Effect of Phenylpyruvate on Pyruvate Dehydrogenase Activity in Rat Brain Mitochondria

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1. The effects of phenylpyruvate, a metabolite produced in phenylketonuria, on the pyruvate dehydrogenase-complex activity were investigated in rat brain mitochondria. 2. Pyruvate dehydrogenase activity was measured by two methods, one measuring the release of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ pyruvate and the other measuring the acetyl-CoA formed by means of the coupling enzyme, pigeon liver arylamine acetyltransferase (EC 2.3.1.5). In neither case was there significant inhibition of the pyruvate dehydrogenase complex by phenylpyruvate at concentrations below 2 mM. 3. However, phenylpyruvate acted as a classical competitive inhibitor of the coupling enzyme arylamine acetyltransferase, with a K_i of $100\text{ }\mu\text{M}$. 4. It was concluded that the inhibition of pyruvate dehydrogenase by phenylpyruvate is unlikely to be a primary enzyme defect in phenylketonuria.

Phenylketonuria, an inborn error of phenylalanine metabolism (Harris, 1959), gives rise to elevated plasma and tissue concentrations of both phenylalanine and phenylpyruvate (Jervis & Drejza, 1966). Various estimates are available of the plasma concentration to which phenylpyruvate may rise in phenylketonuria (Harris, 1959; Jervis & Drejza, 1966; Small *et al.*, 1970; Patel, 1972), the highest of these being in the region of 0.5 mM (Patel, 1972). Phenylketonuria may give rise to severe brain mal-function and is particularly characterized by a deficiency in myelin deposition in the nervous tissue. Weber *et al.* (1970) have shown effects of phenylpyruvate on both brain hexokinase and 6-phosphoglucuronate dehydrogenase, establishing an apparent K_i of phenylpyruvate in the range 3–6 mM. They have also demonstrated that phenylalanine inhibits pyruvate kinase with an apparent K_i of 9 mM. Bowden & McArthur (1972) have shown that phenylpyruvate inhibits pyruvate decarboxylation in rat brain homogenates with an apparent K_i of 6 mM. They have suggested that the inhibition of the pyruvate dehydrogenase complex may be a primary enzymic defect characteristic of phenylketonuria.

We have sought to test this hypothesis more rigorously by using a more purified enzyme source (rat brain mitochondrial extract) and an assay system distinct from the decarboxylation assay of Bowden & McArthur (1972). In this assay method the pyruvate dehydrogenase-complex activity is monitored by using a coupling enzyme, the pigeon liver arylamine acetyltransferase (EC 2.3.1.5). Our results indicate that when both assays are used at phenylpyruvate concentrations of up to 2 mM negligible inhibition of the pyruvate dehydrogenase complex *per se* occurs.

However, phenylpyruvate acts as a competitive inhibitor of the coupling enzyme arylamine acetyltransferase, with an apparent K_i of $100\text{ }\mu\text{M}$. Thus additional coupling enzyme must be added to the assay of pyruvate dehydrogenase in the presence of phenylpyruvate to maintain the non-rate-limiting status of the coupling enzyme essential to any coupled assay. Preliminary reports of this work have been published (Land & Clark, 1972).

Materials and Methods

Chemicals

Coenzyme A, NAD^+ and lactate dehydrogenase (EC 1.1.1.27) were purchased from Boehringer und Soehne, Mannheim, Germany. Thiamin pyrophosphate and Alumina C γ were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Phenylpyruvate (sodium salt) was obtained from BDH Ltd., Poole, Dorset, U.K., and *p*-(*p*-aminophenylazo)benzenesulphonic acid (sodium salt) from Fluka AG, Buchs, Switzerland. Sodium $[1-^{14}\text{C}]$ pyruvate (sp. radioactivity $14.4\text{ }\mu\text{Ci/mmol}$) was from The Radiochemical Centre, Amersham, Bucks., U.K., and non-radioactive pyruvic acid from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Pyruvic acid was twice distilled under vacuum and stored at -20°C before use. Acetyl-CoA was prepared from CoA and acetic anhydride essentially by the method of Ochoa (1955) and the purity was assayed by titration of free thiol groups with 2,2'-bipyridyl disulphide. All other compounds were of the highest quality available commercially and reagents were made up in double-glass-distilled water.

Animals

Male albino rats of the Wistar strain (155–175 g body wt.) were used for all experiments and were fed *ad lib.* on laboratory diet no. 1 (Spratts, Reading, Berks., U.K.). Mitochondria were prepared from rat brain cortex by the method of Clark & Nicklas (1970). Pyruvate dehydrogenase activity was measured in mitochondrial preparations that had been frozen and thawed three times.

Pyruvate dehydrogenase assay

By decarboxylation of [1-¹⁴C]pyruvate. The method used was adapted from that of Wieland *et al.* (1971) by W. Neilson (personal communication). The reaction was run in the main chamber of a conical flask with a centre well containing 0.5 ml of Hyamine (1M, in methanol) with continuous shaking at 25°C. The reaction was stopped after 30 min by adding 2 ml of 2M-HCl to the main chamber and the contents of the flask were shaken for a further 60 min to facilitate complete absorption of the released ¹⁴CO₂ by the Hyamine. Hyamine extraction was accomplished by washing with five 0.5 ml batches of methanol. The ¹⁴CO₂ was counted for radioactivity in a Packard 2425 liquid-scintillation counter in a scintillator mixture of 4 g of 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)-thiophen/litre of toluene. The main reaction chamber contained the following components in a final volume of 3 ml (final concentrations): 100 mM-Tris-HCl buffer, pH 8.0, 5 mM-MgCl₂, 16.7 mM-mercaptoethanol, 6.7 mM-NAD⁺, 2.7 mM-thiamin pyrophosphate, 1 mM-CoA, 500 µg of a crystalline suspension of lactate dehydrogenase, 8.3 mM-pyruvic acid and 0.2 µCi of [1-¹⁴C]pyruvate. The reaction was started by adding a portion (100 µl) of mitochondrial extract. Preliminary experiments showed that this assay followed linear kinetics up to 3 mg of mitochondrial protein (approx. 1 mg was used as a routine) and for at least 30 min (the time used as a routine).

By coupling with pigeon liver arylamine acetyltransferase. This assay couples the acetyl group of acetyl-CoA formed by pyruvate dehydrogenase to an azo dye and the formation of the acetyl-*p*-(*p*-aminophenylazo)benzenesulphonic acid is monitored by the decrease in *E*₄₆₀. The acetyl transfer is catalysed by pigeon liver arylamine acetyltransferase, which was prepared from pigeon liver acetone-dried powder as described by Tabor *et al.* (1953), except that 20 mM-potassium phosphate buffer, pH 7.8, was required to elute the enzyme from the alumina gel (mainly in the third and fourth washings; each wash was 10 ml of eluent, and enzyme concentration was approx. 2.5 mg of protein/ml) and was assayed by the method of Jacobson (1961).

The reaction mixture was essentially as described by Coore *et al.* (1971). In a final volume of 2 ml the following were present (final concentrations): 100 mM-

Tris-HCl, pH 8, 0.5 mM-EDTA (potassium salt), 5 mM-MgCl₂, 1 mM-thiamin pyrophosphate, 0.5 mM-NAD⁺, 0.1 mM-CoA, 30 mM-mercaptoethanol, 0.3 mM-*p*-(*p*-aminophenylazo)benzenesulphonic acid, arylamine acetyltransferase (approx. 200 µl of 2.5 mg of protein/ml of soln.) and mitochondrial extract 5–10 µl of 15 mg of protein/ml of soln.). The reaction

Table 1. *Effect of phenylpyruvate on [1-¹⁴C]pyruvate decarboxylation by rat brain mitochondria*

The results are expressed as nmol of [1-¹⁴C]pyruvate decarboxylated/min per mg of mitochondrial protein. Each value is the mean of two separate experiments. The assay conditions were as described in the Materials and Methods section and the runs were performed at 25°C. The enzyme source was a preparation of rat cerebral cortex mitochondria frozen and thawed three times.

Rate of [¹⁴ C]pyruvate decarboxylation (nmol/min per mg of protein)	Concn. of phenylpyruvate (mM)
32.5	0
32.9	0.5
33.3	1
30.9	2
23.9	5
19.3	10

Fig. 1. *Activity of pyruvate dehydrogenase complex at various pyruvate concentrations*

Pyruvate dehydrogenase-complex activity was measured at 25°C by the coupled-enzyme assay as outlined in the Materials and Methods section. The activity (*v*) is expressed as nmol of acetyl-CoA transferred/min per mg of protein and is plotted as a double-reciprocal plot (1/*v* against 1/[S]). The results have been subjected to computer analysis to derive the best-fit straight line and the various enzyme parameters. ●, 10 mM-Mg²⁺; ○, 5 mM-Mg²⁺.

was started by adding pyruvate. Sufficient acetyltransferase was added to ensure maximum activity of the pyruvate dehydrogenase complex (i.e. so that the acetyltransferase was not rate-limiting) and this was done as a preliminary titration for each mitochondrial preparation used. Control cuvettes containing no acetyltransferase but all the other components were run simultaneously. Preliminary experiments indicated that this assay followed linear kinetics with time (up to 20 min) and amount of mitochondrial extract. Protein was determined by the biuret method (Gornall *et al.*, 1949).

Evaluation of results

In all cases where appropriate, the best lines through a series of points have been calculated by linear regression methods with the aid of a computer program.

Results

Effect of phenylpyruvate on brain pyruvate dehydrogenase-complex activity as measured by decarboxylation of [1-¹⁴C]pyruvate

The results of the experiments are presented in Table 1, which shows that no significant inhibition of pyruvate decarboxylation by phenylpyruvate occurs up to concentrations of 2 mM-phenylpyruvate. At higher concentrations some inhibition is apparent, more than 40% inhibition occurring at 10 mM-phenylpyruvate at saturating pyruvate concentrations. It is unlikely therefore that in phenylketonuria phenylpyruvate has any direct effect on pyruvate decarboxylation, since the highest recorded tissue phenylpyruvate concentrations are an order of magnitude below this (Patel, 1972). The K_i of 6 mM for phenylpyruvate on pyruvate decarboxylation in brain homogenates reported by Bowden & McArthur

Fig. 2. Effect of phenylpyruvate and added acetyltransferase on the activity of pyruvate dehydrogenase complex

Pyruvate dehydrogenase-complex activity was measured at 25°C by the arylamine acetyltransferase coupled-enzyme method (see the Materials and Methods section). The initial rates are expressed as nmol of acetyl-CoA transferred/min per mg of mitochondrial protein. (a) Effect of increasing concentrations of phenylpyruvate on the pyruvate dehydrogenase-complex activity measured in the presence of 250 μ M-pyruvate (final concn.). (b) Effect of adding back to this system increasing amounts of additional acetyltransferase (i.e. additional to that initially required in the absence of phenylpyruvate to achieve maximal pyruvate dehydrogenase activity) (concentration of stock acetyltransferase added was approx. 2.5 mg of protein/ml). The first point in (b) represents the same conditions as the last point in (a), i.e. the activity of the pyruvate dehydrogenase complex in the presence of 250 μ M-pyruvate and 3 mM-phenylpyruvate (final concn.).

(1972) may be due to an increased acetyl-CoA/CoA or NADH/NAD⁺ ratio, since in the method of assay (Bowden *et al.*, 1971) used by them no exogenous CoA nor any NADH-reoxidizing system is present. It is well established in other tissues that increases in either of these ratios inhibit pyruvate dehydrogenase activity (Hansen & Henning, 1966).

Effect of phenylpyruvate on pyruvate dehydrogenase-complex activity as measured by the coupling enzyme arylamine acetyltransferase

Fig. 1 shows a classical reciprocal plot of rat brain pyruvate dehydrogenase-complex activity at various pyruvate concentrations as assayed by the coupled assay of Coore *et al.* (1971). This gives a K_m for pyruvate of $89.73 \pm 4.83 \mu\text{M}$ (6), which is Mg^{2+} -independent,

Fig. 3. Effect of phenylpyruvate on pigeon liver arylamine acetyltransferase

The pigeon liver arylamine acetyltransferase was prepared essentially as described by Tabor *et al.* (1953) and assayed by the method of Jacobson (1961). In the final volume of 2 ml, the following were present (final concentrations): 100 mM-Tris-HCl, pH 8, 10 mM-EDTA, 30 mM-mercaptoethanol, various concentrations of acetyl-CoA and enzyme. This was pre-incubated at room temperature for 5 min and the reaction started by adding the azo dye [50 μM -*p*-(*p*-aminophenylazo)benzenesulphonic acid]. The reaction was monitored by measuring the decrease in E_{460} by using the extinction coefficients for the azo dye and its acetyl derivative quoted by Jacobson (1961). Enzyme activity (v) is expressed as units, where 1 unit is the amount of enzyme that transforms 1 μmol of substrate/min. The reciprocal of the initial velocities is plotted against phenylpyruvate concentration at three different concentrations of acetyl-CoA: ●, 29 μM ; ▲, 36 μM ; ■, 43 μM .

dent, and a V_{max} of 42.2 ± 1.3 (5) and 20.85 (2) nmol/min per mg of mitochondrial protein in 5 and 10 mM- Mg^{2+} respectively. The K_m value for pyruvate of approx. 90 μM reported here for the rat brain mitochondrial pyruvate dehydrogenase is higher than that observed for the purified enzyme from pig brain (45 μM ; Siess *et al.*, 1971) and ox brain (17 μM ; Blass & Lewis, 1973). However, the value of V_{max} in the presence of 5 mM- Mg^{2+} is compatible with the maximal oxygen uptakes of 200 ± 20 ng atoms/min per mg of protein, consistently observed by us for these rat brain mitochondria oxidizing pyruvate and malate under optimum conditions (J. M. Land, unpublished work; Nicklas *et al.*, 1971). There is, however, some discrepancy between the V_{max} value reported here and the much higher oxygen uptakes for rat brain mitochondria oxidizing pyruvate reported recently by Cremer *et al.* (1972).

If pyruvate dehydrogenase-complex activity is measured in the presence of phenylpyruvate no inhibition of the pyruvate dehydrogenase complex *per se* is observed, provided that the coupling enzyme is maintained in excess (see Fig. 2). To obtain this it is necessary to add additional amounts of coupling enzyme; for example, in the presence of 3 mM-phenylpyruvate a total of 1.5 mg of coupling-enzyme protein (600 μl) has to be present for full expression of pyruvate dehydrogenase-complex activity, as opposed to only 0.5 mg of coupling-enzyme protein (200 μl) required in the absence of any inhibitor. Fig. 2(a) shows the decrease in the rate of acetyl-CoA transfer in the coupled assay in the presence of increasing concentrations of phenylpyruvate, with the restoration of the original activity on the addition of further coupling enzyme (Fig. 2b). These results suggest that phenylpyruvate is acting as an inhibitor of the coupling enzyme.

Effect of phenylpyruvate on pigeon liver arylamine acetyltransferase

The hypothesis that phenylpyruvate was inhibiting the acetyltransferase was further supported by studies with the acetyltransferase alone. The activity of this enzyme was measured in the presence of various concentrations of phenylpyruvate at three different acetyl-CoA concentrations. The results are plotted in Fig. 3 and show a classical competitive inhibition with an apparent K_i of 100 μM for phenylpyruvate. Studies with other acetyl donors, e.g. *p*-nitroacetanilide, also show inhibition by phenylpyruvate.

Discussion

The results reported here do not lend support to the hypothesis that the inhibition of pyruvate dehydrogenase by phenylpyruvate is a primary enzymic defect in phenylketonuria (Bowden & McArthur,

Scheme 1. Ionization states of phenylpyruvic acid

1972). Both methods of assay indicate that phenylpyruvate concentrations in excess of 2–3 mM are required before any inhibition (direct or indirect) of the pyruvate dehydrogenase complex occurs. Such concentrations of phenylpyruvate are unlikely to occur in phenylketonuria (Patel, 1972).

However, the observations reported here on the effects of phenylpyruvate on acetyl-group transfer by the pigeon liver arylamine acetyltransferase may have important metabolic implications for phenylketonuria as well as being of interest in themselves. Evidence for this is presented in the accompanying paper (Land & Clark, 1973).

The nature of the competition between phenylpyruvate and the acetyl group must lie in common structure and functional features. Phenylpyruvate may theoretically exist in a number of different forms, shown in Scheme 1. The titration of phenylpyruvate reveals two apparent $\text{p}K$ values, one at $\text{pH} 6.5$ and the other at $\text{pH} 12.1$. The former probably represents a conglomerate of dissociation and equilibrium constants associated with the formation of the carboxyl anion and the keto/enol equilibrium. However, the $\text{p}K$ at 12.1 is associated with the appearance of a large absorption maximum at 320 nm and probably represents the formation of the enolate anion (III) (Errara & Greenstein, 1947; J. M. Land & J. B. Clark, un-

published work). Spectral studies on phenylpyruvate at $\text{pH} 9$ and 12.75 suggest that the form (IIC) of phenylpyruvate does not exist to any appreciable extent and that the keto form of the anion (IIA), is the predominant species present at $\text{pH} 9$. As the enol form (IIB) contributes to the absorption at 320 nm but the keto form (IIA) does not (Errara & Greenstein, 1947) it is possible to calculate that the keto form (IIA) of the phenylpyruvate anion predominates (96%) at the pH at which the assays were run. Two possibilities therefore exist: (a) that the inhibition is a property of the predominant species present, the keto form (IIA) of phenylpyruvate, or (b) that a very effective inhibition is occurring by means of the small concentration of the enol form (IIB) present (i.e. the inhibition constant of the enol form must be almost two orders of magnitude smaller than the keto form). As phenylpyruvate inhibits the acetyltransferase with similar inhibition constants when both acetyl CoA and *p*-nitroacetanilide are used as acetyl donors, it is possible that the competition is occurring with respect to the acetyl group of acetyl-CoA. Both the keto form of phenylpyruvate (IIA) and the two acetyl donors (acetyl-CoA and *p*-nitroacetanilide) have in common the $\text{X}-\text{CH}_2-\text{CO}-\text{Y}$ spatial grouping. Further preliminary studies with phenylacetaldehyde, which also contains this grouping, show that this compound is

also an effective inhibitor of acetyl transfer, thus lending support to the hypothesis that it is the keto form of phenylpyruvate (IIA) that is the inhibitory species.

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