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Effect of Phenylalanine Metabolites on the Activities of Enzymes of Ketone-Body Utilization in Brain of Suckling Rats

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1. The effects of phenylalanine and its metabolites (phenylacetate, phenethylamine, phenyl-lactate, *o*-hydroxyphenylacetate and phenylpyruvate) on the activity of 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30), 3-oxo acid CoA-transferase (EC 2.8.3.5) and acetoacetyl-CoA thiolase (EC 2.3.1.9) in brain of suckling rats were investigated. 2. The 3-hydroxybutyrate dehydrogenase from the brain of suckling rats had a K_m for 3-hydroxybutyrate of 1.2mM. Phenylpyruvate, phenylacetate and *o*-hydroxyphenylacetate inhibited the enzyme activity with K_i values of 0.5, 1.3 and 4.7mM respectively. 3. The suckling-rat brain 3-oxo acid CoA-transferase activity had a K_m for acetoacetate of 0.665mM and for succinyl (3-carboxypropionyl)-CoA of 0.038mM. The enzyme was inhibited with respect to acetoacetate by phenylpyruvate ($K_i = 1.3$mM) and *o*-hydroxyphenylacetate ($K_i = 4.5$mM). The reaction in the direction of acetoacetate was also inhibited by phenylpyruvate ($K_i = 1.6$mM) and *o*-hydroxyphenylacetate ($K_i = 4.5$mM). 4. Phenylpyruvate inhibited with respect to acetoacetyl-CoA both the mitochondrial ($K_i = 3.2$mM) and cytoplasmic ($K_i = 5.2$mM) acetoacetyl-CoA thiolase activities. 5. The results suggest that inhibition of 3-hydroxybutyrate dehydrogenase and 3-oxo acid CoA-transferase activities may impair ketone-body utilization and hence lipid synthesis in the developing brain. This suggestion is discussed with reference to the pathogenesis of mental retardation in phenylketonuria.

The basic biochemical defect of phenylketonuria, an inborn error of phenylalanine metabolism, is the genetically linked deficiency in the activity of hepatic phenylalanine 4-hydroxylase (EC 1.14.16.1) (Jervis, 1953). This lack of activity leads to a considerable accumulation of phenylalanine and consequently to an increased concentration of phenylalanine metabolites, namely phenylpyruvate, phenylacetate, phenyllactate and *o*-hydroxyphenylacetate, in the tissues of patients with uncontrolled phenylketonuria.

It is well established that ketone bodies in developing brain are an important fuel for respiration, as indicated by the measurements of arterio-venous differences for 3-hydroxybutyrate and acetoacetate across the brain of suckling rats (Hawkins *et al.*, 1971) and the high activities of the enzymes involved in ketone-body utilization (Page *et al.*, 1971; Tildon *et al.*, 1971; Page & Williamson, 1971). Further, as Klee & Sokoloff (1967) suggested, ketone bodies have another metabolic role in the developing brain, providing the acetyl-CoA required for the synthesis of lipids involved in the myelination process.

Since the period of maximum rate of myelination occurs during the early stages of brain development (Davison & Dobbing, 1968), any inhibition of ketone-body utilization during this period might have detrimental effects on this process and thus might be responsible for mental retardation in phenylketonuria. It is noteworthy that impaired myelination (Shah *et al.*, 1972*b*) and progressive brain dysfunction can be observed in patients with untreated phenylketonuria (Knox, 1972).

Kinetic studies on purified 3-oxo acid CoA-transferase (EC 2.8.3.5) from the brain of weanling rats (Tildon & Sevdalian, 1972) and acetoacetyl-CoA thiolase (EC 2.3.1.9) of suckling-rat brain (Middleton, 1973) have been reported. The present paper reports the effects of phenylalanine and its metabolites on the enzyme activities of ketone-body utilization in brain of suckling rats. The results indicate that phenylpyruvate markedly inhibits both 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and 3-oxo acid CoA-transferase, the latter being the key enzyme for ketone-body utilization (Williamson *et al.*, 1971).

Materials and Methods

Animals

Suckling rats of either sex (20–21 days old) and of the Wistar strain were used. All the rats were kept with their mother until they were used for the experiments.

Chemicals

3-Hydroxybutyrate dehydrogenase was supplied by Boehringer Corp., Mannheim, Germany. Nicotinamide dinucleotides, CoA, DL-3-hydroxybutyrate, succinate, acetoacetate, L-phenylalanine, phenylacetate, phenethylamine, phenyl-lactate, *o*-hydroxyphenylacetate and phenylpyruvate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Acetoacetyl-CoA was prepared from diketen and CoA as described by Wieland & Rueff (1953). The acetoacetyl-CoA concentration of the preparation was determined by the method of Decker (1965). Succinyl-CoA (3-carboxypropionyl-CoA) was prepared from succinic anhydride and CoA (Simon & Shemin, 1953). The succinyl-CoA concentration of the ether-extracted solution was determined by a coupled assay with 3-oxo acid CoA-transferase and 3-hydroxyacyl-CoA dehydrogenase (Williamson, 1974).

Preparation of homogenates

The rats were killed by decapitation. Brains were rapidly removed, weighed, minced finely with scissors and homogenized in 10vol. of 0.25M-sucrose (Lehninger *et al.*, 1960) with a motor-driven Potter–Elvehjem all-glass homogenizer. After centrifugation at 800*g* for 5min the supernatant was treated in an MSE ultrasonic disintegrator for five 30s periods at about 20kHz. The tube containing the sample was immersed in a beaker filled with ethanol cooled to −20°C. During ultrasonic treatment the temperature of the ethanol was kept between −5°C and −10°C by occasional addition of liquid N_2. The whole homogenate after ultrasonic treatment was used for the assay of 3-hydroxybutyrate dehydrogenase. For the assay of 3-oxo acid CoA-transferase, 10vol. of 0.3% deoxycholate in 1mM-2-mercaptoethanol/50mM-sodium phosphate buffer, pH7.4, was used for homogenization. After centrifugation for 20min at 30000*g*, enzyme activities were measured in the supernatant. For the assay of acetoacetyl-CoA thiolase, 4vol. of a medium containing 0.25M-sucrose in 1mM-2-mercaptoethanol/10mM-Tris/HCl buffer, pH7.4 (Williamson *et al.*, 1971), was used for homogenization. After centrifugation at 30000*g* for 20min, the cytoplasmic-enzyme activity was determined in the supernatant. The pellet was resuspended in 4vol. of a medium containing 0.3% deoxycholate, 1mM-mercaptoethanol and 5mM-sodium phosphate buffer, pH7.4, and centrifuged at 30000*g* for 20min. Mitochondrial-enzyme activity was determined in the supernatant. Lactate dehydrogenase (EC 1.1.1.27) and glutamate dehydrogenase (EC 1.4.1.2) activities were assayed as marker enzymes for cytoplasmic and mitochondrial fractions respectively.

Determination of enzyme activities

3-Hydroxybutyrate dehydrogenase was assayed by the method of Lehninger *et al.* (1960). The assay mixture (final volume 3.1ml) contained: Tris/HCl buffer, pH8.5 (250μmol), sodium DL-3-hydroxybutyrate (30μmol), NAD^+ (7μmol), nicotinamide (125μmol), cysteine (30μmol) and NaCN (3μmol). The reaction was started by adding the enzyme sample (50–100μl, about 1mg of protein) to the reaction mixture. 3-Oxo acid CoA-transferase was assayed in both directions by measuring the rate of formation of acetoacetyl-CoA from succinyl-CoA and acetoacetate, and that of disappearance of acetoacetyl-CoA in the presence of succinate as described by Williamson *et al.* (1971). Acetoacetyl-CoA thiolase was determined as described by Williamson *et al.* (1971).

Lactate dehydrogenase activity was measured by the method of Bergmeyer *et al.* (1965) and glutamate dehydrogenase was assayed as described by Williamson *et al.* (1967). Enzyme activities are expressed as μmol of product formed or substrate transformed/min per g fresh wt. of tissue. All enzyme activities were measured at 25°C.

When the effects of phenylalanine and its metabolites were investigated, these compounds were added immediately before starting the reaction, the pH being determined at the beginning and the end of the measurements.

Results

3-Hydroxybutyrate dehydrogenase activity

The effects of phenylalanine and its metabolites on the activities of the enzymes of ketone-body utilization in the developing rat brain are shown in Table 1. The activity of 3-hydroxybutyrate dehydrogenase was strongly inhibited by phenylpyruvate. Phenylacetate and *o*-hydroxyphenylacetate also inhibited the activity of 3-hydroxybutyrate dehydrogenase of rat brain. However, phenylalanine showed no inhibitory effect on this enzyme.

Lineweaver–Burk plots of the brain 3-hydroxybutyrate dehydrogenase were linear and gave a K_m value for DL-3-hydroxybutyrate of 1.2mM (Table 2). When the activity of 3-hydroxybutyrate dehydrogenase was determined in the presence of phenylpyruvate, the Dixon plot indicated that phenylpyruvate was an inhibitor of the enzyme, with K_i 0.5mM (Fig. 1). Similar studies on the effects of phenylacetate and *o*-hydroxyphenylacetate indicated that these compounds were also acting as inhibitors of the enzyme. The inhibition constants obtained from the Dixon plots for each compound were 1.3mM for phenylacetate and 4.7mM for *o*-hydroxyphenylacetate.

Table 1. *Effects of phenylalanine and its metabolites on the activities of the enzymes involved in ketone-body utilization in brain of suckling rats*

The values were calculated from the mean of at least four observations, taking the activity of control without addition to be 100%. Control values shown in parentheses are means ± S.E.M. of at least six observations and are expressed as μmol of product formed or substrate transformed/min per g fresh wt. of tissue. All added compounds were at a concentration of 10mM. For full details, see the Materials and Methods section.

	Relative activity (as % of control without addition)				
		3-Oxo acid CoA-transferase		Acetoacetyl-CoA thiolase	
Addition	3-Hydroxybutyrate dehydrogenase	Acetoacetate ↓ Acetoacetyl-CoA	Acetoacetyl-CoA ↓ Acetoacetate	Cytoplasmic	Mitochondrial
None	(1.2±0.1)	(1.09±0.05)	(10.2±0.82)	(1.35±0.11)	(3.64±0.22)
Phenylalanine	100	91	97	84	89
Phenylacetate	68	83	94	96	115
Phenylethylamine	72	95	70	92	89
Phenyl-lactate	80	80	79	100	100
o-Hydroxyphenylacetate	60	61	25	96	104
Phenylpyruvate	<10	<10	30	65	58

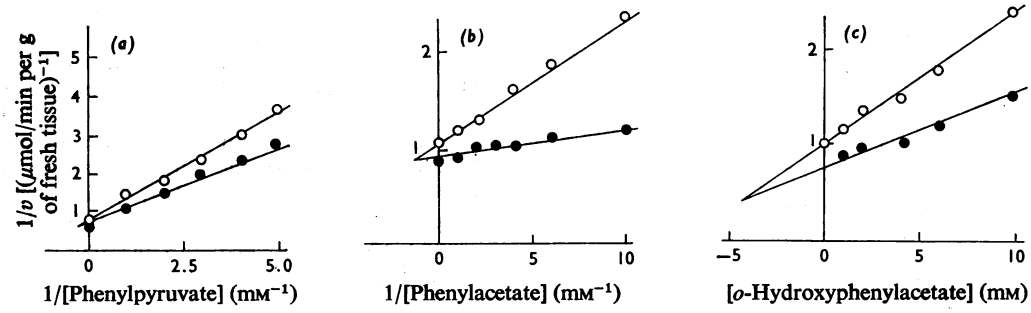

Fig. 1. *Effect of phenylpyruvate, phenylacetate and o-hydroxyphenylacetate on the activity of 3-hydroxybutyrate dehydrogenase from suckling rat brain*

The 3-hydroxybutyrate dehydrogenase activity from the brain of 20–21-day-old rats was assayed as described in the Materials and Methods section, except that two different 3-hydroxybutyrate concentrations (○, 5mM; ●, 20mM) were used in the presence of different concentrations of phenylpyruvate (*a*), phenylacetate (*b*) or *o*-hydroxyphenylacetate (*c*). Each point represents the mean value of results from at least four duplicate experiments.

3-*Oxo acid CoA-transferase activity*

The activity of the 3-oxo acid CoA-transferase, a mitochondrial enzyme existing only in extrahepatic tissues, was studied in both physiological (acetoacetate → acetoacetyl-CoA) and reverse directions. Phenylalanine itself and phenylacetate, phenethylamine and phenyl-lactate were without effect on the activity of enzyme from brain of suckling rats. However, *o*-hydroxyphenylacetate and, to a greater extent, phenylpyruvate inhibited the enzyme activity (Table 1). Kinetic studies of the brain 3-oxo acid CoA-transferase yielded K_m values for acetoacetate of 0.665mM and for succinyl-CoA of 0.038mM (Table 2). When the activity of the brain 3-oxo acid CoA-transferase was determined in the presence of several phenylpyruvate concentrations, the Dixon plot (Fig. 2) showed that phenylpyruvate acts as an inhibitor, with K_i 1.3mM. Similar studies for *o*-hydroxyphenylacetate gave a K_i value of 4.5mM. The inhibition constants obtained for the reverse reaction (acetoacetyl-CoA → acetoacetate) were 1.6 and 4.5mM for phenylpyruvate and *o*-hydroxyphenylacetate respectively.

Acetoacetyl-CoA thiolase activity

The effects of phenylalanine and its metabolites on the cytoplasmic and mitochondrial acetoacetyl-CoA thiolase from the brain of suckling rats are shown in

Table 2. *Michaelis constants of the enzymes involved in ketone-body utilization in brain of suckling rats*

Enzyme activities from the brain of 20–21-day-old rats were extracted and measured as described in the Materials and Methods section. The K_m values were determined by double-reciprocal plot of activity against substrate concentration.

Enzyme	Substrate	K_m (mM)
3-Hydroxybutyrate dehydrogenase	DL-3-Hydroxybutyrate	1.2
3-Oxo acid CoA-transferase	Acetoacetate	0.665
	Succinyl-CoA	0.038
Cytoplasmic acetoacetyl-CoA thiolase	Acetoacetyl-CoA	0.021
	CoA	0.025
Mitochondrial acetoacetyl-CoA thiolase	Acetoacetyl-CoA	0.009
	CoA	0.022

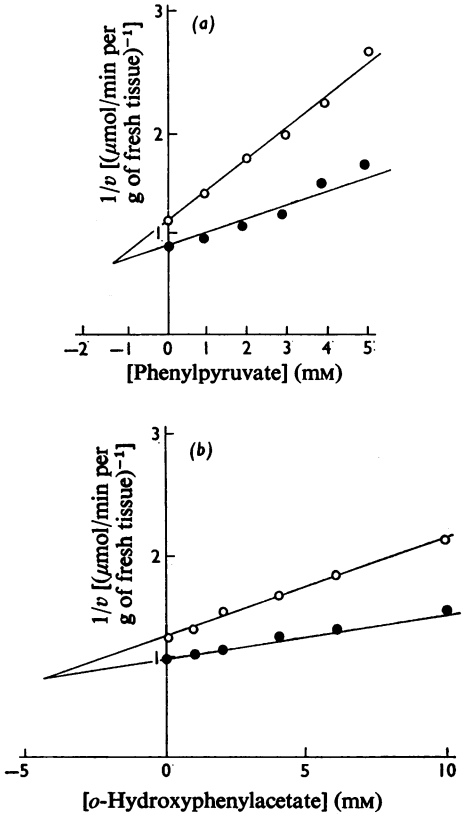

Fig. 2. *Effect of phenylpyruvate and o-hydroxyphenylacetate on the activity of 3-oxo acid CoA-transferase from suckling rat brain*

The 3-oxo acid CoA-transferase activity from the brain of 20–21-day-old rats was measured as described by Williamson *et al.* (1971), except that two different acetoacetate concentrations (○, 2.0mM; ●, 10.0mM) were used in the presence of different concentrations of phenylpyruvate (*a*) or *o*-hydroxyphenylacetate (*b*). Each point represents the mean value of results from at least four duplicate experiments.

Table 1. The results indicate that only phenylpyruvate strongly inhibits the enzyme regardless of its origin. The kinetic behaviour of the mitochondrial enzyme from rat brain shows that substrate inhibition by acetoacetyl-CoA but not CoA occurs with the mitochondrial enzyme, whereas substrate inhibition by CoA occurs with the cytoplasmic enzyme. The apparent K_m values for acetoacetyl-CoA and CoA respectively were 0.021 and 0.025mM for the cytoplasmic acetoacetyl-CoA thiolase and 0.009 and 0.022mM for the mitochondrial enzyme (Table 2), in good agreement with those values obtained by Middleton (1973) for the purified enzymes. Kinetic studies using the Dixon plot (Fig. 3) yielded K_i values for phenylpyruvate of 3.2 and 5.2mM for the acetoacetyl-CoA thiolase activities derived from mitochondria and cytoplasm respectively.

Discussion

Phenylpyruvate has been reported to be an inhibitor of a number of enzyme activities. Hexokinase (EC 2.7.1.1) of adult and foetal human brain (Weber, 1969; Weber *et al.*, 1970) and rat brain (Weber *et al.*, 1970) are inhibited by phenylpyruvate, with K_i values between 2.0 and 6.8mM. Phosphogluconate dehydrogenase (EC 1.1.1.44) is also inhibited by phenylpyruvate, with a K_i value 1.3mM (Weber *et al.*, 1970). Patel (1972) has shown that rat brain pyruvate carboxylase (EC 6.4.1.1) is inhibited by phenylpyruvate. Bowden *et al.* (1972) found that 2-oxoglutarate dehydrogenase activity is inhibited by phenylpyruvate, with a K_i value of 4mM. Land & Clark (1973) have reported the inhibition of citrate synthase (EC 4.1.3.7), acetyl-CoA carboxylase (EC 6.4.1.2) and fatty acid synthetase with K_i values for phenylpyruvate of 0.7, 10.0 and 0.25mM respectively.

Whether phenylpyruvate in the brain arises from the blood or is derived from phenylalanine in the brain by means of the phenylalanine aminotransferase (Benuck *et al.*, 1971) is a matter of uncertainty. However, despite the lack of information on the

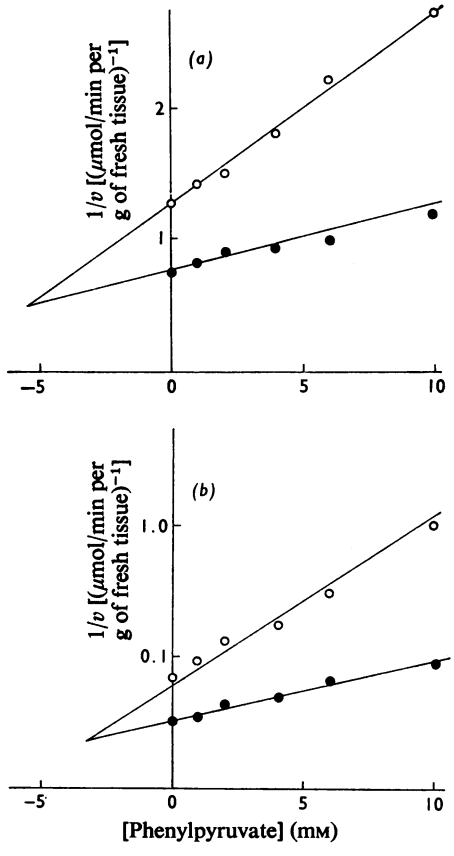

Fig. 3. *Effect of phenylpyruvate on the activity of acetoacetyl-CoA thiolase from suckling rat brain*

The acetoacetyl-CoA thiolase activity from cytoplasm (*a*) and mitochondria (*b*) of 20–21-day-old rat brain was measured as described by Williamson *et al.* (1971), except that two different concentrations of acetoacetyl-CoA (○, 0.05mM; ●, 0.1mM) (*a*) or CoA (○, 0.13mM; ●, 0.4mM) (*b*) were used in the presence of six different concentrations (0, 1, 2, 4, 6 and 10mM) of phenylpyruvate. Each point represents the mean value of results from at least four duplicate experiments.

actual phenylpyruvate concentration in the brain of phenylketonuric individuals, the high phenylalanine concentration in brain (McKean & Peterson, 1970) and the blood phenylpyruvate concentration, which reaches values up to 0.5mM (Knox, 1972), make it likely that this compound is present in the brain of phenylketonuric individuals at concentrations that are able to cause the inhibition of enzymes such as 3-hydroxybutyrate dehydrogenase (Fig. 1) and, to a smaller extent, 3-oxo acid CoA-transferase (Fig. 2). Since the concentrations of 3-hydroxybutyrate and acetoacetate in the blood are about 0.9 and 0.2mM respectively and they are lower still in the brain (Hawkins *et al.*, 1971), calculations indicate that the inhibition of 3-hydroxybutyrate dehydrogenase and 3-oxo acid CoA-transferase could be relevant in the physiopathology of phenylketonuria. The fact that 3-oxo acid CoA-transferase is the key enzyme for the extrahepatic tissue utilization of ketone bodies (Williamson *et al.*, 1971) makes the inhibition of this enzyme even more important.

Land & Clark (1973) have reported that phenylpyruvate inhibits citrate synthase activity competitively. The same authors (Land & Clark, 1974) have suggested an inhibition of the transport of 3-hydroxybutyrate across the inner mitochondrial membrane on the basis of the inhibition of the mitochondrial oxidation of 3-hydroxybutyrate by phenylpyruvate. It is not possible to decide whether the effect of phenylpyruvate on the 3-hydroxybutyrate oxidation is due to the inhibition of its mediated transport across the mitochondrial membrane (Land & Clark, 1974) or to an inhibition on the metabolic pathways involved in the mitochondrial utilization of ketone bodies. Of course, it may be attributed to a combination of both mechanisms. Nevertheless, it should be noted that phenylpyruvate also inhibits the transport of pyruvate into brain mitochondria (Land & Clark, 1974; Halestrap *et al.*, 1974). This effect, by decreasing the availability of acetyl-CoA from pyruvate, would mean that lipid synthesis was more dependent on carbon provided by ketone bodies.

A general impairment of ketone-body metabolism may occur in phenylketonuria. The inhibition of 3-hydroxybutyrate dehydrogenase and 3-oxo acid CoA-transferase activities in the brain, together with the inhibitions of 3-hydroxybutyrate transport across the mitochondrial membrane (Land & Clark, 1974) and of citrate synthase activity (Land & Clark, 1973) by phenylpyruvate would lead to a decrease in the availability of acetyl-CoA for respiration inside the mitochondria, as well as for the supply of carbon skeleton for synthesis of cholesterol and fatty acids in the cytosol. Evidence for this has been obtained from studies on more integrated systems, e.g. experiments *in vivo* in rats with experimentally induced phenylketonuria and brain-slice studies (J. Benavides, unpublished work). Patel & Owen (1976) reported that the incorporation of ketone bodies into brain lipids was inhibited by phenylpyruvate. However, they found that the phenylalanine and phenylpyruvate had no effect on the enzymes involved in ketone-body utilization in brain. This lack of agreement with the present work may be due to the fact that they used adult rat brain enzymes and a commercial preparation of 3-hydroxybutyrate dehydrogenase for the inhibitor studies. We also observed (results not shown) that phenylpyruvate at concentrations up to 10mM did not inhibit the activity of the commercial 3-hydroxy-

butyrate dehydrogenase assayed as described by Patel & Owen (1976).

There is almost general agreement that phenylketonurics are mentally normal at birth and that a progressive loss of intelligence occurs during the first years of life, when the myelination process is also occurring. The highest activities of myelination (Davison & Dobbing, 1968) and brain ketone-body utilization (Williamson & Buckley, 1973) are parallel during brain development. If there is a relationship between both processes, any effect of ketone-body utilization would lead to an impairment of myelination and hence brain dysfunction. Shah *et al.* (1972*a*) suggested that the primary event that could account most satisfactorily for the hypomyelination associated with hyperphenylalaninaemia was the decrease in the rate of cholesterol synthesis. Klee & Sokoloff (1967) suggested that ketone bodies provide acetyl-CoA for synthesis of cholesterol and fatty acids in the cytosol and Edmond (1974) showed that ketone bodies are incorporated into lipids in the brain of suckling rats.

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