Regulation of Rat Liver Pyruvate Kinase

THE EFFECT OF PREINCUBATION, pH, COPPER IONS, FRUCTOSE 1,6-DIPHOSPHATE AND DIETARY CHANGES ON ENZYME ACTIVITY

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1. Preincubation of partially purified rat liver L-type pyruvate kinase at 25° for 10 min. causes a marked increase in co-operativity with respect to both the substrate, phosphoenolpyruvate, and the allosteric activator, fructose 1,6-diphosphate. 2. The results are consistent with the existence of two forms of liver L-type pyruvate kinase, designated forms L_A and L_B . It is postulated that form L_A has a low K_m for phosphoenolpyruvate (about 0.1 mM) and is not allosterically activated, whereas form L_B is allosterically activated by fructose 1,6-diphosphate, exhibiting in the absence of the activator sigmoidal kinetics with half-maximal activity at about 1 mM-phosphoenolpyruvate. In the presence of fructose 1,6-diphosphate, form L_B gives Michaelis-Menten kinetics with K_m less than 0.1 mM. It is further postulated that preincubation converts form L_A into form L_B . 3. The influence of pH on the preincubation effect was studied. 4. The inhibition of pyruvate kinase by Cu^{2+} was studied in detail. Though phosphoenolpyruvate and fructose 1,6-diphosphate readily protect the enzyme against Cu^{2+} inhibition, little evidence of significant reversal of the inhibition by these compounds could be found. 5. The effects of starvation, fructose feeding and preincubation on the pyruvate kinase activity of crude homogenates of various tissues of the rat were also studied.

Hess, Haeckel & Brand (1966) showed that FDP† stimulated yeast pyruvate kinase with kinetics characteristic of allosteric activation. A similar result was reported by Taylor & Bailey (1967) for the pyruvate kinase of the 30–40%-saturated ammonium sulphate fraction of rat liver homogenate. This fraction corresponds to the L-type pyruvate kinase distinguished immunologically by Tanaka, Harano, Morimura & Mori (1965) and shown by them to be under dietary control. Muscle pyruvate kinase (M-type described by Tanaka *et al.* 1965) was not influenced by FDP (Hess *et al.* 1966; Taylor & Bailey, 1967). Passeron, de Asua & Carminatti (1967) reported that the L-type pyruvate kinase, but not the M-type, from mouse liver was inhibited by Cu^{2+} and that this inhibition was 'reverted' by FDP. It was suggested that the stimulation of pyruvate kinase in the absence of added Cu^{2+} might be due to FDP overcoming inhibition by Cu^{2+} present as impurity in the reagents, and further that Cu^{2+} present in the liver

might exert a physiological control over the activity of this enzyme *in situ*.

The initial purpose of the present study was to investigate more fully the interaction of Cu^{2+} and FDP on pyruvate kinase, with a view to assessing their importance in controlling the enzyme. However, the preliminary finding that the action of these agents was profoundly affected by experimental conditions necessitated the extension of the study to include the effects of preincubation and pH.

EXPERIMENTAL

Materials. All reagents used were of analytical grade or the purest available. The following chemicals were obtained from Boehringer Corp. (London) Ltd. (London, W. 5): ADP, $NADH_2$, PEP (tricyclohexylammonium salt), FDP and lactate dehydrogenase. Other chemicals were supplied by British Drug Houses Ltd. (Poole, Dorset).

Preparation of pyruvate kinase from rat liver. Fresh rat liver (230 g.) was homogenized in a Waring Blender with 4 vol. of a solution containing 0.05 M-KCl and 5 mM-EDTA adjusted to pH 7.5. This and all subsequent procedures, including centrifugation, were carried out at 3–5°. The crude homogenate was centrifuged for 1 hr. at 15000 g_{av}. (MSE Mistral centrifuge, 6 × 250 ml. head, 12000 rev./min.)

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† Abbreviations: FDP, fructose 1,6-diphosphate; PEP, phosphoenolpyruvate.

and the protein of the supernatant was fractionated by differential $(\text{NH}_4)_2\text{SO}_4$ precipitation. The $(\text{NH}_4)_2\text{SO}_4$ concentration was successively raised to 25, 45, 55 and 70% saturation, and at each stage the precipitate was sedimented by centrifugation at $15000g_{av}$ for 1 hr. after the mixture had been allowed to stand in the cold-room at the new concentration of $(\text{NH}_4)_2\text{SO}_4$ for at least 2 hr. The precipitates sedimenting at 25–45% and 55–70% saturation were designated L- and M-type pyruvate kinase respectively (Tanaka *et al.* 1965). Testing for stimulation by FDP as described below showed strong activation of the L fraction but none with the M fraction. Each fraction was redissolved in a volume of water corresponding to 30% of the volume of liver used. Both samples were stable indefinitely when stored at -20° .

In those experiments (Tables 2 and 3) concerned with a comparison of the effects of FDP and Cu^{2+} on the pyruvate kinase of different tissues and with the influence of diet, the preparation used was the supernatant fluid from the centrifugation of a 1:20 (w/v) homogenate of the tissue made in water. For all such samples centrifugation was carried out for 30 min. at $20000g_{av}$. (MSE Superspeed 50, $10 \times 10\text{ml. head}$, 20000rev./min.).

Animals and diet. In the dietary experiments male Wistar rats (150–200 g.) were fed on Oxoid diet 86 [H. C. Styles (Bewdley) Ltd., Bewdley, Worcs.] *ad libitum*. Unlimited access to fluid was permitted, water for the control rats and 20% (w/v) fructose for the 'fructose-treated' rats.

Assay of pyruvate kinase activity. Pyruvate kinase activity was assayed by the method of Bücher & Pfeleiderer (1955). The reaction was coupled with lactate dehydrogenase to give oxidation of NADH_2 , which was followed continuously by measuring the change in E_{340} with a Beckman DB spectrophotometer and Sargent SR chart recorder. The reaction was carried out at 25° . The final volume of the reaction mixture was always 3.0 ml., and except where it was necessary to vary conditions for the purpose of a particular experiment the final concentrations were as follows: triethanolamine-HCl buffer, pH 7.5, 40 mM; KCl, 70 mM; NADH_2 , 0.15 mM; ADP, 1.0 mM; MgSO_4 , 8.0 mM; lactate dehydrogenase (rabbit muscle), 10 units. The concentrations of PEP, FDP and Cu^{2+} , together with details of the incubation procedure, are noted in the legends to the Tables and Figures. Except where stated otherwise the results shown are for L-type pyruvate kinase.

RESULTS

Effect of preincubation on enzyme activity. Fig. 1 shows the effect of preincubation at 25° on the activity of L-type pyruvate kinase at pH 7.5 in the presence of 3 mM-EDTA and 0.1 mM-PEP, conditions normally used to demonstrate stimulation by FDP (Taylor & Bailey, 1967). Preincubation of the enzymes in the assay mixture, complete except for the PEP used to start the reaction, results in a 50% decrease in activity in 1.5 min. and an 85% decrease in 10 min.

The effect of FDP was tested in two ways. In the first, ΔE_{340} was measured for 1 min. after the reaction had been started by the addition of PEP,

Fig. 1. Effect of preincubation at 25° on L-type pyruvate kinase activity. The reaction mixture including enzyme was preincubated at 25° , and the reaction was started by addition of PEP to give a final concn. of 0.1 mM. ●, No FDP; ▲, FDP (0.5 mM) present during the incubation; ■, FDP (0.5 mM) added after the reaction had been started with PEP. EDTA (final concn. 3 mM) was always present, and other conditions were as described in the Experimental section.

FDP (0.5 mM) was then added, and the new rate was determined. In the second, FDP was added at the same time as the enzyme and was therefore present throughout the preincubation. Without preincubation (zero time in Fig. 1) FDP stimulates activity whether it is present initially or added after the substrate, PEP, being rather more effective in the former case.

Decrease in enzyme activity is not observed when FDP is present in the reaction mixture before preincubation, and if it is added after the PEP the activity is instantaneously raised to a value equal to or in excess of that found when FDP is added under the same conditions without preincubation. The extra activity, observed only when FDP was added after the preincubation period, increases with the duration of the incubation.

The preincubation effect can be demonstrated regardless of whether lactate dehydrogenase is added before or after preincubation, thus showing that the change is not due to an alteration in the activity of this enzyme.

The effect of preincubation on the enzyme's response to FDP is more clearly illustrated by

comparing the plots of enzyme activity against FDP concentration obtained with and without preincubation (Fig. 2). Though half-maximal stimulation is obtained at about $1\text{--}2\text{ }\mu\text{M}$ in both cases, the shapes of the two curves are clearly different. The curve obtained after preincubation is sigmoidal, but without preincubation the response is more

Fig. 2. Influence of preincubation on the response of L-type pyruvate kinase to increasing FDP concentration. The reaction was started by addition of PEP to give a final concn. of 0.1 mM , and preincubations were carried out at 25° . ▲, Without preincubation; ●, reaction started after 10 min. preincubation. FDP was added at the same time as the PEP. EDTA (final concn. 3 mM) was always present, and other conditions were as described in the Experimental section.

complex, showing some signs of saturation at very low concentrations of FDP, up to $1\text{ }\mu\text{M}$, and then increasing above this concentration to reach a maximum at about $3\text{--}4\text{ }\mu\text{M}$. Such curves are a consistent finding. At concentrations of FDP below $1\text{ }\mu\text{M}$ preincubation also decreases activity, whereas at concentrations above $1\text{ }\mu\text{M}$ activity is increased by preincubation.

The effect of preincubation on the relationship between enzyme activity and PEP concentration is shown in Fig. 3; there are several similarities with the corresponding FDP curves (Fig. 2). Thus the curve showing the response to increasing PEP without preincubation is complex and similar in shape to that obtained when FDP concentration is the variable. In particular, signs of saturation are apparent at concentrations of PEP below 0.5 mM , though above this concentration the rise in activity with concentration is resumed and continues up to the highest concentration tested, 5 mM . Though preincubation converts this response into a regular sigmoidal form, half-maximal activity with and without preincubation lies in the range $1.0\text{--}1.5\text{ mM}$ -PEP. The addition of saturating concentrations of FDP, however, transforms both curves into Michaelis-Menten forms with half-maximal values at about $10\text{ }\mu\text{M}$ -PEP.

In the absence of FDP there is a cross-over point, 0.6 mM -PEP, below which preincubation decreases activity and above which activity is increased by

Fig. 3. Influence of preincubation on the relationship between L-type pyruvate kinase activity and PEP concentration. ○, No preincubation, no FDP; ●, no preincubation, FDP (0.5 mM) present; △, 10 min. preincubation, no FDP; ▲, 10 min. preincubation, FDP (0.5 mM) present. When FDP was present it was added after the reaction had been started with PEP. Preincubation was carried out at 25° . EDTA (final concn. 3 mM) was always present, and other conditions were as described in the Experimental section.

Fig. 4. Effect of pH on L-type pyruvate kinase activity with and without preincubation. Potassium phosphate buffer (final concn. 50 mM) was used throughout. FDP, when present, was added, to give a final concn. of 0.5 mM, after the reaction had been started with PEP. EDTA (final concn. 3 mM) was always present, and other conditions were as described in the Experimental section. (a) 1 mM-PEP without preincubation, and (b) 0.1 mM-PEP without preincubation: $\circ$, no FDP; $\bullet$, FDP (0.5 mM) present; (c) 1 mM-PEP with 10 min. preincubation, and (d) 0.1 mM-PEP with 10 min. preincubation: $\triangle$, no FDP; $\blacktriangle$, FDP (0.5 mM) present.

preincubation (cf. Fig. 2, showing a similar cross-over point for FDP). The increase in activity that occurs on preincubation in the presence of FDP and previously noted in Fig. 1 is clearly seen here.

Effect of pH and nature of buffer. The experiments described so far were all carried out at pH 7.5. In view of the marked influence of pH on the extent of stimulation by FDP, previously noted by Taylor & Bailey (1967), it was decided to determine whether or not the changes induced by preincubation are also pH-dependent. Fig. 4 shows the effect of pH on pyruvate kinase activity in the presence and absence of FDP, with and without preincubation at two concentrations of PEP. As noted above, at pH 7.5 preincubation tends to decrease activity at low concentrations of PEP and to increase it at high concentrations. Comparison of Figs. 4(b) and 4(d) (0.1 mM-PEP) shows that high pH favours inactivation, this being complete in 10 min. at pH 8.0, whereas no inactivation occurs below pH 7.0. Qualitatively similar but less marked changes are also found with 1.0 mM-PEP (Figs. 4a and 4c), i.e. at concentrations nearer saturation.

The influence of FDP on the pH-activity curves is most striking at low concentrations of PEP. In the presence of saturating concentrations of FDP, at 0.1 mM-PEP, preincubation has little effect on activity regardless of pH, and under these conditions activity varies little between pH 6.0 and 8.0. However, at the same concentration of PEP but in the absence of FDP an optimum occurs at pH 6.5–7.0.

To distinguish between the effects of different media on the changes induced by preincubation and direct influences on the enzyme assay, a series of experiments was carried out in which samples of enzyme were pretreated under various conditions and then assayed by the standard procedure, namely triethanolamine buffer, pH 7.5, 0.1 mM-PEP etc. (Fig. 5). There is no decrease in enzyme activity after preincubation of the preparation diluted 1:1 with water, 0.2 M-potassium chloride or 0.2 M-potassium phosphate buffer, pH 6.0, but inactivation does occur after preincubation when the diluting agent is 0.2 M-triethanolamine buffer, pH 7.5, or 0.2 M-potassium phosphate buffer, pH 7.0

Fig. 5. Effect of preincubation under various conditions on the activity of L-type pyruvate kinase. Samples (25 μ l.) of the L-type pyruvate kinase fraction were diluted with an equal volume of either water, 0.2M-KCl, 0.2M-triethanolamine-HCl buffer, pH 7.5, or potassium phosphate buffer, pH 6.0, 7.0 or 8.0, and preincubated for 10 min. at 25°. Samples (10 μ l.) taken before and after preincubation were assayed under standard conditions in triethanolamine-HCl buffer, pH 7.5, with 0.1mM-PEP and 3mM-EDTA as described in the Experimental section. Open columns (□), initial activity; closed columns (■), activity after 10 min. preincubation. Above each of these, the column enclosed by broken lines indicates activity in the presence of FDP (0.5mM).

or 8.0. In each case where preincubation decreased the activity in the absence of FDP, activity was increased in the presence of FDP.

Effect of Cu^{2+} on pyruvate kinase. To study the inhibition of pyruvate kinase by Cu^{2+} it is necessary to omit EDTA from the reaction mixture. This omission of itself generally causes a slight decrease in enzyme activity and alterations in the response to changing substrate concentration. At low PEP concentrations (0.1mM) and in the absence of EDTA or FDP, Cu^{2+} inhibits pyruvate kinase activity (Fig. 6), half-maximal inhibition occurring at 30–40 μM - Cu^{2+} . The same half-maximal concentration is found for Cu^{2+} inhibition in the presence of saturating concentrations of FDP. Stimulation by FDP, though still apparent at 25 μM - Cu^{2+} , is not seen at higher concentrations, 50 μM - and 0.1mM- Cu^{2+} . It is possible, however,

Fig. 6. Influence of PEP concentration on inhibition of L-type pyruvate kinase by Cu^{2+} . The reaction was started by the simultaneous addition of enzyme, Cu^{2+} and PEP. No EDTA was present. FDP, when present, was added 1–1.5 min. after the reaction had been started. ○, PEP (0.1mM), no FDP; ●, PEP (0.1mM), FDP (0.5mM); △, PEP (5mM), no FDP; ▲, PEP (5mM), FDP (0.5mM).

that some stimulation does occur at these high concentrations but is not seen because of the difficulty of detecting differences when activity is low. In the presence of high PEP concentrations (5mM) inhibition by Cu^{2+} is much decreased (Fig. 6) regardless of the presence or absence of FDP. Thus 0.1mM- Cu^{2+} , which is sufficient to abolish activity at low concentrations of PEP, causes respectively only 24 and 17% inhibition in the presence and absence of FDP at the high concentration of PEP. In these experiments Cu^{2+} and PEP were added simultaneously to the enzyme mixture, there was no preincubation, initial rates were plotted and FDP was added last. Under these conditions FDP does not appear to decrease inhibition, whereas increasing the concentration of the substrate PEP does.

Fig. 7 shows the influence of FDP on Cu^{2+} inhibition under two conditions: first, when the FDP is added before Cu^{2+} (Fig. 7a), and, secondly, when it is added last (Fig. 7b). Raising the FDP concentration by 200-fold (5 μM to 1mM) causes a scarcely significant decrease in the Cu^{2+} inhibition in either case, but clearly pretreatment with FDP is always more effective than adding it after the Cu^{2+} .

The influence of PEP concentration on Cu^{2+} inhibition is shown more clearly in Fig. 8. At high concentrations of PEP, FDP (0.5mM) restores activity in the presence of 50 μM - Cu^{2+} to 86% of the control value. This concentration of FDP has little

Fig. 7. Effect of FDP on the inhibition of L-type pyruvate kinase by Cu^{2+} . The reaction was started by the simultaneous addition of enzyme, PEP and, where appropriate, Cu^{2+} to the reaction mixture. No EDTA was present. In (a) FDP was added at the same time as the PEP and enzyme. In (b) FDP was added 1 min. after the reaction had been started. ●, No CuSO_4 ; ▲, $10 \mu\text{M-CuSO}_4$; ■, $30 \mu\text{M-CuSO}_4$.

Fig. 8. Effect of PEP on the inhibition of L-type pyruvate kinase by Cu^{2+} . The reaction was started by the simultaneous addition of PEP, enzyme and Cu^{2+} to the reaction mixture. FDP, when present, was added 1-1.5 min. after the reaction had been started to give a final concn. of 0.5 mM . No EDTA was present; other conditions were as described in the Experimental section. ●, $50 \mu\text{M-CuSO}_4$, no FDP; ○, no CuSO_4 , no FDP; ▲, $50 \mu\text{M-CuSO}_4$, 0.5 mM-FDP ; Δ, no CuSO_4 , 0.5 mM-FDP .

Fig. 9. Influence of preincubation on the inhibition of L-type pyruvate kinase by Cu^{2+} . The reaction mixture including enzyme and, where appropriate, Cu^{2+} was preincubated at 25° , and the reaction was started by the addition of PEP to give a final concn. of 0.1 mM . No EDTA was present; other conditions were as indicated in the Experimental section. ○, No additions; ●, $10 \mu\text{M-CuSO}_4$; Δ, FDP (0.5 mM) added before PEP; ▲, $10 \mu\text{M-CuSO}_4$ and FDP (0.5 mM) added before PEP; □, FDP (0.5 mM) added 1-1.5 min. after PEP; ■, $10 \mu\text{M-CuSO}_4$ and FDP (0.5 mM) added 1-1.5 min. after PEP.

effect on Cu^{2+} inhibition at 0.1 mM-PEP . Experiments of this kind were carried out at pH 6.0, 7.0, 7.5 and 8.0 with essentially similar results. How-

ever, the higher the pH the higher was the minimum concentration of PEP to permit stimulation by FDP in the presence of Cu^{2+} .

The influence of Cu^{2+} on the effects of preincubation is shown in Fig. 9. In the absence of FDP the loss of activity that normally occurs

Table 1. *Influence of EDTA on pyruvate kinase activity*

The reaction was started by the simultaneous addition of PEP (final concn. 0.1 mM), enzyme and, where appropriate, Cu^{2+} to the reaction mixture. FDP, where indicated, was added 1–1.5 min. after PEP to give a final concn. of 0.5 mM. EDTA when present was at a final concentration of 3 mM and, except where indicated, was present in the reaction mixture before the addition of enzyme.

Duration of preincubation (min.)	Concn. of Cu^{2+} (mM)	Pyruvate kinase activity ($\Delta E_{340}/\text{min.}$)			
		– EDTA		+ EDTA	
		– FDP	+ FDP	– FDP	+ FDP
0	0	0.19	0.50		
0	0			0.30	0.64z
10	0	0.01	0.60		
10	0			0.16	1.08
0	0.1			0.28	0.54
0	0.1	0.05		0.06*	0.22*
0	0.1	0.07	0.12		0.18†

* EDTA added after PEP but before FDP.

† EDTA added after FDP.

Table 2. *Effect of preincubation, FDP and Cu^{2+} on the pyruvate kinase activity of various organs*

The reaction was started by the simultaneous addition of PEP (final concn. 0.1 mM), enzyme and, where appropriate, CuSO_4 (final concn. 0.1 mM) to the reaction mixture, except in preincubation experiments, where the reaction mixture and enzyme were maintained at 25° for 10 min. before the addition of PEP. FDP (final concn. 0.5 mM), where required, was added 1–1.5 min. after the reaction had been started. EDTA, where indicated, was always present in the reaction mixture before addition of enzyme. Other conditions were as described in the Experimental section. Crude extracts were the supernatant fluids from the centrifugation of 1:20 (w/v) homogenates made in water.

Pyruvate kinase sample	Pyruvate kinase activity ($\Delta E_{340}/\text{min.}$)							
	With EDTA				Without EDTA			
	No preincubation		10 min. preincubation		No preincubation		No preincubation + CuSO_4 (0.1 mM)	
	– FDP	+ FDP	– FDP	+ FDP	– FDP	+ FDP	– FDP	+ FDP
Liver M-type	0.110	0.110	0.065	0.065	0.045	0.045	0.035	0.035
Crude extracts								
Liver	0.156	0.186	0.069	0.186	0.135	0.169	0.012	0.012
Heart	0.250	0.250	0.295	0.295	0.240	0.240	0.245	0.245
Spleen	0.130	0.135	0.128	0.128	0.078	0.078	0.042	0.042
Brain	0.450	0.400	0.425	0.340	0.400	0.400	0.250	0.280
Kidney	0.115	0.145	0.030	0.050	0.025	0.115	0.008	0.04
Erythrocyte	0.039	0.042	0.038	0.038	0.030	0.024	0.002	0.002

during preincubation is more rapid. (The greater inactivation in the control experiments compared with those in Fig. 1 is due to the presence of EDTA in the latter.) This result does not necessarily indicate that Cu^{2+} inhibition is time-dependent. However, time-dependence of Cu^{2+} inhibition is obvious when FDP is present, since under these conditions little inactivation occurs in the absence of Cu^{2+} , but, in the presence of $10 \mu\text{M}$ - Cu^{2+} , inhibition increases progressively throughout the incubation period.

Table 1 illustrates the influence of EDTA (3 mM) on the effect of FDP and preincubation on pyruvate kinase activity. The presence of EDTA gives higher activities with or without FDP. In the absence of EDTA and under the conditions specified, namely 0.1 mM-PEP etc., loss of activity on preincubation is more complete than in the presence of 3 mM-EDTA. If EDTA is present before Cu^{2+} is added little inhibition can be detected, whereas the addition of EDTA after Cu^{2+} gives only slight relief of inhibition.

Table 3. *Effects of starvation and feeding of animals on fructose on the pyruvate kinase activity of various organs*

The reactions were carried out as described in Table 2. EDTA (final concn. 3mM) was always present. The results are the means from at least four animals, and the ranges of results are given in parentheses. Starved rats were deprived of food overnight. Fructose-fed rats were given 20% fructose for 3 days and consumed 12.1g. of carbohydrate/day/rat (7.1g. from diet 86 and 5.0g. from the 20% fructose). Control rats consumed 11.1g. of carbohydrate/day/rat from diet 86.

		Pyruvate kinase activity (μ moles/min./g. wet wt.)			
Pyruvate kinase sample		No preincubation		10 min. preincubation	
		- FDP	+ FDP	- FDP	+ FDP
Liver	Control	16.3 (13.1-21.2)	18.5 (14.5-24.1)	6.9 (5.5-8.5)	18.6 (14.0-27.0)
	Starved	11.7 (9.3-14.6)	18.1 (15.6-20.5)	2.7 (1.5-3.5)	16.7 (14.5-19.5)
	Fructose-fed	33.5 (24.2-41.0)	33.6 (27.1-48.2)	10.9 (8.0-12.5)	33.4 (28.5-40.0)
Kidney	Control	16.5 (14.5-17.1)	17.6 (14.5-19.3)		
	Fructose-fed	18.5 (14.0-22.2)	20.5 (18.2-22.2)		
Heart	Control	38.9 (24.6-54.0)	38.2 (27.1-49.2)		
	Fructose-fed	33.2 (21.3-40.5)	31.9 (22.4-42.5)		
Spleen	Control	36.6 (34.8-40.4)	34.0 (29.0-40.4)		
	Fructose-fed	30.2 (27.3-34.8)	30.2 (27.3-34.8)		
Brain	Control	43.8 (36.3-48.3)	36.6 (33.8-38.6)		
	Fructose-fed	49.5 (38.6-55.5)	38.7 (36.2-43.5)		

Table 2 shows that the effect of FDP, preincubation and Cu^{2+} on the pyruvate kinase activity of extracts from several organs of the rat. FDP stimulates pyruvate kinase only in the liver and kidney, having no effect on the enzyme from heart, spleen, brain and erythrocyte, nor on the M-type enzyme of liver. Preincubation inactivates the pyruvate kinase in the extracts of liver and kidney and also the M-type enzyme, but only with liver extract was the activity restored by FDP. Cu^{2+} inhibits not only liver pyruvate kinase but also that of spleen, brain, kidney and erythrocyte. Inhibition of erythrocyte pyruvate kinase has previously been reported by Solvonuk & Collier (1955).

The effects of overnight starvation, and of feeding the animals on fructose, on pyruvate kinase activity in crude extracts of several tissues are shown in Table 3. Only in liver does feeding the animals on fructose cause a significant increase in pyruvate kinase activity. The feeding of the rats on 20% fructose was carried out mainly to study the effects of preincubation and FDP on the induced hepatic pyruvate kinase. A more detailed study of the effect of dietary carbohydrate on hepatic pyruvate kinase has been described by Bailey, Taylor & Bartley (1968). Starving the rats overnight results in low hepatic pyruvate kinase activity as measured in the absence of FDP, but addition of FDP (0.5mM) raises activity to the value found in the controls. However, the effects of preincubation are essentially similar for the pyruvate kinase from livers of starved and control rats.

DISCUSSION

The changes occurring on preincubation of L-type pyruvate kinase are characterized by an increase in co-operativity with respect to both the substrate, PEP, and the allosteric activator, FDP. Though the results obtained without preincubation are not quantitatively reproducible from day to day, they invariably yield curves that appear to have two components, one saturated at low concentrations of PEP, and, superimposed on this, another having a form similar to that of the curve obtained after preincubation. This suggested an explanation of the preincubation changes in terms of two kinds of pyruvate kinase and conversion of one into the other.

The following hypothesis is therefore proposed to account for the complex kinetic changes that occur on preincubation. The enzyme can exist in two forms, L_A and L_B . Type L_A conforms to Michaelis-Menten kinetics and has K_m about 0.1mM for PEP; it is not affected by FDP. Type L_B is allosterically stimulated by FDP and therefore gives a sigmoidal response to increasing PEP concentration in the absence of FDP and a half-maximal activity at about 1mM. In the presence of saturating concentrations of FDP the L_B form of the enzyme gives Michaelis-Menten kinetics and K_m 10 μ M. If the allosteric scheme of Monod, Wyman & Changeux (1965) is adopted the form L_B must itself be postulated to exist as two species, $L_B(R)$ and $L_B(T)$. Initially the enzyme as prepared and used here consists of a mixture of forms L_A and L_B .

This hypothesis accounts for the complex shape of the activity-substrate concentration curve obtained without preincubation and the pure sigmoidal response found after preincubation, as well as the change in shape and the displacement of the curves induced by FDP. It also explains why preincubation results in a decrease of activity at low concentrations of PEP, but to explain how it simultaneously causes stimulation of the activity measured at high concentrations of substrate it is necessary to postulate further that a given molar quantity of form L_B has a greater maximal activity than a similar quantity of form L_A . This would also account for the finding that preincubation stimulates activity when saturating concentrations of FDP are present even at low concentrations of PEP.

The striking changes brought about by this relatively mild treatment (10 min. at 25°) are in marked contrast with the remarkable heat-stability of the enzyme as measured by activity at high concentrations of FDP or PEP. Under these conditions only 50% of the activity is lost by incubation at 50° for 60 min. (C. B. Taylor & E. Bailey, unpublished work). Heat-induced changes in allosteric systems are of particular interest in connexion with the phenomena of desensitization (Monod, Changeux & Jacob, 1963). However, the changes occurring during preincubation of pyruvate kinase appear to fall into a different category, since far from causing desensitization this treatment renders the allosteric component more obvious. The possibility that the L_A form of the enzyme is the M-type described by Tanaka *et al.* (1965) seems to be ruled out by the finding that, though our preparation of M-type pyruvate kinase from liver lost some activity on preincubation at 25°, the loss was not restored by FDP.

The experiments give little information on the physical nature of the transition from form L_A to form L_B . But, clearly, this could be a change in the conformation of the enzyme, an alteration in the degree of molecular aggregation, or even removal of a portion of the pyruvate kinase molecule.

The existence of two forms L_A and L_B and their interconversion could be of significance in the control of pyruvate kinase activity, though as yet there is no evidence to show that these two forms are present *in vivo*. However, the phenomenon of heat-inactivation reversible by FDP is found both in crude extracts of tissue and in the partially purified preparations of pyruvate kinase, indicating that forms L_A and L_B are not simply artifacts of the purification procedure.

The interpretation of the pH-activity curves is complicated by the difficulty of distinguishing between the pH characteristics of the various hypothetical forms of the enzyme and the effect of pH on the interconversion of these forms. The experi-

ments in which the enzyme was preincubated at various pH values and then assayed under standard conditions at pH 7.5 are clearly consistent with the conversion of form L_A into form L_B occurring more readily at high pH. This could, however, obscure an effect of pH on the equilibrium between the two allosteric forms of the L_B species. Thus the data in Fig. 4(d) (pH-activity curves after preincubation at low PEP concentration) could indicate that at low pH even in the absence of FDP most of the enzyme is in the activated form, $L_B(T)$, but the results could equally well be interpreted to mean that at high pH the enzyme is predominantly in the L_B form whereas at low pH the L_A form only is present.

Cu^{2+} inhibits L-type pyruvate kinase of rat liver at concentrations of 10 μM and above, the degree of inhibition increasing with time. It has been suggested by Passeron *et al.* (1967) that the inhibition of pyruvate kinase by Cu^{2+} is reversed by FDP. Though our experimental results are for the most part in agreement with those of Passeron *et al.* (1967) where they can be compared, they do not support the views of these authors on the interaction of Cu^{2+} and FDP on pyruvate kinase. In considering substances that antagonize inhibition by Cu^{2+} it is necessary to distinguish between protection and reversal. Thus a concentration of the powerful chelating agent EDTA that will prevent inhibition if added before the Cu^{2+} (protection) will not reverse it after the Cu^{2+} has been added to the enzyme. Moreover, we find little evidence of reversal of Cu^{2+} inhibition by FDP. The demonstration that FDP can still stimulate pyruvate kinase in the presence of Cu^{2+} does not of itself constitute evidence of reversal of Cu^{2+} inhibition. Thus only slight relief of Cu^{2+} inhibition could be demonstrated by increasing the FDP concentration 200-fold. However, since FDP is more effective when added before than after the inhibitor it clearly protects the enzyme from the effects of Cu^{2+} . There are two ways in which this might occur: either FDP chelates Cu^{2+} , or it causes some conformational change in the enzyme that renders the site attacked by Cu^{2+} less accessible. At first sight chelation does not seem likely, since concentrations of FDP stoichiometrically less than the amount of Cu^{2+} present are capable of providing protection (Fig. 7). However, if PEP chelated Cu^{2+} (see below) the effective concentration of free Cu^{2+} would be considerably less than the total concentration present.

Our results suggest that PEP is more effective in protecting against Cu^{2+} inhibition than is FDP. Little inhibition occurs at 5 mM-PEP even with 0.1 mM- Cu^{2+} , though at 0.1 mM-PEP this concentration of Cu^{2+} causes strong inhibition. Further, the stimulation of pyruvate kinase by FDP in the presence of moderate concentrations of Cu^{2+} is

greatly potentiated by raising the PEP concentration (Fig. 6). These effects of PEP could be due to chelation of Cu^{2+} or to conformational changes, as suggested in the case of FDP. It is noteworthy that both substrate and activator appear to protect against Cu^{2+} , and both would cause the same conformational change according to Monod *et al.* (1965). The hypothetical allosteric change from form $\text{L}_B(\text{R})$ to form $\text{L}_B(\text{T})$ might be accompanied by a change in susceptibility to Cu^{2+} . However, electrometric measurements (C. B. Taylor & E. Bailey, unpublished work) have shown that both PEP and FDP remove Cu^{2+} from solution stoichiometrically. Though the concentrations used to demonstrate this are rather higher than in the pyruvate kinase experiments, they suggest that chelation alone may be a sufficient explanation of the inter-relationship between the effects of Cu^{2+} , FDP and PEP on pyruvate kinase. Further, our results suggest that Cu^{2+} inhibition is not reversed readily enough to make it likely that this inhibition is part of the control system regulating glycolysis in the liver.

High-carbohydrate diets, and in particular diets containing fructose, have been shown to increase hepatic pyruvate kinase activities in the rat (Krebs & Eggleston, 1965; Tanaka, Harano, Sue & Morimura, 1967; Bailey *et al.* 1968). The present results indicate that this effect is confined to the liver and is not found in kidney, brain, heart or spleen. This is in accord with the observations that only the L-type pyruvate kinase is influenced by diet, and that of the tissues tested here only liver contains L-type enzyme (Tanaka *et al.* 1967).

The effect of brief (overnight) starvation is of interest since, though maximal activity is un-

affected, activity in the absence of FDP is significantly lower than in controls. Though this could reflect a change in the form of the enzyme, it may indicate a decreased FDP concentration in the liver of the starved rat. This change on brief starvation must be distinguished from the decrease in total pyruvate kinase found after 24–48 hr. starvation (Krebs & Eggleston, 1965; Tanaka *et al.* 1967).

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