Comparative Kinetic Study of Human Pyruvate Kinases Isolated from Adult and Fetal Livers and from Hepatoma†

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ABSTRACT: Adult human liver type L, human hepatoma type M, and human fetal liver type M pyruvate kinases were partially purified. Kinetic studies carried out using these three preparations revealed differences in the kinetic constants with respect to ADP and phosphoenolpyruvate. Moreover, type L pyruvate kinase was found to show allosteric kinetics with respect to P-enolpyruvate, and substrate inhibition by ADP. Hepatoma type M pyruvate kinase showed complex kinetics with respect to P-enolpyruvate, whereas the fetal type M isoenzyme showed simple Michaelis–Menten kinetics. Results of product inhibition and isotope exchange studies showed the mechanism of reaction to be similar for the liver type L and hepatoma type M isoenzymes. A sequential ordered mechanism with P-enolpyruvate adding first and MgATP being released last is consistent with the results.

Two major forms of pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) which display different kinetic properties with respect to P-enolpyruvate have been found in rat liver (Tanaka *et al.*, 1967a,b; Susor and Rutter, 1968; Carminatti *et al.*, 1968). The major or L-type pyruvate kinase displays sigmoidal kinetics, as does the enzyme from yeast (Haeckel *et al.*, 1968; Hess *et al.*, 1966; Hunsley and Suelter, 1969), pyruvate kinase A from adipose tissue (Pogson, 1968), pyruvate kinase I from *Escherichia coli* K_{12} (Malcovati and Kornberg, 1969), *E. coli* B (Maeba and Sanval, 1968), and *Thiobacillus neopolitanus* (Cornish and Johnson, 1971). On the other hand, type M liver pyruvate kinase displays Michaelis–Menten kinetics, as does the enzyme from rat muscle (Tanaka *et al.*, 1967a,b; Taylor *et al.*, 1969), rabbit muscle (McQuate and Utter, 1959; Reynard *et al.*, 1961; Holmsen and Storm, 1968; Mildvan and Cohn, 1966), leucocytes (Koler *et al.*, 1968), rat hepatoma 3924 A (Taylor *et al.*, 1969) and pyruvate kinase B from adipose tissue (Pogson, 1968). The L-type pyruvate kinase is inhibited by high levels of ADP, whereas the M type is not (Tanaka *et al.*, 1967a).

We have shown (Balinsky *et al.*, 1973a) that the type L enzyme from human liver also displays allosteric kinetics, and is subject to activation by fructose 1,6-diphosphate and to allosteric inhibition by ATP, AMP, citrate, and alanine, which is relieved by fructose diphosphate. Human hepatoma has a raised level of pyruvate kinase and an isoenzyme pattern resembling that of muscle or liver type M (Balinsky *et al.*, 1973b). The hepatoma isoenzyme shows altered, less marked allosteric properties, is not activated by fructose diphosphate, and is less susceptible to inhibition by ATP and citrate. The M isoenzyme from fetal liver shows no allosteric properties except for relief of alanine inhibition by fructose 1,6-diphosphate (Balinsky *et al.*, 1973a).

The mechanism of reaction of pyruvate kinase has mainly been studied using rabbit muscle pyruvate kinase. Kinetic, equilibrium binding, and nuclear magnetic resonance studies with this enzyme point to a rapid equilibrium random mechanism in which either ADP or P-enolpyruvate are able to bind to the enzyme (Reynard *et al.*, 1961; Mildvan and Cohn, 1965, 1966; Holmsen and Storm, 1969). However, ATP and pyruvate are thought to bind in a preferred order (Mildvan and Cohn, 1966). In addition, these latter authors showed that Mn^{2+}, MnADP, and free ADP can all bind to the enzyme.

In the present study an attempt was made to elucidate the mechanisms of reaction of human liver type L, and hepatoma and fetal types M pyruvate kinase. The kinetic properties of all three types were found to differ. However, overall studies indicated that the catalytic mechanism of the hepatoma type M and liver type L isoenzymes appears to be similar. A preliminary report of some of these results has been presented (Balinsky *et al.*, 1972).

Methods and Materials

Reagents. Trisodium adenosine 5′-diphosphate, disodium adenosine 5′-triphosphate, monosodium phosphoenolpyruvate, and pig heart lactate dehydrogenase were obtained from Biochemica Boehringer, Mannheim, Germany. The reduced form of nicotinamide adenine dinucleotide was obtained from Sigma and dithiothreitol from Calbiochem. Sephadex G-200 was obtained from Pharmacia, Uppsala. [^{14}C]Pyruvate (12.4 Ci/mol) and [γ-^{32}P]ATP (17 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England. DEAE-cellulose paper (DE-81) was purchased from Whatman. This was dipped into 5 mM Na_2EDTA just before use and dried. Scintillation grade 2,5-diphenyloxazole and 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene were obtained from the Packard Instrument Co.

Tissues. Autopsy material was obtained within 6 hr of death. Normal liver was obtained from a male aged 57 who died of pneumonia and showed no liver pathology. The hepatoma tissue was obtained from a male aged 20, who showed, in addition to primary hepatocellular malignant carcinoma, secondary metastases in the lungs, portal glands, and peritoneum. The precipitating cause of death was intraperitoneal hemorrhage; the cancerous liver at postmortem weighed 5 kg. The cancerous sections were dissected out and carefully separated from morphologically normal tissue. The fetal liver was obtained from an aborted fetus of approximately 20-weeks gestation period. All tissues were stored at −20° until required.

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Assay Procedure. Throughout the purification procedure and in all the kinetic studies pyruvate kinase was assayed spectrophotometrically using a Unicam SP-800 recording spectrophotometer with water at 30° circulating through the cell housing. The pyruvate kinase activity was estimated by coupling the pyruvate formed to lactate dehydrogenase and following the oxidation of NADH at 340 nm.

The standard reaction mixture was similar to that described by Shonk and Boxer (1964). Quartz cuvets of 1-cm light path contained in a final volume of 1 ml, 50 mM triethanolamine-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 5 mM Na_2EDTA (adjusted to pH 7.0), 0.1 M KCl, 3 mM P-enolpyruvate, 3 mM ADP, 4 U of lactate dehydrogenase, 0.15 mM NADH, and a suitable amount of the pyruvate kinase preparation to be assayed.

A unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADH/min assuming a molar absorptivity of NADH at 340 nm of 6.22×10^3 (Horecker and Kornberg, 1948). Specific activity is the activity per milligram of protein. The protein content was determined by the method of Warburg and Christian (1942).

Determination of Isotope Exchange Rates at Equilibrium. The technique of using isotope exchange studies at equilibrium as a means of obtaining further information on enzyme mechanisms was first proposed by Boyer (1959) and has been described in detail by Cleland (1967). All reactants are present at concentrations such that there is no net chemical reaction, but there will be constant turnover of substrates and products. Addition of a labeled reactant to such a system will result in exchange of label with the corresponding product. The concentration of any reactant pair can be altered in a constant ratio without affecting the equilibrium; hence the effect of increasing the concentration of individual reactant pairs on the exchange rate can be measured.

The equilibrium constant for the pyruvate kinase reaction

$$K_{eq} = \frac{[\text{pyruvate}][\text{ATP}]}{[\text{PEP}][\text{ADP}]} \text{ at pH } 9.0$$

was taken to be 370 (McQuate and Utter, 1959). The reaction mixture contained in a total volume of 100 μl, 50 mM glycine–NaOH buffer (pH 9.0), 12.5 mM $MgCl_2$, 0.25 mM EDTA, a suitable amount of enzyme, and pyruvate, MgATP, ADP, and P-enolpyruvate (PEP) in various proportions but always maintaining equilibrium conditions. K^+ was not added as it caused streaking on subsequent chromatography, but sufficient NH_4^+ was present in the enzyme preparation to permit full enzyme activity. For the measurement of exchange between[^{14}C]pyruvate and P-enolpyruvate, 7.4 mM pyruvate, 2 mM P-enolpyruvate, and MgATP and ADP in a 100:1 ratio were used. Exchange between [γ-^{32}P]ATP and P-enolpyruvate was measured in the presence of 2 mM ATP, 2 mM P-enolpyruvate, and pyruvate and ADP in a 370:1 ratio; or alternatively, with 74 mM pyruvate, 0.4 mM ADP, and MgATP and P-enolpyruvate in a 2:1 ratio. After preincubating the reaction mixture at 30°, the exchange reaction was started by adding [^{14}C]pyruvate or [γ-^{32}P]ATP. After quickly mixing, 15-μl aliquots were removed with a Hamilton syringe at suitable time intervals and spotted onto the EDTA-treated DEAE-cellulose chromatography paper (7.5 × 0.5 in.) to the top of which three folds of Whatman's No. 1 chromatography paper had been pinned. Since the enzyme is Mg^{2+} dependent, it was assumed that the EDTA would stop the reaction by chelating the Mg^{2+}. This was confirmed by spectrophotometric study.

The [^{14}C]pyruvate and labeled P-enolpyruvate were separated by ascending chromatography in 0.1 M Tris-HCl buffer (pH 7.4) for 30 min at room temperature. [γ-^{32}P]ATP and labeled P-enolpyruvate were separated by developing the chromatograms for 3 hr at room temperature in 0.1 M ammonium formate–formic acid buffer (pH 3.1) containing 5 mM EDTA, by ascending chromatography.

After drying the chromatograms in a 40° oven, the positions of the phosphorus-containing compounds were located by dipping marker strips into the ammonium molybdate stain described by Thomson (1969). These strips were then exposed to ultraviolet light and the phosphate-containing compounds appeared as blue spots. The nucleotide ATP could be detected by placing the marker strip directly under uv light prior to staining. Pyruvate was detected with the dinitrophenylhydrazine reagent described by Smith and Smith (1969).

After marking, the test strips containing the labeled P-enolpyruvate were cut out, fluted lengthwise, immersed in 15 ml of scintillator (7.5 g of 2,5-diphenyloxazole and 250 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 2.5 l. of toluene) and counted in a Packard Tri-Carb liquid scintillation counter.

Counts per minute were plotted against incubation time to determine the initial velocity of each exchange rate.

Purification Procedure. All operations were performed at 4°C unless otherwise stated.

Partial Purification of Liver Type L Pyruvate Kinase from Normal Human Adult Liver. EXTRACTION. Approximately 5–10 g of liver was removed from the frozen tissue, weighed, and homogenized with an Ultra-Turrax homogenizer in five volumes of a solution containing 0.15 M KCl–0.05 M $KHCO_3$–0.006 M Na_2EDTA–0.001 M dithiothreitol (*cf.* Shonk and Boxer, 1964). The homogenate was then centrifuged at 36,000*g* for 30 min at 4°. This was designated the crude fraction.

FIRST AMMONIUM SULFATE FRACTIONATION. Solid ammonium sulfate was added to the crude fraction to 25% saturation (70 g/100 ml was taken to be equivalent to 100% saturation at 0°; Dawson *et al.*, 1969). After 10 min the precipitated protein was removed by centrifugation at 36,000*g* for 10 min in an MSE high-speed 18 refrigerated centrifuge. The precipitate, which generally contained very little pyruvate kinase, was discarded. The ammonium sulfate concentration in the clear supernatant was then raised to 50% saturation and the precipitate was collected as above, redissolved in 0.025 M cacodylate–NaOH buffer (pH 6.2) containing 0.5 M sucrose, 2.5 mM Na_2EDTA, and 0.1 mM dithiothreitol, and dialyzed for 1 hr against the same buffer.

ACETONE FRACTIONATION. The method used was similar to that described by Tanaka *et al.* (1967a), the only difference being that the enzyme was precipitated at 23–45% v/v acetone instead of 20–45% as described by these authors. The acetone was removed from the precipitate by passing a stream of nitrogen over its surface and the precipitate was redissolved in a minimal volume of 0.25 M Tris-HCl buffer (pH 7.4) containing 0.5 M sucrose, 2.5 mM EDTA, and 0.1 mM dithiothreitol. As this solution was generally turbid it was centrifuged at 36,000*g* for 10 min and the precipitate was discarded. The clear supernatant was found to contain all the pyruvate kinase activity.

SECOND AMMONIUM SULFATE FRACTIONATION. Type L pyruvate kinase was separated from type M by refractionating the enzyme with solid ammonium sulfate as described by Passeron *et al.* (1967). The L type was precipitated between 25 and 37% saturation and the precipitate was redissolved in a minimal

TABLE I: Partial Purification of Normal Adult Human Liver Type L Pyruvate Kinase.

Fraction	Total Act. (Units)	Total Protein (mg)	Sp Act. (Units/ mg of Protein)	Purifcn Factor	Yield (%)
Crude homogenate	660	10,650	0.062	1.0	100
First ammonium sulfate fractionation (25–50% saturation) and dialysis	502	4,180	0.12	1.9	76
Acetone fractionation (25–40%)	354	840	0.42	6.8	54
Second ammonium sulfate fractionation (25–37% saturation)	183	120	1.52	24.5	27
Sephadex G-200	128	60	2.13	34.3	19

TABLE II: Partial Purification of Type M Pyruvate Kinase from Human Hepatoma.

Fraction	Total Act. (Units)	Total Protein (mg)	Sp Act. (Units/ mg of Protein)	Purifcn Factor	Yield (%)
Crude homogenate	810	915	0.88	1.0	100
First ammonium sulfate fractionation (50–70% saturation) and dialysis	576	235	2.45	2.8	71
Acetone fractionation (0–49%)	550	155	3.67	4.2	68
Second ammonium sulfate fractionation (50–60% saturation)	305	49.4	6.18	7.0	38
Sephadex G-200	280	5.1	54.9	62.5	35

volume of 0.025 M Tris-HCl buffer (pH 7.4) containing 0.5 M sucrose, 2.5 mM EDTA, and 0.1 mM dithiothreitol. Starch gel electrophoresis, carried out as described by Balinsky *et al.* (1973b), confirmed the complete separation of the two isoenzymes.

SEPHADEX G-200. The concentrated enzyme was placed onto a Sephadex G-200 column of dimensions 95 × 1 cm^2 and eluted with 0.025 M Tris-HCl buffer (pH 7.4) containing 0.5 M sucrose, 2.5 mM EDTA, 1 mM dithiothreitol, and 0.5 M ammonium sulfate. The ammonium sulfate was added to stabilize the enzyme (Taylor *et al.*, 1969). The fractions of highest specific activity were pooled and the enzyme was stored at 4° until required for kinetic studies. The enzyme was found to be stable for several months in this form.

Partial Purification of Hepatoma Type M Pyruvate Kinase. The cancerous tissue contained no type L pyruvate kinase, as tested by starch gel electrophoresis (Balinsky *et al.*, 1973b). The procedure used to purify type M pyruvate kinase from cancerous tissue of hepatomas was similar to that used to partially purify normal liver type L pyruvate kinase except that most of the pyruvate kinase activity in the first ammonium sulfate fractionation step was found in the 50–70% fraction, and in the 50–60% fraction of the second ammonium sulfate fractionation step. Also, with the acetone fractionation step, the enzyme could only be precipitated at a concentration of 0–49%.

Partial Purification of Fetal Type M Pyruvate Kinase. Fetal liver was found to contain approximately 23% type M and 77% type L pyruvate kinase. The M isoenzyme was partially purified by using the identical procedure to that used for the purification of hepatoma type M in order to determine whether the two have similar properties. The complete separation of the M- from the L-type enzyme was confirmed electrophoretically as above.

Results

Tables I, II, and III show the results obtained with the purification procedures used. Overall purification factors of 34.3-, 62.5-, and 43.5-fold, and yields of 19, 5, and 5% were obtained on purification of the normal liver type L, hepatoma, and fetal types M pyruvate kinases, respectively. The yield of the fetal type M isoenzyme was low because it only comprises 23% of fetal liver pyruvate kinase.

Initial Velocity Patterns. Double-reciprocal plots of initial velocity *vs.* substrate concentration at various constant levels of the second substrate are shown in Figure 1. The values of the kinetic constants are summarized in Table IV.

Liver Type L Pyruvate Kinase. From Figure 1A it is seen that Lineweaver–Burk plots of reciprocal velocity *vs.* reciprocal ADP concentration at various constant levels of P-enolpyruvate gave a series of straight lines that turned up at high ADP concentrations, indicating inhibition at high ADP levels. This effect was more marked at low levels of P-enolpyruvate. Extrapolation of the linear sections of these plots gave a series of lines cutting the abscissa at different points, showing that the Michaelis constant for ADP varied with the P-enolpyruvate level. All the lines met at a point below the abscissa.

Replots of the data as reciprocal velocity *vs.* reciprocal P-enolpyruvate concentration at various constant levels of ADP gave lines that curved upward, reflecting allosteric kinetics (Figure 1B). A secondary plot of the intercepts on the ordinate of the latter plot *vs.* reciprocal ADP concentra-

TABLE III: Partial Purification of Type M Pyruvate Kinase from Human Fetal Liver.

Fraction	Total Act. (Units)	Total Protein (mg)	Sp Act. (Units/mg of Protein	Purifcn Factor	Yield (%)
Crude homogenate	265	3590	0.074	1.0	100
First ammonium sulfate fractionation					
0–50% saturation	197	1650	0.119	1.6	75
50–75% saturation and dialysis	60	540	0.113	1.5	23
Acetone fractionation of 50–70% saturated ammonium sulfate fraction (0–45%)	58	253	0.228	3.1	22
Second ammonium sulfate fractionation (50–60% saturation)	30	83	0.360	4.9	11
Sephadex G-200	13.5	42	3.22	43.5	5

tion (Florini and Vestling, 1957) gave a Michaelis constant for ADP of approximately 0.56 mM, as shown by the lowest, dotted line in Figure 1A. As no reliable Michaelis constant for P-enolpyruvate could be obtained from Figure 1B, data from another experiment were examined using the Hill equation, rearranged to the form

$$\log v/(V_{max} - v) = n \log (S) - \log K$$

where v represents the observed velocity, V_{max} the maximum velocity, n the minimal number of substrate binding sites, and K a constant (Hill, 1913; Atkinson, 1966). On plotting log $v/(V_{max} - v)$ *vs.* the log of the substrate concentration, several parameters can be obtained. The $K_{1/2}$, or P-enolpyruvate concentration at half-maximal velocity, is obtained at log $v/(V_{max} - v) = 0$. The slope of the Hill plot, n_H, gives the interaction coefficient and represents the minimal number of binding sites. A $K_{1/2}$ value of 0.75 mM for P-enolpyruvate at 1 mM ADP concentration was obtained with n_H value of 2.1 (inset).

Hepatoma Type M Pyruvate Kinase. Plots of reciprocal velocity *vs.* reciprocal ADP concentration at various constant levels of P-enolpyruvate for hepatoma type M pyruvate kinase gave a series of straight lines that met at a point on the abscissa, indicating that the Michaelis constant for ADP (0.26 mM) was independent of the P-enolpyruvate level (Figure 1C). However, replots of the data as reciprocal velocity *vs.* reciprocal P-enolpyruvate concentration at various constant levels of ADP gave a series of nonlinear plots (Figure 1D). From a Hill plot (Inset), a $K_{1/2}$ value for P-enolpyruvate of 0.16 mM at 1, 0.45 or 0.25 mM ADP concentration was obtained, with n_H value of 0.8.

Fetal Liver Type M Pyruvate Kinase. On plotting reciprocal velocity *vs.* reciprocal ADP concentration at various constant levels of P-enolpyruvate, a series of straight lines was obtained meeting at a point on the abscissa, as for hepatoma type M pyruvate kinase. Unlike the results for the latter enzyme, however, on replotting the data as reciprocal velocity *vs.* reciprocal P-enolpyruvate concentration, a similar set of straight lines meeting at a point on the abscissa was obtained. This indicates that in this case the Michaelis constants of each of the two substrates were independent of the level of the other. Michaelis constants of 0.28 and 0.11 mM for ADP and P-enolpyruvate were obtained from such plots.

Product Inhibition Studies. With normal liver type L pyruvate kinase, MgATP gave hyperbolic noncompetitive inhibition with ADP as the variable substrate and competitive inhibition when P-enolpyruvate was the variable substrate (Figure 2, upper portion). With hepatoma type M pyruvate kinase, MgATP gave linear noncompetitive inhibition *vs.* ADP with a K_i of 16.6 mM. A nonlinear pattern was obtained for MgATP inhibition when P-enolpyruvate was used as the variable substrate (Figure 2, lower portion). Insufficient fetal enzyme was available to carry out an analogous experiment.

Isotope Exchange Studies. From the plots of reciprocal velocity of exchange *vs.* reciprocal substrate concentration (Figure 3) it can be seen that the rate of exchange between [^{14}C]pyruvate and P-enolpyruvate could be eliminated at high MgATP–ADP level. Exchange between [γ-^{32}P]ATP and P-enolpyruvate could also be eliminated on raising the pyruvate–ADP pair to a high concentration. On the other hand, exchange between [γ-^{32}P]ATP and P-enolpyruvate increased hyperbolically (linear double-reciprocal plot) on raising MgATP–P-enolpyruvate levels in a constant ratio. In all cases, similar results were obtained for both normal liver type L and hepatoma type M pyruvate kinases.

TABLE IV: Kinetic Properties of Pyruvate Kinase from Human Adult and Fetal Liver and Hepatoma.

Source	Michaelis Constant (mM)	
	ADP	P-enolpyruvate
Adult liver type L	0.56	0.75 ($K_{1/2}$)
Fetal liver type M	0.28	0.11
Adult hepatoma type M	0.26	0.16 ($K_{1/2}$)

Discussion

Stable partially purified types L and M pyruvate kinase preparations have been obtained from normal human adult liver, hepatoma, and fetal liver. As with rat liver (Susor and Rutter, 1968) this enzyme could be stabilized with 0.5 M sucrose and by the addition of 0.5 M ammonium sulfate at the stage preceding the Sephadex G-200 step.

The kinetics and Michaelis constants of all three preparations were found to differ. Although they all displayed Michaelis–Menten kinetics with respect to ADP, only normal liver type L pyruvate kinase was inhibited at high ADP levels

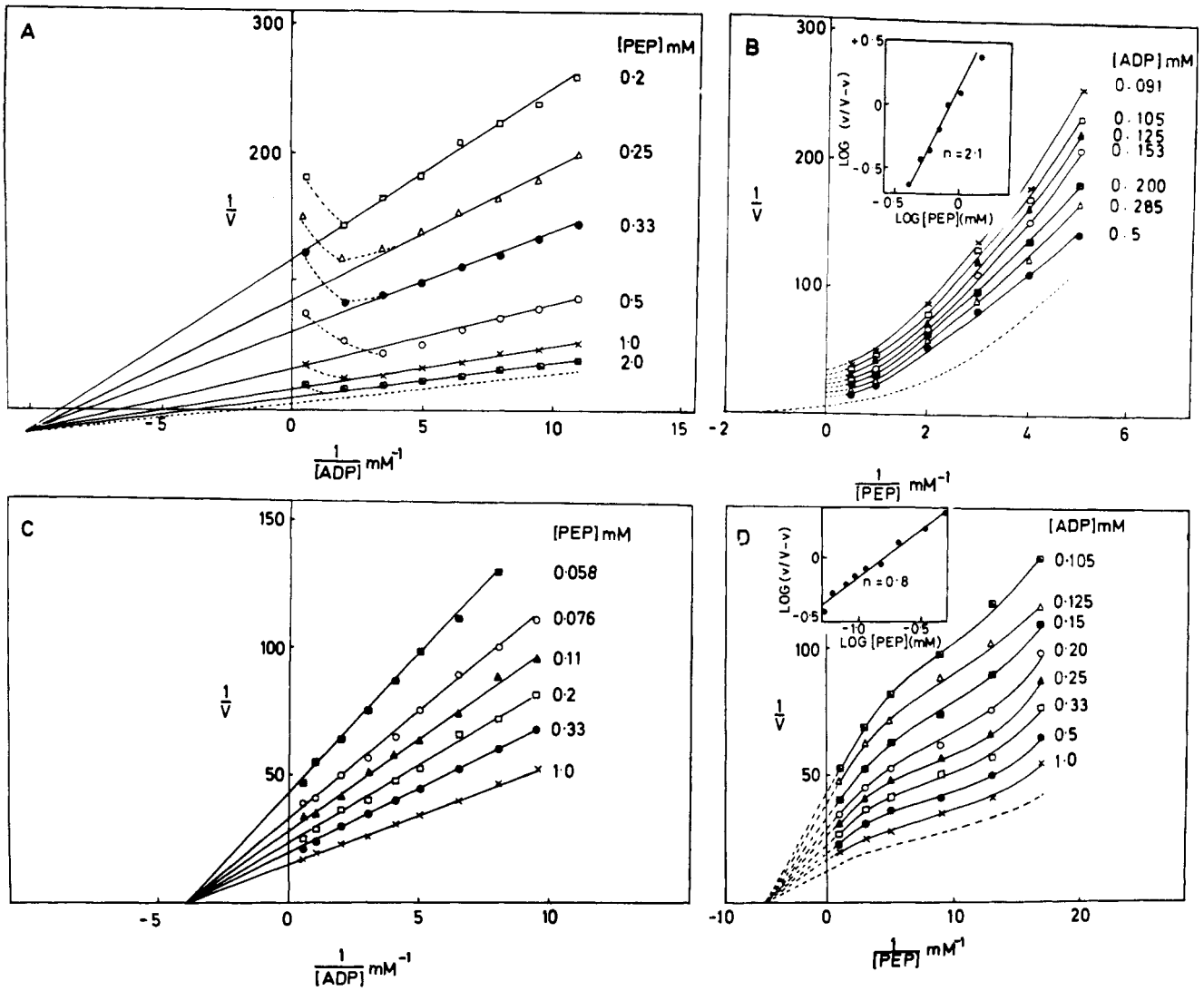


FIGURE 1: Double-reciprocal plots of initial velocity *vs.* substrate concentration at various fixed levels of the second substrate. Reaction mixtures contained 50 mM triethanolamine-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 5 mM EDTA, and 0.1 M KCl in 1.0 ml in addition to P-enolpyruvate, ADP, 0.15 mM NADH, 4 U of lactate dehydrogenase, and enzyme. Velocity is expressed as change of absorbance at 340 nm/min at 30°. The lowest line is the extrapolated line at infinite concentration of variable substrate. Insets are Hill plots. (See text for detailed description.) (A,B) Normal human adult liver type L pyruvate kinase; (C,D) hepatoma type M pyruvate kinase.

and had Michaelis constants for ADP which varied with the P-enolpyruvate level. Similar results have been reported for rat liver type L pyruvate kinase by Tanaka *et al.* (1967a) and for human erythrocyte pyruvate kinase by Staal *et al.* (1971). The limiting Michaelis constant for ADP of normal adult liver type L pyruvate kinase was found to be higher (0.56 mM) than that of the hepatoma (0.26 mM) and fetal (0.28 mM) types M pyruvate kinase. It was also higher than the apparent Michaelis constant for ADP (0.1 mM) obtained by Tanaka *et al.* (1967a) for rat liver pyruvate kinase. However, it was similar to that obtained by Staal *et al.* (1971) for human erythrocyte pyruvate kinase and by Pogson (1968) for rat adipose tissue pyruvate kinase B (0.67 mM). The Michaelis constants for ADP found for the hepatoma and fetal types M pyruvate kinase were similar to those for rat muscle (Tanaka *et al.*, 1967a), rabbit muscle (Reynard *et al.*, 1961; Mildvan and Cohn, 1966; Holmsen and Storm, 1969), rat adipose tissue pyruvate kinase B (Pogson, 1968), and that found for human erythrocytes by Ibsen *et al.* (1968), *viz.*, 0.27–0.33 mM.

Of the three types of pyruvate kinase tested, only the fetal type M isoenzyme displayed simple Michaelis–Menten kinetics with respect to P-enolpyruvate, similar to those reported for rabbit muscle (Holmsen and Storm, 1968; Mildvan and Cohn, 1966; Reynard *et al.*, 1961), rat muscle (Tanaka *et al.*, 1967a), adipose tissue pyruvate kinase B (Pogson, 1968), human leucocyte pyruvate kinase (Koler *et al.*, 1968), and for erythrocyte pyruvate kinase by Ibsen *et al.* (1968). The Michaelis constant of 0.11 mM was also similar to that reported by these authors.

On the other hand, normal liver type L pyruvate kinase displayed sigmoidal kinetics with respect to P-enolpyruvate, giving rise to Lineweaver–Burk plots which were concave upward (Figure 1). This, together with the finding of a high Michaelis constant for P-enolpyruvate (0.75 mM), is consistent with the findings for rat liver pyruvate kinase (Tanaka *et al.*, 1967a; Susor and Rutter, 1968; Rozengurt *et al.*, 1969; Carminatti *et al.*, 1968; Taylor *et al.*, 1969), rat adipose tissue pyruvate kinase A (Pogson, 1968), and for human erythrocytes by Staal *et al.* (1971). The sigmoidal kinetics indicate that there is more than one binding site for P-enolpyruvate on the

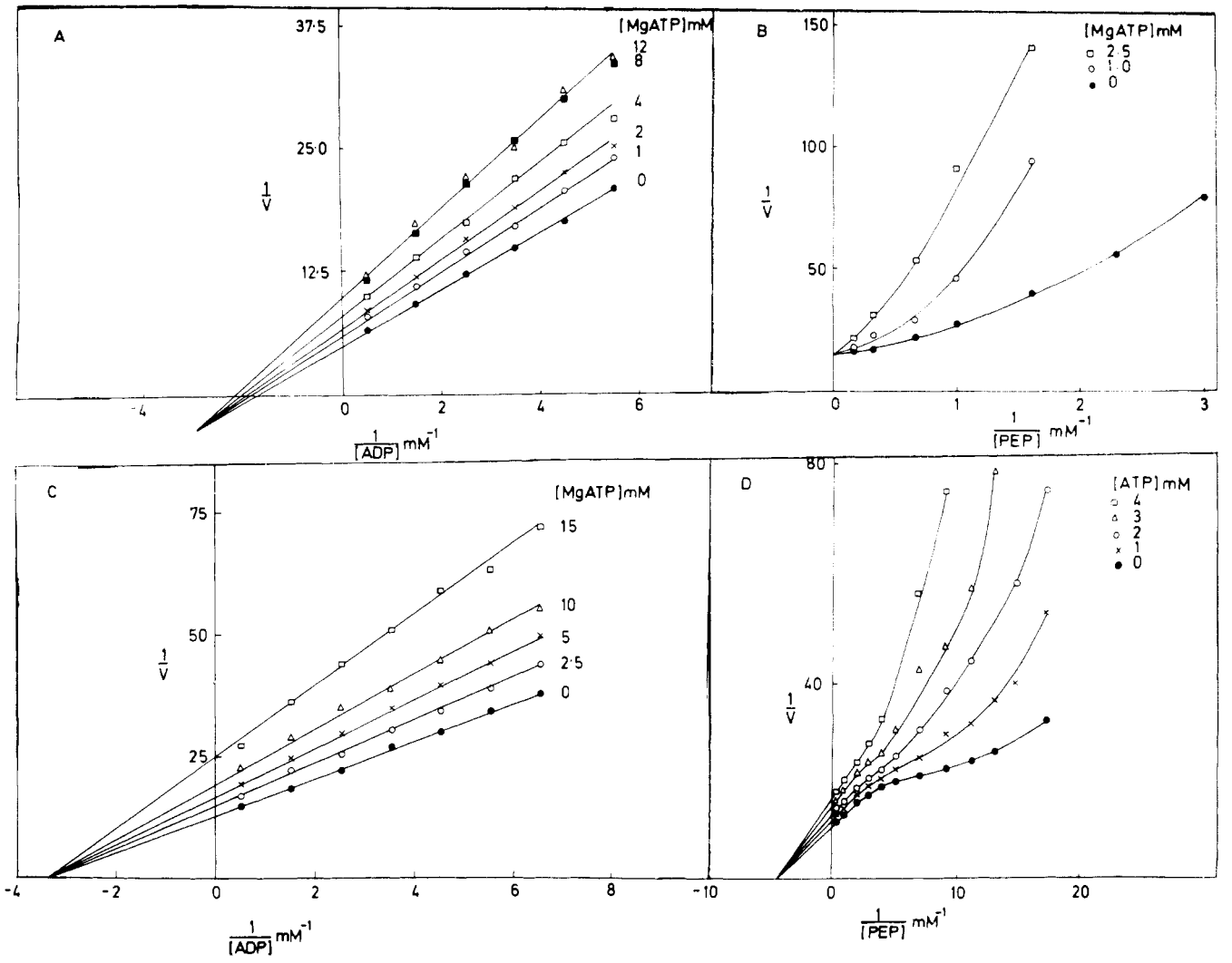


FIGURE 2: Double-reciprocal plots of initial velocity *vs.* ADP or P-enolpyruvate concentration at various fixed levels of MgATP. Conditions were as in Figure 1 except for the presence of MgATP. A,B: Normal adult human liver type L pyruvate kinase. A: P-enolpyruvate was present at 5 mM concentration. B: 0.4 mM ADP was present. C,D: hepatoma type M pyruvate kinase. C: 1 mM P-enolpyruvate was used. D: 1 mM ADP was used.

L-type isoenzyme, as shown in more detail elsewhere (Balinsky *et al.*, 1973a). Occupation of an allosteric site by ADP could explain the inhibition at high ADP concentration.

The results of kinetic studies using hepatoma type M pyruvate kinase not only differed from those for the fetal type M and normal adult liver type L pyruvate kinase, but were also different from those described by Taylor *et al.* (1969) for pyruvate kinase isolated from rat 3924 A hepatoma. In the present study, nonhyperbolic kinetics were obtained, whereas these authors found typical Michaelis–Menten kinetics. The results obtained both from the Lineweaver–Burk and the Hill plots point to the presence of more than one binding site for P-enolpyruvate. The n_H value of 0.8 found indicates that the enzyme may show negative cooperativity with respect to P-enolpyruvate.

It has often been asserted that tumor tissue represents a reversion to a more primitive state; yet from the present results it would appear that it differs from the fetal type. This is confirmed by detailed studies with allosteric inhibitors (Balinsky *et al.*, 1973a). The complex kinetics displayed by the tumor enzyme probably ensure the efficient utilization in the glycolytic pathway of P-enolpyruvate both at high and low levels. Since hepatoma type M pyruvate kinase has a low Michaelis constant for P-enolpyruvate, the enzyme can function efficiently at low levels of P-enolpyruvate, yet at high levels of P-enolpyruvate the enzyme will be activated by its substrate. On the other hand, liver type L pyruvate kinase will function most efficiently only at high P-enolpyruvate levels, thus enabling P-enolpyruvate to be diverted into gluconeogenesis when present at low concentration.

The finding that MgATP inhibition is noncompetitive with respect to ADP in both normal human liver type L and hepatoma type M pyruvate kinase is similar to the results of Holmsen and Storm (1969) for the enzyme from rabbit muscle, but differs from those of Reynard *et al.* (1961), who found MgATP to be a competitive inhibitor *vs.* ADP for the same rabbit muscle enzyme. When replotting the vertical intercepts as a function of the MgATP concentration, a linear plot was obtained with hepatoma type M pyruvate kinase, but the plot with normal adult liver type L pyruvate kinase was hyperbolic. Such a hyperbolic replot might be attributed to MgATP acting at two sites. As shown elsewhere, ATP is an allosteric inhibitor of pyruvate kinase (Balinsky *et al.*, 1973a). The finding that MgATP inhibition against P-enolpyruvate

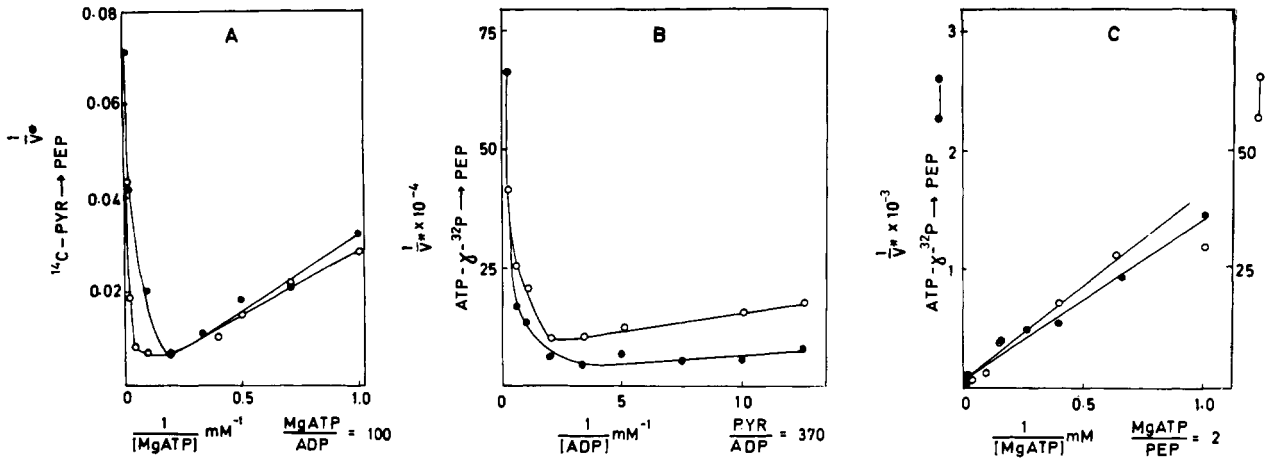


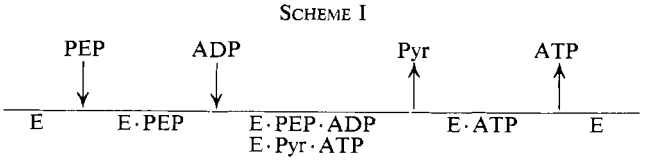
FIGURE 3: Isotope exchange studies with normal liver type L (●) and hepatoma type M (○) pyruvate kinase. Reaction mixtures contained 50 mM glycine–NaOH buffer (pH 9.0), 12.5 mM $MgCl_2$, 0.25 mM EDTA, enzyme, and other additions as shown. (A) Isotope exchange rates of [^{14}C]pyruvate to P-enolpyruvate when varying MgATP–ADP in a 100:1 ratio. 7.4 mM pyruvate and 2 mM P-enolpyruvate were also present. (B) Isotope exchange rates of [γ-^{32}P]ATP into P-enolpyruvate when varying pyruvate–ADP in a 370:1 ratio. 2 mM ATP and 2 mM P-enolpyruvate were also present. C: Isotope exchange rates of [γ-^{32}P]ATP into P-enolpyyruvate when varying MgATP–P-enolpyruvate in a 2:1 ratio. 74 mM pyruvate and 0.4 mM ADP were present.

was competitive in the case of normal adult liver type L pyruvate kinase agrees with the findings for rabbit muscle (Reynard *et al.*, 1961) and erythrocyte pyruvate kinase (Campos *et al.*, 1965). Boyer (1969) later found this inhibition to be of a mixed type but mainly of a competitive nature.

As shown above, these three isoenzymes differ in their allosteric properties and kinetic constants. A close perusal of the patterns obtained for steady-state kinetics and isotope exchange indicates, however, that the catalytic mechanisms of the various isoenzymes examined are probably the same. In all cases, the lines obtained in the initial rate studies meet at a point, indicating that all the isoenzymes have a sequential mechanism, whereby both substrates must add to the enzyme before either product is released (Cleland, 1963). These results do not, however, show whether substrate addition is random or ordered.

The interpretation of product inhibition studies of an allosteric enzyme may be somewhat complicated by interaction at more than one site; nevertheless it is tempting to speculate on the possible mode of substrate addition and product release as indicated by our data. The results showing that MgATP is a noncompetitive inhibitor with respect to ADP for both normal liver type L and hepatoma type M pyruvate kinase, and inhibits competitively *vs.* P-enolpyruvate, might indicate an ordered mechanism with P-enolpyruvate being the first substrate to bind to the enzyme and MgATP the last product to be released, as is shown in Scheme I.

SCHEME I



This scheme does not take into account the various metal ions such as Mg^{2+} and K^+.

This is contrary to the conclusions of Mildvan and Cohn (1966) and Reynard *et al.* (1961), who proposed a random sequential mechanism for rabbit muscle pyruvate kinase. A rapid-equilibrium, random sequential mechanism would be indicated if MgATP were competitive against both P-enolpyruvate and ADP. Our results would not be inconsistent with a rapid-equilibrium random mechanism if the enzyme can form a dead-end E·ADP·ATP complex (Cleland, 1963). However, in view of the large size of ATP and ADP, and the fact that they probably occupy the same site on the enzyme, such a mechanism seems very unlikely.

Further evidence that the mechanism of normal adult human liver type L and hepatoma type M pyruvate kinases may be ordered comes from the results of the isotope exchange experiments. The finding that the [^{14}C]pyruvate to P-enolpyruvate exchange could be eliminated at high MgATP–ADP levels suggests that either one or both of the varied reactants add between the labeled reactants. In the case of a random mechanism, though there would be inhibition at high MgATP–ADP levels, there would not be complete cut-off of the exchange. Furthermore, [γ-^{32}P]ATP to P-enolpyruvate exchange can similarly be eliminated at high ADP–pyruvate levels. The latter finding could, however, also be explained by the formation of the dead-end complex E·ADP·Pyr, but a similar explanation of the former result would again require one to postulate an E·ADP·ATP complex.

In conclusion, it appears that the reaction mechanism of both liver type L and hepatoma type M pyruvate kinase is identical. The ordered mechanism shown in Scheme I fits all available data, but a rapid-equilibrium random mechanism with dead-end complex formation cannot be entirely excluded. The main difference between the two enzymes, as shown here and discussed more fully elsewhere (Balinsky *et al.*, 1973a), is that the liver type L enzyme is allosteric and exists normally in the inactive form, whereas the hepatoma type M enzyme normally occurs in the active form. The hepatoma enzyme differs in some respects from the fetal M isoenzyme, and it would be interesting to find out, for example, from sequence studies of highly purified isoenzymes, whether the hepatoma enzyme is in fact more closely related to the M or the L isoenzyme.

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