Malkin, R., and Rabinowitz, J. C. (1967), *Biochemistry 6*, 3880.
Nakamura, S. (1970), *Biochem. Biophys. Res. Commun. 41*, 177.
Petering, D. H., and Palmer, G. (1970), *Arch. Biochem. Biophys. 141*, 456.
Poe, M., Phillips, W. D., Glickson, J. D., McDonald, C. C., and San Pietro, A. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 68.

Mechanism of Action of 2,3-Diphosphoglycerate-Independent Phosphoglycerate Mutase*

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ABSTRACT: An improved method of purification of the diphosphoglycerate-(DPGA) independent phosphoglycerate mutase from wheat germ is described. By gel filtration the molecular weight was estimated to be 54,000. The reaction may involve (a) an intramolecular migration of phosphate, (b) an intermolecular transfer of phosphate from DPGA (present as contaminant or synthesized by the enzyme) to the substrates, (c) a phosphoenzyme mechanism, or (d) an intermolecular transfer of phosphate between two or more molecules of substrate. There was no induced transport with ^{14}C- or ^{32}P-labeled substrates and ^{32}P- and ^{14}C-labeled substrates at chemical equilibrium exchanged at equal rates. Mechanisms b and c were therefore excluded. A close adherence to Michaelis–Menten kinetics (K_m = 0.3 mM) with no sigmoid element and a lack of cotransport even at low concentration of substrates excluded mechanism d. The absence of binding of the substrates or phosphate by the enzyme in gel filtration studies with columns equilibrated with substrates and the lack of exchange between the substrates and DPGA provided additional evidence excluding these schemes. The enzyme, therefore, catalyzes an intramolecular transfer of phosphate (a). Since there was no counter transport at high substrate concentrations (20 mM) the rate constant for any isomerization of the free enzyme must probably be in excess of 1.0×10^6 sec^{-1}. Cyclic glyceric 2,3-phosphate was not a substrate, activator or inhibitor; exchange between the substrates and the ester could not be demonstrated; and the ester could not be isolated from the enzyme–substrate complex. The participation of the cyclic ester or a cyclic pentacovalent intermediate is therefore considered unlikely. The phosphatase activity of the enzyme involves the formation of an enzyme phosphate since exchange with the substrates of [^{14}C]glyceric acid but not [^{32}P]P_i was shown. The mechanism of the mutase may therefore involve the transient formation of an enzyme phosphate and a free glyceric acid molecule which remains bound to the enzyme until rephosphorylated: the enzyme may thus be related to the phosphatases. The marked difference from the DPGA-dependent enzymes suggests that these are two separate families of mutase enzymes.

The phosphoglycerate mutases from wheat and rice germ and from a number of other vegetable sources are 2,3-diphosphoglycerate (DPGA)[1] independent (Towne *et al.*, 1956; Ito and Grisolia, 1959; Grisolia and Joyce, 1959; Fernandez and Grisolia, 1960; Grisolia, 1968). In contrast, the activity of the phosphoglycerate mutases from muscle, yeast, and a number of other sources is very markedly stimulated by DPGA although there may be some small residual activity in the absence of this cofactor (Grisolia, 1968). In this paper the mechanism of the DPGA-dependent mutase of wheat germ is investigated.

The conversion of 2-phosphoglycerate (2-PGA) to 3-phosphoglycerate (3-PGA) by phosphoglycerate mutase may be represented by the steps

$$n(\text{2-PGA}) + E_1 \underset{k_{-1}}{\overset{k_{-1}}{\rightleftharpoons}} E(PGA)_n \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} + n(\text{3-PGA}) \quad (1)$$

$$E_1 \underset{k_{+3}}{\overset{k_{-3}}{\rightleftharpoons}} E_2 \quad (2)$$

where $E(PGA)_n$ represents the enzyme–substrate complex, and $E_1 \rightleftharpoons E_2$ an isomerization of the free enzyme. "*n*" indicates the number of substrate molecules which must combine with the enzyme before reaction can occur: it will be unity for most mechanisms unless the enzyme possesses more than one active center and there is cooperativity between the centers.

Possible mechanisms for the transfer of phosphate by wheat-germ phosphoglycerate mutase are summarized in Figure 1. Scheme A represents the direct intramolecular transfer of phosphate between two hydroxyl groups. The acid-catalyzed interconversion of 2-PGA and 3-PGA (Ballou and Fisher, 1954) probably proceeds in this manner with the intermediate formation of a cyclic 2,3-phosphoglycerate ester but the DPGA-dependent enzymes probably do not use this mech-

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[1] Abbreviations used are: 3-PGA, D-3-phosphoglyceric acid; 2-PGA, D-2-phosphoglyceric acid; DPGA, D-2,3-diphosphoglyceric acid; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; NADH, reduced nicotinamide-adenine dinucleotide.

anism (Harrison *et al.*, 1955; Pizer and Ballou, 1959). Scheme B, in which there is transfer of phosphate from DPGA to monophosphoglycerate was proposed by Sutherland *et al.* (1949) for muscle phosphoglycerate mutase. Such a scheme might seem to be excluded for the DPGA-independent mutase of wheat germ since the reaction still occurs when rigorously purified substrates are used and since the presence of a DPGA-generating system could not be demonstrated (Grisolia *et al.*, 1961). Nevertheless, it should still be considered since even purified substrates may contain traces of DPGA and it is very difficult to exclude the formation of small traces of DPGA by the enzyme. Scheme C involves an enzyme phosphate. This is similar to that suggested originally for muscle phosphoglucomutase (Najjar and Pullman, 1954). This mechanism requires DPGA since DPGA can dissociate from the enzyme leaving the enzyme in an inactive dephosphorylated form. Although attempts to isolate the wheat-germ enzyme in a phosphorylated form have not been successful (Grisolia *et al.*, 1961), this does not necessarily exclude the mechanism since the enzyme phosphate may be unstable. It is also not excluded by the apparent lack of a DPGA requirement. Further, with this type of mechanism there are two additional possibilities. The phosphoenzyme may be formed from monophosphoglycerate: the observation that the enzyme possesses phosphatase activity may be relevant in this connection (Ito and Grisolia, 1959; Fernandez and Grisolia, 1960). Alternatively, an enzyme–monophosphoglycerate complex may perform the same role as the phosphoenzyme as is illustrated in Scheme C′. Scheme D (Pizer, 1962) involves a concerted transfer of phosphate between two molecules of substrate. For this mechanism as with Scheme A no cofactor is required but it is necessary for two molecules of substrate to be present upon the enzyme molecule simultaneously. The minimum value for "*n*" in eq 1 will, therefore, be two. Grisolia and Joyce (1959) suggested a similar mechanism in which there was a concerted transfer between three substrate molecules.

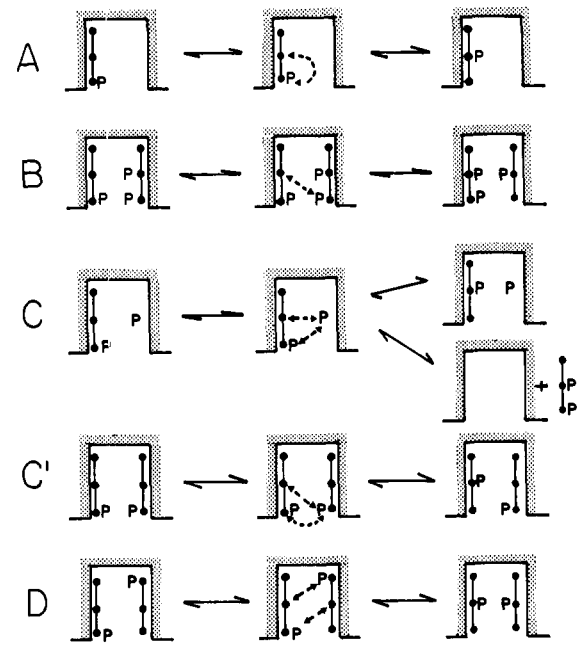

FIGURE 1: Schemes for the phosphoglycerate mutase reaction. Scheme A represents an intramolecular transfer of the phosphate group. Scheme B represents an intermolecular transfer of phosphate from DPGA to monophosphoglycerate. Scheme C represents a phosphoenzyme mechanism: as illustrated the enzyme phosphate is formed from DPGA; alternatively it might be generated by hydrolysis of monophosphoglycerate. Scheme C′ represents a modified phosphoenzyme mechanism in which an enzyme 3-PGA complex performs the role of a phosphoenzyme. Scheme D represents the concerted intermolecular transfer of phosphate between two molecules of substrate.

For Scheme A the transfer of phosphate is intramolecular whereas in all the other it is intermolecular. In some of these intermolecular schemes a labeled molecule of substrate will not be directly converted into a labeled molecule of product. Thus, consider Schemes C and C′ and suppose that the substrate is labeled with ^{32}P. When one molecule of the substrate reacts with the enzyme the labeled phosphate is transferred to the enzyme; and to transfer the phosphate finally to the product a second molecule of substrate (which need not be labeled) must react with the enzyme (Britton and Clarke, 1968, 1969). However, if the substrate is labeled with ^{14}C, the ^{14}C label will appear directly in the product without an intermediate stage. By the same argument for Scheme B there are three intermediate phosphates in the transfer of ^{32}P from substrate to product and one intermediate in the transfer of a ^{14}C label.

Particular use has been made of the induced transport test (Britton, 1965, 1966a,b, 1967; Britton and Clarke, 1968). The test is an application of permeability techniques to enzyme kinetics. It can be regarded as a relaxation technique in which the perturbation is induced by the addition of nonradioactive substrate. Radioactive substrate is incubated with the enzyme until equilibrium is reached and then nonradioactive substrate is added. The unlabeled material may be regarded as a separate species. The equilibrium distribution of the labeled material, therefore, should not be affected by the addition of the unlabeled material and the radioactivity in the substrate (total radioactivity *not* specific activity) should remain constant.[2] In certain cases, however, there may be interaction between the flows of unlabeled and radioactive material between substrate and product. If the flow of nonradioactive material from substrate to product induces a flow of radioactive material in the same sense radioactivity will be carried from substrate to product during the reaction and there will be a transient fall in the radioactivity in the substrate (*cotransport*). If the nonradioactive flow should induce a radioactive flow in the opposite direction there will be a transient rise in the radioactivity in the substrate (*countertransport*). Relatively few molecular mechanisms are able to produce interaction between the flows (Britton, 1965, 1966a,b, 1967) and consequently the interpretation of induced transport data is relatively unambiguous. The indirect transfer of label discussed in connection with Schemes C, C′, and B and certain types of interaction between active centers should produce an isotopic flow in the same sense as the nonisotopic flow. A rate-limiting isomerization of the free enzyme or, conceivably, interaction between active centers of a rather unlikely type should cause a flow in the opposite sense.

Materials

Wheat germ (S-50) was a gift of General Mills. Yeast enolase, yeast, and chicken-breast phosphoglycerate mutase

[2] This assumes that the addition of the substrate does not affect the activity coefficients of substrate and product differentially. This assumption appears to be justified by the fact that the equilibrium constant is not affected by the addition of substrate. Activity coefficients and isotope effects are discussed in Britton and Clarke (1968).

were prepared as previously described (Grisolia *et al.*, 1968; De la Morena *et al.*, 1968; Torralba and Grisolia, 1966). Rabbit muscle lactic dehydrogenase, pyruvate kinase, enolase, crystalline 3-PGA (monobarium and sodium salts), crystalline 2-PGA (sodium salt), and crystalline DPGA (pentacyclohexylammonium salt) were obtained from Boehringer Mannheim Corp. Glycerate (lead salt) was obtained from Aldrich Chemical Co., Inc. [^{32}P]Phosphoric acid in HCl was obtained from Mallinckrodt and HCl free from New England Nuclear. Uniformly labeled D-[^{14}C]glyceric acid, 3-PGA, DPGA, and albumin (bovine plasma, crystallized A grade) were purchased from Calbiochem. β-NADH (grade III) was from Sigma, and ADP was from P-L Biochemicals, Inc. Ribonuclease A (bovine pancreas) was purchased from Sigma, DFP-treated trypsin from Worthington Biochemical. Ovalbumin was a gift of Dr. M. Noelken. Sephadex G-100, G-75, G-25, DEAE A-50, and Blue Dextran were obtained from Pharmacia. Hydroxylapatite (Bio-Gel HTP) and Dowex AG-50W-X8 (200–400 mesh, hydrogen form) were purchased from Bio-Rad and bentonite (USP) from Fisher Scientific Co. Calcium phosphate gel and bentonite suspension were prepared as previously described (Ito and Grisolia, 1959; Grisolia *et al.*, 1961). Spectrophotometric measurements were performed in a Gilford automatic multiple-sample recording spectrophotometer Model 2000, equipped for measurement at constant temperature.

Methods

Preparation of 3-PGA and 2-PGA Free from DPGA. 3-PGA free from DPGA was prepared by the method of Grisolia *et al.* (1969) and to prepare 2-PGA, a micromodification was used with a 10-μl column.

Preparation of [^{32}P]Monophosphoglycerate. [^{32}P]3-PGA was prepared from [^{32}P]DPGA obtained by incubating human blood with [^{32}P]P_i. For material of low specific activity the method of Grisolia *et al.* (1961) was used. To obtain material of high specific activity, the procedure of H. G. Britton and J. B. Clarke (unpublished data) was adopted. The [^{32}P]PGA was chromatographed with the system isopropyl ether (88%)–formic acid (9:6, v/v) (Eggleston and Hems, 1952), methyl Cellosolve–ethyl methyl ketone–3 N NH_3 (7:2:3, v/v) (Mortimer, 1952), and butyl ether–88% formic acid (9:6, v/v). The last, a two-phase system was useful for separating inorganic phosphate from the organic phosphates which remain at the origin.

Preparation of [^{14}C]- and [^{32}P]3-PGA Free from 2-PGA. 2-PGA was removed enzymatically with enolase pyruvate kinase and lactic dehydrogenase (H. G. Britton and J. B. Clarke, unpublished data). To ensure complete removal of radioactive 2-PGA, nonradioactive 2-PGA was added after the initial reaction and the reaction again allowed to go to completion.

Preparation of D-Glyceric Acid 2,3-Phosphate. The cyclic ester was synthesized essentially as described by Pizer and Ballou (1959) from 1 g of 3-PGA (Ba salt) omitting the conversion to the cyclohexylamine salt. Four phosphate peaks were eluted from the cellulose column, pooled, and taken to dryness at 50°. The residues were dissolved in water, adjusted to pH 7, and subjected to thin-layer chromatography (cellulose, solvent III, Table I). The third peak showed a major component with an R_F corresponding to the cyclic phosphate and a minor component corresponding to the monophosphoglycerate or P_i (Khorana *et al.*, 1957). After hydrolysis (30 min, 100°, 1 N HCl) only monophosphoglycerate was found. P_i was measured (Gomori, 1942) before and after hydrolysis with H_2SO_4 (Bartlett, 1959). There was no P_i in the original material; 0.46 mM P_i was found after hydrolysis. Since 40% of 3-PGA is hydrolyzed under these conditions the concentration was ~100 mM.

Separation of Labeled Phosphate Esters, P_i, and Glyceric Acid by Chromatography. These were separated by paper chromatography (Grisolia and Cascales, 1966; Jacobs and Grisolia, 1966) and by thin-layer chromatography on cellulose (Table I). Nonradioactive compounds (0.1, 0.2, 0.3, and 0.2 μmole of DPGA, 3-PGA, P_i, and glyceric acid, respectively) were spotted before the application of the sample. The phosphate compounds were detected by the method of Wade and Morgan (1953). Glyceric acid was detected with bromocresol or acridine reagents (Smith, 1960). The spots were cut out and the radioactivity measured by scintillation (Grisolia and Cascales, 1966).

Determination of [^{14}C]2-PGA. The procedure was based upon that of H. G. Britton and J. B. Clarke (unpublished data). The 2-PGA is converted to lactate and the 3-PGA is removed by Zn and Ba. Samples (100 μl) were added to 100 μl of 0.6 M $HClO_4$ in a cuvet. Buffer (2.5 ml; ethanolamine-Cl^- (pH 7.6, 0.04 M)–KCl (6 mM)–$MgCl_2$ (6.4 mM)–NaOH (0.024 M)), ADP (50 μl; 10 mM in 0.2 M triethanolamine buffer, pH 7.6), NADH (50 μl; 10 mM in 0.2 M triethanolamine buffer, pH 7.6), 2 μl of lactic dehydrogenase (5 mg/ml), and 2 μl of pyruvate kinase (10 mg/ml) were added. The optical density at 340 nM was read, 2 μl of enolase (Boehringer, 10 mg/ml) was added, and the optical density redetermined when the reaction had gone to completion (40 min). The solution (1.9 ml) was mixed with 0.1 ml of 16 mM sodium lactate. Without delay 0.25 ml of $ZnSO_4$ (approximately 5% w/v) was added followed by 0.25 ml of $Ba(OH)_2$ [approximately 0.15 M; 9.8–9.9 ml should give a faint pink color with phenolphthalein when added to 10 ml of the $ZnSO_4$ solution (Somogyi, 1945; Nelson, 1944)], and the suspension was thoroughly agitated. After standing overnight at 4°, it was filtered and 1 ml was transferred to a scintillation vial. Scintillation fluid (10 ml; 5.5 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene dissolved in 667 ml of toulene mixed with 333 ml of Triton X-100) was added to the vial. Less than 1% of [^{14}C]3-PGA (probably largely radiochemical impurities) was left in solution after treatment with Zn and Ba.

Determination of [^{32}P]2-PGA. The procedure was based upon that of H. G. Britton and J. B. Clarke (unpublished data). After conversion of the 2-PGA to lactate and ATP, as described for the determination of [^{14}C]2-PGA, 1.8-ml samples were transferred to conical centrifuge tubes containing 0.2 ml of 2.55 M $HClO_4$. The tubes were heated in a boiling-water bath for 30 min to hydrolyze the ATP, cooled, and 0.7 ml of phosphate-precipitating mixture was added (0.8 M ammonium molybdate–0.2 M triethylamine hydrochloride–H_2O, 2:1:4, v/v, made up just before use). After standing at 4° overnight and centrifugation, the precipitates were washed three times with a washing mixture (0.8 M ammonium molybdate–0.2 M triethanolamine hydrochloride–H_2O 2.55 M–$HClO_4$, 2:1:22:2, v/v) to which a trace of phosphate had been added. The precipitates were dissolved in 0.3 ml of 1 N NaOH at 80° and the solutions were transferred quantitatively with three washings of H_2O (0.2, 0.2, and 0.3 ml, respectively) to scintillation vials. Scintillation mixture (10 ml) was added (see determination of [^{14}C]2-PGA) in which 4% Cab-O-Sil had been suspended.

Simultaneous Determination of [^{14}C]- and [^{32}P]2-PGA. In the exchange experiments at chemical equilibrium both iso-

TABLE I: R_F Values of Phosphate Esters, P_i, and Glyceric Acid Separated by Paper Chromatography and Thin-Layer Chromatography on Cellulose.[a]

Support	Solvent	Development (hr)	R_F Values: 2,3-Diphosphoglycerate	3-Phosphoglycerate	Glyceric Acid 2,3-Phosphate	P_i	Glyceric Acid
Whatman No. 1 paper	I, ethyl acetate–acetic acid–water (3:3:1, v/v) (Mortimer, 1952)	15	0.30	0.40	0.40	0.52	0.62
Whatman No. 1 paper	II, isopropyl alcohol–ammonia–water (7:1:2, v/v) (Markman and Smith, 1952a)	20	0.20	0.24	0.50	0.24	0.60
Thin-layer cellulose	I	15	0.25	0.34	0.37	0.43	0.56
Thin-layer cellulose	III, isopropyl alcohol–ammonia–water (7:0.5:2.5, v/v) (Pizer and Ballou, 1959)	20	0.06	0.06	0.27	0.06	

[a] Ascending development at room temperature (approximately 25°) was used. Whatman No. 1 paper was washed as previously described (Grisolia and Cascales, 1966).

topes were determined simultaneously. 2-PGA was converted to lactate and ATP as described, but the buffer was increased to 2.7 ml; 0.9 ml was taken to determine [^{32}P]ATP with half-quantities of the reagents; 1.85 ml was taken to estimate [^{14}C]-lactate as described. The isotopes were completely separated.

Determination of [^{14}C]- *and* [^{32}P]*3-PGA and -2-PGA.* The procedures described for 2-PGA were used, except that 3 units of wheat-germ mutase was added.

Phosphoglycerate Mutase Assay. The enolase-coupled assay (Rodwell *et al.*, 1956) was used at pH 8.7 for the wheat-germ enzyme and at pH 7.0 for the DPGA-dependent enzymes. For the kinetic experiments, the amount of enolase was increased to 25 units to eliminate an induction period. $MgCl_2$ was added to correct for 3-PGA binding, assuming an apparent binding constant of 298 M^{-1} at pH 8.7 (H. G. Britton and J. B. Clarke, unpublished data). One unit of enzyme activity in 3 ml in a 1-cm cell gave an increase of 0.1 in optical density per min at 240 nm and at 30° (Rodwell *et al.*, 1956).

Phosphoglycerate Phosphatase Assay. The following were mixed in 0.25 ml: 25 μmoles of the appropriate buffer (see Results), 1 μmole of [^{32}P]3-PGA, and 15–50 units of mutase. After incubation at 30 or 37°, the mixture was cooled, 0.25 ml of 40 mM sodium phosphate and 0.85 ml of phosphate-precipitating mixture (0.6 M perchloric acid (0.35 ml)–0.8 M ammonium molybdate (0.1 ml)–0.2 M triethylamine (0.05 ml)–water (0.35 ml); Sugino and Miyoshi, 1964) were added. After 10 hr at 4°, the precipitate was centrifugated, washed, dissolved, and counted as in the determination of [^{32}P]2-PGA.

Protein was estimated by the methods of Mokrasch *et al.* (1956) and Lowry *et al.* (1951). 2-PGA and 3-PGA were assayed as previously described (Rodwell *et al.*, 1957).

Induced Transport Tests. The substrates (in approximately equilibrium proportion) were incubated with the enzyme for a short period before adding radioactive substrate. After a further period for equilibration, samples were taken and non-radioactive 2-PGA dissolved in a small quantity of buffer was added. Samples were taken during the chemical reaction which followed. To correct for radioactivity present in radiochemical impurities and to allow for any phosphatase activity, samples were taken throughout the procedure and assayed without enolase. The radioactivity in these samples which amounted to 20–25% of the total radioactivity found in the 2-PGA was subtracted to determine the radioactivity in the 2-PGA alone. The total radioactivity in the 2-PGA and 3-PGA was estimated from samples taken at the end of the reaction. Approximately equilibrium proportions of the substrates were used and the initial equilibration period was allowed to reduce any induced transport of the radioactive label.

Exchange at Chemical Equilibrium. The substrates were incubated with the enzyme until equilibrium was reached and a mixture of [^{14}C]- and [^{32}P]3-PGA free from labeled 2-PGA was added. The radioactivity-labeled material represented less than 2% of the 3-PGA present. Samples were taken to assay [^{32}P]2-PGA and [^{14}C]2-PGA and total radioactivity in the substrates. To calculate the fluxes, the decimal logarithm of the differences between the counts in the individual samples and the final equilibrium value were plotted against time and the best straight line drawn. The fluxes were derived from the slope of the line and from the expression

$$\text{flux} = 2.303V(\text{slope}) \Big/ \left(\frac{1}{[\text{2-PGA}]} + \frac{1}{[\text{3-GPA}]}\right)(\text{E}) \quad (3)$$

where V = volume of solution, (E) = total quantity of enzyme, and [2-PGA] and [3-PGA] represent the equilibrium concentrations of the substrates.

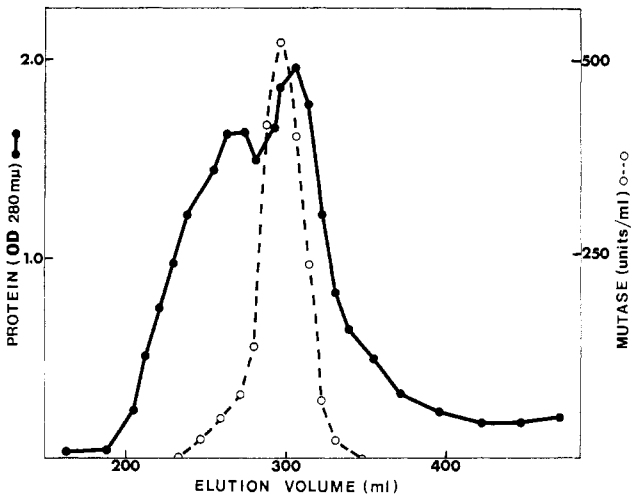

FIGURE 2: Elution pattern of wheat-germ phosphoglycerate mutase on Sephadex G-100. 18 ml of fraction V (221 mg of protein, specific activity 94) was applied to the column (2.8 × 120 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.2), and eluted with the same buffer at a flow rate of 12 ml/hr. 4-ml fractions were collected and assayed for protein (optical density at 280 mμ) (●) and mutase (○). Fractions eluted from 260 to 320 ml were pooled to give fraction VI (105 mg of protein, specific activity 170).

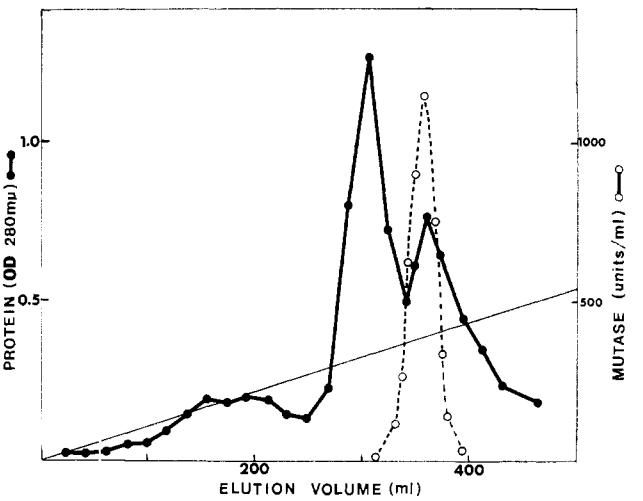

FIGURE 4: Elution pattern of wheat-germ phosphoglycerate mutase on DEAE A-50 Sephadex. 44 ml of fraction VII (88 mg of protein, specific activity 384) was applied to the column (2 × 40 cm) equilibrated with sodium–potassium phosphate buffer (pH 8; I = 0.05). The column was eluted with a linear gradient consisting of 250 ml of phosphate buffer–0.45 M NaCl (pH 8) in the reservoir vessel and 250 ml of equilibrating buffer in the mixing chamber. The flow rate was 15 ml/hr and 5-ml fractions were collected and assayed for protein (●) and mutase activity (○). Tubes eluted from 345 to 375 ml were pooled to give fraction VIII (25 mg of protein, specific activity 1025). Diagonal line represents the gradient.

Results

Enzyme Purification. Unless indicated otherwise, all operations were carried out at 0–5° and centrifugations were for 10 min at 7000*g*. Ammonium sulfate solutions were saturated at pH 5.5. All volumes refer to the original volume for the particular step. The method of Ito and Grisolia (1959) was used with the following modifications. Wheat germ was extracted with 4 volumes of water for 30 min and then centrifuged. To each 100 ml of the supernatant fluid (fraction I) was added 60 ml of $(NH_4)_2SO_4$. After 10 min, the solution was centrifuged and 66 ml of $(NH_4)_2SO_4$ was added to the supernatant. It was allowed to stand 10 min, centrifuged, and the precipitate was dissolved in water (0.75 volume of fraction I) to give fraction II (protein concentration 14 ± 2 mg per ml). About 1.4 volumes of calcium phosphate gel (35 mg/ml) were added to this fraction. The mixture was stirred occasionally for 10 min, centrifuged, and the precipitate was discarded. The supernatant (fraction III) was stirred with ~0.07 volume of bentonite (50 mg/ml) for 10 min and centrifuged. (The exact proportions of calcium phosphate and bentonite should be checked for each batch.) The enzyme was precipitated from the supernatant fluid (fraction IV) by the addition of 0.516 g/ml of solid ammonium sulfate. After standing for 30–60 min the preparation was centrifuged for 20 min. The loosely packed precipitate was transferred to a 40-ml centrifuge tube and centrifuged at 15,000*g* for 30 min. The pellet was taken up in a minimum of water (20–30 ml) and centrifuged at 15,000*g* to remove insoluble material. The supernatant (fraction V) was applied to a column of Sephadex G-100 and eluted with 0.01 M sodium phosphate buffer (pH 7.2) (Figure 2). Tubes containing mutase were pooled (fraction VI) and concentrated ~6 times in a Model 50 Diaflo ultrafiltration assembly equipped with a UM-2 membrane. Fractions containing 50 ± 10 mg of protein were then applied to a hydroxylapatite column and eluted with a linear gradient of phosphate buffer (pH 7.2) (Figure 3). The specific activity of the eluted enzyme (fraction VII) with different batches of hydroxylapatite varied between 300 and 1000 units per mg. To obtain a higher specific activity fraction VII was adjusted to pH 8 (1 N NaOH), applied to a column of DEAE A-50 Sephadex, and eluted with a linear gradient of sodium–potassium phosphate buffer (pH 8.0) (Figure 4). Tubes with mutase were pooled and concentrated to 5000 units of mutase/ml (~10 times) as described above (fraction VIII). The enzyme is reasonably stable. Fractions

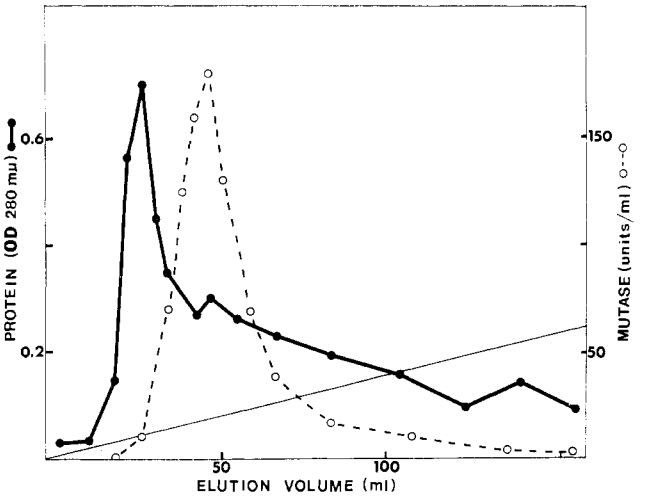

FIGURE 3: Elution pattern of wheat-germ phosphoglycerate mutase on hydroxylapatite. 10 ml of concentrated fraction VI (38 mg of protein, specific activity 135) was applied to the column (1 × 15 cm) equilibrated with 0.01 M sodium phosphate buffer (pH 7.2). The column was developed using a linear gradient consisting of 250 ml of 0.1 M sodium phosphate buffer (pH 7.2) in the reservoir vessel and 250 ml of equilibrating buffer in the mixing chamber. With a flow rate of 10 ml/hr, 1.5-ml fractions were collected and protein (●) and enzyme (○) were estimated. Tubes eluted from 31 to 65 ml were pooled to give fraction VII (7.5 mg of protein, specific activity 425). Diagonal line represents the gradient.

TABLE II: Purification of Wheat-Germ Phosphoglyceric Acid Mutase.[a]

Fraction	Vol (ml)	Total Act. (Units)	Total Protein (mg)	Sp Act.	Yield (%)
I	1330	101,080	37,900	2.6	100
II	1000	96,000	11,550	8.3	94
III	2000	88,950	3,600	24	88
IV	1900	78,842	1,336	59	78
V	42	75,600	1,134	66	74
VI	133	43,100	319	135	42
VII	40	22,156	68.4	324	22
VIII	33	17,180	16.7	1027	17

[a] From 400 g of wheat germ.

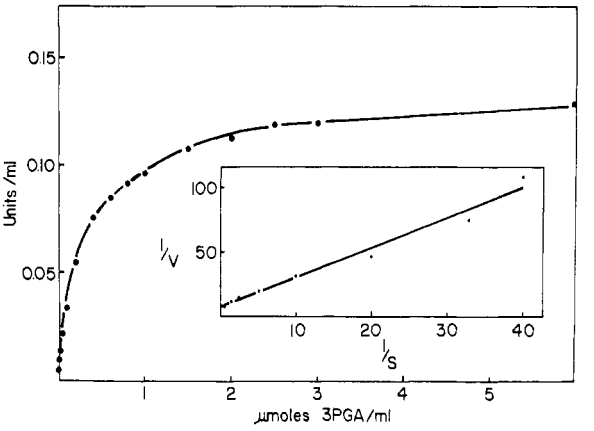

FIGURE 5: The effect of substrate concentration upon velocity assayed by the enolase coupled method (Rodwell *et al.*, 1956) at pH 8.7 and 30°. For conditions, see the text.

III–VI can be kept frozen for several weeks. Fractions VII and VIII are not stable to freezing in phosphate buffer, but can be kept at 0–4° for 2 or 3 weeks without loss of activity. They become stable to freezing if 0.7% bovine serum albumin is added. Fractions VII and VIII are free of enolase (<0.1%). Table II summarizes the purification.

Molecular Weight Estimation. The molecular weight was estimated by gel filtration (Andrews, 1964) with a column of Sephadex G-75 fine (2.5 × 45). Ribonuclease A, DFP-treated trypsin, ovalbumin, and bovine serum albumin were used as standards. Samples (4.5 mg) of each standard and 1 mg of mutase in 4 ml of 50 mM Tris-Cl buffer (pH 7.5) were applied to the column and it was eluted with the same buffer at a flow of 12 ml/hr. Fractions (1 ml) were collected. The wheat-germ mutase was eluted between ovalbumin and bovine serum albumin and the molecular weight was estimated to be 54,000.

Initial Velocities. Initial velocities were measured between 10 μM and 6 mM 3-PGA at pH 8.7 and at 30°. The curve (Figure 5) closely followed Michaelis–Menten kinetics with no evidence of any sigmoid characteristics as might have been expected for Scheme C, C′, and D. The apparent K_m, 3.3×10^{-4} M, is lower than previously reported (Ito and Grisolia, 1959). Other measurements were made at pH 8.7 at 24 and 20° and at pH 7 at 20°. The velocity–concentration curves again closely followed Michaelis–Menten kinetics. The respective K_m's were 3.7×10^{-4}, 3.8×10^{-4}, and 2.9×10^{-4} M. As reported by Ito and Grisolia (1959) the optimum pH was found to be 8.9.

Induced Transport Tests at Intermediate Substrate Concentrations. An induced transport test with ^{32}P-labeled substrates is illustrated in Figure 6 which also shows the results with the DPGA-dependent enzyme from yeast. With the wheat-germ enzyme there was essentially no change in the radioactive 2-PGA after the addition of unlabeled substrate. In contrast, with the yeast enzyme there was a marked fall followed by a return in the radioactivity in the 2-PGA, reflecting cotransport of ^{32}P from 2-PGA to 3-PGA. Identical results were obtained in a duplicate experiment and in another experiment in which the substrate concentrations were doubled. In a fourth experiment 50 μM DPGA was added to the reaction mixture. A similar result was again obtained despite the addition of the cofactor. If it is assumed that isomerization of the free enzyme is rapid (eq 2), lines A, B, and C (Figure 6) represent the expected behavior of the radioactivity in the 2-PGA for Schemes A, B, and C (or C′). These theoretical lines do not depend upon the rate constants and apply generally. The experimental result for the wheat-germ enzyme thus corresponds closely with that expected for Scheme A. If the isomerization of the free enzyme were rate limiting, the theoretical amount of cotransport for Schemes B and C would be reduced. Line B could be displaced toward the position of line C in Figure 6 and line C toward Line A. For Scheme A countertransport would occur. Scheme B would still give a degree of cotransport which would be clearly distinguishable from the experimental result. However, for Scheme C the amount of cotransport might be reduced to such an extent that this distinction could not be made. Further experiments were therefore carried out with ^{14}C-labeled substrates.

An experiment with ^{14}C substrates is shown in Figure 7 together with an identical experiment with the yeast enzyme. With both enzymes there was no induced transport. Similar results were obtained in a duplicate experiment and in a third experiment in which the concentrations were doubled. Lines A&C and B (Figure 7) represent the expected patterns for the different Schemes provided that any isomerization of the free enzyme is rapid. If the isomerization of the enzyme were rate limiting the cotransport shown by Scheme B would be reduced and line B would be displaced toward the position of line A & C and both Schemes A and C would show countertransport. Scheme B has already been excluded by the experiments with ^{32}P substrates and Scheme C would only be compatible with the ^{32}P data if there were a rate-limiting isomerization of the free enzyme which would be sufficient to give very marked countertransport with the ^{14}C substrates. For example, if the ^{32}P data followed line C* in Figure 6 then the ^{14}C data should follow line C* in Figure 7. Thus, only Scheme A is compatible with both sets of data. Further, any isomerization of the free enzyme must not be substantially rate limiting under the conditions of the experiments.

Induced Transport Tests at Very High and Very Low Substrate Concentrations. The enzyme in Scheme D binds two molecules of substrate and it will behave as if it were polyvalent (Britton, 1965, 1966). Therefore, at low concentrations of substrates, cotransport (which should be the same with both ^{14}C- and ^{32}P-labeled substrates) should occur and the maximum should be that shown by curve D (Figure 8). However, if the addition of the first molecule of substrate or the last molecule of product to the enzyme should be rate limiting, or if there should be a rate-limiting isomerization

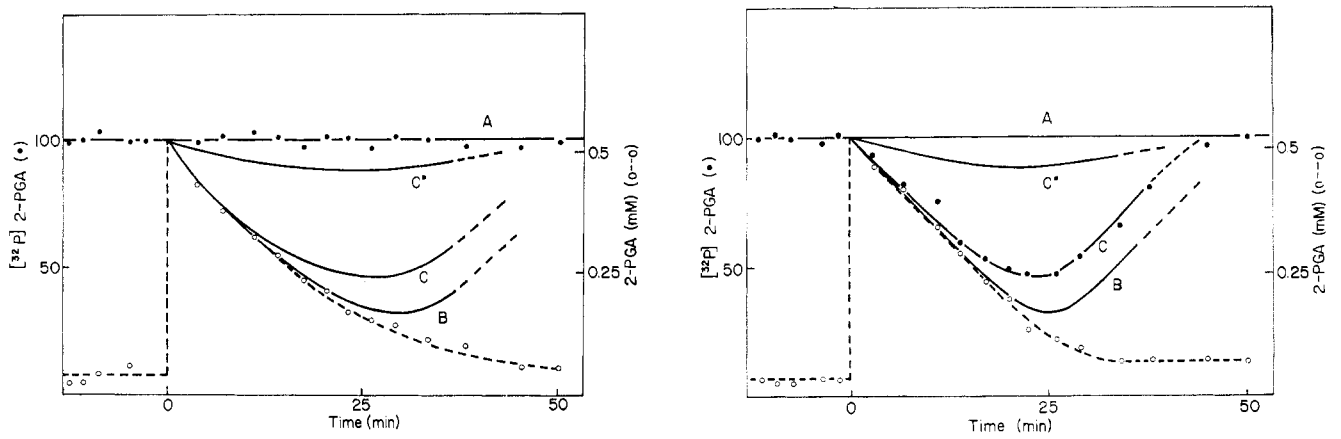

FIGURE 6: Induced transport tests with ^{32}P-labeled substrates. (A, left) With wheat-germ phosphoglycerate mutase. (B, right) With yeast phosphoglycerate mutase. (○) Concentration of 2-PGA (millimolar) and (●) [^{32}P]2-PGA expressed as per cent of the initial values before the addition of nonradioactive 2-PGA. The continuous lines are the theoretical curves for the radioactive 2-PGA for the different schemes in Figure 1 (see text). (A) In the experiment illustrated in part A, 10 μl of wheat-germ phosphoglycerate mutase (0.25 unit), 3.49 ml of Tris-Cl^- buffer (pH 7.4) (Tris (16.7 mM)–$MgCl_2$ (3.33 mM)–bovine serum albumin (0.02%)), and 50 μl of a solution of 3-PGA and 2-PGA (3-PGA: 2-PGA, 10:1, total concentration 31.2 mM, dissolved in the Tris buffer) were incubated at 30° for 39 min. [^{32}P]3-PGA (~0.3 μCi) was then added in a volume of 50 μl. After a further 39 min of incubation, seven samples of 100 μl were taken and at zero time 100 μl of 14.6 mM 2-PGA (dissolved in the Tris buffer) was added to the 2.9 ml of solution. 100-μl samples were taken during the ensuing chemical reaction. The samples were assayed for 2-PGA and [^{32}P]2-PGA as already described. The concentrations before the addition of 2-PGA were 2-PGA (0.0366 mM) and 3-PGA (0.415 mM), and immediately after the addition 2-PGA (0.521 mM) and 3-PGA (0.402 mM). (B) The experimental details and concentrations were as described in 7A except that yeast phosphoglycerate mutase (2.1 units) was used and DPGA was added. The concentration of DPGA after the addition of 2-PGA was 1.01 μM.

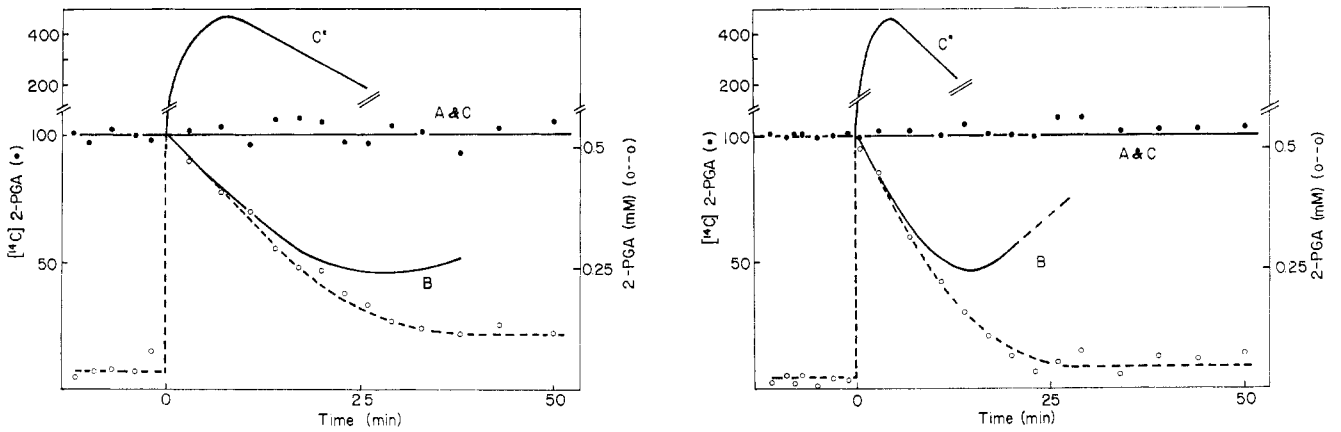

FIGURE 7: Induced transport tests with ^{14}C-labeled substrates. (A, left) With wheat-germ mutase. (B, right) With yeast mutase. (○) 2-PGA concentration (mM) and (●) [^{14}C]2-PGA expressed as per cent of initial values before addition of nonradioactive 2-PGA. The continuous lines are theoretical curves for the radioactive 2-PGA for Schemes A, B, and C (Figure 1, see text). (A) The details of the procedure and the concentration were those described in Figure 7A except that 50 μl of [^{14}C]3-PGA (1 μCi approximately) was added instead of [^{32}P]3-PGA and the samples were assayed for the ^{14}C isotope. (B) The details of the procedure were those described in Figure 7B except that [^{14}C]3-PGA was used.

of the free enzyme, the amount of cotransport should fall as the substrate concentrations are raised and at sufficiently high concentrations countertransport should occur. At intermediate concentrations, therefore, there may be little induced transport in either direction. Experiments (Figures 8 and 9) were carried out with ^{14}C substrates at high and very low substrate concentrations. The experiments at high concentrations were at pH 8.7 to minimize pH changes during the reaction which would affect the equilibrium constant (due to the difference in ionization constants of 2-PGA and 3-PGA). Magnesium was also omitted to avoid the complications of magnesium binding. There was no induced transport at either concentration and duplicate experiments gave identical results. These experiments covered a 200-fold range of concentrations, and it must be concluded that it is very unlikely that the reaction proceeds by Scheme D.

Scheme A would give rise to countertransport at high substrate concentrations (Figure 9) if there were a rate-limiting isomerization of the free enzyme. Curve A* is the theoretical curve for $\alpha = 0.03$ mM^{-1} (see eq 6 and Appendix). If the K_m is 0.3 mM this value of α corresponds to a rate constant for the isomerization of about 1.0×10^6 sec^{-1} (assuming a turnover number for the enzyme of 1.6×10^4 min^{-1}). Any isomerization of the enzyme must thus have a larger rate constant. If the apparent K_m is affected by the high ionic strength due to the substrates the quantitative calculation will be affected but the conclusion that the isomerization of the free enzyme is not substantially rate limiting will still apply.

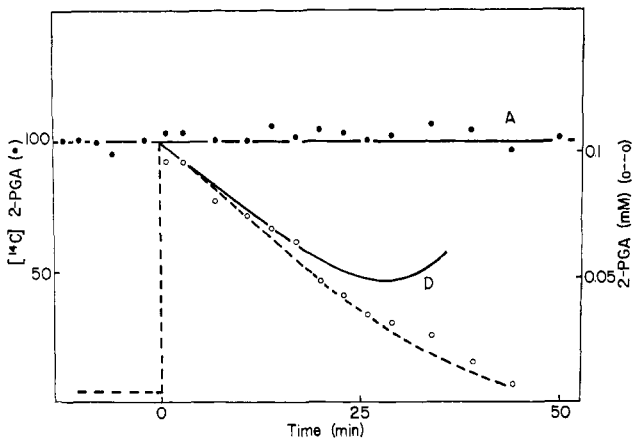

FIGURE 8: Induced transport test with ^{14}C-labeled substrates at low substrate concentrations. (○) Concentrations of 2-PGA (millimolar) and (●) [^{14}C]2-PGA expressed as per cent of initial values before the addition of nonradioactive 2-PGA. The continuous lines are theoretical curves for the radioactive 2-PGA for the schemes in Figure 1 (see text). 3.55 ml of Tris-Cl^- buffer (pH 7.4) (Tris (16.7 mM)-$MgCl_2$ (3.33 mM)-bovine serum albumin (0.02%)), 10 μl of a mixture of 3-PGA and 2-PGA (3-PGA:2-PGA 10:1, total concentration 31.2 mM, dissolved in the Tris buffer), and 7.5 μl of wheat-germ phosphoglycerate mutase (~0.06 unit) were incubated for 40 min. 25 μl of [^{14}C]3-PGA (~1 μCi) was then added and the mutase incubated a further 40 min. Seven samples of 100 μl were taken and at zero time 10 μl of 29.2 mM 2-PGA (dissolved in Tris buffer) was added. Samples (100 μl) were taken during the chemical reaction that followed. Concentrations before addition of 2-PGA were 2-PGA (7.38 μM) and 3-PGA (83.6 μM) and immediately after the addition 2-PGA (108 μM) and 3-PGA (83.3 μM).

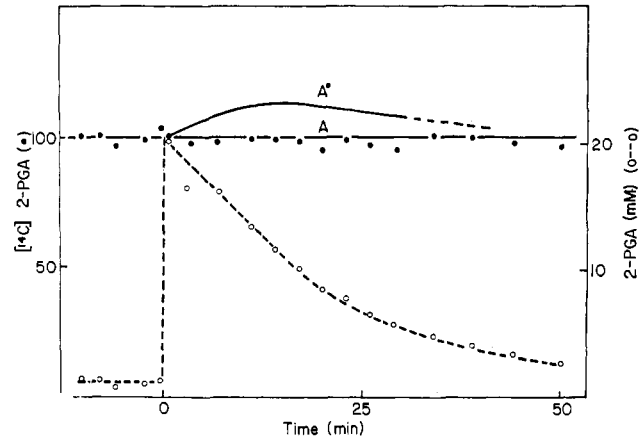

FIGURE 9: Induced transport with ^{14}C-labeled substrates at high substrate concentrations. (○) Concentration of 2-PGA (millimolar) and (●) [^{14}C]2-PGA expressed as per cent of initial values before the addition of nonradioactive 2-PGA. The continuous lines are theoretical lines (see text). 0.7 ml of Tris-Cl^- buffer (pH 8.7, 44 mM containing 0.02% bovine serum albumin), 100 μl of a mixture of 3-PGA and 2-PGA (3-PGA:2-PGA 10:1, total concentration 200 mM, dissolved in 33.3 mM Tris buffer and adjusted to pH 8.7), and 17 μl of wheat-germ enzyme (6.8 units) were incubated for 18 min at 30°. 200 μl of [^{14}C]2-PGA (4 μCi) was added and the mixture incubated for a further 30 min. Seven samples of 10 μl were taken and at zero time 100 μl of 2-PGA (200 mM dissolved in 33.3 mM Tris buffer and adjusted to pH 8.7) was added to the solution. 10-μl samples were taken during the subsequent chemical reaction. 2-PGA and [^{14}C]2-PGA were estimated as described in Methods. The concentrations before the addition of nonradioactive 2-PGA were 2-PGA (1.59 mM) and 3-PGA (18.1 mM). After the addition the concentrations were 2-PGA (20.5 mM) and 3-PGA (16.4 mM). The buffer concentration was 33.5 mM.

Exchange of ^{14}C- and ^{32}P-Labeled Substrates at Chemical Equilibrium. For Scheme C at chemical equilibrium

$$\frac{\text{flux of [}^{14}\text{C]-3-PGA to 2-PGA}}{\text{flux of [}^{32}\text{P]-3-PGA to 2-PGA}} = 2 + \alpha[\text{A}] \qquad (4)$$

and for Scheme B at chemical equilibrium

$$\frac{\text{flux of [}^{14}\text{C]3-PGA to 2-PGA}}{\text{flux of [}^{32}\text{P]3-PGA to 2-PGA}} = \frac{3 + 2\alpha[\text{A}]}{2 + \alpha[\text{A}]} \qquad (5)$$

where

$$\alpha = k_{+1}k_{+2}/k_{-3}(k_{-1} + k_{+2}) \qquad (6)$$

α will be zero if the isomerization of the enzyme is rapid. Schemes B and C should thus yield flux ratios equal to or greater than 3:2 and 2:1, respectively, whereas Schemes A and D should give flux ratios of unity. With the wheat-germ enzyme, a flux ratio close to one was found (Table III) excluding Schemes B and C. In contrast, the DPGA-dependent enzyme of yeast gave a flux ratio of 2.

Attempts to Demonstrate Substrate Binding. To explain the Michaelis–Menten kinetics observed Schemes C, C′, and D would require one molecule of phosphate or 3-PGA to be very tightly bound. To test for such binding, gel filtration chromatography with columns equilibrated with the substrate (Hummel and Dreyer, 1962) was used. A mixture (1 ml) of 0.05 mM wheat-germ mutase and 0.01 mM 3-PGA in 0.01 M glycine buffer (pH 8.5) was applied to a column of Sephadex G-25 fine (0.9 × 55 cm) equilibrated with 0.01 mM [^{32}P]3-PGA in 0.01 M glycine buffer (pH 8.5) at 4°. The column was eluted with equilibrating buffer at a flow of 30 ml/hr. Fractions (1 ml) were collected and assayed for mutase and radioactivity. There was no evidence of a radioactive peak associated with the enzyme or trough following the enzyme, and the radioactivity in the individual samples remained with 5% of each other. A second experiment gave a similar result.

TABLE III: Fluxes of Glycerate and Phosphate at Chemical Equilibrium Determined by the Use of ^{14}C- and ^{32}P-Labeled Substrates.[a]

Quantity of Enzyme Added (Units)	^{14}C Flux[b]	^{32}P Flux[b]	$\frac{^{14}C\text{ Flux}}{^{32}P\text{ Flux}}$
0.09	0.131	0.137	0.95
0.075 (approximately)	0.139	0.147	0.95

[a] 1.5 ml of buffer (pH 7.4) (Tris-Cl^- (16.7 mM)-$MgCl_2$ (3.33 mM)-bovine serum albumin (0.02%)), 25 μl of a solution of 26.4 mM 3-GPA and 2.64 mM 2-PGA dissolved in the same buffer, and 2–6 μl of enzyme were incubated at 30° for 30 min. 30 μl of a solution of [^{32}P]3-PGA and [^{14}C]3-PGA was then added (representing less than 2% of the 3-PGA already present). The radioactivity in the 2-PGA and the fluxes were determined as described in the Methods section. The concentrations of 2-PGA and 3-PGA were 0.0379 and 0.428 mM in the final solution. [b] μmole/min per unit.

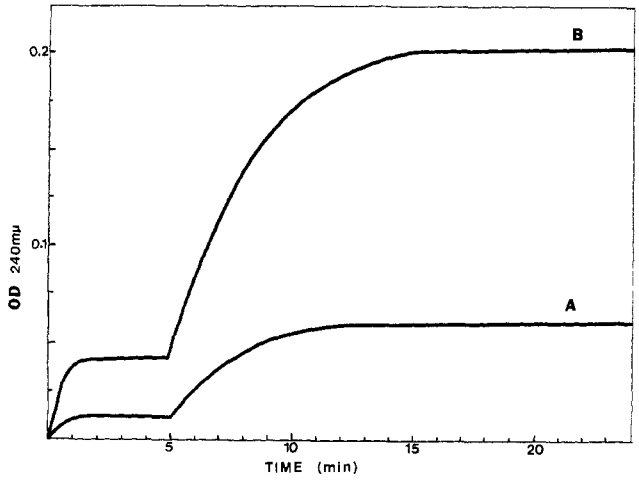

FIGURE 10: The effect of wheat-germ mutase on glyceric acid 2,3-phosphate before and after hydrolysis. Each cuvet (A and B) contained, in a volume of 3 ml, 3.3 mM $MgSO_4$ and 33.3 mM Tris-Cl^- (pH 7). 3.3 mM glyceric acid 2,3-phosphate was added to cuvet A and 0.8 mM of hydrolyzed ester (30 min at 100° in 1 N HCl) was added to cuvet B. At 0 and 5 min, respectively, 10 units of enolase and 1 unit of mutase were added. The absorbance at 240 mμ was recorded at 30°.

Exchange Experiments between [^{32}P]3-PGA and DPGA. Schemes B, C, and C′ (Figure 1) may bring about an exchange of ^{32}P between the substrates and DPGA. Table IV summarizes some experiments to test for such exchange. Large quantities of enzyme were used and in some KCl was added since it greatly increases the rate of exchange between 3-PGA and DPGA with the DPGA-dependent enzymes (Grisolia and Cascales, 1966). There was no exchange under any of the conditions although exchange with chicken-breast mutase was readily demonstrated.

Tests for an Intermediate Cyclic Glyceric 2,3-Phosphate. Scheme A is the only mechanism compatible with the data. The following experiments were carried out to test whether this intramolecular transfer involves the cyclic ester formation.

KINETIC EXPERIMENTS. Glyceric acid 2,3-phosphate (10 μmoles) was assayed as substrate for the wheat-germ mutase (Figure 10). On the addition of enolase there was a small increase in optical density (equivalent to 0.1 μmole of 2-PGA contaminant) and when the mutase was added there was a further increase of 0.052 in optical density (equivalent to 0.47 μmole of 3-PGA contaminant), but thereafter the optical density remained constant. With the same quantity of 3-PGA an increase in optical density of 0.1/min would have been observed, with a total change of 1.0. Figure 10 also shows that in contrast the hydrolyzed ester acts as a substrate both for mutase and enolase.

The experiments were repeated with the DPGA-dependent enzyme from chicken breast. No reaction was observed, confirming the observation of Pizer and Ballou (1959) on the enzyme from rabbit muscle.

To test if the cyclic phosphate activates or inhibits, cyclic phosphate (0.083, 0.33, 0.67, 3.3, and 6.7 mM) was incubated at 30° with 3-PGA (3.3 mM), $MgSO_4$ (3.3 mM), Tris-Cl^- at pH 8.7 (33 mM), enolase (7 units/ml), and wheat-germ mutase (0.12 unit/ml). No significant effect of the cyclic phosphate upon the rate of the reaction was observed.

EXCHANGE EXPERIMENTS. Although glyceric acid 2,3-phosphate appears not to be a substrate, inhibitor, or activator, it might be a transient intermediate which could be released occasionally from the enzyme. To test for this possibility, wheat-germ mutase (4 and 80 units per ml) was incubated with [^{32}P]3-PGA (1.8 mM) and glyceric acid 2,3-phosphate (10 mM) at pH 8.7 (Tris-Cl^-, 100 mM) and at 40°. Samples were chromatographed (solvent II, Table I) to determine incorporation of radioactivity in the cyclic ester. No ^{32}P (<1.5%) was incorporated in either of these experiments over a 60-min period despite the very large amounts of enzyme used, or in a third experiment at pH 7 with 90 units/ml of enzyme. A similar lack of incorporation of radioactivity into the cyclic compound was found in two experiments with the DPGA-dependent enzyme from chicken breast (4 and 80 units per ml; Tris-Cl^-, 100 mM, pH 7.0).

ATTEMPTS TO ISOLATE CYCLIC GLYCERIC 2,3-PHOSPHATE FROM THE ENZYME–SUBSTRATE COMPLEX. The cyclic ester might be an intermediate if it were unable to leave the enzyme. To test for this, a concentrated solution of the enzyme was incubated briefly with freshly rechromatographed [^{14}C]3-PGA. The first sample was deproteinized with ethanol; the remaining samples were applied directly to the chromatograph paper. After 40-sec incubation (Table V), less than 0.008% of the radioactivity was present in the cyclic ester. Subsequent samples showed traces of radioactivity in the cyclic phosphate, probably due to [^{14}C]glyceric acid which chromatographs immediately ahead of the cyclic compound. The K_m for 3-PGA is 0.3 mM, and 22% of the radioactive 3-PGA should have been present as an enzyme–substrate complex. The phosphate buffer may increase the K_m somewhat by competition but it is clear that no cyclic ester is released when the enzyme is denatured. The appearance of glyceric acid was due to the phosphatase activity of the enzyme (Ito and Grisolia, 1959; Grisolia *et al.*, 1961).

Monophosphoglycerate Phosphatase Activity. The wheat-germ mutase retains phosphatase activity after extensive

TABLE IV: Exchange Reactions between [^{32}P]3-PGA and DPGA.[a]

Mutase Added	KCl Added (mM)	% ^{32}P into DPGA 0 min	30 min
None	None	9	9
Wheat germ	None	11 (8)	7 (11)
Wheat germ	100	8 (15)	8 (9)
Wheat germ	200	11 (6)	11 (11)
Wheat germ	1500	19 (2)	17 (11)
Chicken breast	None	10	21
Chicken breast	100	14	88
Chicken breast	200	10	95
Chicken breast	1500	14	75

[a] Each tube contained, in 2.5 ml, 1.5 mM [^{32}P]3-PGA (1500 cpm/μmole), 6.5 mM DPGA, and the indicated KCl. 2 units of phosphoglycerate mutase was added at zero time. All reagents and the enzyme were adjusted to pH 7. The incubation was at 30°. At time 0 and 30 min, 1-ml portions were withdrawn, deproteinized (Grisolia and Cascales, 1966), and 50-μl aliquots chromatographed with solvent I (Table I). The data in parentheses are from similar experiments carried out with 200 units of mutase. The wheat-germ mutase retains at least 85% of its activity in the presence of 6.5 mM DPGA.

TABLE V: Attempts to Isolate Cyclic Glyceric 2,3-Phosphate from the Enzyme–Substrate Complex.[a]

Sample Taken at	% ^{14}C into	
	Glyceric 2,3-Phosphate	Glyceric Acid
40 sec	0.008 (0.000)	0.22
2 min	0.017 (0.005)	0.43
4 min	0.02	1.11

[a] A mixture containing, in 0.2 ml, approximately 0.140 mM wheat-germ mutase in 0.1 M phosphate buffer at pH 7.2 (1800 units of fraction VII) and 0.2 mM [^{14}C]3-PGA (214×10^6 dpm/μmole) was incubated at room temperature. After 40 sec an aliquot was mixed with 3 volumes of cold absolute ethanol, centrifuged, and 40-μl aliquots of the supernatant applied on the chromatograph paper. The incubation mixture was transferred to a water–ice bath and at 2 and 4 min 10-μl aliquots were withdrawn and applied to the paper without deproteinization (solvent II, Table I). The values in parentheses were obtained when the radioactivity corresponding to glyceric 2,3-phosphate was eluted and rechromatographed under the same conditions.

TABLE VI: Exchange Reactions between [^{14}C]Glyceric Acid and 3-PGA.[a]

Mutase	Units	pH	% ^{14}C into 3-PGA		
			0 min	75 min	120 min
None		7.35	0.19	0.15	0.10
Chicken breast	100	7.0	0.19		0.43
Yeast	100	7.0	0.22		0.49
Wheat germ	28	7.35	0.22	0.78	1.4
	56	7.35	0.24	1.3	2.2
	112	7.35	0.28	2.1	3.7
	28	8.7	0.3	0.97	1.4
	56	8.7	0.3	3.5	3.9
	112	8.7	0.34	7.4	9.2

[a] Each tube contained, in a total volume of 0.25 ml, 48 μM [^{14}C]glyceric acid (3×10^7 cpm/μmole), 1.3 mM 3-PGA, and 80 mM Tris-Cl$^-$ at pH 7.0, 7.35, or 8.7. The incubation was at 38°. Phosphoglycerate mutase was added at time zero. At the indicated times, 50 μl was withdrawn and chromatographed with solvent I (Table I).

purification (Grisolia *et al.*, 1961). This activity has been further investigated.

KINETIC EXPERIMENTS. 3-PGA phosphatase activity at different pH was assayed with acetate (pH 4–6.5), Tris-Cl$^-$ (pH 7–8.6), and bicarbonate buffer (pH 9.6–11.2). The optimum pH was 5.8, similar to the optimum for the nonspecific acid phosphatase from wheat germ (Joyce and Grisolia, 1960). The ratio mutase:phosphatase was 2500 at pH 5.8 and it rose to 100,000 at pH 9.6. As with the nonspecific phosphatase, Mg^{2+} (20 mM), at pH 5.6, stimulated (300%) and P_i (20 mM) inhibited (70%). PP_i, 2-phosphoglycolate, and bisulfite (20 mM) at pH 5.6, which stimulate the DPGA phosphatase activity of the DPGA-dependent mutases (Diederich *et al.*, 1971), were inhibitory (93, 70, and 50%, respectively). KCl (0.9 M) and urea (2 M) inhibited (15 and 60%, respectively).

EXCHANGE EXPERIMENTS. Table VI shows that in the presence of wheat-germ mutase [^{14}C]glyceric acid is incorporated into 3-PGA with a maximum at pH 8.7. At 48° the rate of incorporation increased about threefold. With the chicken, breast and yeast enzymes there was no incorporation. Phosphate was present in the enzyme preparation but the incorporation was not due to a reversal of the reaction. Wheat-germ mutase (200 units/ml) incubated with [^{14}C]glyceric acid (32 μM) and P_i (0.4, 4.0, and 20 μM) in Tris-Cl$^-$ (100 mM pH 8.7) at 48° for 60 min yielded no [^{14}C]3-PGA (<0.08%). P_i was not incorporated into 3-PGA when wheat-germ mutase (200 units/ml) was incubated at 48° with [^{32}P]P_i (20 mM), 3-PGA (4 mM), and Tris-Cl$^-$ (100 mM, pH 8.7) for 60 min. No exchange was also found between [^{14}C]glyceric acid (4 μM) and the cyclic ester (3.3 mM) with the wheat-germ or chicken-breast enzymes (160 units/ml) incubated at pH 8.7 and 7, respectively, at 38° for 60 min.

Discussion

The procedure for isolating wheat-germ phosphoglycerate mutase is very reliable (it has been reproduced over 25 times); the specific activity is no higher than previously reported but the yield is substantially greater (Grisolia *et al.*, 1961). Its molecular weight (54,000) is comparable with the DPGA-dependent enzymes from chicken breast, rabbit muscle, and pig kidney and about one-half that of the yeast enzyme (Torralba and Grisolia, 1966; Edelhoch *et al.*, 1957; Pizer, 1962; Diederich *et al.*, 1970; Sasaki *et al.*, 1966).

Five mechanisms were considered (Figure 1). Scheme A is an intramolecular transfer of phosphate. The other Schemes (B, C, C′, and D) involve an intermolecular transfer. All of the latter schemes have been excluded by two or more tests. Schemes B, C, and C′ were excluded by the induced transport tests and by the relative rates of exchange of ^{32}P and ^{14}C substrates at chemical equilibrium. These tests excluded not only the particular mechanisms illustrated but related mechanisms (Britton and Clarke, 1969). Further support for the exclusion of these three schemes was provided by the lack of exchange between [^{32}P]3-PGA and DPGA and by the lack of binding in gel filtration with [^{32}P]3-PGA. The latter findings confirm and extend the observations of Ito and Grisolia (1959) who found no phosphate in the enzyme protein, and Grisolia *et al.* (1961) who found no radioactivity associated with the enzyme after incubation with [^{32}P]DPGA or [^{32}P]-3-PGA and precipitation by salt or filtration through a Dowex column.

Scheme D, the concerted transfer of phosphate between two (or more) molecules of substrate, would demand sigmoid kinetics. However, no sigmoid element was detected even at very low concentrations and under a variety of conditions. Further in the induced transport tests cotransport should have occurred at low substrate concentrations. If one molecule of substrate was bound tenaciously to the enzyme, the sigmoid element and cotransport would be found only at extremely low substrate concentrations but the fact that no [^{32}P]3-PGA binding was detected by gel filtration with columns equilibrated with substrate appears to exclude this possibility. Scheme A thus appears to be the only possible mechanism for the wheat-germ enzyme. All of the data is compatible with this scheme provided that any isomerization of the free

enzyme, if such a step should exist, is not rate limiting. From the induced transport data at high concentrations of substrate the rate constant for isomerization must be higher than 10^6 sec^{-1}.

Cyclic 2,3-monophosphoglycerate did not act as a substrate, inhibitor, or activator for the wheat-germ enzyme. There was no exchange between [^{32}P]3-PGA and the cyclic phosphate and the cyclic ester could not be isolated from the enzyme–substrate complex. Thus it is unlikely that the cyclic ester is involved in the enzyme reaction. The reaction might involve a pentacovalent cyclic complex. However, the two hydroxyl oxygens of the glyceric acid would occupy equatorial positions in the bitrigonal pyramid of phosphorus. The bonds at the apices are most liable to attack and the complex should dehydrate at least occasionally to the cyclic phosphate. The present evidence, therefore, makes such a cyclic pentacovalent intermediate unlikely. The cyclic ester is additionally made improbable by the energy required to form the diester bonds. The formation of a cyclic ester in the ribonuclease reaction (Markham and Smith, 1952b) is probably not comparable since the substrate is a diester already.

Even the most highly purified preparations of wheat-germ mutase have phosphatase activity. Although a nonspecific phosphatase is present in the wheat germ (Joyce and Grisolia, 1960), this phosphatase, unlike the phosphatase activity of the mutase, is inhibited by mercuric ions (Joyce and Grisolia, 1960; Grisolia and Tecson, 1967). The pH optimum (5.8) for the phosphatase activity of the mutase was similar to that of the nonspecific phosphatase, and it was stimulated by Mg^{2+}. In an attempt to stimulate the phosphatase activity by inducing a conformational change, high concentrations of urea and KCl were used. Both, however, produced inhibition. The exchange between the monophosphoglycerates and [^{14}C]glyceric acid and the lack of exchange found with P_i indicates that the phosphatase activity of the mutase involves the formation of an enzyme phosphate. This suggests that the mutase reaction may also proceed with the intermediate formation of a phosphoenzyme and free glyceric acid which remains tightly bound to the enzyme until it is rephosphorylated. Thus, the mutases and the phosphatases may be evolutionarily related.

Isotope studies upon the DPGA-dependent mutases from rabbit muscle (Britton and Clarke, 1969), pig kidney, and yeast (H. G. Britton, J. Carreras, and S. Grisolia, unpublished data) have excluded the intramolecular transfer of phosphate and indicate a mechanism of the phosphoenzyme type (Scheme C). Although a sequential mechanism (Scheme B) has also been suggested for the yeast enzyme (Chiba *et al.*, 1970), it is clear that the DPGA-dependent and -independent enzymes have very different mechanisms. This suggests that the two groups, as with aldolase I and aldolase II, are phylogenetically analogous rather than homologous enzymes (Rutter, 1964).

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Appendix

Calculation of Induced Transport Curves. Induced transport curves were computed by the method of H. G. Britton and J. B. Clarke (unpublished data) based upon Britton and Clarke (1968). To take into account Mg^{2+} binding, the free [Mg^{2+}] concentration is calculated and then the effective equilibrium constant is derived from the expression

$$K = K^0\frac{1 + K_B[Mg^{2+}]}{1 + K_A[Mg^{2+}]}$$

where K^0 = equilibrium constant in the absence of Mg^{2+}, K_B = binding constant for 2-PGA, K_A = binding constant for 3-PGA, and [Mg^{2+}] = concentration of free ionized Mg^{2+}. At pH 7.4 under conditions described in Figures 7, 8, and 9, K^0 = 1/11.92 K_B = 286 mM^{-1}, K_A = 255 mM^{-1} (H. G. Britton and J. B. Clarke, unpublished data).

For curves A, B, C, and C* the expressions for the flux ratios given for the corresponding schemes for phosphoglucomutase were used (Britton and Clarke, 1968). For curve D the expression for curve C was employed (Britton, 1965, 1966,a,b, 1967).

For curve A*, Figure 10 an equilibrium constant of 1/11 was used. The ratio of the total counts in the substrate to the counts in 2-PGA gave a value of 1/10.9 in the experiment shown in Figure 10 and a value of 1/11.1 was obtained for the second experiment not illustrated.

Calculation of the Rate Constant for Isomerization. To calculate the rate constants, eq 32 of the Appendix of the paper of Britton and Clarke (1968) was used. The Michaelis constant for 2-PGA was calculated from the constant for 3-PGA using the Haldane relationship. V_F/V_R = 1 (Ito and Grisolia, 1959).

References

Andrews, P. (1964), *Biochem. J. 91*, 222.
Ballou, C. E., and Fisher, H. O. L. (1954), *J. Amer. Chem. Soc. 76*, 3188.
Bartlett, G. R. (1959), *J. Biol. Chem. 234*, 466.
Britton, H. G. (1965), *Nature (London) 205*, 1323.
Britton, H. G. (1966a), *Nature (London) 209*, 296.
Britton, H. G. (1966b), *Arch. Biochem. Biophys. 117*, 167.
Britton, H. G. (1967), *Arch. Biochem. Biophys. 121*, 522.
Britton, H. G., and Clarke, J. B. (1968), *Biochem. J. 110*, 161.
Britton, H. G., and Clarke, J. B. (1969), *Biochem. J. 112*, 10P.
Chiba, H., Sugimoto, E., Sasaki, R., and Hirose, M. (1970), *Agr. Biol. Chem. 34*, 498.
De la Morena, E., Santos, I., and Grisolia, S. (1968), *Biochim. Biophys. Acta 151*, 526.
Diederich, D., Khan, A., Santos, I., and Grisolia, S. (1970), *Biochim. Biophys. Acta 212*, 341.
Edelhoch, H., Rodwell, V. W., and Grisolia, S. (1957), *J. Biol. Chem. 228*, 891.
Eggleston, L. V., and Hems, R. (1952), *Biochem. J. 52*, 156.
Fernandez, M., and Grisolia, S. (1960), *J. Biol. Chem. 235*, 2188.
Gomori, G. (1942), *J. Lab. Clin. Med. 27*, 955.
Grisolia, S. (1968), *in* Homologous Enzymes and Biochemical Evolution, van Thoai, N., and Roche, J., Ed., New York, N. Y., Gordon and Breach, p 167.
Grisolia, S., Bogart, D. B., and Torralba, A. (1968), *Biochim. Biophys. Acta 151*, 298.
Grisolia, S., and Cascales, M. (1966), *Biochem. Biophys. Res. Commun. 2*, 200.
Grisolia, S., and Joyce, B. K. (1959), *J. Biol. Chem. 234*, 1335.
Grisolia, S., Joyce, B. K., and Fernandez, M. (1961), *Biochim. Biophys. Acta 50*, 81.

Grisolia, S., Moore, K., Luque, J., and Grady, H. (1969), *Anal. Biochem. 31*, 235.
Grisolia, S., and Tecson, J. (1967), *Biochim. Biophys. Acta 132*, 56.
Harrison, W. H., Boyer, P. D., and Falcone, A. B. (1955), *J. Biol. Chem. 215*, 303.
Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63*, 530.
Ito, N., and Grisolia, S. (1959), *J. Biol. Chem. 234*, 242.
Jacobs, R. J., and Grisolia, S. (1966), *J. Biol. Chem. 241*, 5926.
Joyce, B. K., and Grisolia, S. (1960), *J. Biol. Chem. 235*, 2278.
Khorana, H. G., Tener, G. M., Wright, R. S., and Moffatt, J. G. (1957), *J. Amer. Chem. Soc. 79*, 430.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randell, R. J. (1951), *J. Biol. Chem. 193*, 265.
Markham, R., and Smith, J. D. (1952a), *Biochem. J. 52*, 552.
Markham, R., and Smith, J. D. (1952b), *Biochem. J. 52*, 558.
Mokrasch, L. C., Davidson, W. D., and McGilvery, R. W. (1956), *J. Biol. Chem. 222*, 179.
Mortimer, D. C. (1952), *Can. J. Chem. 30*, 653.
Najjar, V. A., and Pullman, M. E. (1956), *Science 119*, 631.
Nelson, N. (1944), *J. Biol. Chem. 153*, 375.
Pizer, L. I. (1962), *Enzymes 6*, 179.
Pizer, L. I., and Ballou, C. E. (1959), *J. Biol. Chem. 234*, 1138.
Rodwell, V. W., Towne, J. C., and Grisolia, S. (1956), *Biochim. Biophys. Acta 20*, 394.
Rodwell, V. W., Towne, J. C., and Grisolia, S. (1957), *J. Biol. Chem. 228*, 875.
Rutter, W. J. (1964), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 23*, 1249.
Sasaki, R., Sugimoto, E., and Chiba, H. (1966), *Arch. Biochem. Biophys. 115*, 53.
Smith, I. (1960), *in* Chromatographic and Electrophoretic Techniques, Smith, I., Ed., Vol. I, New York, N. Y., Interscience Publishers, p 279.
Somogyi, M. (1945), *J. Biol. Chem. 160*, 62.
Sugino, Y., and Miyoshi, Y. (1964), *J. Biol. Chem. 239*, 2360.
Sutherland, E. W., Posternak, T. Z., and Cori, C. T. (1949), *J. Biol. Chem. 179*, 501.
Torralba, A., and Grisolia, S. (1966), *J. Biol. Chem. 241*, 1713.
Towne, J. C., Rodwell, V. W., and Grisolia, S. (1956), *J. Biol. Chem. 226*, 777.
Wade, H. E., and Morgan, D. M. (1953), *Nature (London) 171*, 529.

Inactivation of Nicotinamide–Adenine Dinucleotide Glycohydrolase from Livers of Different Mammalian Species by Nicotinamide–Adenine Dinucleotide*

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ABSTRACT: Nicotinamide–adenine dinucleotide glycohydrolase (EC 3.2.2.5) from microsomes and nuclei of mouse Ehrlich ascites cells is rapidly inactivated by its substrate, NAD^+, at pH 8.0. The possibility that NADase from tissues of other mammalian species may be similarly inactivated by NAD^+ has been investigated. NADases from mouse, rat, and rabbit livers were all significantly inactivated by NAD^+ at pH 8.0, whereas the NADases from dog, bovine, and pig livers were insensitive to the inactivation. Mouse, rat, and rabbit liver NADase had pH optima of 6.3–6.8 and molecular weights of 69,000 or higher while dog, bovine, and pig liver NADases had pH optima of around 9.0 and had molecular weights of 40,000 or less. Again, the mouse, rat, and rabbit liver enzymes were reversibly inactivated by 6 M urea. In contrast, the dog, bovine, and pig liver enzymes were irreversibly inactivated by this reagent. Purified rat liver microsomal NADase could be dissociated into two subunits of about 38,000 each and reassociated into an active form of the enzyme with a molecular weight of 70,000 by decreasing the concentration of urea to 1.5 M urea or lower. The results of this present work indicate that mammalian liver microsomal NADases may be divided into two classes on the basis of the following criteria: (a) inactivation by NAD^+ at pH 8.0, but not at pH 6.0; (b) pH–activity relationship; (c) molecular weight; (d) effect of urea.

Tissue levels of cellular nicotinamide–adenine dinucleotide glycohydrolase (EC 3.2.2.5) (NADase) have been reported to change markedly in certain diseases (Goldman *et al.*, 1970) and after the use of certain agents (Green, 1966; Tsukagoshi *et al.*, 1968). Although Swislocki *et al.* (1967) reported variations in the properties of this enzyme from different tissues, no information is available concerning a property recently reported by us (Green and Dobrjansky, 1971) on the interaction of NAD glycohydrolase with the substrate, NAD^+. We found that the enzyme NADase from mouse Ehrlich ascites cell nuclei or microsomes was inactivated by its substrate, NAD^+, and that this inactivation was very rapid at pH 8.0. The present paper is concerned with determining the generality of this observation. The effect of NAD^+ at pH 8.0 on microsomal NADase from mouse liver as well as from the livers of other mammalian species has been investigated in order to examine the relationship between this NAD^+ effect and the properties of the individual NADases.

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