PROTEOLYSIS AND INHIBITION OF A β-AMYLASE

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

John Phillip Riehm

1961

This is to certify that the

thesis entitled

Proteolysis and Inhibition
of a β-Amylase

presented by

John Phillip Riehm

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Chemistry

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Major professor

Date November 22, 1961

PROTEOLYSIS AND INHIBITION OF A β-AMYLASE

By

John Phillip Riehm

AN ABSTRACT OF A THESIS

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1961

ABSTRACT

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By John Phillip Riehm

The inhibitory effect of various reagents which are known to react with sulfhydryl groups has been examined on the enzyme sweet potato β-amylase with the hope of specifically labeling the "active site" of the molecule. Both N-ethyl maleimide and p-chloromercuribenzoate are effective inhibitors. N-ethyl maleimide reacts with four sulfhydryl groups in aqueous solution and 15 to 16 in 8M urea. The inactivation of β-amylase by p-chloromercuribenzoate which is reversible, involves the reaction of six sulfhydryl groups. Fifty percent inactivation of β-amylase activity by both reagents corresponds to reaction of one sulfhydryl group, suggesting that only one sulfhydryl group is required for catalysis.

The reaction of sweet potato β-amylase with the exopeptidases has been examined with the hope of degrading the molecule to a smaller active fragment. β-Amylase appears to be resistant to proteolysis catalyzed by carboxypeptidase A and leucine aminopeptidase. Nevertheless, carboxypeptidase B does catalyze a small degradation of the molecule, thus indicating the presence of either a lysine or arginine residue at the carboxyl-terminus of the molecule. The combined action of carboxypeptidases A and B results in much more degradation; nevertheless, this treatment has no effect on the amylase activity of the β-amylase molecule.

John Phillip Riehm

A new method has been devised for the quantitative determination of reaction between N-ethyl maleimide and sulfhydryl groups of proteins This method depends on the determination of S-cysteinosuccinic acid produced from acid hydrolysates of the N-ethyl maleimide-treated proteins.

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ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. John C. Speck, Jr., whose interest, co-operation and guidance have greatly facilitated the progress of the present study.

Acknowledgment is also due to the Department of Chemistry and the National Institutes of Health for funds provided in support of this work.

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PROTEOLYSIS AND INHIBITION OF A β-AMYLASE

I. INTRODUCTION

Knowledge concerning the catlytic properties of an enzyme is one of the intriguing puzzles of biology. Enzymatic activity undoubtedly relies on the structure, the conformation and the specific chemical groupings which are present in the molecule, and an understanding of enzymatic catalysis will not be complete until these factors are interrelated. Protein chemistry, in the past few years, has made great strides towards a solution of this problem as evidenced by the elucidation of certain active sites of certain enzymes, the complete amino acid sequence of another (ribonuclease), and by experiments with model systems. Nevertheless, it appears probable that a solution of the intimate mechanism of action for any enzyme will require much ingenious experimentation.

On reviewing the numerous avenues for obtaining information into the mechanism of enzyme catalysis, two approaches have appeared to warrant study. These are: 1. an investigation of the group(s) essential for catalytic activity, and 2. a study of active fragments resulting from the proteolytic degradation of a native molecule. The results of such inquiries could lead to the labeling of the active site and to a feasible mechanism which could be tested with the aid of model compounds. The observations reported in this text extend previous investigations carried out in this laboratory on the enzyme sweet potato β-amylase (1).

β-amylases appear to be restricted to heigher plants. They have been crystallized from barley, wheat, soybeans and sweet potatoes. The occurrence of a β-amylase in sweet potatoes was shown in 1920 by Gore (2). Gore observed that slow heating of sweet potatoes in the 60° to 80°C range yielded a very high conversion of the starch into maltose. In 1946 Balls et al (3) succeeded in crystallizing this enzyme by ammonium sulfate fractionation; two years later they reported a refinement in their isolation (4). Balls and co-workers noted that the enzyme is only slowly denatured at 65°C and utilized this property in one of the initial steps in the purification. The pH versus activity curves with citrate and acetate buffers showed that the optimum pH is 4.5 (4). Singer and England (5) studied the physiochemical properties of the molecule and found the protein to be electrophoretically homogeneous. Nevertheless, an impurity of approximately 3 percent was detected in the ultracentrifuge. A molecular weight of 152,000 was calculated from these ultracentrifuge data.

The work of England and Singer (6) indicated that sweet potato β-amylase is inhibited by substances known to attack sulfhydryl groups. Low concentrations (10^{-6}M) of p-chloromercuribenzoate, silver nitrate, and mercuric chloride gave rapid inactivation. A twenty-fold excess of glutathione could partially reverse (to the extent of 50 percent) such inhibition. Oxidation of the sulfhydryl groups by o-iodosobenzoate also led to inactivation. This inactivation appeared to involve no polymerization, since the sedimentation properties of the inactivated enzyme were identical with those of the native molecule. Hellerman, Chinard and Ramsdell (7) have studied the reaction between cysteine and o-iodosobenzoate.

Since no reports on the total amount of cysteinyl residues nor on the number of sulfhydryl groups required for enzymatic activity of sweet potato β-amylase are to be found in the literature, investigations into this important feature of the molecule have now been carried out. Other work having to do with this enzyme and described in this text has involved 1. purification of the enzyme and 2. a quantitative determination of its amino acid content.

Mechanism of β-Amylase Action

The following discussion will not attempt to differentiate between the various β-amylases since no variations have been noted in their mechanism of action. Contrary to the usual connotation in carbohydrase nomenclature, the name β-amylase should not suggest the presence of β-linkages in the substrate. In general, it may be stated that these enzymes catalyze the hydrolysis of starch (α-1,4-glucosidic linkages) to the disaccharide, β-maltose. It has been demonstrated that hydrolysis catalyzed by β-amylase commences from the non-reducing end of the polysaccharide and continues until the entire chain is converted into maltose or until enzyme action is blocked by chain branching.

The presence of β-maltose as the end-product has been cited by Kuhn (8) and Freeman(9). These workers noted that the products of the reaction mutarotated upwards. Since maltose was identified as the end-product, β-maltose, which would mutarotate upwards to the equilibrium mixture, was believed to be the sole initial product. Recently, Thoma and Koshland (10) pointed out that an upward mutarotation need not imply a quantitative release of β-maltose; it merely indicates that the initial products contain more of the β-isomer than does the

equilibrium mixture. Accordingly, Thoma and Koshland carried out a study on the extent of inversion. The rotation of a maltotetraose solution immediately after hydrolysis catalyzed by β-amylase indicated that β-maltose is indeed quantitatively released. This observation apparently rules out the possibility of cleavage by a free carbonium ion mechanism.

Chemical alterations of the reducing terminus of oligiosaccharides have demonstrated the attack of β-amylase at the non-reducing terminus. Hydrolysis of methyl-α-maltotrioside yielded maltose and methyl-α-glucoside (10). Hydrolysis of maltoheptanoic acid, catalyzed by the enzyme, gave maltose and maltotrionic acid (11).

As the hydrolysis of starch and glycogen catalyzed by β-amylase progresses towards branches in the chains, the hydrolysis rate becomes much slower. Hence, amylopectins and glycogens following β-amylase treatment give high molecular weight "limit dextrins". These dextrins, though resistant to hydrolysis catalyzed by β-amylase, are rapidly cleaved under the influence of α-amylases. Chemical and enzymatic investigations have shown that all the branches in the original substrate are found in the limit dextrin (12).

Peat and co-workers treated a limit dextrin with an "R-enzyme" preparation (13) ("R-enzymes" are specific for the hydrolysis of (1-6)-α-glucosidic linkages). The outer "stubs" of the limit dextrins were shown to consist entirely of maltose and maltotriose, indicating that β-amylase removes all but the three innermost glucose units from the odd-membered outer chains, and a single maltose unit from the even-membered chains.

Studies in which starch or glycogen were hydrolyzed in the presence of H_2O^{18} have shown that β-amylase catalyzes the cleavage of the 1-4-glycosidic bond between the C1 and the oxygen bridge (14,15). Accordingly, Mayer and Larner (14) proposed a mechanism for the catalytic hydrolysis based on this observation. This mechanism involves the following steps: 1. orientation of the substrate on the enzyme surface, 2. protonation of the bridge oxygen to form an oxonium ion which is cleaved on the C1 side, and 3. stereospecific hydration of the resulting carbonium ion. Koshland (16) has explained β-amylase catalysis as an SN_2 reaction in which water attacks the potential aldehyde carbon from the backside, displacing the R-O group and, inverting the configuration at C1.

Thoma and Koshland have put forward evidence that β-amylase catalyzes hydrolysis through an induced-fit mechanism (10,17). The induced-fit theory postulates that the substrate induces changes in the conformation of the enzyme and that these changes are required for enzymatic activity. In contrast to this, the older template theory proposes that the enzyme, due to its conformation, dictates the type of substrate to be hydrolyzed. The facts used in proposing the induced-fit theory are the following: 1. the Schardinger dextrins, cyclohexa- and cycloheptaamylose are not cleaved in the presence of β-amylase but are strong competitive inhibitors of the enzyme, and 2. the hydrolysis of maltose catalyzed by β-amylase is very slow, that of maltotriose is slow, whereas that of maltotetraose is quite rapid. Thoma and Koshland's model postulates that reaction of a group, X, on the enzyme with the substrate, induces the enzyme to fit closely to the substrate. In a reactive enzyme-substrate complex the two enzyme catalytic groups, A and B, are brought into a favourable proximity with the substrate. It

is assumed that the enzyme forms a complex with the Schardinger dextrins, in which the two groups, A and B, are not able to complete the catalytic process.

The Exopeptidases

Since previous experiments having to do with the action of various endopeptidases on sweet potato β-amylase (1) did not yield active fragments, it was decided to test the action of the exopeptidases on the enzyme. Exopeptidases catalyze hydrolysis of peptide bonds adjacent to terminal α-amino or terminal α-carboxyl groups. The best characterized of these enzymes are the carboxypeptidases, A and B, and leucine aminopeptidase. It is worth noting that these enzymes have been instrumental in the determination of amino acid sequences (33,34,35). Hirs, Stein and Moore (33) elucidated the primary structure of ribonuclease with the aid of both carboxypeptidases A and B. Dixon, Kaufman and Neurath (35) used leucine aminopeptidase to determine the sequence around the phosphoserine residue of trypsin modified by reaction with diisopropyl phosphofluoridate. It is interesting also that these exopeptidases have been used to modify native proteins. The action of carboxypeptidase A on various proteins shows a wide range of effects from the liberation of one amino acid as in its action on tobacco mosaic virus protein (36), to many when it acts on glucagon (37). In the hydrolysis of yeast enolase catalyzed by carboxypeptidase A, it has been reported (38) that over 150 amino acid residues are removed without loss of enzymatic activity. Drechsler and Boyer (39) have reported that carboxypeptidase A catalyzes the hydrolysis of three tyrosyl residues from the aldolase molecule with a concomitant decrease in

activity of 93 percent towards the substrate fructose 1,6-diphosphate.

Perhaps the most outstanding example of exopeptidase degradation of native proteins is that reported by Hill and Smith (40,41,42). Leucine aminopeptidase degrades approximately two-thirds of the mercuripapain molecule, liberating free amino acids, without altering the enzymatic activity of papain when it is reactivated with cysteine. A homogeneous active fragment was obtained from the proteolysis mixture. This fragment contains 76 of the original 185 residues and differs from intact papain in ultraviolet spectrum, amino end group and molecular weight, but shows similar behaviour with respect to denaturation by heat, acid and urea. Malmström (43) has reported that leucine aminopeptidase releases about 150 amino acids from the N-terminus of enolase without loss of enolase activity.

Carboxypeptidase B is a relatively new tool in the hands of the biochemist. Its presence in bovine pancreas was first reported in 1956 by Folk (44) and, a homogeneous preparation was described in 1960 (45). The enzyme specifically catalyzes the hydrolysis of lysine and arginine from the carboxyl terminus of proteins. Folk and Gladner (46) studied the effect of carboxypeptidase B on trypsin and noted that 3.5 moles of arginine were released in 2 hours.

These experiments indicate that it is possible to degrade a native enzyme and obtain a smaller unit which may still contain part or all of the original activity. With this thought in mind, the proteolytic action of these three exopeptidases on sweet potato β-amylase has been examined.

Inhibitors

Inhibitors of enzyme catalysis have proven to be a useful aid in determining the type of groups essential for catalytic activity. They also provide a means of labeling amino acids at the so-called "active site" of the molecule.

A well known case of inhibition is that by diisopropyl phosphofluoridate in abolishing completely and irreversibly the proteolytic activity of chymotrypsin and trypsin (18). In each instance a crystalline inactive derivative containing a single diisopropyl phosphoryl group can be isolated (19). Acid hydrolysis of these protein derivatives produces significant amounts of phosphoserine (20,21). Proteolysis of these derivatives followed by amino acid sequence studies have led to the elucidation of the amino acid sequences about these serine residues (22,23). The phosphoserine residues in trypsin and chymotrypsin are found in the sequence: glycyl, asparty1, seryl, glycyl (24).

In the past decade, the inhibition of enzymes by reagents which are known to react with thiols has received much attention. The analytical demonstration of sulfhydryl groups in an enzyme, and the reversible inactivation of the enzyme by a reagent such as p-chloromercuribenzoate has been taken as evidence that one or more cysteine residues are involved in the catalytic process. Recently Vallee, Combs, and Hock (27) have reported that the activity of carboxypeptidase A depends on a zinc mercaptide. One zinc ion appears to be bound to the only titratable sulfhydryl group of the zinc-free protein. Replacement of zinc with the sulfhydryl group reagents, p-chloromercuribenzoate or silver ion, renders the enzyme inactive. Removal of these reagents

by cysteine and replacement of the zinc restores the carboxypeptidase A activity. These observations indicate that it should be possible to explore the chemical nature of the "active site" of carboxypeptidase A by labeling this sulfhydryl group.

The possibility of labeling the "active site" of an enzyme has initiated studies into the effect of various reagents which are known to react with sulfhydryl groups on the molecule sweet potato β-amylase. The observations reported in this text attempt to correlate inhibition of β-amylase activity with the number of sulfhydryl groups undergoing reaction.

Inhibitors which attack sulfhydryl groups have been thought to be specific reagents for thiol compounds. Nevertheless, it is known that ribonuclease which contains no sulfhydryl groups is inhibited by iodoacetic acid, one of the classical sulfhydryl reagents. Gundlach, Stein and Moore (25) found that inactivation of ribonuclease by iodoacetic acid resulted in the alkylation of different amino acids at various pH values. At a pH of 8.5 the ε-amino groups of lysine were alkylated. At pH 5.5 a nitrogen of histidine (presumably a ring nitrogen) was alkylated, and at pH 2.8 the sulfur of methionine underwent alkylation. With the aid of C^{14}-labeled bromoacetic acid Barnard and Stein (26) have demonstrated that the histidine which is alkylated is the one nearest the carboxyl-terminus of ribonuclease.

The reaction of N-ethyl maleimide with proteins was also believed to be specific for sulfhydryl groups, and the spectrophotometric method for determining thiols with N-ethyl maleimide (28) has been employed in many instances for the quantitative determination of cysteinyl residues in proteins (29,30). The method of Alexander depends on the decrease in

absorbance at 300 mμ resulting from a presumed addition of the thiol across the double bond of N-ethyl maleimide. Nevertheless, Smyth, Nagamatsu and Fruton have shown that N-ethyl maleimide reacts with imidazoles and the amino groups of peptides in, possibly, an N-acylation reaction. In the case of imidazoles, reaction proceeds to the formation of a polymer, possibly consisting of N-ethylmaleamic acid. Recently Riggs (32) has shown that a decrease in absorbance is observed when N-ethyl maleimide(10^{-3}M) is added to 0.1M solutions of various amino acids. These results give rise to doubts concerning the reliability of Alexander's spectrophotometric method. It is true that the conditions of Riggs and Smyth et al are much more rigorous (higher concentrations of N-ethyl maleimide and amino acids) than those employed by Alexander, nevertheless, there is the possibility of acylation reactions in conjunction with the alkylation of sulfhydryl groups. Portions of the observations reported in this text are attempts to clarify this uncertainty. N-ethyl maleimide reacts with cysteine to form S-cysteino-(N-ethyl)-succinimide (31). On acid hydrolysis, S-cysteino-(N-ethyl)-succinimide is converted to S-cysteinosuccinic acid. Reaction of N-ethyl maleimide with sulfhydryl groups of proteins and acid hydrolysis of the protein derivatives should also lead to the production of S-cysteinosuccinic acid also. These reactions are shown in Chart I.

The uptake of N-ethyl maleimide in the presence of various proteins was studied in two ways: (1) by the spectrophotometeric method outlined by Alexander, and (2) by determining the amount of S-cysteinosuccinic acid produced from an acid hydrolysate of the proteins treated with N-ethyl maleimide. Comparison of the two methods should indicate, within experimental error, the presence of reactions other than reaction with cysteine residues.

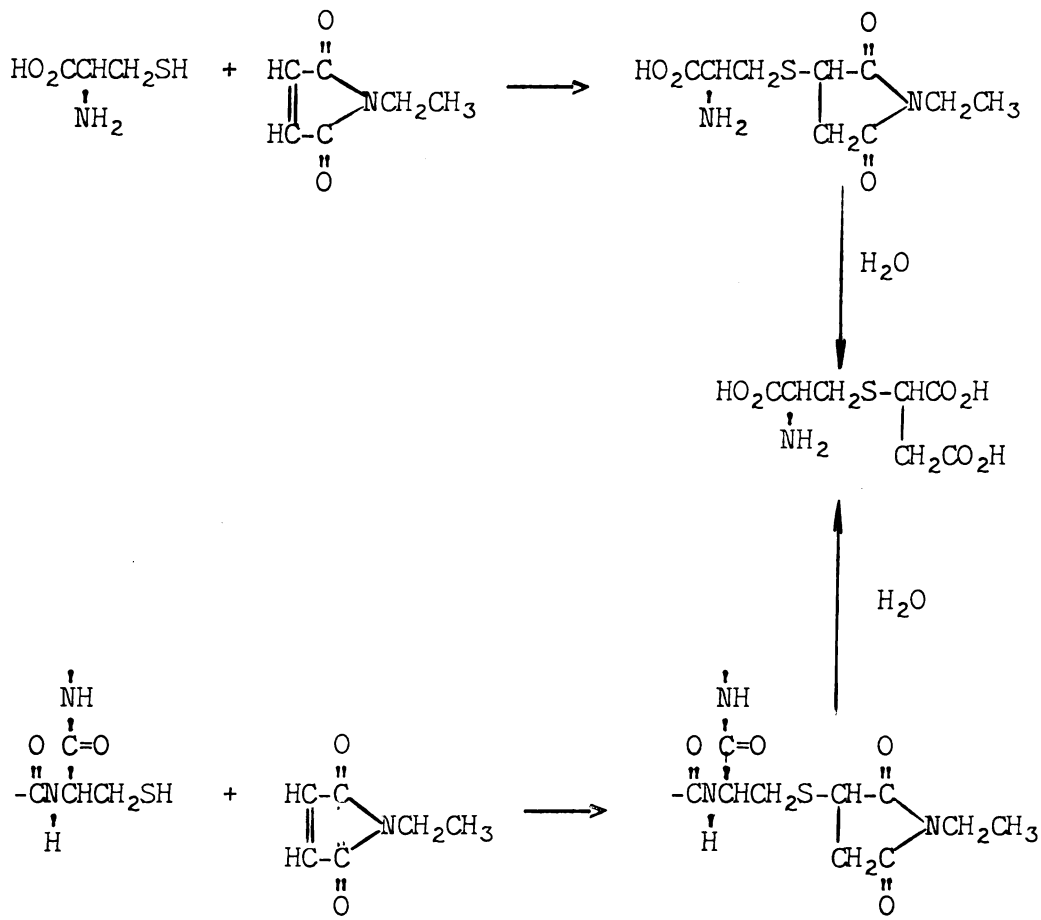


CHART I. Formation of S-Cysteinosuccinic Acid.

II. EXPERIMENTAL

1. Apparatus

Spectrophotometers.- Absorbance measurements in the ultraviolet range were carried out with the Beckman Model DU spectrophotometer. Controlled reaction temperatures were obtained by fitting the instrument with a thermospacer assembly and by connection to a circulating bath. A Beckman Model B spectrophotometer was used for measurements in the visible range. This instrument was adapted for test tubes by replacing the cell compartment with the test tube compartment.

Columns.-

(a) Protein Chromatography--Columns fitted with glass wool plugs and packed with the desired resin were employed in the purification of enzyme solutions.

(b) Amino Acid Chromatography--The separation of the neutral and acidic amino acids required a 0.9 x 165 cm jacketed column. A condenser with an inner diameter of 0.9 cm and fitted with a sintered plate was used for the basic amino acids. This column also proved useful in the preliminary investigations of the compound S-cysteinosuccinic acid.

Sterile Apparatus.- Proteolytic digestions of longer than six hours were carried out in a sterile apparatus. The apparatus consisted of a Pyrex bacterial filter attached to a 20 ml filter tube and had a side arm to permit withdrawal of samples during the reaction without contamination. The apparatus was sterilized at 15 pounds pressure for 30 minutes prior to each run.

Standardized Test Tubes.- Soft glass test tubes were standardized according to the procedure of Stein and Moore (47).

2. Reagents

Proteins.- The sweet potato β-amylase was a twice crystalline product obtained from Worthington Biochemical Corp. The enzyme is prepared according to Balls et al (4). Carboxypeptidase A (lot 601), prepared according to the method of Anson (48) was also obtained from Worthington Biochemical Corp. Aldolase (lot B-1894) which is isolated from rabbit muscle according to the method of Taylor, Green and Cori (49) was purchased from Mann Research Laboratories. Egg albumin which was crystallized five times (lot F 63) and thrice crystallized β-lactoglobulin (lot F 26) were purchased from Pentex Incorp.

Resins.- Amberlite MB-1 (lot 700613) and Amberlite CG-120 Type 2 (lot 785993) were obtained from Fischer Scientific Co. Sephadex G-75 was purchased from Pharmacia. Diethylaminoethyl cellulose (lot 107418, 0.78 meq. per g) was obtained from the California Corporation for Biochemical Research.

Chemicals.- N-ethyl maleimide (lot c2282) and L-cysteine hydrochloride monohydrate (lot B2056) were acquired from Mann Research Laboratories. p-Chloromercuribenzoate (sodium salt, lot 102735) was purchased from the California Corporation for Biochemical Research. Carbobenzoxy-glycyl-L-phenylalanine (lot B1310) and hippuryl-L-arginine (lot F2911) were products of Mann Research Laboratories. L-leucinamide hydrochloride (lot 6534) was purchased from the Nutritional Biochemical Corporation.

Ninhydrin Color Reagent.- The reagent was prepared by dissolving 2 g of ninhydrin (Nutritional Biochemical Corp.) and 0.3 g of hydrindatin in 75 ml of methyl cellosolve (Fischer Scientific Co.). Twenty-five ml of 4M acetate buffer (pH 5.25) was then added to this solution. The reagent was always freshly prepared. The hydrindatin was prepared according to the method of Stein and Moore (50). Twenty g of ascorbic acid in 100 ml of water at 40°C was added to 500 ml of water at 90°C, and containing 20 g of ninhydrin. On standing, the hydrindatin crystallized. The product was filtered and dried over phosphorus pentoxide. The yield was 18.5 g.

3. Correlation of Protein Concentration to Absorbance at 280 mμ

Approximately 10 mg of β-amylase was dialyzed in the cold for 24 hours against 0.1M phosphate buffer (four changes of buffer). The enzyme solution was centrifuged to remove any denatured protein and was diluted to 5 ml. Aliquots were removed and diluted for optical density measurements at 280 mμ. Kjeldahl nitrogen determinations were performed on 0.5-ml samples. Since the dry protein contains 15.41 percent nitrogen (1), nitrogen values were converted to mg of protein. The results are reported in Table 1(see Results).

4. Assay of β-Amylase Activity

The method of Noelting and Bernfeld (51,52) was employed for assaying β-amylase activity. This procedure is based on the formation of a colored product from the reaction of maltose and 3,5-dinitrosalicylic acid in alkaline solution. The concentration of this colored material is measured spectrophotometrically at 540 mμ.

Reagent.- Five g of 3,5-dinitrosalicylic acid was moistened with a few ml of water and 100 ml of 2N sodium hydroxide was added. The suspension was brought to a volume of 250 ml by the addition of water and stirred until the 3,5-dinitrosalicylic acid dissolved. One-hundred and fifty g of Rochelle salt was then added to this solution and the solution finally diluted to a volume of 500 ml.

Assay Procedure.- One ml of a one-percent starch solution (pH 4.6; 0.01M acetate buffer) was placed in a 25 ml volumetric flask and equilibrated (at 30°C) in the constant temperature bath. One ml of enzyme solution was then added and the reaction allowed to proceed for 185 seconds. Reaction was stopped by the addition of 2 ml of the 3,5-dinitrosalicylic acid solution, and the flask placed in a boiling water bath for 5 minutes, cooled, diluted to 25 ml and the absorbance read at 540 mμ against that of a blank. Enzyme concentrations were adjusted to approximately 0.001 mg per ml. In inhibition studies, enzyme concentrations were raised tenfold when inhibition neared 90 percent.

5. Purification of β-Amylase on Sephadex G-75

Singer (5) reported that ultracentrifugation of an eight times recrystallized enzyme preparation revealed the presence of a slower moving component. This impurity amounted to approximately 2 to 3 percent of the total protein. The commercially available enzyme also contains this impurity, as shown by ultracentrifugation, and it was thought that purification was a prerequisite to studies on the enzyme. The following procedure was employed in the purification step. A sample of β-amylase was dialyzed for 24 hours in the cold, against redistilled water (two changes). The solution was added in the cold, to a 1 x 15 cm

column of Sephadex G-75 which had been equilibrated with water. Employing water as the eluting solvent, 2.3 ml fractions were collected. Samples containing β-amylase activity were pooled and perevaporated in the cold to a concentration of approximately 1 mg per ml. This sample was dialyzed against 0.1M phosphate buffer (pH 6.0), centrifuged to remove dust and examined in the ultracentrifuge for the presence of impurities.

6. Quantitative Amino Acid Analysis

This report is a continuation of previous results (1) wherein analysis is reported for 13- and 72-hour hydrolysates. Analyses have now been performed on 48-hydrolysates (impure enzyme) and on 72-hour hydrolysates (purified sample).

Preparation of the Samples.- Solutions of sweet potato β-amylase were exhaustively dialyzed in the cold against redistilled water (several changes of water). The enzyme solutions were placed in hydrolysis ampules and lyophillized. Constant boiling hydrochloric acid (4 to 5 ml) was added, the ampules were evacuated, sealed and placed in a 105°C oven for either 48 or 72 hours. The samples were removed from the ampules and dried *in vacuo* over sodium hydroxide. One ml of water was added to the residue and the samples were again brought to dryness. This drying procedure was repeated twice to insure complete removal of the hydrochloric acid. Ten ml of buffer (either pH 3.25 or 5.28) was added to the residue, and the resulting solutions were stored in the frozen state until they were analyzed for amino acid content.

Operation of Columns.- (a) One-hundred and Fifty-cm Column. One ml

of the protein hydrolysate, in pH 3.25 buffer, was added to the top of the column and allowed to flow into the resin. This was followed by two, 1-ml aliquots of buffer which were also allowed to flow into the resin packing. The column was connected to a separatory funnel, containing pH 3.25 buffer, and air pressure of 4 to 5 pounds was applied. The effluent was collected in 2-ml fractions while maintaining the temperature of the jacketed column at 50°C throughout the run. An eluant of pH 4.25 buffer was introduced in time to allow valine to emerge with the new buffer. Stein and Moore (53) suggest making this change at an effluent volume 2.15 times that at which aspartic acid emerges from the column. This procedure resulted in poor resolution of cystien and valine. The best separations between these two amino acids were obtained by introducing pH 4.25 buffer at an effluent volume 2.15 times the peak of aspartic plus 40 ml. Upon the elution of phenylalanine from the column, 0.2N sodium hydroxide was passed through the column overnight. This operation was followed by re-equilibration of the column with pH 3.25 buffer. This procedure readied the column for the next run.

(b) Fifteen-cm Column.- The operation of this column is practically identical to that of the 150-cm column except that the eluting buffer is pH 5.28. Upon the appearance of arginine the column may be used immediately for another sample.

Leucine Standard Curve.- For the preparation of this standard curve, 131 mg of L-leucine was dissolved in 10 ml of redistilled water, and aliquots of this solution were diluted to concentrations of 0.05 to 1.0 micromoles per 2 ml. One ml of the ninhydrin reagent was added to 2-ml

aliquots of the solutions in previously standardized test tubes. The test tubes were stoppered and placed in a boiling water bath for 15 minutes. On cooling, 10 ml of 50 percent ethanol was added to each tube and the absorbance was read at 540 mμ against a blank. When tubes had an optical density greater than 0.600, an additional 5 ml of 50 percent ethanol was added. The amount of amino acid present in each tube of effluent was determined in a similar manner, except for proline which was read at 440 mμ. The amount of amino acid present was calculated in terms of leucine equivalents which when divided by its color yield (50) gave the concentrations of the amino acid.

7. The Exopeptidases

Experiments with Carboxypeptidase A

Preparation of the Carboxypeptidase A Solution.- Approximately 0.1 ml of the carboxypeptidase A suspension was dissolved in a few ml of 0.01N soidum hydroxide, and the resulting solution was diluted to 25 ml with phosphate buffer (pH 8.0, 0.1M). The absorbance at 280μ divided by 2.3 gave the mg of carboxypeptidase per ml (39). The activity of the carboxypeptidase solution was measured on the substrate, carbobenzoxy-glycyl-L-phenylalanine.

Action of Carboxypeptidase A on β-Amylase.- Solutions of β-amylase containing approximately 30 mg of the enzyme, were dialyzed in the cold against redistilled water and passed through a 1 x 10 cm column of mixed-bed resin (Amberlite MB-1). This treatment freed the protein of any absorbed amino acids. Initially, experiments were carried out which involved no purification of the enzyme. The resulting solutions were perevaporated in the cold to a volume of approximately 5 ml, and

were dialyzed against phosphate buffer (pH 8.0, 0.1M). Carboxypeptidase A was added to the resulting solutions so that the ratio of carboxypeptidase to protein was 1 to 50, and proteolysis was allowed to proceed at 30°C. At various times 0.1 ml aliquots of the reaction mixture were withdrawn for β-amylase activity measurements. At the same time, aliquots were removed for the determination of amino acids released. This amino acid analysis was carried out in the following manner. To 1 ml of the protein solution was added 1 ml of 5 percent trichloroacetic acid. The resulting mixture, containing the suspended, denatured protein, was allowed to stand in the refrigerator for 30 minutes before centrifuging. Aliquots (1.5 ml) of the supernatant solution were then mixed in test tubes with 1 ml of the ninhydrin reagent. The tubes were boiled for 15 minutes, diluted with 10 ml of 50 percent ethanol, and the absorbance of the solutions measured at 570 mμ.

Experiments with Carboxypeptidase B

Isolation of the Enzyme.- The enzyme was prepared according to Folk et al (45). This procedure involves preparation of an acetone powder from swine pancreas, extraction of the powder with water, and fractionation of the extract with ammonium sulfate. The fraction of protein precipitating between 0.35 and 0.60 ammonium sulfate saturation was purified on diethylaminoethyl cellulose (0.78 m.e. per g). Fifty g of acetone powder gave 280 mg of protein in a total volume of 15 ml. The activity of the preparation was determined on the substrate hippuryl-L-arginine and contained 1,200 units per mg of protein.

Action of Carboxypeptidase B on β-amylase.- Samples of β-amylase which were purified as outlined in the carboxypeptidase A experiments

were dialyzed against 0.1M phosphate buffer (pH 8.0, 0.1M NaCl). Solutions of carboxypeptidase B were added so that the ratio of carboxypeptidase B to protein was 1 to 25, and proteolysis was allowed to proceed at 30°C. At various times, aliquots were removed for 1. β-amylase activity measurements, and 2. ninhydrin color yields of the trichloroacetic acid soluble fraction. The results of this investigation are reported in Table VI (see Results). One experiment was performed in which the cummulative effect of carboxypeptidases A and B was studied. Carboxypeptidase B was added to 5 ml of a β-amylase solution in pH 8.0 phosphate buffer (0.1M) containing 20 mg of protein. The reaction was allowed to proceed for 4 hours at 30°C and then carboxypeptidase A was added.

Experiments with Leucine Aminopeptidase

Preparation of the Enzyme.- The enzyme was prepared according to Smith et al (54). The procedure involves : 1. preparation of an acetone powder from swine kidney, 2. extraction with water and ammonium sulfate fractionation of the extract between 0.50 and 0.70 saturation, 3. a heat treatment at 70°C, and 4. electrophoresis on a starch column. The enzyme had a specific activity of 51 as measured on the substrate leucinamide hydrochloride.

Action of Leucine Aminopeptidase on β-Amylase.- A sample of sweet potato β-amylase (10 mg in 4 ml) was dialyzed against pH 8.5 tris(hydroxymethyl)aminomethane buffer (0.005M). Leucine aminopeptidase was added to the β-amylase solution so that the ratio of the leucine aminopeptidase concentration to that of β-amylase was 1 to 50. At various times, aliquots were removed for β-amylase activity measurements. At the same

time, aliquots were removed and examined for released amino acids. This amino acid analysis was carried out in an identical manner to that described in the carboxypeptidase A experiments.

8. Inhibitors

Inhibition of β-Amylase by p-Chloromercuribenzoate.– The spectrophotometric determination of sulfhydryl groups in proteins as developed by Boyer (55) was used in these studies. This method is based on the increase in absorbance at 250 mμ of a mixture of the protein and p-chloromercuribenzoate as a consequence of reaction of p-chloromercuibenzoate with sulfhydryl groups. Solutions of the enzyme were prepared by dialyzing the samples against the desired buffer. These dialyzed solutions contained approximately 1 mg of protein per ml and the reaction was studied at three pH values, 4.5 (0.1M acetate), 6.8, and 8.0 (0.1M phosphate). The p-chloromercuribenzoate solutions were prepared by dissolving 50 to 60 mg of the solid in the least amount of 0.1M sodium hydroxide, and diluting to approximately 5×10^{-4}M with the appropriate buffer. In a typical experiment, 0.05 ml of the p-chloromercuribenzoate solution was added from a microburette to a 3-ml aliquot of the β-amylase solution. The solution was carefully mixed and allowed to stand for 30 minutes in the sample compartment of the Beckman DU spectrophotometer before determining the increase in absorbance. The sample compartment of the spectrophotometer was maintained at 30°C during the run by means of a circulating thermostat. The addition of 0.05-ml aliquots of p-chloromercuribenzoate was repeated until the increase in absorbance at 250 mμ was constant. Reactions were carried out under identical conditions of concentration and temperature for the determination of β-amylase activity.

Reactivation of β-Amylase Inhibited by p-Chloromercuribenzoate and Mercuric Chloride.— A β-amylase solution, which contained 1.68 mg of the enzyme per ml was dialyzed against pH 8.0 phosphate buffer. One ml of either a p-chloromercuribenzoate or mercuric chloride solution (0.075 μmoles per ml) was added to 1 ml of the enzyme solution, and the mixture was allowed to stand at 30°C. Thirty minutes later, either 1 ml of versene (0.75 μmoles per ml), cysteine (7.5 μmoles per ml) or a combination of both was added. These solutions were allowed to stand another 30 minutes before β-amylase activity was determined.

Inhibition of β-Amylase by N-Ethyl Maleimide.— Preliminary investigations involved studies, over a wide pH range, into the effect of 10^{-3}M N-ethyl maleimide on the catalytic activity of β-amylase. Samples of sweet potato β-amylase, containing approximately 1 mg per ml were dialyzed overnight against the desired buffer. Studies were carried out in pH 4.5, 0.1M acetate buffer and 0.1M phosphate buffer pH 6.8, 7.0, 7.5, 8.0, and 8.5. An equal volume of 2×10^{-3}M N-ethyl maleimide solution, in the appropriate buffer, was added to the protein solution and the reaction was allowed to proceed at 30°C. At various times, aliquots of the reaction were removed and tested for β-amylase activity.

Spectrophotometric Determination of Sulfhydryl Groups by N-Ethyl Maleimide.— The procedure of Alexander (28) was employed in these studies. A sample of the purified enzyme was perevaporated, in the cold, to approximately 6 to 8 mg per ml and dialyzed against phosphate buffer (pH 8.0, 0.1M). An aliquot of N-ethyl maleimide was added to the enzyme solution, making the final concentration of the N-ethyl maleimide 1×10^{-3}M, and the decrease in absorbance at 300 mμ was followed

spectrophotometrically in the Beckman spectrophotometer. The sample compartment of the spectrophotometer was maintained at 30°C by means of a circulating thermostat. At various times, aliquots of the reaction were removed and tested for β-amylase activity. From a knowledge of the amount of enzyme present, the decrease in absorbance at 300 mμ, and the percent inactivation, Figure 4 was plotted (see Results).

N-ethyl maleimide uptake in the presence of 8M urea was studied by adding 0.5 ml of the enzyme solution to 3 ml of 9.3M urea which was 1×10^{-3}M in N-ethyl maleimide. Again the decrease in absorbance at 300mμ was measured. The urea solutions were always freshly prepared in the desired buffer, tested for cyanate (56) and contained 1×10^{-4}M ethylenediaminetetraacetic acid.

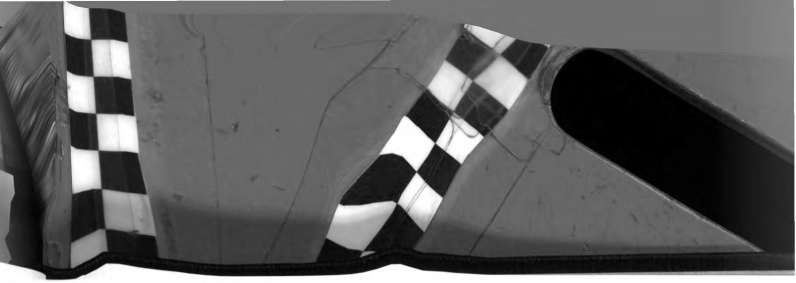
Action of Other Inhibitors on β-Amylase.— β-Amylase solutions containing approximately 1 mg per ml were dialyzed against the desired buffer and an equal volume of either iodoacetic acid (1×10^{-2}M, pH 4.5, 0.1M acetate and pH 7.5, 0.1M phosphate), maleic acid (10^{-3}M, pH 8.0, 0.1M phosphate), or sodium arsenite (1×10^{-2}M, pH 6.8, 0.1M phosphate) was added. At various intervals, aliquots were removed for activity measurements.

9. Quantitative Determination of S-Cysteinosuccinic Acid on the Stein-Moore Column

Preparation of S-Cysteinosuccinic Acid.— This compound was prepared according to the procedure of Morgan and Friedman (57,58). To 200 ml of water, 9.3 g of maleic acid was added, and the pH was adjusted to 7.4. Cysteine (2.42 g) was added to the resulting solution, and the

flask containing the mixture was evacuated and placed in a 37°C oven for 8 hours. The solution then was made acidic to congo red by the addition of dilute sulfuric acid, and 6 grams of mercuric sulfate was added. The precipitate which formed was removed by centrifuging, decomposed with hydrogen sulfide, and the resulting mixture was filtered. Cadmium acetate (2 ml of a 10 percent solution) was added to the filtrate to precipitate excess cysteine. The mixture was filtered. The mercuric sulfate treatment was repeated on the filtrate. The precipitate was decomposed again with hydrogen sulfide, and the mixture was filtered. The sulfate ion was quantitatively removed from this filtrate by the addition of a 4 percent solution of barium hydroxide. The barium sulfate was filtered off, and the filtrate was reduced in a flash evaporator to approximately 50 ml. The remaining water was removed by lyophillization. The residue which was a white powder was washed in ethanol and dried in vacuo over phosphorus pentoxide. The yield was 3.93 g (83 percent of theory). The specific rotation, $[\alpha]_D^{20}$ in water solution (c = 1 percent) was -29.40. Recrystallization of 0.4 g of this product from 10 ml of glacial acetic acid yielded 195 mg (48 percent of theory). The melting point was 135-136°C and the specific rotation, $[\alpha]_D^{20}$ in water solution (c = 1 percent) was -.13. The percent nitrogen found was 5.87±0.13, calculated for $C_7H_{11}O_6NS$ N was 5.87.

To 100 ml of citrate buffer (pH 3.25) was added 23.7 mg of the recrystallized S-cysteinosuccinic acid. One ml of this solution was placed on the 150-cm Stein-Moore column (this reporesents 1 μmole of the compound), and its position of emergence from the column was observed. Since diastereoisomers should be formed on the addition of cysteine to the double bond of maleic acid, attempts were made to resolve



the isomers. This was performed by lowering the pH of the eluting buffer to pH 3.0.

The amount of color obtained when S-cysteinosuccinic acid is reacted with ninhydrin, in a manner identical to that described for amino acid analysis is shown in the Results in Table XII.

Preparation of S-Cysteino-(N-Ethyl)-Succinimide.– This procedure differed from that reported by Smyth and co-workers (31). L-cysteine hydrochloride monohydrate (875 mg) was added to 25 ml of water and the pH was adjusted to 6.0 by the addition of 1N sodium hydroxide. To this solution was added 625 mg of N-ethyl maleimide. This reaction mixture was allowed to stand at room temperature for 1 hour and then brought to dryness in a flash evaporator at 30°C. The solid residue was crystallized from 90 percent ethanol. The yield was 950 mg (77 percent of theory). Five-hundred mg of this product was twice recrystallized from 80 percent ethanol. The recovery was 300 mg. The percentage composition found for this substance was: C, 44.18; H, 5.60; s, 12.93; N, 11.20. That calculated for $C_9H_{14}O_4N_2S$ was C, 43.90; H, 5.69; S, 13.01; N, 11.38.

Conversion of S-Cysteino-(N-Ethyl)-Succinimide to S-Cysteino-succinic Acid.– To 10 ml of water was added 24.6 mg of S-cysteino-(N-ethyl)-succinimide. One ml aliquots of the resulting solution were placed in Pyrex hydrolysis tubes and lyophillized to dryness. Three ml of constant boiling hydrochloric acid was added to each residue, and the tubes were evacuated, sealed and placed in a 105°C oven for varying lengths of time. On completion of the hydrolysis, the solutions were evaporated to dryness in vacuo over sodium hydroxide. These samples were dissolved in 10 ml of pH 3.25 citrate buffer. One ml samples

then were placed on the Stein-Moore column and the amount of S-cysteinosuccinic acid determined from the elution patterns.

Yields of S-Cysteinosuccinic Acid from N-Ethyl Maleimide-Treated Sweet Potato β-Amylase.- The enzyme solutions were dialyzed against phosphate buffer (pH 8.0, 0.1M). To the enzyme solutions (15 to 20 mg in 5 to 10 ml of solution) was added an equal volume of 2×10^{-3}M N-ethyl maleimide. The reactions were allowed to proceed to approximately 95 percent inhibition. The reaction period was then followed by either: 1. Dialysis against redistilled water, 2. passing the solution through a 1 x 10 cm column of mixed-bed resin (amberlite, MB-1), or 3. adjusting the pH of the solution to 4.5, followed by dialysis against 0.1M acetate buffer (pH 4.5) and then dialysis against redistilled water. The resulting solutions were placed in hydrolysis tubes and lyophillized. Constant boiling hydrochloric acid then was added to the residues, and the tubes were evacuated, sealed and placed in a 105°C oven for 72 hours. The hydrolysates were brought to dryness in vacuo over sodium hydroxide and made to a known volume with pH 3.25 citrate buffer. A sample of these solutions was placed on the Stein-Moore column and another fraction was used for micro-Kjeldahl nitrogen determinations.

The reaction mixtures from the spectrophotometric analysis of N-ethyl maleimide and β-amylase in 8M urea were exhasutively dialyzed against redistilled water and treated in a similar manner for amino acid analysis.

Experiments with N-Ethyl Maleimide on other Proteins

Experiments with Aldolase.- Approximately 1 ml of the aldolase

suspension was dialyzed against a pH 7.8 ethylenediaminetetracetic acid buffer (10^{-3}M) and passed through a 1 x 10-cm column of mixed-bed resin (Amberlite, MB-1) (39). The effluent protein solution was perevaporated in the cold to 3 or 4 ml, and dialyzed against a pH 6.8 phosphate buffer (0.1M). To 2 ml of the enzyme solution was added 1 ml of a 3 x 10^{-3}M N-ethyl maleimide solution, and the decrease in absorbance at 300mμ and 30°C was followed in the Beckman DU spectrophotometer. The concentration of aldolase was determined from its absorbance at 280 mμ since the absorbance at 280 mμ divided by 0.91 equals mg of aldolase per ml (39). Each reaction mixture of 3 ml volume contained approximately 10 mg of aldolase.

Experiments in 6M urea were performed by adding 1 ml of the enzyme solution to 2 ml of 9M urea which was 1.5 x 10^{-3}M in N-ethyl maleimide, and the reactions followed in the Beckman DU spectrophotometer. On completion of the reactions as determined spectrophotometrically, the protein solutions were exhaustively dialyzed against redistilled water and lyophillized. Constant boiling hydrochloric acid (3 to 4 ml) was added to the residue and the tubes were evacuated, sealed, hydrolyzed for 72 hours in a 105°C oven and the samples diluted to a known volume with pH 3.25 citrate buffer. Aliquots of these solutions were placed on the Stein-Moore column and the amounts of S-cysteinosuccinic acid determined. The protein content of these solutions was calculated from: 1. the amino acid analysis and correlation of these values to the values reported by Rutter (59), and 2. micro-Kjeldahl nitrogen determinations. A value of 16.8 percent nitrogen was used in calculating mg of protein from mg of nitrogen (60).

Experiments with Ovalbumin.– Samples of ovalbumin (10 to 20 mg in 5 ml) were dialyzed in the cold against the desired buffer. To 2 ml aliquots of the protein solutions were added 1 ml aliquots of 3×10^{-3}M N-ethyl maleimide and the reactions followed in the Beckman DU spectrophotometer at 300mμ. Experiments were performed at pH 4.5 in 0.1M acetate buffer, and at pH 6.8 in 0.1M phosphate buffer.

Experiments in 8M urea were performed by first adjusting the pH of the urea solution to pH 4.5. Approximately 25 mg of ovalbumin was dissolved in 10 ml of the urea solution. To 3 ml of this protein solution was added 0.5 ml of 3.5×10^{-3}M N-ethyl maleimide and the reaction was followed spectrophotometrically at 300 mμ in the Beckman spectrophotometer. A standard curve of protein concentration versus absorbance at 280 mμ was prepared and served for determining the amount of protein present in a reaction. The method for determining the amount of S-cysteinosuccinic acid was identical to that followed in the aldolase experiments. Protein concentrations of the hydrolysates were calculated from the amino acid concentrations and the amino acid composition reported in the literature (61). A molecular weight of 44,500 for ovalbumin was used in determining the moles of sulfhydryl that reacted per mole of protein.

Experiments with β-Lactoglobulin.– Experiments were carried out in 8M urea, pH 6.8, 0.1M phosphate, and the conditions were identical to the ovalbumin experiments. The molecular weight was taken to be 35,500, the absorbance at 280 mμ of a 1 percent solution as 9.6 (55), and the percent nitrogen as 15.6 (62).

One experiment was performed in which the concentration of N-ethyl maleimide was 10^{-1}M. Approximately 25 mg of protein was dissolved in 10 ml of phosphate buffer (0.2M, pH 8.0) and 125 mg of N-ethyl maleimide was added. The reaction was allowed to proceed at 30°C for 24 hours. After this the solutions were dialyzed against redistilled water and treated as previously described for amino acid analysis.

Experiment with Polylysine.– A sample of polylysine (146 mg in 10 ml of phosphate buffer, 0.2M, pH 7.5) was added to 62.5 mg of N-ethyl maleimide and the reaction was allowed to proceed at 30°C for 24 hours. The solution was exhaustively dialyzed against redistilled water, lyophillized, hydrolyzed for 72 hours and an aliquot of the hydrolysate placed on the Stein-Moore column.

III. RESULTS AND DISCUSSION

1. Correlation of Protein Concentration to Absorbance at 280 mμ

Table I. Correlation of Protein Concentration to Absorbance at 280 mμ

Mg of Protein per ml	Absorbance at 280 mμ
0.057	0.082
0.096	0.142
0.192	0.276
0.384	0.564

From these results it was calculated that the absorbance of a 1 percent solution is 14.4. This figure is not in agreement with that reported by Englard and Singer (5). These workers obtained a value of 17.1 based on a value of 13.5 for the percent nitrogen of the enzyme. When Englard and Singer's data is recalculated using 15.41 as the value for the percent nitrogen, the absorbance value for a 1 percent solution becomes 14.9. Balls et al (4) reported that sweet potato β-amylase contains 15.1 percent nitrogen. This figure agrees favorably with the value of 15.41 which was employed in these studies (1). The correlation of protein concentration to absorbance at 280mμ provided a rapid and accurate method of determining protein concentrations, and was employed throughout as a means of determining protein content.

2. Purification of β-Amylase on Sephadex G-75

Figure 1 shows that a purification of the enzyme is obtained on elution from the dextran gel, Sephadex G-75, with a small, inactive

peak appearing after the active component. This operation increases the activity of the β-amylase approximately 15 percent and yields a preparation which is homogeneous in the ultracentrifuge.

3. Results of the Amino Acid Analysis

The results of the amino acid analysis are reported in Tables II and III. Varying the length of hydrolysis indicated that threonine, serine, and tyrosine undergo a significant amount of decomposition. Therefore, the amounts of these amino acids were calculated by extrapolation to zero time. On the other hand, valine and isoleucine increased with time, and the true values were taken to be the 72-hour values.

Half-cystine and histidine are present in the lowest amounts. The molecule contains 22 half-cystine and 19 histidine residues. The acidic amino acids, aspartic acid and glutamic acid, comprise 20 percent of the molecule. Assuming that the ammonia arises from hydrolysis of the amides, glutamine and asparagine, it can be stated that approximately one-half of glutamic acid and aspartic acid is present as these amides.

4. Action of the Exopeptidases on Sweet Potato β-Amylase

Carboxypeptidase A.- Carboxypeptidase A is a pancreatic exopeptidase which catalyzes the hydrolysis of peptide bonds from the carboxyl terminus of proteins. The enzyme shows maximal activity towards carboxyl-terminal aromatic amino acids but is completely inactive toward substrates containing carboxyl-terminal arginine, lysine, or proline. It was reported previously (1) that proteolytic action of carboxypeptidase A

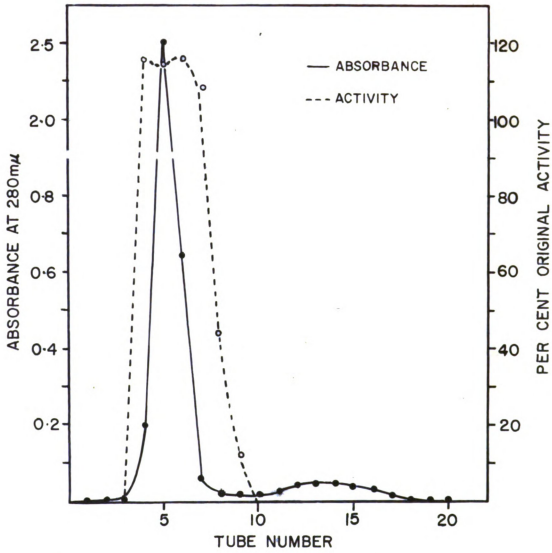


Figure 1. Purification of β-amylase on Sephadex G-75.

Table II. Amino Acid Content of Sweet Potato β-Amylase

Acid	13 hour[1]	48 hour	72 hour[1]	Purified 72 hour	Average
Aspartic	14.18±.23	14.29±.12	14.16±.16	13.98±.06	14.16±.23
Threonine	3.41±.06	2.97±.02	2.77±.14	2.78±.11	3.56[2]
Serine	3.31±.15	2.90±.03	2.64±.20	2.77±.07	3.48[2]
Glutamic	11.08±.23	11.15±.09	11.02±.15	11.09±.56	11.11±.27
Proline	5.04±.12	5.11±.22	5.20±.17	5.05±.24	5.10±.17
Glycine	4.15±.18	4.22±.04	4.30±.09	4.22±.14	4.23±.10
Alanine	5.31±.11	5.18±.04	5.13±.15	5.02±.16	5.14±.16
1/2 Cystine	1.51±.13	1.44±.13	1.43±.18	1.61±.15	1.50±.15
Valine	5.12±.12	5.32±.11	5.68±.19	5.69±.20	5.69[3]
Methionine	4.14±.26	4.16±.06	3.96±.13	4.31±.18	4.14±.22
Isoleucine	4.06±.11	4.23±.15	4.46±.09	4.44±.29	4.45±.25[3]
Leucine	8.14±.10	8.10±.15	8.14±.15	7.91±.18	8.06±.16
Tyrosine	6.33±.44	6.04±.13	5.85±.22	5.92±.22	6.52[2]
Phenylal	6.14±.25	6.17±.13	6.01±.12	6.21±.29	6.13±.22
Lysine	6.27±.15	6.36±.10	6.23±.06	6.32±.08	6.31±.10
Histidine	1.61±.05	169 ±.08	169 ±.05	1.80±.06	1.68±.08
Arginine	4.72±.18	4.41±.08	4.44±.10	4.59±.08	4.54±.15
Ammonia -	1.61±.08	1.85±.06	2.22±.08	1.86±.06	1.49[2]

[1]Taken from (I).

[2]Extrapolated to zero time.

[3]72 hour values taken.

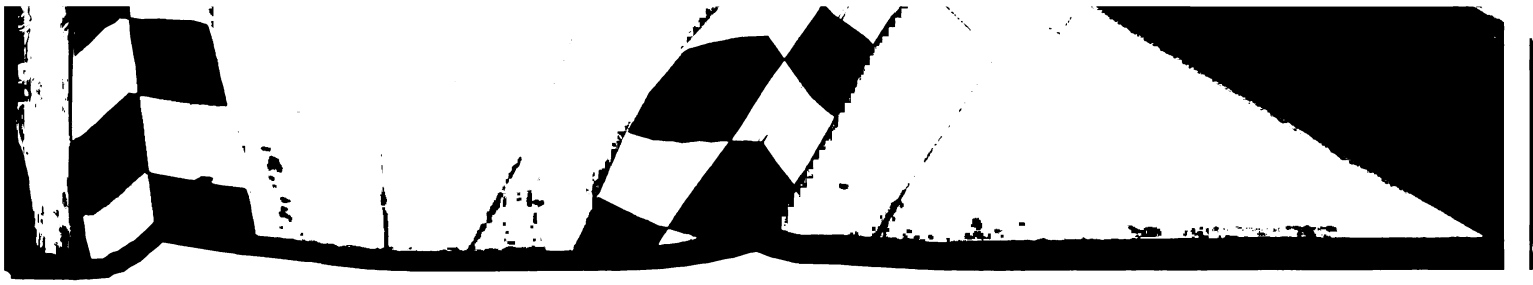


Table III. Amino Acid Content of Sweet Potato β-Amylase

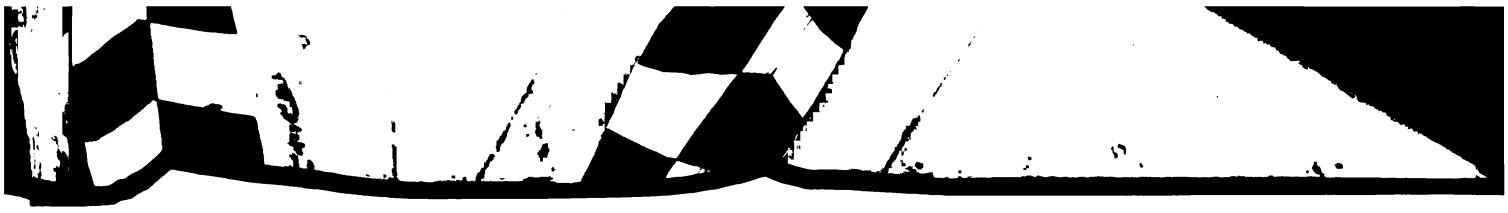
Amino Acid	Gm. of A.A. Residues Per 100 gm. Protein	Percent of Total Nitrogen	Number of Residues per mole(152,000)
Aspartic	14.16	11.16	187
Serine	3.56	3.19	54
Threonine	3.48	3.63	61
Glutamic	11.11	7.82	131
Proline	5.10	4.76	80
Glycine	4.23	6.75	113
Alanine	5.14	6.55	110
Valine	5.69	5.23	87
1/2 Cystine	1.50	1.35	22
Methionine	4.14	2.87	48
Isoleucine	4.45	3.56	60
Leucine	8.06	6.47	108
Tyrosine	6.52	3.63	61
Phenylalanine	6.13	3.78	63
Lysine	6.31	8.95	75
Histidine	1.68	3.34	19
Arginine	4.54	10.45	44
Ammonia	1.49	8.43	142
TOTALS	97.29	101.92	1,323

on β-amylase resulted in a 20 to 25 percent increase in β-amylase activity. It was desirable to check this observation and, if possible, to determine the amino acids released by carboxypeptidase A action on β-amylase. Table IV summarizes the study of the action of carboxypep tidase A on the unpurified enzyme.

Table IV. Action of Carboxypeptidase A on Unpurified β-Amylase

Digestion Period (Hours)	Activity (Absorbance at 540 mμ)	Non-Protein Nitrogen (Absorbance at 570 mμ)
	(Run No. 1)	
0	.148 .139	.160
1/2	.172 .168	.185
1	.175	.195
2	.185 .192	.222
	(Run No. 2)	
0	.395 .405	.530
1 1/2	.470 .480	.558
6	.480	.580

These results support the previously reported fact (1), that the action of carboxypeptidase A on unpurified β-amylase causes a striking increase in β-amylase activity. There was also an increase in non-protein nitrogen. Chromatograms of these reaction mixtures showed the release of tyrosine, serine, and asparagine. It was noted, as may be seen from the zero time non-protein nitrogen values that the β-amylase contained impurities. A zero time chromatogram (no carboxypeptidase A)

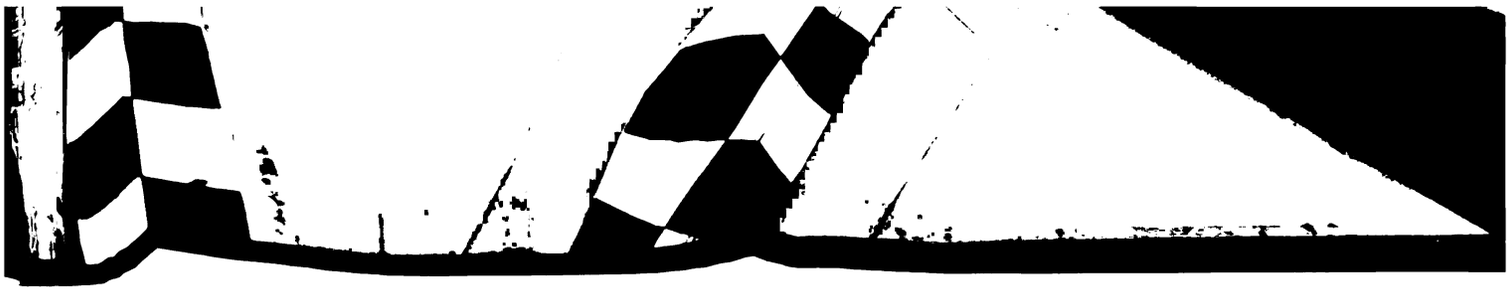


indicated the presence of small amounts of tyrosine, alanine and valine. In order to obtain more meaningful results, it was necessary to obtain a purer protein substrate. Therefore, solutions of β-amylase were passed through Sephadex G-75, followed by a mixed bed resin treatment (Amberlite MB-1).

Table V. Action of Carboxypeptidase A on Purified β-Amylase

Digestion Time (Hours)	Activity (Absorbance at 540 mμ)	Non-Protein Nitrogen (Absorbance at 570 mμ)
	(Run No. 1)	
0	.258 .260	
2	.256 .258	
4	.256 .260	
6	.260 .258	
	(Run No. 2)	
0	.235 .240	.145
1	.240 .245	.160
2	.235 .235	.140
4	.240 .235	.125
6	.225 .235	.160

Purified β-amylase appears to be unchanged upon carboxypeptidase A treatment, since no noticeable alteration in activity occurs and no non-protein nitrogen is released on such treatment. The increase in activity noted with the impure β-amylase may be explained by assuming the presence of an impurity. It is reasonable to postulate that the impurity acts as an inhibitor by masking the active site. On degradation



of this inhibitor by carboxypeptidase A the active site is rendered free for the catalytic process.

The lack of degradation of purified β-amylase by carboxypeptidase A may be explained in either of two ways: 1. the tertiary structure of β-amylase does not permit hydrolysis by carboxypeptidaseA, or 2. the carboxyl-terminal group does not conform to the specificity requirements of the enzyme. To test the latter thought it was decided to test the action of carbosypeptidase B on β-amylase.

Carboxypeptidase B.- Table VI indicates that carboxypeptidase B has no effect on the catalytic activity of β-amylase. Nevertheless, carboxypeptidase B catalyzes the hydrolysis of amino acids from the carboxyl-teminus of β-amylase as evidenced by the large increase in the ninhydrin color reaction of the non-protein nitrogen fraction. The combined effect of carboxypeptidases A and B is even more striking. Once again the catalytic activity of the β-amylase is unaffected, but the ninhydrin color reaction of the non-protein nitrogen fraction indicates the release of approximately 30 moles of amino acids per mole of β-amylase.

Further investigations into the exact extent of degradation by these carboxypeptidases could prove to be very fruitful, since it may be possible to obtain a pure substance which would possess β-amylase activity and differ from the native enzyme in many physical properties

Leucine Aminopeptidase.- An attempt at degrading sweet potato β-amylase from the amino-terminus of the molecule is reported in Table VII.

Table VI. Action of Carboxypeptidase B on β-Amylase

Digestion Time (Hours)	Activity (Absorbance at 540 mμ)		Non-protein Nitrogen (Absorbance at 570 mμ) β-Amylase	Carboxypeptidase
	(Run No. 1)			
0	.450	.455		
.5	.452	.444		
2	.438	.435		
3	.448	.435		
	(Run No. 2)			
0	.220	.210	.220	
.5	.220	.220		
1	.215	.220		
2	.215			
4	.220	.210	1.35	.710
4[1]	.190	.195		
5	.180	.185		
6	.195	.190	1.55(diluted)	.640
8	.180	.185	1.65	.650

Table VII. Action of Leucine Aminopeptidase on Sweet Potato β-Amylase

Digestion Time (Hours)	Activity (Absorbance at 540 mμ)		Non-Protein Nitrogen (Absorbance at 570 mμ)	
			Leucine Aminopeptidase	-amylase
0	.268		.185	.125
.5	.270	.274		
1	.266	.264		
2	.272	.274		
4	.268			
6	.266	.262	.200	.115
12	.280	.264	.215	.130

Leucine aminopeptidase appears to have no effect on β-amylase. The resistance of the β-amylase to such proteolysis can be attributed to the tertiary structure of the β-amylase molecule. Hill and Smith (63) report that lysozyme and ribonuclease are completely resistant to the catalytic hydrolysis of leucine aminopeptidase. Nevertheless, when these proteins are oxidized by performic acid and then treated with leucine aminopeptidase, considerable amino acid release is noted..

5. Inhibitors

Inhibition of Sweet Potato β-Amylase by p-Chloromercuribenzoate.- β-Amylase, as is shown in Figure 2 is completely inactivated by p-chloromercuribenzoate. The plots of inactivation at pH 4.5, 6.8, and 8.0 are different and this probably indicates a varying selectivity of reaction with the available sulfhydryl groups. At pH 4.5, the protein precipitated from solution as the alkylation by p-chloromercuribenzoate

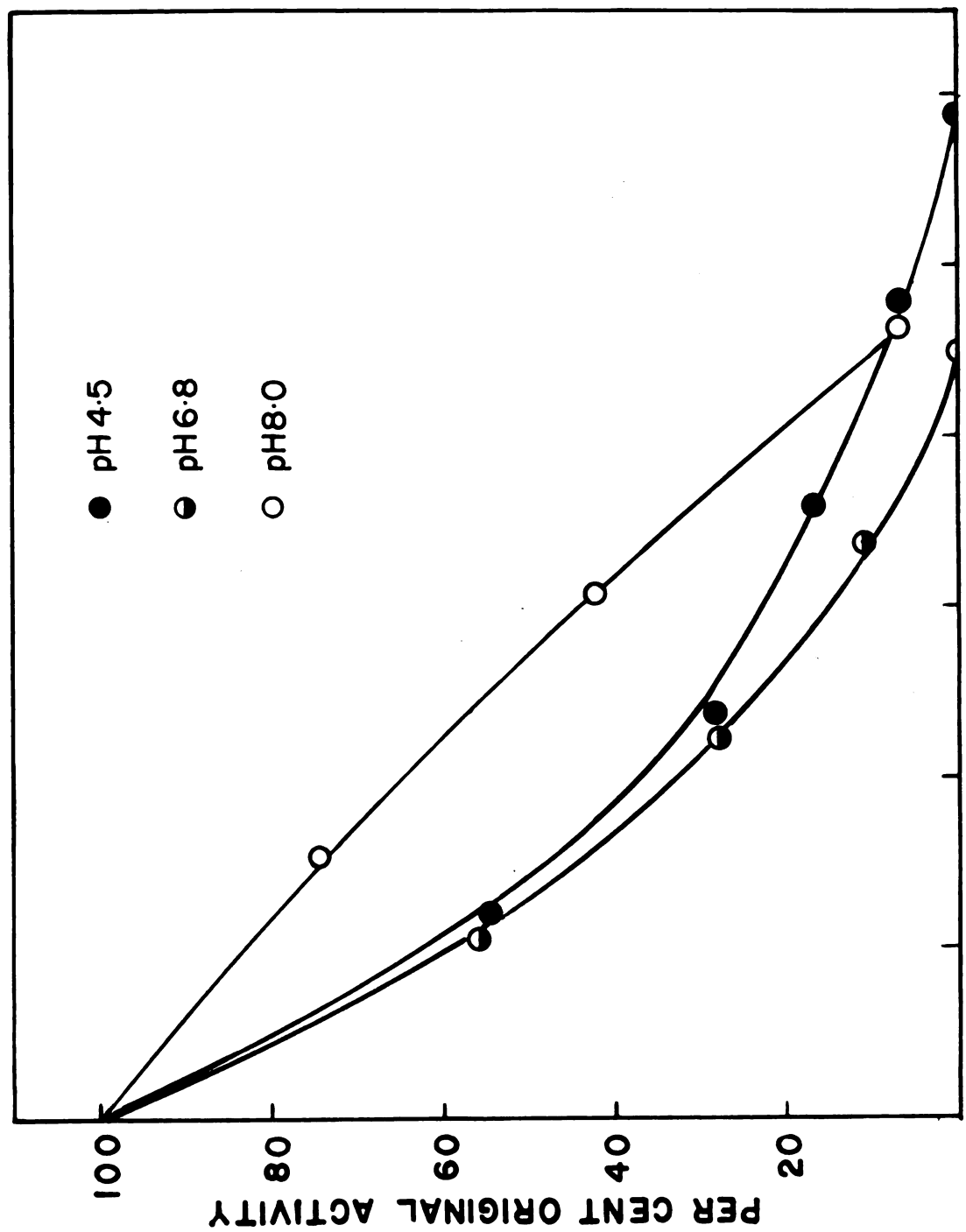
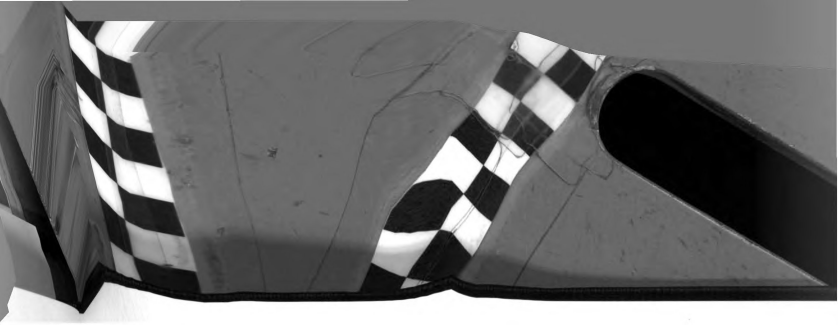


Figure 2. Correlation of sulfhydryl group reaction with inhibition of β-amylase by p-chloromercuribenzoate.



approached six sulfhydryl groups. Results from the pH 6.8 and 8.0 plots indicate that 50 percent inactivation of β-amylase corresponds to the alkylation of one sulfhydryl group.

Table VIII. Spectrophotometric Determination of p-Chloromercuribenzoate with β-Amylase

pH	Number of Sulfhydryl Groups Undergoing Reaction
8.0	6.0, 5.9, 6.3
6.8	6.1, 6.0
4.5	5.9, 5.0

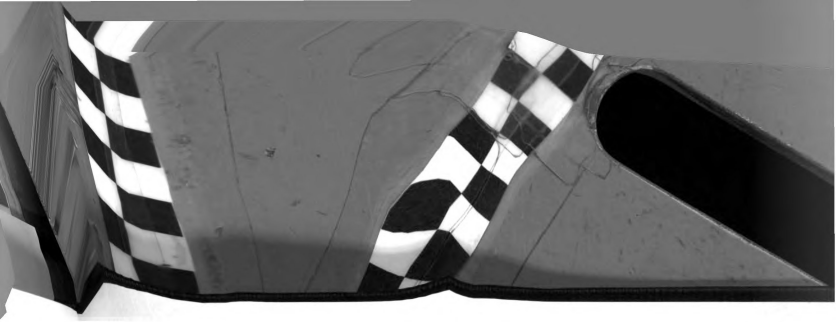
Table VIII shows that the maximum number of sulfhydryl groups as determined with this reagent is six. This fact should not be interpreted as indicating the absence of unreacted sulfhydryl groups. Boyer (55) noted that reaction between aldolase and p-chloromercuribenzoate resulted in the alkylation of 8 cysteine residues in aqueous solution, and 28 in 6M urea.

Reactivation of p-Chloromercuribenzoate and Mercuric Chloride Inhibited β-Amylase.– Englard and Singer (6) reported that 50 percent reactivation was obtained when a twenty-fold excess of glutathione to mercuric chloride was added to the mercuric chloride inactivated β-amylase. It was of interest to examine the possibility of 100 percent reactivation since this would support the theory that inactivation was a result of sulfhydryl group reaction. As may be seen in Table IX, 100 percent reactivation was obtained when a mixture of versene and cysteine was added to the inactive enzyme.

Table IX. Reactivation of β-Amylase Inhibited by p-Chloromercuribenzoate and Mercuric Chloride

β-Amylase Conc. Moles per 1	Mercuric Chloride Conc. Moles per 1	Versene Conc. Moles per 1	Cysteine Conc. Moles per 1	Activity Absorbance 540 mμ	Percent Reactivation
3.3×10^{-6}	0	0	0	.148	
5.5×10^{-6}	3.75×10^{-5}	0	0	0	
$3.3\times.0^{-6}$	2.5×10^{-5}	2.5×10^{-4}	0	0	0
3.3×10^{-6}	2.5×10^{-5}	0	2.5×10^{-3}	.132,.136	90
3.3×10^{-6}	2.5×10^{-5}	2.5×10^{-4}	2.5×10^{-3}	.148	100
β-Amylase Conc. Moles per 1	p-Chloromercuri-benzoate Conc. Moles per 1	Versene Conc. Moles per 1	Cysteine Conc. Moles per 1	Activity Absorbance 540 mμ	Percent Reactivation
3.3×10^{-6}	0	0	0	.146	
5.5×10^{-6}	3.75×10^{-5}	0	0	0	
3.3×10^{-6}	2.5×10^{-5}	2.5×10^{-4}	0	0	0
3.3×10^{-6}	2.5×10^{-5}	0	2.5×10^{-3}	.132	90
3.3×10^{-6}	2.5×10^{-5}	2.5×10^{-4}	2.5×10^{-3}	.146	100

Inhibition of β-Amylase by N-Ethyl Maleimide.– Figure 3 shows that the most rapid inactivation of the enzyme by N-ethyl maleimide occurs at pH 8.0 and 8.5. Alexander has shown that N-ethyl maleimide reaction with sulfhydryl compounds can be followed spectrophotometrically at 300 mμ (28). The decrease in absorbance at 300 mμ can be used as an assay method since, when present in excess, N-ethyl maleimide reacts stoichiometrically with sulfhydryl compounds. It was of interest to employ this method to obtain a correlation of inhibition to the number of sulfhydryl groups undergoing reaction with N-ethyl maleimide. These



results are shown in Figure 4, and again it is noted that 50 percent inactivation arises when one sulfhydryl group has reacted.

Table X. Spectrophotometric Determination of N-Ethyl Maleimide with β-Amylase

pH	Number of Sulfhydryl Groups Undergoing Reaction
8.0	3.7, 3.8

As may be seen from Table X, N-ethyl maleimide reacts with 4 cysteine residues of β-amylase. This is a lower value than that obtained with p-chloromercuribenzoate. Roberts and Rouser (64) found that N-ethyl maleimide reacted with only 60 percent as many cysteine residues in serum albumin as did p-chloromercuribenzoate.

Action of Other Inhibitors on β-Amylase.— Iodoacetic acid, maleic acid and sodium arsenite show no inhibitory effect on the catalytic activity of β-amylase.

Table XI. Action of Other Inhibitors on β-Amylase

Time	Iodoacetic Acid (10^{-2}M) pH 4.5	pH 7.5	Maleic Acid (10^{-3}M) pH 8.0	Sodium Arsenite (10^{-2}M) pH 6.8
0	.340	.352	.446	.242
.5			.450	.248
1	.334	.348	.446	.240
2	.332	.351	.455	.245
4	.336	.349	.454	

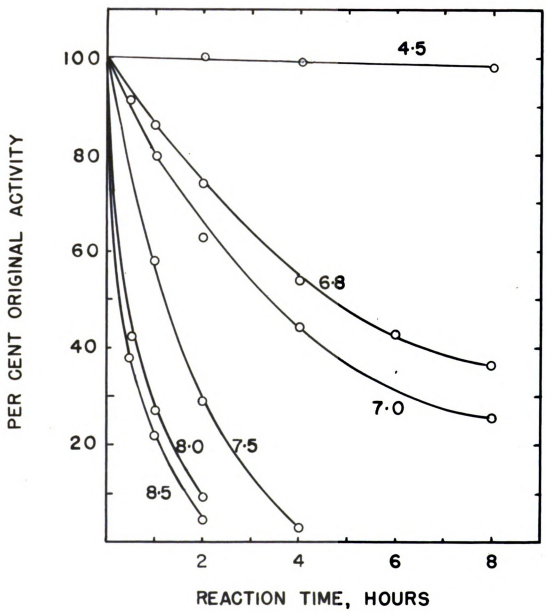


Figure 3. Inhibition of sweet potato β-amylase by N-ethyl maleimide.

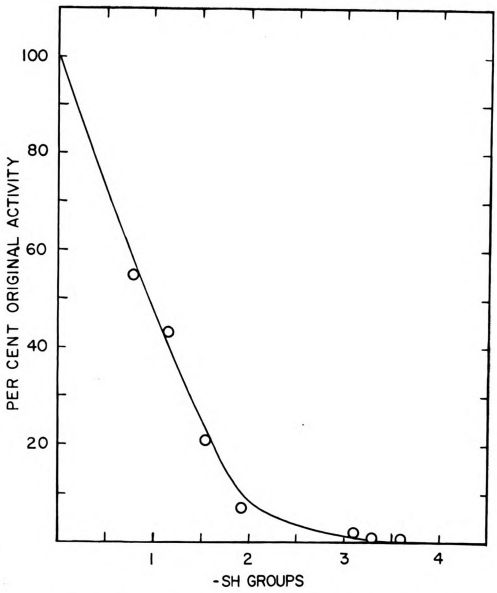


Figure 4. Correlation of sulfhydryl group reaction with inhibition of β-amylase by N-ethyl maleimide at pH 8.0.

6. Quantitative Determination of S-Cysteinosuccinic Acid from N-Ethyl Maleimide-treated Proteins

These experiments involved: 1. preparation of S-cysteinosuccinic acid and a study of its elution in the Stein-Moore analysis for amino acids, 2. preparation of S-cysteino-(N-ethyl)-succinimide and studies of its conversion to S-cysteinosuccinic acid under conditions employed for protein hydrolysis, and 3. treatment of various proteins with N-ethyl maleimide and observations into the yield of S-cysteinosuccinic acid in the hydrolysates of the N-ethyl maleimide-modified proteins.

Studies on S-Cysteinosuccinic Acid.- Figure 5 illustrates the appearance of 1 μmole of S-cysteinosuccinic acid with respect to aspartic acid (0.5 μmole) on elution in the Stein-Moore separation from a 150-cm column. It should be noted that S-cysteinosuccinic acid is eluted approximately 50 ml prior to the appearance of aspartic acid, at which position there should be no interference from other ninhydrin positive compounds arising from a normal protein hydrolysate.

Since the addition of cysteine across the double bond of maleic acid should give rise to diastereoisomers, two ninhydrin positive peaks may be expected when S-cysteinosuccinic acid is separated in the Stein-Moore analysis. Nevertheless, as shown in Figure 5, only one peak is found. Attempts to separate the single peak into two entities by lowering the pH of the eluting buffer to pH 3.0 resulted in tailing and no separation.

Table XII shows the amount of color produced when standard solutions of S-cysteinosuccinic acid are reacted with ninhydrin. These readings

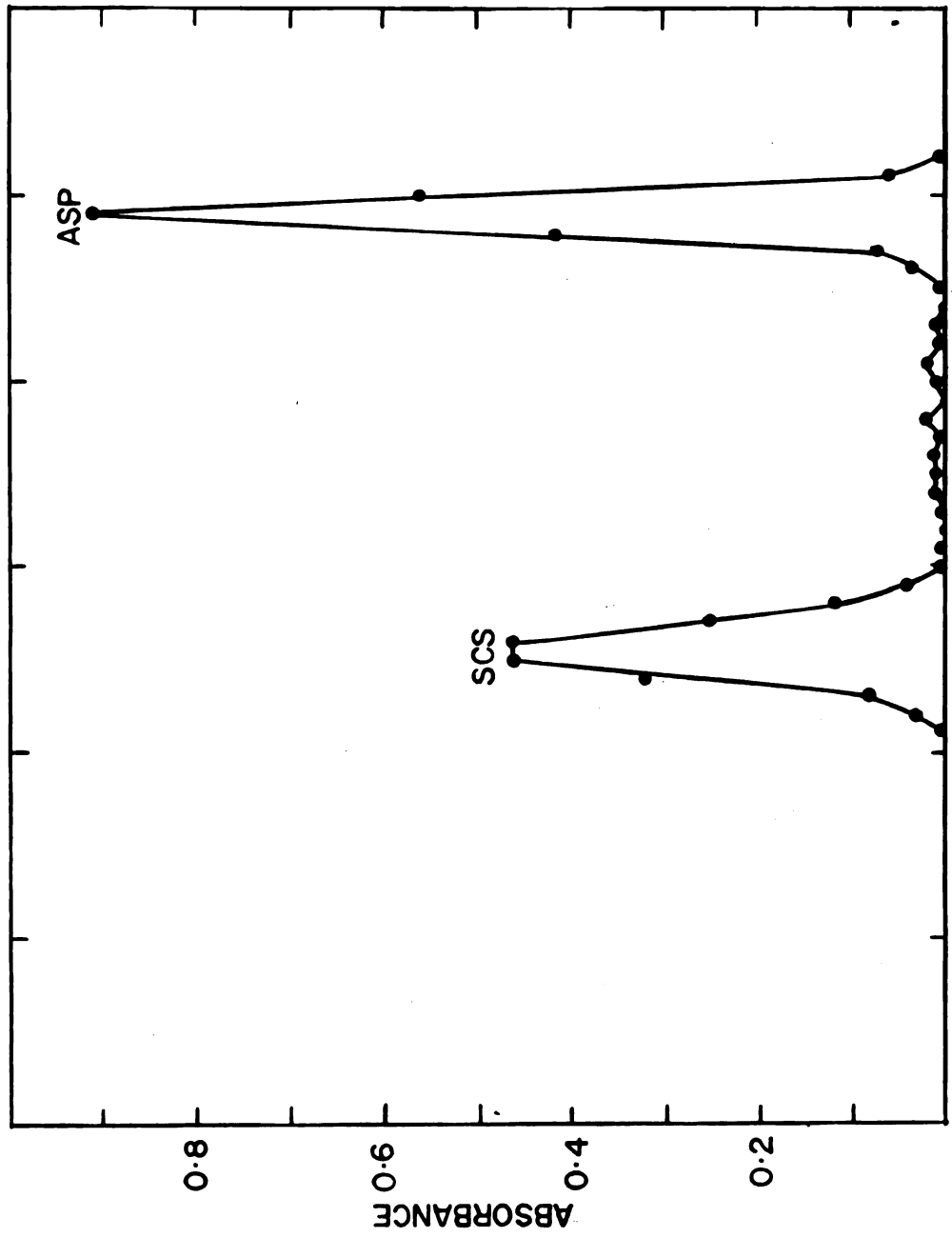


Figure 5. Elution curve of S-cysteinosuccinic acid from a 150-cm Stein-Moore column.

were placed in a graph and all unknown amounts of S-cysteinosuccinic acid were calculated from this curve.

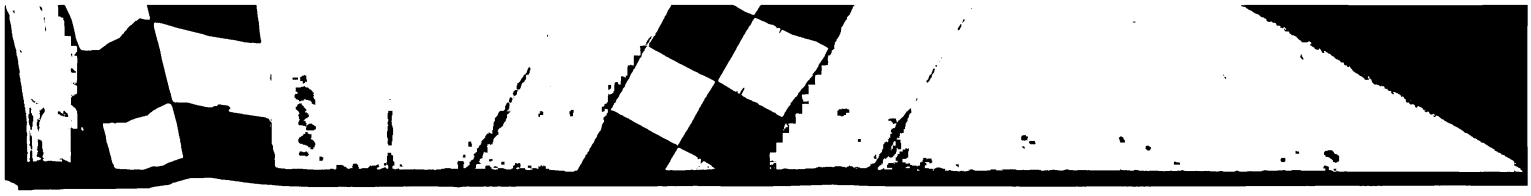
Table XII. Color Yield from the Reaction Between Ninhydrin and S-cysteinosuccinic Acid

Micromoles of S-Cysteinosuccinic Acid	Absorbance at 570 mμ	
	Diluted with 10 ml of 50 percent Ethanol	Diluted with 15 ml of 50 percent Ethanol
0.1	.187, .190	
0.2	.365, .375	
0.3	.540, .545	.385, .385
0.6		.775, .780

Studies on S-Cysteino-(N-ethyl)-succinimide.- Elution patterns of one μmole of S-cysteino-(N-ethyl)-succinimide from a 30-cm column of Amberlite CG-120 show the presence of two ninhydrin positive peaks. Hence it is postulated that the diastereoisomers resulting from the addition of cysteine to the double bond of N-ethyl maleimide are separable. These peaks are approximately of equal size.

Table XIII. Conversion of S-Cysteino-(N-ethyl)-succinimide to S-Cysteinosuccinic Acid

Time of Hydrolysis (Hours)	Percent Conversion to S-Cysteinosuccinic Acid
24	44
72	87
120	94



A study of the conversion of S-cysteino-(N-ethyl)-succinimide to S-cysteinosuccinic acid is reported in Table XIII. These data indicate that S-cysteino-(N-ethyl)-succinimide is quite stable to acid hydrolysis. An elution pattern from a 30-cm column of Amberlite CG-120 supported this fact. Figure 6 is an elution pattern of a 72 hour hydrolysate of 1 μmole of S-cysteino-(N-ethyl)-succinimide. The first ninhydrin positive compound to appear is S-cysteinosuccinic acid and it represents 87 percent conversion from S-cysteino-(N-ethyl)-succinimide. Aspartic acid, which was added as a tracer, follows S-cysteinosuccinic acid, and finally the diastereoisomers of S-cysteino-(N-ethyl)-succinimide are eluted. Since the protein hydrolyses were carried out for 72 hours, all S-cysteinosuccinic acid values were divided by 0.87 to correct for incomplete hydrolysis of S-cysteino-(N-ethyl)-succinimide.

Reaction of N-Ethyl Maleimide with Proteins.– Table XIV reports the results of the spectrophotometric method, and the yields of S-cysteinosuccinic acid from hydrolysates of N-ethyl maleimide-treated proteins. In every instance the two methods compare favorably, indicating that under conditions which are suited for the spectrophotometric analysis, the reaction between N-ethyl maleimide and proteins appears to be limited to reaction with sulfhydryl groups. Complete acidic and neutral amino acid elution patterns were performed on all of the N-ethyl maleimide treated proteins. In each case the only new peak was S-cysteinosuccinic acid.

The sulfhydryl content of aldolase as calculated with this reagent compares favorably with the values obtained by the action of p-chloromercuribenzoate on this molecule (55). β-Amylase, in 8M urea, shows the presence of 15 to 16 cysteine residues. Amino acid analysis indicates

Table XIV. Comparison of the Spectrophotometric Method with Yields of S-Cysteinosuccinic Acid

Protein	Reactions Conditions	Number of Sulfhydryl Groups Undergoing Reaction as Measured: Spectrophotometrically	By Yields of S-Cysteinosuccinic Acid
Ovalbumin	0.1M acetate buffer, pH 4.5	0	0
Ovalbumin	0.1M phosphate buffer, pH 6.8	0	--
Ovalbumin	8M urea, 0.1M acetate buffer, pH 4.5	2.9 2.9	2.9 3.0
β-Lactoglobulin	8M urea, 0.1M phosphate buffer, pH 6.8	2.0 2.1	2.1 1.9
Aldolase	0.1M phosphate buffer, pH 6.8	7.7 7.8	7.9 8.1
Aldolase	6M urea, 0.1M phosphate buffer, pH 6.8	27.1 27.3	26.2 --
β-Amylase	0.1M phosphate buffer, pH 8.0	3.7 3.8	4.1 3.9
β-Amylase	8M urea, 0.1M phosphate buffer, pH 8.0	15.2 15.5	15.5 15.0

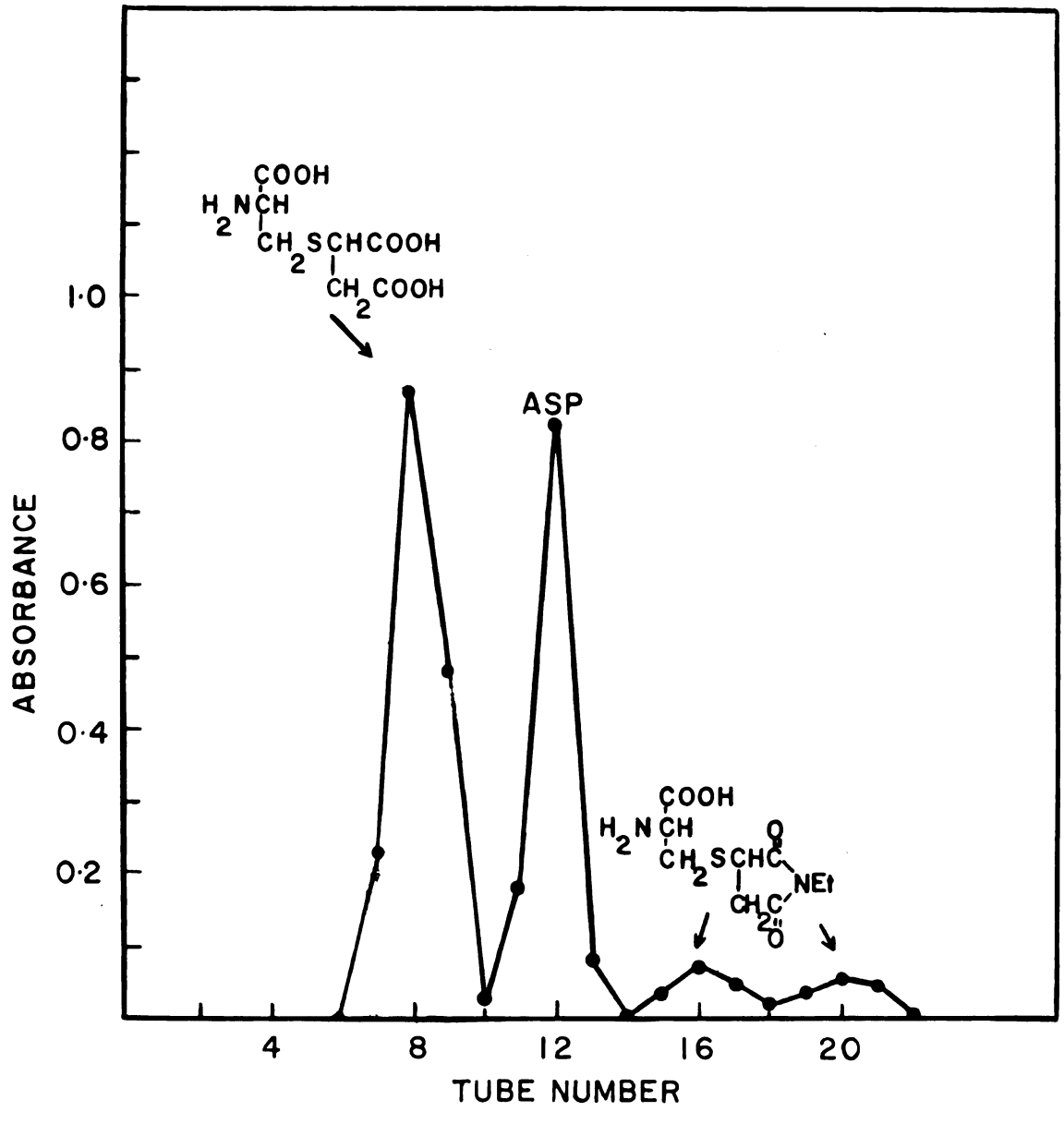
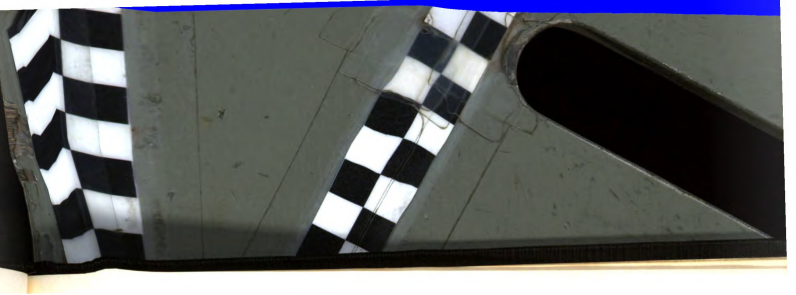


Figure 6. Elution curve of a 72-hour acid hydrolysate of S-cysteino-(N-ethyl)-succinimide from a 30-cm Stein-Moore column.

the presence of 22 half-cystine residues. Hence, it can be concluded that the β-amylase molecule contains 3 disulfide linkages.

Reactions in 0.1M N-Ethyl Maleimide. Chromatography on an Amberlite CG-120 column of acid hydrolysates of β-lactoglobulin which were treated with 0.1M N-ethyl maleimide showed the appearance of a new peak which was eluted directly after proline. Polylysine, treated in an identical manner also gives rise to this peak. It is postulated that this entity arises from the alkylation of the ε-amino group of lysine; however, further investigation will be required before this can be established.



IV. SUMMARY

1. Purification of sweet potato β-amylase has been accomplished by elution of the enzyme from a Sephadex G-75 column.

2. A complete amino acid analysis of β-amylase is reported.

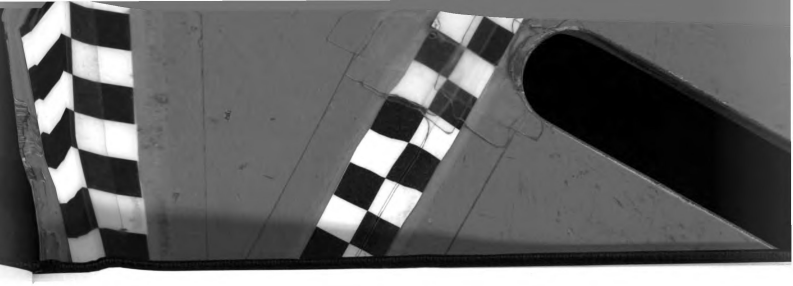
3. Sweet potato β-amylase is resistant to the proteiolytic attack of carboxypeptidase A and leucine aminopeptidase. This is illustrated by the complete retention of β-amylase activity and by the absence of free amino acids when these enzymes are allowed to react on β-amylase.

4. Carboxypeptidase B catalyses the hydrolysis of approximately 4 amino acids from the carboxyl-terminus of β-amylase. The combined action of carboxypeptidases A and B leads to a release of approximately 30 amino acids. Nevertheless, the activity of the β-amylase molecule is not affected.

5. p-Chloromercuribenzoate inhibits sweet potato β-amylase activity. This inhibition may be reversed by the addition of a mixture of cysteine and versene. A spectrophotometric analysis of the reaction between p-chloromercuribenzoate and β-amylase in aqueous solution, indicates that six sulfhydryl groups are attacked. These results also suggest that only one of these sulfhydryl groups is required for activity.

6. N-ethyl maleimide also inhibits the catalytic activity of β-amylase. Fifty percent inactivation in aqueous solution is characterized by the reaction of one sulfhydryl group. N-ethyl maleimide reacts with four sulfhydryl groups in aqueous solution, and 15 to 16 sulfhydryl groups in 8M urea.

7. The possibility of other reactions between N-ethyl maleimide and proteins, under conditions which are suited for the spectrophotometric analysis of sulfhydryl groups, has been excluded in the case of aldolase, β-amylase, ovalbumin, and β-lactoglobulin. In each instance the number of sulfhydryl groups undergoing reaction as determined spectrophotometrically compares favourably with the yields of S-cysteinosuccinic acid produced from acid hydrolysates of N-ethyl maleimide treated proteins.



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