Biochimica et Biophysica Acta, 481 (1977) 6—24

BBA 68061

L-GLYCEROL-3-PHOSPHATE DEHYDROGENASE FROM THE INSECT *CERATITIS CAPITATA*

PURIFICATION, PHYSICOCHEMICAL AND ENZYMIC PROPERTIES

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(Received July 26th, 1976)

Summary

Soluble L-glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate: NAD^+ 2-oxidoreductase, EC 1.1.1.8) from the Mediterranean fruit fly *Ceratitis capitata* has been purified 130-fold with an overall yield of about 40%. The final preparation had a specific activity of about 200 μmol NADH/min/mg protein.

The enzyme preparation has been shown to be homogeneous throughout disc gel electrophoresis, dodecyl sulphate gel electrophoresis, isoelectric focusing and ultracentrifugation.

The K_m values for dihydroxyacetone phosphate, NADH, L-glycerol-3-phosphate and NAD^+ were respectively 0.33, 0.018, 0.74 and 0.26 mM. L-glycerol-3-phosphate dehydrogenase from the insect had a maximal activity around pH 6.6 for the oxidation of NADH and pH 10.0 for the reduction of NAD^+. It was stable from pH 6.0 to pH 9.0 at 20°C for 1 h and remained active after incubating at 30°C for 30 min at pH 6.6. The enzyme was completely inactivated by incubating at 60°C for 5 min. Enzyme stability versus ionic strength as well as the dependence of the reaction velocity on temperature are also reported.

The active enzyme was found to have a minimum molecular weight of approx. 63 000. Molecular weight determinations by sodium dodecyl sulphate gel electrophoresis gave subunit weights of 33 500. The isoelectric point of the protein was determined by electrofocusing and found to be 5.75 ± 0.05. The extinction coefficient at 278 nm was calculated by dry weight measurements to be $E^{1\,cm}_{1\,mg/ml} = 0.42 \pm 0.1$. Sedimentation velocity studies on ultracentrifuge indicated a dependence of the sedimentation coefficient on the enzyme concentration.

The amino acid composition of the enzyme was determined. The protein has no free N-terminal residue and the digestion with carboxypeptidases gave the C-terminal sequence: -Ala-Gly-Ser.

All these data are discussed in relation to the properties of the enzyme from other sources.

Introduction

NAD^+-linked cytoplasmic L-glycerol-3-phosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD^+ 2-oxidoreductase, EC 1.1.1.8) is one of the two enzyme activities of the α-glycerol-phosphate shuttle necessary for the transport of the NADH electrons from the cytosol to the mitochondria and plays a crucial role in the energy-producing metabolism of insects, mainly in the flight muscles. These facts are supported by the observation that there is a marked rise in the specific activity of the L-glycerol-3-phosphate dehydrogenase in flight muscle immediately before the insect becomes able to fly [1,2]. This sharp rise in the activity of the enzyme does not occur in leg muscles or fat body. The importance of the cytoplasmic L-glycerol-3-phosphate dehydrogenase to flight energetics is consistent with the fact that some mutants [3] which lack this enzyme activity cannot fly. Further support for the role of the soluble enzyme in flight muscle metabolism comes from studies on flightless insects where low levels of the enzyme were found in the thoracic muscles [4,5].

The high utilization of oxygen in insect flight muscles which use carbohydrates as fuel supply overcomes the utilization of lactate dehydrogenase for reoxidation of NADH produced by the D-glyceraldehyde-3-phosphate dehydrogenase, in such a way that insect flight muscles almost lack lactate dehydrogenase activity.

Thus, the α-glycerol-phosphate cycle serves the regeneration of NAD^+ as fast as it is formed and hydrogen is being transported to the sarcosomes by means of α-glycerol-phosphate instead of by NADH which cannot penetrate into the mitochondria. The α-glycerol-phosphate cycle is an adaptation to a fast oxidative utilization of carbohydrates and since it appears to be one of the most important metabolic specializations in insects, we thought it of interest to obtain information on the structural and regulatory properties of α-glycerol-phosphate dehydrogenase from recently emerged adults of the Dipterous *Ceratitis capitata*, the lipid metabolism of which has been studied throughout different stages of development [6—10].

This paper deals with the isolation, purification and description of physicochemical and enzymic properties of this enzyme.

Materials and Methods

Rearing of insects

The Dipterous *Ceratitis capitata* (Wiedeman) was cultured according to the conditions previously described [11]. Adult insects (1—2 days after emergence) were killed by immersing them in liquid nitrogen immediately after collection and stored at −20° C until required.

Chemicals

DL-glycerol 3-phosphate, dihydroxyacetone phosphate (as dimethylketal di-monocyclohexylamine), NAD^+, NADH, rabbit muscle L-glycerol-3-phosphate dehydrogenase, horse liver alcohol dehydrogenase, rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle fructose-1,6-biphosphate aldolase, rabbit muscle triose phosphate isomerase, α-amylase from *Bacillus*

subtilis, hen egg-white lysozyme, sperm whale myoglobin, horse serum albumin and horse heart cytochrome *c* were purchased from Sigma Co. (St. Louis, Mo., U.S.A.). Bovine pancreas chymotrypsinogen A, ovalbumin, *E. coli* alkaline phosphatase and bovine liver catalase were obtained from Boehringer (G.F.R.). Carboxypeptidase A, bovine pancreas trypsin and pig stomach pepsin were obtained from Worthington (Freehold, U.S.A.), 5,5'-Dithiobis(2-nitrobenzoic acid) was purchased from Calbiochem (Los Angeles, Calif. U.S.A.). Sephadex G-200 and Blue Dextran were purchased from Pharmacia (Sweden). Ampholines for electrofocusing and Ultrogel AcA-44 were from LKB (Sweden). Sephraphore III strips were from Gelman Inst. Co. (U.S.A.). All other reagents (analytical grade) were purchased from Merck (Darmstadt, G.F.R.).

Preparation of the enzyme

Homogenization. Adults insects (50 g) were homogenized in 150 ml of 0.1 M Tris · HCl, pH 7.6 (at 4°C), containing 0.01 M EDTA and 0.05% streptomycin sulphate, in an Omni Mixer (Sorvall, U.S.A.) for 45 s. The homogenate was then filtered and the residue reextracted with 75 ml of the same buffer for 45 s. The combined filtrates were centrifuged at 15 000 rev./min (Sorvall, rotor SS-34) for 45 min at 4°C.

Heat treatment. The supernatant obtained after centrifugation was brought to ionic strength 0.1 with KCl, heated in a water bath for 30 min at 30°C and then cooled down to 4°C. The suspension was clarified by centrifuging at 27 000 × *g* for 30 min at 4°C.

Ammonium sulphate fractionation. The clear supernatant was brought to 40% saturation with powdered $(NH_4)_2SO_4$ (243 g/l). After complete addition of ammonium sulphate, stirring was continued for 2 h. After removing the precipitate by centrifugation at 27 000 × *g* for 30 min at 4°C, the supernatant was brought up to 60% saturation by renewed addition of solid ammonium sulphate (132 g/l) and stirring maintained for 2 h. The suspension was centrifuged at 27 000 × *g* for 30 min at 4°C and the precipitate was collected.

Ultrogel column chromatography. The precipitate was dissolved in the minimum volume of 25 mM Tris · HCl, pH 7.5 (at 25°C). The undissolved material was removed by centrifugation and the solution was applied to a column (2.6 × 100 cm) of Ultrogel AcA-44 equilibrated in 25 mM Tris · HCl, pH 7.5 (at 25°C). Elution was performed with the same buffer at 30 ml/h and 5-ml fractions were collected. Fractions with a glycerol-3-phosphate dehydrogenase higher than 40 units/ml* were pooled and precipitated by adding solid ammonium sulphate up to 65% saturation under magnetic stirring for 1 h. The suspension was centrifuged at 27 000 × *g* for 30 min at 4°C and the precipitate collected and dissolved in the minimal volume of 25 mM Tris · HCl, pH 7.5. The supernatant was rechromatographied (undissolved material was removed by centrifugation prior to application) in the same column and eluted under the same conditions. Fractions showing a dehydrogenase activity higher than 40 units/ml were collected and pooled.

DEAE-cellulose column chromatography. The pooled fractions from Ultrogel chromatography were applied directly to a 2.6 × 40-cm column of DEAE-

* See Enzyme Assays.

cellulose (DE-52 Whatmann) equilibrated with 25 mM Tris · HCl, pH 7.5, containing 10 mM 2-mercaptoethanol. After loading the protein on the DEAE-cellulose, the column was washed with 400 ml of the same buffer, and a linear gradient was applied. Starting buffer: 25 mM Tris · HCl, pH 7.5, containing 10 mM 2-mercaptoethanol; limiting buffer: 25 mM Tris · HCl, pH 7.5, containing 10 mM 2-mercaptoethanol and 0.05 M ammonium sulphate. Total gradient volume, 800 ml. 5-ml Fractions were collected at a flow rate of 40 ml/h. Fractions exhibiting glycerol-3-phosphate dehydrogenase activity were combined.

Extinction coefficient. The extinction coefficient of the enzyme was determined in triplicate on a dry weight basis. A solution of the enzyme in 5 mM sodium bicarbonate buffer, pH 6.4, was passed through a Millipore filter and the absorbances at 278 and 280 nm were determined in a 1-cm path-length quartz cuvette with a Varian Techtron (model 635D) spectrophotometer. Dialysed 3-ml aliquots were pipetted into clean preweighed vials and dried at 90°C and then at 108°C until constant weight.

Protein determination

Protein estimation was performed according to the method of Lowry [12] using bovine serum albumin as standard. When pure, the enzyme concentration was determined assuming an extinction coefficient ($E^{1\,cm}_{1\,mg/ml}$) of 0.414 at 278 nm.

Enzyme assays

L-glycerol-3-phosphate dehydrogenase was routinely assayed by monitoring the change in absorbance resulting from the oxidation of NADH in the presence of dihydroxyacetone phosphate. The decrease in absorbance of NADH at 340 nm was linear versus time for at least 2 min following initiation of the reaction . The standard assay mixture contained 0.2 mM NADH and 0.5 mM dihydroxyacetone phosphate in 0.025 M histidine and 0.025 M Tris, pH 6.6, to a final assay volume of 1 ml. Substrate was prepared from the dicyclohexylamine salt dimethyl ketal monohydrate according to the method of Ballou [13]. Reaction was started with 10 µl of enzyme sample. Unless otherwise stated, all assays were run at 25°C. One unit of enzyme activity is equivalent to the oxidation of 1µmol of NADH per min, based on a molar extinction coefficient for NADH of $6.22 \cdot 10^3$ at 340 nm [14]. Assays using NAD^+ (0.55 mM) and DL-glycerol 3-phosphate (3.0 mM) as substrates were carried out in 0.05 M glycine/NaOH buffer, pH 10.0, in a final volume of 1 ml.

Acrylamide gel electrophoresis

Acrylamide gel electrophoresis was performed according to the procedure of Davis [15]. Electrophoresis was carried out in 7.5% acrylamide gels with Tris/glycine buffer, pH 8.5, and a constant current of 4 mA per gel. Proteins were observed after staining with Coomassie Blue dye. Location of dehydrogenase activity was performed by incubating gels at 37°C in 0.078 M glycine/NaOH buffer, pH 9.0, containing 0.154 M DL-glycerol 3-phosphate (45 mg/ml), NAD^+ (1.56 mg/ml), phenazine methosulphate (0.625 mg/ml) and Nitroblue Tetrazolium (0.234 mg/ml). Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn

[16]. Samples were incubated at 100°C in 0.01 M sodium phosphate, pH 7.0, containing 1% SDS and 1% 2-mercaptoethanol for 4 min. 10% acrylamide solutions were used for the preparation of the gels. Electrophoresis was carried out for 5 h at a constant current of 8 mA per gel.

Cellulose acetate electrophoresis

Electrophoresis using cellulose acetate strips was carried out in a buffer system of 0.06 M sodium barbital, pH 8.6, containing 10 mM 2-mercaptoethanol and 0.05 M Tris · HCl, pH 7.5, containing 10 mM 2-mercaptoethanol. 5 mA per strip were applied during 1 h at 4°C. Proteins were stained on the strips using 0.5% (w/v) Ponceau Red in 5% trichloroacetic acid. Enzyme activity was located on the strips by means of the method used for the acrylamide gels. Strips were made transparent by treating with acetic acid (10%, v/v) in methanol for 20 min and then the strips were heated at 60°C until they became transparent. Gels and cellulose strips were scanned using a Canalco Spectrophotometer Model G, equipped with an integrator for the automatic recording of the bands.

Electrofocusing

Enzyme samples were dialyzed against 1% aqueous glycine prior to application. Isoelectric focusing was performed on a LKB-8100-1 electrofocusing column (110 ml) at 4°C with 2% Ampholine in a sucrose gradient from 60% (w/v) to 5% (w/v). Electrofocusing was carried out with Ampholine ranges of of pH 3.5—10.0, 4.0—8.0 and 4.0—6.0. Samples were applied to the column in the light gradient solution. Initial applied voltage was 300 V (10 h) and then 500 V for 62 h. The column was emptied at 42 ml/h and 3-ml fractions were collected. Enzyme activity was determined after neutralization of the samples. Proteins were determined by the absorbance at 280 nm.

Gel chromatography

L-glycerol 3-phosphate dehydrogenase from the insect and suitable marker proteins were chromatographed at 3°C on a Sephadex G-200 column (1.3 × 76 cm) using 0.05 M Tris · HCl, pH 7.3, containing 0.1 M KCl and 0.02% sodium azide as eluant. The void volume was 35 ml. The reference proteins in the effluents were detected by absorbance and/or enzyme activities. Values in the Results are average of three separate determinations.

Ultracentrifugation

(A) The sedimentation experiments were carried out at 20°C in a Spinco Model E ultracentrifuge (Beckman Instruments) equipped with schlieren optics. The enzyme was dissolved in 5 mM Tris · HCl, pH 7.5, containing 2 mM 2-mercaptoethanol. Experiments were run at 0.5, 1, 1.5, 3 and 5 mg/ml as determined by absorbance measurements at 278 nm. Photographs of the Schlieren patterns were taken at 2—4-min intervals during the whole experiment. Distances from the boundary to the reference line were measured on photographic plates using a Nikon Shadowgraph supplied with a micro-comparator. For low protein concns., ultraviolet absorption was used.

(B) Sedimentation velocity measurements according to the method of Martin

and Ames [17] were carried out with a Spinco L4 (Beckman Instruments) ultracentrifuge using an SW 65 rotor. A linear 5—20% (w/v) sucrose gradient in 0.05 M Tris · HCl buffer, pH 7.4, was used. Centrifugations were carried out for 16 h and 3°C at 50 000 rev./min using suitable protein markers. L-glycerol-3-phosphate dehydrogenase was studied at different concentrations.

Partial specific volume determination

The partial specific volume of the enzyme from the insect was determined on a digital Paar Precision Density-meter DMA 02D (A. Paar KG, Graz, Austria) according to the method of Kratky et al. [18]. Concentrations of 0.3, 0.7 and 1.3 mg/ml were used in this determination.

Carboxymethylation

Reaction of L-glycerol-3-phosphate-dehydrogenase with iodoacetate was carried out essentially as described by Craven et al. [19].

Amino acid analyses

The protein was hydrolyzed with 5.7 M HCl, containing 0.1% (w/v) phenol, in evacuated sealed tubes, at 108°C for different lengths of time (24, 48, 72, 96 and 120 h). The hydrolysates were analysed on a Durrum amino acid analyser (model D 500). For each hydrolysis time, at least three different determinations were performed. Half-cystine was determined as carboxymethylcysteine after reduction and carboxymethylation. In order to minimize destruction of carboxymethylcysteine, constant-boiling HCl, containing 1% mercaptoacetic acid, was ocasionally used for acid hydrolysis. Total sulphydryl groups were estimated by reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) [20] in 0.1 M Tris · HCl buffer, pH 7.5, containing 1 mM EDTA and 2% sodium dodecyl sulphate. Freshly prepared solutions of reduced glutathione were used as standards. The molar extinction coefficient of 13 600 $M^{-1} \cdot cm^{-1}$ at 412 nm for 5-thio-2-nitrobenzoate was employed for calculation. Tryptophan was determined spectrophotometrically in 0.1 NaOH [21] or 6 M guanidine hydrochloride [22].

N-terminal analysis

N-terminal analysis was performed according to the method of Gray [23]. Dansyl derivatives were chromatographed on polyamide layers using the solvent systems: 90% (v/v) formic acid/H_2O (3 : 200, v/v); benzene/acetic (9 : 1, v/v) and methanol/ethyl acetate/acetic acid (1 : 20 : 1, v/v/v). N-terminal sequence analyses were also carried out using an automatic (Beckman Model 890B) protein sequencer. About 300 nmol of carboxymethylated L-glycerol 3-phosphate dehydrogenase were applied to the sequencer and degraded by a procedure similar to that of Edman and Begg [24]. Conversion of the 2-anilino-5-thiazolinone derivatives into phenylthiohydantoins and subsequent identification was done as described in ref. 25.

C-terminal analysis

L-glycerol-3-phosphate dehydrogenase (3.1 mg) was dissolved in 0.2 ml of 0.1 M Tris · HCl buffer, pH 8.5, containing 0.1% sodium dodecyl sulphate

(w/v). This solution was incubated at 37°C with 5 µl of diisofluorophosphate-treated carboxypeptidase A suspension (50 mg/ml) previously washed free from amino acids. Samples were taken at suitable time intervals, diluted immediately with 0.1 M HCl and applied to the amino acid analyzer. Norleucine was used as internal standard.

Results and Discussion

Purification of the enzyme

Table I summarizes the procedure utilized in the purification of L-glycerol-3-phosphate dehydrogenase from *Ceratitis capitata*. The overall yield is 39%; the final purification is 130-fold and the specific activity is approx. 200 units/mg.

The addition of streptomycin sulphate to the homogenization mixture was necessary to avoid a serious protein degradation due to the presence of endogenous bacteria, previously identified through antibiograms.

Bewley et al. [26] described the existence in crude extracts of *Drosophila* adults of several forms of L-glycerol-3-phosphate denydrogenase, one of which is heat labile and is carried over from the larval stage contributing 25% of the total activity. We estimated that this larval form contributes only approx. 10% of the total activity in the crude extracts from *Ceratitis capitata* adults and that it can be practically eliminated by heating the extract to 30°C for 30 min. Additionally this heat step eliminates about 25% of the toal protein in the crude extract.

The enzyme was precipitated in the range of 40—60% saturation of ammonium sulphate; about 15% of the total enzyme content was lost in the ammonium sulphate fractionation.

The use of Ultrogel AcA 44 as the first chromatographic step (Fig. 1) in the purification of the enzyme was due to the observation that an important loss of

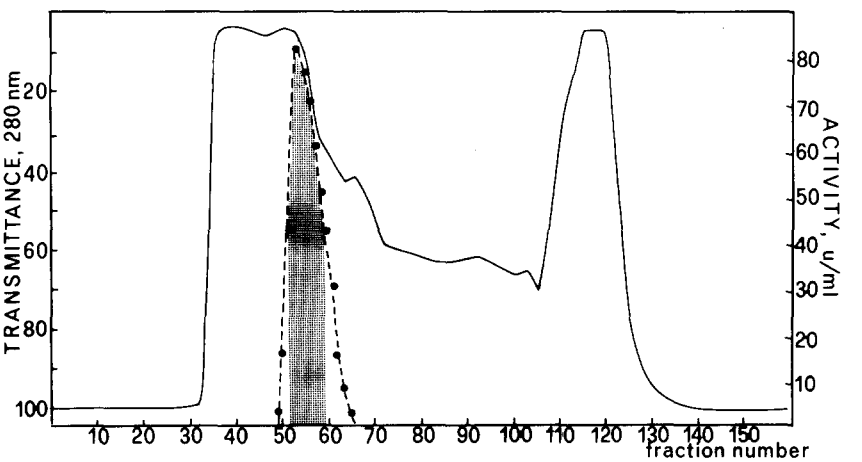

Fig. 1. Elution profile of L-glycerol-3-phosphate dehydrogenase of *Ceratitis capitata* from the first Ultrogel AcA 44 column. Details of the elution are given under Materials and Methods. The shaded zone corresponds to the pooled enzyme fractions.

TABLE I

PURIFICATION OF L-GLYCEROL-3-PHOSPHATE DEHYDROGENASE FROM *CERATITIS CAPITATA* ADULTS

Estimation of the enzyme was carried out using dihydroxyacetone phosphate and NADH as substrates according to the procedure described in Materials and Methods.

Step	Volume (ml)	Total units	Units/ ml	Total protein		Specific activity (units/mg)	Purification (-fold)	Yield (%)
				(mg)	mg/ml			
Crude homogenate	180	4600	25.6	3000	16.6	1.53	—	100
Heat treatment	170	4300	25.3	2200	12.9	1.95	1.28	93
40% satd. $(NH_4)_2SO_4$ supernatant	190	4100	21.6	1300	6.8	3.15	2.06	89
60% satd. $(NH_4)_2SO_4$ sediment	18	3600	200.0	700	38.9	5.14	3.36	78
First Ultrogel AcA44	45	2900	64.5	120	2.7	24.16	15.79	63
Second Ultrogel AcA44	35	2500	71.4	80	2.3	31.25	20.42	54
DEAE-cellulose (25 mM Tris · HCl, pH 7.5, 10 mM 2-mercaptoethanol)	45	1790	39.8	9	0.2	198.88	129.99	39

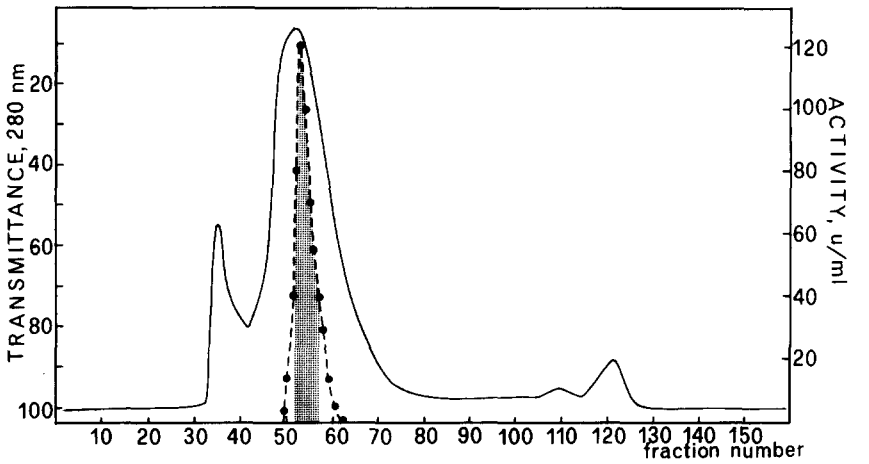

Fig. 2. Elution profile of L-glycerol-3-phosphate dehydrogenase of *Ceratitis capitata* from the second Ultrogel AcA44 column. Details of the elution are given under Materials and Methods. The shaded zone corresponds to the pooled enzyme fractions.

enzyme activity takes place if an ion exchange chromatography is carried out before gel chromatography as it was used by other authors [27]. A rechromatography on Ultrogel AcA 44 (Fig. 2) of the pooled enzyme fractions increased notably the purification. In this second Ultrogel column chromatography a minor peak of denatured enzyme emerged at the void volume. Relationships between this minor peak and the major peak of recovered enzyme activity agree with previous results of Marquardt and Brosemer [23]; also, if the enzyme solution were concentrated about 10-fold using Amicon PM10 membrane prior to the rechromatography on the second Ultrogel AcA 44, a much higher peak emerged at the void volume and more pronounced loss in recovered enzyme activity was observed.

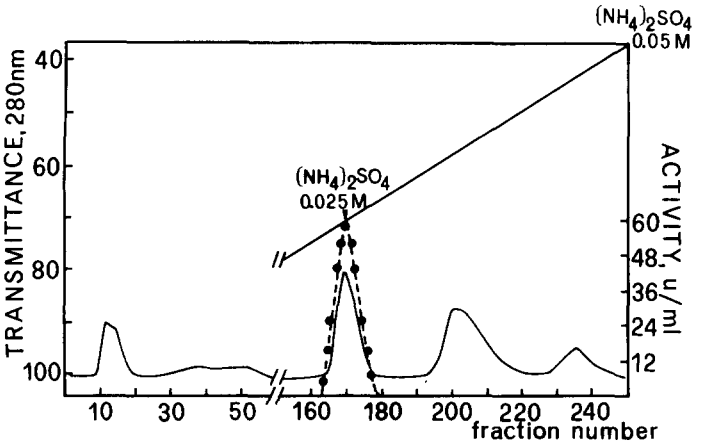

Fig. 3. DEAE-cellulose column elution profile of L-glycerol-3-phosphate dehydrogenase from *Ceratitis capitata*. Details of the elution are given under Materials and Methods. The concentration of $(NH_4)_2SO_4$ for the peak tube of maximum activity is indicated.

The pooled fractions showing enzyme activity from the second Ultrogel chromatography were chromatographied on a DEAE-cellulose column with a linear gradient of ammonium sulphate. The elution pattern from the DEAE-cellulose column is shown in Fig. 3.

All chromatographic procedures were assayed regarding to the inclusion in the buffers of various concentrations of the reducing agent 2-mercaptoethanol. In the absence of 2-mercaptoethanol, no loss of enzyme activity was observed in gel filtration chromatography; however, when the enzyme was chromatographied on DEAE-cellulose, it was necessary to add 10 mM 2-mercaptoethanol to the buffers in order to preserve the enzyme activity.

Any other subsequent purification step did not increase the specific activity of the preparation.

Homogeneity

After the last step of the purification procedure, the enzyme appeared as a single band on polyacrylamide gel and cellulose acetate strip electrophoresis under the conditions previously described. L-glycerol-3-phosphate dehydrogenase activity was coincident with the single protein bands obtained. No impurities were detected after densitometration of both gels and strips.

Only one protein band was also found in sodium dodecyl sulphate electrophoresis.

Isoelectric focusing of the final preparation of the enzyme in sucrose gradient gave a single and symmetrical protein peak coincident with the enzyme activity.

The enzyme also appeared to be monodisperse in the schlieren patterns of a sedimentation run, showing a single symmetrical peak.

All these criteria proved that the purification procedure results in a homogeneous L-glycerol-3-phosphate dehydrogenase preparation and were repeatedly obtained from different batches of the enzyme. Amino acid composition of the enzyme from different batches was also coincident.

Kinetic parameters

The apparent Michaelis constant (K_m) for the four different substrates is given in Table II.

K_m value for dihydroxyacetone phosphate (0.33 mM) in the presence of 0.2 mM NADH is identical to the value described for the enzyme from *Apis mel-*

TABLE II

K_m VALUES OF L-GLYCEROL-3-PHOSPHATE DEHYDROGENASE OF THE INSECT *CERATITIS CAPITATA* FOR THE DIFFERENT SUBSTRATES

Substrate		
Variable concentration	Fixed concentration	K_m (mM)
Dihydroxyacetone phosphate	0.2 mM NADH	0.330
NADH	0.5 mM dihydroxyacetone phosphate	0.018
L-glycerol 3-phosphate	0.55 mM NAD^+	0.740
NAD^+	1.5 mM L-glycerol-3-phosphate	0.260

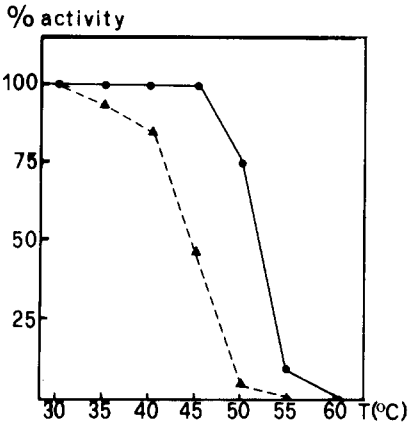

Fig. 4. Effect of temperature on the stability of L-glycerol-3-phosphate dehydrogenase of *Ceratitis capitata*. Aliquots of 0.15 mg/ml were incubated for 5 min (●———●) and 30 min (▲- - - - -▲) at the given temperature. The buffer used for the incubation was 0.1 M histidine, 0.1 M Tris pH 6.6. Details of the experiment are given under Materials and Methods.

lifera thoraces [29]. Dihydroxyacetone phosphate concentrations above 0.5 mM no longer fits a Lineweaver-Burk plot as observed by Brosemer and Marquardt [29] for the honeybee enzyme.

K_m value for NADH (0.018 mM) in the presence of 0.5 mM dihydroxyacetone phosphate is somewhat higher than the values previously reported for the L-glycerol-3-phosphate dehydrogenase from honeybee (0.010 mM) (30), chicken breast muscle (0.006 mM) (31) and *E. coli* (0.010 mM) (32).

The comparative values of the Michaelis constants obtained for the nucleotides indicate that the *Ceratitis capitata* enzyme binds NADH much more strongly than NAD^+. This result is consistent with those obtained with L-glycerol-3-phosphate dehydrogenase preparations from beef liver [33] and chicken breast muscle [31] as well as with most NAD^+-linked dehydrogenases.

The maximum turnover number per 33 500 daltons are 188 s^{-1} for dihydroxyacetone phosphate reduction and 32 s^{-1} for the L-glycerol-3-phosphate oxidation. These values are notably higher than those reported for the beef liver enzyme [33].

Effect of pH and temperature on enzyme stability

L-glycerol-3-phosphate dehydrogenase from *Ceratitis capitata* was stable between pH 6.0 and pH 9.0 at 20° C for 1 h. The same range of pH stability was observed at 20° C for 16 h. A complete inactivation of the enzyme occurred at pH 5.0 and pH 10.0 for 1 h at 20° C.

At pH 6.6, the enzyme was stable at 30° C for 30 min as well as at 45° C for 5 min. A complete inactivation of the enzyme was observed at 55° C for 30 min as well as at 60° C for 5 min (Fig. 4). Thus, the thermal stability of the enzyme from *Ceratitis capitata* is similar to the stability described for the *Drosophila virilis* and rabbit enzyme preparations [34].

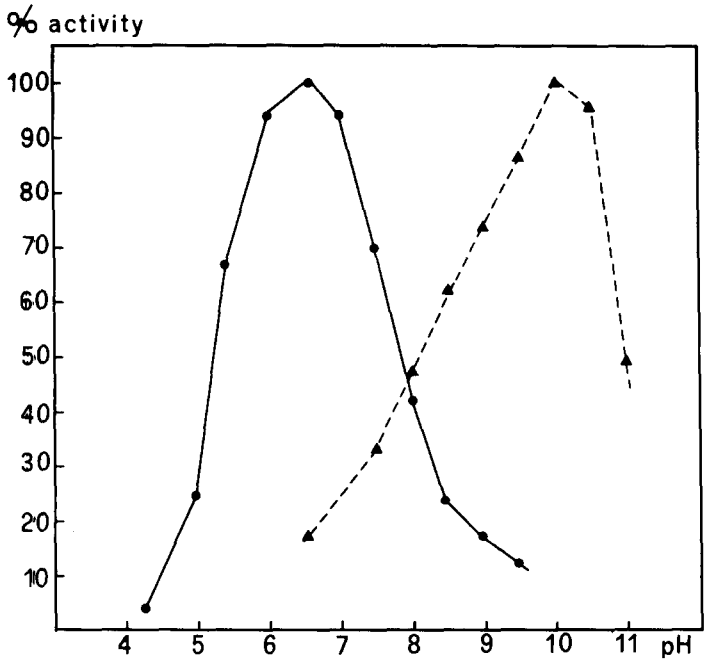

Fig. 5. Effect of pH on the activity of L-glycerol-3-phosphate dehydrogenase of *Ceratitis capitata*. Dehydrogenase activity was assayed using dihydroxyacetone phosphate (●———●) and L-glycerol-3-phosphate (▲- - - - -▲) as substrates according to the standard procedures, except that pH was adjusted to the adequate values with HCl or NaOH.

Effect of pH and temperature on enzyme activity

The optimum pH for the NADH oxidation was about pH 6.6, whereas the optimum pH for the NAD^+ reduction was about pH 10.0 (Fig. 5). The first

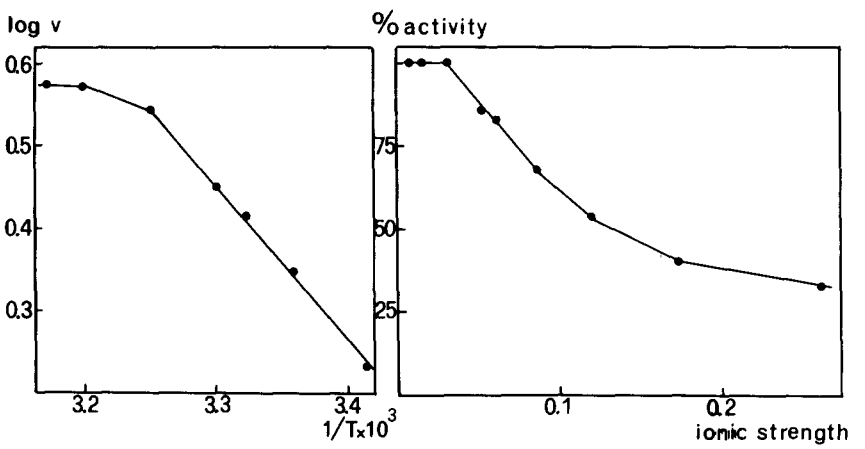

Fig. 6. Effect of temperature on the activity of L-glycerol-3-phosphate dehydrogenase of *Ceratitis capitata*. The standard dihydroxyacetone phosphate assay was carried out. For all temperatures, pH of assay buffer was 6.6 (at 31°C).

Fig. 7. Effect of ionic strength on the activity of L-glycerol-3-phosphate dehydrogenase of *Ceratitis capitata*. The standard dihydroxyacetone phosphate assay was carried out except that the ionic strength was adequately modified.

value is identical to that described for the honeybee enzyme preparation [30] and it differs from the optimum pH reported for L-glycerol-3-phosphate dehydrogenase from chicken breast muscle (7.5—8.0) [31], *E. coli* (7.5) [32] and rabbit (7.5) [33].

The dependence of the reaction velocity on temperature is shown in Fig. 6. The log velocity vs. reciprocal temperature plot was linear between 20 and 35°C. The Arrhenius activation energy for the catalysis of the conversion of NADH-dihydroxyacetone phosphate into NAD^+-L-glycerol-3-phosphate by *Ceratitis capitata* L-glycerol-3-phosphate dehydrogenase was calculated from the slope of the linear portion of the graphic (Fig. 6) to be 8800 cal/mol. The temperature coefficient, Q_{10}, in the linear interval was 1.7.

Effect of the ionic strength on enzyme activity

Maximum activity for the NADH oxidation was found at ionic strengths lower than 0.03. At 0.3 ionic strength, the activity was about 30% of that observed at $\Gamma/2 = 0.03$ (Fig. 7). Thus, the enzyme from the insect was inhibited in solutions of high ionic strength as it has been evidenced for the dehydrogenase from other sources [32,35].

Ultraviolet spectrum and extinction coefficients

The ultraviolet spectrum of the enzyme (Fig. 8) shows three shoulders around 259, 265 and 270 nm that have been already described for bee and rabbit L-glycerol-3-phosphate dehydrogenase [28].

In accordance with the low aromatic amino acid content, the molar extinction coefficient for the pure enzyme at 278 nm is 26.1 $mM^{-1} \cdot cm^{-1}$. It is lower

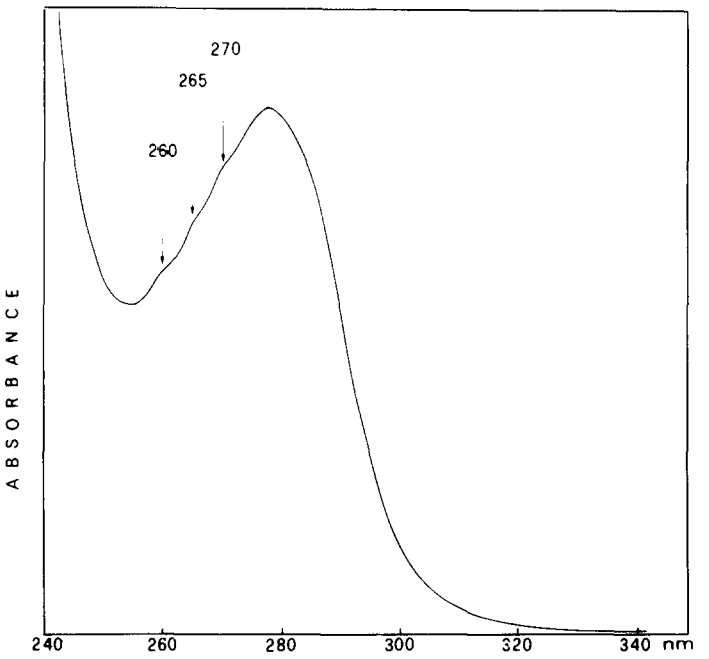

Fig. 8. Ultraviolet spectrum of L-glycerol-3-phosphate dehydrogenase of the insect *Ceratitis capitata*.

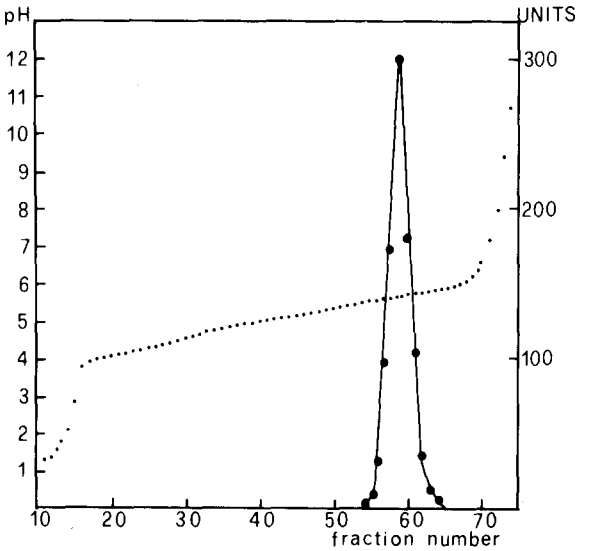

Fig. 9. Isoelectric focusing of L-glycerol-3-phosphate dehydrogenase from the insect *Ceratitis capitata* in a pH 4—6 gradient. Enzyme activity was checked in the standard assay. pH is represented by the dotted line.

than the coefficient described for the chicken breast enzyme (31.0 $mM^{-1} \cdot cm^{-1}$) [36] and for the rabbit muscle enzyme (38, 42 and 44 $mM^{-1} \cdot cm^{-1}$) [37—39].

The extinction coefficient at 278 nm is coincident with that calculated from the amino acid composition of the enzyme on the basis of one tryptophan residue per subunit.

Isoelectric point

The isoelectric point was estimated by electrofocusing of purified L-glycerol-3-phosphate dehydrogenase in the pH ranges 4—8 and 4—6. One sharp and symmetrical peak of enzyme activity exhibited the maximum at pH 5.75 (Fig. 9); thus, the p*I* of the enzyme from *Ceratitis capitata* is 5.75 ± 0.05.

Molecular properties

Molecular weight. The molecular weight of L-glycerol-3-phosphate dehydrogenase of *Ceratitis capitata* has been investigated by gel filtration chromatography, sedimentation velocity and sucrose gradient ultracentrifuge.

TABLE III

ENZYME CONCENTRATION DEPENDENCE OF THE SEDIMENTATION COEFFICIENT

Enzyme concentration (mg/ml)	$s_{20,w}$ (S)
0.5	4.50
1.0	4.80
1.5	5.05
3.0	5.65
5.0	6.30

Gel filtration chromatography was carried out on Sephadex G-200. Samples (0.3 ml) containing 0.5—1.0 mg of the enzyme were applied to the calibrated Sephadex column. A slight variation in the elution volume of the dehydrogenase depending on the concentration of the protein applied to the calibrated Sephadex column was observed. The elution volume varied in a V_e range corresponding to 69 000—76 000 daltons estimated from the calibration plot. Identical elution positions were obtained for *Ceratitis capitata* (0.7 mg/0.3 ml applied to the column) and rabbit muscle (0.7 mg/0.3 ml applied to the column) L-glycerol-3-phosphate dehydrogenases.

Dodecyl sulphate polyacrylamide gel electrophoresis of the enzyme revealed a single sharp band, indicating a molecular structure composed of two subunits of identical molecular weight 33 500.

Sedimentation velocity studies on the analytical ultracentrifuge in the conditions described in Materials and Methods showed a clear dependence of $s_{20,w}$ on enzyme concentrations (Table III). Higher protein concentrations were not studied because of limiting solubility. Plot of these values and extrapolation to zero concentration gave a $s_{20,w}$ of 4.2 S. The same value for the sedimentation coefficient has been described for the enzyme of rabbit muscle [38]. In all runs a single symmetrical peak was observed in agreement with the sedimentation behaviour reported for the enzyme of rabbit muscle [40], chicken muscle and liver [41] and *Drosophila virilis* (34). L-glycerol-3-phosphate dehydrogenase from *Ceratitis capitata* resembles the enzyme from the honeybee in that both dehydrogenases exhibit a marked dependence of sedimentation velocity on protein concentration. However, Marquardt and Brosemer [28] obtained a broad, slightly asymmetrical boundary for honeybee dehydrogenase at several enzyme concentrations whereas no anomalous sedimentation behaviour was detected with the L-glycerol-3-phosphate dehydrogenase from *Ceratitis capitata* at the various protein concentrations studied by Schlieren optics.

Concerning to the stoicheiometric constitution of the quarternary structure of the functionally active species of the enzyme, gradient centrifugations at different protein concentrations were performed in swinging-bucket tubes according to the method of Martin and Ames [17]. The lowest enzyme concentration applied to the tubes with the preformed gradients was 0.2 mg/ml. At this concentration the peak of L-glycerol-3-phosphate dehydrogenase of *Ceratitis capitata* has a $s_{20,w}$ of 4.3 S in close agreement with the value obtained by the sedimentation velocity studies, thus indicating a dimeric state of the protein oligomer as the smallest active form of the enzyme.

Molecular radius. The molecular radius of the native enzyme was calculated according to the procedure of Ackers [42]. We obtained an average value of 34.5 Å as the equivalent hydrodynamic radius (Stokes radius) of the enzyme for the concentration studied.

Diffusion coefficient. If one assumes a Stokes radius of 34.5 Å, a diffusion coefficient $D = 6.22 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1} was obtained.

Partial specific volume. The partial specific volume of the enzyme was determined in a digital densitometer at 20° C. A value of 0.741 ml/g was obtained, that agrees well with the calculated value from the amino acid composition (v = 0.736 ml/g). These values for the partial specific volume of the *Ceratitis capitata* enzyme are in accordance with the results of Fondy et al. [38] for the rabbit muscle enzyme.

TABLE IV

AMINOACID COMPOSITION OF L-GLYCEROL-3-PHOSPHATE DEHYDROGENASE FROM THE INSECT *CERATITIS CAPITATA* AND OTHER SOURCES

Amino acid	*Ceratitis capitata*			Residues per 100 residues			
	Residues per 100 residues *	Residues per 63 000-dalton dimer	Integral even values	*Apis mellifera* ‡‡	*Vespula vulgaris* ‡‡	*Bombus nevadensis* ‡‡	Rabbit muscle ‡‡‡
Asx	10.74	61.9	62	10.6	10.1	9.9	8.0
Thr **	6.16	35.5	36	6.5	4.8	5.9	8.9
Ser **	5.54	31.9	32	3.0	4.0	3.3	3.0
Glx	11.39	65.7	66	10.3	10.9	10.7	12.0
Pro	4.50	25.9	26	3.2	4.3	3.2	4.3
Gly	7.99	46.0	46	7.4	7.7	8.7	10.7
Ala	8.15	46.9	46	8.8	8.3	7.8	9.1
Cys ***	2.20	12.7	12	3.3	2.8	3.3	3.2
Val **	7.17	41.3	42	8.7	8.3	9.6	9.1
Met	1.73	10.0	10	1.0	1.5	1.7	1.7
Ile **	6.45	37.2	38	8.9	8.0	7.3	7.9
Leu	8.97	51.7	52	9.0	8.7	8.8	8.8
Tyr	2.35	13.5	14	1.8	1.2	2.1	1.2
Phe	3.80	21.9	22	3.9	4.1	3.5	4.2
His	2.11	12.2	12	1.6	1.8	1.9	2.5
Lys	6.94	40.0	40	6.7	8.9	7.3	7.8
Arg	3.77	21.7	22	5.1	4.5	4.8	2.5
Trp	—	2.9 ‡	2	(1) ‡	ND ‡‡	ND ‡‡	(2) ‡

* Average for three hydrolysates at each hydrolysis time (24, 48, 72, 96 and 120 h). To avoid computational errors due to rounding off, two decimal places are retained throughout this column. However, the overall accuracy is not higher than ±1.5%.

** Residues Ser and Thr were calculated independently for each hydrolysis time and the given value is obtained by extrapolation to zero time of hydrolysis. Given values for Val and Ile are the average of the three hydrolysates at 120 h.

*** Average of three 24-h hydrolyses of carboxymethylated enzyme as carboxymethylcysteine.

‡ Determined by the method of Edelhoch [22].

‡‡ From ref. 45.

‡‡‡ From ref. 38.

‡ Number of Trp residues per subunit.

‡‡ Not described.

Using a $\bar{v}$ = 0.741 ml/g, a = 34.5 Å, $s_{20,w}$ = 4.2 S, the enzyme from the insect has a molecular weight of 63 100, applying the calculations of Siegel and Monty [43]. The calculated frictional ratio (f/f_0) is 1.293.

Amino acid composition

The amino acid composition of *Ceratitis capitata* L-glycerol-3-phosphate dehydrogenase is given in Table IV. The number of amino acid residues per molecule is given assuming a molecular weight of 63 000. The molecular weight for the enzyme calculated from the integral even values is 63 370. The molecular weight of the enzyme calculated from the less abundant amino acids present in the hydrolysate (Met = 10, His = 12, Tyr = 14) is in agreement with the minimum value obtained by ultracentrifugation.

The partial specific volume calculated on the basis of the amino acid composition [44] is 0.738 ml/g, in close concordance with the value determined experimentally on the digital densitometer.

The extinction coefficient calculated also from the amino acid composition is $E^{1\,cm}_{1\,mg/ml}$ = 0.47, in good agreement with the value obtained spectrophotometrically from the protein dry weight measurements.

Table IV shows also the amino acid composition of L-glycerol-3-phosphate dehydrogenase from other animal species, mainly other insect species. The great similarity among the amino acid composition of the enzyme from *Ceratitis capitata* and those from the insects *A. mellifera*, *V. vulgaris* and *B. nevadensis* [45] suggests a high degree of homology for this enzyme; a less homology is observed, as might be expected, with the enzyme from rabbit muscle [38].

N- and C-terminal residues

Both dansyl and Edman's degradation methods failed to show the presence of a free N-terminal amino acid residue on L-glycerol-3-phosphate dehydrogenase of *Ceratitis capitata*. The enzyme from honeybee [46] and rabbit [47] has also a blocked N-terminal residue, although the nature of the blocking group has not been demonstrated.

The results of a time-course experiment using carboxypeptidase A are given in Table V. It shows the presence of a C-terminal sequence -Ala-Gly-Ser. The enzyme hydrolysis gave 84 nmol of serine per 50 nmol of dehydrogenase (mol. wt. 63 000) for 6-h incubation; it indicates, therefore, the identity of the end-terminal sequence for both subunits of the enzyme and, possibly, the identity of both subunits.

-SH group estimation

L-glycerol-3-phosphate dehydrogenase, freshly prepared and showing a high catalytic activity, was denaturated in 2% sodium dodecyl sulphate and titrated with 5,5'-dithiobis(2-nitrobenzoic acid). A value of about 8 -SH groups per mol was obtained. It was reported previously that about 12 mol -SH/78 000 daltons were found in the rabbit muscle enzyme [48] on iodine. Van Eys et al. [47] obtained a value of 11.8 mol -SH/78 000 daltons with *p*-mercuribenzoate in 8 M urea whereas values of 7.8 mol/78 000 daltons were obtained without urea. Taking into account that 12 and 20 are the total numbers of half-cystine residues obtained, respectively, for the dehydrogenase of the insect and rabbit

TABLE V

QUANTITATIVE ESTIMATION OF C-TERMINAL GROUPS IN L-GLYCEROL-3-PHOSPHATE DEHYDROGENASE OF THE INSECT *CERATITIS CAPITATA*.

Values are expressed as nmol of amino acid liberated per 50 nmol of protein mol.wt. 63 000.

Time (h)	Amino acid		
	Serine	Glycine	Alanine
0.5	73	39	34
1	79	44	40
3	80	50	46
6	84	57	55

muscle, the conclusion must be reached that all the -SH groups are not available to -SH reagents in the enzyme from both sources. The possibility of the presence of -S-S- groups was rejected [47] by experiments of oxidation and dinitrophenylation of the rabbit muscle enzyme.

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