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A Comparison of the Regulation of Pyruvate Dehydrogenase in Mitochondria from Rat Brain and Liver

By RICHARD JOPE and J. P. BLASS
Departments of Biological Chemistry and Psychiatry and the Mental Retardation Research Center, UCLA School of Medicine, Los Angeles, Calif. 90024, *U.S.A.*

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The total activity of pyruvate dehydrogenase in mitochondria isolated from rat brain and liver was 53.5 and 14.2nmol/min per mg of protein respectively. Pyruvate dehydrogenase in liver mitochondria incubated for 4min at 37°C with no additions was 30% in the active form and this activity increased with longer incubations until it was completely in the active form after 20min. Brain mitochondrial pyruvate dehydrogenase activity was initially high and did not increase with the addition of Mg^{2+} plus Ca^{2+} or partially purified pyruvate dehydrogenase phosphatase or with longer incubations. The proportion of pyruvate dehydrogenase in the active form in both brain and liver mitochondria changed inversely with changes in mitochondrial energy charge, whereas total pyruvate dehydrogenase did not change. The chelators citrate, isocitrate, EDTA, ethanedioxybis(ethylamine)tetra-acetic acid and Ruthenium Red each lowered pyruvate dehydrogenase activity in brain mitochondria, but only citrate and isocitrate did so in liver mitochondria. These chelators did not affect the energy charge of the mitochondria. Mg^{2+} plus Ca^{2+} reversed the pyruvate dehydrogenase inactivation in liver, but not brain, mitochondria. The regulation of the activation–inactivation of pyruvate dehydrogenase in mitochondria from rat brain and liver with respect to energy charge is similar and may be at least partially regulated by this parameter, and the effects of chelators differ in the two types of mitochondria.

The pyruvate dehydrogenase complex is an intramitochondrial multienzyme complex that catalyses the conversion of pyruvate into acetyl-CoA and CO_2. It is regulated in mammals by a phosphorylation–dephosphorylation cycle catalysed by a pyruvate dehydrogenase kinase and a pyruvate dehydrogenase phosphatase (Linn *et al.*, 1969, 1972). The pyruvate dehydrogenase kinase, which is tightly bound to the pyruvate dehydrogenase complex, inactivates pyruvate dehydrogenase specifically by transferring a phosphate group from $MgATP^{2-}$ to a serine moiety of pyruvate dehydrogenase (Barrera *et al.*, 1972). Known inhibitors of pyruvate dehydrogenase kinase include pyruvate ($K_i = 1$ mM) and ADP ($K_i = 0.1$ mM, competitive with ATP) (Hucho *et al.*, 1972; Burgett, 1972). The pyruvate dehydrogenase phosphatase, which is loosely bound to the pyruvate dehydrogenase complex, reactivates pyruvate dehydrogenase in the presence of Mg^{2+} and Ca^{2+} by removing the phosphate (Severson *et al.*, 1974). The pyruvate dehydrogenase phosphatase is inhibited by NaF and is maximally activated by 10mM-$MgCl_2$ (Hucho *et al.*, 1972). The properties of this regulatory system have been extensively studied and found to be similar for isolated enzymes from several tissues, including brain (Linn *et al.*, 1969; Burgett, 1972; Blass & Lewis, 1973). This regulatory system has also been shown to be important *in vivo* in some tissues (Wieland *et al.*, 1971, 1972; Siess *et al.*, 1971). When rats were starved, Wieland and co-workers found that the proportion of pyruvate dehydrogenase in the active form, but not the total amount of pyruvate dehydrogenase, decreased in liver, heart and kidney but did not change in brain.

Regulation of pyruvate dehydrogenase in the brain is of special interest for several reasons. The brain usually depends on carbohydrate utilization for its energy requirements (McIlwain & Bachelard, 1971; Hawkins *et al.*, 1971), and there is relatively little excess of pyruvate dehydrogenase activity available compared with pyruvate flux in the brain (Cremer & Teal, 1974). Also, we have reported that impairment of pyruvate oxidation has widespread effects on biosynthetic processes in the brain *in vitro* (Gibson *et al.*, 1975). Finally, functional abnormalities of pyruvate oxidation have been described, including patients with a partial deficiency of the pyruvate dehydrogenase complex (Blass *et al.*, 1970, 1975).

We have investigated and compared the regulation of pyruvate dehydrogenase activity in isolated rat brain and liver mitochondria by studying the relation between changes in pyruvate dehydrogenase activity and changes in energy charge and other parameters.

Experimental

Materials

Male Sprague–Dawley rats weighing 150–220g were from Mission Supply Corporation, Los Angeles, Calif., U.S.A. Sodium [1-^{14}C]pyruvate (2–10Ci/mol)

was from New England Nuclear Corporation, Boston, Mass., U.S.A. It was stored dry at 0°C until immediately before use. Lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40) and myokinase (EC 2.7.4.3) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Firefly extract was from Sigma Chemical Co. or from Worthington Biochemical Corporation, Freehold, N.J., U.S.A.

Methods

The pyruvate dehydrogenase phosphatase was partially purified from pig and cow hearts by the method of Denton *et al.* (1972). Rats were decapitated and brain mitochondria were isolated by the method of Clark & Nicklas (1970). Liver mitochondria were prepared by the method of Schneider & Hogeboom (1950) with an isolation medium containing 75mM-sucrose, 225mM-mannitol and 0.1mM-EDTA (Walajtys *et al.*, 1974). Liver mitochondria prepared as for brain mitochondria did not differ in pyruvate dehydrogenase activity or adenine nucleotide concentrations from the usual liver mitochondrial preparation.

Mitochondria were incubated at 37°C, with constant shaking, in 130mM-KCl, 20mM-Tris–HCl and 5mM-KH_2PO_4, pH7.2 (Walajtys *et al.*, 1974), with additions as noted in the legends. Incubations lasted 4min except where noted.

To measure pyruvate dehydrogenase activity in mitochondria after incubations, the incubation mixture was rapidly centrifuged at 0°C for approximately 2min to a maximum of 20000*g*. The mitochondrial pellet was resuspended in 20mM-potassium phosphate buffer, pH7.4, containing 1mM-EDTA, 30mM-nicotinamide and 2mM-$MgCl_2$. The samples were quickly frozen and thawed twice and then sonicated for 10s. Control experiments indicated that freezing and thawing (up to six times) and alterations in the time and conditions of sonication did not change the activity of pyruvate dehydrogenase in brain mitochondria. The pyruvate dehydrogenase was activated by incubating either intact mitochondria with 10mM-$MgCl_2$ plus 1mM-$CaCl_2$ or disrupted mitochondria with partially purified pyruvate dehydrogenase phosphatase plus 10mM-$MgCl_2$. The pyruvate dehydrogenase activity was assayed by measuring production of $^{14}CO_2$ from sodium [1-^{14}C]pyruvate by the method of Taylor *et al.* (1973), except that the reaction time was 4min and 10mM-mercaptoethanol was used instead of dithiothreitol.

Adenine nucleotides were measured on samples of the incubated solution of mitochondria, which were added to 0.1vol. of 2.5M-$HClO_4$. The ATP was measured by the luciferase method of Stanley & Williams (1969) as modified by Dowdall *et al.* (1974). AMP and ADP were measured by the method of Adam (1963). The recovery of nucleotides, determined by the addition of standards to the $HClO_4$-treated tissue were: ATP, 96%; ADP, 90%; AMP, 88%. The values reported below were not corrected for recovery.

Mitochondrial protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Statistical significance was calculated by Student's *t*-test.

Results

Total pyruvate dehydrogenase activity

In 11 preparations each of brain and liver mitochondria, pyruvate dehydrogenase activity was 53.5±3.3 and 14.16±1.11nmol/min per mg of protein respectively, after activation by incubation with 10mM-$MgCl_2$ plus 1mM-$CaCl_2$ for 5min. Maximum activation of liver mitochondrial pyruvate dehydrogenase was achieved within this period (Fig. 1), and the addition of partially purified pyruvate dehydrogenase phosphatase produced no further activation (13.41±0.91nmol/min per mg of protein in seven preparations). In brain mitochondria, pyruvate dehydrogenase activity did not increase after incubation with 10mM-$MgCl_2$ plus 1mM-$CaCl_2$ (Table 1), with or without pyruvate dehydrogenase phosphatase, nor with 1 or 100mM-$MgCl_2$, nor on the omission of $CaCl_2$. Incubation of liver or brain mitochondria with Mg^{2+} plus Ca^{2+} (see Fig. 1) or with pyruvate dehydrogenase phosphatase for longer intervals produced no further activation.

Incubation of disrupted mitochondria with 1mM-ATP for 5min decreased pyruvate dehydrogenase activity to 8.6±1.6nmol/min per mg of protein in 12 preparations of brain mitochondria (16% active pyruvate dehydrogenase) and to 1.57±0.32nmol/min per mg of protein in six preparations of liver mitochondria (11% active pyruvate dehydrogenase).

Effects of additions to mitochondria on pyruvate dehydrogenase activity

Brain mitochondrial pyruvate dehydrogenase was completely in the active form after 4min of incubation without added substrate (Table 1). The activity decreased significantly in the presence of certain oxidizable substrates, including pyruvate plus malate, 2-oxoglutarate, citrate and glutamate plus malate. Neither dinitrophenol nor amobarbital significantly changed the pyruvate dehydrogenase activity. In liver mitochondria incubated for 4min without added substrate, 30% of the pyruvate dehydrogenase was in the active form (Table 1). Addition of pyruvate, which inhibits pyruvate dehydrogenase kinase, activated pyruvate dehydrogenase maximally, whether or not malate was present. Citrate,

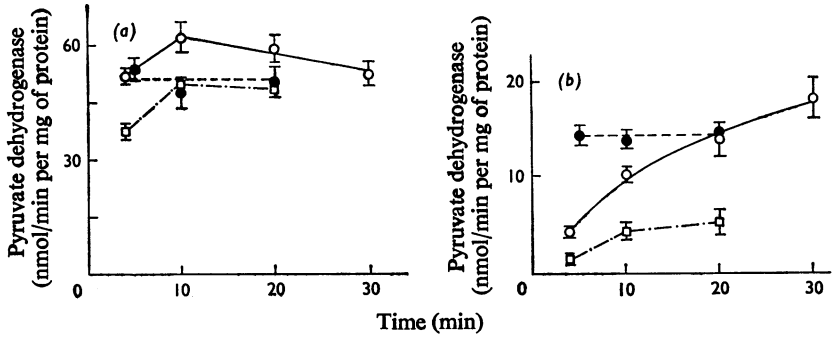

Fig. 1. *Changes in pyruvate dehydrogenase activity in (a) brain and (b) liver mitochondria*

Mitochondria were incubated with: ○, no addition; ●, 10mM-$MgCl_2$ and 1mM-$CaCl_2$; □, 1mM-malate and 5mM-glutamate; and then treated as described in the Experimental section. Values are means±S.E.M. of triplicate determinations on 3 to 15 mitochondrial preparations.

Table 1. *Effects of various compounds on pyruvate dehydrogenase activity in rat brain and liver mitochondria*

Mitochondria were incubated with the indicated materials and the pyruvate dehydrogenase activity was measured as described in the Experimental section. Activity was determined in triplicate on the number of preparations given in parentheses. Values are means±S.E.M. of all determinations. N.D., Not determined. *$P<0.001$.

Additions	Pyruvate dehydrogenase activity (nmol/min per mg of protein)	
	Brain mitochondria	Liver mitochondria
None	51.8±2.1 (14)	4.35±0.41 (15)
$MgCl_2$ (10mM)+$CaCl_2$ (1mM)	53.5±3.3 (11)	14.16±1.11 (11)*
Pyruvate (5mM)	45.0±5.1 (4)	19.16±1.76 (3)*
Pyruvate (5mM)+malate (1mM)	35.8±0.7 (3)*	18.25±2.36 (2)*
β-Hydroxybutyrate (5mM)+malate (1mM)	42.4±4.2 (4)	3.51±0.73 (4)
Malate (1mM)	48.5±2.0 (4)	3.44±0.41 (4)
Succinate (5mM)	50.9±3.4 (7)	3.16±0.28 (4)
Glutamate (5mM)+malate (1mM)	37.5±2.0 (6)*	1.41±0.15 (6)*
2-Oxoglutarate (5mM)+malate (1mM)	40.4±0.6 (2)*	N.D.
2-Oxoglutarate (5mM)	36.2±3.1 (5)*	1.41±0.07 (3)*
Citrate (5mM)	39.0±1.6 (10)*	1.06±0.15 (8)*
Dinitrophenol (0.1mM)	56.4±3.0 (8)	15.54±1.31 (8)*
Amobarbital (2mM)	46.4±0.8 (3)	19.44±0.54 (3)*

2-oxoglutarate and glutamate plus malate significantly decreased pyruvate dehydrogenase activity below that found with no added substrate; but β-hydroxybutyrate, malate and succinate did not. Incubation of liver mitochondria with dinitrophenol or amobarbital activated pyruvate dehydrogenase.

Mitochondrial pyruvate dehydrogenase after longer incubations

Brain mitochondrial pyruvate dehydrogenase maintained a high activity for at least 20min when incubated with 10mM-$MgCl_2$ plus 1mM-$CaCl_2$ (Fig. 1*a*). Without added substrate, pyruvate dehydrogenase in brain mitochondria remained maximally activated during 30min of incubation. The addition of 5mM-glutamate plus 1mM-malate produced an initial decrease in pyruvate dehydrogenase activity which was reversed with longer incubation to a pyruvate dehydrogenase activity equal to that obtained with Mg^{2+} and Ca^{2+} but lower than that in mitochondria incubated for the same time without additions.

When liver mitochondria were incubated without added substrate up to 30min, pyruvate dehydrogenase activity gradually increased to a maximum value (Fig. 1*b*). Incubation with 10mM-$MgCl_2$ plus 1mM-$CaCl_2$ activated pyruvate dehydrogenase in liver mitochondria within 4min. The addition of 5mM-glutamate plus 1mM-malate decreased the

Table 2. *Effect of time of incubation and dinitrophenol on the concentrations of ATP, ADP and AMP and on pyruvate dehydrogenase activity in rat liver and brain mitochondria*

Mitochondria were treated and metabolites assayed as described in the Experimental section. Values are means±S.E.M. for three to six mitochondrial preparations. Energy charge = $\frac{ATP+\frac{1}{2}ADP}{ATP+ADP+AMP}$ (Atkinson, 1968).

Addition	Time (min)	ATP (nmol/mg of protein)	ADP (nmol/mg of protein)	AMP (nmol/mg of protein)	Energy charge	Pyruvate dehydrogenase (nmol/min per mg of protein)
Liver mitochondria						
None	4	5.8±1.2	4.9±0.8	2.6±0.3	0.60±0.03	2.7±0.2
None	10	3.7±0.9	4.9±0.5	2.4±0.2	0.55±0.04	7.4±0.6
None	20	0.9±0.4	5.9±1.5	2.4±0.3	0.41±0.03	13.3±1.9
Dinitrophenol (0.1mM)	4	1.7±0.6	7.9±0.5	2.8±0.3	0.45±0.02	14.0±1.0
Brain mitochondria						
None	4	3.1±0.4	4.1±0.6	3.9±0.3	0.46±0.01	42.2±2.8
None	10	2.2±0.4	3.6±0.1	3.5±0.7	0.43±0.02	43.4±1.0
None	20	2.4±0.6	3.8±0.9	3.9±1.0	0.43±0.04	48.7±3.3
Dinitrophenol (0.1mM)	4	1.2±0.1	7.5±1.8	5.5±0.6	0.34±0.02	55.1±3.1

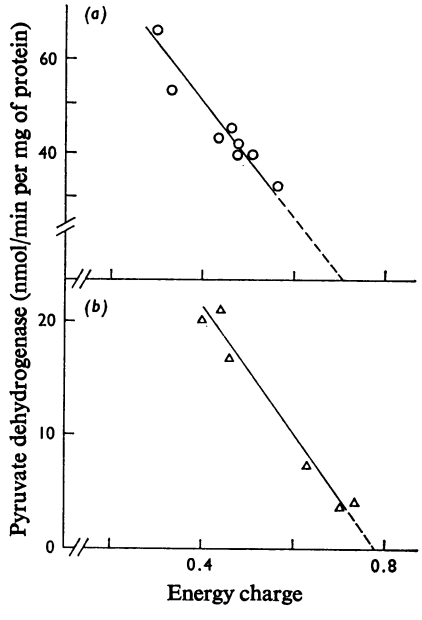

Fig. 2. *Variation of energy charge and pyruvate dehydrogenase activity in rat brain (a) and liver (b) mitochondria*

Mitochondria were incubated for 4 to 20min with or without added substrate and then treated as described in the Experimental section. Results from one preparation of mitochondria from brain and one from liver are shown. Pyruvate dehydrogenase activity was determined in triplicate.

initial pyruvate dehydrogenase activity and decreased the rate of increase in pyruvate dehydrogenase activity during incubation.

Adenine nucleotide concentrations and pyruvate dehydrogenase activity

Mitochondria were incubated without substrate for 4, 10 and 20min or with dinitrophenol for 4min and the concentrations of ATP, ADP and AMP as well as the pyruvate dehydrogenase activity were measured in the same mitochondrial samples (Table 2). Liver mitochondrial pyruvate dehydrogenase activity increased as the adenylate energy charge decreased. The average activity of pyruvate dehydrogenase in brain mitochondria was similar in preparations incubated without substrate for 4, 10 and 20min (see Fig. 1), as was the average energy charge. In these experiments dinitrophenol increased pyruvate dehydrogenase activity and decreased energy charge in both brain and liver mitochondria, compared with values after incubation for 4min without any additions.

When values for pyruvate dehydrogenase activity and energy charge were compared in individual preparations of mitochondria incubated under a variety of conditions including several substrates (as in Table 1 and Fig. 1), similar and inverse relationships between pyruvate dehydrogenase activity and energy charge became evident for brain and liver (Figs. 2 and 3). The day-to-day variation in the absolute activities of pyruvate dehydrogenase and energy charge in brain mitochondria incubated without substrate presumably obscured that relationship when results for several days were averaged, as for Fig. 1 and Table 1. In Fig. 2, results are shown for a single preparation of brain mitochondria and one from liver. In Fig. 3, results with four preparations of brain mitochondria are compared with three preparations from liver, by expressing the value of pyruvate dehydrogenase as a percentage of the maximum activity observed in each preparation.

Effects of chelators on mitochondrial pyruvate dehydrogenase

Brain mitochondrial pyruvate dehydrogenase activity decreased in the presence of each of the five compounds that chelate covalent cations. There was no increase in activity when 10mM-$MgCl_2$ and 2mM-$CaCl_2$ were present (Table 3). In liver mitochondria, citrate and isocitrate lowered pyruvate dehydrogenase activity below that found without added substrate, whereas other chelators did not. Addition of 10mM-$MgCl_2$ plus 2mM-$CaCl_2$ with liver mitochondria and any of these chelators increased the pyruvate dehydrogenase activity. The energy charge in mitochondria treated with ethanedioxybis(ethylamine)tetra-acetate or citrate was approximately the same as in mitochondria incubated without added substrate in two preparations of both brain and liver mitochondria.

Discussion

Maximum pyruvate dehydrogenase activity in brain mitochondria is three- to four-fold that in liver mitochondria. This value for total pyruvate dehydrogenase activity in liver mitochondria agrees with that reported by others (Walajtys *et al.*, 1974; Portenhauser & Wieland, 1972) using different procedures to assay pyruvate dehydrogenase. The difference in total activity in mitochondria from the two tissues agrees with the relative activities found in homogenates of brain and liver tissues (Wieland *et al.*, 1972; Siess *et al.*, 1971). Also, with no additions to the mitochondrial incubation medium, brain pyruvate dehydrogenase is totally in the active form whereas in liver it is only 30% in the active form. The brain has a higher capacity to oxidize pyruvate through pyruvate dehydrogenase than does liver in terms of both the active, and total, amounts of pyruvate

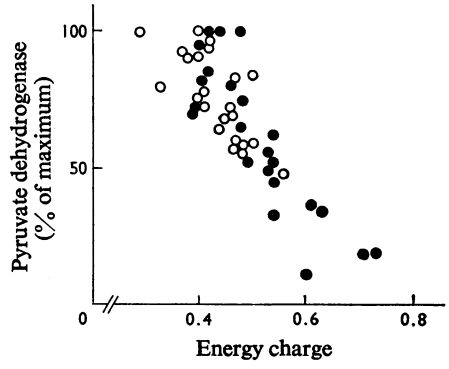

Fig. 3. *Variation of pyruvate dehydrogenase activity and energy charge in rat brain* (○) *and liver* (●) *mitochondria*

Pyruvate dehydrogenase values are shown as a percentage of the maximum pyruvate dehydrogenase activity of each mitochondrial preparation. Each point represents the average of a triplicate determination of pyruvate dehydrogenase activity. Results from three different preparations from liver (●) and four from brain (○) are shown. In these experiments, the average value for maximum pyruvate dehydrogenase activity for mitochondria from brain was 62.3nmol/min per mg of protein and for those from liver was 16.6nmol/min per mg of protein.

Table 3. *Effects of chelators and Mg^{2+} and Ca^{2+} on pyruvate dehydrogenase activity in rat brain and liver mitochondria*

Mitochondria were incubated with the indicated materials for 4min and pyruvate dehydrogenase activity was measured as described in the Experimental section. Values are means±S.E.M. Activity was determined in triplicate on the number of preparations given in parentheses. *$P<0.001$; †$P<0.01$ versus control value; ‡$P<0.001$; §$P<0.01$ versus the value for the appropriate chelator.

Additions	Pyruvate dehydrogenase activity (nmol/min per mg of protein)	
	Brain mitochondria	Liver mitochondria
None (control)	56.3±3.1 (5)	3.55±0.36 (5)
$MgCl_2$ (10mM)+$CaCl_2$ (1mM)	55.9±4.3 (4)	13.73±1.79 (5)*
Citrate (1mM)	35.1±1.1 (5)*	1.47±0.21 (4)*
Citrate (1mM)+$MgCl_2$ (10mM), $CaCl_2$ (2mM)	39.6±1.9 (5)*	14.87±1.95 (5)*‡
Citrate (1mM)+dinitrophenol (0.1mM)	44.1±6.4 (2)	6.97±1.88 (2)§
Isocitrate (1mM)	38.9±0.8 (2)*	1.24±0.03 (2)*
Isocitrate (1mM)+$MgCl_2$ (10mM)+$CaCl_2$ (2mM)	36.8±3.5 (2)*	7.61±1.40 (2)†‡
EDTA (1mM)	35.9±5.1 (2)†	4.32±0.14 (2)
EDTA (1mM)+$MgCl_2$ (10mM)+$CaCl_2$ (2mM)	38.0±2.8 (2)*	5.43±0.36 (2)†
EGTA (1mM)	41.8±2.4 (4)†	3.17±0.47 (2)
EGTA (1mM)+$MgCl_2$ (10mM)+$CaCl_2$ (2mM)	44.0±3.1 (4)†	9.54±1.27 (3)*‡
Ruthenium Red (0.5μg/ml)	33.2±4.2 (2)*	6.17±0.88 (2)
Ruthenium Red (0.5μg/ml)+$MgCl_2$ (10mM) +$CaCl_2$ (2mM)	40.2±1.1 (2)*	13.52±2.03 (2)*§

dehydrogenase available. This is not surprising since the brain normally relies on carbohydrate metabolism for energy production, whereas the liver regularly oxidizes fatty acids and other compounds.

The inhibitory effect of citrate on pyruvate dehydrogenase activity has been considered in several recent investigations (Walajtys *et al.*, 1974; Taylor & Halperin, 1973). Citrate chelates divalent cations and therefore might lower pyruvate dehydrogenase activity by chelating the Mg^{2+} or Ca^{2+} required by pyruvate dehydrogenase phosphatase. However, Taylor & Halperin (1973) found that addition of Mg^{2+} and Ca^{2+} did not reverse citrate inhibition of pyruvate dehydrogenase activity in disrupted muscle mitochondria. We found that pyruvate dehydrogenase activity in brain mitochondria was decreased by each of the chelators tested, whereas only citrate and isocitrate decreased its activity in liver mitochondria. Addition of $MgCl_2$ plus $CaCl_2$ did not overcome this inhibition in brain mitochondria but did do so in liver mitochondria. In this regard, the control of pyruvate dehydrogenase activity in liver appears to be different from that in two excitable tissues, brain and muscle.

Pyruvate dehydrogenase activity in both brain and liver mitochondria increased as energy charge decreased. For brain mitochondria, increased pyruvate dehydrogenase activity correlated better with energy charge than with mitochondrial ATP content or ATP/ADP ratio. For liver mitochondria, correlations of pyruvate dehydrogenase activity related well to all three parameters. The energy-charge expression contains two types of useful information with regard to the control of pyruvate dehydrogenase activity. First, it can express changes of ATP relative to ADP, which act competitively to activate or inhibit pyruvate dehydrogenase kinase respectively. Secondly, the concentrations of free Mg^{2+} and Ca^{2+}, which are required for pyruvate dehydrogenase phosphatase activity, increase as the energy charge falls due to lower affinities for bivalent cations as the number of phosphates attached to the adenosine decreases. Thus a relationship between pyruvate dehydrogenase activity and energy charge may be due to either the competitive effect of ATP and ADP on pyruvate dehydrogenase kinase or to the regulation of pyruvate dehydrogenase phosphatase activity by chelation of divalent ions, or both. It is interesting that, although pyruvate dehydrogenase in *Escherichia coli* is apparently not subject to phosphorylation–dephosphorylation, its activity also changes inversely with energy charge (Shen *et al.*, 1968; Reed, 1974).

The activity of pyruvate carboxylase, the other mitochondrial enzyme which acts on pyruvate, increases as mitochondrial energy charge increases (Patel & Tilghman, 1973; Morikofer-Zwez *et al.*, 1973). This change in activity relative to energy charge is opposite to that found with pyruvate dehydrogenase and indicates that energy charge may be a factor regulating the pathway of pyruvate utilization in brain and in liver mitochondria.

Direct experiments may indicate the significance of these mechanisms in controlling pyruvate dehydrogenase activity in more physiological preparations of brain and *in vivo*.

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