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A STUDY OF PHOSPHOGLYCERATE KINASE IN HUMAN ERYTHROCYTES

I. ENZYME ISOLATION, PURIFICATION AND ASSAY

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Summary

The enzyme ATP-3-phospho-D-glycerate-1-phosphotransferase (EC 2.7.2.3) (phosphoglycerate kinase) has been isolated from human red cells in crystalline form by a modification of the method of Yoshida and Watanabe ((1972) J. Biol. Chem. 247, 440—445).The crystalline enzyme was further purified by electrofocusing using carrier ampholytes (pH 7—9). The isoelectric point of phosphoglycerate kinase was estimated to be 8.75. The specific activity of purified phosphoglycerate kinase from electrofocusing was 2200 units per mg of protein at pH 8.3 (37°C).

Enzyme activity was assayed in the forward direction leading from 1,3-diphosphoglycerate to a 3-phosphoglycerate using a fluorimetric procedure for NAD-coupled enzymes for the measurement of the reaction rate at very low substrate concentrations.

The auxiliary indicator enzymes were added in excess to yield true initial velocity kinetics, i.e. with no time lag upon addition of substrate (1,3-diphosphoglycerate). This was established theoretically using a mathematical model and confirmed experimentally. Further phosphoglycerate kinase was shown to be the rate-limiting step when the assay conditions were varied.

Introduction

Phosphoglycerate kinase (EC 2.7.2.3) catalyzes the reversible transfer of the phosphoryl group from the carbonyl group of 1,3-diphosphoglycerate to ADP.

Phosphorylphosphate + ADP $\rightleftharpoons$ 3 phosphoglyceric acid + ATP

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Abbreviation: PGM, phosphoglyceromutase (EC 2.7.5.3).

Purified phosphoglycerate kinase has been crystallized from yeast, muscle and human red cells [1—5]. It has also been partially purified from pea seeds [6], *Escherichia coli* [7] and liver [8].

This enzyme has been assayed in the backward direction and by indirect means the forward direction [2,9]. This may have been due to difficulties in carrying out the assay in the forward direction i.e. utilizing an auxiliary and indicator chain of enzymes, low substrate concentration. The use of several enzymes in an assay system is not unique in the estimation of enzyme activity, i.e. the studies on phosphofructokinase [10—12].

In the present study the enzyme phosphoglycerate kinase was isolated from human red cells in a highly purified form. An assay procedure was developed to measure phosphoglycerate kinase activity in the forward direction leading from 1,3-diphosphoglycerate to 3-phosphoglycerate. The efficacy of the assay system was demonstrated both theoretically and experimentally.

Materials and Methods

Human blood (2—3-weeks-old preserved in acid/citrate dextrose) was obtained from the Blood Bank, Victoria Hospital, London, Canada. DL-glyceraldehyde 3-phosphoric acid (diethyl-acetal, monobarium salt); adenosine 5′-diphosphate (disodium salt from equine muscle' grade I); dithiothreitol (Cleland's reagent); alcohol dehydrogenase (from yeast; twice crystallized); phosphoglycerate mutase (from rabbit muscle); L-cysteine hydrochloride hydrate and NADH were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase (from muscle) and lactate dehydrogenase were purchased from Boehringer Mannheim Corp., New York, U.S.A.

Polyacrylamide gel electrophoresis was carried out according to the method of Davis [13].

Diethyl aminoethyl-Sephadex-A50 (anion exchanger) and carboxymethyl C-50 (cation exchanger) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ampholine (carrier ampholytes) pH 3—10 and 7—9 were obtained from LKB Produkter AB, Sweden.

Electrofocusing was carried out in a electrofocusing column (LKB Model 8101) using carrier ampholytes (pH ranges 3—10 and 7—9, respectively) at 3.5°C. The electrofocusing was run for 3 and 5 days when using carrier ampholytes pH 3—10 and 7—9, respectively.

Substrate 1,3-diphosphoglycerate

Substrate 1,3-diphosphoglycerate was synthesized according to the method of Negelein [14]. The procedure was slightly modified: a larger volume of the enzymes glyceraldehyde-3-phosphate dehydrogenase and alcohol dehydrogenase were used (1.0 ml each of 0.75 and 0.6%, respectively instead of 0.6 ml as reported). All pH measurements were done electrometrically at 0°C with a Radiometer 28, pH meter (Radiometer, Copenhagen). 1,3-Diphosphoglycerate was stored in liquid nitrogen in order to retard the hydrolysis of the substrate.

Phosphoglycerate kinase activity was measured fluorimetrically by the procedure of Maitra and Estabrook [15] in the forward reaction leading from 1,3-di-

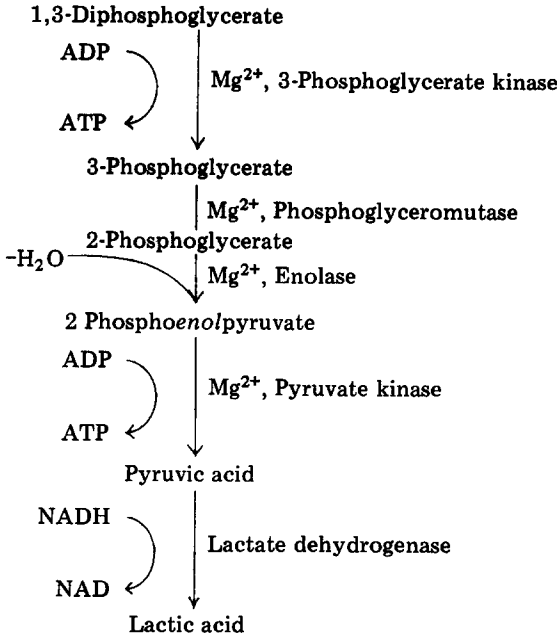

Fig. 1. The scheme for assaying the purified phosphoglycerate kinase by utilizing lactate dehydrogenase as the indicator reaction. Conditions as described in the text.

phosphoglycerate to 3-phosphoglycerate (Fig. 1). The reaction mixture consisted of $34 \cdot 10^{-3}$ M Tris buffer, pH 8.5; $5 \cdot 10^{-3}$ M phosphate buffer, pH 7.3; $6 \cdot 10^{-3}$ M cysteine · HCl, pH 7.0; $4 \cdot 10^{-4}$ M ADP; $14 \cdot 10^{-6}$ M NADH $1 \cdot 10^{-3}$ M $MgCl_2$; $10 \cdot 10^{-6}$ M 1,3 diphosphoglycerate; $87 \cdot 10^{-8}$ mg phosphoglycerate kinase ($2.5 \cdot 10^{-3}$ units/ml of assay mixture); $42 \cdot 10^{-4}$ mg PGM * (5.5 units/ml of assay mixture); $4 \cdot 10^{-2}$ mg enolase (7.15 units/ml of assay mixture) $17 \cdot 10^{-3}$ mg pyruvate kinase (7.26 units/ml of assay mixture) and $6 \cdot 10^{-3}$ mg lactate dehydrogenase (7.26 units/ml of assay mixture). The assay was carried out in fluorimeter, Eppendorf 1101M, equipped with a temperature-controlled cell housing and recorder, Brinkman Model 2543.

The above-listed ingredients were added to a pair of cuvettes of 1.0 cm light path to a final volume of 1.0 ml and at a final pH of 8.3. The reaction mixture was preincubated at 37° C for 15 min for temperature equilibration before the addition of 1,3-diphosphoglycerate. A series of blanks were run in which one of the following ingredients, respectively, was omitted from the assay mixture, ADP, Mg^{2+}, 1,3-diphosphoglycerate, and phosphoglycerate kinase. No appreciable change in fluorescence was observed when one of the above ingredients was omitted. A typical phosphoglycerate kinase assay is shown in Fig. 2. The change in fluorescence (ΔR) was standardized using NADH, whose concentration had been previously determined spectrophotometrically. One unit is defined as the quantity of enzyme that will convert 1μM of substrate per min (I.U.) at 37° C. Enzyme activity was expressed on the basis of change in fluorescence (ΔR/min).

In order to demonstrate that a lag period can be avoided by using the proper

* See footnote p. 74.

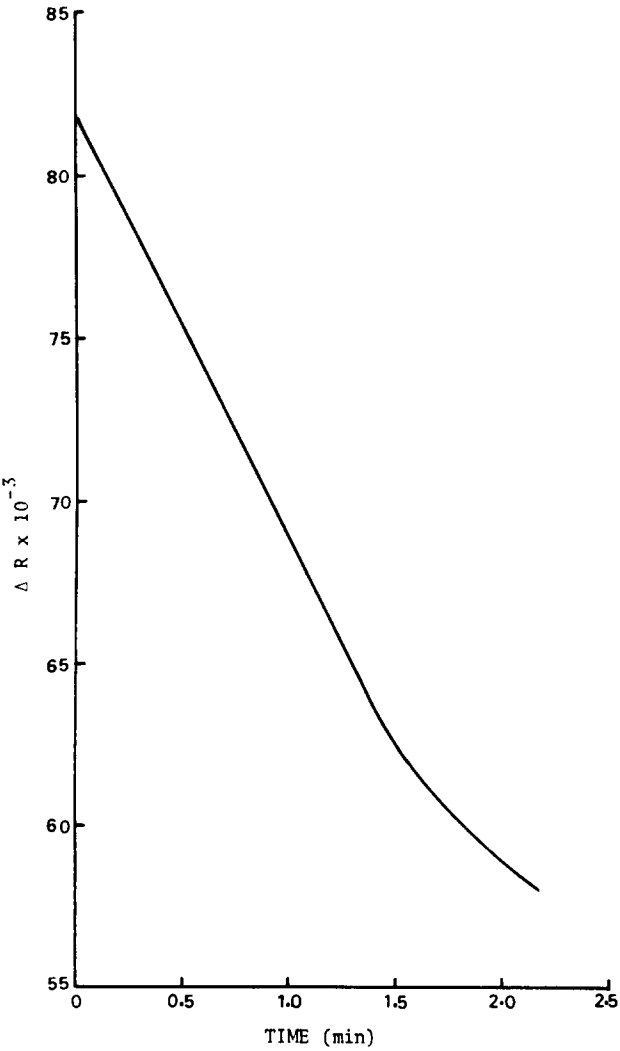

Fig. 2. A typical phosphoglycerate kinase assay. Conditions as described in the text.

ratio of auxiliary-indicator enzymes with respect to the test enzyme (phosphoglycerate kinase) a theoretical treatment was undertaken. A Fortran IV computer programme was written and employed to determine the concentration of substrate, product and the reaction velocity at each step in a sequence of five steps of uncatalyzed irreversible first-order reactions. The calculations were done using a PDP-10 computer of the Department of Computer Science, University of Western Ontario, London, Ontario, Canada.

Isolation of erythrocytes enzyme

Some of the purification steps were the same as those reported by Yoshida and Watanabe [1]. Two steps (Sephadex G-75 gel filtration and carboxymethyl-Sephadex column chromatography) were eliminated and replaced by $(NH_4)_2SO_4$ precipitation and electrofocusing. Electrophoresis of the crystalline

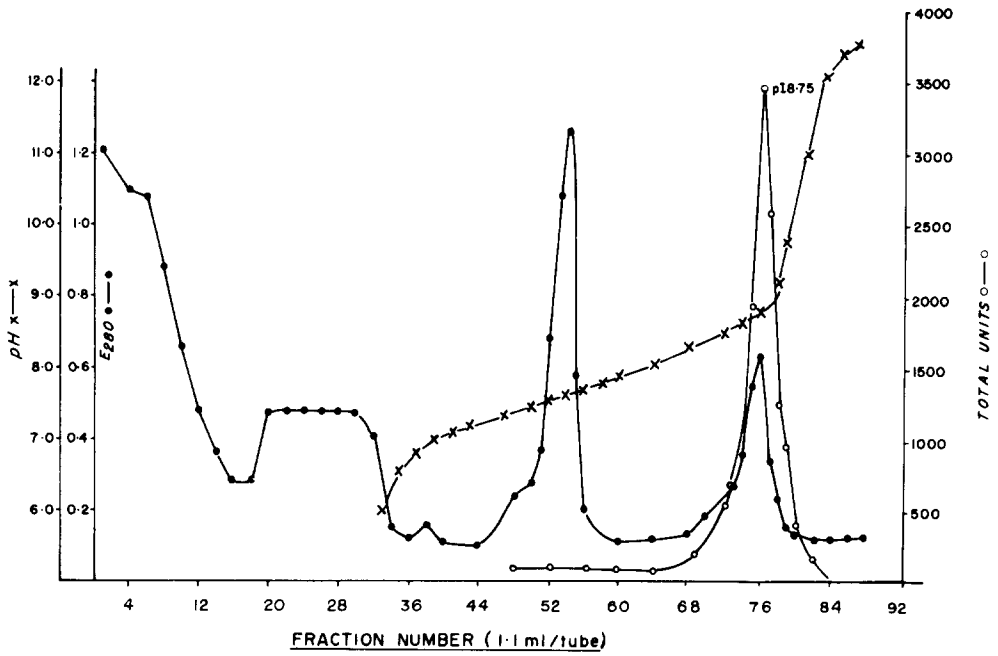

Fig. 3. Separation of phosphoglycerate kinase by electrofocusing pH ranges between 7 and 9. Conditions as described in the text.

enzyme on polyacrylamide gel demonstrated two major and two minor protein components. The slower migrating protein band appeared to contain the enzyme activity. The preliminary experiments demonstrated the crystalline preparation contained other proteins in addition to the active enzyme (phosphoglycerate kinase).

Further purification of the crystalline protein by electrofocusing procedures using carrier ampholytes (pH 3—10) demonstrated a similar pattern that obtained in the analytical gel electrophoresis. Further purification over a narrower pH range (pH 7—9) effected a higher resolution of phosphoglycerate kinase activity since it appeared to separate more discretely from other protein components (Fig. 3). An isoelectric point of 8.75 was estimated in both ranges. The aliquots which contained the highest phosphoglycerate kinase activity were diluted several times with 0.01 M phosphate buffer, pH 7.0, and concentrated by ultrafiltration in order to remove the ampholine and sucrose. The concentrated purified enzyme was stored with added cysteine (12.0 mM) at −20° C.

Further crystallization of the purified phosphoglycerate kinase obtained from electrofocusing was carried out by a procedure described earlier [1] except that dialysis was carried out for 48 h rather than 24 h against increasing concentration of $(NH_4)_2SO_4$. The final concentration was 85% (instead of 65% as reported by Yoshida and Watanabe [1]).

Results and Discussion

Phosphoglycerate kinase activity was sharply defined in a small volume when the enzyme was subjected to electrofocusing (Fig. 3). Table I lists in sequence

TABLE I

THE PURIFICATION OF PHOSPHOGLYCERATE KINASE FROM HUMAN BLOOD

A unit of phosphoglycerate kinase is defined as the amount of enzyme which catalyzes the formation of 1 μmol of 3-phosphoglycerate per min. Specific activity is expressed as units per mg of protein. Conditions as described in the text.

Fraction	Specific activity	Yield (%)
Hemolysate	1.07	100
Supernatant after elimination of hemoglobin with ethanol/ chloroform	45.5	71
Precipitate with ethanol dissolved in phosphate buffer	52.00	62
Eluate from CM-Sephadex concentrated by ultrafiltration	132.00	38
Eluate from DEAE-Sephadex, concentrated by ultrafiltration	219.00	33
Crystallization by $(NH_4)_2SO_4$	475.00	28
Purification by isoelectrofocusing	2260.00	27

the purification of phosphoglycerate kinase from human erythrocytes.

Recently it has been reported by Jacobs [16] that the electrofocusing procedure affects the amino acid composition of proteins. However, there was no significant loss in phosphoglycerate kinase activity (Table I) as a result of the electrofocusing procedures followed in this investigation (with added dithiothreitol 10 mg/100 ml).

Purified phosphoglycerate kinase from human erythrocytes sedimented as a single peak in the analytical ultracentrifuge. On polyacrylamide gel electrophoresis a single sharp protein band was observed. A similar estimation of molecular weight was obtained by ultracentrifugation (mol. wt. 48 000) as reported before [1].

To test the efficacy of the assay system the level at which each enzyme in the reaction sequence would be rate limiting was investigated. The concentration of one enzyme was varied in the assay mixture while the concentrations of the other enzymes were maintained at a constant level. The concentration of phosphoglycerate kinase throughout the experiment was kept constant ($2.53 \cdot 10^{-3}$ units per ml of assay mixture). The first auxiliary enzyme in the series, i.e. PGM, responsible for the conversion of 3-phosphoglycerate to 2-phosphoglycerate was assayed at varying concentrations. PGM in excess of one unit per ml of assay mixture ceased to be rate limiting (Fig. 4a). Increasing this enzyme up to 22 units per ml of assay mixture did not effect a further increased rate in the assay of phosphoglycerate kinase. The arrow, in Fig. 4a, indicates the concentration of PGM (5.5 units per ml of assay mixture) utilized throughout in the assay procedure; this represents a minimum of a 5-fold excess of PGM over its rate-limiting concentrations (the ratio of PGM activity with respect to the phosphoglycerate kinase assayed was greater than 2000 times).

Similarly enolase (Fig. 4b), pyruvate kinase (Fig. 5a) and lactate dehydrogenase (Fig. 5b) were rate limiting at concentrations less than 1.5 units per ml of assay mixture. Excess concentrations of each of these enzymes, respectively (up to 29 units per ml of assay mixture were tested) did not effect a further increase in the rate in the assay. Enolase, pyruvate kinase and lactate dehydrogen-

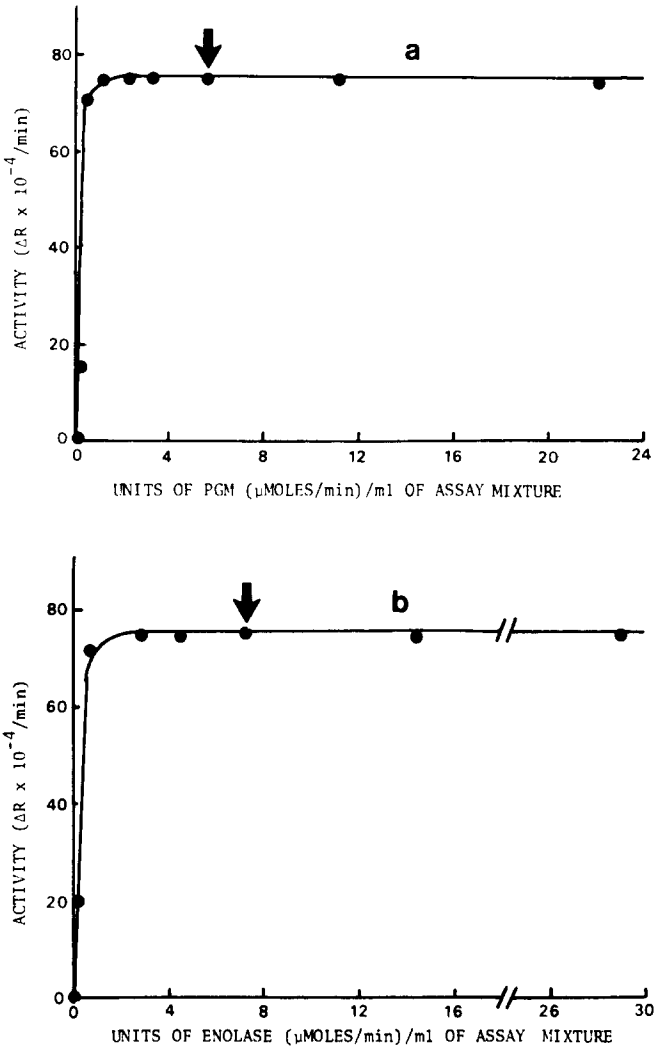

Fig. 4. Assay of phosphoglycerate kinase activity at varying concentrations of a, phosphoglyceromutase (PGM); b, enolase. Conditions as described in the text.

ase were maintained throughout at a concentration of 7.15, 7.26 and 7.26 units per ml of assay mixture, respectively. These enzymes were used in excess over their rate-limiting concentrations (the ratios of enolase, pyruvate kinase and lactate dehydrogenase activities with respect to the phosphoglycerate kinase assayed were greater than 2800 times).

In addition to ascertaining that the auxiliary-indicator enzymes were added in excess to the assay mixture, tests were carried out to determine the effect of varying substrates (1,3-diphosphoglycerate and ADP, Figs. 6 and 7, respectively) and cofactor (Mg^{2+}, Fig. 8) concentrations on these enzymes in order to establish whether phosphoglycerate kinase was rate limiting under these conditions. The auxiliary-indicator chain activity far exceeded the phosphoglycerate kinase activity (Figs. 6—8). This is further proof that the auxiliary-indicator en-

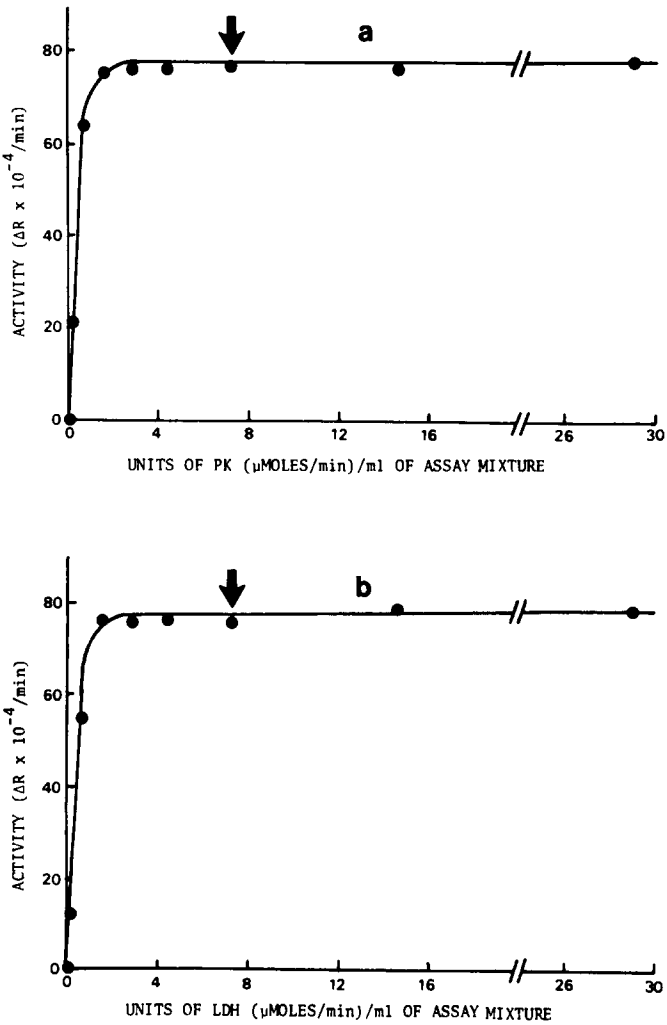

Fig. 5. Assay of phosphoglycerate kinase activity at varying concentrations of a, pyruvate kinase (PK); b, lactate dehydrogenase (LDH). Conditions as described in the text.

zymes were not rate limiting in these systems. The allosteric effects of ADP and 1,3-diphosphateglycerate on phosphoglycerate kinase activity [19] were not observed with the auxiliary-indicator enzymes when 3-phosphoglycerate was the added substrate. In summary it may be stated that the phosphoglycerate kinase assay described and utilized herein has been shown to be reliable since: (a) the auxiliary-indicator enzymes have been shown to be added in excess, (b) the observed effects of variation in concentrations of substrates and cofactor are an expression of phosphoglycerate kinase activity.

A theoretical treatment of the requirement of a five-enzyme chain of test, auxiliary and indicator enzymes was undertaken to further establish the efficacy of the assay system. Bergmeyer [17] has previously dealt with a two-step system.

If in a chain of continuous reactions, A is the starting material and F is the

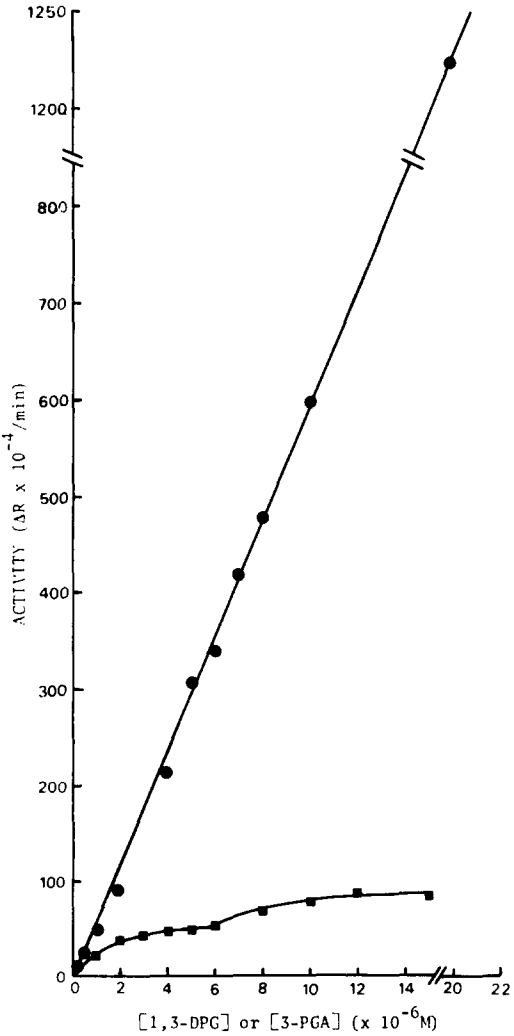

Fig. 6. Parallel assays using 1,3-diphosphoglycerate (DPG) and 3-phosphoglycerate (PGA) as substrate. Substrate: ■———■, 1.3-diphosphoglycerate (DPG): ●———●, 3-phosphoglycerate (PGA). Conditions as described in the text.

end product with B, C, D and E as the intermediate stages, then this can be represented symbolically as:

$$A \overset{k_1}{\rightarrow} B \overset{k_2}{\rightarrow} C \overset{k_3}{\rightarrow} D \overset{k_4}{\rightarrow} E \overset{k_5}{\rightarrow} F \qquad (1)$$

where k_1 is the rate of test enzyme, k_2, k_3 and k_4 are the rates of auxiliary enzymes and k_5 is the rate of indicator enzyme. It is assumed that when time $t = 0$

$$C_B = C_C = C_D = C_E = C_F = 0 \qquad (2)$$

If a is the initial concentration of A, then by definition, after time t when all

species are present:

$$a = C_A + C_B + C_C + C_D + C_E + C_F \tag{3}$$

and hence

$$C_F = a - C_A - C_B - C_C - C_D - C_E \tag{4}$$

where C_A, C_B, C_C, C_D, C_E and C_F are the concentrations of the respective reactants in Expression 1.

Assuming that the reaction is irreversible and of first order in nature, the differential equations expressing the reaction velocities at any instant t read as:

$$\frac{dC_A}{dt} = -k_1 C_A \tag{5}$$

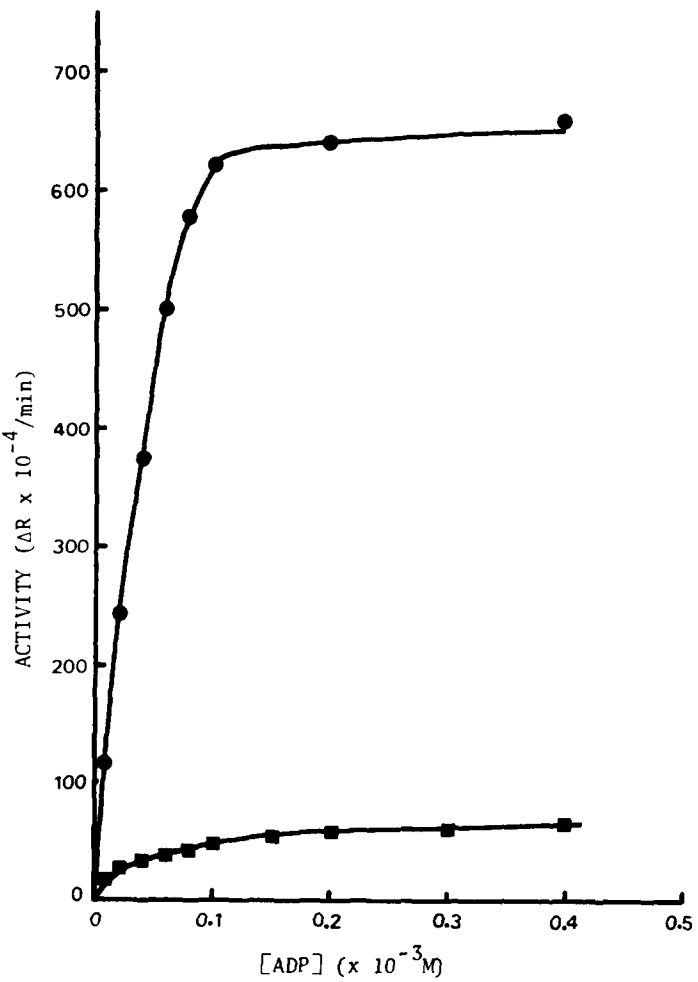

Fig. 7. Parallel assays. The effect of varying ADP concentrations. Substrate: ■———■, $10 \cdot 10^{-6}$ M 1,3-diphosphoglycerate; ●———●, $10 \cdot 10^{-6}$ M 3-phosphoglycerate. Conditions as described in the text.

$$\frac{dC_B}{dt} = k_1 C_A - k_2 C_B \tag{6}$$

$$\frac{dC_c}{dt} = k_2 C_B - k_3 C_C \tag{7}$$

$$\frac{dC_D}{dt} = k_3 C_C - k_4 C_D \tag{8}$$

$$\frac{dC_E}{dt} = k_4 C_D - k_5 C_E \tag{9}$$

and

$$\frac{dC_F}{dt} = k_5 C_E \tag{10}$$

Solving this system of linear first-order differential equations with constant coefficients, see for example ref. 18, yields:

$$C_F(t) = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + A_3 e^{-k_3 t} + A_4 e^{-k_4 t} + A_5 e^{-k_5 t} + a \tag{11}$$

where

a = initial concentration of A (12)

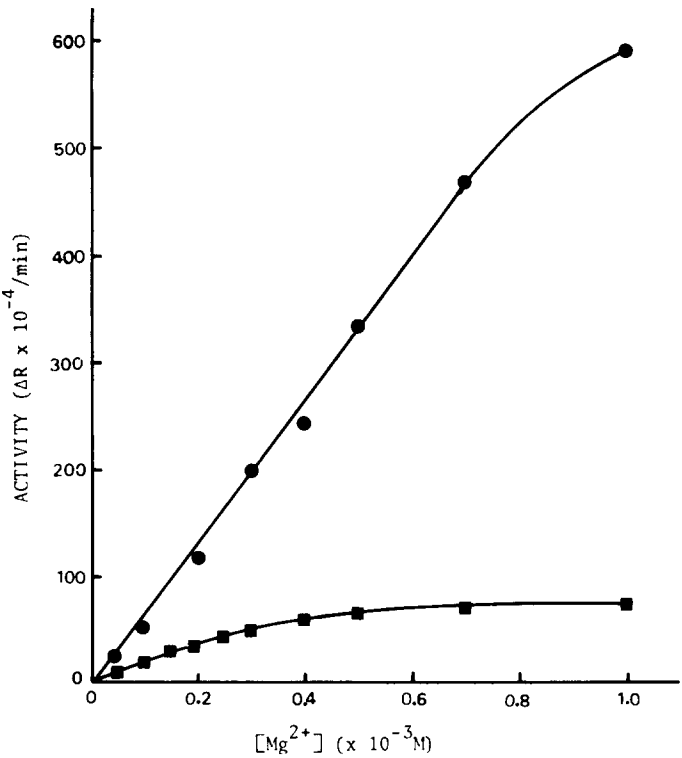

Fig. 8. Parallel assays. The effect of varying Mg^{2+}concentrations. Substrate: ■———■, $10 \cdot 10^{-6}$ M 1,3-diphosphoglycerate; ●———●, $10 \cdot 10^{-6}$ M 3-phosphoglycerate. Conditions as described in the text.

$$\beta = ak_1k_2k_3k_4k_5 \tag{13}$$

$$A_1 = \frac{-\beta}{(k_2-k_1)(k_3-k_1)(k_4-k_1)(k_5-k_1)k_1} \tag{14}$$

$$A_2 = \frac{-\beta}{(k_1-k_2)(k_3-k_2)(k_4-k_2)(k_5-k_2)k_2} \tag{15}$$

$$A_3 = \frac{-\beta}{(k_1-k_3)(k_2-k_3)(k_4-k_3)(k_5-k_3)k_3} \tag{16}$$

$$A_4 = \frac{-\beta}{(k_1-k_4)(k_2-k_4)(k_3-k_4)(k_5-k_4)k_4} \tag{17}$$

and

$$A_5 = \frac{-\beta}{(k_1-k_5)(k_2-k_5)(k_3-k_5)(k_4-k_5)k_5} \tag{18}$$

Defining velocity of the reaction as:

$$V = \frac{dC_F}{dt} \tag{19}$$

Eqn 11 yields on differentiation with respect to time t

$$v = [A_1k_1e^{-k_1t} + A_2k_2e^{-k_2t} + A_3k_3e^{-k_3t} + A_4k_4e^{-k_4t} + A_5k_5e^{-k_5t}] \tag{20}$$

Hence if A = starting material (1,3-diphosphoglycerate), B = product of A (3-phosphoglycerate), C = product of B (2-phosphoglycerate), D = product of C (2-phosphoenolpyruvate), E = product of D (pyruvic acid), and F = product of E, i.e. final product to be measured (lactic acid), then k_1 = rate of test enzyme (phosphoglycerate kinase), k_2 = rate of auxiliary enzyme (PGM), k_3 = rate of auxiliary enzyme (enolase), k_4 = rate of auxiliary enzyme (pyruvate kinase), and k_5 = rate of indicator enzyme (lactate dehydrogenase).

Where k is the corresponding rate of enzyme activity (specific activity × protein concentration) used experimentally expressed as μmol per min (37°C): $k_1 = 2.53 \cdot 10^{-3}$, $k_2 = 5.5$, $k_3 = 7.15$, $k_4 = 7.26$, $k_5 = 7.26$.

A Fortran IV computer programme was written and employed to determine the concentration of product and the reaction velocity using Eqns. 11 and 20, respectively, in a sequence of five steps of uncatalyzed irreversible first-order reactions.

In a sequence of five uncatalyzed first-order reactions it was possible to demonstrate the ratio of auxiliary-indicator enzymes compared to test enzyme required to give true initial velocity kinetics of the test enzyme. The ratio between the test and auxiliary-indicator enzyme activities were also used to determine a theoretical family of curves describing the reaction rates at different levels of intermediate enzyme activities (i.e k_2, k_3, k_4, and k_5). The following ratios were calculated from the experimental values used with respect to the test enzyme from the activities (μmol/min) of the individual enzymes in the sequence. The ratios of the individual rates are: $k_1 : k_2 : k_3 : k_4 : k_5 = 1 : 2178 : 2838 : 2882 : 2882$. These are the minimum ratios of the individual rates calculated with respect to k_1. The activity of k_1 was calculated from the highest

TABLE II

THE EFFECT OF VARYING VALUES OF k_2 ON THE PHOSPHOGLYCERATE KINASE ASSAY

When k_1 = 1, k_2 = variable, k_3 = 2838, k_4 = 2882, and k_5 = 2882. Conditions as described in the text.

Varying values of k_2	Formation of product at time 1	Decrease in concentration of '*A*' at time 1
8712	0.6316	0.3683
4356	0.6316	0.3683
2178	0.6315	0.3684
217.8	0.6300	0.3699
21.78	0.6140	0.3859
2.178	0.4154	0.5845
0.2178	0.0740	0.9259

phosphoglycerate kinase activity fraction obtained from electrofocusing. The ratios of the rates may be higher depending on the activity of the test enzyme (k_1).

The rate of activity of each k other than test enzyme (i.e. k_2, k_3, k_4 and k_5)

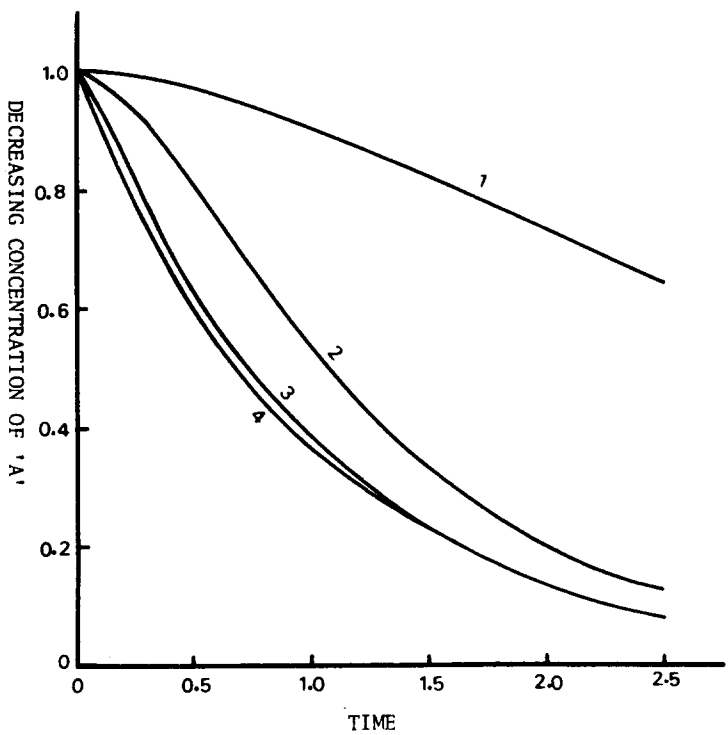

Fig. 9. A theoretical family of curves at varying values of k_5 when, k_1 = 1, k_2 = 2178, k_3 = 2838, k_4 = 2882, and k_5 = variable. Conditions as described in the text.

Curve No.	Values of k_5
1	0.2882
2	2.882
3	28.82
4	288.2, 2882, 5764 and 11528

was varied individually while the remainder were constant (e.g. $k_1 = 1$, $k_2 = 2178$, $k_3 = 2838$, $k_4 = 2882$ and $k_5 = 0.2882$, 2.882, 28.82, 288.2, 2882 and 11528. Similarly k_4, k_3 and k_2 was varied, respectively, and the other were constant). In this way it was possible to determine the ratio of the rates of auxiliary-indicator enzyme to the test enzyme (k_1) required to give the true initial velocity of k_1.

The formation of product (F) at varying values of k_2 at time 1 are given in Table II (this table is an abbreviated version of a computer printout dealing with seven different rates of k_2 and the rate of conversion of substrate to the indicator product). It may be seen from Table I that the formation of product at time 1 for different values of k_2 (Table II) reached a maximum at a rate of k_2 between 21.78 and 217.8. Rates of k_2 below this level resulted in the decreased formation of product at time 1 (see Table II). The formation of product at time 1 was not affected by a further increase in the rate of k_2 (i.e. four times rate used experimentally). Similar results were obtained when k_3, k_4 and k_5, respectively, were varied while maintaining the others constant.

A theoretical family of curves at different rates of k_5 (i.e. 0.01, 0.1, 1.0 .... 400% of 2882) were produced with the aid of the computer (Fig. 9). With rates $< 1\%$ (i.e. 0.2882 and 2.882) of the amount employed experimentally was used a prolonged lag period was observed. A very short lag period was observed at 1.0% of the k_5 rate. No lag period was observed at 10% or greater (10% of 2882) of k_5. Further increases in the rate of k_5 (several times over the experimental values) did not alter the nature of the curve. A similar family of curves was obtained when the rate of each enzyme (k) was varied, respectively, i.e. k_2, k_3, and k_4. The curves are similar to those obtained by Bergmeyer [17] for a sequence of two uncatalyzed first order reactions.

These theoretical results demonstrate that a lag phase in a sequence of five first-order reactions can be avoided by using appropriate ratios of enzyme activities, i.e. k_2, k_3, k_4 and k_5 and this was demonstrated experimentally with phosphoglycerate kinase. Using a mathematical model for a sequence of five uncatalyzed first-order reactions it is possible to predict the occurrence of a lag phase based on the ratio of activities of this test and auxiliary enzymes in an assay system.

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