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Pyruvate Kinase

Classes of Regulatory Isoenzymes in Mammalian Tissues

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Kinetic studies of the pyruvate kinases of rat and human tissues have led to the identification of three classes of isoenzymes with qualitative differences in regulatory properties.

Class L, the major component in liver extracts and a minor component of kidney extracts, shows markedly sigmoidal kinetics with respect to the concentration of phospho*enol*pyruvate, allosteric inhibition by ATP and alanine, and activation by fructose 1,6-bisphosphate. Class A, present in adipose tissue, and the major component in kidney and the minor one in liver, shows slightly sigmoidal substrate saturation curves and is allosterically inhibited by alanine and activated by fructose 1,6-bisphosphate. Class M, present in muscle and brain, has none of the above regulatory properties.

The activities of the pyruvate kinases of classes L and A respond immediately to changes in the concentration of the effectors alanine and fructose 1,6-bisphosphate.

The two regulatory isoenzymes are strongly inhibited by three amino acids: alanine, cysteine, and phenylalanine. Cysteine is the stronger inhibitor for class L, while phenylalanine is the stronger one for class A. The allosteric inhibition is stereospecific for the L-amino acids in contrast with the weak isosteric inhibition of muscle pyruvate kinase by L- or D-alanine as analogue of the product.

The allosteric inhibition of pyruvate kinase L by ATP is highly specific in contrast with a rather wide specificity of this enzyme for nucleoside diphosphates as acceptor substrates.

Evidence for the occurrence of several isoenzymes of pyruvate kinase in higher animals, particularly in the rat, has been reported from several laboratories [1–3]. While most of the classifications have been based on electrophoretic evidence, a marked contrast in regulatory properties of the major component of pyruvate kinase activity in liver ("L") with respect to the older known muscle enzyme ("M") has also been amply demonstrated [2,4–8]. A systematic screening for key kinetic properties of the pyruvate kinase activity in certain rat tissues and fractions, prompted by the interest in the physiological significance of the potential control of the pyruvate kinase reaction in different types of metabolism, has given results which indicate that there are at least three classes of isoenzymes of pyruvate kinase differing in regulatory properties in rat and human tissues: the highly regulatable class L, the apparently unsophisticated class M, and a regulatable class of intermediate complexity, inhibited by alanine and activated by fructose 1,6-bisphosphate, which we propose to designate as class "A" because it was first observed in adipose tissue [9][1]. Class A, rather than M as formerly reported, is the major component of the pyruvate kinase activity in kidney extracts and the minor one in liver extracts. A comparative study of the allosteric properties of the classes A and L from kidney and liver is also reported here.

Abbreviations. Fru-P_2, fructose 1,6-bisphosphate; *P*-pyruvate, phospho*enol*pyruvate.

Enzyme. Pyruvate kinase (EC 2.7.1.40).

MATERIALS AND METHODS

Wistar rats fed on a standard laboratory diet were used. Tissues were homogenized in the cold (2–4 °C) with 3 vol. of 0.25 M sucrose (except in the case of adipose tissue where 7 vol. was used) in a Kontes Dual-Grinder homogenizer, or, for the muscular tissues, a Virtis homogenizer. A sample of human liver from a surgical biopsy was obtained from Dr V. Rojo

[1] This grouping of isoenzymes in classes does not interfere with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [10] for the designation of individual isoenzymes.

(Centro Nacional de Investigaciones Médico-Quirúrgicas de la Seguridad Social, Madrid). The homogenates were centrifuged at 30000 × *g* for 30 min. Ammonium sulphate fractionation of tissue extracts was carried out by addition, to the latter, of a saturated solution of ammonium sulphate (at 2—4 °C, neutralized) to give the desired percentage saturations. The 30 to 45% and the 55—70% saturation precipitates, collected by centrifugation at 20000 × *g* for 15 min, were dissolved in 25 mM Tris-HCl pH 7.4 containing 0.1 mM EDTA and 1 mM dithioerythritol and dialyzed for 2 h against the same mixture. The 30 to 45% fraction was routinely preincubated 5 min at 38 °C just before assay [8].

Pyruvate kinase activity was assayed spectrophotometrically following the decrease in absorbance at 340 nm, at room temperature (approx. 25 °C) in a reaction mixture containing, unless indicated otherwise, 50 mM imidazole-HCl pH 7.0, 0.1 M KCl, 5 mM $MgCl_2$, 0.15 mM NADH, 1 mM MgADP, 1 U/ml lactate dehydrogenase, and 5 mM phospho*enol*-pyruvate. Standard concentrations of potentially regulatory metabolites added were 2 mM for alanine and MgATP and 10 μM for Fru-P_2.

Amino acids were of the L-series, unless indicated otherwise. They were obtained from Calbiochem, Mann, Fisher and Sigma. Muscle pyruvate kinase and auxillary enzymes were obtained from Boehringer. Nucleotides were obtained from Sigma, except CDP which was from Boehringer.

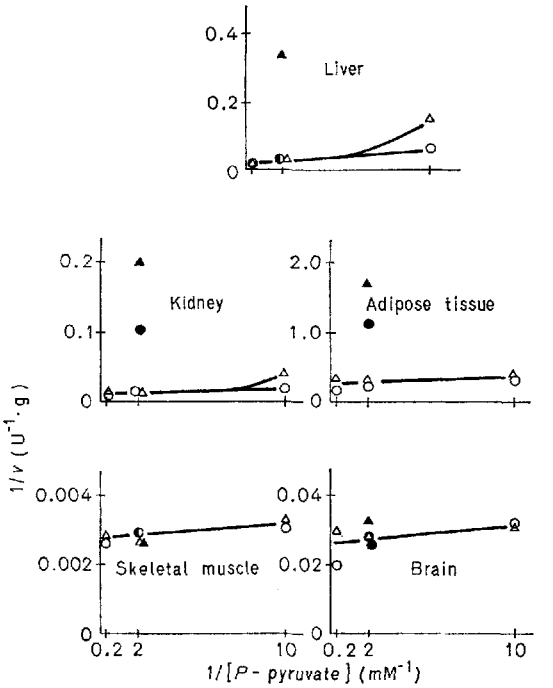

Fig. 1. *Effects of the concentration of* P-*pyruvate, alanine, and cold pretreatment on the pyruvate kinase activity of certain rat tissues.* Extraction and assay as indicated in Materials and Methods. Extracts were prewarmed at 38 °C for about 5 min (triangles) or kept at 0 °C (circles) just until assay at room temperature. Filled symbols: assay in the presence of 2 mM alanine; open symbols, without added alanine

RESULTS

Effects of Alanine on the Pyruvate-Kinase Activity of Extracts and Fractions of Different Tissues

While class L pyruvate kinase is allosterically inhibited by both ATP and alanine [8], it was found that the enzyme from adipose tissue [9] and kidney extracts was strongly inhibited by alanine but not by ATP. A combination of alanine and cold pretreatment [8] has led to the distribution of major tissues of the rat in three groups. As shown in Fig. 1, the pyruvate kinase activity in liver extracts is strongly inhibited by 2 mM alanine, at moderate concentrations of phospho*enol*pyruvate if the extract is preincubated at 37 °C before the assay; kidney and adipose tissue extracts are inhibited by alanine irrespective of temperature and skeletal muscle and brain (as well as heart and uterus) extracts are never inhibited by 2 mM alanine.

Liver and kidney extracts were fractionated by precipitation with ammonium sulphate to separate the two isoenzymes known to occur in each of these tissues, and the fractions corresponding to the two isoenzymes were characterized with respect to allosteric properties by the use of a few purposely selected experimental conditions. The results in Table 1 show for the lower ammonium sulphate fraction a behaviour consistent with the allosteric properties of pyruvate kinase L: (a) homotropic cooperativity for phospho*enol*pyruvate, inhibition by (b) ATP and (c) alanine, and (d) activation by Fru-P_2. In contrast, the higher ammonium sulphate fraction of both tissues, is inhibited by alanine but not by ATP, is activated by Fru-P_2, and exhibits slight cooperativity with respect to phospho*enol*pyruvate in the absence of alanine. The effect of the concentration of alanine on the inhibition of pyruvate kinase in the two higher ammonium sulphate fractions of liver and kidney and in tissue extracts is shown in Fig. 2. In the three cases the effect of the concentration of alanine on the activity with 0.5 mM phospho*enol*pyruvate reached 50% inhibition at about 0.1 mM and was about 90% at 5 mM. These results suggest that the three cases involve the same type of regulatory, isoenzyme of pyruvate kinase, which henceforth will be designated as class A. This conclusion is reinforced by other kinetic parallelism between the major component of kidney extracts and the minor one of liver extracts that will be reported below, but leaves quite open the possibility of each class involving several individual isoenzymes that could differ in other properties.

Table 1. *Kinetic characterization of the pyruvate kinase isoenzymes in rat liver and kidney fractions*
Extract of rat liver and kidney were obtained and fractionated, and pyruvate kinase activity was assayed as described in Materials and Methods except for the concentrations of phospho*enol*pyruvate (*P*-pyruvate) and effectors as indicated. All fractions were preincubated for about 5 min at 37 °C just before assay. Results with the 55—70% fraction from liver in the presence of added Fru-P_2 were corrected for a small blank value caused by this metabolite with this aldolase-rich fraction

Additions	Liver				Kidney			
	30—45%		55—70%		30—45%		55—70% $(NH_4)_2SO_4$	
	U/g tissue							
Fru-P_2 addition, 10 μM	—	+	—	+	—	+	—	+
P-pyruvate, 0.1 mM	0.5	10	0.4	0.6	0.3	1.1	5	6
P-pyruvate, 0.5 mM	7	11	0.8	0.9	1.0	1.6	10	10
P-pyruvate, 0.5 mM + alanine, 2 mM	0.5	12	0.2	0.9	0.1	1.3	1	9
P-pyruvate, 0.5 mM + MgATP, 2 mM	1	13	0.6	0.7	0.4	1.4	10	10
P-pyruvate, 5 mM	12	13	1.2	1.1	2.0	2.2	12	13

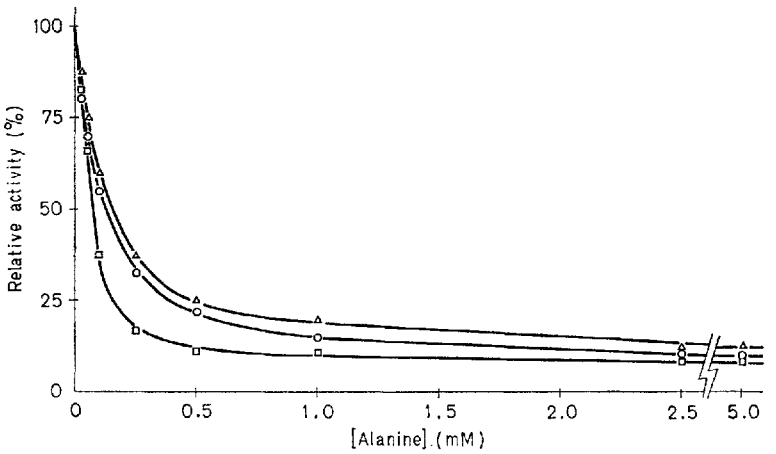

Fig. 2. *Effect of the concentration of alanine on the activity of pyruvate kinase A from rat kidney, liver, and adipose tissue.* 55—70% ammonium sulphate fractions of extracts from liver (△), kidney (○), and adipose tissue (□) were obtained and assayed as indicated in Materials and Methods, with 0.5 mM phospho*enol*pyruvate

Time Course of the Inhibition by Alanine and the Activation by Fru-P_2 *of Pyruvate Kinases L and A*

Llorente *et al.* [8] showed that the strong inhibition of pyruvate kinase L by ATP started without detectable lag and disappeared on removal of ATP with an enzymatic trap. Fig. 3 shows that the inhibition of pyruvate kinases L and A by alanine and its counteraction by Fru-P_2 also appear and disappear very rapidly after addition and removal, respectively. The marked transition visible in the figure after the Fru-P_2 trap corresponds to the contribution of the enzymatic trap during the removal of Fru-P_2; because of the high affinity of the enzyme for Fru-P_2, the initial rate of NADH disappearance is increased since the Fru-P_2 trap is acting before the pyruvate kinase runs short of activator. Observations of Hess and Kutzbach [11] on electrophoretic behaviour of pyruvate kinase L that suggested the possibility of considerable hysteresis in the disappearance of the conformational change induced by Fru-P_2 should be ascribed rather to the very high affinity of the enzyme for Fru-P_2 when saturated with phospho*enol*pyruvate. (B. Hess, personal communication). As previously emphasized for pyruvate kinase L [8], full activation of pyruvate kinase A, even in the presence of concentrations of alanine in the upper physiological range, can be achieved by a concentration of Fru-P_2 as low as approx. 10 μM, within the physiological range of free Fru-P_2 in liver.

Specificity of the Allosteric Inhibition by Alanine of Pyruvate Kinases L and A from Liver and Kidney

The 20 amino acids common to proteins were tested as effectors of pyruvate kinases of classes L and A with preparations from both liver and kidney. From the results summarized in Table 2 it appears that there are three amino acids of particular interest: alanine, cysteine and phenylalanine. Stronger inhibitors than alanine are cysteine for pyruvate kinase L and phenylalanine for pyruvate kinase A. Some

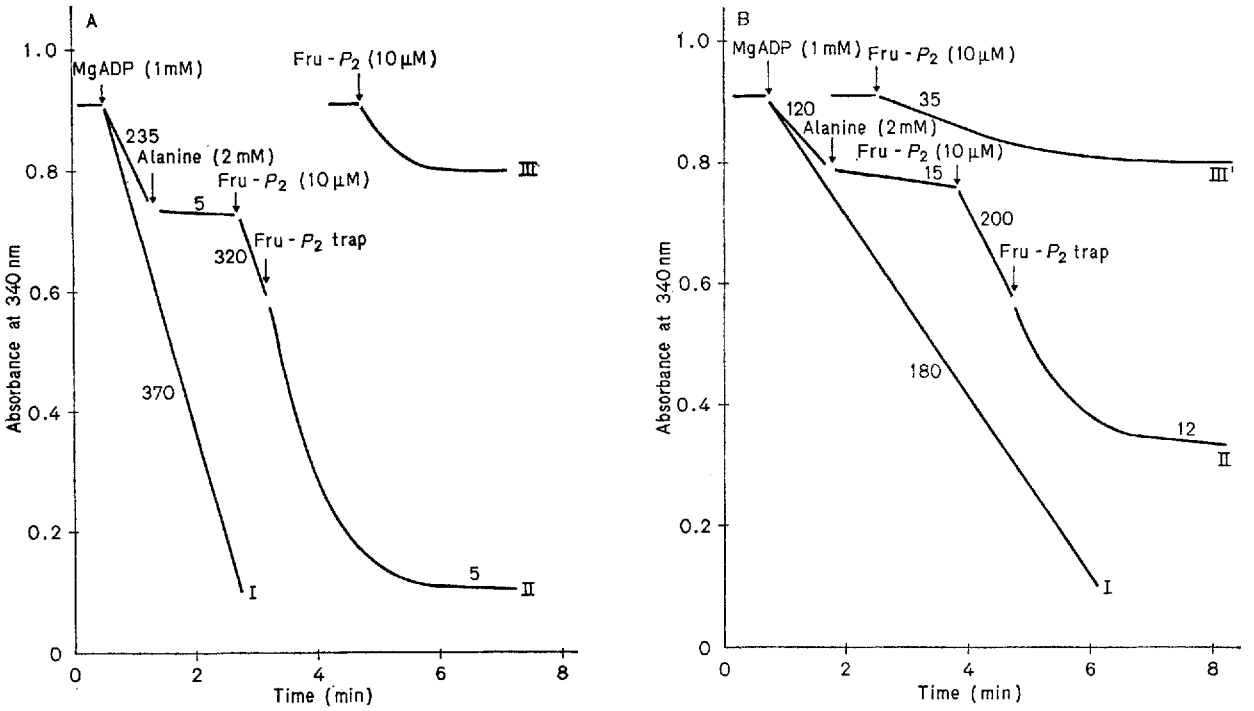

Fig. 3. *Time course of the inhibition by alanine and the activation by Fru-*P_2 *of pyruvate kinases L and A from rat liver.* 30—45% (A) and 55—70% (B) ammonium sulphate fractions from rat liver, obtained as described in Materials and Methods, were used for pyruvate kinases of class L and A respectively. Pyruvate kinase activity was assayed in I as described in Materials and Methods, and in II after 5-min preincubation at 37 °C and with phospho*enol*pyruvate at 0.5 mM in the case of class L and at 0.2 mM in that of class A, plus additions (in about 10-μl volumes) to give final molarities as indicated in brackets; the Fru-P_2 trap was a mixture of 2 U aldolase, 10 U glycerol-3-phosphate dehydrogenase, and 60 U triosephosphate isomerase. Insert III is a test of the Fru-P_2 trap system without liver fraction; insert III' is a test of the utilization of Fru-P_2 by the corresponding liver enzymes present in the 55—70% fraction, in the absence of added MgADP. The numbers accompanying lines indicate reaction rates in 10^3 (ΔA_{340}/min)

Table 2. *Inhibition by amino acids of the pyruvate kinases of classes L and A from rat liver and kidney*
30—45% and 55—70% ammonium sulphate fractions of rat liver and kidney extracts, obtained as described in Materials and Methods, were used for classes L and A respectively and assayed with 0.5 mM *P*-pyruvate. The class L fractions were preincubated 5 min at 37 °C prior to assay. Results are expressed as a percentage of the activity of controls without added amino acids. Control values were per cuvette: 180 U and 95 U for class L from liver and kidney respectively, and 120 U for class A from both liver and kidney

Amino acid added	Class L				Class A			
	Liver		Kidney		Liver		Kidney	
	1 mM	5 mM	1 mM	5 mM	1 mM	5 mM	1 mM	5 mM
	%	%	%	%	%	%	%	%
Control	100		100		100		100	
Alanine	10	2	20	5	20	15	15	10
Phenylalanine	70	45	65	30	15	5	10	5
Cysteine	5	2	25	15	40	35	40	35
Valine	85	50	70	45	70	40	85	45
Isoleucine	—	70	—	—	80	35	65	45
Proline	55	15	65	15	60	20	55	40
Tryptophan	—	85	—	—	50	20	50	30
Methionine	—	65	—	90	60	25	55	40
Glycine	—	55	80	70	—	95	—	—
Serine	70	50	80	70	—	120	—	110
Threonine	—	60	—	—	50	40	45	40
Tyrosine	80	55	75	60	75	45	85	60
Asn, Leu, Gln, Asp, Glu, Lys, Arg, His	>70				>70			

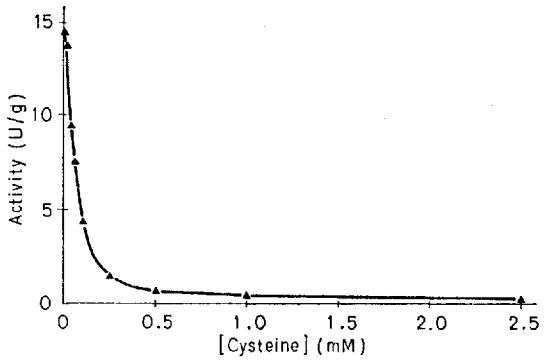

Fig. 4. *Effect of the concentration of cysteine on the activity of pyruvate kinase L from rat liver.* A 30—45% ammonium sulphate fraction of rat liver extract was obtained and assayed as described in Materials and Methods, with 0.25 mM phospho*enol*pyruvate and cysteine as indicated

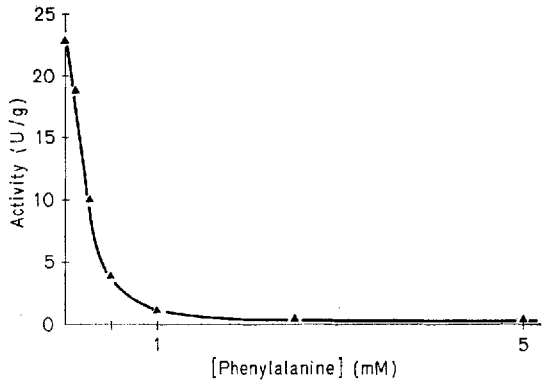

Fig. 5. *Effect of the concentration of phenylalanine on the activity of pyruvate kinase A from rat kidney.* A 55—70% ammonium sulphate fraction of rat liver extract was obtained and assayed as described in Materials and Methods, with 0.5 mM phospho*enol*pyruvate and phenylalanine as indicated

Table 3. *Effects of alanine, phenylalanine, cysteine, and mixtures of them on pyruvate kinases of classes L and A*
Experimental conditions as in Table 2. All amino acids were used at 5 mM concentrations. Results are expressed as a percentage of the activity of the controls

Effectors	Class L (liver)	Class A (kidney)
	%	%
None	100	100
Alanine	4	18
Phenylalanine	75	8
Cysteine	2	40
Alanine + phenylalanine	4	—
Alanine + cysteine	—	34
Phenylalanine + cysteine	2	20

Table 4. *Stereospecificity of the alanine effects on pyruvate kinases of classes L and M, as either an allosteric or an isosteric effector respectively*
Enzyme preparations were obtained as indicated in Materials and Methods. Class L was assayed as indicated in Materials and Methods, while class M was assayed by coupling to hexokinase (0.7 U) and glucose-6-phosphate dehydrogenase (1.5 U) with 1 mM glucose and 0.4 mM NADP. KCl was 0.15 M except when testing the effect of pyruvate, which was used as the potassium salt. Activities are expressed as a percentage of controls

Effector	Concn	Class L (from rat liver)	Class M (from rabbit muscle)	
		0.5 mM	0.1 mM	2 mM *P*-pyruvate
	mM	%	%	%
None		100	100	100
L-Alanine	1	21	—	—
D-Alanine	1	100	—	—
L-Alanine	10	<5	—	—
D-Alanine	10	90	—	—
L-Alanine	300	—	52	100
D-Alanine	300	—	56	110
Pyruvate	150	—	11	69

differences in apparent sensitivity according to the source of the enzyme preparation might not be significant because of the probability of some contamination, particularly with class A in the case of the lower ammonium sulphate fraction of rat kidney.

The effect of the concentration of cysteine on the activity of pyruvate kinase L from rat liver is illustrated in Fig. 4. At 0.25 mM phospho*enol*pyruvate, as little as 0.06 mM cysteine is enough for 50% inhibition. The strong inhibition by cysteine is not shared by other compounds with free —SH groups, since neither dithioerythritol, glutathione, DL-homocysteine or even D-cysteine, at 1 mM, were inhibitory in similar conditions. Fig. 5 illustrates the strong inhibition of pyruvate kinase A by phenylalanine.

The above three strongly inhibitory amino acids were assayed in pairs on the two classes of regulatory pyruvate kinases, as shown in Table 3. With pyruvate kinase L the strong inhibition by either cysteine or alanine were not appreciably affected by equimolar phenylalanine. Nevertheless, with pyruvate kinase A, equimolar mixtures of either phenylalanine or alanine with cysteine were less inhibitory than the former amino acids alone. These observations might contribute to the ascertaining of the number of amino acid regulatory sites, which will be discussed below.

Differential stereospecificity eliminates the possibility that the inhibition by alanine of pyruvate kinases L and A could be related to the fact that alanine is a rather close structural analogue of the other product, pyruvate. The results in Table 4 indicate that alanine is indeed a potential isosteric inhibitor of pyruvate kinase, but that there are drastic differences both quantitative and qualitative between the strong and stereospecific inhibition of pyruvate kinase L (as well as that of type A, not

Table 5. *Substrate and allosteric specificity for nucleotides of pyruvate kinase L from rat liver*

A 30—45% ammonium sulphate fraction of rat liver extract, obtained as described in Materials and Methods and virtually free of adenylate kinase, was used as enzyme preparation. Assays were carried out as indicated in Materials and Methods with substrates and effectors as indicated. Results are expressed in relative values, taking as 100 that obtained with the higher concentration of donor and acceptor substrate

(A) Nucleoside diphosphates as substrates	Relative activities (with 2.5 mM *P*-pyruvate)		(B) Nucleoside triphosphates as inhibitors (5 mM)	Relative activities (with 0.25 mM *P*-pyruvate and 1 mM MgADP)	
	1 mM	5 mM		— Fru-P_2	+ Fru-P_2 10 μM
			None	35	70
MgADP	86	100	MgATP	1	62
MgADP[a]	29	69	MgGTP[a]	36	50
MgIDP	15	50	MgITP	26	72
MgUDP[a]	25	65	MgUTP[a]	19	19[b]
MgCDP[a]	1	4	MgCTP[a]	22	66

[a] To rule out or eliminate ATP impurities, yeast hexokinase (1.5 U) and 2-deoxyglucose (10 mM) were added to the reaction mixture prior to pyruvate kinase.

[b] Raising the concentration of added Fru-P_2 to 0.1 mM did not affect the inhibition by UTP.

included in the Table) by L-alanine. and the weak isosteric inhibition of pyruvate kinase M by either L- or D-alanine. The inhibitions by phenylalanine and cysteine are also stereospecific for the L-isomer and can be counteracted by low concentrations of Fru-P_2.

Allosteric Inhibition of Pyruvate Kinase L by ATP

The strong inhibition by ATP of native pyruvate kinase L has been reported to be an allosteric effect subject to reversible desensitization [8]. Nucleotide specificity studies with the 30—45% ammonium sulphate fraction of rat liver, fully confirm this conclusion, since there is a marked difference between a wide specificity for nucleoside diphosphates as acceptors (ADP, GDP, UDP, and IDP) and a narrow one for Fru-P_2 reversible inhibition by nucleoside triphosphates, which is highly specific for ATP (Table 5). This observation fulfills a major requirement of those outlined by Sols [12] to identify as allosteric certain effects by primary products. Although this allosteric inhibition can be reversed by increasing the concentration of phospho*enol*pyruvate it is not related to the inhibition of muscle pyruvate kinase, well characterized by Reynard *et al.* [13] as competitive respect to both ADP and phospho*enol*-pyruvate because of steric hindrance on the active subsites.

The lack of significant allosteric inhibition by ATP of the class A isoenzyme has been substantiated in several ways. Lowering the phospho*enol*pyruvate concentration to 0.1 mM, while increasing the inhibition of pyruvate kinase activity in a kidney extract by 2 mM alanine to about 95%, gave only a weak (≈30%) inhibition by 2 mM ATP of total pyruvate kinase activity in the presence as well as in the absence of Fru-P_2. In mixtures of extracts of liver and kidney there was inhibition by ATP as expected for the proportion of pyruvate kinase L present in the mixtures.

*Effect of the Concentration of Phospho*enol*pyruvate on the Activity of Pyruvate Kinase A after Different Treatments*

Total pyruvate kinase activity in kidney extracts, of which the major component belongs to class A as shown above, gave nearly hyperbolic saturation curves for phospho*enol*pyruvate with $[S]_{0.5}$ of ≈0.1 Mm. Nevertheless, after fractionation with ammonium sulphate the $[S]_{0.5}$ went up to 0.6 mM. The results in Fig. 6 indicate that the difference is related to the combined effect of the use of a cold buffer containing EDTA to dissolve the pyruvate kinase A after its precipitation with ammonium sulphate. In the absence of this treatment isolated pyruvate kinase A from rat kidney exhibited a K_m of 0.1 mM. The increase in K_m induced by the EDTA-containing buffer can be reversed by preincubation of the preparation at 37 °C. The EDTA-mediated effect is reminiscent of the observations of changes in behaviour of the pyruvate kinase in extracts of adipose tissue [14], a tissue that seems to contain pyruvate kinase of class A only [9].

Characterization of Classes L and A of Pyruvate Kinase in Human Liver

A sample of human liver obtained by surgical biopsy was homogenized and the extract fractionated with ammonium sulphate essentially as indicated in Materials and Methods. Fig. 7 gives the kinetic characterization of the 25—45% ammonium sulphate fraction. The following $[S]_{0.5}$ and n (Hill coefficient) values for phospho*enol*pyruvate were calculated: without additions, 0.2 mM and 1.9; with 2 mM MgATP, 0.4 mM and 3.3; with 2 mM alanine, 0.7 mM and 3.6; with 2 mM cysteine, 1.1 mM and ≈4.0; and with 10 μM Fru-P_2, <0.05 mM. These results correspond to pyruvate kinase of the L class, as defined above.

The kinetic characterization of the 55—75% ammonium sulphate fraction is summarized in Table 6. The results clearly indicate that human liver, like that of the rat, contains, in addition to a class L pyruvate kinase, a class A isoenzyme which is allosterically inhibitable by alanine (and phenylalanine) but not by MgATP, and which is strongly activable by Fru-P_2.

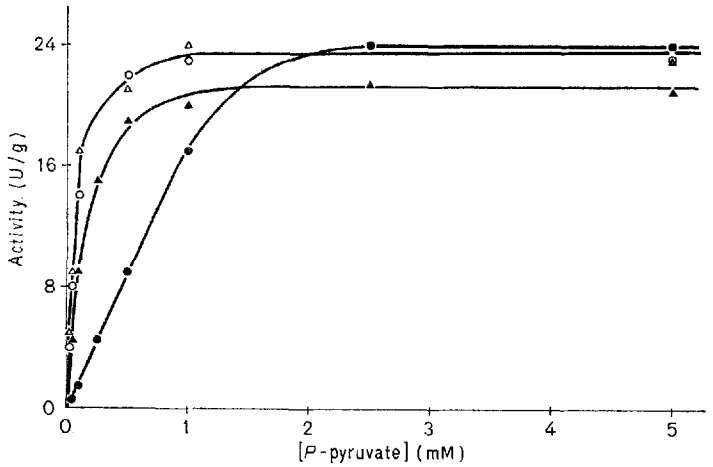

Fig. 6. *Effects of EDTA and preincubation temperature on the activity of rat-kidney pyruvate kinase A at different concentrations of phosphoenolpyruvate.* A 55—70% ammonium sulphate fraction of rat kidney was obtained and assayed as described in Materials and Methods, except that the precipitated proteins were dissolved in either 0.25 M sucrose (open symbols) or in 50 mM Tris pH 7.4, 0.1 mM EDTA, 1 mM dithioerythritol, and assayed without (circles) or with 5-min preincubation at 37 °C (triangles)

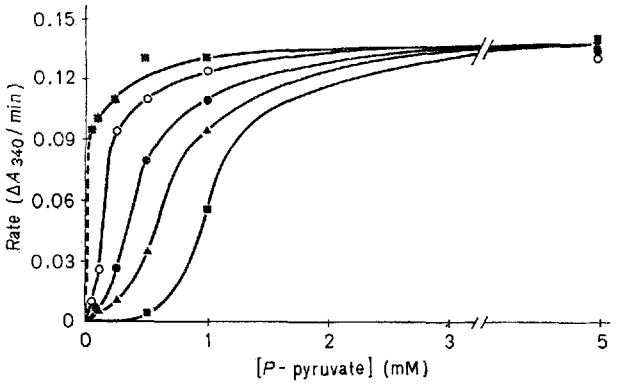

Fig. 7. *Allosteric properties of class L pyruvate kinase from human liver.* A 25—45% ammonium sulphate saturation fraction of human liver was obtained and assayed as described in Materials and Methods, at different concentrations of phospho*enol*pyruvate and with additions as follows: (○) no additions; (●) 2 mM MgATP; (▲) 2 mM alanine; (■) 2 mM cysteine; (*) 10 μM Fru-P_2

Table 6. *Allosteric characterization as class A of the pyruvate kinase activity in the 55—75% ammonium sulphate fraction of human liver*

A human liver sample was homogenized, fractionated and assayed essentially as described in Materials and Methods with 0.25 mM *P*-pyruvate and additions as indicated

Inhibitors (2 mM)	Fru-P_2 (10 μM)	Activity	Effect
		U/mg	(%)
None	—	65	
None	+	135[a]	activation (107)
Alanine	—	20[b]	inhibition (67)
Alanine	+	130	
Phenylalanine	—	8[c]	inhibition (88)
Phenylalanine	+	135	
MgATP	—	67	no inhibition

[a, b, c] Lowering the $[S]_{0.5}$ for *P*-pyruvate from (a) 0.4 to <0.05 mM b) to 0.8 mM and (c) to 1.0 mM.

DISCUSSION

The increasing number of recognizable distinct forms of pyruvate kinase in animal tissues [15—17] makes it convenient for studies of metabolic control at the enzyme level to group them in classes according to regulatory properties. The present work gives evidence that in higher animals there are at least three classes of isoenzymes of pyruvate kinase differing in regulatory properties in a well-defined and consistent pattern. These classes are not mere conformational varieties of a given isoenzyme [11]. On the contrary, each of these classes includes, or might include, several different isoenzymes, even in the restricted sense of genetically determined differences in primary structure [10].

Of the three classes, two have well-defined allosteric properties suitable for metabolic control in physiological conditions. These two, the L and A classes, share a number of regulatory properties but differ in the allosteric inhibition by ATP, which occurs in class L but not in class A. ATP as a primary product of the pyruvate kinase reaction in its physiological direction is an isosteric inhibitor, as shown by Reynard *et al.* [13] with the muscle enzyme. The L class is not merely more sensitive to the same type of inhibition but has a specific mechanism for inhibition by ATP which is clearly allosteric on the basis of the convergence of the following criteria, among those outlined by Sols [12]: (a) it is indepen-

dent of the concentration of ADP; (b) there is marked differential specificity, particularly with respect to the guanosine nucleotides; (c) the enzyme can be reversibly desensitized by standing in the cold; (d) it is counteracted by the allosteric effector Fru-P_2 and (e) it does not occur in enzymes from other sources such as, the M and A classes, as well as the yeast enzyme. Minor differences in reported K_i values for ATP between muscle pyruvate kinase and the non-L component in liver extracts [18] are not likely to involve any significant non-isosteric component.

The difference between the A and the L classes of isoenzymes of pyruvate kinase implies more than the restriction to the latter of the allosteric inhibition by ATP. Although these two regulatory isoenzymes are both allosterically inhibited by alanine within the physiological range of concentrations, there are marked differences in the specificity of inhibition by amino acids. Class L is more sensitive to inhibition by cysteine than by alanine and much less sensitive to phenylalanine, while class A is more strongly inhibited by phenylalanine and only weakly by cysteine. The results do not permit a conclusion as to whether a single site with a different specificity pattern in each class of isoenzyme accounts for all these inhibitions or if two or three different amino acid inhibition sites are involved in each isoenzyme. The fact that serine is appreciably inhibitory for class LI, which is the ones trongly inhibited by cysteine and the behaviour of mixtures of amino acids (Table 3) tend to suggest a site for cysteine in class L. Nevertheless, some other type of information would be required to ascertain this matter. In any case, of these three amino acids only alanine is known to occur in liver at concentrations suitable for clearly important inhibitions *in vivo* [19—21]. Carminatti and coworkers [22—24] and Tanaka and coworkers [16,17] have independently observed the strong inhibition by alanine of the non-L component of pyruvate kinase in liver and kidney extracts. The independent observation of Tanaka's and Garminatti's groups of certain regulatory properties of the non-L fraction of liver and kidney is reassuring. Their failure to observe counteraction by Fru-P_2 of the inhibition by alanine of what we refer to as class A (Tanaka's M_2, Carminatti's kidney I) suggests desensitization in their highly purified preparations. Pyruvate kinase L and A are not significantly activated by any of the protein amino acids, including histidine [25].

All available evidence indicates that muscle pyruvate kinase is not a regulatory enzyme. The inhibition by phenylalanine described by Carminatti *et al.* [26] has concentration requirements and pH dependence orders of magnitude outside the physiological range. From their data and the reported physiological concentration of phenylalanine in muscle of about 0.15 mM [27] the possible inhibition *in vivo* would be less than 5%. The significance of the potential inhibition by phenylalanine of the pyruvate kinase M from muscle (the binding has been recently studied by Kayne and Price [28]), as well as that from brain [29], remains to be ascertained. Another marginal phenylalanine effect, the inhibition of the intestinal isoenzyme of alkaline phosphatase, has been shown to be neither regulatory nor allosteric [30][2].

Alanine is the predominant amino acid going from muscle to liver in fasted animals [31], partly substituting for lactate in the classical Cori cycle. It has been characterized as a major precursor of carbon skeletons for gluconeogenesis [32,33]. Also, it has been found to be a powerful stimulant of glucagon secretion [24]. These facts make alanine a most suitable candidate for the negative control of pyruvate kinase activity essential to prevent large-scale diversion of phospho*enol*pyruvate in gluconeogenesis.

We have no plausible explanation for the occurrence of two different classes of allosteric, alanine-inhibitable, fructose-bis-phosphate-activatable pyruvate kinases in animal tissues, nor for the occurrence of a regulatable pyruvate kinase in non-gluconeogenic tissues. A similar problem concerns phospho*enol*-pyruvate carboxykinase in these tissues. For adipose tissue the suggestion has been made that it could be related to glycerogenesis involving the lower part of the "gluconeogenic pathway", although the large excess of pyruvate kinase makes unlikely an efficient control of its activity for such a purpose. And not even such a hypothetical role is apparent for the non-parenchymal cells of the liver, where the pyruvate kinase seems to be also of class A [35,36]. It might not be fortuitous that there is apparent paralellism between the distribution of a regulatable pyruvate kinase and that of phospho*enol*pyruvate carboxykinase, well characterized in adipose tissue and whose absence in skeletal muscle seems also well established. Glutamate, glutamine, aspartate and asparagine constitute about one fourth of the protein pool that can lead to intermediate of the Krebs cycle through the action of glutamate dehydrogenase, glutamic oxalacetic transaminase, the urea cycle, and the purine nucleotide cycle [37]. For substantial use of these carbon skeletons for energy or fatty acid synthesis there is need of an ancillary pathway to acetyl-CoA. The pair phospho*enol*pyruvate carboxykinase—pyruvate kinase can insure such a conversion by an NADPH-independent system. One is tempted to speculate that once regulatable as well as non-regulatable classes of pyruvate kinase have been established in an organism, the former could be located in the genome in such relation to phospho*enol*pyruvate carboxykinase that express-

[2] The pyruvate kinase of *Rhodotorula glutinis* is not appreciably affected by phenylalanine at 10 mM (J. M. Gancedo, personal communication).

ibility of the latter in certain tissues would tend to be accompanied precisely by that of a regulatable pyruvate kinase, regardless of its ultimate value in a given tissue. In any case the occurrence of a regulatable pyruvate kinase in these tissues might be useful to buffer the levels of glycolytic intermediate from Fru-P_2 to phospho*enol*pyruvate.

Pyruvate kinase activity in rat liver was shown to vary with nutritional conditions [38], the changes being due to variations in the level of the L isoenzyme [2]. In kidney, pyruvate kinase L behaves also as an adaptive enzyme (increased by high fructose diet), while class A does not change in either organ [39].

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