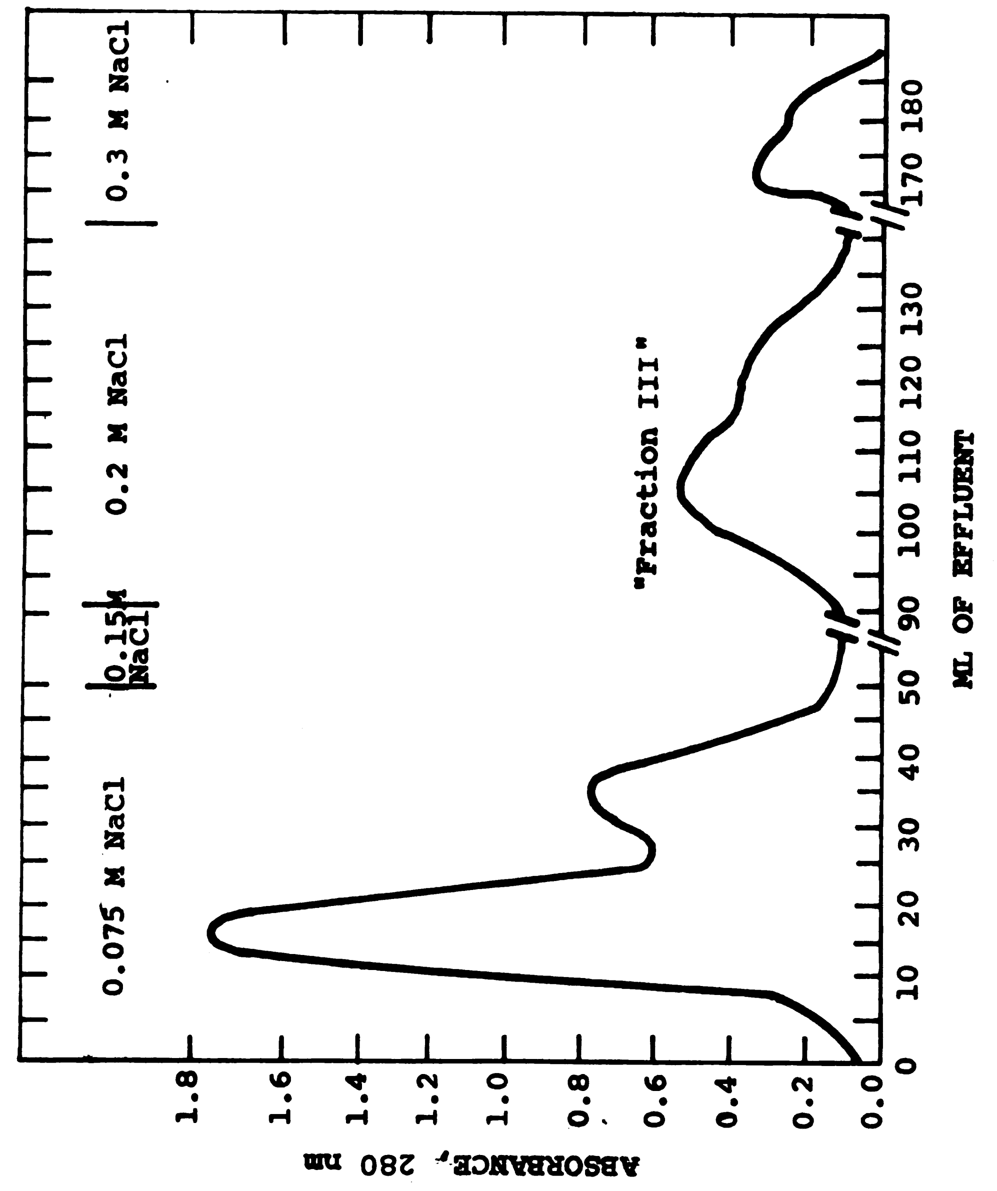

0.075 M NaCl
0.15 M NaCl
0.2 M NaCl
0.3 M NaCl
"Fraction III"
ABSORBANCE, 280 nm
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
ML OF EFFLUENT
0
10
20
30
40
50
90
100
110
120
130
170
180

Figure 4.--Standard curve for the fluorescent determination of histidyl-leucine after reaction with o-phthalaldehyde. Activation and fluorescence monochromators were set at 365 nm and 495 nm, respectively.

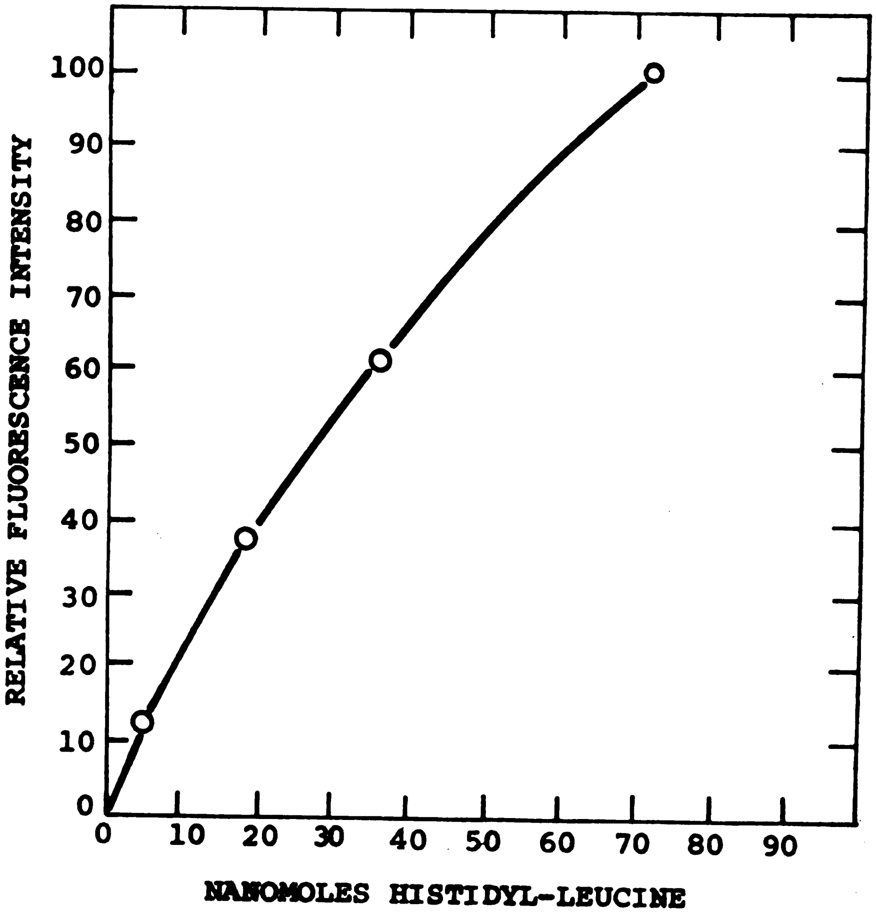

RELATIVE FLUORESCENCE INTENSITY
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30
40
50
60
70
80
90
NANOMOLES HISTIDYL-LEUCINE

Figure 5.--Diagramatic representation of polyacrylamide gel electrophoresis patterns of DEAE-Sephadex A-50 (Fractions I - IV), Fraction II from Sephadex G-200, and porcine plasma converting enzyme preparation. 0.37 M tris, pH 8.8 in gel; 0.05 M borate buffer, pH 9.2 in electrode vessel.

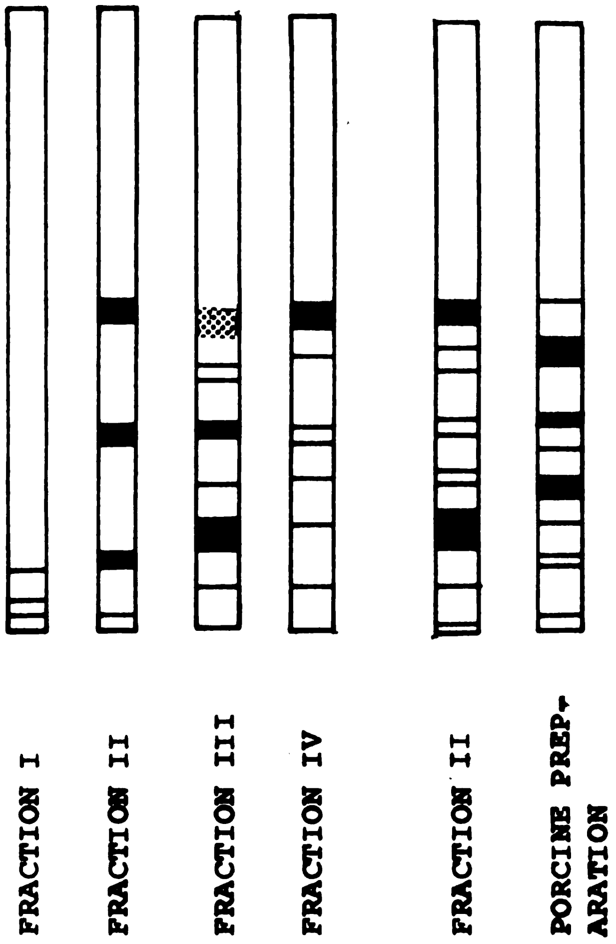
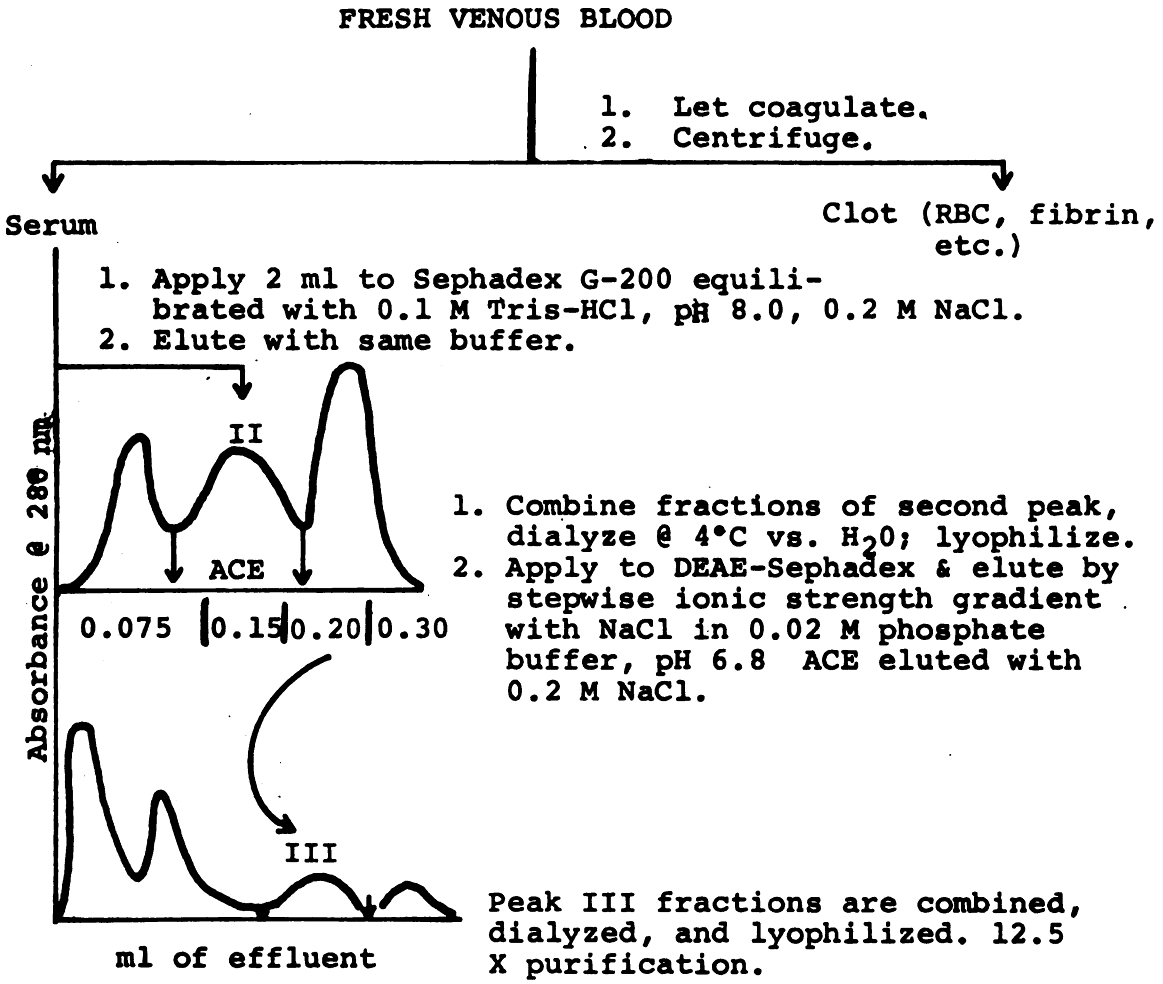

FRACTION I
FRACTION II
FRACTION III
FRACTION IV
FRACTION II
PORCINE PREP-
ARATION

Table I. Purification of Angiotensin Converting Enzyme

	Fold Purification	mg Protein	Total Activity	Sp. Ac. Units/mg	% Recovery
Plasma	1.0	150	12	0.08	100
Sephadex G-200	3.1	41	10	0.25	66
Sephadex-DEAE	12.5	5.5	5.5	1.00	33

Converting Enzyme unit: 1 nmole of histidyl-leucine released from angiotensin I/min @ 37°C, 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride.

SCHEME II.--Isolation of Angiotensin Converting Enzyme (ACE)



1. Let coagulate.
2. Centrifuge.

1. Apply 2 ml to Sephadex G-200 equilibrated with 0.1 M Tris-HCl, pH 8.0, 0.2 M NaCl.
2. Elute with same buffer.

1. Combine fractions of second peak, dialyze @ 4°C vs. H_2O; lyophilize.
2. Apply to DEAE-Sephadex & elute by stepwise ionic strength gradient with NaCl in 0.02 M phosphate buffer, pH 6.8 ACE eluted with 0.2 M NaCl.

Peak III fractions are combined, dialyzed, and lyophilized. 12.5 X purification.

ion exchange chromatography.

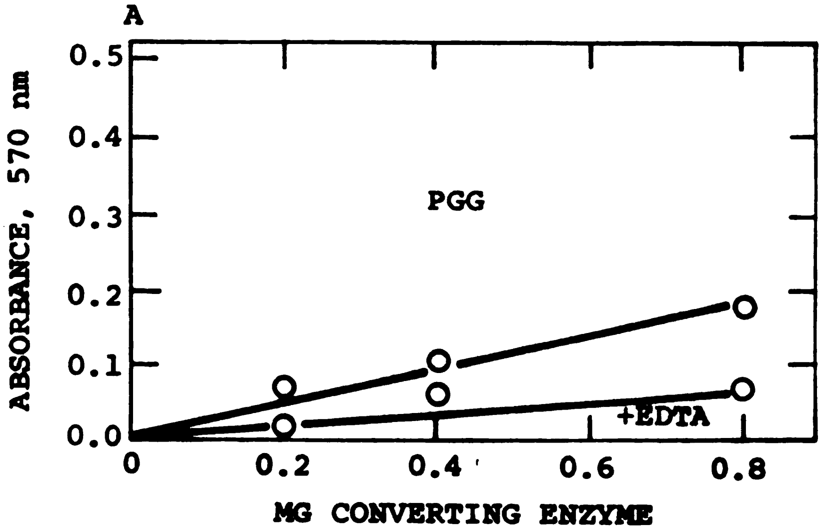
d. Angiotensin Converting Enzyme in the Cohn Alcohol Fractions of Human Plasma. By the ninhydrin procedure, Cohn Fractions I and IV-1 catalyzed the hydrolysis of Hippuryl-Gly-Gly to give a rate of 0.55 and 0.45 nmoles Gly-Gly/min/mg of protein. Fraction V had weak activity but the reproducibility was poor. No activity could be detected in Fractions II + III and IV-4.

The fluorometric assay using Gly-His-Gly as substrate confirmed that converting enzyme activity occurred primarily in Fraction IV-1. Further proof that this activity was attributable to angiotensin converting enzyme was obtained from experiments using known inhibitors of the enzyme. Thus the activity in Fraction IV-1 was inhibited by inorganic pyrophosphate and 8-hydroxyquinoline and unaffected by diisopropylfluorophosphate.

2. Cofactor Requirements for Angiotensin Converting Enzyme Activity

a. Divalent Metal Cation. Since angiotensin converting enzyme required calcium or a divalent metal ion for activity, it was found also that incubation with 1.0 mM ethylenediaminetetraacetate produced inhibition with the new substrate analogs used in this study. Figures 6 and 7 graphically depict that the activity was proportional to the quantity of enzyme and that the hydrolysis of four different substrates

Figure 6.--Effect of EDTA (lower curves) on the hydrolysis of Phe-Gly-Gly (Figure 6A) and Hippuryl-Gly-Gly (Figure 6B). The enzyme in 0.5 M phosphate buffer, pH 7.25 containing 0.1 M sodium chloride, was preincubated overnight at 4°C with 1.0 mM EDTA. The experiment was initiated by the addition of substrate. The controls (upper curves) contained no inhibitor.


A
ABSORBANCE, 570 nm
0.0
0.1
0.2
0.3
0.4
0.5
0
0.2
0.4
0.6
0.8
MG CONVERTING ENZYME
PGG
+EDTA

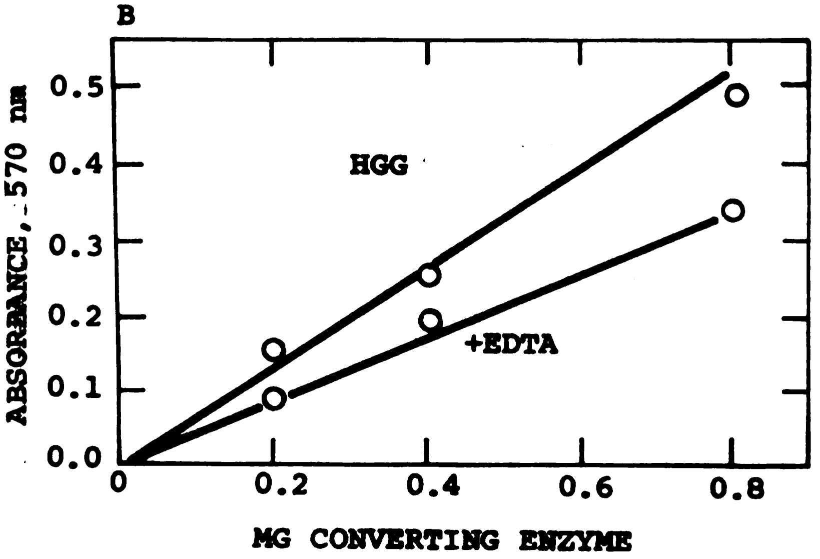
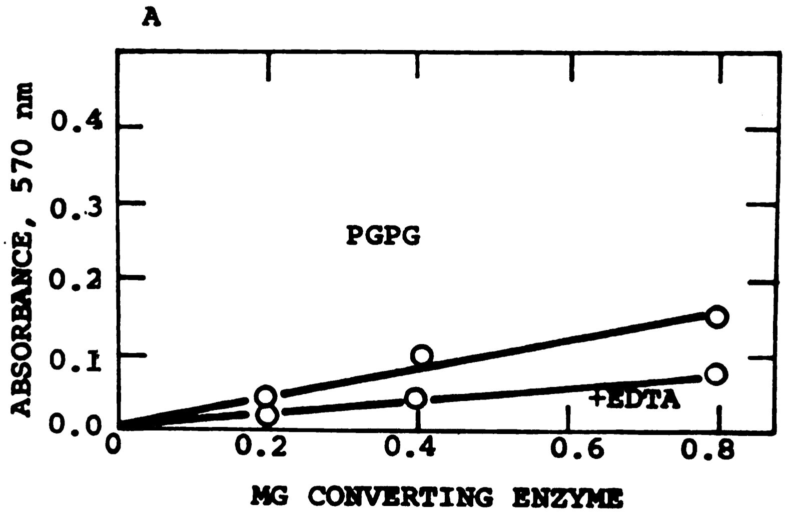
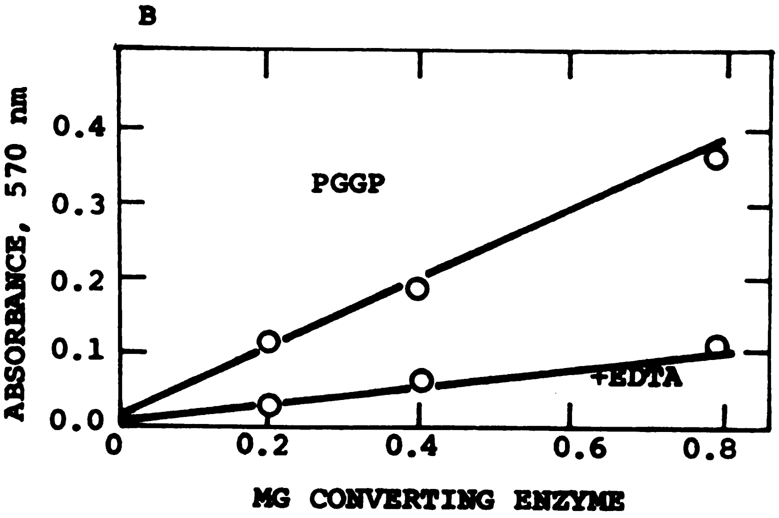

B
ABSORBANCE, 570 nm
0.0
0.1
0.2
0.3
0.4
0.5
0
0.2
0.4
0.6
0.8
MG CONVERTING ENZYME
HGG
+EDTA

Figure 7.--Effect of EDTA (lower curves) on the hydrolysis of Phe-Gly-Phe-Gly (Figure 7A) and Phe-Gly-Gly-Phe (Figure 7B). The enzyme, in 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride was preincubated overnight at 4°C with 1.0 mM EDTA. The experiment was initiated by the addition of substrate. The controls (upper curves) contained no inhibitor.


A
ABSORBANCE, 570 nm
0.4
0.3
0.2
0.1
0.0
PGPG
+EDTA
0
0.2
0.4
0.6
0.8
MG CONVERTING ENZYME


B
ABSORBANCE, 570 nm
0.4
0.3
0.2
0.1
0.0
PGGP
+EDTA
0
0.2
0.4
0.6
0.8
MG CONVERTING ENZYME

was inhibited by EDTA. Gly-His-Gly (not shown in Figures 6 and 7) produced similar results.

b. Monovalent Anion. Studies have shown that chloride is also necessary for maximum converting enzyme activity and as seen in Figures 8 and 9 deletion of chloride ions diminished enzyme activity but not to the extent that had been reported for angiotensin I. The enzymatic effect of angiotensin converting enzyme on the hydrolysis of Phe-Gly-Gly and Gly-His-Gly (not shown) was independent of chloride ion concentration.

3. Substrate Analogs for Angiotensin Converting Enzyme. The ninhydrin assay was used to follow the hydrolysis of some of the substrate analogs; the sensitivity and linearity of the method using Gly-Gly standards, may be observed in Figure 10.

With the aid of thin-layer chromatography, Gly-Gly (R_f, 0.11) and Phe (R_f, 0.49) from Phe-Gly-Gly (R_f, 0.30); Phe-Gly (R_f, 0.37) from Phe-Gly-Phe-Gly (R_f, 0.49); Phe-Gly and Gly-Phe (R_f, 0.35; isomers not resolved) from Phe-Gly-Gly-Phe; Gly-Gly (R_f, 0.11) from Hippuryl-Gly-Gly; His-Leu (R_f, 0.52) from angiotensin I (R_f, 0.09); and His-Gly (R_f, 0.21) and glycine (R_f, 0.38) from Gly-His-Gly were identified as products arising from the incubation of substrates with human and porcine plasma converting enzyme preparations. The detection of a terminal amino histidine residue by fluorescence also indicated cleavage at the position indicated.

Figure 8.--Effect of chloride ion on the activity of converting enzyme using substrate analog Phe-Gly-Gly-Phe. Varying concentrations of enzyme in 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride (upper curve), and without sodium chloride (lower curve), were incubated with the substrate for 3 hours at 37°C and the liberated products determined using the ninhydrin procedure.

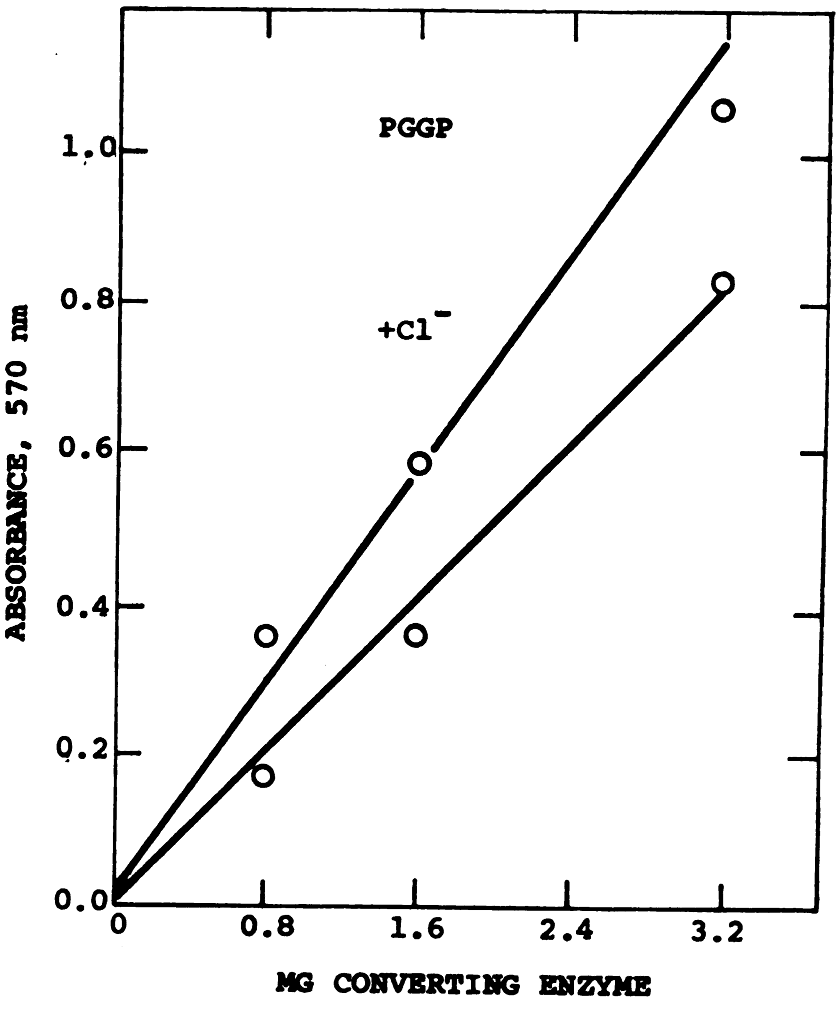

PGGP
+Cl$^-$
ABSORBANCE, 570 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
0.8
1.6
2.4
3.2
MG CONVERTING ENZYME

Figure 9.--Effect of chloride ion on the activity of converting enzyme using substrate analogs Phe-Gly-Phe-Gly and Hippuryl-Gly-Gly. Varying concentrations of enzyme in 0.5 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride (upper two curves, +Cl^-, and without sodium chloride (lower two curves), were incubated with substrate for 3 hours at 37°C, and the liberated products determined using the ninhydrin procedure.

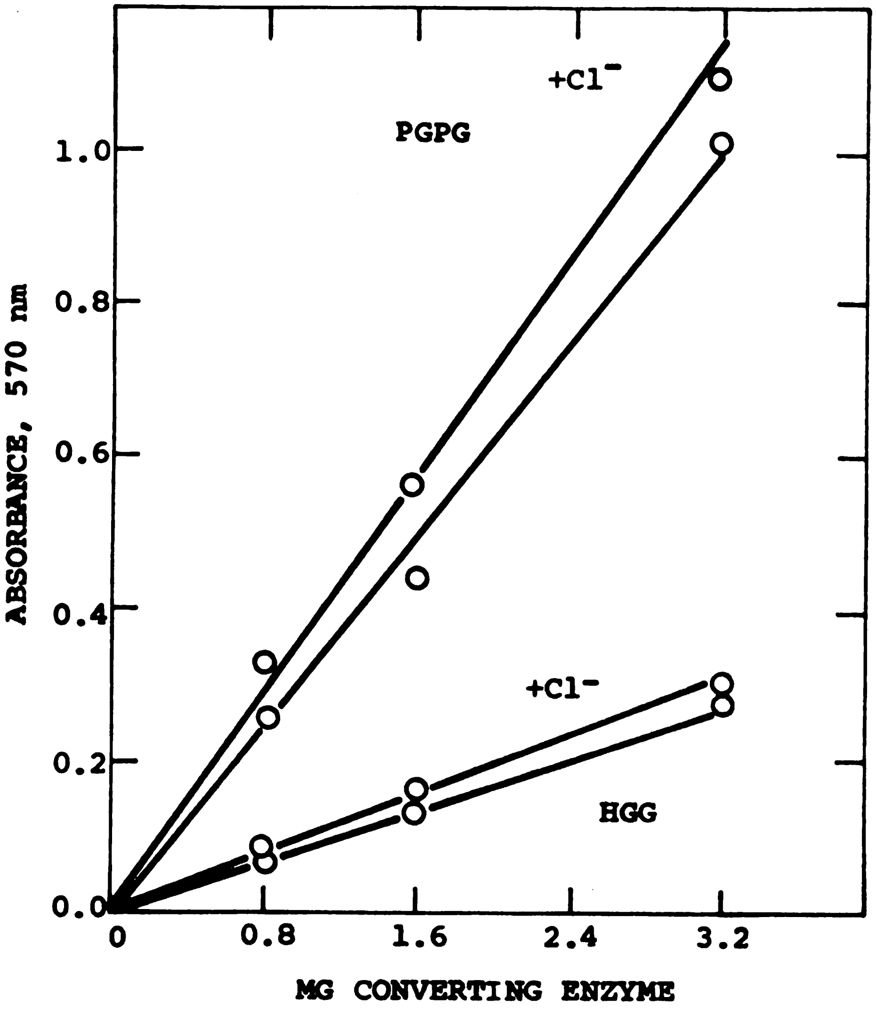
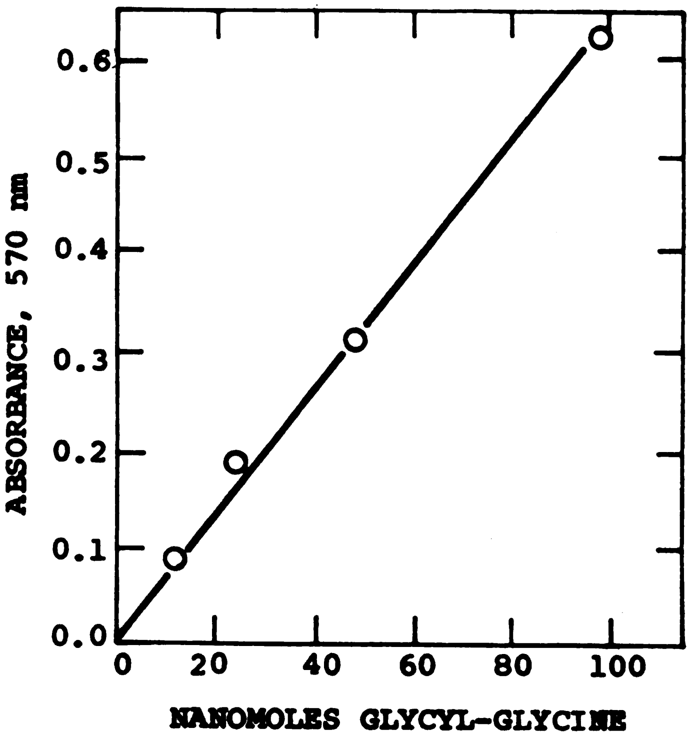

+Cl$^-$
PGPG
+Cl$^-$
HGG
ABSORBANCE, 570 nm
0.0
0.2
0.4
0.6
0.8
1.0
0
0.8
1.6
2.4
3.2
MG CONVERTING ENZYME

Figure 10.--Glycyl-glycine standard curve using the ninhydrin procedure for the colorimetric assay. To varying concentrations of glycly-glycine contained in 1.0 ml was added 0.5 ml of ninhydrin, and the solutions vigorously boiled for 15 minutes and then cooled. After adding 2.5 ml of water to each the absorbance was read at 580 nm.


ABSORBANCE, 570 nm
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
20
40
60
80
100
NANOMOLES GLYCYL-GLYCINE

In search for possible new substrate analogs for angiotensin converting enzyme, a large number of small peptides was tested, and Table II A and II B show a compilation of all those investigated. Initial investigations involved the ninhydrin assay, as developed by Dorer et al. (20) for the determination of peptidase activity. Later studies used the newly developed converting enzyme assays. The DEAE preparation of plasma angiotensin converting enzyme was incubated with the substrate tested and the amount of hydrolysis was determined using spectrophotometric and fluorometric methods. Table II B lists peptides which were not hydrolyzed by converting enzyme.

4. Fluorometric Assay of Angiotensin Converting Enzyme Using OPT and βME. o-Phthalaldehyde generally reacts with amino acids and peptides in alkaline medium in the presence of β-mercaptoethanol to give strongly fluorescing compounds (77,116). It occurred to us that this reaction might be suitable for the assay of converting enzyme provided the lytic products were fluorescent.

To determine peptides in aqueous solution, borate buffered OPT was added to the peptide in borate buffer, allowed to stand 5 to 25 minutes, and the fluorescence measured. Figure 11 compares the fluorescence obtained for Hippuryl-Gly-Gly, Phe-Gly-Phe-Gly, and their lytic dipeptides. In a typical assay procedure, 1.0 mM Phe-Gly-Phe-Gly was mixed with converting enzyme of various concentrations

Table II-A.--Substrates Tested for Dipeptidyl Carboxypeptidase Activity with Angiotensin Converting Enzyme

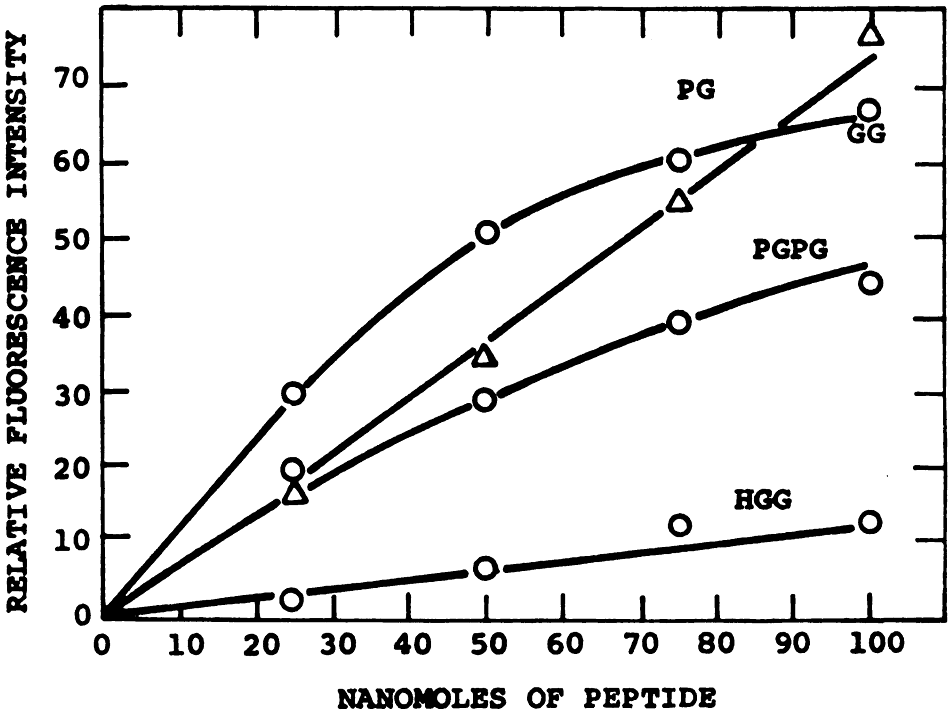
Substrate	Estimated* Relative Rate	TLC for Dipeptide	Investigators
Z-Phe-His-Leu	1000	His-Leu	Piquilloud _et al._
Gly-His Gly	200	His-Gly	Summary _et al._
Z-Pro-Phe-His-Leu	100	His-Leu	Piquilloud _et al._
Angiotensin I	100	His-Leu	Summary _et al._
Phe-Gly-Gly-Phe	40	Phe-Gly/ Gly-Phe	Summary _et al._
Phe-Gly-Phe-Gly	20	Phe-Gly	Summary _et al._
Phe-Gly-Gly	20	Gly-Gly	Summary _et al._
Hippuryl-Gly-Gly	20	Gly-Gly	Yang _et al._
Hippuryl-His-Leu	9	His-Leu	Cushman and Cheung
Boc-Phe (NO_2)-Phe-Gly	8	Phe-Gly	Yang _et al._
Z-Pro-Leu-Gly	5	Leu-Gly	Yang _et al._
Z-Gly-Gly-Gly	5	Gly-Gly	Summary _et al._
Ac-Ser-Pro-Phe-Arg	3	Phe-Arg	Yang _et al._
Bradykinin	(Not reported)	Phe-Arg	Yang _et al._

*Angiotensin I as 100

Table II-B.--Peptides Unhydrolyzed by Angiotensin Converting Enzyme

Peptide	Investigators
Hippuryl-His	Cushman and Cheung
Hippuryl-Leu	Cushman and Cheung
Hippuryl-Gly	Cushman and Cheung
t-Boc-His-Leu	Cushman and Cheung
Z-Gly-Gly	Yang *et al*.
Hippuryl-Phe-NH_2	Yang *et al*.
Z-Pro-Phe-NH_2	Yang *et al*.
t-Boc-Phe(NO_2)-Phe	Yang *et al*.
Ala-Gly-Gly	Summary *et al*.
Phe-Phe-Phe-Phe	Summary *et al*.
Z-Gly-Gly-Phe	Summary *et al*.
Z-Gly-Gly-Leu	Summary *et al*.
t-Boc-$(Pro)_4$	Summary *et al*.

Figure 11.--Fluorometric determination of peptide hydrolysis using o-phthalaldehyde and β-mercaptoethanol. Standard solutions of Phe-Gly-Phe-Gly, Hippuryl-Gly-Gly, and their lytic dipeptides were prepared, and their relative fluorescence intensities determined after reaction with o-phthalaldehyde and β-mercaptoethanol in 0.05 M borate buffer, pH 9.5.


RELATIVE FLUORESCENCE INTENSITY
NANOMOLES OF PEPTIDE
PG
GG
PGPG
HGG
0
10
20
30
40
50
60
70
0
10
20
30
40
50
60
70
80
90
100

and incubated for 3 hours. An aliquot of each was added to buffered OPT and the fluorescence intensities were measured in a spectrophotofluorometer. Figure 12 shows the change in fluorescence as a function of enzyme concentration using DEAE-Sephadex "Fraction III" as the source.

5. Spectrophotometric and Spectrophotofluorometric Assays of Angiotensin Converting Enzyme Using Glycyl-Histidyl-Glycine As Substrate

a. Converting Enzyme Assay in Partially Purified Preparations of Human Serum. The spectrophotometric and fluorometric methods developed for the assay of converting enzyme were all quite suitable since the substrates were commercially available. The substrate Gly-His-Gly was particularly attractive since its hydrolysis was catalyzed at a rate equal to twice that of the natural substrate angiotensin I (Table II A), thus shortening the incubation times. In addition, one had the option of selecting either a spectrophotometric or spectrofluorometric procedure, or if desired, they could be run sequentially.

The production of a yellow color resulting from the reaction under alkaline conditions of OPT with the imidazole group of histidyl peptides allowed spectrophotometric and hence quantitative determination of His-Gly.

Proteins in the enzyme preparation also reacted to produce a yellow chromophore, but the blank values were low.

Figure 12.--Activity of human converting enzyme as a function of protein concentration. The enzyme, partially purified on Sephadex G-200 and subfractionated on DEAE-Sephadex A-50, was incubated at 37°C in phosphate buffer, pH 7.25, containing 0.1 M sodium chloride, with 500 nmoles of Phe-Gly-Phe-Gly in total volume of 1.0 ml. Phenylalanyl-glycine was measured by the fluorogenic reaction of the dipeptide with o-phthalaldehyde in the presence of β-mercaptoethanol.

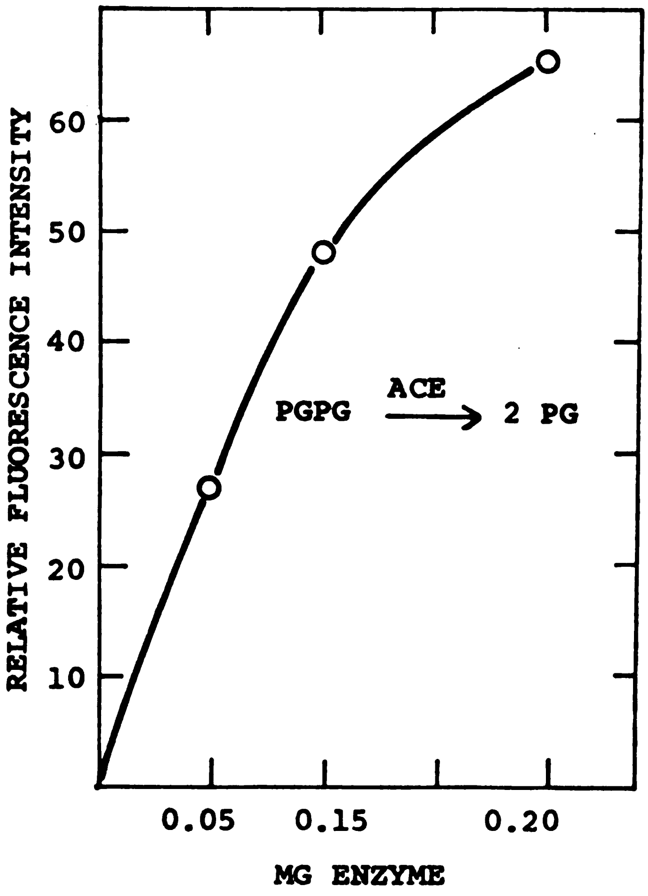

RELATIVE FLUORESCENCE INTENSITY
60
50
40
30
20
10
0.05
0.15
0.20
MG ENZYME
PGPG
ACE
2 PG

Figure 13 shows the absorption spectra of the various components of the reaction mixture. At 420 nm, the absorbance of His-Gly was quite satisfactory, and the absorbance from the protein chromophore was minimized. The color due to the His-Gly-OPT adduct reached maximum intensity from 5 to 10 minutes after addition of OPT; however, the reaction with proteins was not complete until 30 to 40 minutes after the addition of OPT reagent. Readings were made in this time frame, since the color slowly began to decay after 40 minutes. The fluorometric method also measured the amount of His-Gly liberated by angiotensin converting enzyme; however, for analysis OPT was reacted with the imidazole moiety in the presence of 2 N sodium hydroxide, and then the fluorescence was measured under acid conditions. Since proteins did not form a fluorophore under these conditions, there was no background fluorescence.

The hydrolysis of Gly-His-Gly was found to be proportional to both the time of incubation at fixed enzyme and substrate concentrations and to the enzyme concentration (Figures 14 A and 14 B). Figure 15 depicts the hydrolysis of Gly-His-Gly as a function of substrate concentration. The Lineweaver-Burk plot inset indicated a Michaelis constant of 6.0×10^{-4} M. There was a close correlation between the percent hydrolysis as shown by spectrophotometric and spectrofluorometric methods (Figure 16).

Incubation of the enzyme with Gly-His-Gly at various

Figure 13.--Protein (curve B) and histidylglycine (curve A) spectral absorbance curves after reaction with OPT. o-Phthalaldehyde was reacted with a preparation of angiotensin converting enzyme (0.5 mg/ml, Fraction II) and a mixture of hydrolytic products of Gly-His-Gly, 0.10 mM His-Gly and 0.10 mM Gly. The absorbance of the resulting chromophores were determined as a function of wavelength.

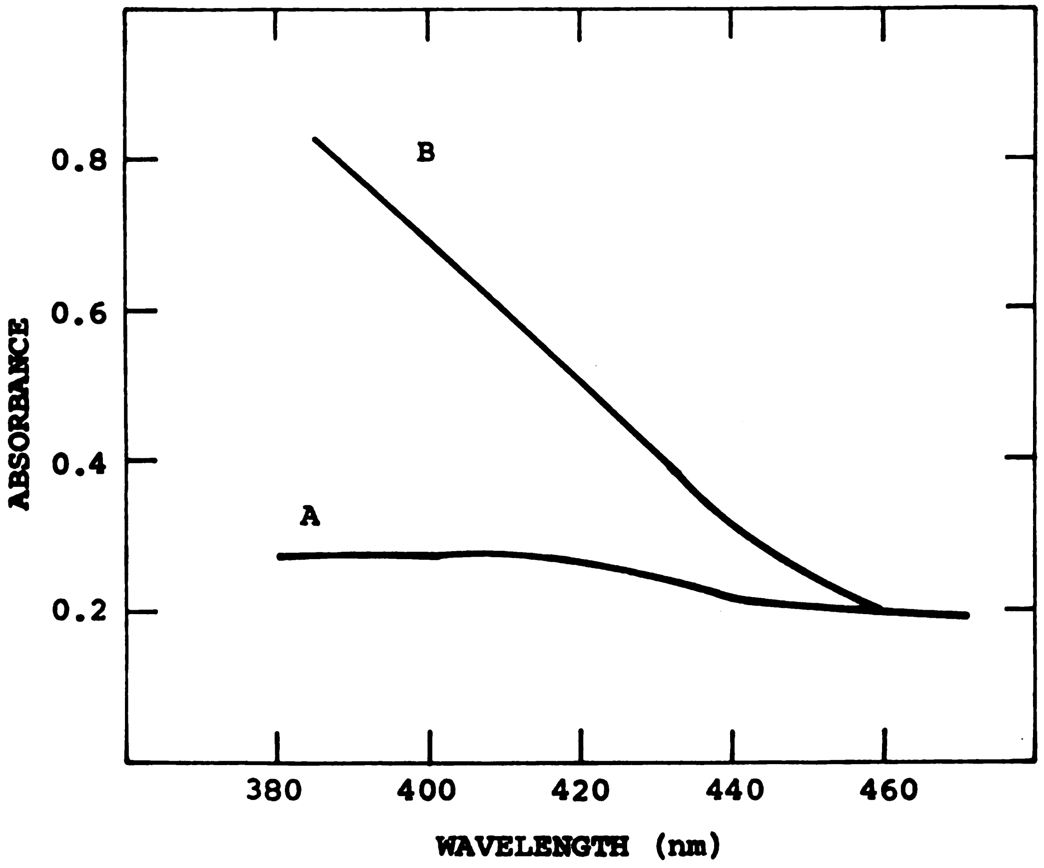

B
A
0.8
0.6
0.4
0.2
ABSORBANCE
380
400
420
440
460
WAVELENGTH (nm)

Figure 14.--Hydrolysis of glycyl-histidylglycine by plasma angiotensin converting enzyme. For the hydrolysis of Gly-His-Gly as a function of time (Figure 14A) the substrate was incubated with varying concentrations of enzyme at 37°C. Aliquots were removed at the times indicated and the histidyl-glycine quantitated spectrophotometrically. For the hydrolysis of substrate as a function of enzyme concentration((Figure 14B) the substrate was incubated at 37°C for 90 minutes with varying concentrations of enzyme and the liberated dipeptide quantitated, as above, using o-phthalaldehyde.

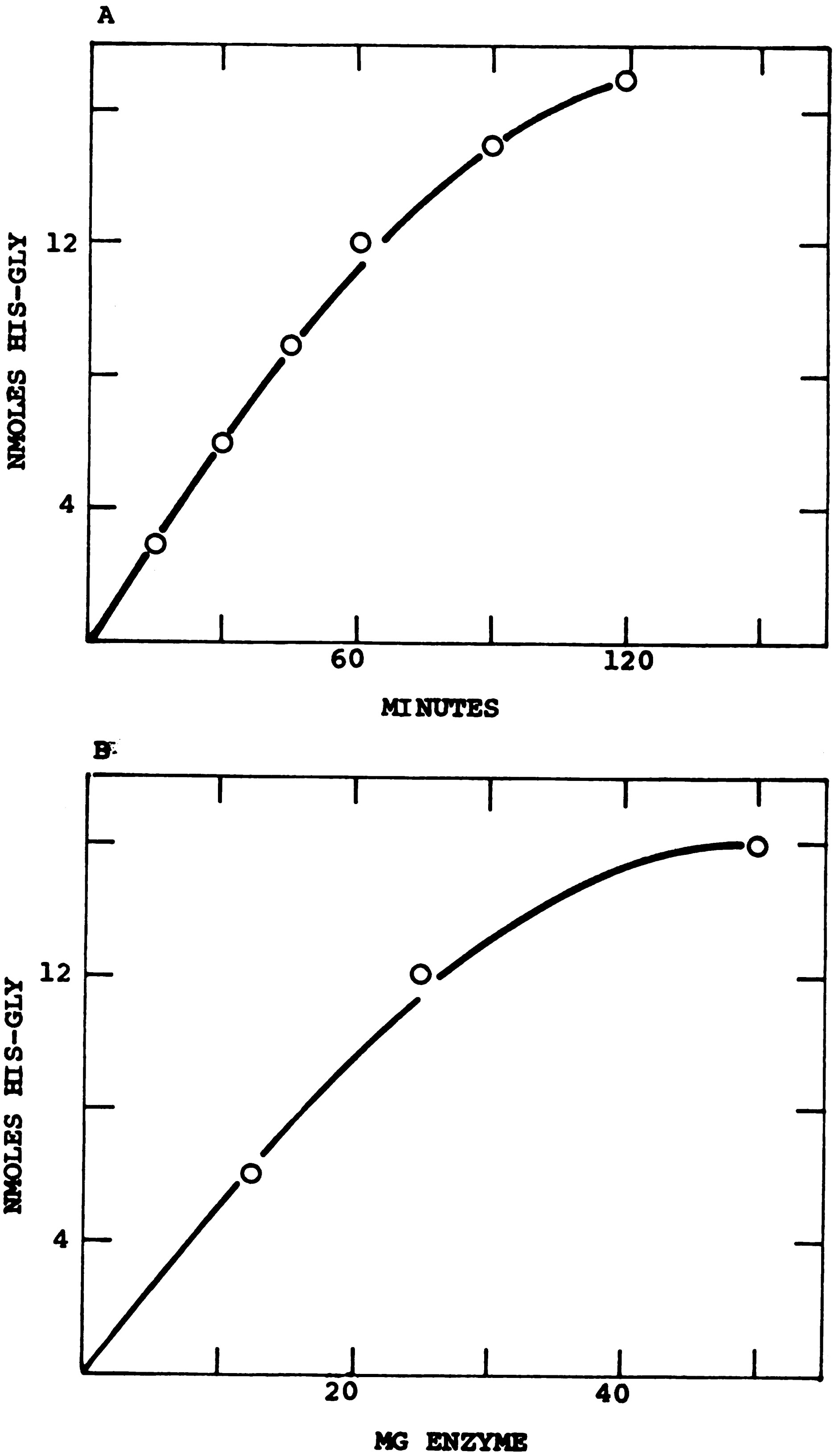

A
NMOLES HIS-GLY
12
4
60
120
MINUTES
B
NMOLES HIS-GLY
12
4
20
40
MG ENZYME

Figure 15.--The effect of substrate concentration on the activity of angiotensin converting enzyme of plasma, measured by the rate of hydrolysis of glycyl-histidyl-glycine. Gly-His-Gly, 25 - 200 nmoles, was incubated at 37°C with 0.10 mg of DEAE Fraction III enzyme preparation in 1.0 ml of 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride. The liberated histidyl-glycine was determined fluorometrically using o-phthalaldehyde. The inset shows a Lineweaver-Burk plot of the same data.

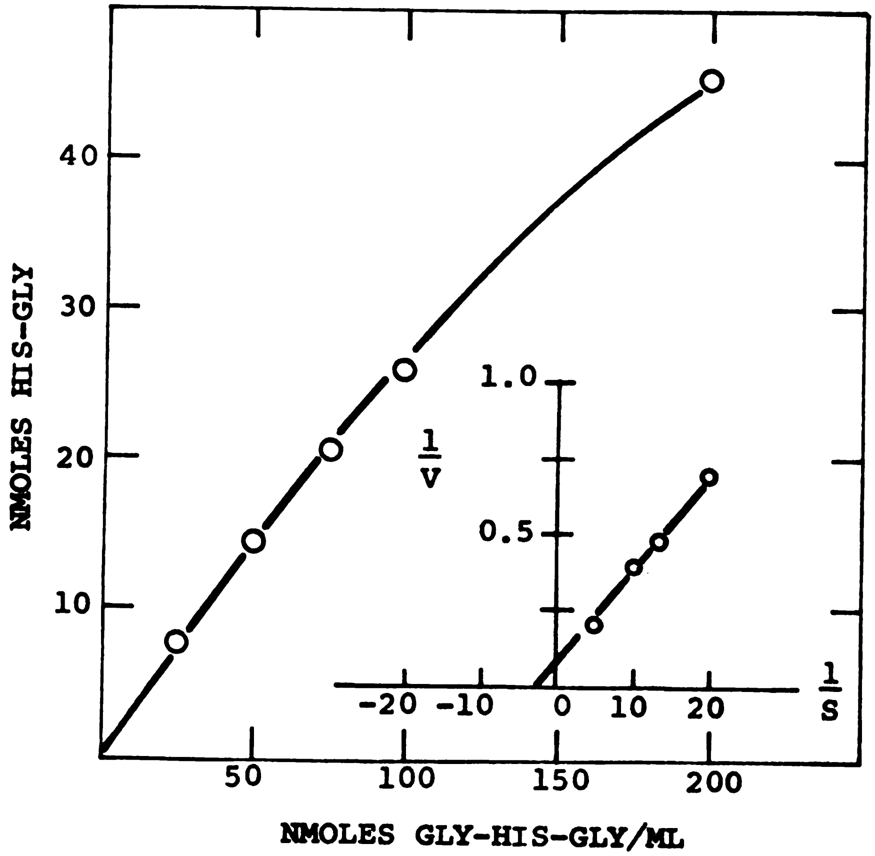
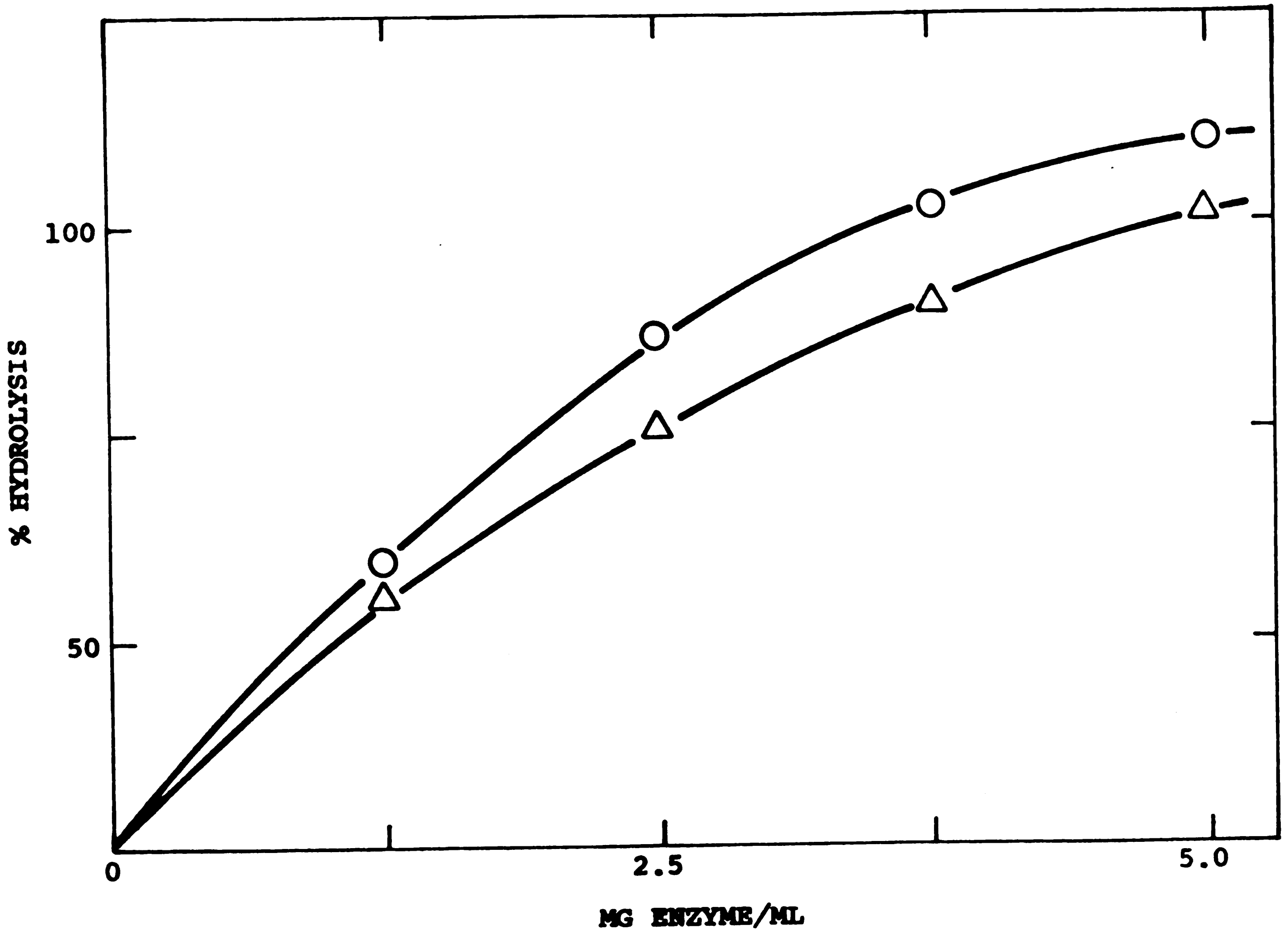

NMOLES HIS-GLY
40
30
20
10
50
100
150
200
NMOLES GLY-HIS-GLY/ML
1.0
0.5
1/V
-20
-10
0
10
20
1/S

Figure 16.--Comparison of spectrophotometric and spectrofluorometric assays for angiotensin converting enzyme as a function of enzyme concentration. The enzyme preparation (1.25-5.0 mg of Fraction II, human plasma) in 1.0 ml of 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride was incubated with 0.5 mM Gly-His-Gly, and aliquots of the liberated histidyl-glycine were quantitated colorimetrically and fluorometrically using o-phthaldehyde.


% HYDROLYSIS
100
50
0
2.5
5.0
MG ENZYME/ML

temperatures indicated that the temperature of maximum hydrolysis was 43°. Similarly, incubation in 0.05 M phosphate buffer, with the pH adjusted between 6 and 9, showed an optimum at pH 7.25. This agrees with the value of pH 7.25 reported by Huggins _et al_. (56) for plasma angiotensin converting enzyme.

Human plasma converting enzyme, as revealed by amido black staining, was a rapidly moving protein component during polyacrylamide gel electrophoresis. Table III shows converting enzyme activity as a function of the distance of migration along the gel. Both Gly-His-Gly and angiotensin I were hydrolyzed by the same regions. The activity at the origin is associated with protein which failed to enter the gel. The faintness of the bands associated with the active regions confirms the low levels of plasma converting enzyme as was found by H-J Lee _et al_. (105).

Since angiotensin converting enzyme required a divalent metal ion for activity, incubation with 0.20 mM EDTA produced 100% inhibition. As previously reported (56), the sulfhydryl agent, p-chloromercuribenzoate, also was a potent inhibitor of the enzyme (Table IV).

Pyrophosphate was found to be comparable to 8-hydroxyquinoline as an inhibitor. Aldosterone, an important mineralcorticoid regulator of kidney function, was found to be a good inhibitor (more than 50%) at relatively low concentrations.

Table III.--Hydrolysis of Gly-His-Gly and Angiotensin I by Polyacrylamide Gel Electrophoresis Fractions

Section (cm from origin)	Converting Enzyme Activity[a] Substrate		Intensity of Bands Present[b]
	AI	GHG	
0.0 - 0.5	+++	+++	-
0.5 - 1.0	-	+	+
1.0 - 1.5	-	-	+++
1.5 - 2.0	-	-	++
2.0 - 2.5	-	-	-
2.5 - 3.0	-	-	++
3.0 - 3.5	++	+	++
3.5 - 4.0	++	++	+
4.0 - 4.5	+++	++++	++

[a] Comparative activity of sections:

4+ = good hydrolysis
3+ = moderate hydrolysis
2+ = weak hydrolysis
1+ = slight hydrolysis
- = no hydrolysis

[b] See Figure 5, p. 53

Table IV.--Inhibition of Human Plasma Angiotensin Converting Enzyme[+]

Compound	Pre-incubation	Percent Inhibition				
		0.5	1.0	1.5	2.0	2.5 (mM)
EDTA *	-	100	100	100	100	100
8-Hydroxyquinoline	-	20	43	61	80	96
p-Chloromercuribenzoate	-	40	70	100	100	100
N-Ethylmaleimide	15 minutes, 37°C	9	19	28	38	47
Iodoacetic acid	15 minutes, 37°C	0	0	0	1	3
Pyrophosphate*	-	30	55	75	91	100
Angiotensin II*	-	23	49	67	90	100
		5.0	10.0	15.0	20.0	(µl)
Adrenal glands extract	15 minutes, 37°C	19	14	-	-	
B. Jararaca venom extract*	15 minutes, 37°C	21	21	-	-	
Human urinary factor*	-	70	84	87	90	
Plasma inhibiting factor*	-	55	79	100	100	
		0.075		0.15	(mM)	
Aldosterone	15 minutes, 37°C	52		64		

[+] 100 nmoles of Gly-His-Gly were incubated for 90 minutes with 0.5 mg of Sephadex G-200 fractionated human plasma angiotensin converting enzyme preparation and the indicated amount of inhibitor in a total volume of 0.3 ml. The liberated His-Gly was determined spectrophotometrically.

[*] Confirmed using angiotensin I as the substrate. Angiotensin I (35 nmoles) was incubated as described above and the liberated His-Leu was determined spectrophotometrically.

A crude extract of Bothrops Jararaca venom, a specific inhibitor of angiotensin converting enzyme (108,110), was found to inhibit the enzyme when either angiotensin I or Gly-His-Gly was the substrate. The acetone extract of dog adrenal glands, presumably containing adrenal steroids, gave comparable inhibition of Gly-His-Gly hydrolysis.

Angiotensin II, the natural octapeptide product of angiotensin I hydrolysis, caused 49% inhibition of Gly-His-Gly hydrolysis at 1.0 mM concentration. Product inhibition was confirmed using the natural substrate angiotensin I.

The low activity in partially purified preparations of angiotensin converting enzyme prompted a look for effectors in human plasma and urine. Following dialysis of both urine and of plasma and passage of the dialysate over a cation exchange column (Dowex 50W-X8), the components bound to the resin were found to inhibit plasma converting enzyme (e.g. 100% inhibition by 15 μl of Plasma Inhibiting Factor, Table IV). The active component could not be extracted into ether or benzene and was only slightly soluble in chloroform/methanol (3/2). Human Urinary Factor was also bound by an anion exchange resin (Dowex 1-X8). Acid hydrolysis of either the urine or plasma preparations did not affect their inhibitory ability.

b. Angiotensin Converting Enzyme Assay in ACD Plasma. The spectrophotometric assay developed in this study was a rapid, specific and sensitive method for the determination of angiotensin converting enzyme activity in small amounts of plasma or serum. As shown in Figure 17, the percent hydrolysis was a linear function of time at fixed plasma (0.10 ml) and substrate concentrations (100 nmoles), provided the time of incubation did not exceed 30 minutes. As is characteristic of enzyme catalyzed reactions, the percent hydrolysis of Gly-His-Gly was proportional to the plasma (angiotensin converting enzyme) concentration at a fixed substrate concentration (Figure 18). A potent peptidase inhibitor, diisopropylfluorophosphate, incorporated into the incubation mixture had no effect on the hydrolytic reaction.

Since His-Gly, the product of Gly-His-Gly hydrolysis, as well as the plasma proteins, reacted with OPT, the assay procedure required the preparation of a protein free filtrate prior to addition of the OPT reagent. Further, the amber color of serum or plasma, similar in color to the OPT-His-Gly chromophore, absorbed blue light (420 nm) strongly. Protein precipitation with tungstic acid reduced the background color to negligible levels, permitting the measurement of nanomole quantities of His-Gly.

Inhibition studies (Table IV) and polyacrylamide gel electrophoresis experiments (Table III) using purified human serum preparations demonstrated that Gly-His-Gly was a suitable substrate analog for the assay of angiotensin converting enzyme. Thus substrate modification, small peptide assays, and inhibition experiments were undertaken to determine

Figure 17.--ACD plasma catalyzed hydrolysis of Gly-His-Gly as a function of time. ACD plasma (0.10 ml) was incubated with 100 nmoles of Gly-His-Gly in 0.05 M phosphate buffer, pH 7.25, containing 0.1 M sodium chloride, and the liberated His-Gly was determined spectrophotometrically with OPT at the indicated times.

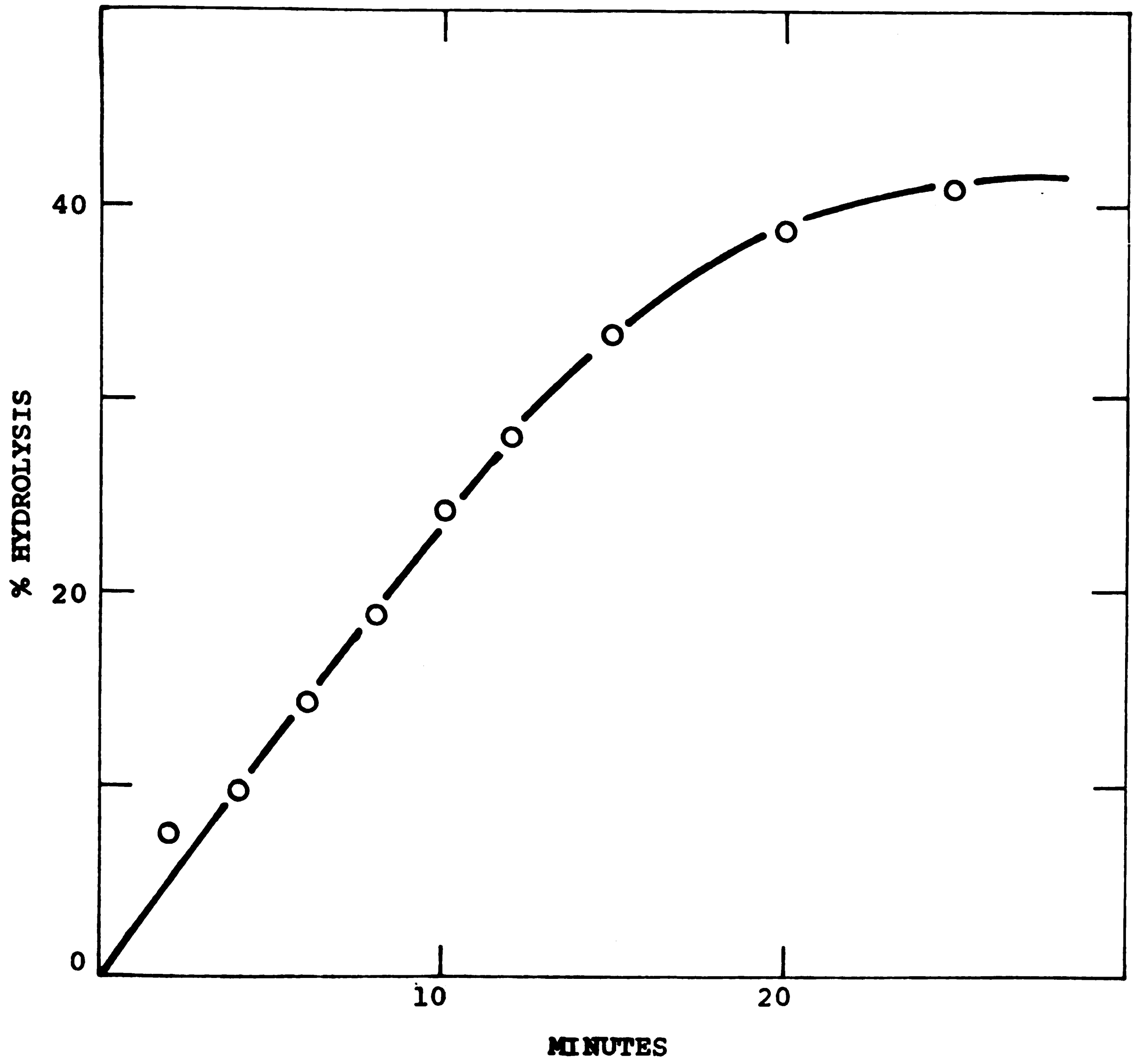
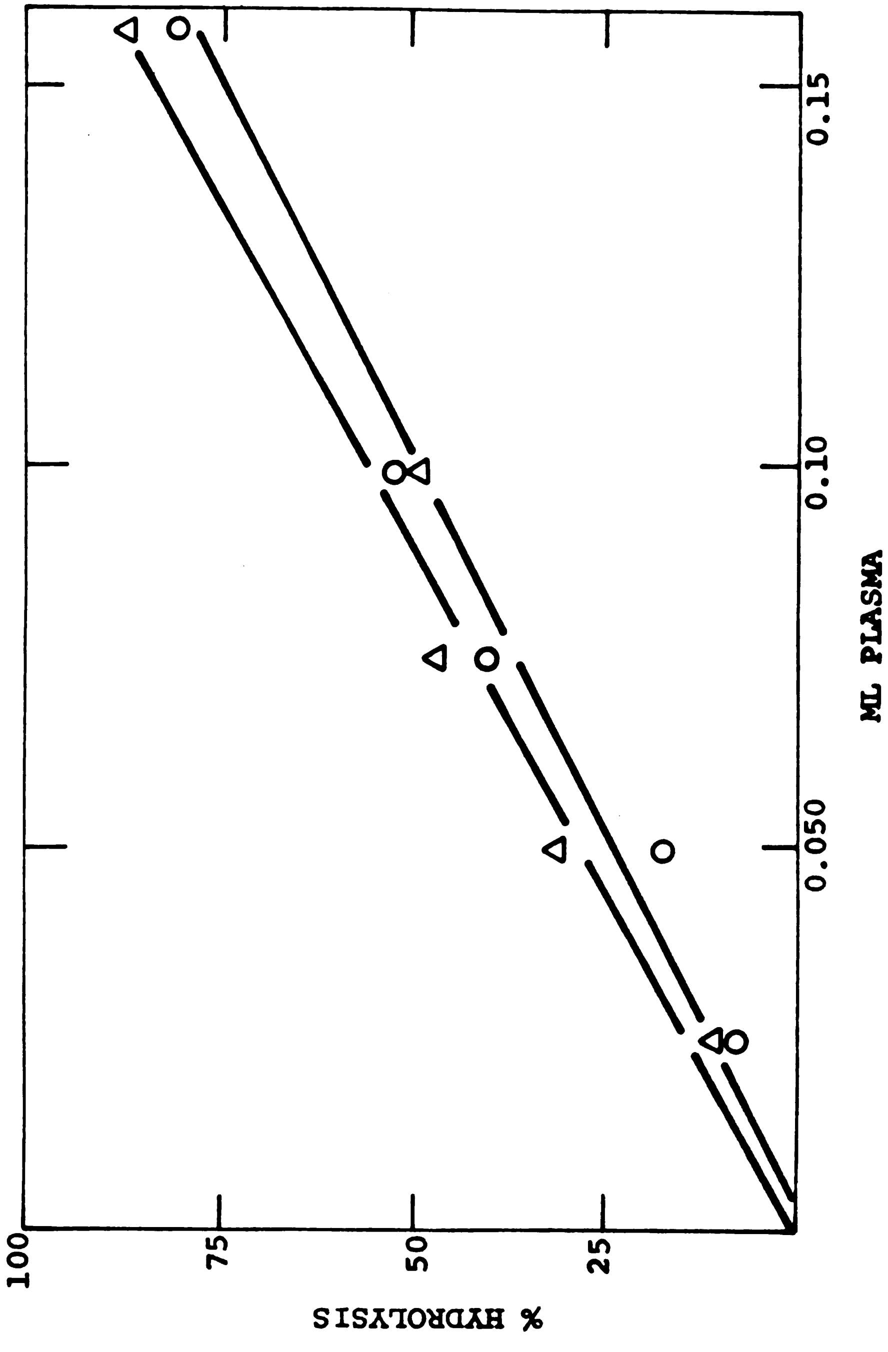

% HYDROLYSIS
40
20
0
10
20
MINUTES

Figure 18.--Activity of human angiotensin converting enzyme as a function of plasma concentration. The indicated amount of plasma, with and without DFP (triangles and circles respectively), was incubated with Gly-His-Gly for 10 minutes at 37° in 0.05 M phosphate buffer, pH 7.25, containing 0.10 M sodium chloride, and the His-Gly determined spectrophotometrically with OPT.


% HYDROLYSIS
100
75
50
25
ML PLASMA
0.050
0.10
0.15

whether the catalyzed hydrolysis of Gly-His-Gly by plasma was due to angiotensin converting enzyme or plasma peptidases.

Since it was reported that Hippuryl-His-Leu (106) and Hippuryl-Gly-Gly (107) were catalytically hydrolyzed by converting enzyme preparations, we acetylated the amino group of Gly-His-Gly in order to preclude aminopeptidase catalyzed hydrolysis of the substrate. N-acetyl-Gly-His-Gly was hydrolyzed at an appreciably reduced rate in both the purified angiotensin converting enzyme preparation and in plasma (Table V).

The effect of esterification of the natural substrate, angiotensin I, was not reported, although the enzyme was termed a "dipeptidyl carboxypeptidase" (107). The ethyl esters of both Gly-His-Gly and angiotensin I were found to be hydrolyzed at rates approximately equal to those of the unesterified substrates (Table V).

If the hydrolysis of Gly-His-Gly by the partially purified preparation of human serum comverting enzyme and by plasma was catalyzed by the same enzyme, then both sources should produce comparable results when incubated with small peptides and substrate analogs. Table V lists the small peptides that were tested and semiquantitatively tabulates the amount of hydrolysis observed with plasma and the partially purified converting enzyme preparation. The partially purified preparation of converting enzyme and plasma catalyzed the hydrolysis of the same substrate analogs.

Table V.--Effect of Angiotensin Converting Enzyme Preparations and ACD Plasma on Small Peptides

Peptide	Porcine Plasma Converting Enzyme Preparation	Human Plasma
Gly-His-Gly	+++++	+++++
Gly-His-Gly, ethyl ester	+++++	+++++
Phe-Gly-Phe-Gly	++++	++++
Phe-Gly-Gly-Phe	++++	+++++
Hippuryl-Gly-Gly	++	0
N-acetyl Gly-His-Gly	+	+
Z-Gly-Gly-Gly	+	++
Phe-Phe-Phe-Phe	0	+
Z-Gly-Gly-Phe	0	0
Z-Gly-Gly-Leu	0	0
t-Boc-Pro-Pro-Pro-Pro	0	0

	Nanomoles of peptide hydrolyzed	
	per 90 min*	per 10 min
Angiotensin I	19	20
Angiotensin I, ethyl ester	18	-
Gly-His-Gly	40	40
Gly-His-Gly, ethyl ester	37	38
N-acetyl Gly-His-Gly	5	4

*Fraction II of Human Serum, Sephadex G-200 preparation

5+ = good hydrolysis
4+ = moderate hydrolysis
2+ = weak hydrolysis
1+ = slight hydrolysis
0 = no hydrolysis

Inhibition experiments provided additional proof that the Gly-His-Gly assay was measuring angiotensin converting enzyme activity in plasma. As is shown in Table VI, fluoride, citrate, oxalate and diisopropylfluorophosphate, compounds known to inhibit peptidases, had little effect on the enzyme catalyzed hydrolysis of Gly-His-Gly, while inhibitors of converting enzyme (Table IV, VI) produced identical effects in the purified angiotensin converting enzyme preparation and in plasma.

B. FINDINGS ON THE MECHANISM OF THE FLUOROGENIC REACTION OF AMINO ACIDS, PEPTIDES AND PROTEINS WITH O-PHTHALALDEHYDE AND β-MERCAPTOETHANOL

1. Ultraviolet Spectral Changes. As noted from Figure 19, o-phthalaldehyde and β-mercaptoethanol must initially interact for a strongly fluorescing product to develop upon the addition of amino acid, peptide or protein. Thus the very prominent transition at 200 nm, attributable to the carbonyl moiety of OPT, is reduced and a new weak transition at 340 nm appears when βME reacts with OPT. Upon the addition of glycine to form the fluorescent adduct, an intense transition is evidenced in the 225 nm region due to the formation of an imine group or a charge transfer band. It is accompanied by enhancement of the transition in the 340 nm region upon binding of the amino acid to OPT.

The OPT-βME adduct showed intense fluorescence

Table VI.--Effect of Compounds and Extracts on Angiotensin Converting Enzyme Activity in Human Plasma and Serum Preparation

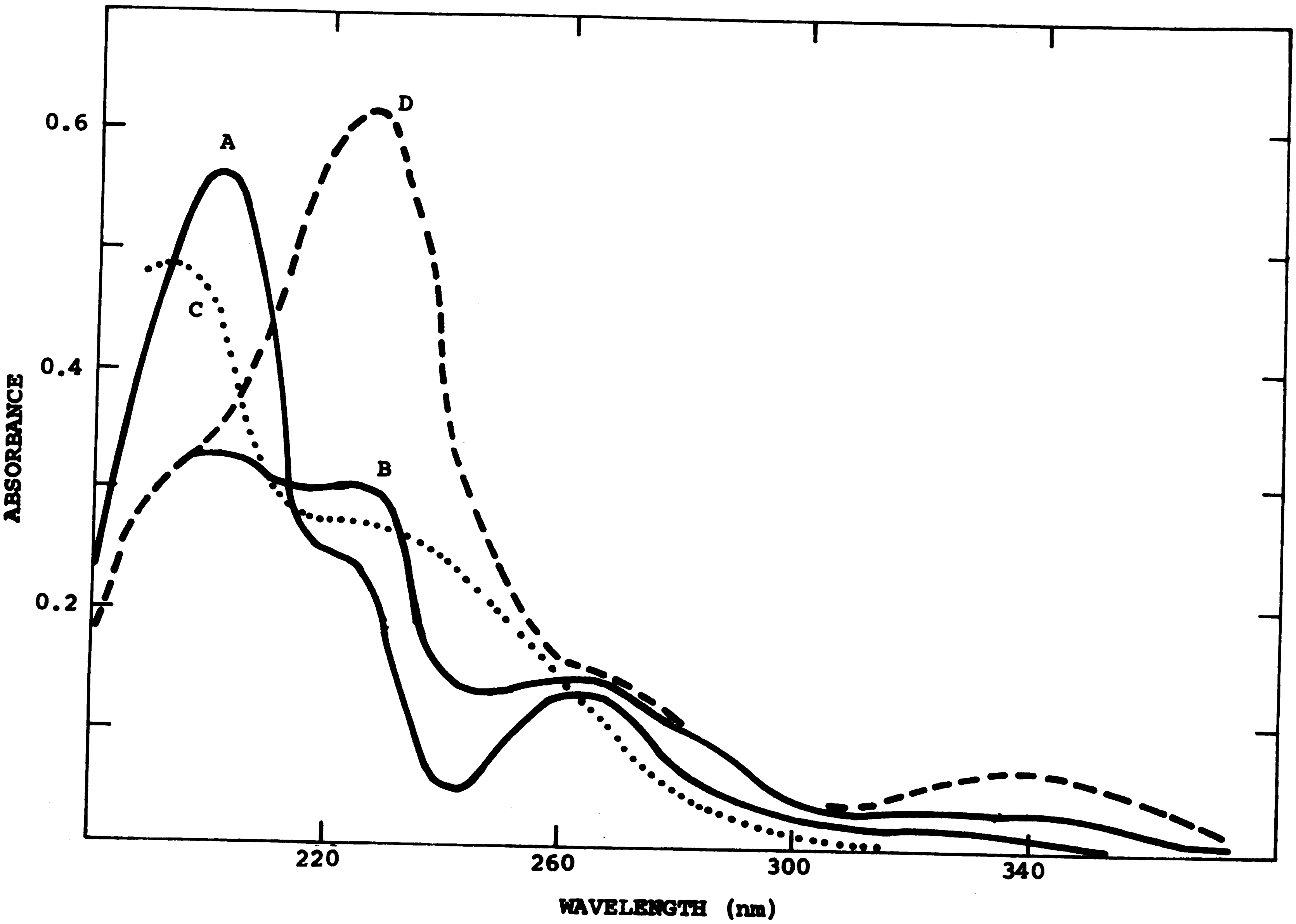
Compounds or Extracts	Fraction II* Preparation		ACD Plasma		
	1.0	2.0	1.0	2.0	(mM)
EDTA	+++++	+++++	+++	++++	
p-Chloromercuribenzoate	++++	+++++	+++++	+++++	
8-Hydroxyquinoline	+++	++++	+++	++++	
Angiotensin II	+++	++++	+++	+++	
Fluoride	0	0	0	0	
Citrate	0	0	0	0	
Oxalate	0	0	0	0	
	5.0	10.0	5.0	10.0	(μl)[+]
Diisopropylfluorophosphate	0	0	+	+	
Bothrops Jararaca Venom Extract	+	++	+	++	
Adrenal Gland Extract	-	++	-	+++	

*Fraction II of Human Serum, Sephadex G-200 preparation

[+] Aliquots were preincubated with Fraction II or plasma prior to initiation of the enzymatic reaction.

5+ = almost total inhibition
4+ = strong inhibition
3+ = moderate inhibition
1+ = slight inhibition
0 = no inhibition

Figure 19.--Ultraviolet spectra of the reaction involving o-phthalaldehyde, β-mercaptoethanol and glycine were 1.86 x 10^{-5}, 1.45 x 10^{-4}, and 2.0 x 10^{-5} M respectively. (A) o-phthalaldehyde; (B) o-phthalaldehyde + β-mercaptoethanol; (C) o-phthalaldehyde + glycine; (D) o-phthalaldehyde + β-mercaptoethanol + glycine.



A
B
C
D
0.6
0.4
0.2
ABSORBANCE
220
260
300
340
WAVELENGTH (nm)

before removal of the methanol solvent; however, slow evaporation and subsequent redissolving in borate buffer of pH 11.0 caused a reduction in fluorescence. This was in agreement with other observations that fluorescence in dilute solution decays after several hours. The adducts that were analyzed were chromatographically pure by thin-layer chromatography, thus indicating little side product formation or degradation during synthesis. If the OPT-βME glycine adduct was chromatographed by thin-layer chromatography in acidic medium, several degradation products were visible including one ninhydrin positive component. The ultraviolet spectrum of the crystalline OPT-βME-glycine adduct is shown in Figure 20.

2. Infrared Spectroscopy. Infrared spectroscopy of the adducts showed that one of the carbonyl groups of OPT disappeared upon the addition of glycine, since there was a reduction in intensity of the band attributable to the C-H stretch on the aldehyde group. Further, the OPT-glycine adduct showed no free amino group. The OPT-βME-glycine adduct showed additional change in the carbonyl region and further reduction of the C-H stretch. No S-H bands could be detected. The imine region was obscured by the Nujol preparation.

3. Nuclear Magnetic Resonance Spectroscopy. The NMR spectra of the adducts are shown in Figure 21. The OPT-glycine adduct showed four separate proton environments. The

Figure 20.--Ultraviolet spectra of the crystalline o-phthalaldehyde, β-mercaptoethanol and glycine adduct.

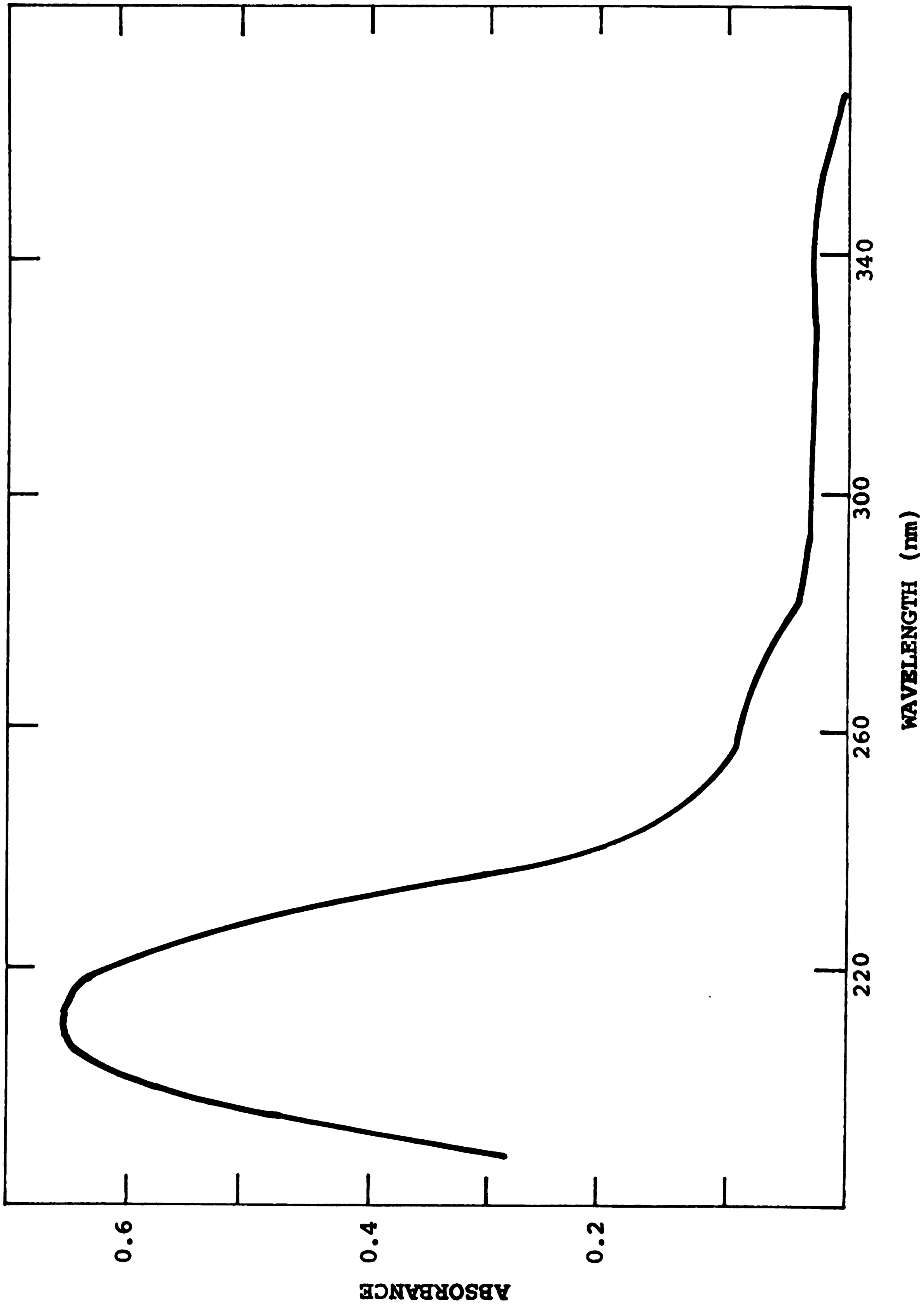
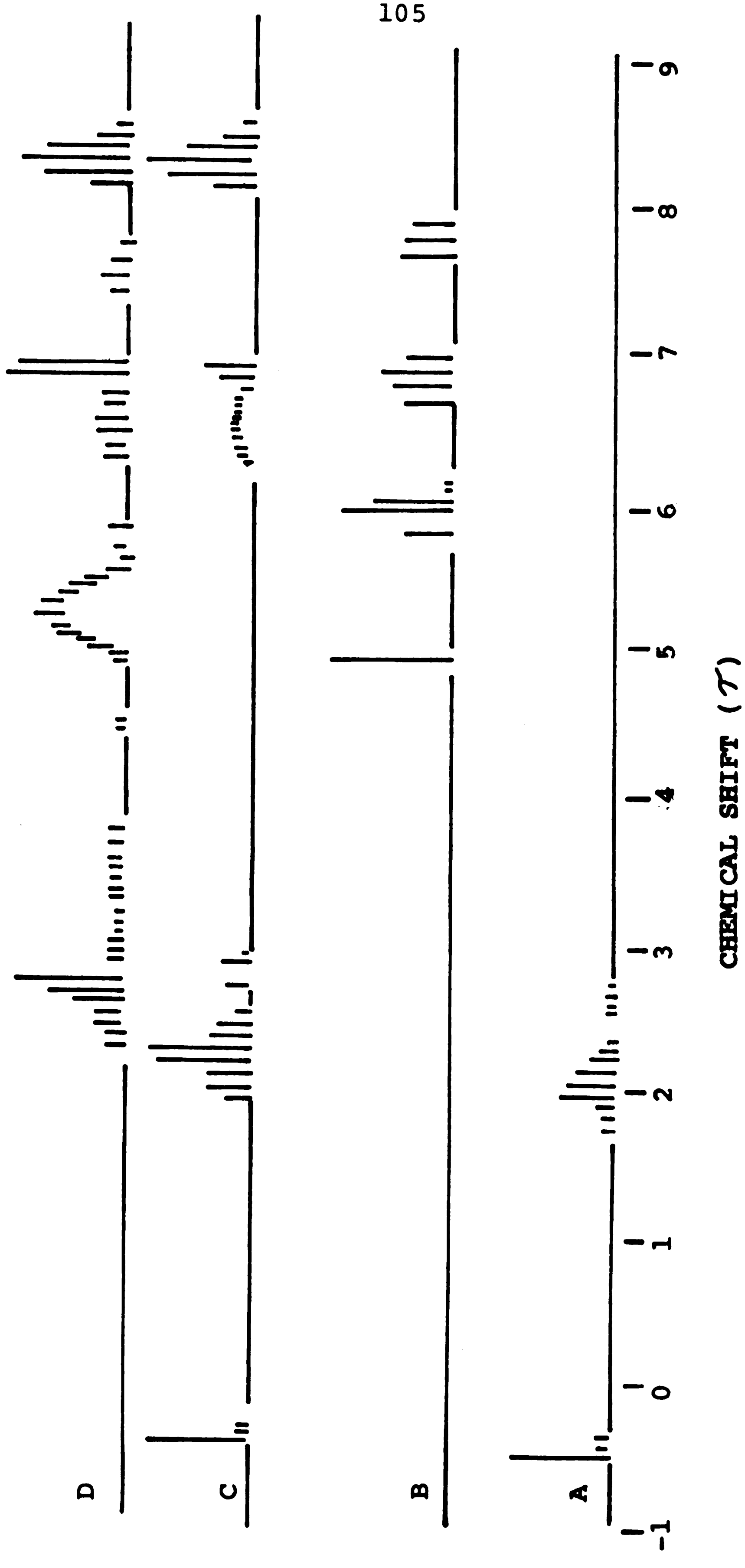

0.6
0.4
0.2
ABSORBANCE
220
260
300
340
WAVELENGTH (nm)

Figure 21.--Diagramatic nuclear magnetic resonance spectra of (A) o-phthalaldehyde, (B) β-mercaptoethanol, (C) o-phthalaldehyde + glycine, (D) o-phthalaldehyde + β-mercaptoethanol + glycine.


D
C
B
A
-1
0
1
2
3
4
5
6
7
8
9
CHEMICAL SHIFT (τ)

one aldehyde proton and the four phenylic protons were seen at t less than 6. The broad band at t = 7 was characteristic of the N-H bond. The doublet which overlayed this broad signal may be interpreted as belonging to the proton on the alpha carbon of glycine. The integrated area attributed to the phenylic protons was set at four, and the rest of the areas evaluated relative to the phenylic region. The number of protons indicated that one molecule of OPT was bound to one molecule of glycine; further, one molecule of βME was bound to this adduct when βME was a co-reagent. The OPT-βME, glycine adduct showed seven proton environments, and loss of the signal due to the aldehyde moiety. The broad band at t = 5 was due to two alcohol groups.

4. Effect of Carboxyl Group Proximity on Fluorescence.

Table VII shows fluorescence observed under a variety of conditions. Thus it may be seen that the carboxyl group has little effect on the fluorescence of the product, as the number of methylene groups which separated it from the aromatic ring were increased. In addition, glycine ethyl ester fluoresced intensely.

5. Influence of Oxygen, Reducing Agents and Alkylating Agents on Fluorescence.

Since βME can reduce dissolved oxygen in solution, the effects of dissolved oxygen on fluorescence were tested. As can be observed from Table VII, the presence or absence of dissolved oxygen had no effect on

Table VII.--Equimolar Fluorescence Intensities of Products Derived From Selected Amino Compounds After Reaction With OPT, βME, or Analogs[a]

Reactants[b]	Fluorescence[c]
Glycine + OPT + βME	100
Ethyl Glycinate + OPT + βME	100
Glycine + OPT + βME + PCMB	87
Glycine + OPT + DTT	50
Glycine + OPT + NaBH$_4$	35
Glycine + OPT + NaHSO$_3$	6
Glycine + OPT + βME + O$_2$	100
Glycine + OPT + N$_2$	6
Glycine + OPT	6
OPT + βME (blank)	2
Asparagine + HMB + βME	0.3
Asparagine + HMB + NaBH$_4$	0.2
Asparagine + HMB	0
HMB + βME (blank)	0
Asparagine + DMB + βME	0
Asparagine + DMB	0
DMB + βME (blank)	0
β-Alanine + OPT + βME	98
Phenylalanine + OPT + βME	150
(Phe)$_4$ + OPT + βME	14
Gly-Gly + OPT + βME	60
Hipp-Gly-Gly + OPT + βME	3
Phe-Gly-Gly + OPT + βME	18
Cysteine + OPT	15
Homocysteine + OPT	25
GSH + OPT	1,180
AMP + OPT + βME	3
BSA + OPT	810
BSA + OPT + βME	10,700

[a] Excitation wavelength 340 nm; fluorescence at 455 nm.

[b] Concentrations of reactants: amino acids, peptides, nucleotide or proteins were 20 μM; OPT, HMB, or DMB were 0.187 mM; and βME, NaHSO$_3$, NaHBH$_4$, DTT or PCMB were 1.45 mM in 0.05 *M* borate buffer of pH 9.5.

[c] Fluorescence spectrophotometer readings were relative to the glycine fluorophor 100 fluorescent units.

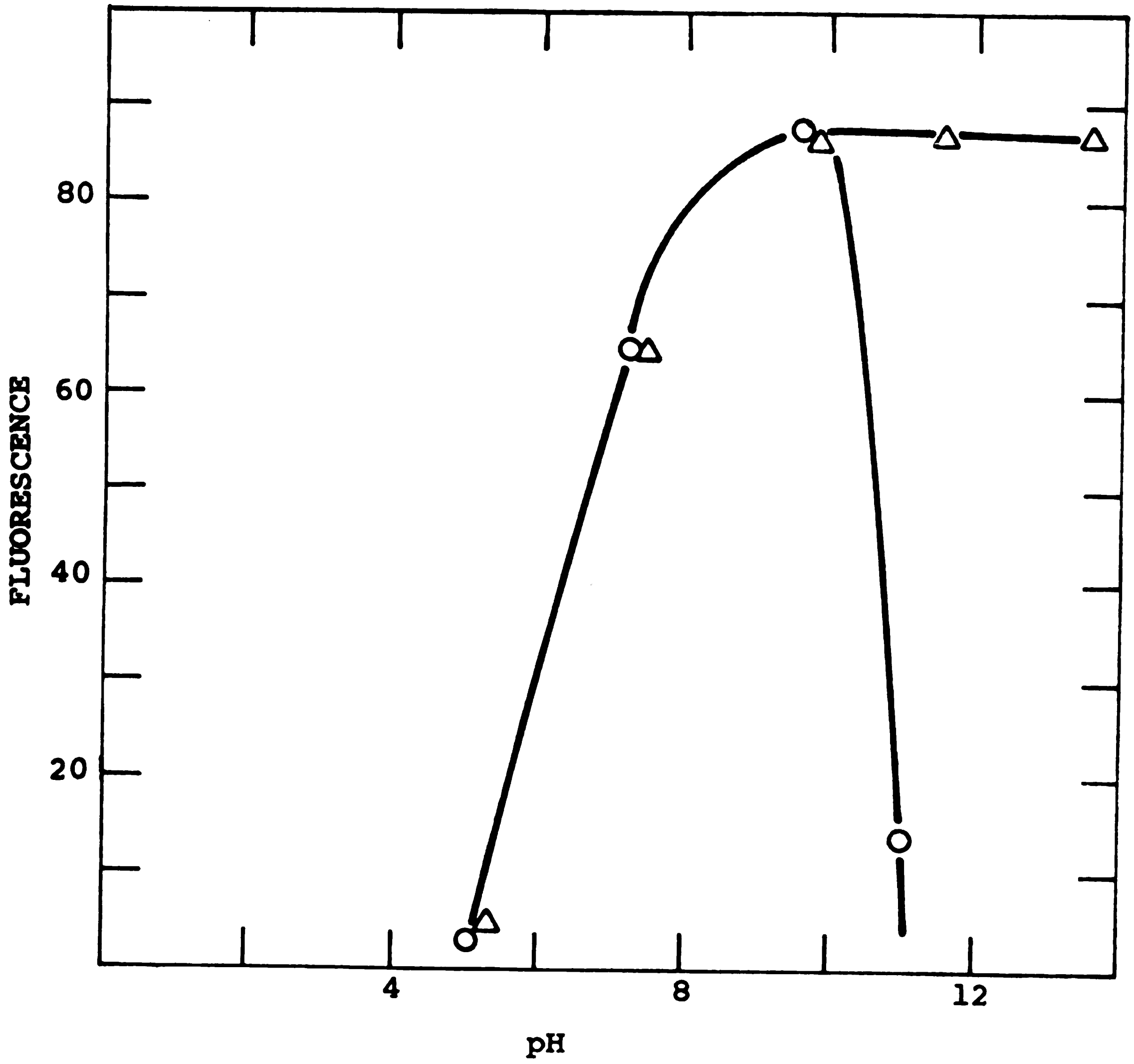
fluorescence. Sodium borohydride substituted effectively for βME, while sodium bisulfite was ineffective in producing fluorescence. p-Mercuribenzoate did not inhibit production of fluorescence until after at least two moles of p-mercuribenzoate were added per mole of βME.

6. Postulated Intermediates. A postulated intermediate, o-hydroxymethylbenzaldehyde, gave very poor fluorescence when substituted for OPT and reacted with glycine. No fluorescence occurred at all with HMB when βME was deleted. Dihydroxymethylbenzene produced no fluorescence with glycine when reacted with or without reducing agents.

7. Fluorescence of Peptides. Di-, tri-, and tetra peptides indicated in Table VII reacted with buffered OPT-βME reagent to give fluorescent products. The relative fluorescence of a few peptides are compared in Table VII and shows that the fluorescence decreased with an increase of peptide bonds.

8. Fluorescence of Proteins. The binding of OPT and βME to proteins was accompanied by a bright blue flourescence which was quite sensitive to pH changes. The pH profile of the bovine serum albumin fluorophore is plotted in Figure 22 and indicates a maximum fluorescence at pH greater than 9.0. This is similar to the relationship observed with amino acids (77). Highly alkaline pH did not favor the formation of the fluorescent adduct involving serum albumin.

Figure 22.--pH profile of fluorescence reaction. Triangles--Fluorescence of bovine serum albumin as a function of pH after the fluorophore had already been formed at pH = 9.5. Circles--fluorescence of bovine serum albumin as a function of the pH at which the reactants were mixed.


FLUORESCENCE
80
60
40
20
4
8
12
pH

The excitation and emission spectra for the reaction of buffered OPT-βME with bovine serum albumin and glycine may be seen in Figures 23 and 24. Note (Figure 25) that the partial quantum efficiency of buffered OPT-albumin adduct was negligible when compared to the OPT-βME-albumin adduct in the 280 nm region.

A sensitive fluorometric method which required only a single reagent, namely buffered OPT-βME, was developed for the quantitative determination of proteins. The fluorescence was directly proportional to the protein concentration for several proteins as shown in Figure 26.

A very interesting parameter of protein fluorescence was the proportionality of the fluorescence to the molecular weight. Shown in Figure 27 is the fluorescence of aldolase (Mol. wt. 158,000), bovine serum albumin (67,000), ovalbumin (45,000), and chymotrypsinogen (25,000) as a function of molecular weight.

The fluorescence of the reaction was proportional to the concentration of bound ligand in a system in which the concentration of ligand was not in excess, thus providing a simple and direct method for the examination of the binding process. When bovine serum was titrated with OPT or βME, and the fluorescence plotted as a function of the OPT or βME concentration, rectangular hyperbolic curves were obtained (Figure 28). Scatchard plots (Figure 29) revealed that 55 molecules of OPT bind per molecule of albumin, and 14

Figure 23.--Corrected fluorescence excitation and emission spectra of (A) bovine serum albumin, λ_{fl} = 335 nm, ,λ_{ex} = 277 nm; (B) o-phthalaldehyde + bovine serum albumin, λ_{fl} = 410 nm, λ_{ex} = 340 nm; (C) o-phthalaldehyde + β-mercaptoethanol + bovine serum albumin, λ_{fl} = 435 nm, λ_{ex} = 340 nm (asterisk refers to emission spectra). The concentrations and scaling factors employed for the various scans are different.

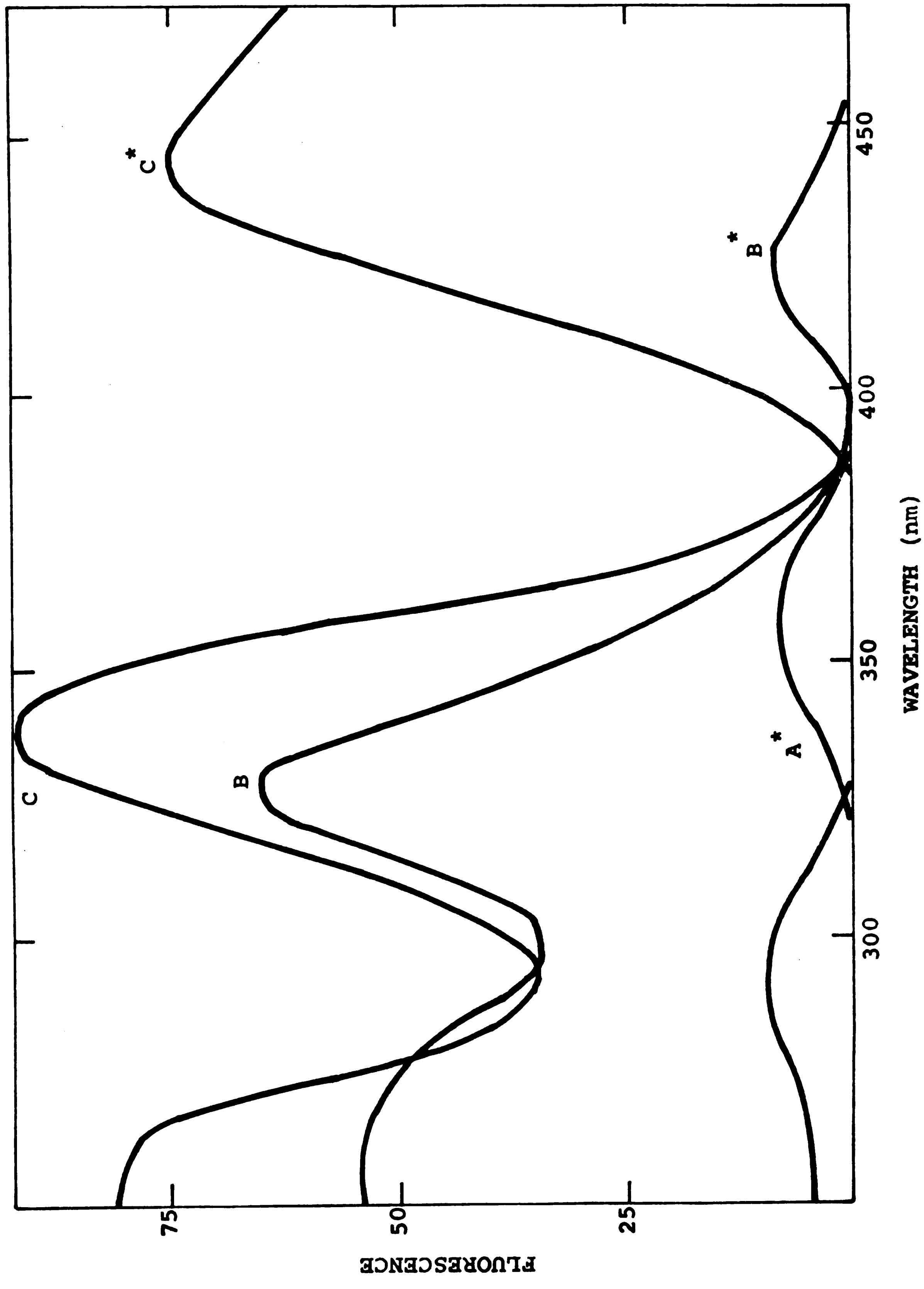

FLUORESCENCE
75
50
25
WAVELENGTH (nm)
300
350
400
450
C
B
A*
B*
C*

Figure 24.--Fluorescence excitation and emission spectra for (A) o-phthalaldehyde + β-mercaptoethanol, λ_{fl} = 450 nm, λ_{ex} = 340 nm; (B) o-phthalaldehyde + β-mercaptoethanol + glycine, λ_{fl} = 450 nm, λ_{ex} = 340 nm (asterisk refers to emission spectra). The concentrations and scaling factors employed for the various scans are different.

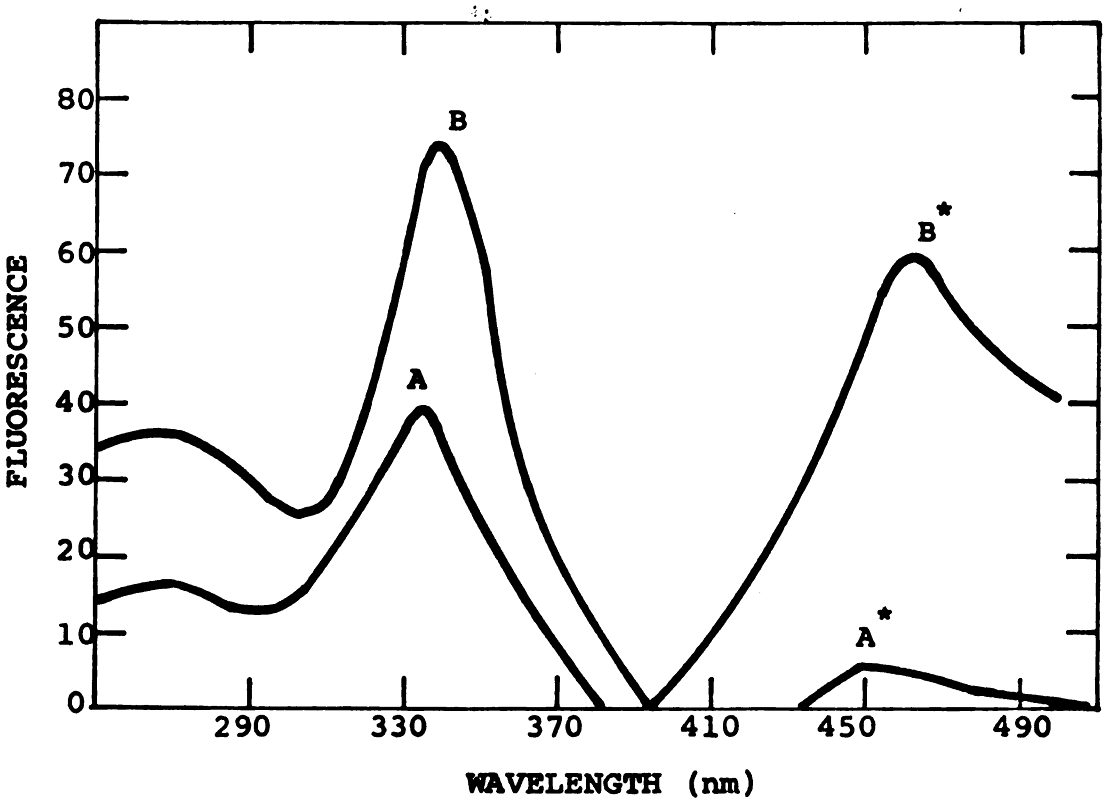

FLUORESCENCE
0
10
20
30
40
50
60
70
80
290
330
370
410
450
490
WAVELENGTH (nm)
B
A
B*
A*

Figure 25.--Partial quantum efficiency of fluorescence as a function of wavelength for (A) o-phthalaldehyde + β-mercaptoethanol + glycine, (B) o-phthalaldehyde + bovine serum albumin, (C) o-phthalaldehyde + β-mercaptoethanol + bovine serum albumin.

ency
n for
gly-
albumin,
bovine

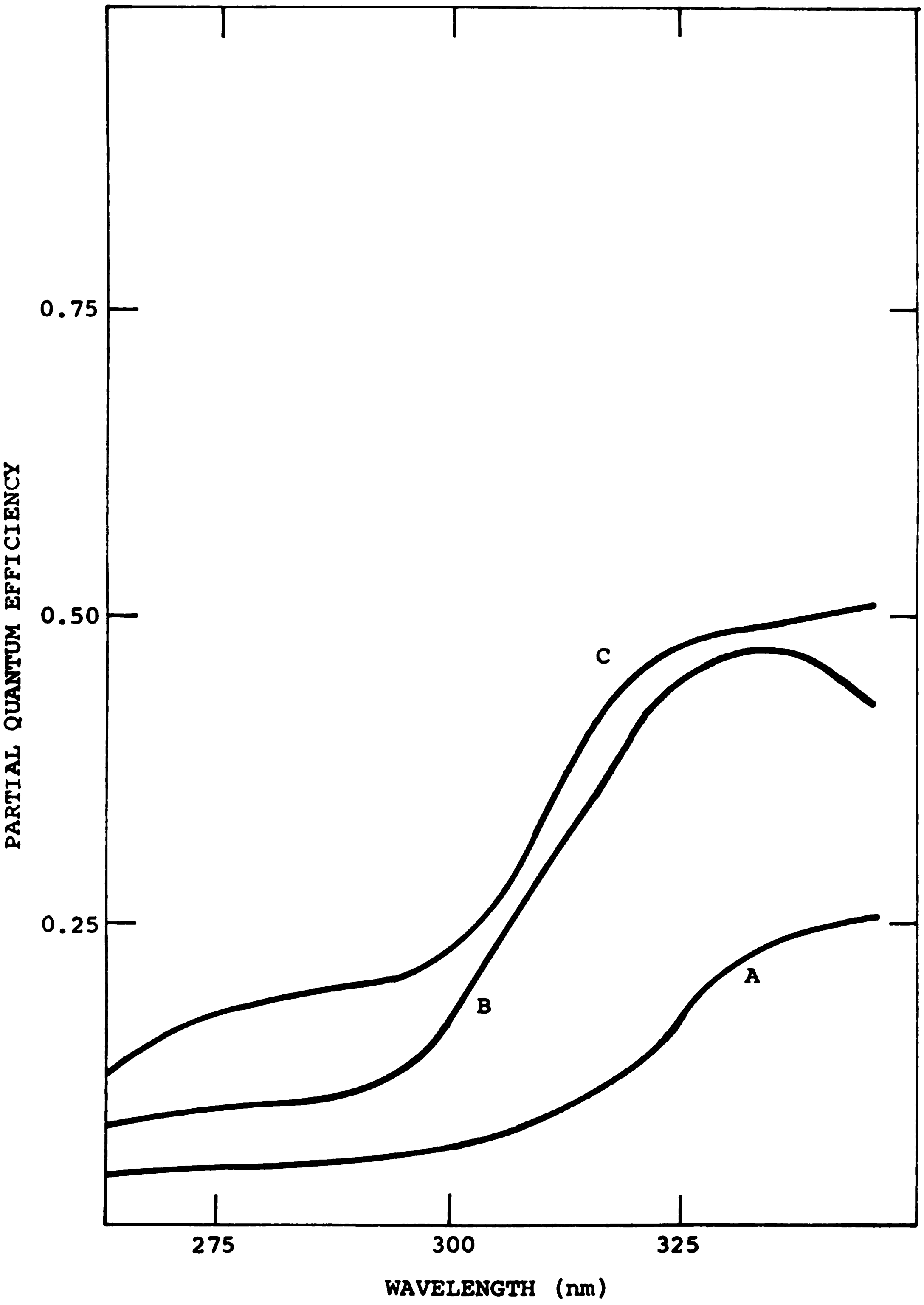


Figure 26.--Fluorescence intensity as a function of concentration for several proteins following reaction with o-phthalaldehyde and β-mercaptoethanol. (A) aldolase, (B) BSA, (C) chymotrypsinogen A, (D) ovalbumin.

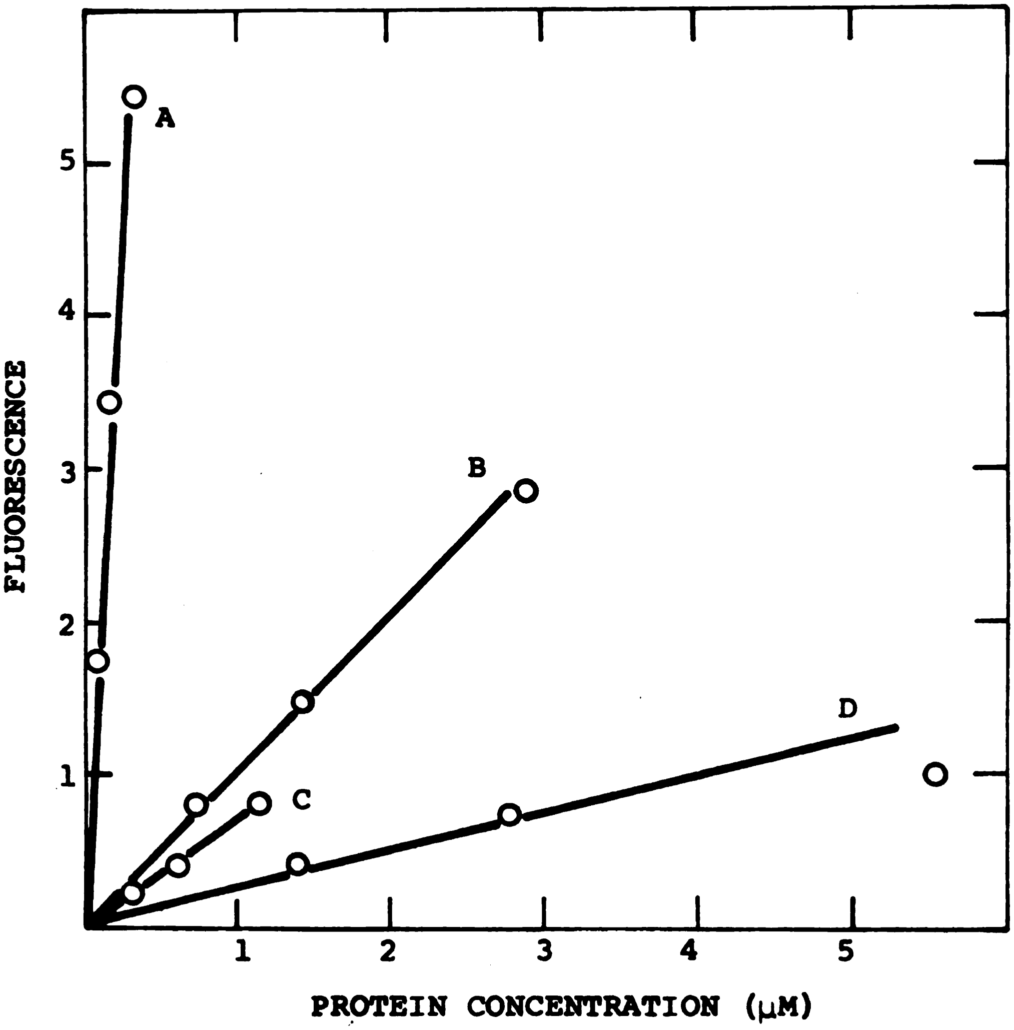

FLUORESCENCE
PROTEIN CONCENTRATION (μM)
1
2
3
4
5
A
B
C
D

Figure 27.--Relative fluorescence intensity of equimolar solutions of several proteins following reaction with o-phthalaldehyde and β-mercaptoethanol. The upper curve contained twice the amount of protein.

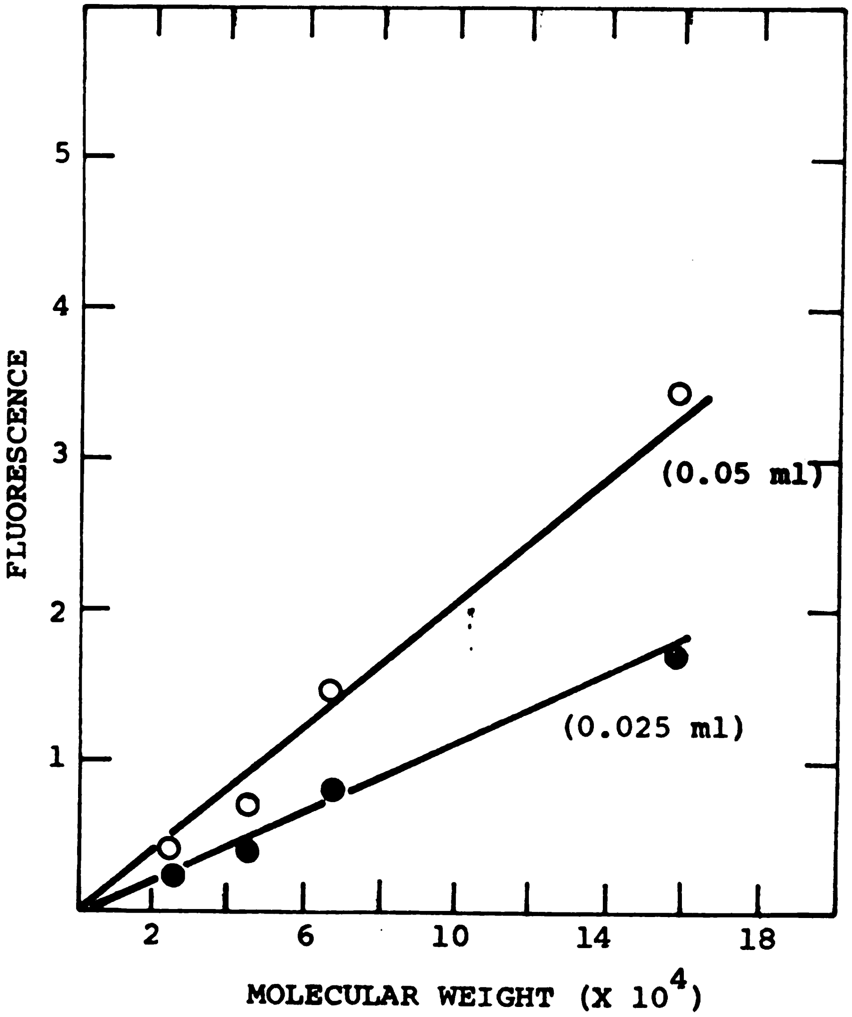

FLUORESCENCE
5
4
3
2
1
2
6
10
14
18
MOLECULAR WEIGHT (X 10^4)
(0.05 ml)
(0.025 ml)

Figure 28.--Binding of o-phthalaldehyde to bovine serum albumin (1.5 nmoles) in the presence of excess β-mercaptoethanol, and the binding of β-mercaptoethanol to o-phthalaldehyde and bovine serum albumin (1.5 nmoles) (OPT concentration/BSA concentration = 55). Upper abscissa refers to triangles or β-mercaptoethanol binding. Lower abscissa refers to circles or o-phthalaldehyde binding.

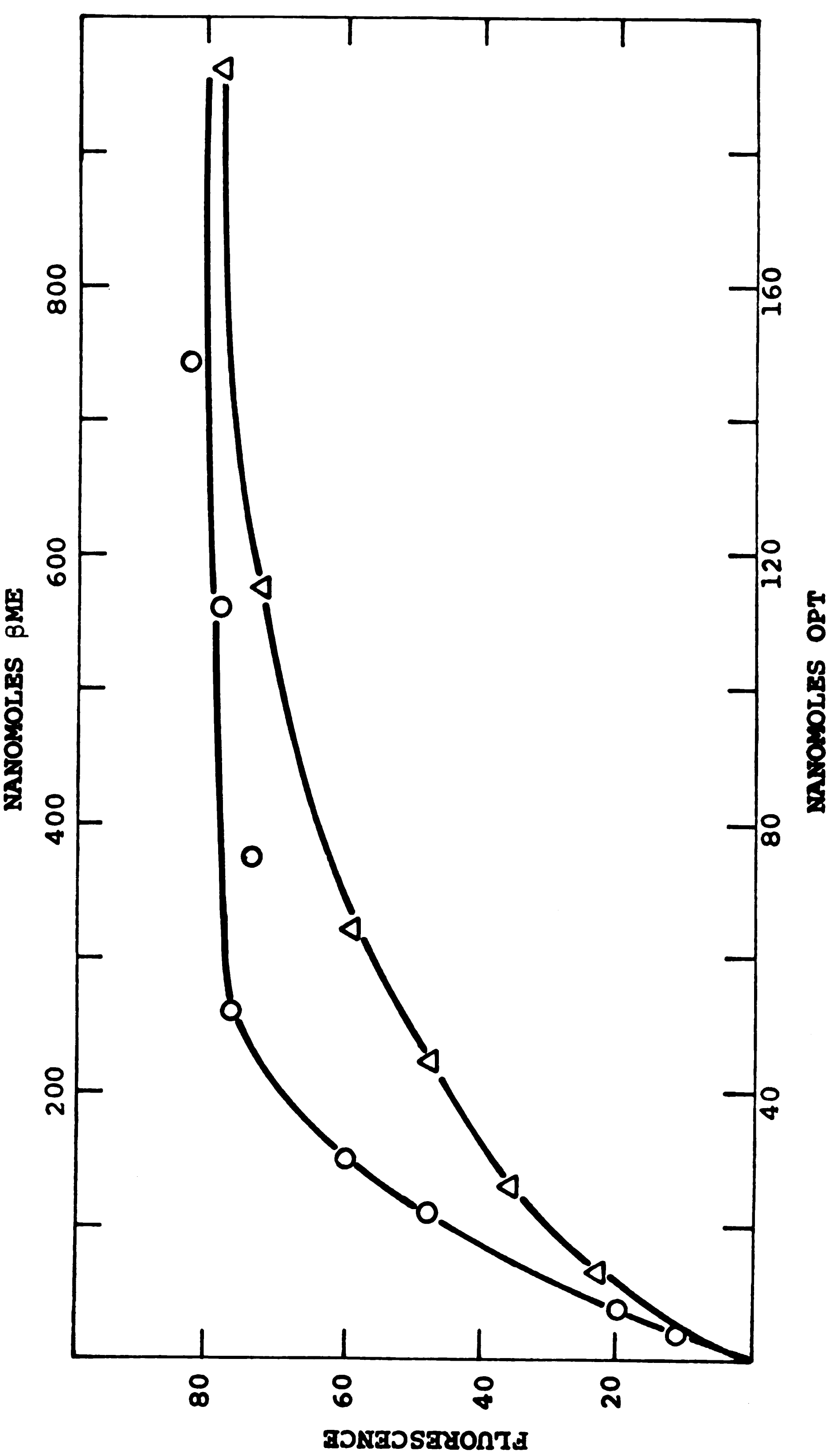
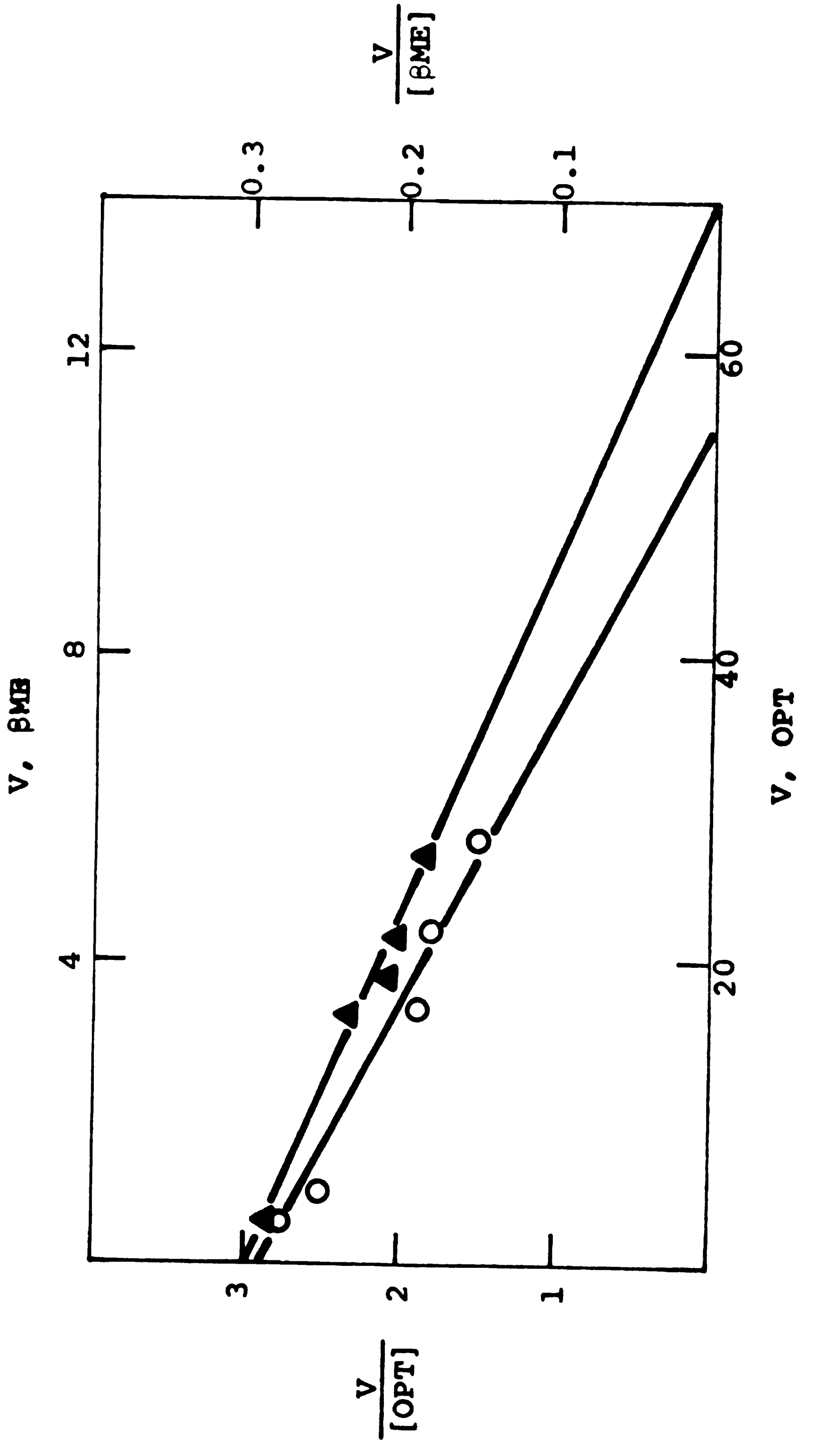

NANOMOLES βME
200
400
600
800
NANOMOLES OPT
40
80
120
160
FLUORESCENCE
20
40
60
80

Figure 29.--Scatchard plot of the binding of o-phthalaldehyde to bovine serum albumin and of β-mercaptoethanol to o-phthalaldehyde. Upper abscissa and left ordinate refer to triangle or β-mercaptoethanol binding to o-phthalaldehyde. Lower abscissa and right ordinate refer to circles or the binding of o-phthalaldehyde to bovine serum albumin. V is the ratio of binding ligand to the complex at the saturation point. V (OPT) = OPT conc./BSA conc., V (βME) = conc./OPT conc.


V, βME
4
8
12
V/[βME]
0.3
0.2
0.1
V/[OPT]
3
2
1
V, OPT
20
40
60

molecules of βME were bound per molecule of OPT. Since the plots were linear, the sites were non-interacting (78). A direct plot of V versus concentration of OPT (not shown) was non-sigmoidal, also indicating non-interacting sites.

V. DISCUSSION

A. ACTION OF ANGIOTENSIN CONVERTING ENZYME ON SUBSTRATE ANALOGS

The first synthetic substrates for converting enzyme, tripeptides benzoyl-glycyl-glycyl-glycine (79) and benzoyl-glycyl-histidyl-leucine (55), were cleaved rather slowly compared to angiotensin I. The site of enzymic action was the peptide bond to which glycine or histidine contributed the NH group. A decisive advance was made by Piquilloud *et al.* (52) who showed that shortening the peptide chain of angiotensin I from the amino end to form Z-phenylalanyl-histidyl-leucine does not abolish its susceptibility to hydrolytic cleavage by converting enzyme. Piquilloud's work was the first indication that the action of converting enzyme on small substrate analogs is favored by the presence of an aromatic side chain on the carbonyl side of the sensitive peptide bond.

Since 1969 studies have shown that the action of angiotensin converting enzyme on small peptides could involve the cleavage of many kinds of peptide bonds and, with our present work, converting enzyme can be considered an enzyme of rather broad side chain specificity (52,55,75,79,106,107).

The lack of inexpensive and readily obtainable substrate analogs, as well as the uncertain state of the problem of the enzymic action of converting enzyme, prompted us to study the hydrolytic action of this enzyme of synthetic substrates. Our first objective was to secure peptides whose structure could be compared to those known to be hydrolyzed by converting enzyme. Table II A incorporates the new substrate analogs and shows the relative rate of hydrolysis of various peptides, using the natural substrate, angiotensin I, as one hundred.

Although the converting enzyme preparations used in our substrate analog studies were not homogenous as demonstrated by polyacrylamide gel electrophoresis, the following evidence strongly supports the conclusion that the conversion of angiotensin I to angiotensin II and the hydrolysis of Phe-Gly-Gly, Phe-Gly-Gly-Phe, Phe-Gly-Phe-Gly, hippuryl-Gly-Gly, and Gly-His-Gly are catalyzed by the same enzyme. First, the enzyme we have purified on its ability to cleave substrate analogs is also found to have increased specific activity with angiotensin I as substrate. Certainly Table II A demonstrates that angiotensin converting enzyme cleaves a variety of peptides. Secondly, in agreement with Yang (107), converting enzyme liberates glycyl-glycine from hippuryl-Gly-Gly. The dipeptides liberated from the substrate analogs were identified by thin-layer chromatography and quantitatively determined by the ninhydrin reaction, except for histidyl dipeptides liberated from angiotensin I and Gly-His-Gly. These dipeptides were reacted with o-phthalaldehyde in alkaline solution and measured spectrophotometrically or,

upon acidification, the fluorescence was measured in a spectrofluorometer. Thirdly, since converting enzyme requires a divalent metal ion for activity, perhaps calcium, incubation of the new substrate analogs with EDTA and 8-hydroxyquinoline produced inhibition. Thus Figures 6 and 7 graphically depict that the activity is proportional to the quantity of enzyme, and the hydrolysis of four different substrates is inhibited by EDTA. In addition, all other studies have shown that Gly-His-Gly hydrolysis is inhibited by the same agents which affect the hydrolysis of angiotensin I, especially the ethanoic extract of *Bothrops Jararaca* venom (Tables IV and VI), which is a specific inhibitor of converting enzyme (108-111). The potent inhibition by p-chloromercuribenzoate of angiotensin I and Gly-His-Gly hydrolysis indicates the presence of a sulfhydryl group at the active site. Studies have shown that chloride ions are necessary for maximum activity, and assays performed in chloride-free medium diminished converting enzyme activity; that is, chloride ion stimulated the hydrolysis of Phe-Gly-Gly-Phe, Phe-Gly-Phe-Gly and hippuryl-Gly-Gly, while Gly-His-Gly and Phe-Gly-Gly hydrolysis was independent of the chloride ion concentration. Finally, hydrolysis of angiotensin I and Gly-His-Gly by the same polyacrylamide gel slices after electrophoretic separation and the absence of peptidases (104) in the DEAE-Sephadex preparation leads to the conclusion that Gly-His-Gly and the other substrate analogs presented here are hydrolyzed by the same enzyme which catalyzes the cleavage of angiotensin I.

The plasma and urine inhibiting factors exhibited similar properties. The cationic and anionic nature of this substance as evidenced by binding to Dowex 50 and Dowex 1, and the lack of acid hydrolysis under conditions which would destroy any peptide, implies that the inhibitor may be an amino acid. Lipid soluble components were eliminated since the inhibitor is not chloroform or ether extractable. Histidine, a major amino acid component of blood and urine, was suspected; however, inhibition of converting enzyme by 2.5 mM histidine could not be demonstrated.

The inhibition of converting enzyme by very low concentrations of aldosterone (Table IV) leads one to postulate that a feed-back control mechanism is operative between the mineralcorticoid/renin angiotensin system. Since angiotensin II directly stimulated the production of aldosterone by the adrenal cortex (112,113) the subsequent increased levels of aldosterone could inhibit converting enzyme and thus lower angiotensin II levels. Inhibition of converting enzyme by the acetone extract of adrenal glands lends additional support to this observation.

The micro-colorimetric assay using Gly-His-Gly is the best method employed thus far in this laboratory. Quantitation of 20 to 100 nanomoles of histidyl-glycine liberated in 90 minutes by a crude preparation of plasma converting enzyme is easily accomplished. The plasma assay for angiotensin converting enzyme is just as sensitive and more rapid. The fluorometric analysis is preferable to the spectrophotometric method when great sensitivity is desired, since 100 to 1000 picomoles of histidyl-glycine per ml can be detected. A disadvantage is the turbidity produced upon acidification, thus requiring centrifugation prior to analysis.

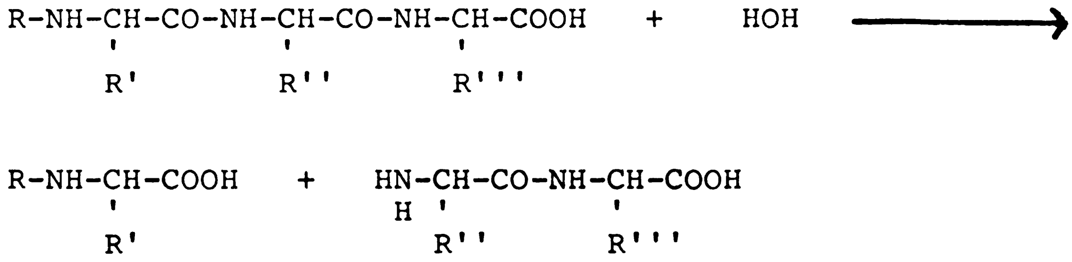
Care must be exercised in the selection of compounds to be incorporated into the assay solution since they may absorb the excitation beam and diminish fluorescence. Appropriate reagent blanks are necessary to correct fluorescence values should this occur.

Chromophore formation often accompanies acidification of the assay mixture, with the concurrent absorption of fluorescence. This problem, as well as the one described above, can be partially avoided by bringing the concentration of all components (enzyme and effectors) to the same level following the termination of the enzyme reaction with sodium hydroxide. The concentration of the cleavage products, however, cannot be made equimolar. Since these components can form chromophores, some quenching will occur which can be diminished by using low substrate concentrations.

B. SIDE CHAIN SPECIFICITY OF ANGIOTENSIN CONVERTING ENZYME

Converting enzyme catalyzes the hydrolysis of peptides at the second peptide bond from the carboxyl end (i.e., a dipeptidyl carboxypeptidase), although the ionized carboxyl group is not required for the hydrolysis of angiotensin I or

Gly-His-Gly. Thus the ethyl ester of angiotensin I and Gly-His-Gly are cleaved at the same rate as the unesterified substrate. (Table V) Substrates are most efficiently hydrolyzed if R' is aromatic as in the amino acid phenylalanine, and R'' is an imidazole moiety of histidine:



$$\text{R-NH-}\underset{\text{R}'}{\text{CH}}\text{-CO-NH-}\underset{\text{R}''}{\text{CH}}\text{-CO-NH-}\underset{\text{R}'''}{\text{CH}}\text{-COOH} + \text{HOH} \longrightarrow$$

$$\text{R-NH-}\underset{\text{R}'}{\text{CH}}\text{-COOH} + \underset{\text{H}}{\text{HN}}\text{-}\underset{\text{R}''}{\text{CH}}\text{-CO-NH-}\underset{\text{R}'''}{\text{CH}}\text{-COOH}$$

The systematic modification of the nature of the N-terminal amino acid residues of the type X-glycylglycine are shown in Table VIII. In all cases where cleavage was found, the site of enzymatic action was restricted to the X-glycyl peptide bond. It will be noted that the first two dipeptides are not cleaved while the latter three tripeptides are, indicating that a minimal chain length equivalent to a tripeptide is required for hydrolysis. A tripeptide substrate containing only aliphatic amino acids, alanyl-glycyl-glycine, is not hydrolyzed, but upon the introduction of an aromatic amino acid, as in phenylalanyl-glycyl-glycine, peptide bond cleavage occurs. Note also that the first eight peptides unhydrolyzed by converting enzyme (Table II B) are dipeptides and, as indicated above, Ala-Gly-Gly is completely aliphatic. The completely aromatic peptide, tetra-L-phenylalanine, is not hydrolyzed, indicating that the enzymic site

TABLE VIII.--N-terminal Modified Substrate Analogs Tested for Converting Enzyme Activity

Substrate Analog X-Gly-Gly	Estimated* Relative Rate	Investigators
Benzoyl-Gly-Gly	0	Yang *et al.*
Benzyloxycarbonyl-Gly-Gly	0	Yang *et al.*
Alanyl-Gly-Gly	0	Summary *et al.*
Phenylalanyl-Gly-Gly	20	Summary *et al.*
Benzoylglycyl-Gly-Gly	20	Yang *et al.*
Benzyloxycarbonylglycyl-Gly-Gly	5	Summary *et al.*

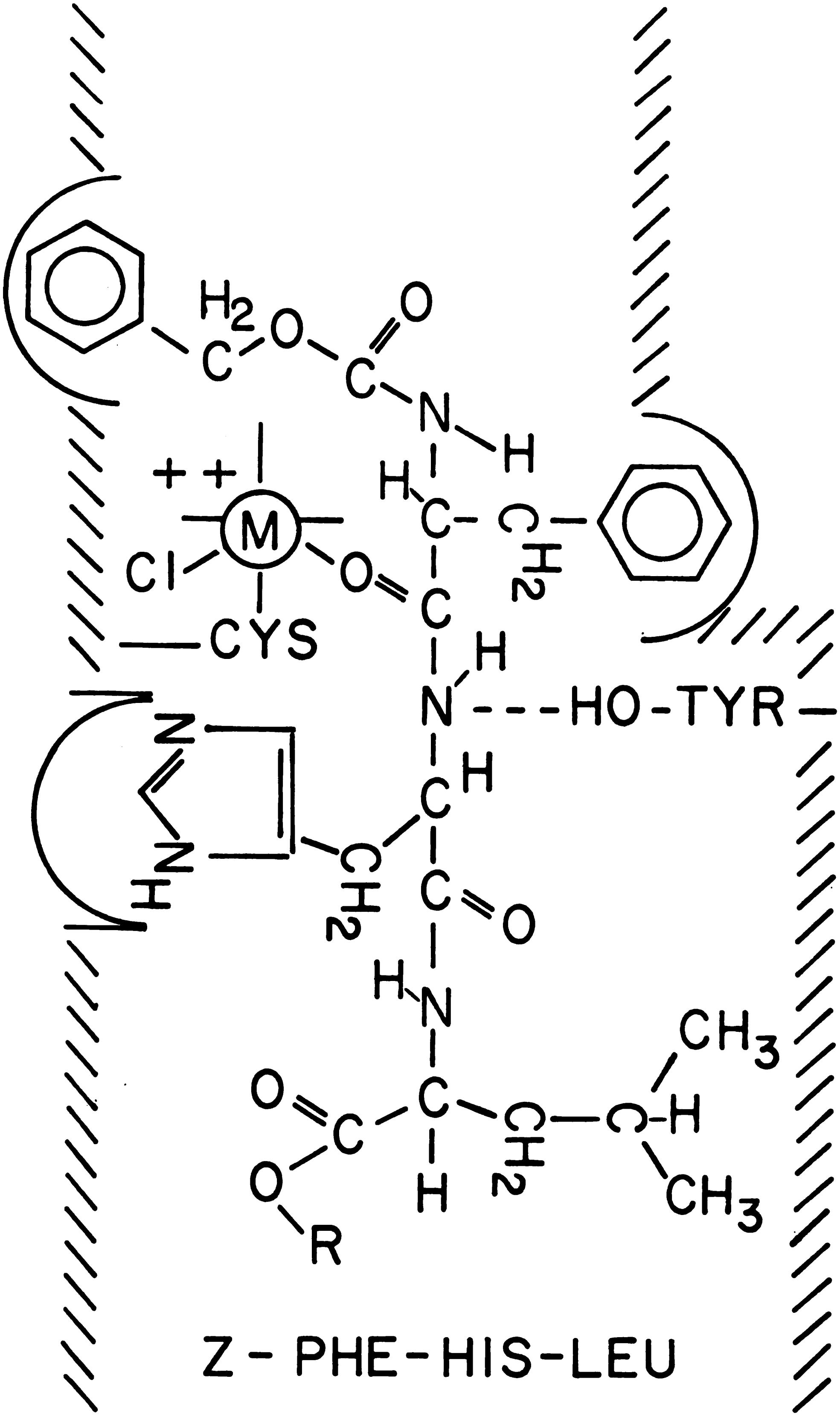
* Angiotensin I as 100

cannot bind four planar aromatic groups, perhaps due to steric hindrance. In the substrates tested for activity with converting enzyme (Table II A), the four that are cleaved most rapidly, except for Gly-His-Gly, have an aromatic residue at the R' position and an imidazole moiety at the R'' position. The favorable effect of the aromatic and planar substitutent at the beta carbon of the third amino acid from the carboxyl end, is emphasized by the fact that when R' is a hydrogen the relative rate of hydrolysis is one fifth that for the phenylalanyl residue. The data in Table II A also indicate that the prolyl residue in Z-Pro-Phe-His-Leu and angiotensin I disrupts the interaction of that portion of the enzymic region that binds planar aromatic groups. Deletion of proline from Z-Pro-Phe-His-Leu increases the rate of hydrolysis by a factor of ten.

In comparing Z-Phe-His-Leu and Phe-Gly-Gly, observe that the introduction of the benzyloxycarbonyl("Z") group causes a large (50-fold) increase in the rate of cleavage of the substrate, assuming, of course, the effect of the terminal amino acids (histidyl-leucine vs. glycyl-glycine) to be minimal. (Note only the slight two-fold increase in hydrolysis of hippuryl-Gly-Gly vs. hippuryl-His-Leu.) These results indicate that the introduction of the hydrophobic group may cause significant increases in the susceptibility of the phenylalanyl-histidyl bond to converting enzyme action and suggests that the "Z" unit may play a special role in the catalytic process. If one speculates that the primary binding energy comes from the interaction of the phenylalanyl-histidyl unit with the enzyme, similar to the phenylalanyl-phenylalanyl unit with pepsin (80), then the hydrophobic group may also interact with a locus on the enzyme and increase catalytic efficiency, perhaps by exerting conformational changes at the active site. These presumed secondary hydrophobic interactions are not necessarily reflected in the binding energy for the interaction of the entire substrate molecule with the enzyme, nor should it be construed that hydrophobic groups are necessary for increased catalytic efficiency. For if one compares the relative rate of hydrolysis of Gly-His-Gly with hippuryl-His-Leu, one may conclude that the apparently weaker binding of Gly-His-Gly enhanced the catalytic efficiency.

Thus it may be concluded for the synthetic substrates studies so far, that enzymic cleavage by converting enzyme should not only be viewed in terms of the amino acid residues that flank the sensitive peptide bond, but also the secondary hydrophobic interactions to aid in binding, as well as the disruptive influence of the prolyl residue, that are removed from the sensitive peptide bond. Figure 30 depicts the active site of angiotensin converting enzyme and illustrates the binding of Z-Phe-His-Leu prior to hydrolytic cleavage.

Figure 30.--Proposed active site of angiotensin converting enzyme based upon substrate analog and inhibitor experiments.


H2
O
C
O
C
N
H
++
M
Cl
CYS
H
C
CH2
O
C
H
N
HO-TYR
N
N
H
CH2
C
H
C
O
H
N
CH3
O
C
C
CH2
C
H
O
H
CH3
R

Z - PHE - HIS - LEU

C. THE FLUORESCENCE REACTION OF AMINO ACIDS, PEPTIDES, AND PROTEINS WITH O-PHTHALADEHYDE

IR, NMR, and UV data indicate the formation of a Schiff base between OPT and glycine. To explain the disappearance of an intense glycine proton resonance peak at tau = 4.4 and the appearance of an intense peak at tau = 2.0, it was necessary to assume that the double bond shifted to the alpha-carbon side of the amino acid nitrogen atom. This rearrangement occurs readily in alkaline solution (100).

The structural requirements for the formation of a fluorescent complex between OPT in the absence of βME and indoles, imidazoles, polyamines, guanidino amines, glutathione, and amino acids are contingent upon the temperature and pH of the fluorogenic reaction and the pH during fluorescence measurement. Thus the fluorogenic reaction of 5-hydroxytryptamine requires 60 minute boiling period at pH less than 1, while histidine, histamine, agmatine, spermidine, and α, ε-diaminopimelic acid are dependent on strongly alkaline conditions. In the alkaline group (Table IX), only agmatine requires heat (88°) for the fluorogenic reaction to occur. The fluorophore is stable in acid solution for OPT derivatives of indolakylamines, imidazoles, and polyamines, but guanidinoamines and glutathione require an alkaline pH for fluorescence. The fluorescent product from OPT with βME and amino acids is labile under acidic conditions and shows a pH dependence similar to the guanidinoamines and glutathione.

Table IX.--Amino Compounds and Conditions Necessary for Their Fluorophore Formation and Fluorescence Upon Reaction with O-Phthalaldehyde[a]

A. Fluorophore Formation

Heat and Acid pH	Neutral pH	Alkaline pH
Indolalkylamines:	Proteins[b]	Imidazoles:
Serotonin		Histidine
5-Methoxytryptamine		Histamine
5-Hydroxytryptamine		Guanidinoamines:
		Arginine
		Agmatine
		Citrulline
		Polyamines:
		Spermidine
		Iminobispropylamine
		Diethylenetriamine
		Amino Acids[b]
		Glutathione

B. Fluorescence of Fluorophore Produced

Acid pH	Alkaline pH
Indolalkyamines	Guanidino Amines
Imidazoles	Amino Acids
Polyamines	Glutathione
	Proteins

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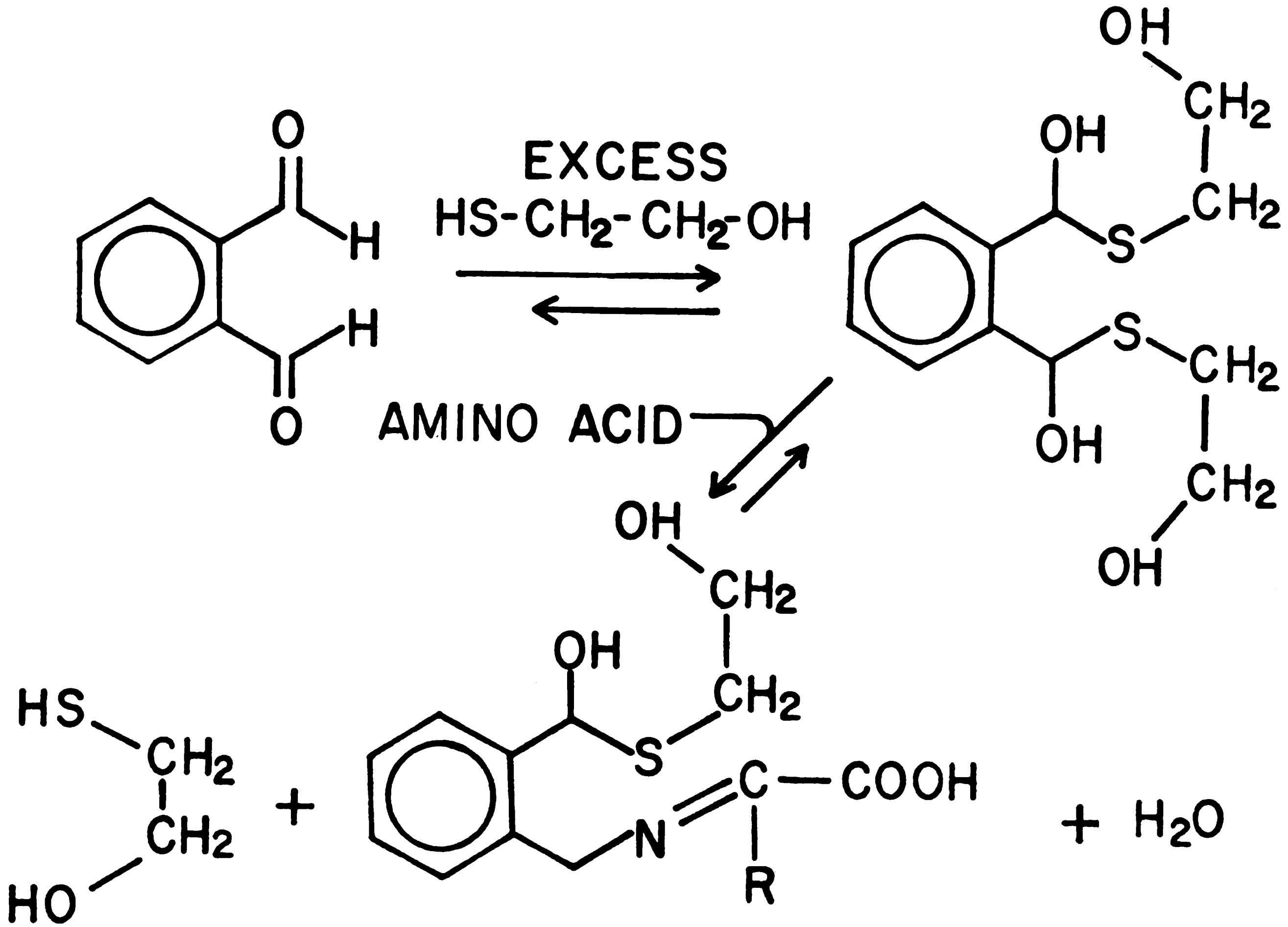
[a] Literature references to all of the compounds listed here are given in the introductory part of this paper.

[b] Require the presence of excess βME.

Shore et al. (90) postulated that the condensation reaction of histamine and OPT formed a three-membered ring system, because alkylation of the amino group prevented fluorophore formation, and m-phthalaldehyde could not replace OPT. Cohn and Shore (91) assumed that the reaction of OPT with the guanidinoamine, agmatine, terminated with the Schiff base, since acid was not necessary for fluorophore formation. Cohn and Lyle (95) suggested that the hemimercaptal was formed with glutathione since the pK of the sulfhydryl group is at the point of optimal fluorescence.

Similarly, the addition of βME to OPT allows the base catalyzed formation of the hemimercaptal, and a Schiff base is formed upon the reaction of the remaining aldehyde group with the amino acid. The proposed reaction is shown in Figure 31. Hemimercaptal formation is evidenced from spectra by the disappearance of the carbonyl and sulfhydryl groups and the appearance of one or more alcohol groups. The nucleophilic addition of βME to the imine bond was not indicated as evidenced by the appearance of the new strong transition in the UV spectra. If addition to the imine bond occurred, then the appearance of a new chromophore would not be seen. The lack of change in the tau = 8.0 and 6.6 signals upon the addition of βME is indicative that no further change occurs in the imine bond region. The proximity of the sulfur and nitrogen atoms suggests an interaction which may lead to an enhancement of the native fluorescence of the imine bond.

Figure 31.--Proposed reaction of o-phthalaldehyde with β-mercaptoethanol and amino acids.


EXCESS
HS-CH2-CH2-OH
AMINO ACID
O
H
OH
CH2
S
R
C
COOH
N
HS
HO
+
+ H2O

Movement of the imine bond relative to the sulfur atom should be minimized in order for strong fluorescence to be observed. A molecule of water, forming strong hydrogen bonds with the electron rich nitrogen and sulfur atoms will tie these two side chains together. This would account for the decrease in fluorescence upon addition of ethanol to the buffered OPT-βME amino acid mixture.

As shown in Table VIII glutathione reacts with OPT alone to produce a strongly fluorescent derivative, while cystine does not. The increase in fluorescence upon insertion of a carbon in the side chain (homocysteine) suggests that the chain is not flexible enough to allow hemimercaptal formation with cysteine. In the case of glutathione, there is sufficient flexibility to allow hemimercaptal formation and hence, fluorescence.

Small shifts in the fluorescence spectra maxima indicate that the addition of amino acid or protein to buffered OPT-βME slightly alters the resulting fluorophore. Since the absence of carbonyl groups adjacent to the aromatic ring appears to be necessary for fluorescence, the dihemimercaptal is probably the OPT-βME fluorophore. The molar ratio of OPT to βME (8:1) in buffered OPT-βME reagent is favorable for dihemicaptal formation. The slight difference in the excitation and emission maxima between the OPT-βME fluorophore and the OPT-βME amino acid fluorophore suggests that a π to π^* transition is involved.

This was concluded due to the lack of a possible n to π^* transition in the dihemimercaptal adduct. The excitation maximum (325 nm) and emission maximum (435 nm) for the OPT (no βME) BSA adduct indicates a considerably different fluorophore.

The reaction of malonaldehyde with amines was studied by Chio and Tappel (98), and they attributed the fluorescence to formation of a Schiff base. Crowell and Varsel (97) investigated twenty-six substituted aromatic aldehydes and found that they generally did not fluoresce even when other ring substitutents were capable of promoting fluorescence. This indicates that the aldehyde group exerts a strong quenching effect on the fluorescence property of the aromatic system. When methyl hemiacetals were formed, most of them exhibited appreciable fluorescence. The acetals were shown to have the same excitation and fluorescence maxima as the corresponding alkyl derivatives. Likewise, the effects of the carboxylic acid group on the fluorescence of benzoic acid was investigated by Tournon and El-Bayoumi (99), and they found that the group reduced the fluorescence when compared to that of toluene. A similar effect is noted with OPT. The carbonyl group must be removed by either reduction by $NaBH_4$ or hemimercaptal formation with βME.

Varying the number of methylene groups between the amino and carboxyl ends of the amino acid suggests that the carboxyl group does not participate in the reaction itself,

but does have an integral role in the fluorescence. The reduction of fluorescence with histamine, as compared to histidine, in the presence of the buffered OPT-βME reagent supports this conclusion (77). With the glycine-OPT-βME adduct in alkaline solution, the dissociated form of the carboxylic acid if fluorescent, but upon protonation with hydrochloric acid there is a marked depression in fluorescence. Since the glycine ethyl ester adduct also showed fluorescence equal to glycine, it appears unlikely that the carboxylate ion is essential for fluorescence. Under acid conditions the fluorophore is also labile, degrading into colored products and a ninhydrin positive component as demonstrated by TLC. As emphasized by Roth (77), alkaline pH was essential for fluorophore formation but the fluorophore was stable from pH 6.0 to 11.5. There is a much greater decrease in fluorescence when the glycyl-OPT-βME adduct is acidified as compared with the ethyl ester. The undissociated carboxylic acid group can quench the fluorescence of the adduct. Possibly the quenching of fluorescence may be due to proton transfer between the acid group and either the aromatic ring or the imine bond. The pH effect observed may also be due to protonation of the nitrogen in the imine bond.

Fluorescence spectra of the amino acids, which had varying distances between the amino and carboxyl ends, showed no changes in the partial quantum efficiency of fluorescence. The UV spectral scans of the amino acids with OPT and βME were all the same (except for the aromatics) indicating that despite a variance in the relative fluorescence among amino acids as indicated by Roth (77) they all react in the same manner.

The various amino acid alkyl and aryl groups may extend quenching and enhancement contributions to the basic fluorophore. An examination of the data allows the following generalizations to be made: 1) the presence of a conjugated system (tyrosine) or a double bond, such as a carbonyl group in glutamine, tends to enhance the fluorescence over the glycine standard; 2) the presence of a lone pair of electrons, such as on an amine (ornithine), tends to quench the fluorescence; and 3) increasing the hydrophobic content of the group also decreases the fluorescence. Structural models of the product show that the amino acid side chains are free to extend close to the imine bond region of the molecule.

Another factor affecting the relative fluorescence of the various amino acids is competition between the separate groups on one amino acid for OPT. In the case of lysine and arginine, the epsilon amino and guanidino groups may also form Schiff bases. This would tend to limit the number of possible fluorophores.

Fluorescence spectra obtained with BSA, OPT, and βME, show the same excitation and emission maxima as is seen for the amino acid derivatives, and the conclusion is that basically the same reaction is occurring with proteins.

Fluorescence is much more intense after reaction with OPT in the presence of βME than in the absence of βME, so the reaction involving histidine without βME was disregarded as the source of fluorescence. The pH profile for the BSA fluorescent derivative (Figure 20) shows that highly alkaline conditions prevent fluorophore formation, but the fluorophore, once formed, is stable at high pH as with amino acids.

The binding of 14 molecules of βME to one of OPT is rather high, but this may be the amount necessary to shift the equilibria in favor of the hemimercaptal derivative. This number for βME is not corrected for reduction of any disulfide linkages in BSA.

The degree of protein crosslinking by the OPT-βME reagent is dependent on concentration. In high concentrations of reagent, BSA precipitates and the centrifuged product is a rubbery pellet. If the concentrations of OPT and βME are kept slightly higher than saturation levels (55 OPT/1 BSA), no such polymerization occurs and it is assumed that little crosslinking characteristic of dialdehydes is occurring.

Studies with oligopeptides indicated that the peptide bond quenches fluorescence when compared with glycine; yet, on an equimolar basis, bovine serum albumin produces a fluorescence that is a hundred fold greater than glycine. Quenching by the peptide bond may be the result of fewer carboxylic acid groups to enhance fluorescence. The enhancement of fluorescence seen with the bovine serum albumin derivative may be accounted for by the fact that the amino acids in bovine serum albumin, which react with OPT, may have a greater relative fluorescence compared to glycine. The fluorescence spectra (Figures 23 and 24) give evidence for a secondary mechanism of fluorescence, that is energy transfer from tyrosine and tryptophan to the OPT-βME adduct. The primary mechanism is direct excitation of the fluorophore at 340 nm and flourescence emission at 454 nm. Excitation at 270-280 nm will allow energy absorption by the typtophan and tyrosine residues in bovine serum albumin and fluorescence emission at 340 nm. This emission band overlaps the excitation band for the OPT-βME fluorophore. Energy transfer from tyrosine to the OPT-βME fluorophore does not occur since the partial quantum efficiency for excitation at 280 nm is about 20% of that observed at 340 nm when emission is observed at 450 nm, thus indicating a possible transfer of energy to the OPT adduct. The partial quantum efficiency of the glycine adduct, under conditions specified above, shows a baseline value.

The OPT-βME reaction has been used as an analytical method for fluorescent determination of amino acids (77). This reaction is used extensively in this laboratory as a quantitative method for the determination of amino acids, peptides and proteins in place of the ninhydrin reaction. The simplicity of the method allows tests to be made within 10 to 15 minutes, and the sensitivity allows one nanomole of amino acid or one hundredth of a nanomole of protein to be detected.

The reagent may be used for the determination of total protein, especially where low concentrations and small amounts of sample are available, as in cerebrospinal fluid. OPT in conjunction with $NaBH_4$ or βME may be used as a fluorescent probe for detection of protein conformation changes. Measurements would be limited to a few hours; however, the label can be reacted with dilute protein solutions in several minutes. Several of the current probes require considerable time and dialysis to prepare the specimen.

Bifunctional reagents such as o-phthalaldehyde undergo complex reactions with proteins apparently involving many different side-chains. For example, glutaraldehyde was used to confer structural rigidity on carboxypeptidase (119). Since a primary amino group is required for Schiff base formation, o-phthalaldehyde probably reacts with lysine, arginine, and terminal amino groups.

The molecular weight relationship of the four purified proteins to fluorescence intensity permitted quick estimation of their individual molecular weights, which were determined by generally more lengthy methods. Thus using the independently determined molecular weight of a protein, a solution equimolar to that of other standard protein solutions may be prepared. Then the fluorescence of its OPT-βME derivative may be compared to other protein standards as depicted on the standard curve in Figure 27. The reason for the linear relationship between fluorescence intensity and molecular weight of the four proteins is unknown, and the dependence must be established for other proteins.

Although the fluoreecence obtained with adenosine monophosphate was only about a hundredth that of glycine on an equimolar basis, the reagent shows the possibility of its use as a fluorescent probe in nucleic acids.

The OPT-βME reagent can be used for the assay of any peptidase or protease activity, since cleavage of peptide bonds results in a net increase in the number of free amino groups and thus an increase in derivable fluorescence.

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