Kinetic Properties of the Partially Purified Pyruvate Dehydrogenase Complex of Ox Brain

By JOHN P. BLASS and CAROLE A. LEWIS

*Mental Retardation Center and Departments of Biological Chemistry and Psychiatry,
University of California Los Angeles Medical School, Los Angeles, Calif. 90024, U.S.A.*

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The properties of a purified preparation of the pyruvate dehydrogenase complex from ox brain have been compared with those of a similar preparation from ox kidney. A broad pH optimum around 7.8, similar dependence on ionic strength, and independence of the nature of the buffer anions or cations characterized preparations from both tissues. Michaelis constants for the binding of pyruvate, thiamin pyrophosphate, NAD⁺ and CoA were also similar. Enzyme from both tissues was inhibited by NADH, by copper and other heavy metals, by high concentrations of tricarboxylic acid-cycle intermediates, and by preincubation with ATP. Acetyl-CoA itself did not appear to inhibit these preparations, although some commercial preparations of acetyl-CoA did contain an inhibitor. Although oxaloacetate and α -oxobutyrate were weak inhibitors, a number of other α -oxo acids including phenylpyruvate did not inhibit. The properties of the pyruvate dehydrogenase complex from brain and kidney appeared similar.

Recent studies have demonstrated the existence of partial genetic defects affecting the pyruvate dehydrogenase complex in patients with neurological disease (Blass *et al.*, 1970, 1971*a,b*, 1972). The enzyme defects have been defined in cultured skin fibroblasts and other non-neural cells. In an attempt to investigate the mechanisms by which such enzymatic defects may interfere with the function of the brain, and specifically to see whether the same enzyme is present in brain as in other tissues, we have compared in detail the kinetic properties of purified preparations of this enzyme complex from ox brain and from ox kidney. As described below, no differences were apparent between the two tissues. These studies also indicated that α -oxo acids did not effectively inhibit the purified pyruvate dehydrogenase complex even at concentrations one to two orders of magnitude higher than those found in the cerebrospinal fluid of patients with phenylketonuria or maple-syrup-urine disease, and thus do not support the suggestion that inhibition of the pyruvate dehydrogenase complex by such acids is an important pathophysiological mechanism in these disorders (Bowden *et al.*, 1971; Bowden & McArthur, 1972). Further, our results suggest that the inhibition by acetyl-CoA of the pyruvate dehydrogenase complex, which has been widely reported (Bremer, 1969; Erfle & Sauer, 1969; Wieland *et al.*, 1969; Siess *et al.*, 1971; Harding *et al.*, 1970; Kanazaki *et al.*, 1969), may be due to impurities contaminating commercial preparations of this compound.

Materials and Methods

Preparation of enzyme

Mitochondria from ox brain cortex were prepared by the method of Cuzner & Davison (1968), and mitochondria from ox kidney cortex were prepared by the same method except that gradient centrifugation was omitted. The pyruvate dehydrogenase complex was purified by a modification of the method of Reed & Willms (1966) from mitochondria that had been disrupted by repeated freezing and thawing and sonication. The disrupted mitochondria were suspended in 20mM-potassium phosphate-1mM-dithiothreitol, pH7.0, to a concentration of about 10mg of mitochondrial protein per ml, and the pH was adjusted to 6.80 by the dropwise addition of 1% acetic acid. Protamine fractionation was then carried out, with the brain complex generally precipitating between 0.01 and 0.02% of protamine and that from kidney between 0.02 and 0.03%. Differential centrifugation of the protamine pellets as described by Reed & Willms (1966) yielded the enzymically active 'yellow pellet.' Ammonium sulphate fractionation provided further purification of some preparations and was done at 4°C by addition of appropriate volumes of a saturated solution of ammonium sulphate. The pyruvate dehydrogenase complex precipitated between 25 and 45% saturation, i.e. after the addition of 0.33 vols. but before the addition of a total of 0.82 vols. of saturated ammonium sulphate. Enzyme free of thiamin was prepared

by the method of Holzer *et al.* (1963), which gave preparations with similar properties to those prepared by dialysis against alkaline buffer or passage over a Sephadex G-25 column equilibrated with such buffer.

Assays for the pyruvate dehydrogenase complex

Purified preparations were assayed spectrophotometrically as described by Reed & Willms (1966). The reaction mixture contained 50mM-potassium phosphate buffer, pH7.8, 1mM-MgCl₂, 2.3mM-NAD⁺, 0.1mM-CoA, 3mM-cysteine, 0.2mM-thiamin pyrophosphate, 1mM-sodium pyruvate, enzyme and other compounds as indicated. The pH was determined and where necessary adjusted to pH7.8 by the addition of dilute HCl or KOH. Addition of pyruvate or of a mixture of pyruvate and CoA initiated the reaction.

For crude preparations, a modification of the radiochemical assay of Schwartz & Reed (1970) was used. The reaction mixture was the same as for the spectrophotometric assay except that 30mM-nicotinamide was present (McIlwain & Rodnight, 1962). Assays were in triplicate and ¹⁴CO₂ was collected as described previously (Blass *et al.*, 1970).

Other assays

Measurements of the activity of the α -oxoglutarate dehydrogenase complex (Mukherjee *et al.*, 1965), of lactate dehydrogenase (Kornberg, 1955) and of phosphotransacetylase (Stadtman, 1953) were by published methods. Protein concentrations were determined by the biuret method or by the method of Lowry (Layne, 1957) using crystalline bovine serum albumin as the standard.

Materials

Sodium pyruvate (Sigma Chemical Co., St. Louis, Mo., U.S.A.), stored in a vacuum desiccator over P₂O₅, contained more than 98% of the expected amount of pyruvic acid by assay with lactate dehydrogenase (Von Korff, 1964). Other α -oxo acids were from Sigma. Phenylpyruvic acid and α -oxo- δ -methylhexanoic acid (α -oxoisohexanoic acid) contained no significant impurities when analysed by g.l.c. and mass spectrometry of the methoxime methyl esters. T.l.c. on silica gel or cellulose (in butan-1-ol-formic acid-water, 19:1:4 by vol.) revealed impurities only in the α -oxobutyric acid, which contained a small amount of less-polar material that stained with I₂ vapour but not with Bromocresyl Green.

Sodium [1-¹⁴C]pyruvate from New England Nuclear Corp., Boston, Mass., U.S.A. (5.9Ci/mol) or from Amersham Searle, Des Plaines, Ill., U.S.A.

(15.2Ci/mol) and sodium [2-¹⁴C]pyruvate from either New England Nuclear Corp. (5.3Ci/mol) or Amersham Searle (13.6 and 17.4Ci/mol) were stored either as dry powders at -20°C in desiccators containing pyrogallol and KOH or in dilute solutions containing excess of HCl (Von Korff, 1964). Radio-purity of the pyruvate preparations varied from 41% to 100%, as measured by t.l.c. in the system described above followed by elution and scintillation counting.

Preparations of acetyl-CoA were from Sigma, from Calbiochem (Los Angeles, Calif., U.S.A.), or from P-L Biochemicals, Inc. (Milwaukee, Wisc., U.S.A.), and contained from 41 to 91% acetyl-CoA on a weight basis, as assayed with phosphotransacetylase (Stadtman, 1957).

Cyclic nucleotides from Sigma contained no impurities on t.l.c. or paper chromatography in six solvent systems. Materials used without further analysis included CoA, NAD⁺, NADH, ATP, ADP, AMP, acetyl phosphate, protamine sulphate, dithiothreitol and tricarboxylic acid-cycle intermediates from Sigma; cysteine from Calbiochem; crystalline bovine serum albumin from Pentex, Kankakee, Ill., U.S.A.; and lactate dehydrogenase (360 μ mol/min per mg of protein) and phosphotransacetylase (1000 μ mol/min per mg of protein) from Boehringer (Mannheim) Corp., New York, N.Y., U.S.A. Other reagents were of analytical grade or of the highest purity available commercially.

Results

Table 1 shows the results of a typical purification of the pyruvate dehydrogenase complex from brain and kidney. In the course of these studies, it became apparent that addition of 1mM-EDTA as well as of 1mM-dithiothreitol to all solutions resulted in higher yields and, in general, higher specific activities than in earlier preparations. Our best preparations from brain had specific activities of 2.0 μ mol/min per mg, representing a more than 7000-fold increase in specific activity compared with crude brain homogenates. Calculations of recovery are not exact, since some crude homogenates contained an inhibitor of the enzyme complex, which led to apparent recoveries of more than 100%. The specific activities of our preparations are similar to those reported by several other workers (Bremer, 1969; Erfle & Sauer, 1969; Wieland *et al.*, 1969; Wieland & Siess, 1970; Hayakawa *et al.*, 1964, 1966; Ishikawa *et al.*, 1966; Scriba & Holzer, 1961) but lower than those obtained by Linn *et al.* (1969). We did not succeed in obtaining enzyme of greater specific activity by their procedure.

Assays for the pyruvate dehydrogenase complex appeared to measure specifically the activity of that

Table 1. *Purification of the pyruvate dehydrogenase complex from ox brain and kidney*

The results of a typical preparation from brain and kidney are shown. The method is described in detail in the text. Fractionation with $(\text{NH}_4)_2\text{SO}_4$ provided no further purification of the particular preparations described in this table.

Fraction	Brain		Kidney	
	Total activity ($\mu\text{mol/min}$)	Specific activity (nmol/min per mg of protein)	Total activity ($\mu\text{mol/min}$)	Specific activity (nmol/min per mg of protein)
Crude homogenate	20.2	0.3	62.0	0.8
Crude mitochondria	7.8	0.9	30.6	3.0
Yellow pellet	3.5	220.0	24.6	330.0

complex, even though the enzyme preparation was not pure. Activity was absolutely dependent on the addition of pyruvate and CoA. The production of NADH measured spectrophotometrically was the same as the production of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ pyruvate ($104 \pm 11\%$, S.E.M. of assays on three enzyme preparations) and of the production of radioactive acetyl-CoA (Lipmann & Tuttle, 1945; Hele *et al.*, 1957) from $[2-^{14}\text{C}]$ pyruvate ($101 \pm 7\%$, S.E.M. of assays on three enzyme preparations). Addition of Na_2AsO_3 abolished activity. Kinetic characteristics of the enzyme did not differ significantly among preparations of different specific activity.

The binding constants for pyruvate, thiamin pyrophosphate, NAD^+ and CoA did not differ significantly between preparations from brain and from kidney (Table 2) and were similar to values reported by others in various tissues and species (Garland & Randle, 1964; Garland *et al.*, 1967; Bremer, 1969; Wieland *et al.*, 1969; Siess *et al.*, 1971; Hayakawa *et al.*, 1964, 1966; Scriba & Holzer, 1961). Preparations from brain and from kidney showed an identical dependence on pH, with a broad optimum about pH 7.8, and on ionic strength, with a broad optimum about $I = 0.10$. At constant ionic strength, substitution of Na^+ for K^+ ions (Nicklas *et al.*, 1971) or of different buffer ions had no more than slight effects on activity (Table 3). A variety of heavy metal ions had inhibitory effects which were the same for preparations from brain or from kidney (Table 4); copper, as either Cu^{2+} or Cu^+ ions, was effective even in the presence of a 20-fold excess of cysteine. Citrate as well as a variety of other tricarboxylic acid-cycle intermediates inhibited the complex from brain and from kidney (Silbert & Martin, 1968), but at concentrations far above the physiological values for such compounds (Fig. 1). High concentrations of a number of α -oxo acids slightly inhibited preparations from either tissue (Table 5); in these studies, the balance cuvette of the double-beam spectrophotometer contained the α -oxo acid being tested, so that only oxidation of pyruvate

Table 2. *Michaelis-Menten constants for substrates and cofactors with the pyruvate dehydrogenase complex from ox brain and kidney*

The values represent means $\pm$ S.D. (The numbers of different enzyme preparations examined are in parentheses.) See the text for details of the enzyme preparation and assay.

	Brain (μM)	Kidney (μM)
Pyruvate	17.00 ± 5.00 (3)	27.00 ± 10.00 (3)
Thiamin pyrophosphate	0.18 ± 0.07 (5)	0.19 ± 0.06 (4)
NAD^+	36.00 ± 16.00 (3)	22.00 ± 7.00 (5)
CoA	4.70 ± 2.40 (4)	5.50 ± 1.60 (4)

and not that of other α -oxo acids was measured. Preparations from both brain and kidney were inhibited by NADH, which appeared to compete with NAD^+ (Fig. 2). The K_i for NADH for the brain enzyme was $0.26 \pm 0.08 \text{ mM}$ (S.D., three preparations) and that for kidney was $0.44 \pm 0.05 \text{ mM}$ (S.D., three preparations). The small difference in the K_i for NADH may have resulted from the small amounts of NADH oxidase activity which contaminated some of these preparations.

Some, but not all, commercial preparations of acetyl-CoA inhibited the pyruvate dehydrogenase complex in our studies. The inhibition, when it occurred, appeared to be competitive with CoA; the K_i for acetyl-CoA appeared to be about 10^{-4} M . However, one preparation did not inhibit at all although it contained 78% acetyl-CoA by weight, as determined by assay with phosphotransacetylase. In another preparation, which inhibited weakly, the inhibitory activity remained intact after treatment with phosphotransacetylase under conditions which converted half of the acetyl-CoA into acetyl phosphate. Further, acetyl-CoA that had been prepared

Table 3. *Effect of buffer anions and cations on the activity of the pyruvate dehydrogenase complex from ox brain and kidney*

The reaction mixtures contained 0.1 M-KCl and 0.02 M concentrations of the various buffer anions shown. The ionic strength of the reaction mixtures was essentially constant owing to the high concentration of KCl. When NaCl was substituted for KCl, 0.02 M-potassium phosphate buffer was present.

Buffer	Specific activity			
	Brain ($\mu\text{mol}/\text{min}$ per mg of protein)	(%)	Kidney ($\mu\text{mol}/\text{min}$ per mg of protein)	(%)
Phosphate (with KCl)	0.316	100	0.452	100
Phosphate (with NaCl)	0.318	101	0.440	97
Glycylglycine	0.336	106	0.421	93
Glycine	0.386	122	0.457	101
N-Tris[hydroxymethyl]- methylglycine (Tricine)	0.389	123	0.457	101
Tris	0.316	100	0.412	91

Table 4. *Effect of heavy metals on the activity of the pyruvate dehydrogenase complex from ox brain*

Activity after 10 min of preincubation of the enzyme with the reaction mixture containing 50 μM concns. of each of the heavy metals is shown, with or without 3 mM-cysteine. Results were similar with two other preparations from brain and one from kidney.

Addition	Specific activity ($\mu\text{mol}/\text{min}$ per mg of protein)	
	Cysteine present	Cysteine absent
None	1.49	1.08
CaCl_2	1.49	0.63
CoCl_2	1.37	0.43
CuCl	0.40	0
CuSO_4	0.40	0
FeSO_4	1.04	0.23
FeCl_3	0.93	0.39
HgCl	1.53	0.35
HgCl_2	1.01	0
MnCl_2	1.81	0
$\text{Pb}(\text{NO}_3)_2$	0.96	0.31
ZnSO_4	0.99	0

enzymically by using acetyl phosphate and phosphotransacetylase (Stadtman, 1953, 1957) did not appear to inhibit effectively. Our preparations of enzyme did not contain significant acetyl-CoA hydrolase activity, as judged by the decrease in absorption due to the thio-ester bond at 232 nm (Stadtman, 1957).

Incubation with ATP in the absence of pyruvate inactivated the enzyme (Fig. 3) with half-maximal inhibition occurring at 0.1–1.0 μM -ATP. We were

Fig. 1. *Effect of tricarboxylic acid-cycle intermediates on activity*

The activity of a typical preparation from brain in the presence of various concentrations of tricarboxylic acid-cycle intermediates is shown. The intermediates were: oxaloacetate, $\square$; *cis*-aconitate, $\blacksquare$; malate, $\triangle$; citrate, $\bullet$; isocitrate, $\circ$; fumarate, ∇ ; succinate, $\blacktriangle$. The activity of the uninhibited preparation was 0.34 $\mu\text{mol}/\text{min}$ per mg of protein. Similar results were obtained with another preparation from brain and one from kidney.

unable to show a reproducible effect of 3':5'-cyclic AMP, 3':5'-cyclic GMP, or energy charge on this process at any stage of purification of the enzyme (Wieland & Siess, 1970; Shen *et al.*, 1968).

Table 5. Effects of α -oxo acids on the activity of the pyruvate dehydrogenase complex from ox brain and kidney

Preparations were incubated with various α -oxo acids in the standard reaction mixture for 10min before addition of sodium pyruvate to initiate the reaction. The final concn. of sodium pyruvate was 0.1 mM, a value within the physiological range for brain (Lowry *et al.*, 1964). The concentration of phenylpyruvic acid was 5mM and of other α -oxo acids 20mM. Results with another preparation from brain were similar. See the text for details.

α -Oxo acid	Specific activity			
	Brain (μ mol/min per mg of protein)	(%)	Kidney (μ mol/min per mg of protein)	(%)
None	1.29	100	0.39	100
α -Oxobutyric acid	0.03	3	0.01	1
α -Oxovaleric acid	1.01	78	0.30	78
α -Oxo- γ -methylvaleric acid	1.01	78	0.34	88
α -Oxo- β -methylvaleric acid	1.13	88	0.34	88
α -Oxohexanoic acid	0.44	34	0.17	44
α -Oxo- δ -methylhexanoic acid	1.25	96	0.40	102
Phenylpyruvic acid	1.21	94	0.37	94

Fig. 2. Effect of NADH on activity of the pyruvate dehydrogenase complex from ox brain

The activity of a typical preparation from brain in the presence of various concentrations of NAD⁺ and NADH is shown. The concns. of NADH were: none, ●; 25 μ M, □; 50 μ M, ▲; 100 μ M, ○. Similar results were obtained with another preparation from brain and a preparation from kidney. Units are μ mol/min. For K_i values, see the text.

Discussion

No significant differences were evident between the properties of the purified preparations of the pyruvate dehydrogenase complex from brain and from kidney. Our results confirm and extend the observations of Siess *et al.* (1971) with brain extracts. These results are not sufficient to establish that the pyruvate dehydrogenase complex is identical in the brain and in

non-neural tissues, but they are consistent with that assumption.

The suggestion has been made that direct inhibition of the pyruvate dehydrogenase complex by α -oxo acids is an important mechanism leading to the brain damage in phenylketonuria and in maple-syrup-urine disease (Dreyfus & Prensky, 1967; Lysiak *et al.*, 1970; Bowden *et al.*, 1971; Bowden &

Fig. 3. Effect of preincubation with various concentrations of ATP on the activity of the pyruvate dehydrogenase complex

The effect of various concentrations of ATP on the activity of purified preparations from brain (●) and from kidney (○) is shown. Enzymes were pre-incubated with ATP for 10 min in 20 mM-potassium phosphate buffer, pH 7.8, and then assayed. These conditions were chosen so that maximal concentrations of ATP would cause approximately 50% inhibition. The vertical broken lines indicate the ATP concentration at approximately half-maximal inhibition.

McArthur, 1972). However, our results indicate that phenylpyruvic acid, which accumulates in phenylketonuria, and the branched chain α -oxo acids, which accumulate in maple-syrup-urine disease, are relatively weak inhibitors even at concentrations one to two orders of magnitude above those found in the blood or cerebrospinal fluid of such patients. These observations agree with those of Kanazaki *et al.* (1969) with the purified pig kidney enzyme. They do not support the postulated significance of this effect in phenylketonuria or in maple-syrup-urine disease unless unusually high concentrations of the α -oxo acids are found to accumulate in specific parts of the brain.

The lack of consistent inhibition by acetyl-CoA in our studies contrasts with results reported by a number of other workers (Bremer, 1969; Erfle & Sauer, 1969; Wieland *et al.*, 1969; Siess *et al.*, 1971;

Harding *et al.*, 1970; Kanazaki *et al.*, 1969). We have not examined the contaminants of commercial preparations of CoA, and it is not clear whether the discrepancy reflects technical factors or a biologically significant difference.

The mechanisms controlling the brain pyruvate dehydrogenase complex generally appear similar to those for other tissues (Bremer, 1969; Wieland *et al.*, 1969, 1971; Wieland & Siess, 1970; Siess *et al.*, 1971; Harding *et al.*, 1970; Linn *et al.*, 1969; Denton *et al.*, 1971; Soeling *et al.*, 1971). The K_m for the binding of pyruvate is similar to that for intact brain mitochondria (Nicklas *et al.*, 1971) and within the range of concentrations reported for pyruvate in neural tissues frozen *in situ* (Goldberg *et al.*, 1966; Lowry *et al.*, 1964; Lolley, 1971). In at least some neural tissues a major factor controlling the rate of pyruvate oxidation may be the concentration of pyruvate itself. The reported stimulation by K^+ of pyruvate utilization by brain mitochondria (Nicklas *et al.*, 1971) does not appear to be an effect on the pyruvate dehydrogenase complex itself. The inhibition of this thiol enzyme by heavy metals is well known (Schwartz & Reed, 1970; Khailova, 1971; Thompson & Whittaker, 1947); the particular sensitivity to copper suggests that an imidazole group may be important to the function of the enzyme. The significance of such inhibition in hepatolenticular degeneration (Wilson's disease), in which copper accumulates, or in mercurial or other heavy metal poisoning, remains speculative at present.

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