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18

PURIFICATION AND PROPERTIES OF TRIOSEPHOSPHATE ISOMERASE FROM SELECTED CHLOROPHYTA

A Thesis

Presented to

the Graduate Faculty

Central Washington State College

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

John C. Meeks

August, 1967

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APPROVED FOR THE GRADUATE FACULTY

Dan L. Willson, COMMITTEE CHAIRMAN

Robert D. Gaines

Virginia P. Harden

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INTRODUCTION

Unicellular green algae have been used extensively for fundamental investigations of plant metabolism. Most of the metabolic pathways common to living cells have been determined as operating in these algae. The pathways have been established primarily through the identification of radioactively tagged intermediate compounds rather than by characterization of the enzyme systems (11). The path of carbon in photosynthesis was determined by Bassham and his associates in 1954 using radioactive tracers with *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (1).

The total protein in some fresh water algae has been determined on a per cent basis of the ash free dry weight. The values vary with species and manner of culturing; some *Chlamydomonas* species contained 36 per cent protein, while *Chlorella pyrenoidosa* displayed 88 per cent when cultured under specific conditions (15). The majority of the total protein is considered to be biologically active rather than structural or particulate (8). The lack of information concerning the qualitative identification of algal proteins appears to result from the low bulk of readily available mass cultured unialgal species, difficulties in rupturing the individual cell walls and isolation of the different proteins.

The predominant storage product of the Chlorophyta or green algae is a starch similar to that found in higher plants. This implies that the enzyme systems for the synthesis of starch in the Chlorophyta may be similar to the systems of higher plants. Amylase and phosphorylase activity have been detected in *Hydrodictyon reticulatum* but not in *Scenedesmus* sp. (11).

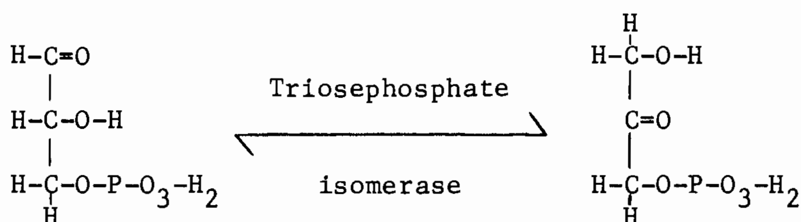
The pathway of monosaccharide degradation in all green algae appears to be similar to that found in higher plants. Crude enzyme preparations from a number of laboratories indicate the activity of those enzymes associated with the Embden-Meyerhof pathway of hexose degradation. In addition, several enzymes of the pentose phosphate cycle and the tricarboxylic acid cycle have been isolated (11).

V. G. Richter, in 1957, reported the presence of triosephosphate isomerase, aldolase and glucose-6-phosphate dehydrogenase in a crude protein extract from a species of Chlorella (17). Richter followed this original study two years later with a comparative study between two algal species. Enzyme activity for glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucoisomerase, transketolase, aldolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase: NADP specific, transaldolase, ribulokinase, and a carboxylating enzyme were present in partially purified extracts from Anacystis nidulans and Chlorella pyrenoidosa (18).

Studies on specific enzyme systems of unicellular green algae have been published in the last year. Two fructose-1, 6-diphosphate aldolases were isolated, purified 30 fold and partially characterized from Chlamydomonas mundana (20). An induced acid phosphatase displaying a wide range of substrate specificity was isolated and partially purified from Euglena gracilis (4). Studies on the activity of a glyoxylate cycle in cell free protein extracts from Chlorella vulgaris (Brannan number one strain) and Chlamydomonas reinhardii have indicated the presence of isocitrate lyase, citrate synthetase and malate synthetase (10).

Triosephosphate isomerase from _Anacystis nidulans_ and _Chlorella pyrenoidosa_ displayed the highest specific activity of the glycolytic enzymes isolated in 1959 by Richter (18). For reasons of its high activity and apparent ease of solubility, triosephosphate isomerase was selected as the enzyme to be investigated by this investigator.

The enzyme triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1) was first discovered in 1934 by Otto Meyerhof (13). Triosephosphate isomerase (TIM or isomerase) catalyzes the reversible isomerization between glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) as shown below.



Triosephosphate isomerase has been studied extensively in the past two decades. It was reported crystallized from calf muscle by Beisenherz in 1955 (2). In addition to describing an assay for activity and techniques for purification, he also reported some of the properties of the enzyme and the reaction. In 1960, isomerase was crystallized from rabbit skeleton muscle by Czok and Bucher (6).

Studying the mechanism of the isomerase reaction, Reider and Rose used the methods of Beisenherz to prepare the enzyme from calf muscle. Running the reaction in tritiated water, they indicated that the interconversion of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate by calf muscle isomerase resulted in the formation of a monotritiated DHAP. This and other data supported the conclusion that the

mechanism results in the formation of an enolated anion bound to the enzyme (19).

In 1966, Burton and Waley studied the enzyme from rabbit muscle. The molecular weight of rabbit isomerase was estimated as 43,000 by exclusion chromatography on Sephadex G-100. Studies on the susceptibility of the enzyme to photooxidation, its reactivity to iodoacetate and the amino acid analysis indicated that histidine functions as the basic group in the active center (5).

Snyder, in 1966, reported the separation of liver isomerase from liver alcohol dehydrogenase by chromatography on a crude CM cellulose column. The degree of inhibition and stimulation of the chromatographically purified isomerase from horse liver was compared with commercial rabbit muscle isomerase (22).

The only reported study of triosephosphate isomerase from higher plants was done on pea seeds when Turner, et al., in 1965, obtained a partially purified preparation (23).

Although there has been a recent increase in the number of investigations of algal enzyme systems, there has yet to appear reports of more than partial purification of an enzyme protein from these organisms. It is the purpose of this study to purify and investigate some of the properties of triosephosphate isomerase from two unicellular green algae, Ankistrodesmus Braunii and Scenedesmus acuminatus, and one filamentous green alga, Chara sp.

The two unicellular algae belong to the Order Chlorococcales of the Chlorophyta. Ankistrodesmus Braunii is identified by relatively

broad fusiform unicells with the lateral margin irregularly convex and narrowed to a short point at the poles. The cells, without a gelatinous envelope, are small, 8 to 10 microns in diameter and from 20 to 56 microns in length (15). Scenedesmus acuminatus is described as having cells arranged in a curved series of 4 cells that are strongly lunate with sharply pointed apices. The convex walls adjoin inwardly with the convex faces directed outward. These unicells are 3 to 7 microns in diameter and 30 to 40 microns in length (15).

The genus Chara has an erect branched thallus differentiated into a regular succession of nodes and internodes. Each node bears a whorl of branches referred to as "leaves." The internodes consist of an internal internodal cell ensheathed or corticated by a layer of vertically elongated cells of much smaller diameter. The macroscopic thallus is attached to the substratum by rhizoids (21).

METHODS AND MATERIALS

Algal Culture and Harvest

Ankistrodesmus Braunii (culture number 245) and Scenedesmus acuminatus (culture number 415), bacteria free unialgal cultures, were obtained from Indiana University and maintained as stock cultures on agar slants of Bristol's medium supplemented with proteose. Chara sp. was collected from a fresh water seepage pond on the east side of the Yakima River 8.5 miles northwest of Ellensburg, Washington.

A. Braunii and S. acuminatus were mass cultured under sterile conditions in a modified aqueous inorganic nutrient medium (12) (Table 1). Culture flasks, two 5 gallon "pyrex" narrow neck containers holding a total volume of 15 liters of nutrient solution per flask, were placed on a culture rack at a controlled temperature of $20^{\circ} \pm 2$. Fluorescent and tungsten light was emitted from the top and bottom at an intensity of a little over 38 lux.

The cultures were aerated with a continuous flow of air containing approximately 3 per cent per volume of carbon dioxide. Airborne contaminants were removed by bubbling the air and CO_2 mixture through 2 gas traps containing concentrated sulfuric acid and a third blank trap. Dry cotton filter plugs were periodically placed in the air lines to prevent any additional contamination by viable spores passing through the acid baths.

Table 1. Complete inorganic medium for mass culture.

Macronutrients		
Compound	Weight (g/liter)	ppm
KNO_3	1.00	1000
$MgSO_4$	0.25	250
KH_2PO_4	0.25	250

Micronutrients		
Compound	Weight (g/liter in stock solution)	0.1 ml/ liter gives ppm of the metal
H_3BO_3	2.860	0.050
$MnCl_2 \cdot 4H_2O$	1.810	0.050
$ZnSO_4 \cdot 7H_2O$	0.222	0.005
$CuSO_4 \cdot 5H_2O$	0.079	0.002
MoO_3	0.015	0.001
$Ca(NO_3)_2 \cdot 4H_2O$	59.000	1.000

Iron Solution and Acetate		
Compound	Weight (g/liter)	1.0 ml/ liter gives ppm of Fe
$FeC_6H_5O_7 \cdot 3H_2O$	5.30*	1.000
$NaC_2H_3O_2 \cdot 3H_2O$	0.25	

*in stock solution

Note: The three macronutrients, the iron and sodium acetate were autoclaved separately. The micronutrients were autoclaved together. The pH of the sterile medium was 6.5.

Aqueous unialgal starter cultures were initiated by inoculating 25 ml of autoclaved inorganic medium with algae from the stock agar slants. Algae were inoculated into the mass culture flasks from 2 to 3 weeks old starter cultures by aseptically pouring 25 ml of the starter solution into 15 liters of the autoclaved medium. Bacterial contamination was checked before inoculation and before harvest by plating on nutrient agar and subsequent incubation at 37°.

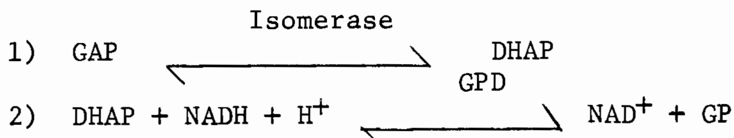
The Chlorococcales were harvested when it became visually apparent that growth had become an excess function of light intensity. S. acuminatus was harvested by three filtrations through Reeve Angel grade number 835 fluted filter paper. The wet residue, consisting of whole algal cells, was scraped from the filter paper then frozen. A. Braunii was harvested in the same manner except Eaton Dikeman grade number 512 fluted filter paper was employed.

The collected Chara sp. was washed with ice cold distilled water, one per cent chlorine water and, again, with ice cold distilled water before freezing(16).

Assay Procedure

The reaction catalyzed by triosephosphate isomerase is a reversible isomerization of two triosephosphates, GAP and DHAP. The equilibrium, as catalyzed by calf muscle isomerase, is in favor of DHAP (2). If one of the reactants is removed, the equilibrium will shift in the direction of the removed compound. The assay procedure for triosephosphate isomerase utilizes this concept by coupling with a second reaction.

This assay is the coupled reaction reported by Beisenherz using GAP as substrate for the isomerase and α-glycerophosphate dehydrogenase (GPD) as the coupling enzyme (2). The action of isomerase transforms GAP to DHAP. The coupling enzyme (GPD), together with the reduced form of the coenzyme nicotinamide adenine dinucleotide (NADH), converts DHAP to α-glyderophosphate (GP) as shown below. DHAP is removed as rapidly



as it is formed, forcing the isomerase reaction essentially to completion. The reaction is followed colorimetrically utilizing the shift of the characteristic absorption of NADH from 340 mμ. Decrease in absorption at this wave length indicates oxidation of NADH and the amount of NADH oxidized is proportional to the amount of GAP transformed to DHAP. The assay mixture used in the purification procedure for triosephosphate isomerase is shown in Table 2.

The reaction is initiated by pipetting into the assay mixture (2.9 ml) 0.1 ml of isomerase solution. The reaction was allowed to proceed for approximately 30 to 70 seconds (change in OD of 0.05 to 0.09 unit) and the time required for a decrease in optical density of 0.1 unit was recorded. Because of the coupled nature of the assay, α-glycerophosphate dehydrogenase was added in excess before the addition of isomerase.

A unit of enzyme activity in this assay is defined as the seconds required for a change of 0.1 OD unit divided into 100. The specific activity of the isomerase is based on the units per mg protein per ml used in the respective reaction.

Table 2. Reaction mixture for assay of triosephosphate isomerase activity

Compound	Concentration per 3.0 ml	Volume in ml
Tris* buffer ph 7.5 containing EDTA	0.025 M 5.4 mM	2.6
α-glycerophosphate dehydrogenase	0.13 mg	0.1
NADH	0.308 μM	0.1
D-glyceraldehyde-3-phosphate	0.150 μM	0.1

* Tris is Tris (hydroxymethyl) aminomethane

Protein determination was by ultraviolet absorption at 276 mμ with Bovine serum albumin as the protein standard. All reactions and protein determinations were run at room temperature on a Beckman model DB spectrophotometer with a one cm light path and in quartz cuvettes.

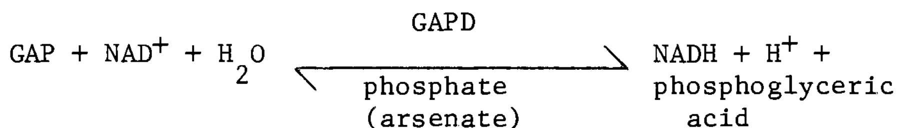
Substrates

NADH was obtained from Calbiochem, Los Angeles, California and stored in a dessicator at 0 to 5°. A stock solution of 5.5 mg in 2.5 ml of distilled water (3.08 μM per ml) was made fresh each week and kept under refrigeration at 0 to 5° when not in use.

Alpha glycerophosphate dehydrogenase was obtained from Calbiochem as a crystalline suspension in 2.0 M ammonium sulfate solution. Weekly, 0.025 ml of the crystalline solution was dissolved in 2.50 ml of Tris buffer (0.025 M, pH 7.5) and dialyzed against the same buffer for 8 hours. The dialyzed solution, free from sulfate ions, contained 0.105 to 0.160 mg protein per ml and was kept under refrigeration at 0 to 5°.

DL-glyceraldehyde-3-phosphate was obtained from the Sigma Chemical Company, St. Louis, Missouri, as the diethyl acetal monobarium salt. Free DL-GAP was obtained by adding 100 mg of the salt to 1.5 g of Dowex 50 resin in the H^+ form in 6.0 ml of distilled water, placing in a boiling water bath for 3 minutes and chilling quickly by transferring to an ice bath. The chilled mixture was centrifuged for ten minutes at high speed in an International model CL clinical centrifuge, the supernatant solution was decanted and the centrifugate washed twice with 2 ml of distilled water and recentrifuged as before. The supernatant fractions were combined and stored frozen in 1.0 ml portions. The final solution contained between 1.1 and 2.1 μM of biologically active D-glyceraldehyde-3-phosphate per ml at pH 2.5. For assays of varying substrate concentration, the solution was concentrated by removing the solvent under a vacuum.

Determination of the concentration of GAP present was carried out using an assay developed by Velick for glyceraldehyde-3-phosphate dehydrogenase (GAPD) (24). The characteristic shift of absorption for the oxidation of NADH is also applicable in this reaction as shown below.



$$GAP + NAD^+ + H_2O \underset{\text{phosphate (arsenate)}}{\overset{GAPD}{\rightleftharpoons}} NADH + H^+ + \text{phosphoglyceric acid}$$

The reaction is forced to completion by replacing the inorganic phosphate by disodium arsenate. The arsenate rather than orthophosphate combines with GAP forming an unstable compound that rapidly dissociates. The assay mixture is shown in Table 3.

Table 3. Assay mixture of glyceraldehydephosphate dehydrogenase

Compound	Concentration per 3.0 ml	Volume in ml
Tris buffer (pH 8.5)	0.20 M	1.50
NaF	0.1 M	0.60
Na_2HAsO_4	0.17 M	0.30
L-cysteine·HCl	0.20 M	0.05
GAP dehydrogenase	0.11 mg*	0.10
GAP solution	0.30 μM*	0.10
H_2O		0.30

* approximate

Addition of 0.1 ml of the GAP solution initiates the reaction and the maximum OD obtained within 5 minutes as recorded. Each micromole of GAP oxidized in the reaction produces one μM of NADH. The final OD at 340 mμ is a measure of the GAP oxidized. The μM of GAP per 3 ml may be determined by the following equation which utilizes the extinction coefficient of NADH (3).

$$\frac{\text{Maximum OD (340 m}\mu\text{) X 3}}{6.22} = \mu\text{M GAP in reaction mixture}$$

Glyceraldehydephosphate dehydrogenase was obtained from Calbiochem as a crystalline suspension in 2.5 M ammonium sulfate solution. A stock solution of 0.1 ml of the crystalline solution was dissolved in 0.9 ml of Tris buffer, pH 8.5, each time a batch of GAP was analyzed. Sodium arsenate, sodium fluoride, and L-cysteine·HCl were obtained from Sigma.

Preparation and Purification of Triosephosphate Isomerase From A. Braunii and S. acuminatus

The purification procedures of triosephosphate isomerase from the Clorococcales, A. Braunii and S. acuminatus, were the same although enzyme activity differed. All purification steps were at solution temperatures of 0 to 10°. The distilled water used for dialysis and as a solvent for the enzyme contained 5.4 X 10^{-3} M of ethylenediaminetetra-acetic acid (EDTA), as did the aqueous saturated ammonium sulfate solutions. Ammonium sulfate fractionations, unless otherwise stated, remained standing for 4 hours at 5° before centrifugation.

Fraction 1. The harvested algae, thawed and mixed into a wet cell paste with 0.03 M sodium pyrophosphate buffer (pH 7.1, containing 5.4 X 10^{-3} M EDTA), were macerated in a chilled mortar with an equal mixture of fine washed sand and levigated alumina. An equal volume of the buffer was added to the macerated cells and the resulting mixture allowed to stand for 4 hours before centrifugation at 39,000 X g for one hour. The supernatant solution was decanted and retained. The pellet was resuspended in sufficient buffer to again make a wet cell paste then remacerated and centrifuged as before. The supernatant fractions from both centrifugations were combined and the pellet, containing predominantly cellular debris with some whole cells, was discarded.

Fraction 2. Chilled (-10°) acetone was added dropwise over a period of 45 minutes to the Fraction 1 supernatant solution to 30 per cent saturation (3 parts acetone to 7 parts crude extract). The precipitate was collected by centrifugation at 28,500 X g for 45 minutes,

discarded, and the supernatant fraction dialyzed for 18 hours against cold (13°) running tap water.

Fraction 3. The supernatant solution from Fraction 2 (in an ice bath) was taken to 37 per cent saturation by the slow addition of solid enzyme-grade ammonium sulfate. The precipitate was collected by centrifugation at 28,500 X g for 30 minutes and discarded. Ammonium sulfate was added to bring the supernatant fraction to 50 per cent saturation and the precipitate was collected by centrifugation at 39,000 X g for 30 minutes. The supernatant fraction, after dialysis against distilled water, displayed no triosephosphate isomerase activity. The pellet, containing the enzyme, was dissolved in a minimal amount of distilled water-EDTA and dialyzed for 4 hours against distilled water-EDTA.

Fraction 4. The dissolved, dialyzed precipitate from Fraction 3 was taken to 20 per cent saturation with aqueous saturated ammonium sulfate. This solution was allowed to stand for 5 days, then dialyzed against distilled water-EDTA. The protein precipitating during this dialysis was removed by centrifugation at 28,500 X g for 10 minutes. The supernatant fraction was taken to 50 per cent saturation with ammonium sulfate solution and allowed to stand for 72 hours. The precipitate was removed by centrifugation at 28,500 X g for 30 minutes and discarded. The supernatant fraction was made up to 75 per cent saturation with ammonium sulfate solution and, after standing with occasional stirring for 32 hours, was centrifuged at 39,000 X g for 30 minutes. The supernatant fraction was decanted and discarded. The pellet, containing all of the isomerase activity, was dissolved in a minimal amount

of ice cold distilled water-EDTA and then dialyzed for 4 hours against distilled water-EDTA.

Fraction 5. The dialyzed protein from Fraction 4 was then lyophilized. The lyophilized material was dialyzed against 0.025 M Tris buffer (pH 7.5) before assays of triosephosphate isomerase activity.

Preparation and Purification of Triosephosphate Isomerase From *Chara* sp.

Purification of triosephosphate isomerase from *Chara* sp. proved to be considerably different and more difficult than the Chlorococcales species. All purification conditions were as described for *A. Braunii* and *S. acuminatus*.

Fraction 1. The previously washed algae were placed in a chilled (5°) Waring Blender with an equal volume of 0.03 M sodium pyrophosphate buffer, pH 7.1. Ten grams of fine washed sand per 100 ml of the buffered algal solution were added to the blender and the cells homogenized for 5 minutes. The homogenized mixture was allowed to stand for 2 hours, then centrifuged at 39,000 X g for one hour. The supernatant fraction, containing most of the water soluble protein, was stored at 5° and the precipitate, containing cellular debris and sand, was discarded.

Fraction 2. To the Fraction 1 supernatant solution, chilled (-10°) acetone was added dropwise to 30 per cent saturation. The precipitate was collected by centrifugation at 29,000 X g for 30 minutes and discarded. The supernatant fraction was dialyzed for 12 hours against cold (13°) running tap water.

Fraction 3. Ammonium sulfate was added to the Fraction 2 supernatant to 35 per cent saturation. The precipitate was collected by centrifugation at 39,000 X g for 30 minutes and discarded. The supernatant fraction was taken to 50 per cent saturation with ammonium sulfate. The precipitate, collected by centrifugation at 28,500 X g for 30 minutes, was discarded. Ammonium sulfate was added to the supernatant fraction to increase saturation to 65 per cent. The precipitate, collected by centrifugation as above, was dissolved and dialyzed against distilled water-EDTA and stored at 5°. The supernatant fraction displayed isomerase activity comparable to the precipitate so was taken to 90 per cent saturation with ammonium sulfate and this precipitate was collected by centrifugation as before. The 90 per cent precipitate fraction displayed isomerase activity comparable to the 65 per cent precipitate fraction although both were lower than the 50 per cent supernatant fraction. The 65 and 90 per cent precipitate fractions were combined.

Further purification of triosephosphate isomerase from *Chara* sp. by fractionation with ammonium sulfate solution was attempted but not applicable. Lyophilization was not attempted as sufficient purification had not been obtained.

Electrophoretic Studies

Electrophoretic studies on triosephosphate isomerase from *A. Braunii* and *S. acuminatus* were carried out on a Buchler Electrophoresis apparatus. The buffer system, Barbital-Sodium Barbital (0.050 ionic strength, pH 8.6), was replaced after every third or fourth run.

The stationary strips were Sepraphore III cellulose acetate purchased from the Gelman Instrument Co., Ann Arbor, Michigan. The strips were soaked for a minimum of 30 minutes in the freshly prepared buffer, then loaded on to the cellulose acetate carrier, placed in the migration chamber, covered and allowed to equilibrate. Twenty-five thousandths (0.025) ml of a protein solution containing approximately one mg protein per ml was added transversely to the center of the equilibrated strips with a lambda pipette. Runs were for 3 hours duration at 150 volts. The strips were dryed at room temperature, stained for 5 to 7 minutes in Ponceau S and cleared by 3 washings of 3 to 5 minutes in 5 per cent acetic acid.

RESULTS AND DISCUSSION

Purification of Triosephosphate Isomerase

The purifications of the triosephosphate isomerase systems from A. Braunii, S. acuminatus and Chara sp. are shown in Tables 4, 5, and 6 respectively.

The purification procedures, involving acetone and ammonium sulfate fractionations, were similar to the procedure outlined for purification of calf muscle isomerase (2).

Electrophoretic homogeneity of the lyophilized preparations was indicated by a single protein band for the isomerase from both Chlorococcales species. The mobilities of the bands in Barbital-Sodium-Barbital buffer, pH 8.6, at 0.05 ionic strength were -1.2×10^{-5} cm^2 $volt^{-1}$ sec^{-1} and -1.01×10^{-5} cm^2 $volt^{-1}$ sec^{-1} (both uncorrected) from A. Braunii and S. acuminatus.

The level of purification attained for the triosephosphate isomerase systems from A. Braunii (460 fold) and S. acuminatus (313 fold) appears to be the highest, to date, obtained from any botanical speciman (17, 18, 23).

Some properties of the isomerase systems were determined for A. Braunii and S. acuminatus but not for Chara sp. due to insufficient purification and low yields of useable enzyme protein.

Effect of pH

The activity of isomerase in the coupled assay system, determined in 0.025 M Tris-HCl, attained a well defined maximum at pH 7.7 for the enzymes from A. Braunii and S. acuminatus (Figs. 1 and 2). This

Table 4. Purification of triosephosphate isomerase from *A. Braunii*.

Fraction number		volume in ml	mg protein per ml	units per ml	specific activity	total activity
1.	crude extract	540.0	42.00	47.6	1.133	25704.0
2.	30% acetone	850.0	1.56	40.0	25.60	34000.0
3.	ammonium sulfate					
	37% supernatant	830.0	0.12	7.61	63.40	6316.0
	50% precipitate	33.0	0.58	142.80	246.2	5712.0
4.	ammonium sulfate solution - 75% precipitate	7.9	0.36	166.60	461.7	1316.0
5.	lyophilized		0.33	66.60	200.0	526.14

Table 5. Purification of triosephosphate isomerase from *S. acuminatus*.

Fraction number	volume in ml	mg protein per ml	units per ml	specific activity	total activity
1. crude extract	256.0	103.50	57.2	0.553	15158
2. 30% acetone	425.0	2.64	28.4	10.75	16320
3. ammonium sulfate					
37% supernatant	460.0	0.24	18.1	75.4	8347
50% precipitate	25.0	1.65	153.8	94.4	3825
4. ammonium sulfate solution - 75% precipitate	16.0	0.525	90.9	173.1	1454
5. lyophilized		0.270	28.5	105.55	800

Table 6. Purification of triosephosphate isomerase from Chara sp.

Fraction number	volume in ml	mg protein per ml	units per ml	specific activity	total activity
1. crude extract	420.0	70.50	18.50	0.262	7770.0
2. 30% acetone	545.0	0.705	11.20	15.88	6104.0
3. ammonium sulfate					
35% supernatant	570	0.070	4.51	64.40	2372.5
50% supernatant	570.0	0.075	4.95	66.00	2821.5
combined 65 and 90% precipitates	36.0	1.035	50.00	48.30	1800.0

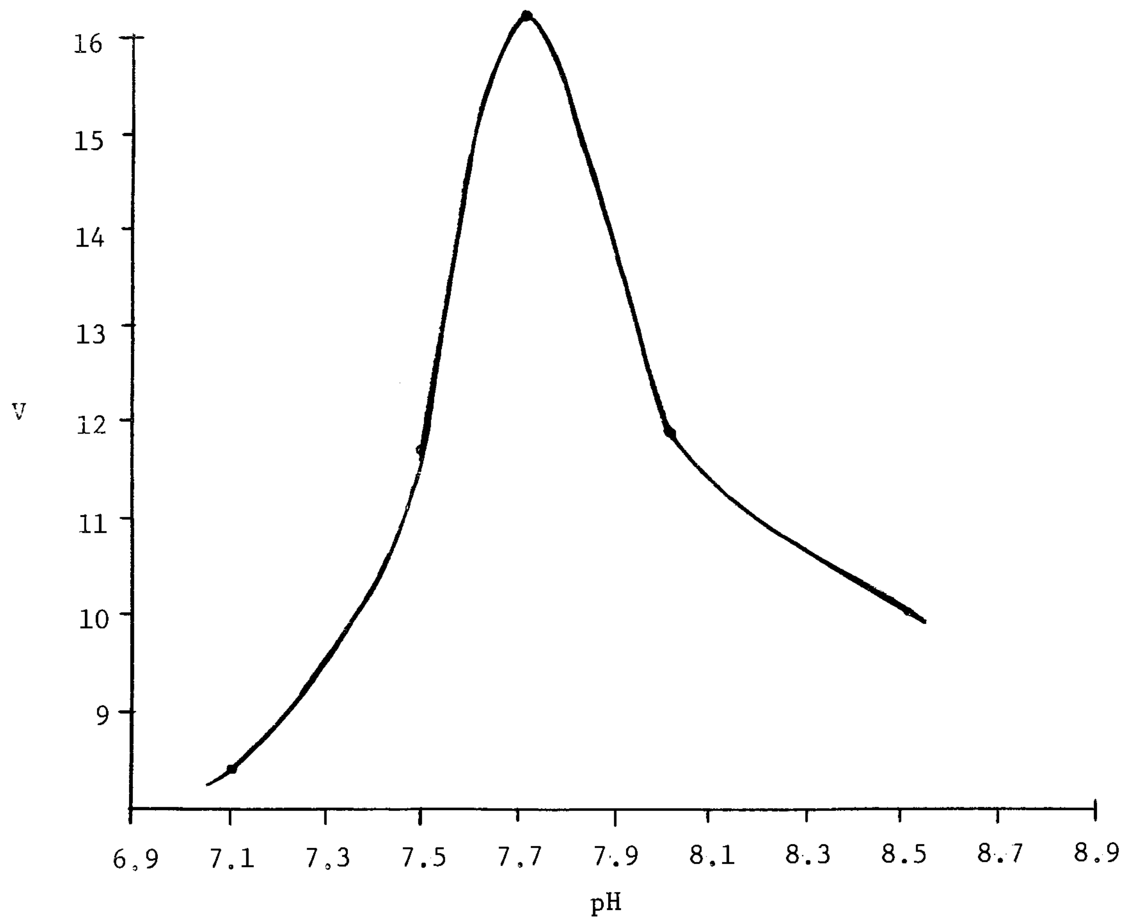


Figure 1. pH optimum for isomerase activity from <u>A</u>. <u>Braunii</u>. V is reported as μM of NADH oxidized per minute X 10^{-2}.

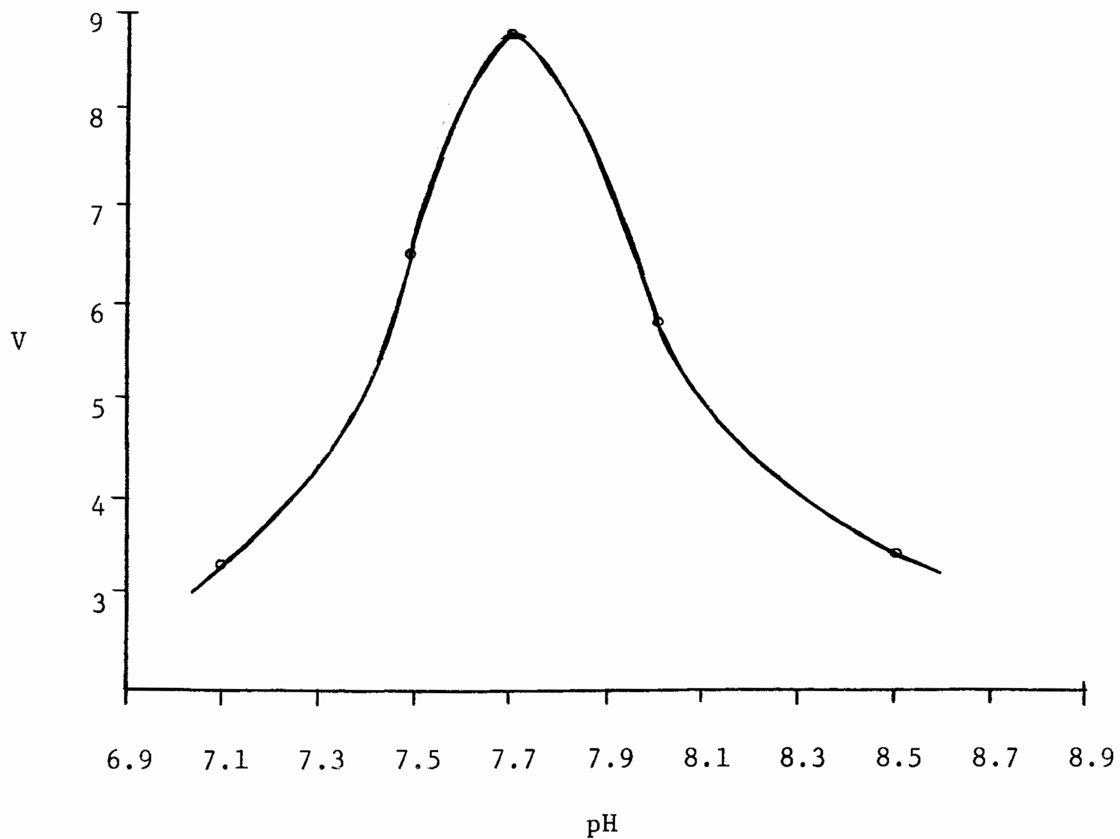


Figure 2. pH optimum for isomerase activity from S. acuminatus, as in Fig. 1

optimum pH differs from the optimum range of pH 7.2 to 8.9 reported for the enzymes from calf muscle (2) and pea seed (23).

The Tris buffer, Tris (hydroxymethyl) aminomethane-HCl, used in assays of enzyme activity in this study differed from those cited in the literature for the reaction coupled with α-glycerophosphate dehydrogenase. Beisenherz (2), Richter (18) and Burton and Waley (5) used 0.020 M triethanolamine-HCl, but Turner, et al. (23) used 0.030 M Tris (hydroxymethyl) aminomethane-acetic acid. The differences in pH optima may be attributed to variations of the buffer solutions.

A large change (1.2 pH unit) in pH of the Tris-HCl system was observed in enzyme assays at high concentrations of GAP (above 2.7×10^{-4} M). This pH change was minimized by increasing the molarity of the Tris buffer to 0.050 M. The observed increase in activity of the enzyme at high substrate concentrations in 0.050 M Tris-HCl buffer appeared to be a function of the stabilized pH rather than the increased molarity of the buffer.

Effect of Substrate Concentration

The determination of the effect of varying GAP concentrations on isomerase activity was carried out as described for the assay procedure using 0.050 M Tris-HCl buffer.

The initial velocities (Vo) for the isomerase activity from A. Braunii and S. acuminatus were obtained at varying substrate concentrations by following the coupled reaction at timed intervals. The velocity of the reaction, reported as micromoles of NADH oxidized per minute, was plotted against time for each substrate concentration and the initial

velocity determined by extrapolation to zero time as shown in Figures 3 and 4.

The effect of D-glyceraldehyde-3-phosphate on the reaction velocity (Michaelis-Menten constant) was determined from a Lineweaver-Burke plot of 1/(Vo) versus 1/(S), where Vo is the initial velocity and (S) is the GAP concentration. The slope and intercept were determined by the method of least squares. The Michaelis-Menten constants (Km), defined as the substrate concentration giving half maximum velocity (7), for the isomerase systems from A. Braunii and S. acuminatus were 4.34 X 10^{-4} M GAP (Fig. 5) and 9.7 X 10^{-4} M GAP (Fig. 6) respectively. These values do not agree closely with those reported for calf muscle (3.9 X 10^{-4} M GAP) (2) and pea seed (3.6 X 10^{-4} M GAP) (23); however, all values are of the same magnitude. Although a similar analysis of the kinetic data was used to determine the Km for the pea seed enzyme, there may be some question concerning the validity of the velocity values used.

According to Dixon and Webb (7), variations in the structure of an enzyme from different species may be sufficient to produce significant differences in the activity of the enzyme reaction. This assumption may explain the differences in the activities of the isomerase systems from the three algae and also the Km differences between A. Braunii and S. acuminatus. It may only be coincidence that the Km from the algae, calf muscle and pea seed are of the same magnitude.

Effect of Inhibitors and Activators

The inhibitory effect on triosephosphate isomerase activity upon the addition of different concentrations of selected salts is shown in

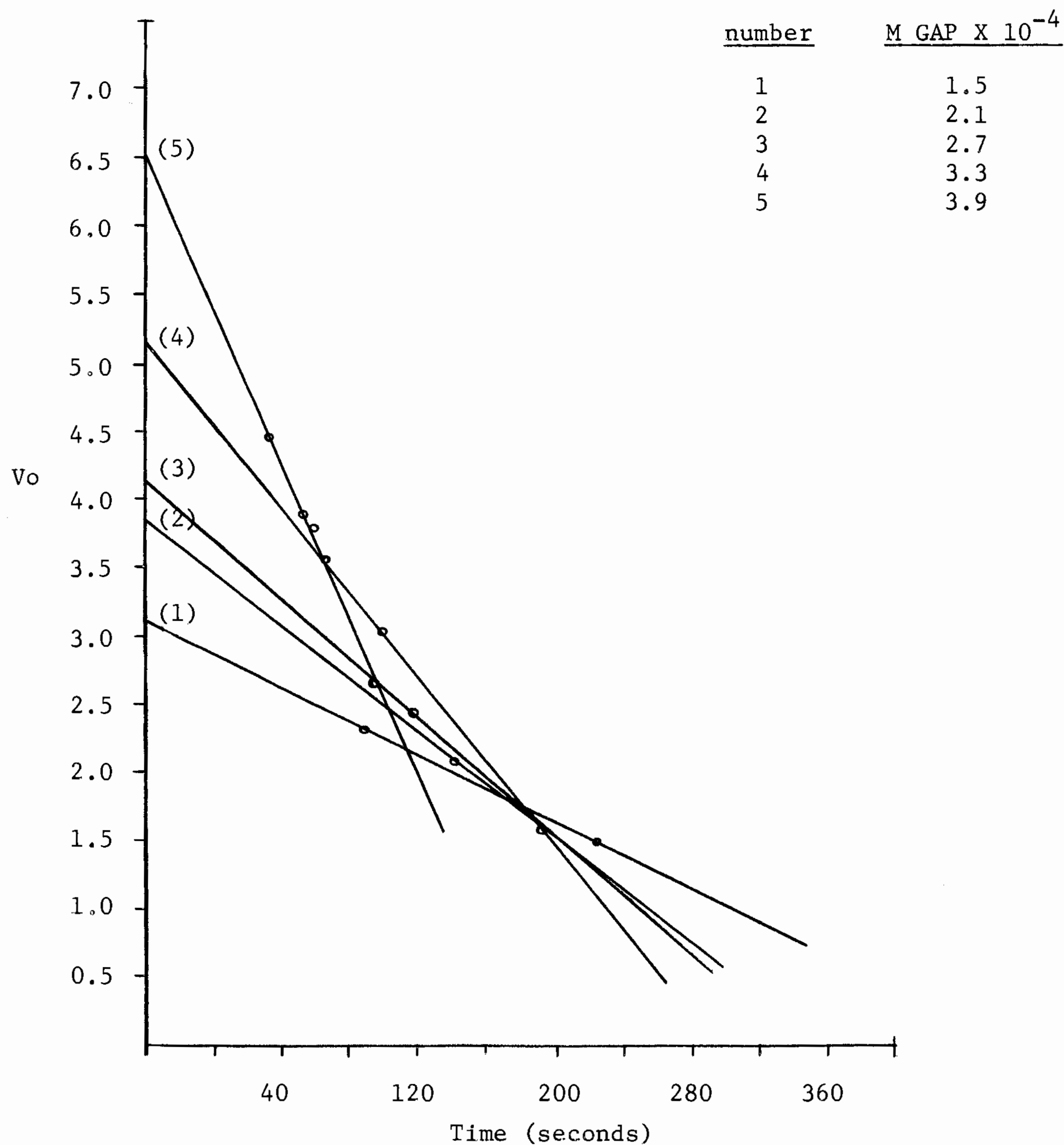


Figure 3. Initial velocity of isomerase activity from A. Braunii at varying GAP concentrations. Vo is reported as μM of NADH oxidized per minute X 10^{-2}. Reaction conditions as described in the text.

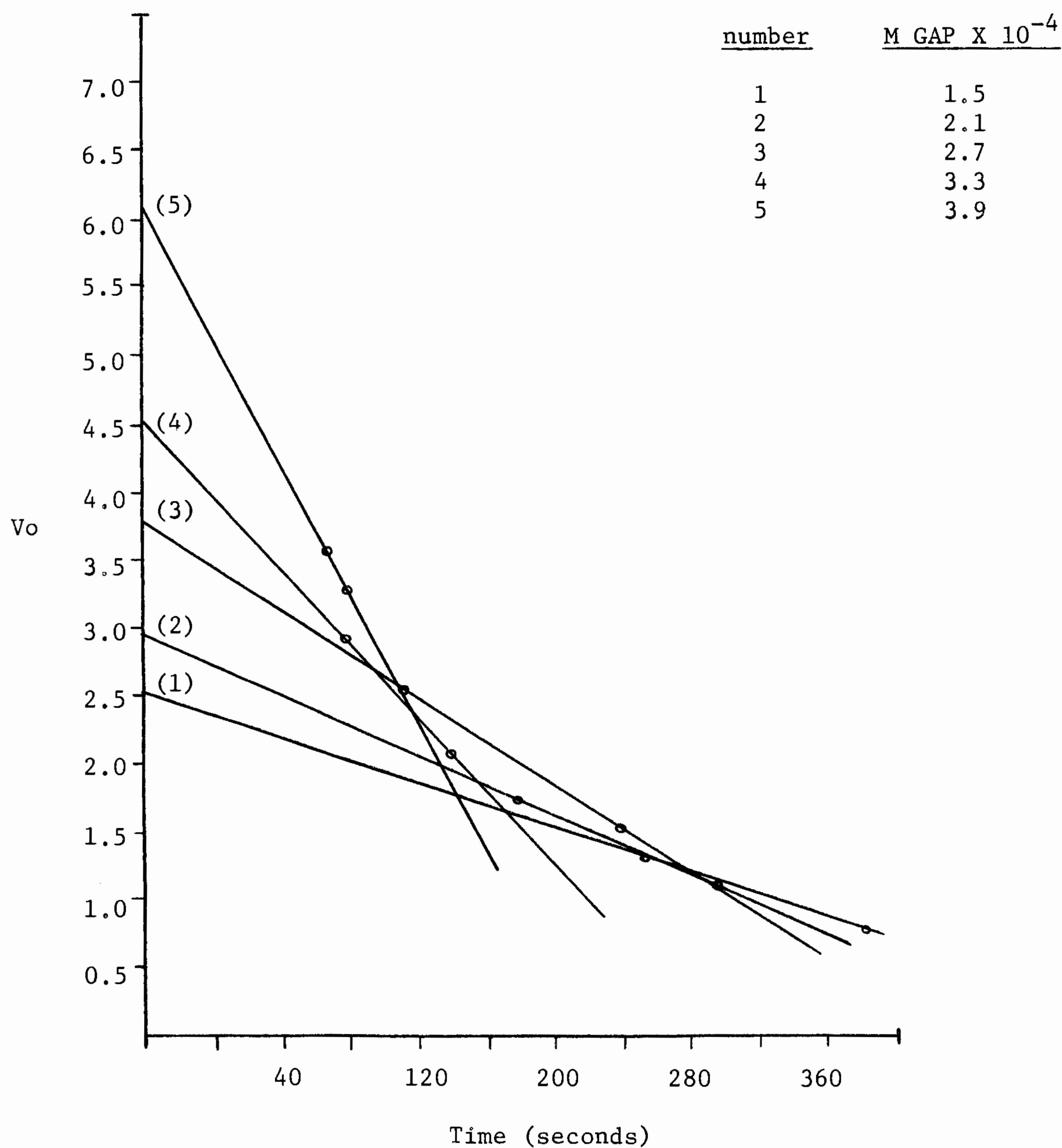


Figure 4. Initial velocity of Isomerase activity from S. acuminatus, as described in Fig. 3.

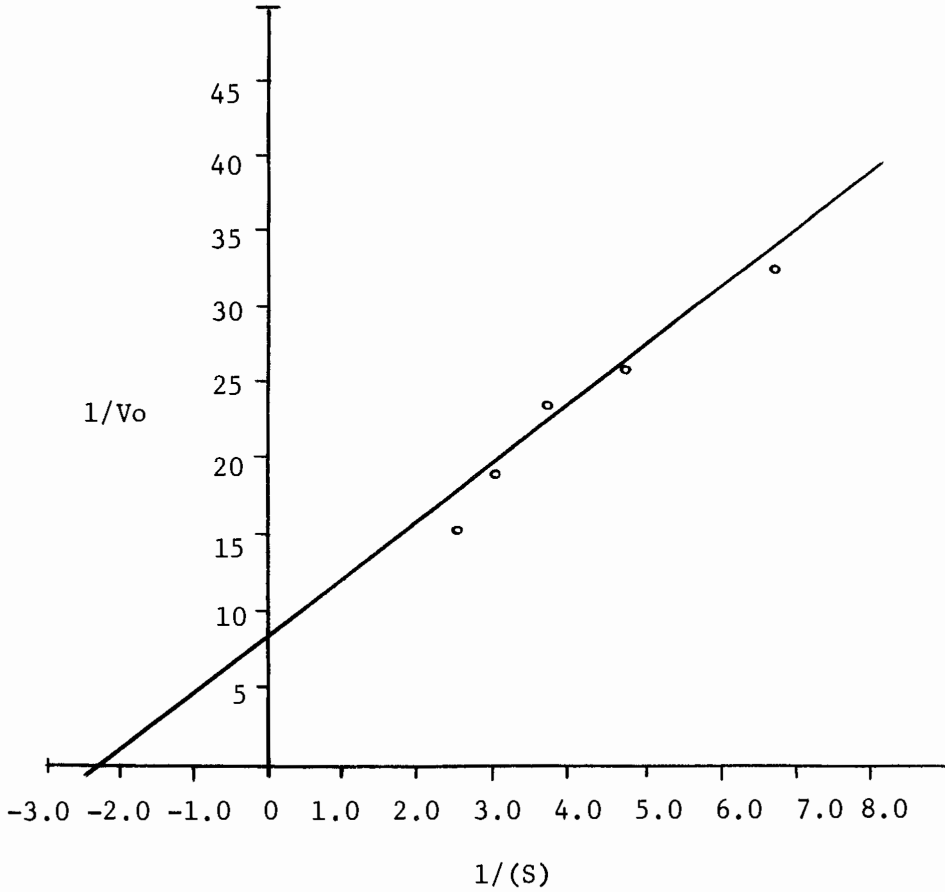


Figure 5. Lineweaver-Burke plot of 1/Vo against 1/(S) for isomerase from A. Braunii. Vo is the initial velocity (μM NADH oxidized/minute); (S) is the GAP concentration (M/liter X 10^{-4}). The intercept is 8.54 and the slope is 3.61. Reaction conditions as described for assay of isomerase activity, with (S) varied as shown.

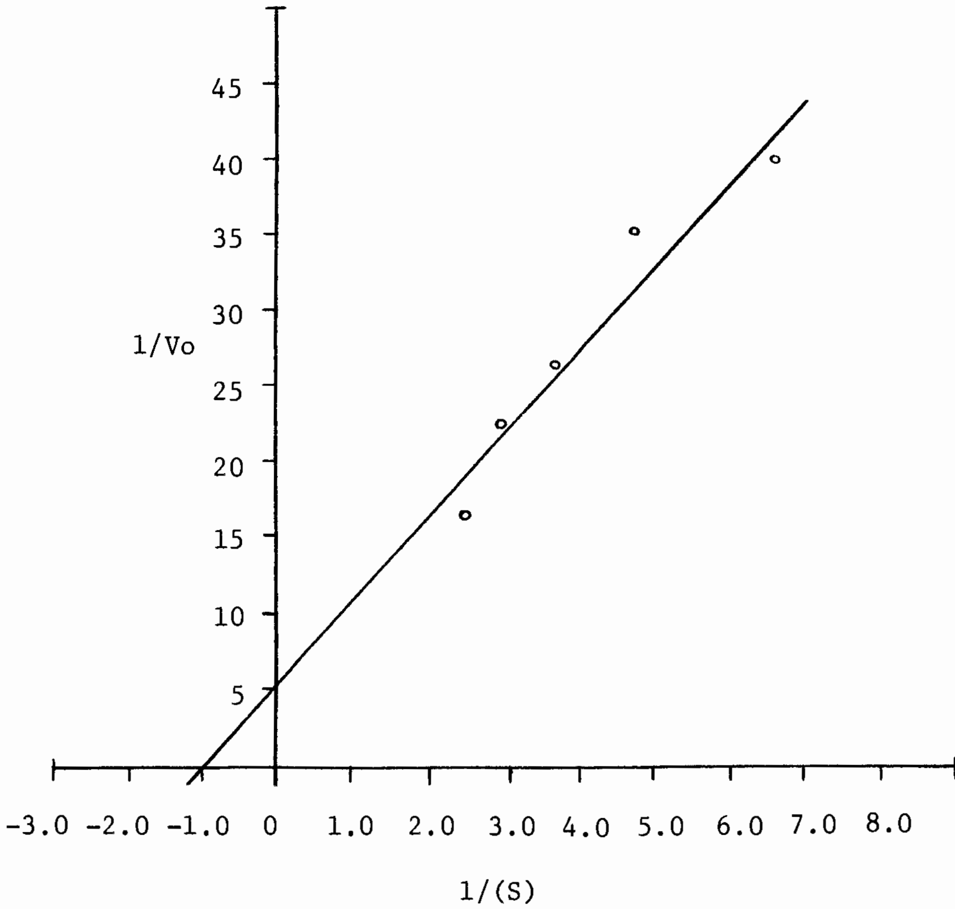


Figure 6. Lineweaver-Burke plot of 1/Vo against 1/(S) for isomerase from S. acuminatus, as described in Figure 3. The intercept is 5.6 and the slope is 5.44.

Tables 7 and 8 for the enzyme from the two algal species. Both sulfate and phosphate ions showed considerable inhibition of the reaction. Inhibition to a lesser degree is observed with chloride ion. Calf muscle isomerase was inhibited 75 per cent by 0.050 M phosphate (2). Pea seed isomerase was inhibited approximately 44 per cent by 0.005 M sulfate and 70 per cent by 0.020 M phosphate (23). Inhibition of the algal isomerase reactions was observed in purification procedures involving ammonium sulfate fractionation.

Inhibition was shown to affect the isomerase activity rather than the α-glycerophosphate dehydrogenase system by using the latter system in a separate reaction. DHAP, formed by the action of aldolase on fructose-1, 6-diphosphate, was transformed to phosphoglyceric acid by α-glycerophosphate dehydrogenase and NADH. The OD shift of NADH in the presence and absence of the salts was the same indicating no inhibitory effect on the α-glycerophosphate dehydrogenase system.

Anion inhibition might be explained in two ways, the first being the formation of a coordination complex between the active site of the enzyme and the anion. An alternate explanation might be the attraction of unlike ionic species and subsequent tieing up of the active site in a competitive inhibition.

The active site of the isomerase enzyme from rabbit muscle is postulated to involve a basic amino acid, histidine (5). Turner, et al. challenged the postulate that this amino acid is the active site in all triosephosphate isomerase enzymes (23). These investigators indicated inhibition to 77 per cent with 10^{-4} M p-Chloromercuribenzoate. This

Table 7. Effects of anions on isomerase activity from A. Braunii.

Reaction conditions were as described for the assay of isomerase activity. Temperature was 23 to 27°, pH 7.7, with 1.08 X 10^{-4} M GAP. The results are expressed as per cent inhibition of control assay.

salt	Final concentration (M) 0.05	0.025	0.005
$(NH_4)_2SO_4$	81.7	70.0	41.7
Na_2SO_4	78.7	64.5	22.8
K_2HPO_4	78.7	58.4	29.5
NH_4Cl	33.0	3.0	

Table 8. Effects of anions on isomerase activity from S. acuminatus.

Reaction conditions as in Table 7.

salt	Final concentration (M) 0.05	0.025	0.005
$(NH_4)_2SO_4$	74.2	70.4	26.8
Na_2SO_4	72.6	63.8	19.8
K_2HPO_4	71.5	63.8	20.9
NH_4Cl	19.8	7.7	

inhibitor, at low concentrations, is selective for thiol groups, such as cysteine, yet Turner's group also indicated 18 per cent inhibition by 4×10^{-3} M cysteine. Cysteine is generally used to reactivate and protect enzymes having a thiol group at the active site or involved in forming the tertiary configuration of the molecule. Free iodoacetate, another inhibitor for thiol groups, displayed only 5 per cent inhibition of pea seed isomerase. Turner, *et al*. reported that the data indicated the presence of thiol groups essential for enzyme activity in pea seed isomerase although the results appear to be somewhat inconsistent with this hypothesis.

Assuming the presence of a thiol group at the active site, incubation with an activator specific for thiol groups would result in reactivation of inactivated isomerase preparations. Czok and Bucher reported inactivated isomerase from rabbit muscle was reactivated by incubation with 0.060 moles of reduced glutathione (6). Algal isomerase, inactivated by freezing in solution, was not reactivated by incubation for 4 and 12 hours at 5° with 0.060 moles of reduced glutathione. Similarly, no increase in activity was noted at any time when cysteine was included in the reaction. The lack of reactivation by incubation with reduced glutathione does not, necessarily, indicate that thiol compounds are not involved in the activity of the enzymes. Inhibitor studies with iodoacetate and p-chloromercuribenzoate should be carried out before a definite conclusion is reached concerning the necessity of a thiol group for isomerase activity from the two unicellular algae.

Snyder and Lee separated horse liver triosephosphate isomerase from alcohol dehydrogenase, then studied the effect of a number of

dicarboxylic acids on the isomerase reaction (22). Horse liver and rabbit muscle isomerase were stimulated by dicarboxylic acids ranging from 2 to 10 carbons in chain length, including glutamic acid. The stimulation, however, occurred only at high substrate concentrations where the isomerase reaction was inhibited by GAP. This substrate inhibition was explained on the basis of competition by two molecules of substrate for two adjacent locations on the enzyme's active site. Alternately, the enzyme may contain a second site, termed an allosteric site, at which the substrate reacts to inhibit isomeration. The stimulation of horse liver and rabbit muscle isomerases did require both carboxyl groups of glutamic acid.

Triosephosphate isomerases from *A. Braunii* and *S. acuminatus* were not stimulated by addition of 0.50 M glutamic acid. The substrate concentrations used (1.4×10^{-4} M GAP), however, were well below that required for half maximum velocity of the reaction.

SUMMARY

1. The triosephosphate isomerase systems from three algal species, Ankistrodesmus Braunii, Scenedesmus acuminatus and Chara sp., have been isolated and purified by a series of solvent and salt fractionations. Maximum activity was obtained by salt fractionation and resulted in a 460 fold increase for A. Braunii, 313 fold for S. acuminatus and 234 fold for Chara sp. Lyophilization of the protein fractions gave solid products, but resulted in a decrease in the specific activity of the enzymes.

2. Electrophoretic studies of the purified systems indicated homogeneous samples from A. Braunii and S. acuminatus. The uncorrected mobilities for these enzymes were -1.2×10^{-5} cm^2 $volt^{-1}$ sec^{-1} and -1.01×10^{-5} cm^2 $volt^{-1}$ sec^{-1} respectively. Contamination of the Chara sp. preparation prevented further characterization of this system.

3. The isomerase activities of A. Braunii and S. acuminatus were maximum at pH 7.7 in 2.5×10^{-2} M Tris-HCl buffer. For both systems, the pH optimum was very sharp and activity dropped off rapidly at lower or higher pH values.

4. Kinetic analysis of the isomerase reactions gave Michaelis-Menten constants (Km) of 4.34×10^{-4} M and 9.7×10^{-4} M GAP respectively for the enzymes from A. Braunii and S. acuminatus. These constants were obtained from Lineweaver-Burke plots using calculated initial velocities.

5. The algal isomerase systems were inhibited by sulfate, phosphate and, to a lesser degree, chloride ions. Ion concentrations of 0.025 to 0.05 M caused inhibition from 58 - 81 per cent. Thiol compounds had no effect on the velocity of the reactions and did not react-

ivate the inactive system. Dicarboxylic acids did not appear to activate the algal systems.

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