PHYSICO - CHEMICAL PROPERTIES OF ASCORBATE OXIDASE ISOZYMES

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ABSTRACT

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By

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The ascorbic acid oxidase (AAO) present in the skin and flesh of the fruit of yellow summer squash (Cucurbita pepo condensa) and green zucchini squash (C. pepo medullosa) was separated by polyacrylamide gel disc electrophoresis into five different molecular forms. By the same technique three AAO isozymic forms were prepared from the cucumber (Cucumis sativus). Repeated electrophoresis of the isolated forms excluded the possibility that these forms were artifacts.

Molecular weight estimates and interconversion studies strongly suggested the presence of a monomeric unit of approximately 30,000 MW in the cucumber and 35,000 MW in the two squashes. A dimer and a tetramer also appeared in the cucumber, while a tetramer, an octamer (8-mer), a dodecamer (12-mer) and an X-mer (MW 670,000-2,000,000) appeared in the two squashes. The monomer represented 50% of the total AAO activity of the cucumber, the dimer 40% and the tetramer 10%. The

dominant AAO form in the two squashes appeared to be the tetramer with 70% of the total activity.

Mild heat (40°C, 5 min. pH 7.0) quantitatively converted the activity of the octamer, dodecamer and X-mer to the activity of the dimer. Depolymerization of some polymers was also effected by treatment with 7M urea, alkali (NaOH, pH 11.0, 30 min. 0°C) or acid (HCl, pH 3.6, 30 min. 0°C).

A reversible association-dissociation of the squash and cucumber isozymes was observed when the ionic strength of the solution was changed. Although the total AAO activity per gram of wet tissue was higher in the skin than in the flesh of all three commodities, the isozyme pattern was the same in the two tissues. The isoelectric point of all multiple forms in both squashes was the same, 5.35; the isoelectric point of the cucumber AAO forms was 6.70. Using a spectrophotometric and a manometric method six different K_m values were determined for the composite AAO forms of the three commodities. Differential and density gradient centrifugations indicated that all AAO forms were present in soluble form.

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INTRODUCTION

More than a decade ago occasional suggestions were made indicating heterogeneity for single enzymes, but these had little impact until simple, easy methods for assessing enzyme heterogeneity were developed and applied in a remarkably fruitful fashion, first to esterases (Hunter and Markert, 1957) and then to other enzymes including the widely studied lactate dehydrogenase (LDH) (Markert and Møller, 1959). These were fortunate choices, because the heterogeneity proved to be extensive and was easily recognized. Differences were found between comparable enzymes in different tissues of the same organism. In addition, a single tissue may yield several enzymes catalyzing the same reaction but having differences in their physical, chemical and kinetic properties. The multiple molecular forms have been distinguished from one another by electrophoresis, chromatography, salt fractionation, ultracentrifugation, immunoelectrophoresis and reaction kinetics.

The following terms have been used by different authors for the multiple forms: isoenzymes, isozymes, iso(enzyme's name) as isoamylases, electroophoretic

variants, multiple forms, multimolecular forms, electrophoretic components and polymorphs.

Since the first conference on multiple molecular forms of enzymes in 1961 an ever-increasing number of enzymes have been reported as existing in more than one molecular form. In fact, it appears that the enzyme existing in only one form is an exception.

The relationship of this phenomenon to the one-gene one-polypeptide theory and to the problem of cellular differentiation poses important biological questions which remain to be solved. The study of isozymes promises to expand our knowledge in a variety of fields ranging from embryology and the studies of evolution to physiology and pathology. The study of them has already proven that it has clinical diagnostic application (Kaplan et al., 1960).

Ascorbate oxidase (AAO), classified as L-ascorbate: O_2 Oxidoreductase, E.C.1.10.3.3, is a copper-containing protein, present in plant tissues and catalyzing the aerobic oxidation of vitamin C. Highly purified preparations of this enzyme from yellow summer squash (Cucurbita pepo condensa), green zucchini squash (C. pepo medullosa) (Dawson, 1966), and cucumber (Cucumis sativus), (Namakura et al., 1968) were reported to be 100 per cent homogeneous in electrophoresis and in ultracentrifugation. Recently, on the basis of gel filtration, the AAO of

cucumbers was separated into three different molecular forms (Porath et al., 1967).

The present study was undertaken in an attempt to elucidate the isozyme nature and properties of ascorbate oxidase.

REVIEW OF LITERATURE

Isozymes

Terminology

It was in 1959 (Markert and Møller) that the term "isozyme" was first suggested as a useful designation for the multiple molecular forms of an enzyme found within a single organism or the members of a single species (Markert, 1968). The term "isozyme" served primarily to focus attention on a significant but neglected biological phenomenon, namely that organisms synthesize many of their enzymes in several different molecular forms, presumably to fulfill specialized needs in different cells or in different parts of the cell's metabolism. A vast literature on isozymes accumulated during the last several years (Weyer, 1968).

Physiological Significance

The tissue specificity of isozyme pattern is convincing evidence that isozymes have biological significance and are not just biochemical curiosities. Nevertheless, the nature of the biological significance is quite ambiguous. Numerous investigations have demonstrated that isozymes differ in various kinetic

properties--reactivity within analogs of NAD, substrate specificity, turnover number, K_m and substrate concentration optimum, thermostability, urea denaturation, inhibition by excessive amounts of substrate (Plagemann et al., 1960; J. Allen, 1961; Kaplan and Ciotti, 1961; Plagemann et al., 1961; Withycomb et al., 1965; Vesell and Yielding, 1966 and 1968; Pesce et al., 1967). It is this last property that is usually selected as the key to biological significance.

A rather vague correlation can be observed between the preponderance of A subunits of LDH in a cell and the exposure of the cell to transient periods of anaerobiosis. Thus, most skeletal muscles of mammals contain a large preponderance of LDH-5 (the A_4 tetramer). On the other hand, those tissues receiving a relatively constant supply of oxygen, such as brain tissue and heart muscle, contain mostly B subunits--that is, mostly LDH-1 and LDH-2. This distribution makes physiological sense, because the A subunits continue to function even though very high concentrations of lactate may have been reached--a condition likely to occur in skeletal muscles. Heart muscle and brain tissues, however, never contain large amounts of lactate, presumably because of the rich supply of oxygen and also because the conversion of pyruvate to lactate by the LDH-1 predominant in these tissues would be progressively inhibited by the accumulating lactic acid. Thus a

negative feedback by-product inhibition would selectively regulate the activity of the individual isozymes and serve to keep the concentration of lactic acid within acceptable limits in terms of cell function.

Origins of Multiple Forms of Proteins

Many proteins formerly considered to be pure can now be resolved using new techniques (Whipple, 1964) into two or more distinct components (Colvin, 1954). Such proteins are said to exhibit "microheterogeneity." Epstein and Schechter (1968) summarized the known origins of this heterogeneity as follows:

A. Evolutionarily unrelated: "convergent" evolution
B. Evolutionarily related
    1. Genetically unrelated: "divergeut" evolution of duplicated genes
    2. Genetically related
        a. Covalent differences
            1) Introduced during translation
            2) Introduced after translation
                a) Deamination
                b) Attachment of carbohydrate
                c) Phosphorylation, sulfation
                d) α- and ε-NH_2acetyls, formyls, Schiff's bases
                e) Oxidation of sulfhydryl groups

f) Oxidation and reduction of prostetic groups

g) Cleavage of peptide chain

b. Mixed multimers

c. Noncovalent differences

1) Aggregation

2) Binding of small molecules

3) "Stable" conformational variants

Ascorbate Oxidase

The enzyme was first detected in 1928 (Szent-Györgyi). It was not until 1938 (Tauber) that the enzyme was sufficiently purified to justify the view that the oxidase was a copper protein. Dawson (1966) has written an excellent review on the physico-chemical properties of this enzyme.

Occurrence and Function

Investigations dealing with ascorbate oxidase activity in a large number of plants, plant products and microorganisms such as apple, barley, cucumber, grape, orange, potato, pea, squash, tomato, Chlorella pyrenoidosa, Aerobacter aerogenes etc. were listed (Dawson, 1966). Most of the research papers have involved studies designed to evaluate the role of the enzyme in the respiratory and metabolic processes.

Many enzyme systems have been found in plant extracts, and these may be subdivided into two categories (Mapson, 1958): (a) those in which the oxidation of ascorbic acid is secondary to the oxidation of a substrate by the enzyme, and (b) those in which there is a direct reaction between enzyme, ascorbic acid, and oxygen. In the latter group there is, as far as our knowledge goes, only one enzyme, AAO, that occurs in the higher plants. The claim that AAO was a terminal oxidase in the respiration of pea seedlings and apples was made both by Davison (1949) and Hackney (1948) on the somewhat inadequate ground that (a) the enzyme was present, and (b) that the respiration was stimulated by the addition of ascorbic acid. Waygood (1950) working with wheat seedlings came to the same conclusion for similar reasons with the added evidence that polyphenolase enzymes were absent, and that cytochrome oxidase could only be detected in the embryonic stage. More positive evidence of the functioning of AAO as a terminal oxidase came from the work of James and his colleagues (1953 and 1955). They found that of the three oxidases, AAO, cytochrome oxidase, and polyphenolase, only the first could be demonstrated in 10 to 17-day-old barley roots. During the development of these roots there appeared to be a gradual replacement of cytochrome oxidase by AAO, as evidenced by a decrease in the respiratory sensitivity to CO and a rise in respiratory sensitivity

towards sodium diethyldithiocarbamate which pointed a progressive change from an iron catalyzed to a copper catalyzed system.

On the basis of inhibition studies, Király (1957) reached the conclusion that cytochrome oxidase, not AAO was the main terminal oxidase in healthy wheat leaves, but that in leaves infected with stem rust, the enhanced respiratory activity became highly sensitive to copper chelating agents, and was paralleled by an increase in AAO activity. The authors suggest that AAO may be present in healthy plants in an inactive state and only becomes operative in infected plants and is the terminal oxidase of the parasitically stimulated respiration. Changes in terminal oxidation under the influence of vernalization (Sisakin and Filippinovic, 1953), illumination (Rubin et al., 1955), and parasitic attack (Rubin and Chetverikova, 1955) have been reported by other workers.

Tamaoki et al. (1960) obtained evidence indicating that, after comparison of the oxidation of ascorbic acid by the mitochondria from normal and crown-gall tomato tissue cultures to the oxidation by an ascorbic acid oxidase preparation, the mitochondria from both tissue cultures contained AAO and responded similarly to co-factors (DPN, cytochrome c) and inhibitors (cyanide, Antimycin A). They further found that the AAO activity was higher in crown-gall than in normal tissue mitochondria.

It is obvious that there remains a good deal of work in relation to the role of AAO in tissue respiration.

No animal source of the enzyme has been described, but it has been reported that the blood copper protein, ceruloplasmin, possesses AAO activity (Osaki et al., 1964) in contradiction to the finding of Morell et al. (1962) who could not detect AAO activity in ceruloplasmin.

Localization of Ascorbate Oxidase

By definition, AAO is an enzyme which is easily brought into solution and hence it is referred to as a soluble oxidase (Bonner, 1957). However, reports have appeared concerning the localization of AAO in various fractions of cell homogenates.

The possibility that AAO could be associated with particulate components of the cell was first raised by Waygood (1950) in his studies of wheat respiration. Bonner (1957) concluded: It is difficult to conceive of a role for a powerful oxidase like AAO in the cell wall, a structure which is relatively inert metabolically; it is conceivable, of course, that the enzyme is in some manner associated with the activities of the cell wall during cell division and cell elongation. In the leaves of buckwheat AAO activity appeared to be present in the cell walls (Mache, 1967).

Mitochondria from tomato tissue cultures contained AAO (Tamaoki, 1960).

Localization of AAO in the grana of spinach chloroplasts indicate a probable close relation of this enzyme to the photosynthetic transport of electrons.

In thalli of the liverwort, Marchantia polymorpha, AAO was found to be strongest in the soluble protein fraction (Van Poucke, 1967).

Molecular Properties of Ascorbate Oxidase

Cucurbita pepo condensa.--The AAO of several plants has been purified, but the most highly purified enzyme has been obtained from the yellow crook-neck squash (Cucurbita pepo condensa) (Stark and Dawson, 1963). Based on sedimentation data, and information concerning the amino acid content of the enzyme, as reported by Stark and Dawson (1962) a molecular weight range of 134,000-140,000 was indicated for the enzyme. By means of the two CU(I)-specific reagents, cuproine and bathocuproine, it has been found that the prostethic copper in AAO exists in a mixed valency state, corresponding to 25% Cu(I) and 75% Cu(II) (Poillon and Dawson, 1963a). This ratio 1:3 corresponds to 2 atoms of Cu(I) and 6 atoms of Cu(II) per enzyme molecule. This same ratio was found for the mixed valency state in the denatured enzyme when the Cu(I) reagent,

bathocuproine, and the Cu(II) reagent, cuprizone, were used simultaneously.

The respective roles of prosthetic Cu(II) and Cu(I) in the function of ascorbate oxidase have been examined with respect to the blue color, activity and inactivation of the enzyme (Poillon and Dawson, 1963b). It has been found that the Cu(I) fraction, representing approximately 25% of the total native enzyme, does not participate in the enzymatic activity or contribute to the blue color. That is to say, the complexing of that fraction of the protein copper by a Cu(I)-specific chelating agent does not affect the activity or the blue color. The Cu(I) fraction existing in the native enzyme cannot be complexed directly with the chelating agent, except when the enzyme is functioning. It is concluded, therefore, that a reversible structural modification in the conformation of the protein moiety occurs during the catalytic cycle, thereby making this non-functional Cu(I) available to the reagent. Furthermore, the configuration of the protein and the binding of its functional Cu(II) fraction, are such that the continually generated Cu(I) component of the reversible Cu(II)$\rightleftarrows$Cu(I) catalytic cycle is at no time available for complexing with the chelating agent. It has been found that the non-functional Cu(I) fraction of the enzyme is responsible for the production of the H_2O_2 that results in the characteristic inactivation of

the enzyme during aerobic function. It was shown that small amounts of H_2O_2 have no inactivating effect on the resting enzyme but are strikingly effective on the functioning enzyme. The enzyme thus inactivated, loses its blue chromophore, but retains its copper. It is suggested that this H_2O_2 effect may involve directly the functional Cu(I) sites or the irreversible oxidation of some critical functional group exposed during the modification in structure of the protein moiety during catalysis.

Analyses of AAO at pH 3.6 showed a loss of both oxidase activity and copper content (Clark et al., 1966). The losses occurred in a non-parallel fashion (activity loss faster than copper loss) and suggest that the enzymatic Cu(II) fraction may be lost at a faster rate than the non-enzymatic Cu(I). However, the non-parallel loss in activity and copper content is consistent with the view that the enzyme is only fully active when the prostethic copper atoms exist and function in specifically oriented groups of two or more. Consequently, the loss of a single copper atom would cause loss in activity proportionately higher than the copper loss. The changes were temperature dependent and were accompanied by an irreversible unfolding (with subsequent aggregation) of the protein moiety of the enzyme.

Cucurbita pepo medullosa.--Ascorbate oxidase, prepared by a procedure which employs DEAE-cellulose

chromatography and starch-column electrophoresis, has been obtained in high purity and in relatively high yield from either yellow or green summer squash (Tokuyama et al., 1965). The enzyme had a specific activity of 3,600 units per mg of protein. The average value for the weight-average molecular weight of the enzyme from sedimentation equilibrium experiments was found to be 140,000.

Cucumis sativus.--Nakamura et al. (1968) purified the AAO of cucumber (Cucumis sativus) and studied its molecular weight and other physico-chemical properties.

This enzyme contains 8 atoms of copper per molecular weight of 132,000 and has a specific activity of 3,500 Dawson's units/mg. Results on spectrophotometric and ESR measurements, as well as those on kinetic analysis and azide inhibition of the enzyme, were also presented.

Commercial preparations of AAO.--The properties of several different highly purified preparations of AAO have been studied using both the Warburg and spectrophotometric methods of assay (Frieden and Maggiolo, 1957). Activating agents were found to affect both the initial rate and maintenance of AAO activity. The enzymic catalysis was increased when oxygen replaced air as the gas phase. The authors reported two K_m values for AAO, 5×10^{-3}M and 3.9×10^{-5}M as determined by the Warburg and spectrophotometric technique, respectively.

Activation of AAO proved to be of two general types. The largest group comprises substances which activate AAO but never inhibit at any concentration. Most of these substances are powerful copper chelators and include representative proteins, amino acids, thyroxine analogs, and nucleic acid components. Activation was also observed with Al+++ and Ca++. Activation at low concentrations but inhibition at high levels was obtained with cyanide, diethyldithiocarbamate, and 8-hydroxyquinoline. Irreversible inhibition was observed with Cu++ and several other metal ions.

Multiple Molecular Forms of AAO

Tokuyama et al. (1965) suggested, after obtaining the values of 140,000 and 147,000 for the weight-average and z-average molecular weights of the enzyme, respectively, that the larger value may be due to the presence of a small amount of a higher molecular weight species of the enzyme.

In cucumber extracts, on the basis of gel filtration, AAO has been found to be present in at least three widely different forms: two large molecular size enzymes (MW 200,000-900,000 if in globular form) and an enzyme of small size (perhaps close to 10,000) (Porath et al., 1967). The designation of the enzymes as ascorbic acid oxidase

is based upon inhibition and activation of the enzyme activity in the presence of quercitin, dehydroquercitin, morin and rutin.

MATERIALS AND METHODS

Enzyme Source

Yellow summer squash (Cucurbita pepo condensa), green zucchini squash (C. pepo medullosa) and cucumber (Cucumis sativus) were used to study the presence of multiple molecular forms of AAO, their physico-chemical properties and the intracellular distribution of the enzyme.

The squashes and cucumbers were grown in Michigan and were obtained from a local market in July, 1968 and July, 1969. Part of the material was used immediately and the rest was frozen and stored at -20°C until used. The storage period did not exceed 8 months. Before preparation of samples for analysis at 2°C, the frozen plant material was thawed at room temperature for one hour.

Extraction and Separation of AAO Isozymes

One part by weight of plant material was homogenized in a Waring blendor for 1 minute at low speed with one part of 0.05M phosphate buffer, pH 7, ionic strength 0.1, containing sucrose at the concentration of 0.25M. After squeezing the homogenate through four thicknesses of cheesecloth the filtrate was centrifuged at

20,000xG (G stands for gravity unit) for 20 minutes at 2°C, and the supernatant was centrifuged a second time at 100,000xG for 2 hours at 2°C (Beckman, Ultracentrifuge, Preparative, Model L-2). The final supernatant was used for electrophoresis.

The method which Davis (1964) developed for serum protein separation using polyacrylamide disc electrophoresis was slightly modified for the separation of the AAO isozymes.

Stock solutions for anodical proteins:*

A. 1 N HCl 48 ml, TRIS (Tris hydroxy methyl aminomethane) 36.3 g, TEMED (N, N, N, N', Tetramethylenediamine) 0.23 ml, and H_2O to make 100 ml (pH 8.8 - 9.0).

B. 1 N HCl 48 ml, TRIS 5.98 g. TEMED 0.46 ml, and H_2O to make 100 ml (pH 6.8).

C. Acrylamide 60.0 g, BIS (N, N-Methylenebiscrylamide monomer) 0.4 g, and H_2O to make 135 ml.

D. Acrylamide 10 g, BIS 2.5 g, and H_2O to make 100 ml.

E. Riboflavin 4.0 mg and H_2O to make 100 ml.

F. Catalyst: Ammonium persulfate 0.14 g and H_2O to make 100 ml.

G. Buffer (dilute to 1/10): TRIS 6.0 g, Glycine 28.8 g, and H_2O to make 1 liter (pH 8.3).

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*All reagents used were Eastman Chemicals products, Rochester 3, N. Y.

H. Protein stain: Aniline black 1 g and 7% acetic acid to make 200 ml.

I. Tracking dye: 0.005% bromphenol blue solution.

Working Solutions

Lower gel: 3% A=2.0ml, C=1.2ml, and H_2O=5.8ml.
4% A=2.0ml, C=1.6ml, and H_2O=5.4ml.
5% A=2.0ml, C=2.0ml, and H_2O=5.0ml.
6% A=2.0ml, C=2.4ml, and H_2O=4.6ml.
7% A=2.0ml, C=2.8ml, and H_2O=4.2ml.
8% A=2.0ml, C=3.2ml, and H_2O=3.8ml.
9% A=2.0ml, C=3.6ml, and H_2O=3.4ml.

In order to form a gel the lower gel is combined with the catalyst F 1:1.

Upper gel: B=2ml, D=4ml, E=2ml, and H_2O=4ml.

Stock solutions for cathodical proteins: (pH 4.3)

A. 1 N KOH 48 ml, Glacial acetic acid 17.2 ml, TEMED 4.0 ml, and H_2O to make 100 ml (pH 4.3).

B. 1 N KOH 48 ml, Glacial acetic acid 2.87 ml, TEMED 0.46 ml, and H_2O to make 100 ml (pH 6.7).

F. Catalyst: Ammonium persulfate 0.28 g and H_2O to make 100 ml.

G. Buffer (dilute to 1/10): Beta alanine 31.2 g, Glacial acetic acid 8 ml, and H_2O to make 1000 ml (pH 5.0).

The rest of the stock solutions and working solutions were identical with those used for the anodical proteins.

The gel tubes used were 7.5 cm long and 0.5 cm in o.d. From bottom to top, 4.5 cm of lower gel was introduced, 0.5 cm of upper gel, and the remainder of the tube was left for the sample and buffer to be introduced.

About 0.3 ml of the dialyzed preparation (9.8 mg total protein per ml), to which sucrose had been added to reach the concentration of 2 per cent (for the purpose of increasing the specific gravity and preventing diffusion into the upper buffer) was layered on top of the upper gel.

The tube was filled with the proper buffer, which was first deprived of oxygen by bubbling nitrogen through it and then made 10^{-3}M in ascorbic acid by adding solid vitamin and stirring carefully. The same buffer was also used in the upper reservoir of the disc electrophoretic apparatus. The lower reservoir contained the same buffer without ascorbic acid.

For cathodical AAO runs, electrophoresis was performed for one hour as for anodical proteins but without the sample. After that period the ascorbic acid which had been added to the upper reservoir had penetrated the separation or lower gel. Then the sample was added on top of the spacer or upper gel, the polarity was reversed,

the buffer in the lower reservoir was made 10^{-3}M in ascorbic acid as described before and the electrophoresis for determination of cathodical molecular forms of AAO started. This technique had been used to guarantee a continuous penetration of the separation gel with ascorbic acid.

The current of 2.75 mA per tube at 2°C was found to be optimum for the separation of the multiple molecular forms of all plant material examined.

The ionic front was allowed to migrate for 4.5 cm in the lower gel (approximately 1.5 hours).

Development of AAO Activity Bands on Polyacrylamide Gel

After the electrophoretic run, the gel was extruded under demineralized water and placed in a tray containing a solution of 25 mg of 2,6-dichloro-benzenoneindophenol (dye) per 100 ml of demineralized water. The tray containing the gel and dye was constantly tilted to allow the aerobic enzymatic reaction to proceed. At the loci of AAO activity, the ascorbic acid, which had penetrated the gel during electrophoresis, was oxidized and the dye remained blue. At all other loci the ascorbic acid decolorized the dye. After the development of the bands (approximately 3-5 minutes) the blue colored columns were rinsed with demineralized water and photographed on high contrast film in tramsmitted light. When a 3% gel was

used it became necessary to leave this very solf gel in the glass tube after it has been detached from the glass walls by means of a needle. Penetration of the dye and O_2 was facilitated by rotating the needle between gel and glass walls.

Inactive proteins were stained with stock solution H, according to Davis (1964). The gel was destained electrically for 15 minutes in 7% acetic acid. Complete destaining was performed by keeping the gel in a 7% acetic acid solution until protein bands appeared.

For recovery studies, the portion of the gel incorporating the multiple forms or form to be recovered was cut off from the rest of the gel with a razor blade and cut into pieces in a small beaker containing 0.5 ml 0.05M phosphate buffer, pH 7. After allowing the gel to stand for 30 minutes at room temperature it was frozen overnight. The following day the gel was thawed and the drip, made 2% in sucrose, was pipetted on to the polymerized gel of the electrophoretic tube for a repeated electrophoresis.

Horizontal starch gel and horizontal polyacrylamide gel electrophoresis did not result in separation of the multiple molecular forms of AAO as satisfactory as the polyacrylamide disc gel electrophoresis.

Estimation of Molecular Size by Gel Filtration

The size of the different molecular forms was estimated by gel filtration (Andrews, 1965). Columns were prepared as follows: to Sephadex G-100 (water regain: 10 ± 1 ml H_2O per g dry Sephadex) demineralized water was added and the gel was allowed to swell on a boiling water bath for 5 hours. The hydrated gel was deaerated under vacuum and the column (1.5 x 84 cm) was filled at 4°C and equilibrated with 0.05M phosphate buffer, pH 7, ionic strength 0.1. The void volume was determined using blue dextran 2,000 (MW $2x10^6$). One ml of the phosphate buffer containing 3 mg of blue dextran was applied on the column.

A second column (1.5 x 60 cm) using Sephadex G-200 (water regain: 20 ± 2 ml H_2O per g dry Sephadex) was also prepared as described above.

The flow rate of the Sephadex G-100 column was 18 ml per hour, that of Sephadex G-200 was 6 ml per hour.

The Sephadex G-100 column was calibrated with chymotrypsinogen A (Beef Pancreas) 6x cryst. salt free (MW 25,000), ovalbumin (2x cryst.) (MW 45,000), albumin (Bovine) cyrst. (MW 67,000), and gamma globulins (Human) (MW 160,000); the Sephadex G-200 column was calibrated with gamma globulins (Human) (MW 160,000), catalase (Beef liver) (MW 250,000), apo-ferritin (Horse) amorphus, salt free (MW 480,000) and thyroglobulin (Bovine)

(MW 670,000). Solutions of 4 mg of each marker per ml of phosphate buffer were separately prepared and applied onto the column. The elution volumes (V_e) of each of the markers were carefully measured using an ISCO UV monitor with recorder (wavelength 254 nm). V_e were plotted versus their molecular weights on semi-log paper. A straight line relationship was obtained. The protein markers were purchased from Mann Research Laboratories, New York, N.Y. 10006; blue dextran 2,000, Sephadex G-100, Sephadex G-200 and the Sephadex columns from Pharmacia Chemical Company, Uppsala, Sweden.

The elution volumes of the multiple molecular forms were determined as fcllows. Eight replicate electrophoretic separations of the AAO isozymes were performed simultaneously in the same apparatus. One of the 8 gels was developed for visualization of the enzymatic bands and then all 8 gels were lined up parallel to each other, with their ends at the same level. This arrangement enabled the dissecting of the gels with a razor blade at the loci of AAO activity. The corresponding loci of the seven gels were combined, triturated in 3 ml phosphate buffer and the extract was applied on Sephadex G-100. An equivalent sample was used for Sephadex G-200. The effluent was collected in 1.5 ml tubes using an automatic fraction collector (Rinco Instruments, Greenville, Illinois). Each fraction was tested for AAO activity

using the following qualitative method: to the 1.5 ml of effluent 0.1 ml of 10^{-3}M ascorbic acid was added, the mixture was shaken for one minute and then 0.1 ml dye (25 mg 2,6-dichlorobenzenoneindophenol per 100 ml water) was added to it. The presence of a blue color after one minute indicated a minimum of 10 micrograms of AAO in the reaction mixture; the basis of this test had been described previously (p. 21).

Intracellular Distribution of AAO Isozymes

The procedure of Tolbert et al. (1968) was modified for the preparation of fractions for differential centrifugation and sucrose density gradient centrifugation.

Differential Centrifugation

Sixty g of skin tissue was chopped into small segments before grinding by hand, on an ice bath, for 1/2 hour, with 60 ml of 0.05M phosphate buffer, pH 7, containing 0.5M sucrose. The homogenate was hand-squeezed through six layers of cheesecloth and 80 ml of the filtrate was fractionated in three steps. It was first centrifuged at 120xG for 20 minutes and a pellet was obtained containing debris. The supernatant of the first centrifugation was spinned at 3,000xG again for 20 minutes, and a pellet containing mostly broken chloroplasts was obtained. The supernatant of this second

centrifugation was subjected to 35,000xG for 20 minutes. The third pellet was considered the mitochondrial fraction; it is the supernatant of this centrifugation that reference is made to when the term supernatant is used in the following. Each pellet was resuspended in 2 ml of the sucrose containing buffer, pH 7. These three suspensions and the supernatant were analyzed for total AAO activity. The mitochondrial fraction was subjected to sucrose density gradient centrifugation.

Sucrose Density Gradient Centrifugation

A non-continuous sucrose density gradient of five layers was prepared at 4°C by pipetting 0.8 ml of 2.5M sucrose (85.5%), 2 ml of 2.0M sucrose (68.4%), 2 ml of 1.5M sucrose (51.3%), and 4 ml of 1.3M sucrose (44.5%). All sucrose fractions contained 0.05M phosphate buffer at pH 7. After layering 1 ml of the mitochondrial fraction on top of the gradient, the samples were centrifuged for 3 hours at 2°C at 25,000 rpm in a Ultracentrifuge, Preparative, International, Model B-60 swinging bucket rotor SB-283. Samples of 1.5 to 4.1 ml were removed from the bottom of the centrifuge tube by piercing with a needle.

Enzyme Assays

Ascorbate Oxidase

The rate of ascorbic acid - AAO reaction was measured manometrically according to the method of Powers et al. (1944) slightly modified. 1.5 ml of 0.2M Na_2HPO_4 - 0.1M citric acid buffer, pH 5.7, 0.5 ml of gelatin solution (750 mg gelatin in 150 ml water), and 0.5 ml of 0.028M ascorbic acid solution was transferred into the main compartment of a Warburg flask. To the sidearm of the flask 0.5 ml of enzyme preparation was pipetted. The flask was connected to the manometer which was used for determining the flask constant, and placed in a constant temperature bath at 30°C. The flask was equilibrated for 5 minutes, then the content of the side arm tilted into the main compartment and the reaction allowed to proceed. Readings were made at 2 minute intervals until no more oxygen was consumed. The activity of one AAO unit was expressed as the amount of enzyme which causes an initial rate of oxygen uptake of 10 microliters per minute (Dawson and Magee, 1955). The specific activity was expressed as units per mg of protein.

Cytochrome c Oxidase

A spectrophotometric procedure had been used for the determination of cytochrome c oxidase activity (Simon, 1958). 0.01 ml of enzyme preparation was

pipetted into a spectrophotometric cell of 1 cm lightpath loaded with 3 ml of a medium containing sucrose (0.2M), phosphate (0.05M, pH 7.1), and cytochrome c which had been reduced with dithionite and then oxygenated to remove the excess of dithionite. Readings of absorbance at 550 nm were made with a Beckman DU spectrophotometer at intervals of 15 sec for 90 sec and then again after the addition of 0.02 ml of 1M potassium ferricyanide to oxidize the cytochrome c. A first order rate constant for the disappearance of reduced cytochrome c was calculated according to Smith (1955).

Glycolate Oxidase

This enzyme was assyaed anoxically by 2,6-dichlorobenzenoneindophenol reduction (Zelitch and Ochoa, 1953). Additions were made to a 3 ml Thunberg Beckman cuvette (1 cm lightpath) in the following order: 2 ml of 0.3M pyrophosphate, pH 8.3, containing KCN in 0.01M NH_4OH (final concentration of KCN in the reaction mixture, $2x10^{-3}M$), 0.1 ml of $2x10^{-3}M$ FMN (final concentration, $0.8x10^{-4}M$), 0.1 ml of enzyme preparation; water so that the final volume would be 2.5 ml; in the side arm, 0.1 ml of 0.125M sodium glycolate (final concentration, $5x10^{-3}M$). The cuvette was evacuated and flushed three times with N_2 which had passed through Fieser's solution to remove

traces of O_2. Dye reduction at 25° was measured at 600 nm by an automatic recording Gilford spectrophotometer.

In the presence of O_2 assays were unreliable since the H_2O_2 generated by the glycolate oxidase could be used by contaminating peroxidases to oxidize any reduced dichlorobenzenoneindophenol which was generated. KCN was left in the assay to ensure against peroxidase activity in case of incomplete removal of O_2.

Catalase

Catalase was assayed by the disappearance of H_2O_2 as measured spectrophotometrically at 240 nm (Luck, 1963).

Protein Determination

The Lowry method (Lowry et al., 1951), in which cyrstalline bovine serum albumin was used as the standard, was the basis of a total protein estimation.

Chlorophyll Determination

Chlorophyll was determined by its absorption at 652 nm (Arnon, 1949). Aliquots from 50 microliters to 1 ml were diluted to 5 ml with water and acetone to make a final concentration of 80% acetone. They were allowed to stand in the dark at 4°C with occasional stirring for five hours to solubilize the chlorophyll. Samples were filtered before reading the absorbance at room temperature.

Isoelectric Point Determination

Polyacrylamide disc gel electrophoresis, as described earlier, had been used to determine the isoelectric point, pI, of the different molecular forms of AAO. The concentration of the gel was 6%, and the D.C. current was 2.75 mA per tube. The tracking dye, bromphenol blue, was allowed to migrate for one hour in the separation gel.

After the first electrophoretic run of the enzyme preparation, containing all isozymic forms, the individual gel bands showing AAO activity were cut off with a razor blade, three identical bands combined, triturated with 0.5 ml phosphate buffer and subjected to a second electrophoretic run.

The color development of the bands was carried out as described above for anodical as well as for cathodical electrophoretic runs.

The electrophoretic mobility was expressed in mm per hour under the conditions of measurement.

The following pH intervals and corresponding buffers had been used for the pI determination:

pH	buffer, ionic strength 0.1
4.5	acetic acid-Na-acetate
5.0	- " -
5.5	- " -

pH	buffer, ionic strength 0.1
6.0	KH_2phosphate-Na_2H-phosphate
6.5	- " -
7.0	- " -
7.5	- " -
8.0	- " -

The stock solution A for anodical proteins for preparation of the separation gel had to be prepared separately for all different pH values. To facilitate gel polymerization within 25 to 30 minutes, the composition of stock solution A was as indicated in Table 1.

K_m and V_{max} Determination

Preparation of Enzyme Extract

Sixty g of peel tissue were blended with 60 ml phosphate buffer, pH 7, ionic strength 0.1, in a Waring blendor at high speed for 1 minute. The homogenate was squeezed through four thicknesses of cheesecloth and the filtrate centrifuged at 25,000 rpm for 30 minutes in a refrigerated (2°C) Beckman Preparative Ultracentrifuge, Model L - 2, using rotor 50.

The supernatants obtained from yellow summer squash and green zucchini squash were diluted with water 1:100, that from cucumber 1:50.

TABLE 1.--Ml TEMED per 100 ml buffer (stock solution A) necessary for polymerization of the separation gel in 30 minutes.

pH of Buffer*	TEMED
4.5	3.60
5.0	3.00
5.5	1.80
6.0	0.80
6.5	0.50
7.0	0.40
7.5	0.35
8.0	0.30

*Stock solution A was adjusted to the proper pH using acetic acid after the addition of TEMED.

Spectrophotometric Method

The spectrophotometric method was adopted from a report by Racker (1952). Four ascorbic acid concentrations were used: $1x10^{-3}M$, $2x10^{-3}M$, $3x10^{-3}M$, and $4x10^{-3}M$.

The components of the reaction mixture were transferred into the Beckman DU spectrophotometer cuvette (1 ml lightpath) in the following order: 0.1 ml ascorbic acid solution, 0.1 ml EDTA ($3x10^{-5}M$), 2.7 ml 0.01M phosphate buffer, pH 7.2, and 0.1 ml of enzyme preparation. The change in A_{265} was measured for 3 minutes using a Ledland Log Converter and a Sargent Recorder.

From the first straight line portion of each tracing the decrease in A_{265} per minute (velocity) was calculated. One chart unit was found to be equivalent to 3.66 moles of ascorbic acid oxidized per liter.

Using the Lineweaver - Burk plot (Christensen and Palmer, 1967), K_m and V_{max} values were determined.

The points were fitted to a straight line by applying the regression analysis.

Warburg Method

The AAO activity was determined as described under Enzyme Assays. The ascorbic acid and enzyme concentrations were the same as in the spectrophotometric method. The initial rates of O_2 uptake were plotted according to

Lineweaver - Burk and the K_m values were determined by regression analysis.

Temperature Treatments of AAO Isozymes

One ml of the supernatant obtained after 100,000xG centrifugation was placed in a 10 ml test tube and treated in a constant temperature water bath for predetermined periods. After immediate cooling of the sample in an ice bath, electrophoresis was started and the effect of temperature on the multiple mulecular forms of AAO was determined. The temperature and time periods used are listed in Results and Discussion.

Chemical Treatments of AAO Isozymes

Urea

Urea had been incorporated into the gel and electrophoresis performed as usual. The urea concentrations are given in Results and Discussion.

2 - Mercapto - Ethanol

One ml of the same supernatant used in the temperature studies was made 0.02M in 2- mercapto - ethanol and incubated for 10 minutes at 0°C; electrophoresis followed immediately.

Acid - Base Treatment

The pH of the enzyme supernatant was increased or decreased by the dropwise addition of NaOH or HCl. In general, the procedure was as follows: 2 ml of the enzyme solution was placed in a 5 ml beaker (at 0°C) equipped with a small Teflon coated stirring bar. After each addition of 1M acid (or base), the pH was measured on a Beckman pH meter. As the solution approached the desired pH, final adjustment was made using the more dilute 0.2M titrant. After incubation with stirring for 30 minutes, electrophoresis was started.

Enzyme Purification

The AAO of yellow summer squash was purified according to Dawson and Magee (1955). Twelve lbs of peel were used as starting material. The rinds were minced to a fine pulp in a meat grinder, and the juice squeezed through cheesecloth. Enough solid $Na_2B_4O_7 \cdot 10\ H_2O$ was added to the juice to bring the pH to about 7.6. The crude juice was then treated with 1M Ba $(C_2H_3O_2)_2$ (10 ml/l of juice) and made 1.6M with respect to $(NH_4)_2SO_4$ (0.3 saturation) by adding the solid salt at room temperature. The precipitate was allowed to settle overnight in the refrigerator so that the supernatant fluid was removeable almost entirely by siphon, requiring centrifugation of only the settled material. The precipitate, instead of being

discarded, was redissolved in 200 ml of cold water, dialyzed overnight at 4°C against 0.05M phosphate buffer, pH 7, ionic strength 0.1 and subjected to polyacrylamide gel electrophoresis. The supernatant was treated with an amount of $(NH_4)_2SO_4$ equal to that previously added, and the resulting precipitate was filtered, and redissolved in 50 ml of cold water. The filtrate of the second ammonium sulfate precipitation, instead of being discarded, was dialyzed as above and subjected to polyacrylamide gel electrophoresis. The redissolved precipitate of the second ammonium sulfate precipitation was also dialyzed and subjected to disc electrophoresis.

Buffers

All buffer solutions were prepared according to Biochemist's Handbook (1961).

RESULTS AND DISCUSSION

Multiple Forms of AAO

A typical pattern of polyacrylamide disc gel electrophoresis of AAO from yellow summer squash skin consisted of five bands, numbered 1, 4, 8, 12, and X, as indicated in Figure 1. In an earlier paper (Amon and Markakis, 1969) the different forms were named A, B, C, D and E. In the new system, where 1 stands for A, 4 for B, 8 for C, 12 for D, and X for E, the letters correspond to the actual molecular subunit structure of the AAO isozymes as it will be discussed later.

The concentration of the polyacrylamide gel played a very important role in the resolution of the multimolecular forms. Different concentrations of separation gel were used ranging from 3% up to 9% (Figure 2). The best resolution was obtained with an 8% gel.

That bands 1 through X represent different sizes of molecules was convincingly demonstrated by changing the molecular sieving properties of the gels. Gels of 9%, 8% and 7% concentration have high sieving ability, the 5% and 4% ones have low sieving ability, while the 3% gel resembles free moving - boundary electrophoresis. The AAO activity was confined to a broad single band using

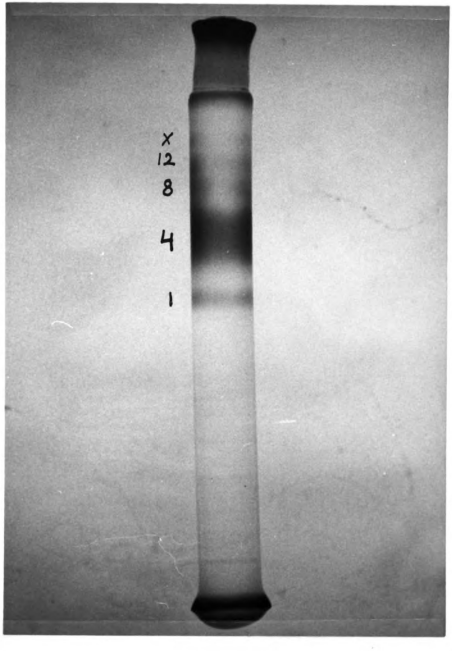


Figure I. --MULTIPLE FORMS OF YELLOW SUMMER SQUASH AAO IN 8% POLYACRYLAMIDE GEL. ELECTROPHORESIS AFTER DIALYSIS OVERNIGHT AGAINST 0.05M PHOSPHATE BUFFER, pH 7.0.

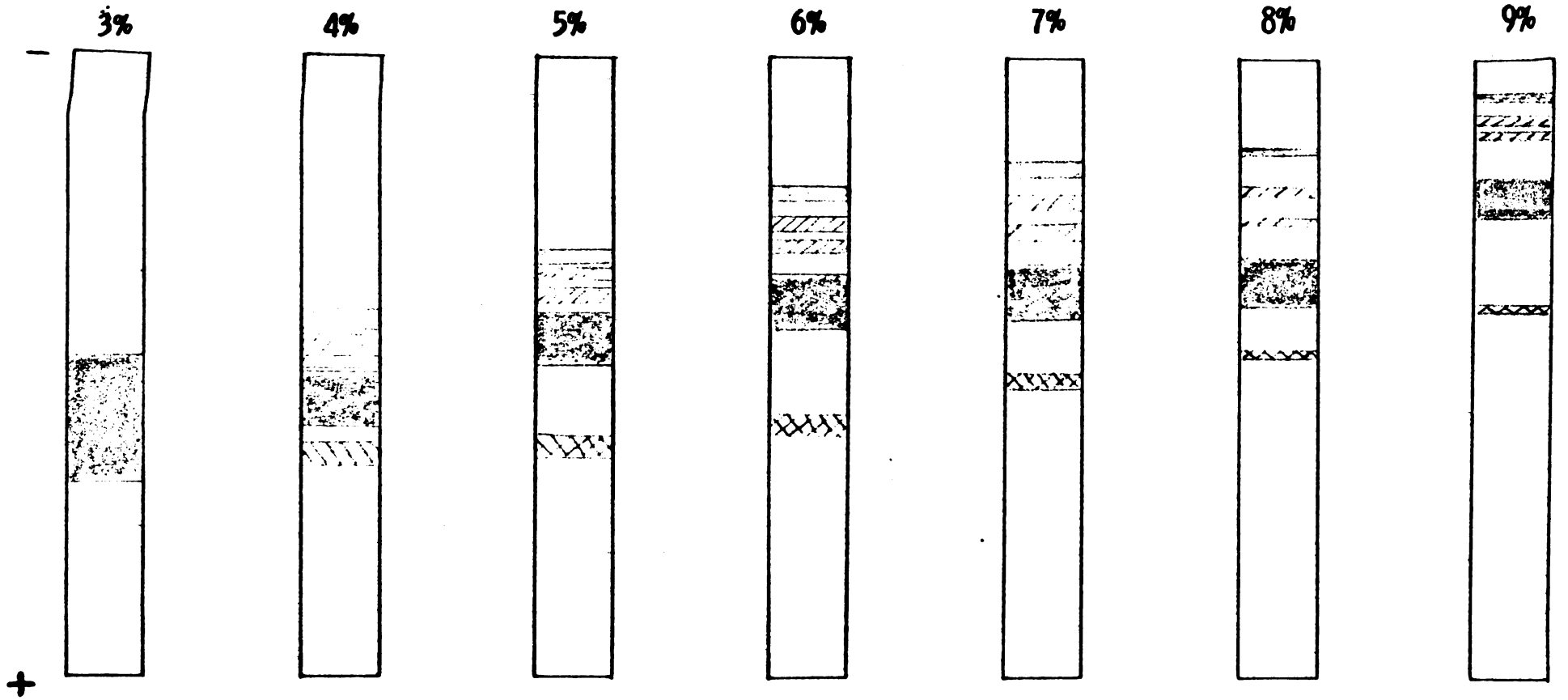


Figure 2.--EFFECT OFGEL CONCENTRATION ON THE RESOLUTION OF THE MULTIPLE FORMS OF AAO IN THE SKIN OF YELLOW SUMMER SQUASH

3% gel, while the higher concentration gels resolved the mixture into the pattern described. Tombs (1965) confirmed the variation of relative mobilities with changing gel concentration for polymeric states of a given protein. On the other hand the small differences in migration velocity in the 3% gel indicate that the isoelectric points of the various AAO forms could not be far apart. This was confirmed by subsequent experiments (Isoelectric point determination).

With this method it was found that there is no difference in the AAO isozyme pattern between yellow summer squash and green zucchini squash (Figure 3). The AAO isozyme pattern of cucumber was very different (Figure 3 and Figure 4).

The protein patterns of the three plant sources examined were different from each other. Each AAO form corresponded to a protein band (Figure 3).

At pH 6.8 in the spacer gel five AAO forms were obtained from each of the squashes, but only one band was obtained from the cucumber. When the pH of the spacer gel was increased to 8.8, again five bands appeared in the electropherograms of the squashes, but three bands were seen in the electropherogram of the cucumber (Figure 4). A probable explanation for this is the differences in the isoelectric points of the isozymes of squash and cucumber. The very small difference between the pH 6.8

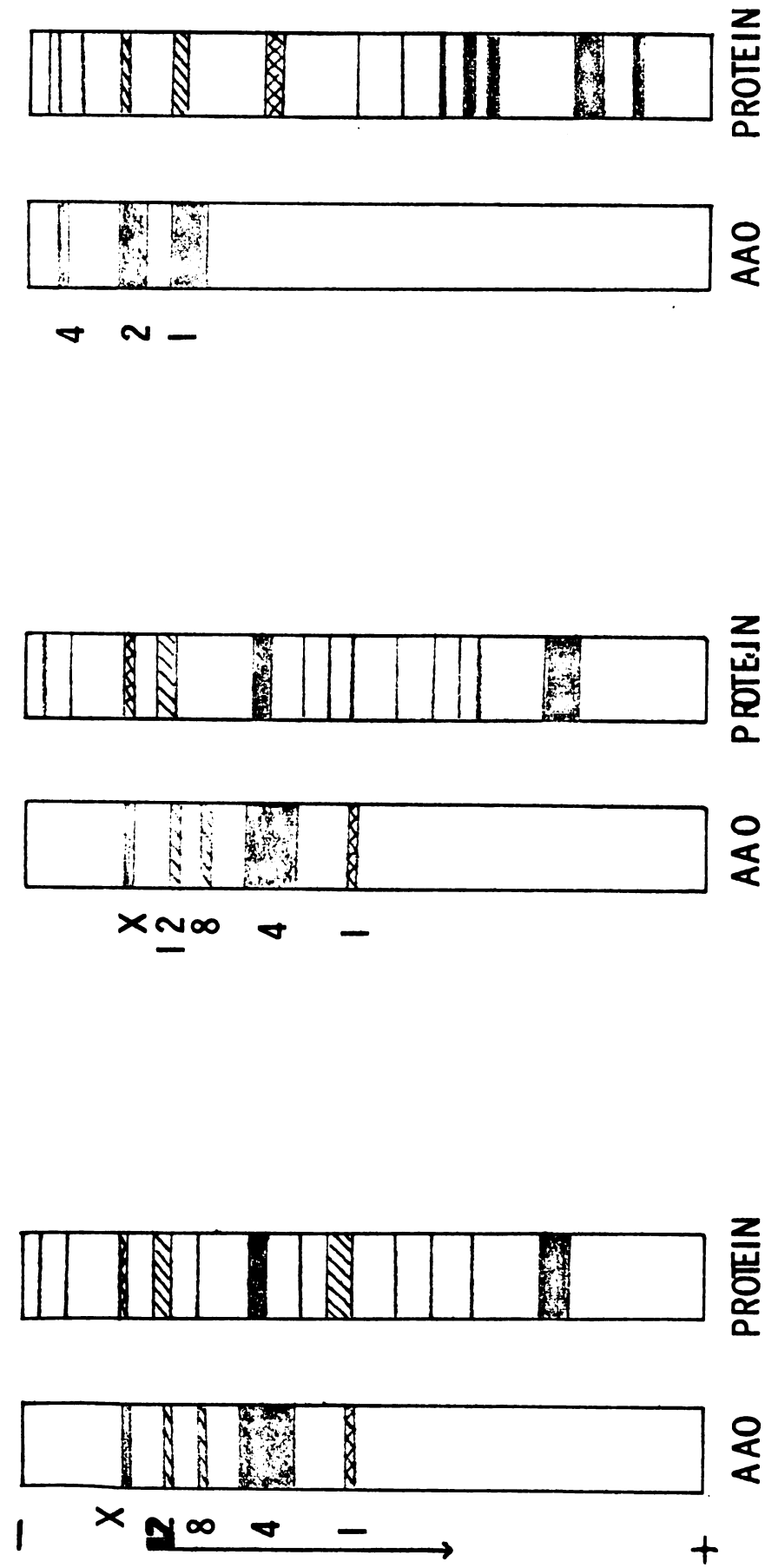


Figure 3.--AAO AND PROTEIN BANDS FROM YELLOW SUMMER SQUASH, GREEN ZUCCHINI SQUASH AND CUCUMBER (8% SEPARATION GEL IN ALL CASES, THE pH OF THE SPACER GEL WAS 6.8 FOR THE SQUASHES AND 8.8 FOR CUCUMBER)

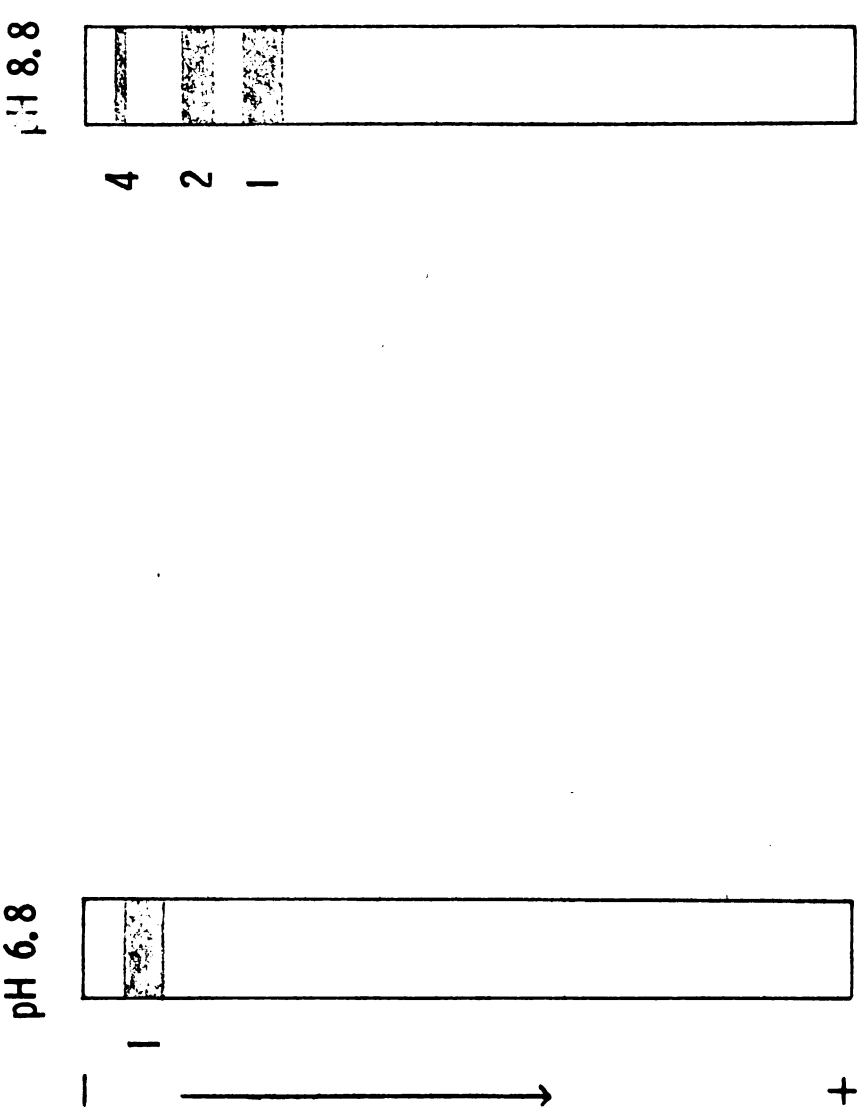


Figure 4.--MULTIPLE FORMS OF AAO FROM CUCUMBER SEPARATED ON 8% GEL AT TWO DIFFERENT pH VALUES OF THE SPACER GEL

(when applied to the spacer gel) and the isoelectric point of the cucumber forms did not allow the heavier forms 2 and 4 to enter the separation gel during the electrophoretic run of approximately 1.5 hours. Molecular weight determination on Sephadex confirmed the identity of band 1 in both electropherograms, those obtained with spacer gel pH 6.6 and pH 8.8.

None of the tissues analyzed showed cathodical forms of AAO using pH as low as 4.3.

The current played an important role in obtaining good band separation. A current of 2.75 mA per tube was found to be optimum for all three species studied.

The recovered enzyme activity from all five zones from yellow summer squash was 80% of that applied on the column, and of the recovered activity 70% was associated with form 4 and 30% with the other forms combined. The same results were obtained with green zucchini squash. In cucumber 50% of the recovered activity were attributed to 1, 40% to 2, and 10% to 4. In another experiment, each of the forms was eluted from several columns and the eluates placed on new gels for electrophoresis. Each form appeared alone in the position expected from the mixed forms. This indicates that the multiple forms separated by polyacrylamide electrophoresis cannot be considered artifacts of preparation.

Tissue Specificity

The skin and flesh of yellow summer squash, green zucchini squash and cucumber were tested in regard to AAO form multiplicity. In all species no difference in isozyme pattern between skin and flesh was apparent. But the skin of all three commodities had the highest concentration of activity (Table 2) and the activity among the three skin tissues was in the order of yellow summer squash, green zucchini squash, cucumber with yellow summer squash showing the highest total activity.

Effect of Extraction Methods on the Total Activity of AAO

Extraction by hand grinding in an all glass grinder (Koutes Glass Corp.) in an ice bath was compared to a Waring blendor extraction at high speed for one minute. Blendor extraction resulted in a 47% more total AAO activity, although when aliquot samples of both extracts were subjected to gel electrophoresis no change in the isozyme pattern was observed.

Effect of Different Extracting Media on the Total Yield in Activity

Water, 0.05M phosphate buffer, pH 7.0, ionic strength 0.05 and 0.2, and 0.05M phosphate buffer pH 8.0, ionic strength 0.05 and 0.2 were used to determine their influence on the AAO activity yield.

TABLE 2.--AAO activity in the skin and flesh of yellow summer squash, green zucchini squash and cucumber.

Commodity	Units per ml final supernatant		Units per g wet tissue		mg protein	
	Skin	Flesh	Skin	Flesh	Skin	Flesh
Yellow summer squash	6.5	4.2	19.5	12.6	0.73	0.25
Green zucchini squash	6.0	3.4	18.0	10.2	0.68	0.21
Cucumber	3.0	0.5	9.0	1.5	0.28	0.04

AAO activity was determined spectrophotometrically. Thirty g of peel tissue from green zucchini squash were homogenized in a Waring blendor for one minute at high speed with 60 ml of extracting medium. The homogenate was squeezed through 4-fold cheesecloth, diluted 1:50 and the AAO activity determined (Table 3). Extraction with phosphate buffer, pH 7.0, yielded the highest activity, water extraction the lowest. A 4-fold increase in ionic strength had little effect on the enzyme yield.

Estimation of Molecular Weights of AAO Isozymes

After trituration of the electrophoretically separated individual AAO forms, their molecular weights were estimated using Sephadex G-100 and Sephadex G-200. The columns were calibrated with proteins of known molecular weights (Figure 5 and Figure 6).

On the basis of at least triplicate Sephadex gel filtrations the following estimates of MW were made.

Yellow summer squash	Molecular form	Molecular weight
	1	30,000 - 42,000
	4	135,000 - 155,000
	8	260,000 - 310,000
	12	390,000 - 460,000
	X	above 670,000 and below 2,000,000

TABLE 3.--Effect of different extraction media and different ionic strength on the AAO activity yield from green zucchini squash.

Extraction medium	Activity*	
	ionic strength	
	0.05	0.2
Phosphate buffer, 0.05M, pH 7.0	0.230	0.265
Phosphate buffer, 0.05M, pH 8.0	0.190	0.205
Water	0.145	

*The activity is expressed as decrease in absorbance at 265 nm.

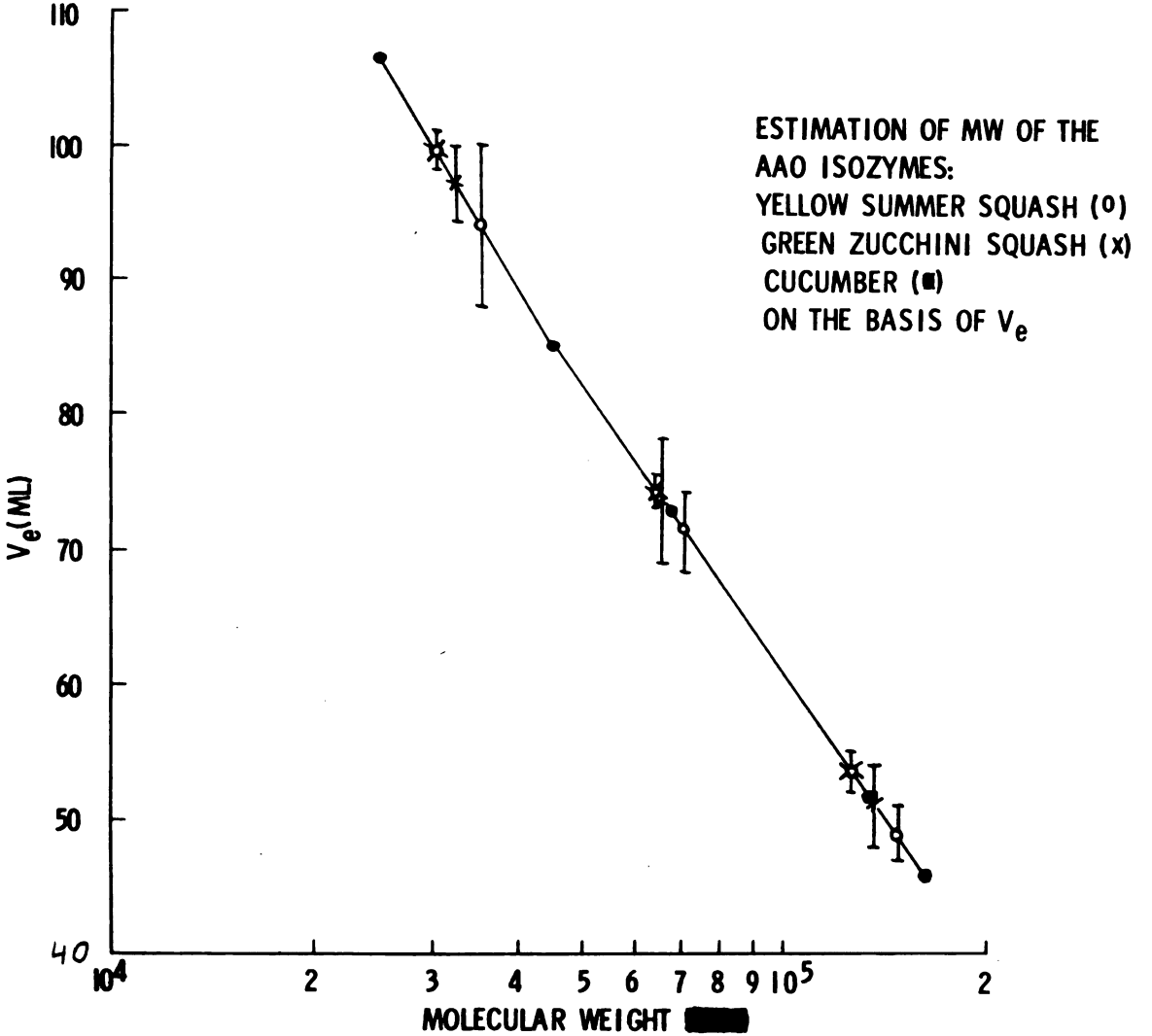


Figure 5. --PLOTS OF ELUTION VOLUMES, V_e, AGAINST [redacted] MOLECULAR WEIGHT[redacted] FOR PROTEINS OF KNOWN MW(•) ON SEPHADEX G-100 COLUMN

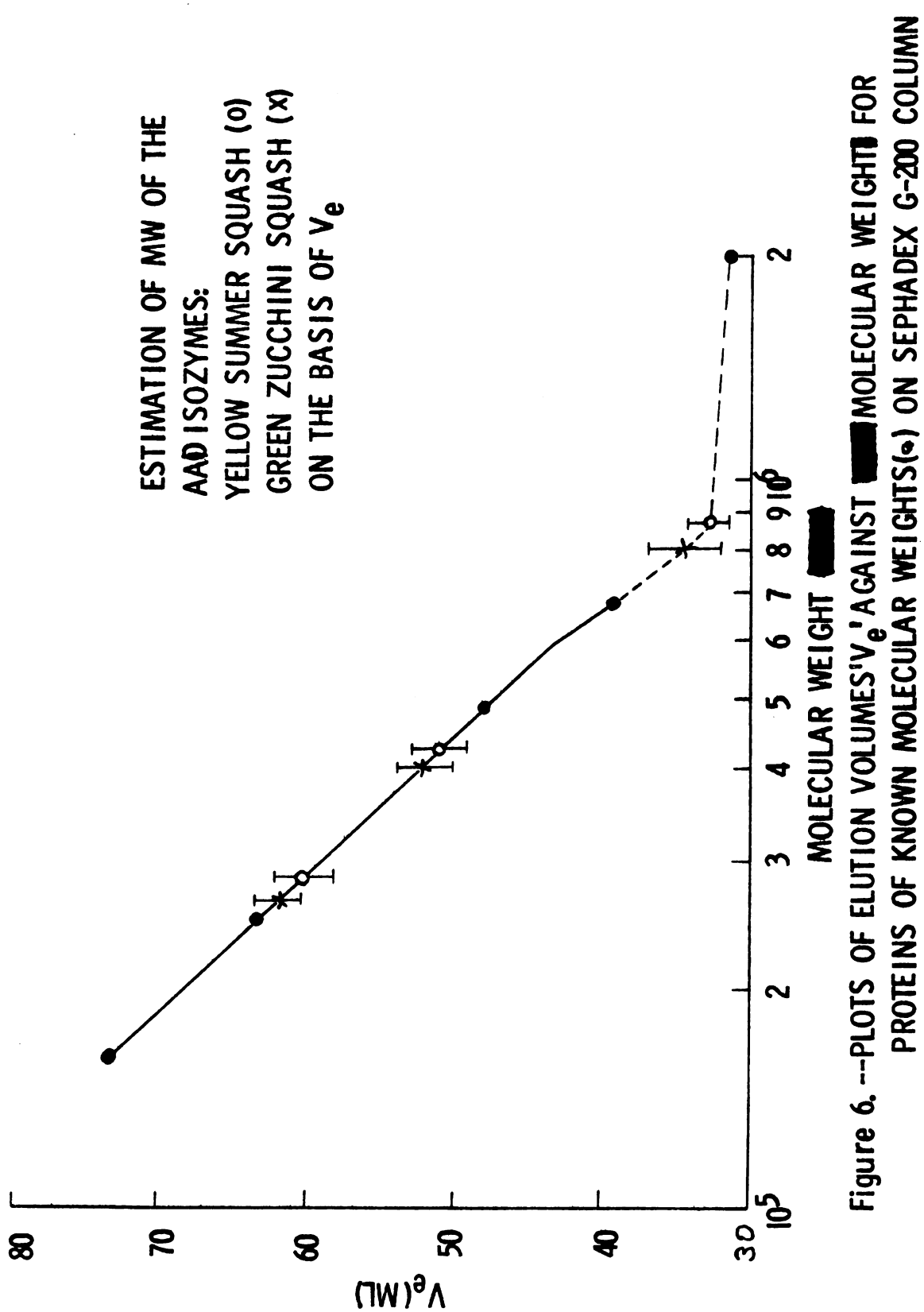


Figure 6.--PLOTS OF ELUTION VOLUMES'V_e' AGAINST MOLECULAR WEIGHT FOR PROTEINS OF KNOWN MOLECULAR WEIGHTS(•) ON SEPHADEX G-200 COLUMN

Green zucchini squash	Molecular form	Molecular weight
	1	30,000 - 35,000
	4	125,000 - 150,000
	8	240,000 - 280,000
	12	370,000 - 440,000
	X	above 670,000 and below 2,000,000
Cucumber	1	29,000 - 32,000
	2	62,000 - 66,000
	4	120,000 - 135,000

Since the heaviest form emerged 2 - 3 ml after the Blue dextran and since the Blue dextran has a MW of approximately 2 Million the heaviest form must be lighter than 2 Million.

Because the highest MW protein marker used was 670,000 the MW of the highest forms of yellow summer squash and green zucchini squash could not be estimated using that technique.

Electrophoresis of the fractions from the peaks of AAO activity immediately after their elution showed that the Sephadex elution fraction corresponding to the lowest molecular weight (form 1) migrated fastest toward the anode and with the same migration velocity as the fastest band when the mixture of all forms was subjected to electrophoresis. Similar agreement between Sephadex

elution fractions and electrophoretic bands was observed for all AAO forms.

The results suggest that the five forms of yellow summer and green zucchini squashes and the three forms of cucumber represent various degrees of aggregation of their monomers. That would justify the new number system, 1 designating the monomer

2	dimer
4	tetramer
8	octamer
12	dodecamer
X	670,000 < X > 2,000,000

In order to symbolize differentially the AAO forms of the 3 commodities the subscripts y, g and c will be used for yellow summer squash, green zucchini squash and cucumber, respectively; e.g. 1_g is the green zucchini squash monomer.

The molecular weights of highly purified AAO reported in the literature are as follows: 134,000 - 140,000 for the AAO of yellow summer squash (Stark and Dawson, 1962); 140,000 for the AAO of green zucchini squash (Tokuyama et al., 1965); 132,000 for the AAO of cucumber (Hakamura et al., 1968). Our molecular weight data for the tetrameric molecular forms are in excellent agreement with those reported earlier. It can be inferred that the highly purified AAO preparations of the

literature represent tetramers. The isozyme form with the highest AAO activity in our studies seemed to be a tetramer for the yellow summer and green zucchini squashes. Tokuyama et al. (1965) suggested the possibility of the presence of a small amount of a higher molecular weight species of AAO from yellow summer and green zucchini squashes. Molecular weight species of AAO from cucumber above 200,000 as reported by Porath et al. (1967) could not be detected in this study. However, this does not exclude the possibility of formation of aggregates of that size (above 200,000) under certain conditions and treatments. As it can be seen in the following experiments, interconversion from lower to higher MW aggregates and vice versa can take place easily.

Interconversion of Molecular Forms of AAO as a Result of Change in Ionic Strength

After making the hypothesis that the different forms shown on electrophoresis represent aggregates, the question was raised whether there is only one basic type of peptide chain involved in these polymers? If so, an interconversion of the different forms should be possible.

When the supernatants of the second centrifugation of the squash and cucumber extracts were dialyzed against demineralized water rather than phosphate buffer, only one zone appeared on the electropherogram (Figure 7 and Figure 8). This zone occupied position 4 for yellow summer

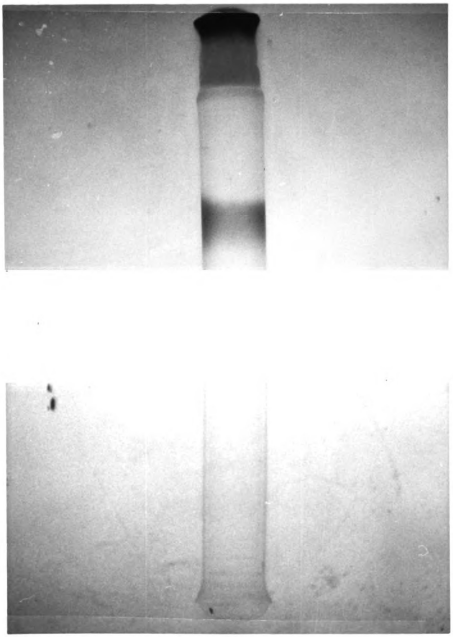


Figure 7. --EFFECT OF IONIC STRENGTH ON THE MULTIPLE FORMS OF YELLOW SUMMER SQUASH AAO(8%GEL). ELECTROPHORESIS AFTER DIALYSIS OVERNIGHT AGAINST DEMINERALIZED WATER.

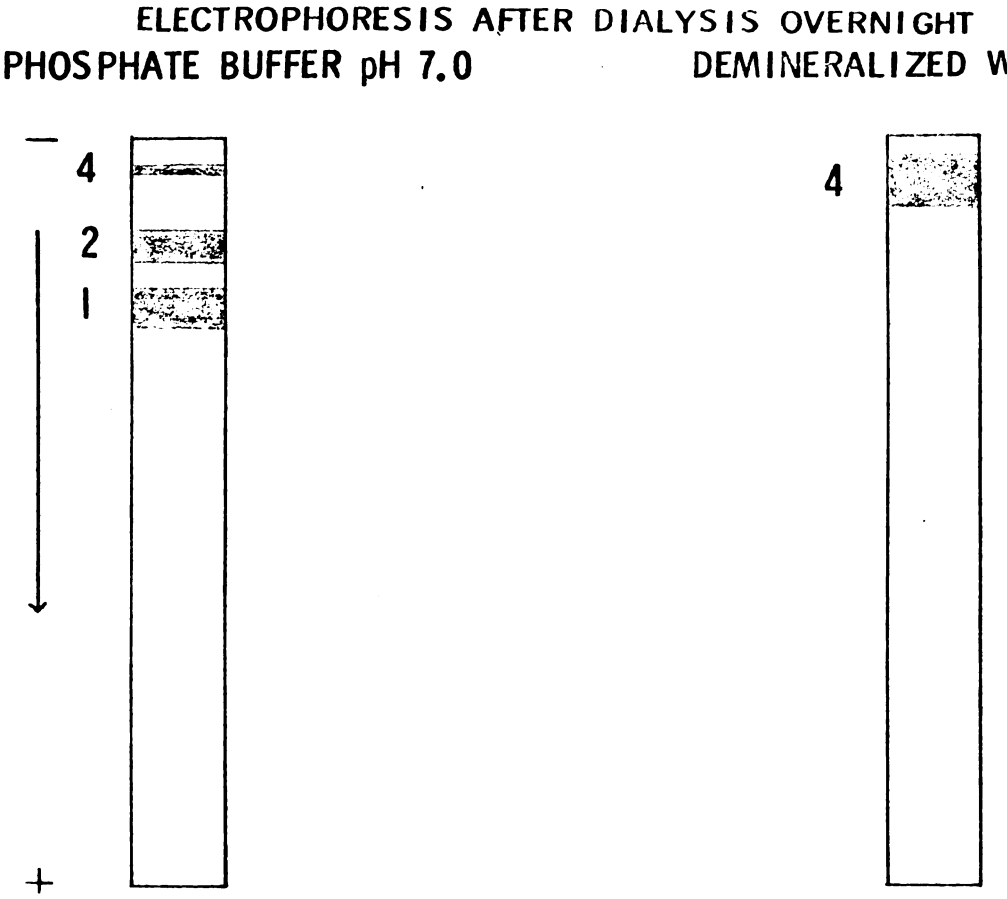


Figure 8.--EFFECT OF IONIC STRENGTH ON THE MULTIPLE MOLECULAR FORMS OF AAO FROM CUCUMBER ON 8% GEL.

and green zucchini squashes, and had the same activity as their zones 1_y, 4_y, 8_y, 12_y, X_y and 1_g, 4_g, 8_g, 12_g, X_g, respectively, together. In cucumber the new zone occupied also position 4 and had the same activity as 1_c, 2_c, 4_c together. Upon elution of this composite zone with 0.05M phosphate buffer, pH 7, ionic strength 0.1, and dialysis against the same buffer all previously observed forms reappeared, including 4. When these forms were again eluted with phosphate buffer from several columns and the combined eluates dialyzed against demineralized water, the single composite form reappeared on the gel. It is clear that a reversible association - dissociation occurs in the isozymes of AAO as a result of change in the ionic strength. A similar effect of the ionic strength had been demonstrated with tyrosinase (Jolly, 1967) and soluble hexokinase (Karpatkin, 1968). According to these observations increased ionic strength results in both association (4 going to 8-, 12- and X-mers) and dissociation (4 going to 1) of forms. Form 4 appears to play a central role in these transformations, although itself is unstable at high ionic strength (0.1).

Effect of Various Temperatures on the Multiple Forms of AAO

Electrophoretic separation on samples stored for 2, 8, 20, and 72 hours at 5°C, showed no change in the activity and polymorphic pattern as indicated in Table 4.

TABLE 4.--Temperature - time effects on the activity and polymorphism of AAO of three commodities (Supernatant, pH 7.0, ionic strength 0.1)

Time	Temperature	Commodity	Molecular form	% loss in total activity
hours	5°C			
2		yellow summer squash	1, 4,8,12,X	0
		green zucchini squash	1, 4,8,12,X	0
		cucumber	1,2,4	0
8		yellow summer squash	1, 4,8,12,X	0
		green zucchini squash	1, 4,8,12,X	0
		cucumber	1,2,4	
20		yellow summer squash	1, 4,8,12,X	0
		green zucchini squash	1, 4,8,12,X	0
		cucumber	1,2,4	0
72		yellow summer squash	1, 4,8,12,X	0
		green zucchini squash	1, 4,8,12,X	0
		cucumber	1,2,4	
hours	25°C			
2		yellow summer squash	1, 4,8,12,X	0
		green zucchini squash	1, 4,8,12,X	0
		cucumber	1,2,4	0
8		yellow summer squash	1, 4,8,12,X	5
		green zucchini squash	1, 4,8,12,X	5
		cucumber	1,2,4	10
20		yellow summer squash	4	50
		green zucchini squash	4	50
		cucumber	none	100

	yellow summer squash	4	50
	green zucchini squash	4	50
	cucumber	none	100
minutes 40°C			
5	yellow summer squash	1,2,4	0
	green zucchini squash	1,2,4	0
	cucumber	1,2,4	0
60	yellow summer squash	2,4	10
	green zucchini squash	2,4	10
	cucumber	2,4	30
minutes 50°C			
5	yellow summer squash	1,2,4	10
	green zucchini squash	1,2,4	10
	cucumber	1,2,4	15
60	yellow summer squash	2,4	30
	green zucchini squash	2,4	30
	cucumber	2,4	70
minutes 60°C			
5	yellow summer squash	4	30
	green zucchini squash	4	30
	cucumber	1,2,4	70
60	yellow summer squash	4	80
	green zucchini squash	4	80
	cucumber	2	98
minutes 70°C			
3	yellow summer squash	4	70
	green zucchini squash	4	70
	cucumber	2	90

Samples stored at room temperature (25°C) showed some activity decrease after 8 hours; but after 20 and 72 hours almost all bands dissappeared except the tetramers of yellow summer and green zucchini squashes. AAO isozymes from cucumber seemed to be more sensitive to storage temperature at 25°C than those of yellow summer and green zucchini squashes.

The treatment at 40°C for 5 minutes produced a new AAO molecular form in the yellow summer and green zucchini squashes. This form was shown to have a MW of 65,000 - 78,000 for yellow summer squash and 58,000 - 77,000 for the green zucchini squash, by Sephadex G-100 gel filtration, and should correspond to a dimer. The formation of the dimer brought about the disappearance of 8-, 12-, and X-forms; and since the total activity did not change a conversion of the 8-, 12-, and X-mer to the 2-mer was proposed. This was confirmed by another experiment: the non-treated 8-, 12-, and X-forms were cut off the gel, combined, triturated, incubated for 5 mihutes at 40°C and put on a new gel; the 2-form alone appeared.

An incubation time of 60 minutes at 40°C destroyed the monomer activity of cucumber AAO and diminished the total activity of all three plant AAO's studied here.

An increased loss of total activity and destruction of the 1-forms was observed following heating at 50°C for 5 and 60 minutes.

At 60°C for 5 minutes only the tetramer survived in the squashes and its activity was 30% less than the total activity of the control. At 60°C for 5 minutes all three AAO forms of the cucumber survived but at a loss of 70% in total activity. When the heating at 60°C was prolonged to 60 minutes, only 20% of the total activity was recovered in the squashes again in form 4 only, and 5% of the total activity of the cucumber, this time in the only surviving form 2.

At 70°C for 3 minutes only 30% of the total activity was left in the squashes (all in form 4) and 10% in the cucumber (all in form 2). After 60 minutes at 70°C no activity was left in any of the AAO's tested.

The one minute treatment at 100°C was sufficient to inactivate AAO completely in all cases.

The temperature - time effect on the AAO forms tested may be summarized as follows:

1) AAO isozymes of yellow summer and green zucchini squashes show the same heat sensitivity pattern throughout the different treatments.
2) The multiple forms of cucumber are more sensitive to heat than those of the other two commodities.

3) The following heat stability order was established for the isozymes:

   a. for yellow summer and green zucchini squashes: 4 > 2 > 1 > 8, 12, X.

   b. for cucumber: 2 > 4 > 1.

Effect of Different Chemicals on AAO Isozymes

Urea

A urea concentration of 5M in the polyacrylamide gel did not alter the total activity of AAO nor the characteristic isozyme pattern of green zucchini squash.

When the urea concentration was increased to 7M, the forms 8, 12 and X disappeared, the 4 activity was decreased and the 1 activity increased. In an 8M urea gel only form 1 remained having approximately 75% of the total activity. The following experiment was designed to explore the origin of 1 after treatment with 8M urea. The untreated 4-form was isolated from a gel and put on a new gel containing 8M urea. Electrophoresis on the new gel resulted in approximately 80% conversion of the tetramer to the monomer and 20% of the activity was lost. In the same way it was shown that 8M urea converted the 8-, 12- and X-forms to the monomer with a 50% loss in total activity.

In order to investigate the differential resistance of the 4-mer and the 12-mer both forms were cut off the gel, triturated separately and an equal amount of activity of either form was applied on a 7M urea gel. It was found that the 12-form completely converted to the monomer, whereas 30% of the tetramer remained unchanged and 70% was converted to the monomer. These results indicate that a 12-mer is not simply composed of three 4-mers.

The loss in activity was probably due to the liberation of copper upon urea treatment (Stark and Dawson, 1963).

The depolymerizing effect of urea may be interpreted on the basis of absence of intersubunit disulfide bonds.

2-mercaptoethanol: 0.02M

When the monomer of green zucchini squash was treated with 0.02M 2-mercaptoethanol for 10 minutes no change in the AAO isozyme pattern was observed. Under similar conditions disulfide bonds of peptide chains are split. It is therefore concluded that the monomer is most likely composed of one peptide chain.

Acid

Approximately 20% of the total AAO activity was left after lowering the pH of the green zucchini squash supernatant to 3.6 by addition of HCl acid and incubating

at 0°C for 30 minutes. A probable unfolding of the enzyme with subsequent loss in activity may have taken place. Upon gel electrophoresis only the tetramer was detected.

When the starting material was diluted with an equal volume of phosphate buffer, pH 7.0, ionic strength 0.1, treated with acid as described above, and an equal volume of sample was applied for gel electrophoresis no activity band was detected on the gel. Since this dilution did not exceed the sensitivity level of the activity measuring method, it can be concluded that at least the tetramer is more acid labile when it is present at lower concentrations.

The acid inactivation of the AAO activity seemed to be irreversible, since no activity was detected after dialysis of the acid-treated sample and adjustment to the initial pH of 7.0.

Relatedly, Clark et al. (1966) reported that treatment of the purified AAO from yellow crookneck squash (Cucurbita pepo), - total activity of 3,300 units per mg of protein - with acid (pH 3.6) after a 3 hour incubation resulted in 95% activity loss and formation of two slower components (S_{20} = 4.4 and 6.3) and some faster sedimenting material as indicated by the sedimentation pattern. A probable unfolding of the enzyme was suggested.

Alkali

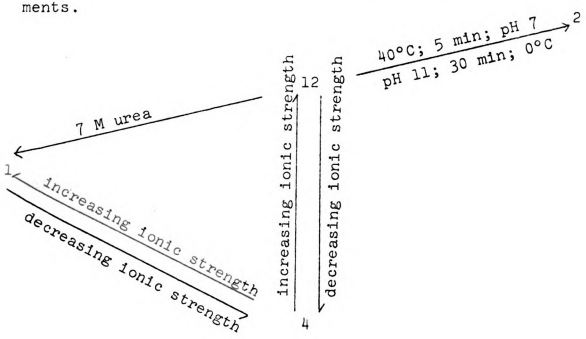
When the green zucchini squash supernatant was adjusted with NaOH to reach the pH 11.0 and remained for 30 minutes at this pH all the original forms of AAO disappeared and a new form appeared. Upon Sephadex gel filtration the new form was shown to have a molecular weight between 58,000 - 77,000 strongly suggesting a dimer.

When each of the original AAO bands was cut off from a gel, triturated with phosphate buffer, pH 7.0, treated for 30 minutes at pH 11.0 and again subjected to electrophoresis, forms 8, 12 and X were converted to the dimer, at a small loss (10 - 20%) of activity. Form 4 and form 1 were not converted to form 2 and apparently were completely destroyed.

Clark et al. (1966) reported that direct molecular weight determinations of the alkali-treated, purified enzyme from yellow summer squash revealed some heterogeneity of the sample. Two new components were found, the major one was calculated to have a molecular weight of about 65,000; the second component (comprising less than 15% of the protein) a MW of about 110,000. They suggested that the native enzyme (MW 135,000 to 140,000) is composed of more than one polypeptide chain, although no further evidence was provided.

The 65,000 MW form of AAO seems to be identical with the dimer found in our investigation, although this dimer appears to have been derived from higher molecular weight species in our conversion experiments. The dimer in the Clark et al. (1966) publication seemed to have its origin in the purified, concentrated tetramer of AAO. Since they conducted their conversion experiments starting with at least 3,000 AAO units of a preparation which was free of molecular forms other than the tetramer, it is possible that they had enough enzyme to detect a derived dimer. In our experiments the original tetramer was only 5 units and a possible small conversion of tetramer to dimer may have gone undetected.

The following scheme summarizes the interconversions of some AAO isozymic forms as a result of various treatments.



Effect of Purification on the Multiple Forms of AAO from Yellow Summer Squash

In order to investigate the reason for the lack of electrophoretic heterogeneity reported earlier (Dawson, 1966), the yellow summer squash AAO was purified according to Dawson and Magee (1955).

Polyacrylamide gel electrophoresis of the filtrate of the second ammonium sulfate precipitation, which was discarded by Dawson and Magee, showed zones 4, 8 and 12 with 4 again being the dominant one. An attempt to trace forms 1 and X of the crude extract among the purification steps failed. It is possible that the 1- and X-forms were either destroyed or interconverted to the other forms during the purification procedure.

It seems obvious that Dawson and Magee's (1955) purification procedure is selective for the purification of the tetramer form of AAO.

Intracellular Distribution of AAO and Other Enzymes

The differential centrifugation of the crude extract of the different plant tissues showed the AAO activity distribution as indicated in Table 5.

The mitochondrial pellet from the differential centrifugation was suspended in 2 ml phosphate buffer, pH 7.0, and tested for protein, AAO cytochrome c oxidase, glycolate oxidase and catalase activities. The results are shown in Table 6.

TABLE 5.--Distribution of AAO activity among differential centrifugation fractions of the skin of three commodities.

Fraction	% Activity		
	yellow summer squash	green zucchini squash	cucumber
Crude extract	100	100	100
Debris: 120xG	10	8	9
Broken chloroplasts 3,000xG	8	8	6
Mitochondria 35,000xG	5	6	3
Supernatant	74	73	87

TABLE 6.--Total protein content and four enzyme activities of the mitochondrial fraction obtained from differential centrifugation of the skin of yellow summer squash, green zucchini squash and cucumber.

		yellow summer squash	green zucchini squash	cucumber
Total protein	mg	26.5	21.2	9.0
AAO	total units	0.22	0.21	0.13
Cytochrome c oxidase	micromoles/min	0.9	0.9	1.4
	specific activity*	0.034	0.043	0.156
Glycolate oxidase	micromoles/min	0.7	0.8	0.3
	specific activity	0.026	0.038	0.033
Catalase	micromoles/min	890.0	815.0	1010.0
	specific activity	33.6	38.4	112.2

*Specific activity = micromoles per minute per mg of total protein.

When sucrose density gradient centrifugation was applied to the mitochondrial fraction, five new fractions were obtained. The relationships between sucrose concentration, fraction volume and type of particles are shown in Table 7.

When the resuspended mitochondria were subjected to sucrose density gradient centrifugation, it was observed that the total AAO activity was located in the supernatant as shown in Table 8. This table also indicates the distribution of the enzymes cytochrome c oxidase, glycolate oxidase and catalase, which were used as indicators (cytochrome c oxidase indicates mitochondria; glycolate oxidase and catalase indicate peroxisomes) for the separation of the different particulate fractions after sucrose density gradient centrifugation (Tolbert et al., 1968 and 1969). Chlorophyll determinations were also used to indicate the chloroplast fraction in the sucrose gradient analysis (Figure 9 and Figure 10).

Table 5 indicates that there may be some small AAO activity associated with particles (debris, chloroplasts and mitochondria). When, however, these particles were resuspended to phosphate buffer, pH 7.0, and centrifuged at 120xG (debris), 3,000xG (chloroplasts) and 35,000xG (mitochondria) the AAO activity was reduced to 4, 2, and 2%, respectively, and after resuspending a second time no activity was detected in any of the particulate fractions.

TABLE 7.--Sucrose density gradient centrifugation of the mitochondrial fraction of the skin of yellow summer squash, green zucchini squash and cucumber.*

Fraction	% Sucrose	Volume ml	Type of particles in band
5	44.5	1.5	Supernatant
4	46.0	4.1	Broken chloroplasts
3	54.5	1.8	Mitochondria
2	64.5	2.1	Peroxisomes
1	73.0	2.2	None

*Equivalent fractions from 2 gradients were combined and the % sucrose determined using a Zeiss Hand Refractometer; the combined fractions were also used for enzyme assays.

TABLE 8.--Enzyme distribution among sucrose density gradient centrifugation fractions of resuspended mitochondria of the skin of yellow summer squash, green zucchini squash and cucumber.

Fraction*	Total protein	AAO	Cytochrome c oxidase		Glycolate oxidase		Catalase	
	mg	Total units	micromoles per min	spec. act.	micromoles per min	spec. act.	micromoles per min	spec. act.
			Yellow summer squash					
5	8.9	0.10	0.10	0.01	0.18	0.02	112.0	12.5
4	0.6	0.00	0.06	0.10	0.00	0.00	8.0	13.3
3	2.2	0.00	0.28	0.13	0.00	0.00	11.0	5.0
2	0.9	0.00	0.00	0.00	0.14	0.16	218.0	242.0
1	0.2	0.00	0.00	0.00	0.01	0.05	81.0	405.0
			Green zucchini squash					
5	7.5	0.10	0.08	0.01	0.20	0.03	98.0	13.1
4	0.4	0.00	0.06	0.15	0.00	0.00	14.0	35.0
3	1.8	0.00	0.30	0.17	0.00	0.00	18.0	10.0
2	0.9	0.00	0.00	0.00	0.18	0.20	200.0	222.2
1	0.2	0.00	0.00	0.00	0.03	0.15	60.0	300.0
			Cucumber					
5	3.1	0.06	0.12	0.04	0.07	0.02	108.0	34.8
4	0.2	0.00	0.06	0.30	0.00	0.00	4.0	20.0
3	0.8	0.00	0.52	0.65	0.00	0.00	9.0	11.3
2	0.4	0.00	0.4	0.10	0.07	0.18	328.0	820.0
1	0.1	0.00	0.00	0.00	0.00	0.00	77.0	770.0

*Fractions are numbered in the order in which they were taken from the bottom of the centrifuge tube.

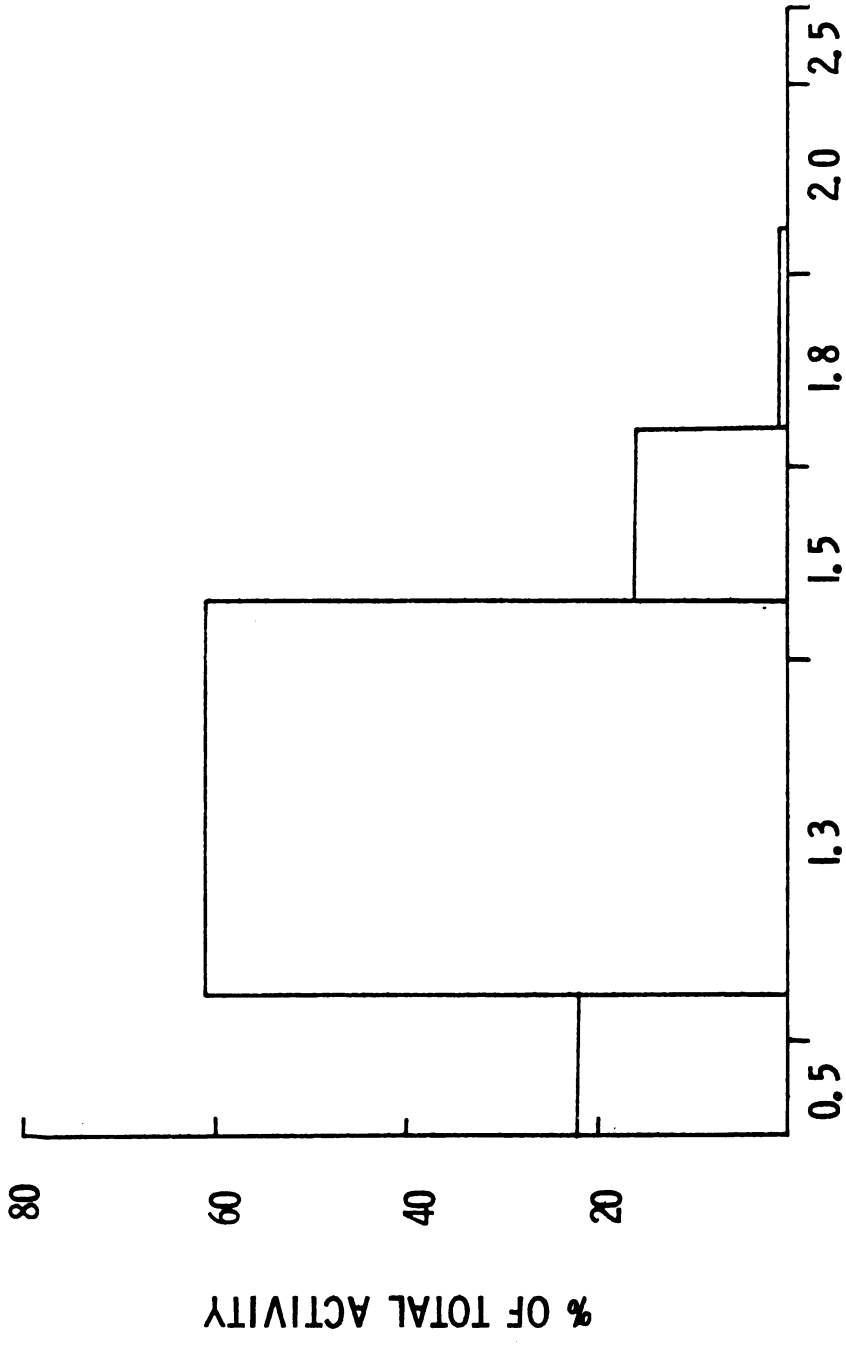


Figure 9.--% DISTRIBUTION OF CHLOROPHYLL FROM THE MITOCHONDRIA FRACTION OF GREEN ZUCCHINI SQUASH SKIN AFTER SUCROSE GRADIENT CENTRIFUGATION

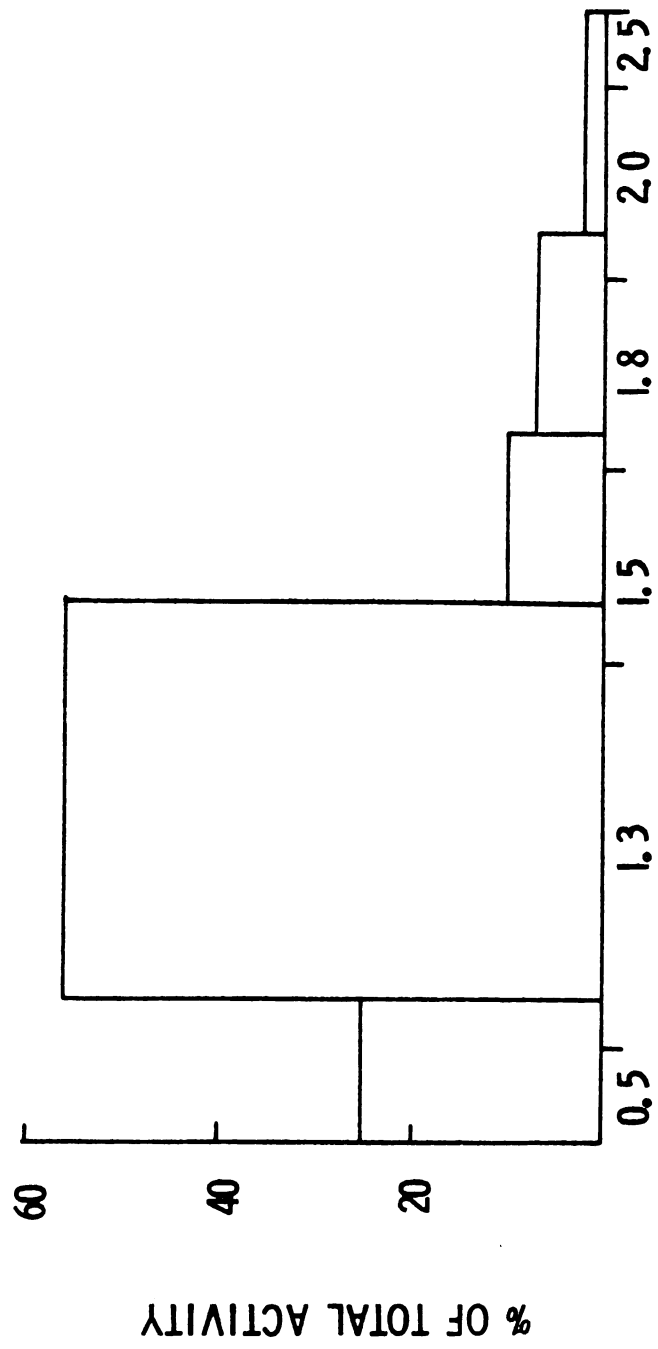


Figure 10. --% DISTRIBUTION OF CHLOROPHYLL FROM THE MITOCHONDRIA FRACTION OF CUCUMBER SKIN AFTER SUCROSE GRADIENT CENTRIFUGATION

It can therefore be concluded that AAO is not a particulate enzyme and it is present in a soluble form.

Isoelectric Points (pI) of AAO Isozymes

The results of the gel electrophoretic separations of the AAO forms at different pH values are summarized in Fibures 11, 12 and 13 for yellow summer squash, green zucchini squash and cucumber, respectively. From these graphs the pI of the various AAO forms can be derived. The pI of AAO from yellow summer squash and green zucchini squash was found to lie at about pH 5.35 and that of the cucumber AAO multiple molecular forms at 6.7.

Dunn and Dawson (1951) estimated the pI of purified AAO from the crookneck squash to be in the region of pH 5.0 to 5.5 using the apparatus of the Tiselius type for electrophoresis.

A report by Nakamura et al. (1968) reported that the difference between enzyme preparations obtained from cucumber AAO and those from summer crookneck squash was that the former is a neutral protein and the latter is an acidic protein as indicated by their electrophoretic behavior. Furthermore he wrote that the isoelectric point of the cucumber enzyme protein seems to be between pH 6.0 and pH 7.8.

Wills (1952) estimated the isoelectric point of AAO from cucumber to be pH 5.1 by suramin inhibition studies.

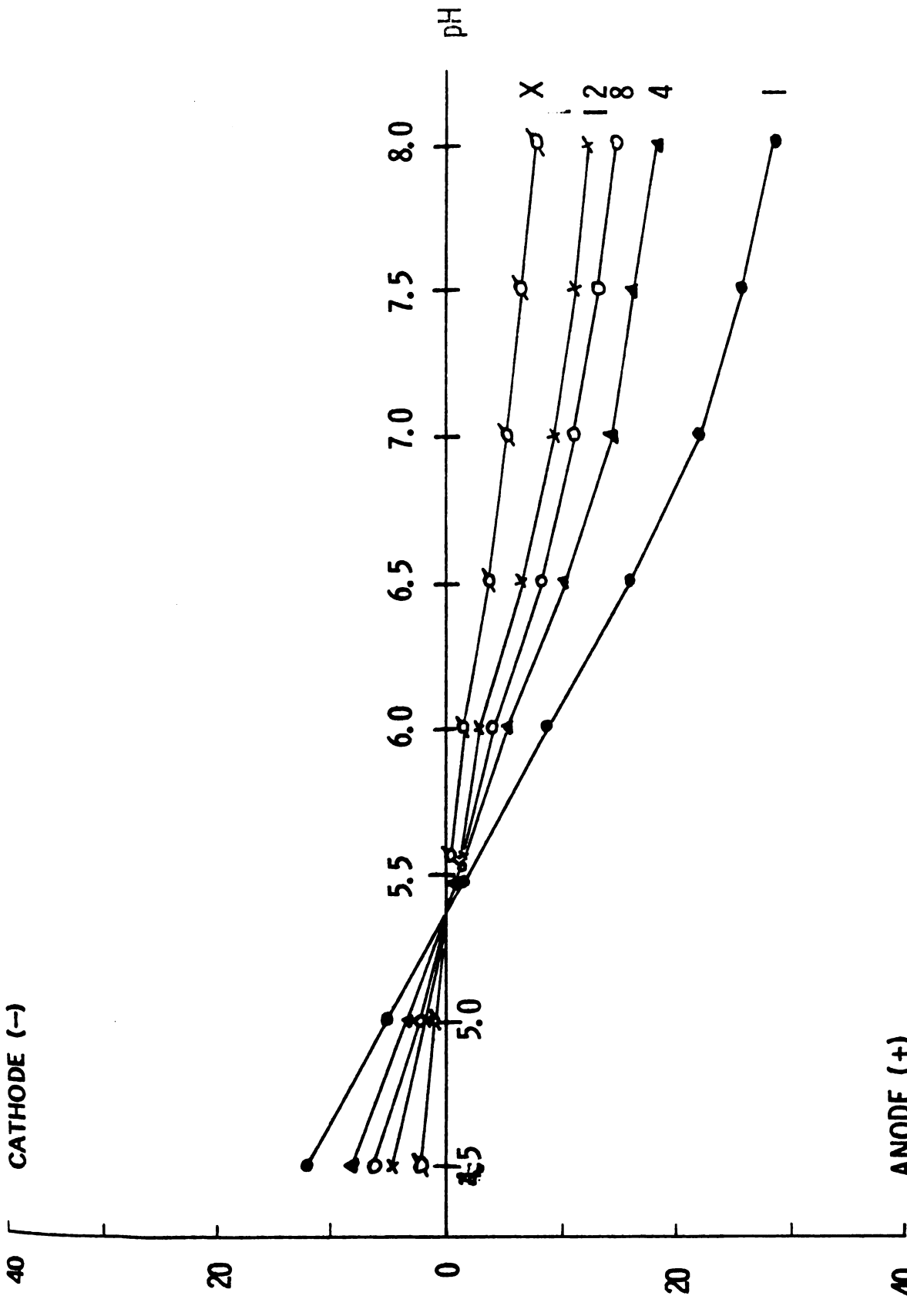


Figure 11.--ACRYLAMIDE GEL ELECTROPHORETIC MOBILITIES OF YELLOW SUMMER SQUASH ISOZYMES AT VARIOUS pH

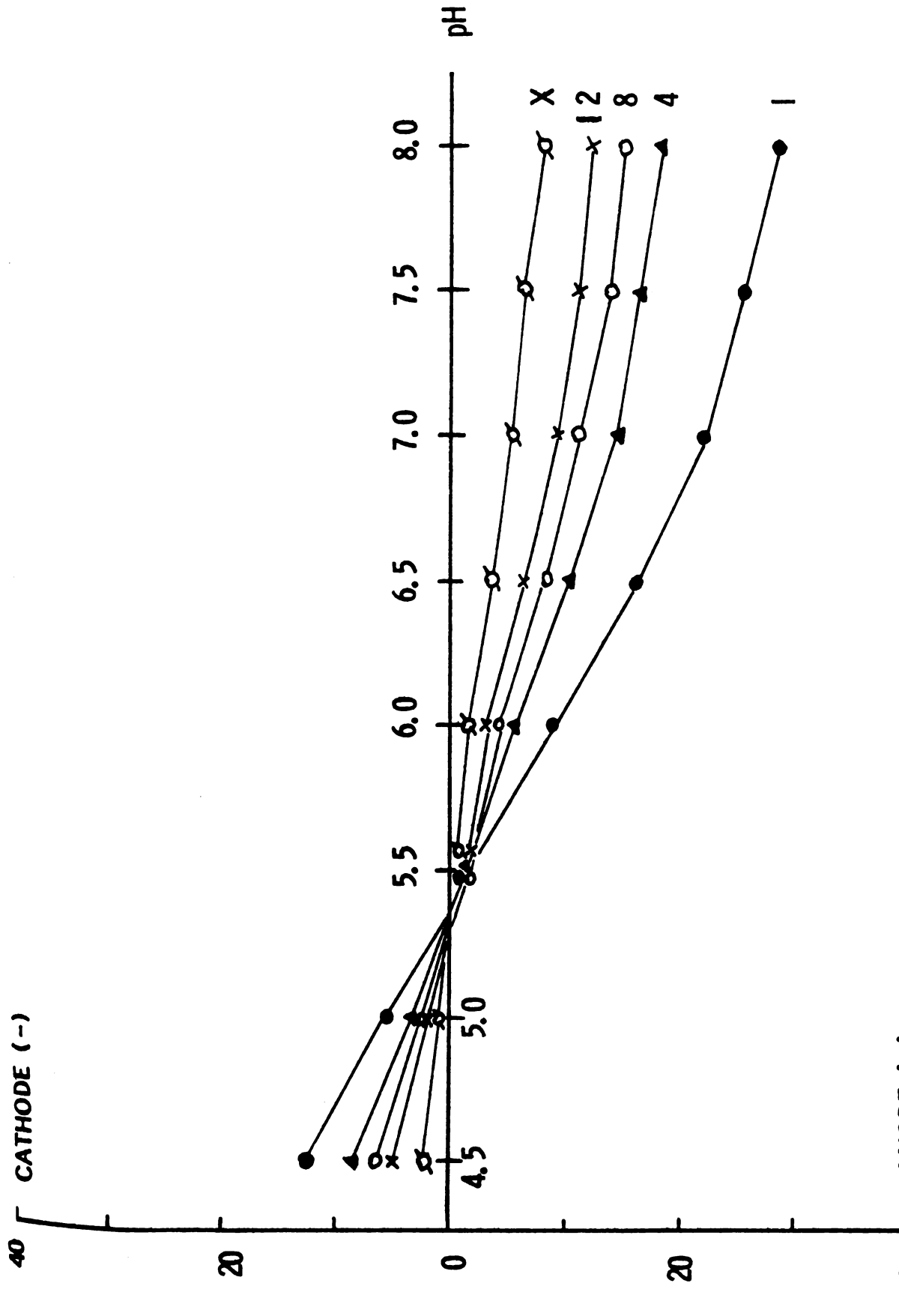


Figure 12 --ACRYLAMIDE GEL ELECTROPHORETIC MOBILITIES OF GREEN ZUCCHINI SQUASH ISOZYMES AT VARIOUS pH

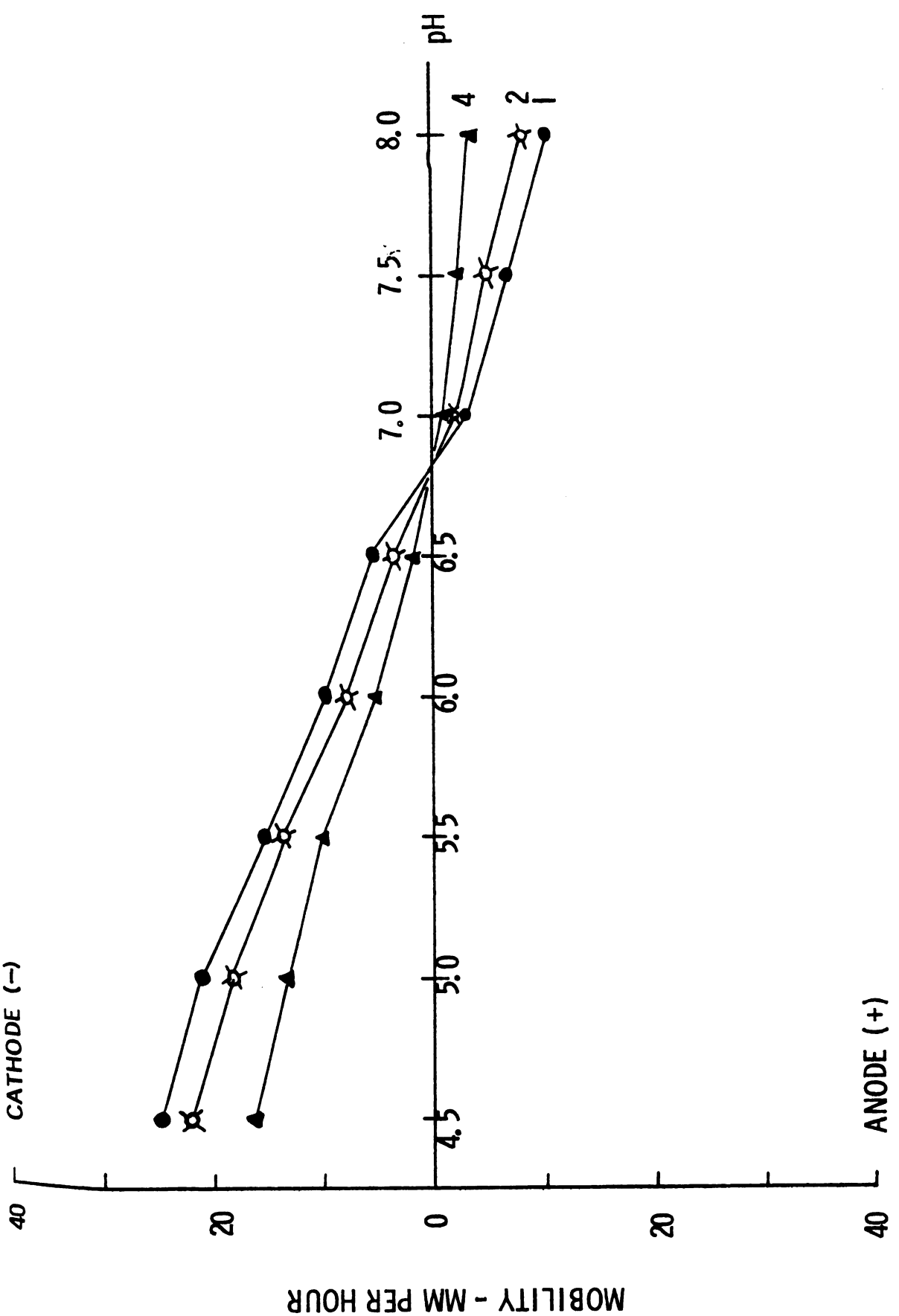


Figure 13.--ACRYLAMIDE GEL ELECTROPHORETIC MOBILITIES OF CUCUMBER ISOZYMES AT VARIOUS pH

K_m and V_{max} of AAO

Frieden and Maggiolo (1957) reported a K_m value of $5x10^{-3}M$ for the AAO of yellow summer squash, using the Warburg technique. They also reported a much lower K_m, $3.9x10^{-5}M$, determined by a spectrophotometric method. They explained this discrepancy on the basis of large differences in enzyme and substrate concentrations in the two test methods.

Our values obtained for K_m using the Warburg and spectrophotometric methods lie relatively close together (Table 9). Our data from the spectrophotometric method are 5 to 10 times higher than those from the Warburg method. The reason for the closer agreement between our Warburg and spectrophotometric values must be due to the identical concentrations used for the substrate in both methods, although the enzyme concentration in the Warburg method had to be higher.

Figures 14, 15 and 16 present the Lineweaver - Burk treatment of the data obtained by the spectrophotometric method.

Other workers reported K_m values of $4.2x10^{-4}M$ at 25° (Schulte and Schillinger, 1952) and $2x10^{-3}M$ at 38° (Shimosaka, 1957) using manometric assays; Cohen (1954) reported a K_m value of $2.4x10^{-4}M$ at 25° using a spectrophotometric assay. All these values do not differ greatly from those obtained in this work.

TABLE 9.--K_m and V_{max} values of the AAO of yellow summer squash, green zucchini squash and cucumber.

	K_m ($x10^4 M$)	V_{max} ($Mmin^{-1}$)($x10^5$)
Yellow summer squash		
Spectrophotometric	1.86	1.06
Warburg	9.80	--
Green zucchini squash		
Spectrophotometric	3.60	0.86
Warburg	22.10	--
Cucumber		
Spectrophotometric	1.81	1.28
Warburg	11.25	--

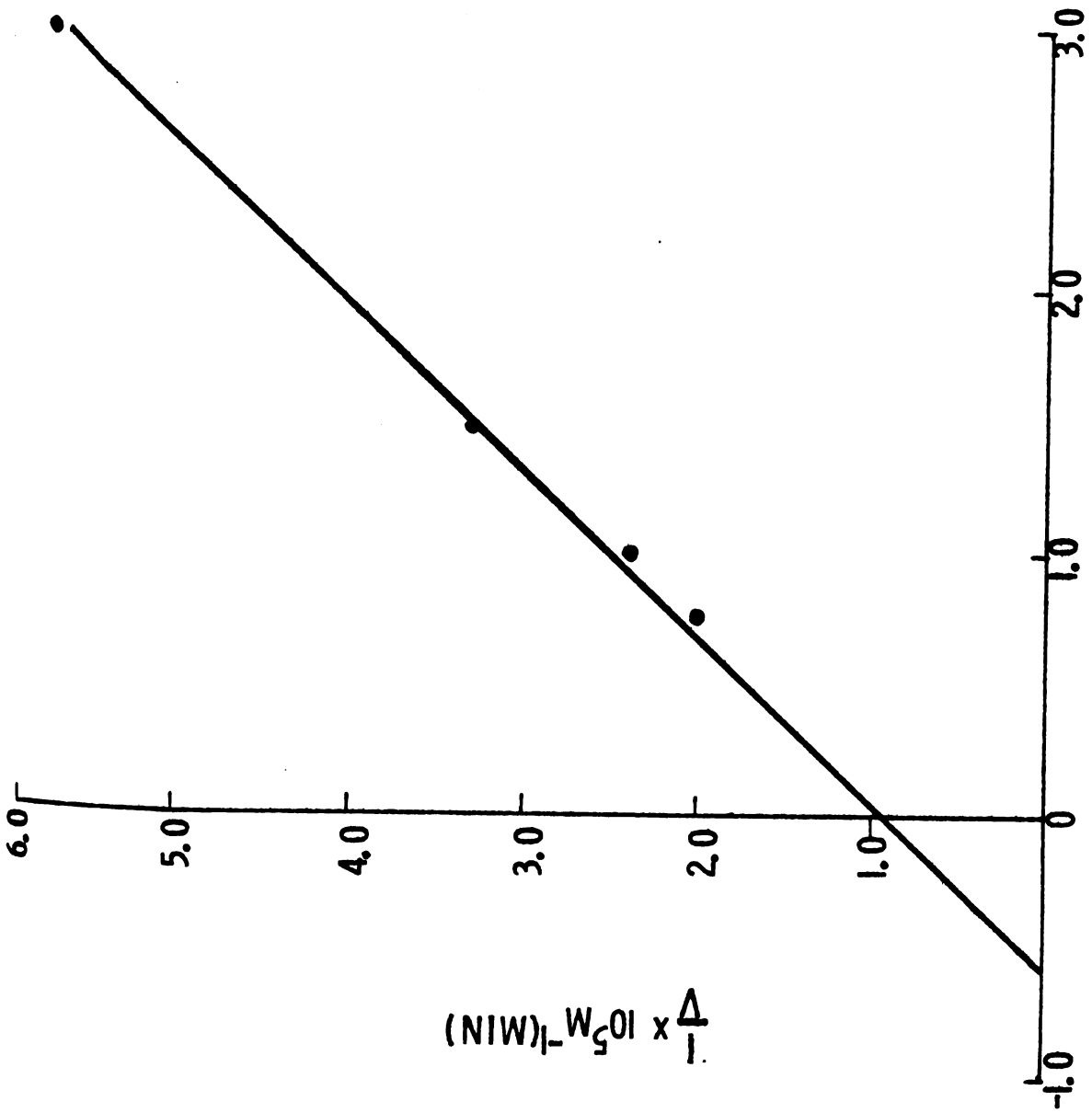


Figure 14. --LINEWEAVER - BURK PLOT OF THE AAO FROM YELLOW SUMMER SQUASH USING A SPECTROPHOTOMETRIC METHOD

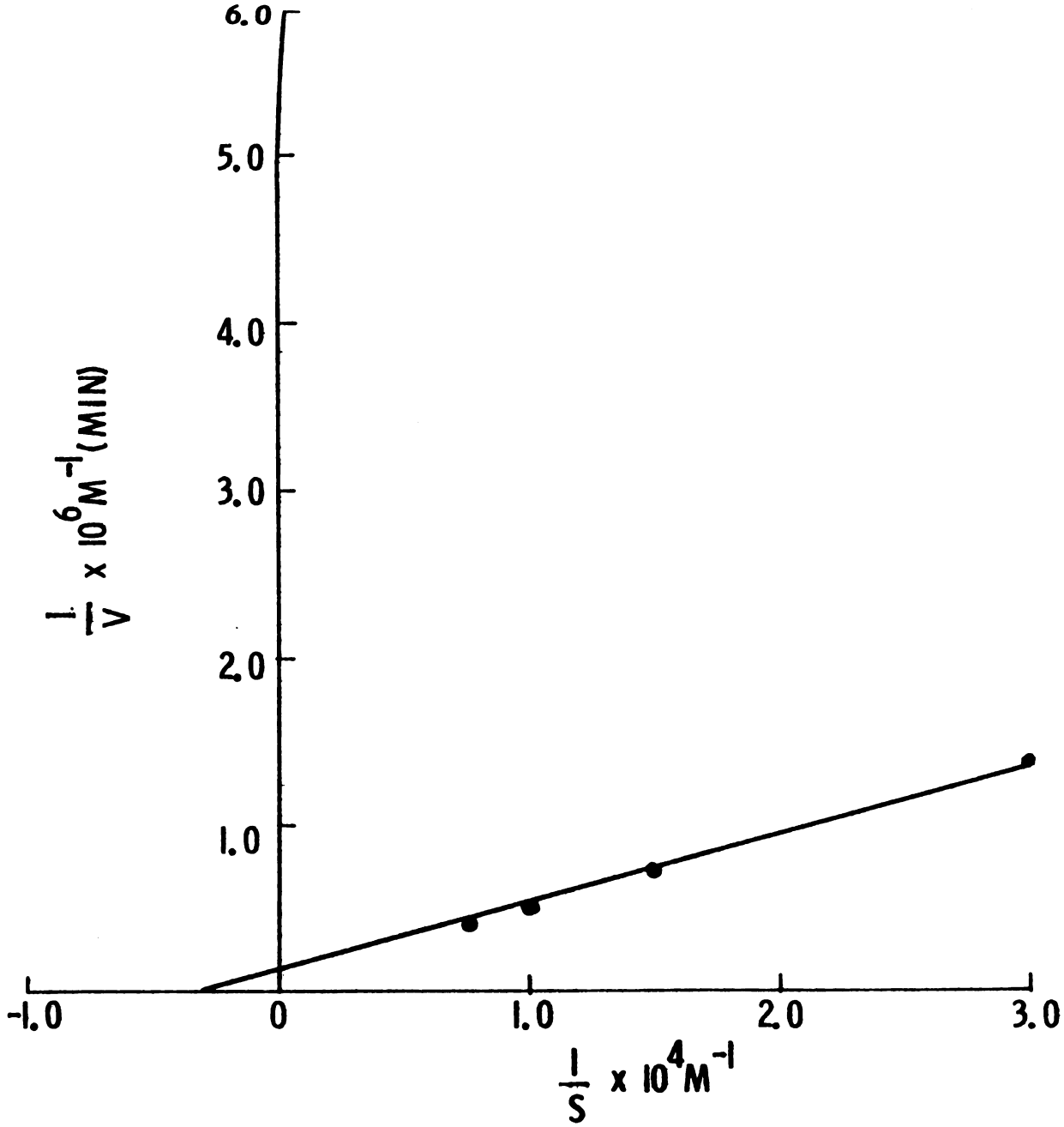


Figure 15.--LINEWEAVER - BURK PLOT OF THE AAO FROM GREEN ZUCCHINI SQUASH USING A SPECTROPHOTOMETRIC METHOD

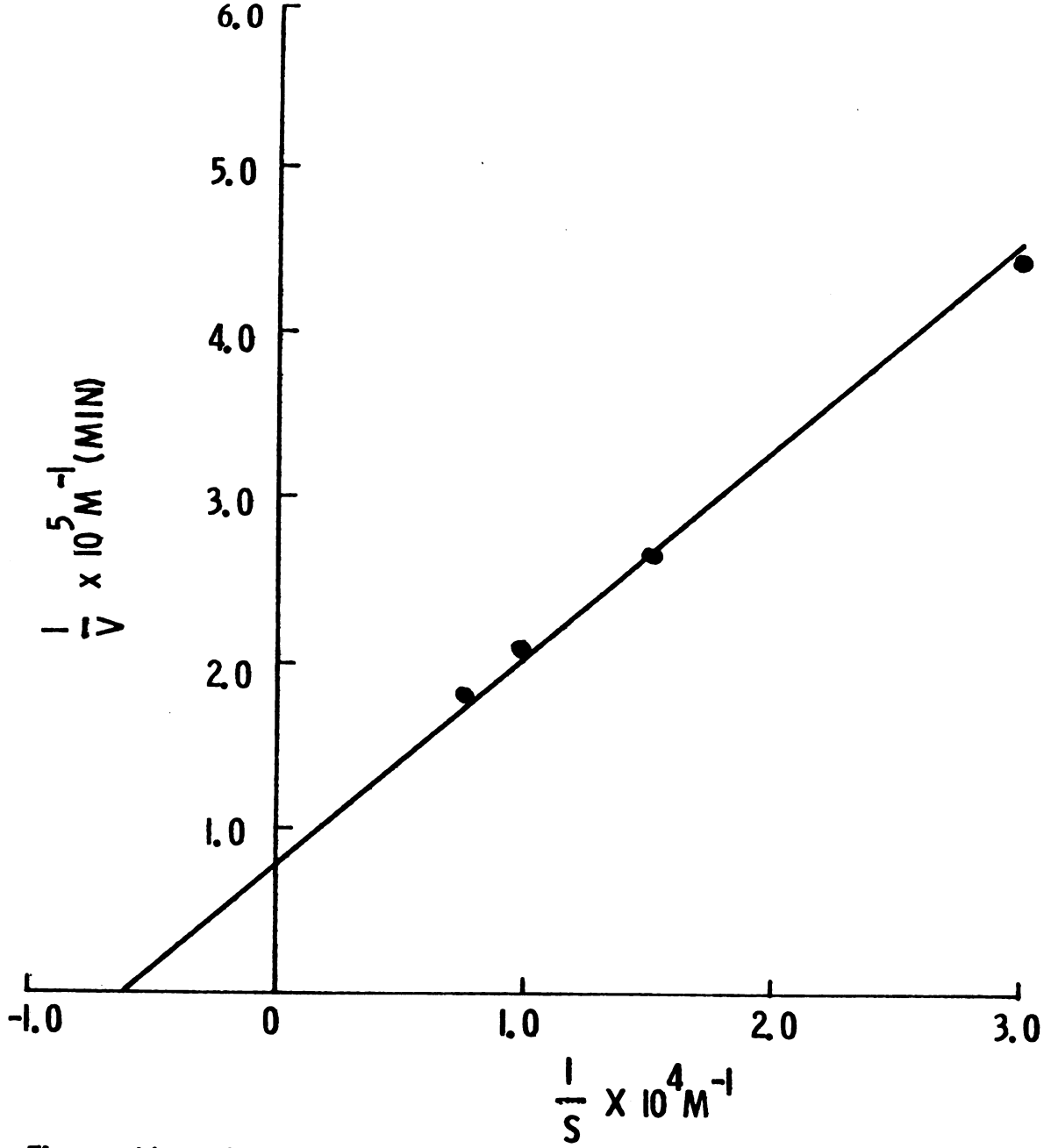


Figure 16.--LINEWEAVER - BURK PLOT OF THE AAO FROM CUCUMBER USING A SPECTROPHOTOMETRIC METHOD

SUMMARY

Disc polyacrylamide gel electrophoresis revealed the existence of five different molecular forms of AAO in yellow summer squash (Cucurbita pepo condensa) and green zucchini squash (C. pepo medullosa) and three such forms in cucumber (Cucumis sativus). Repeated electrophoresis of the isolated forms excluded the possibility that these forms were artifacts, but represents an intracellular state of the enzyme system.

Molecular weight determinations using Sephadex G-100 and Sephadex G-200 strongly suggested the presence of a monomeric unit of approximately 30,000 MW in the cucumber and 35,000 MW in the two squashes. A dimer and a tetramer also appeared in the cucumber, while a tetramer, an octamer (8-mer), a dodecamer (12-mer) and an X-mer (MW 670,000 - 2,000,000) appeared in the two squashes. The MW of the heaviest form, the X-mer, could not be estimated using this technique because the highest MW protein marker used was 670,000.

The monomer represented 50% of the total AAO activity of the cucumber, the dimer 40% and the tetramer 10%. The dominant AAO form in the two squashes appeared to be the tetramer with 70% of the total activity.

Extraction of AAO with phosphate buffer, pH 7.0, yielded the highest activity, followed by phosphate buffer, pH 8.0, and water extraction which yielded the lowest activity. A 4-fold increase in ionic strength had little effect on the enzyme yield.

No difference between skin and flesh in the AAO isozyme pattern was detected in any of the three commodities studied.

A reversible association-dissociation of the squash and cucumber isozymes was observed when the ionic strength of the solution was changed. Increased ionic strength resulted in both association (4 going to 8-, 12- and X-mers) and dissociation (4 going to 1) of forms. The tetramer played a central role in these fransformations, although itself is unstable at high ionic strength.

The isoelectric point of all multiple forms in the yellow summer and green zucchini squashes was 5.35, that of the cucumber AAO forms was 6.70.

Using a spectrophotometric and a manometric method the K_m values were determined for the composite AAO forms of both squashes and the cucumber. The data obtained by the spectrophotometric method were $K_m = 1.86 \times 10^{-4}$M for yellow summer squash, 3.60×10^{-4}M for green zucchini squash and 1.81×10^{-4}M for cucumber; those obtained by the Warburg technique were 9.80×10^{-4}M for yellow summer

squash, 22.10 x 10^{-4}M for green zucchini squash and 11.25 x 10^{-4}M for cucumber.

Differential and density gradient centrifugation indicated that all AAO forms were not particulate proteins.

Mild heat, 40°C, 5 minutes, pH 7.0, quantitatively converted the 8-mer, 12-mer and X-mer to the dimer in the case of the two squashes. An incubation time of 60 minutes at 40°C destroyed the monomer activity of the cucumber AAO. At 60°C for 5 minutes only the tetramer survived in the squashes. When the heating was prolonged to 60 minutes at 60°C, the only surviving form in the cucumber was the dimer. An incubation time of 60 minutes at 70°C destroyed all AAO activity tested.

Treatment of the green zucchini squash isozymic forms with 7M urea resulted in the conversion of the X-, 12-, 8- and 4-forms to the monomer.

Acid treatment (HCl, pH 3.6, 30 min. 0°C) destroyed all the AAO molecular forms from green zucchini squash except the tetrameric form.

It was observed that alkali (NaOH, pH 11.0, 30 min. 0°C) converted forms X-, 12- and 8 of the green zucchini squash AAO to the dimer. The tetramer and the monomer were destroyed upon alkali treatment.

Dawson and Magee's (1955) purification procedure seems to be selective for the purification of the tetramer form of AAO.

BIBLIOGRAPHY

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