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Effect of Phenylpyruvate on Enzymes Involved in Fatty Acid Synthesis in Rat Brain

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1. The activities of, and the effects of phenylpyruvate on, citrate synthase (EC 4.1.3.7), acetyl-CoA carboxylase (EC 6.4.1.2) and fatty acid synthetase derived from the brains of 14-day-old and adult rats were investigated. 2. The brain citrate synthase from 14-day-old rats had a K_m for oxaloacetate of 2.38 μM and for acetyl-CoA of 16.9 μM, and a $V_{max.}$ of 838 nmol of acetyl-CoA incorporation/min per mg of mitochondrial protein. From adult rat brain this enzyme had a K_m for oxaloacetate of 2.5 μM and for acetyl-CoA of 16.6 μM and a $V_{max.}$ of 1070 nmol of acetyl-CoA incorporated/min per mg of mitochondrial protein. Phenylpyruvate inhibited the enzyme from adult and young rat brains in a competitive fashion with respect to acetyl-CoA, with a K_i of 700 μM. 3. The brain acetyl-CoA carboxylase from 14-day-old rats had a K_m for acetyl-CoA of 21 μM and a $V_{max.}$ of 0.248 nmol/min per mg of protein, and from adult rats a K_m for acetyl-CoA of 21 μM and a $V_{max.}$ of 0.173 nmol/min per mg of protein. The enzyme from young and adult rats required citrate (K_a = 3 mM) for activation and were inhibited non-competitively by phenylpyruvate, with a K_i of 10 mM. 4. The brain fatty acid synthetase from 14-day-old rats had a K_m for acetyl-CoA of 7.58 μM and a $V_{max.}$ of 1.1 nmol of malonyl-CoA incorporated/min per mg of protein, and from adult rats a K_m for acetyl-CoA of 4.9 μM and a $V_{max.}$ of 0.48 nmol of malonyl-CoA incorporated/min per mg of protein. Phenylpyruvate acted as a competitive inhibitor with respect to acetyl-CoA with a K_i of 250 μM for the enzyme from 14-day-old rats. 5. These results are discussed with respect to phenylketonuria, and it is suggested that the inhibition of the brain fatty acid synthetase and possibly the citrate synthetase by phenylpyruvate could explain the defective myelination characteristic of this condition.

Phenylketonuria, or phenylpyruvic oligophrenia, is a genetically linked clinical condition, generally accepted as being due to the absence of hepatic phenylalanine hydroxylase (EC 1.14.3.1), which leads to an elevated tissue and plasma phenylalanine concentration (Jervis, 1953). These circumstances cause the metabolism of phenylalanine to occur by pathways which, although always present, are nevertheless normally of minor significance, namely transamination and decarboxylation, giving rise to abnormally high concentrations of certain phenylalanine derivatives, e.g. phenylpyruvate, phenyllactate and phenylacetate. In fact, many of the features of phenylketonuria may be mimicked by inducing experimentally high plasma phenylalanine concentrations (hyperphenylalaninaemia) (Goldstein, 1961; Shah *et al.*, 1970), suggesting that phenylketonuria is caused by high phenylalanine and/or phenylalanine metabolite concentrations. Clinically, phenylketonuric subjects at birth show little abnormality but in the absence of treatment show progressive neurological degeneration and mental retardation. Pathologically most of the changes are confined to the nervous system and show themselves particularly in the defective myelination of the white matter (Shah *et al.*, 1970; Knox, 1972).

During the course of our studies on rat brain pyruvate dehydrogenase (Land & Clark, 1973*b*) it was observed that phenylpyruvate did not inhibit the pyruvate dehydrogenase complex *per se* at concentrations below 2–3 mM, suggesting that pyruvate dehydrogenase was not the site of the primary metabolic lesion in phenylketonuria (cf. Bowden & McArthur, 1972*a*). However, it was also observed that phenylpyruvate competitively inhibited, with a very low K_i of 100 μM, the pigeon liver arylamine acetyltransferase (EC 2.3.1.5), a coupling enzyme used in the assay of the pyruvate dehydrogenase complex. This suggested that in some way phenylpyruvate might interfere with the metabolism of acetyl groups, particularly acetyl-CoA. As the synthesis of fatty acids *de novo* requires acetyl-CoA and inhibition of this synthesis would lead to an impaired myelination, characteristic of phenylketonuria (see Knox, 1972), we have investigated the effects of phenylpyruvate on the brain enzymes involved in

fatty acid synthesis, i.e. citrate synthase (EC 4.1.3.7), acetyl-CoA carboxylase (EC 6.4.1.2) and fatty acid synthetase, in both young (14-day-old) and adult rats. The studies here on 14-day-old rats were particularly pertinent because it is at this period during development that the myelin deposition is occurring (Davison & Dobbing, 1968). Hence any inhibition of the fatty acid-synthesizing enzymes at this stage might have severe effects on myelination. Our results indicate that in animals of both ages citrate synthase and fatty acid synthetase (particularly) may be significantly inhibited by phenylpyruvate at concentrations that might reasonably be expected to exist in the tissue and plasma of phenylketonuric subjects. It is suggested that inhibition of these enzymes may have a major responsibility for the defective myelination and consequent mental retardation observed in such subjects.

Preliminary reports of part of this work have appeared (Land & Clark, 1972, 1973*a*).

Materials and Methods

Materials and animals

Chemicals. NAD^+, CoA and NADPH were purchased from Boehringer und Soehne, Mannheim, Germany; malonyl-CoA and dithiothreitol were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; sodium phenylpyruvate and Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)] were from BDH Ltd., Poole, Dorset, U.K.; 2,2′-bipyridyl disulphide (Aldrithiol II) was supplied by Aldrich Chemical Co., London, U.K. Sodium hydrogen [^{14}C]carbonate (sp. radioactivity 0.1mCi/mmol) was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. 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. Adult rats were male albino animals of the Wistar strain (155–175g body wt.), and they had free access to food (Laboratory Diet no. 1; Spratts, Reading, U.K.) and water up to the time of death. Young animals were 14-day-old unweaned rats of either sex. Animals were always killed at the same time of day (8:30 a.m.), to eliminate any changes in activity due to circadian rhythms. Mitochondria were prepared from rat brain cortex by the method of Clark & Nicklas (1970). Rat brain supernatant fraction (3×10^6*g*-min) was prepared in 0.25M-sucrose–0.5mM-dithiothreitol, pH7.4, and dialysed in 8/32in Visking tubing against 100vol. of 50mM-triethanolamine, pH7.4, containing 2mM-EDTA and 0.25mM-dithiothreitol, for 2h at 4°C, essentially as described by Saggerson & Greenbaum (1970). All enzyme activities were measured at 25°C unless otherwise stated.

Enzyme assays

Citrate synthase. Citrate synthase was measured in rat brain mitochondrial preparations that had been frozen and thawed three times. The assay used was essentially that of Coore *et al.* (1971). The release of free CoA from acetyl-CoA was monitored at 412nm by allowing the liberated thiol groups to react with Ellman's reagent. Each assay was initiated by adding oxaloacetate after any reaction caused by endogenous thiol groups had finished. The reaction mixture consisted of 65nmol of acetyl-CoA, 130nmol of potassium oxaloacetate, pH7.4, 130nmol of Ellman's reagent, 130nmol of Tris–HCl, pH8.0, and 0.08% Triton X-100 in a final volume of 1.3ml. Approx. 20μg of mitochondrial protein was used for each assay and the initial reaction rate measured.

Acetyl-CoA carboxylase. The assay was carried out in test tubes shaken in a water bath at 37°C, essentially as described by Saggerson & Greenbaum (1970). The reaction mixture consisted of 40μmol of triethanolamine buffer, pH6.8, 80μmol of potassium citrate, pH6.8, 20μmol of $MgCl_2$, 8μmol of $MnCl_2$, 30μmol of $KH^{14}CO_3$ (2μCi), 10μmol of ATP, 1μmol of dithiothreitol, 0.2μmol of acetyl-CoA and 2mg of bovine plasma albumin in a final volume of 1ml. At the end of 30min the reaction was stopped by the rapid addition of 0.2ml of acetic acid–HCl mixture (1:10, v/v) and placing the tubes on ice. Samples (200μl) of the reaction mixture were spotted on Whatman no. 1 filter discs (1.8cm diam.) and dried under lamps (disc temperature approx. 60°C) for 1h. The disc was counted for radioactivity in 10ml of scintillation fluid [4g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen/litre of toluene] in a Packard 2425 liquid-scintillation spectrometer. Appropriate corrections were made for quenching from derived standard curves. Preliminary experiments showed the reaction to be linear with time up to 45min and with respect to the volume of supernatant extract used up to 0.3ml (approx. 2mg of protein). As a routine the assay was run for 30min with 0.2ml of supernatant extract.

Fatty acid synthetase. Fatty acid synthetase activity was measured by following NADPH oxidation at 340nm on the addition of malonyl-CoA, essentially by the method of Saggerson & Greenbaum (1970). The reaction cuvette was set up with the following components, in a final volume of 1ml: 100μmol of potassium phosphate, pH6.5, 0.5μmol of NADPH, 50nmol of acetyl-CoA and 5μmol of dithiothreitol. The background oxidation of NADPH was measured for 3min and then the fatty acid synthetase reaction was initiated by the addition of 75nmol of malonyl-CoA.

The blank rate of NADPH oxidation was measured before the addition of malonyl-CoA and was subtracted from the total rate observed in the presence of malonyl-CoA to give the rate of fatty acid synthetase activity. Preliminary experiments showed this rate of reaction to be linear for up to 5 min after addition of malonyl-CoA and with up to 0.35 ml of supernatant extract (approx. 3 mg of protein). The results are expressed as nmol of malonyl-CoA incorporated/min per mg of supernatant protein, assuming 2 mol of NADPH oxidized/mol of malonyl-CoA incorporated (Lynen, 1969).

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

Citrate synthase activity

We have previously reported (Land & Clark, 1973*a*) that citrate synthase from brains of 14-day-old rats was markedly inhibited by phenylpyruvate but not by phenylalanine, phenyl-lactate or *o*-, *m*- or *p*-hydroxyphenylacetate. Similar results were obtained for the brain citrate synthase from adult animals (J. M. Land & J. B. Clark, unpublished work). More detailed studies on the brain citrate synthase from 14-day-old rats indicates a K_m for acetyl-CoA of 16.9 μM (Fig. 1) and for oxaloacetate of 2.38 μM (average of two determinations; see Fig. 2). Figs. 1 and 2 also yield an average value of $V_{max.}$ of 838 nmol/min per mg of mitochondrial protein. If the activity of citrate synthase was studied in the presence of phenylpyruvate at various acetyl-CoA concentrations the results obtained (Fig. 3) indicate that phenylpyruvate was acting as a competitive inhibitor with respect to acetyl-CoA with a K_i of 700 μM. In adult rats similar results were obtained; the K_m for acetyl-CoA was 16.6 μM (Fig. 4) and for oxaloacetate 2.5 μM (Fig. 5), and the mean value of the $V_{max.}$ was 1070 nmol/min per mg of mitochondrial protein. Inhibitor studies with phenylpyruvate on the brain citrate synthase from adult rats again indicated a classical competitive inhibition with respect to acetyl-CoA, with a K_i of 700 μM (Fig. 6). Thus there appears to be very little difference between the citrate synthase from young and adult rat brain, apart from the 25% increase in the $V_{max.}$ in the adult animal.

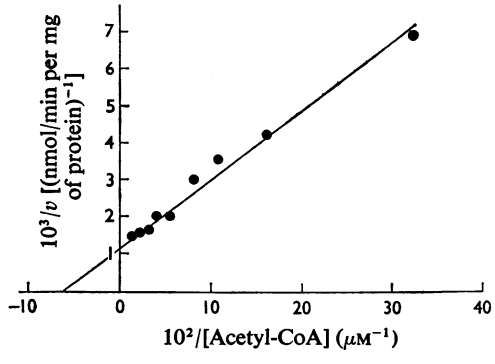

Fig. 1. *K_m for acetyl-CoA of citrate synthase from young rat brain*

Citrate synthase was extracted from the brains of 14-day-old rats and its activity measured at 25°C essentially by the method of Coore *et al.* (1971) (see the Materials and Methods section). The reaction conditions were as described in the Materials and Methods section, except that the concentrations of acetyl-CoA were varied. The enzyme activity (v) is measured as nmol of acetyl-CoA incorporated/min per mg of mitochondrial protein and is plotted as a double-reciprocal plot. The K_m for acetyl-CoA of the citrate synthase is 16.9 μM and the $V_{max.}$ 838 nmol/min per mg of mitochondrial protein. The results have been subjected to computer analysis to derive the best-fit straight line and the various enzyme parameters.

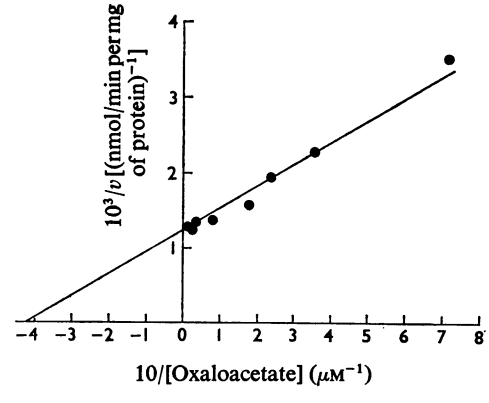

Fig. 2. *K_m for oxaloacetate of citrate synthase from young rat brain*

All conditions were as described in Fig. 1 and in the Materials and Methods section, except that the oxaloacetate concentrations were varied. The activity of the citrate synthase is measured as nmol of acetyl-CoA incorporated/min per mg of mitochondrial protein. The results are plotted as a double-reciprocal plot and yield a K_m for oxaloacetate of 2.38 μM. The results have been subjected to computer analysis to derive the best-fit straight line and the various enzyme parameters.

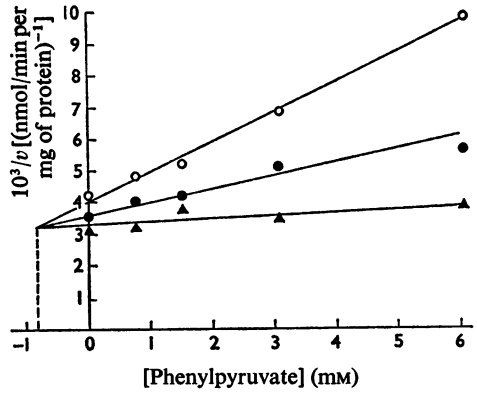

Fig. 3. *Effect of phenylpyruvate on citrate synthase from young rat brain*

The citrate synthase enzyme and activity from brains of 14-day-old rats was measured essentially as outlined in the Materials and Methods section. However, the activity was measured at three different acetyl-CoA (final) concentrations (○, 6.2 μM; ●, 9.3 μM; ▲, 12.4 μM) in the presence of five different phenylpyruvate (final) concentrations (0, 0.77, 1.54, 3.1 and 6.2 mM). The enzyme activity is expressed as nmol of acetyl-CoA incorporated/min per mg of mitochondrial protein and the results are plotted as a single reciprocal plot. By extrapolation of these results a K_i of phenylpyruvate for citrate synthase of 700 μM may be determined. The data have been subjected to computer analysis to derive the best-fit straight line and the various enzyme parameters.

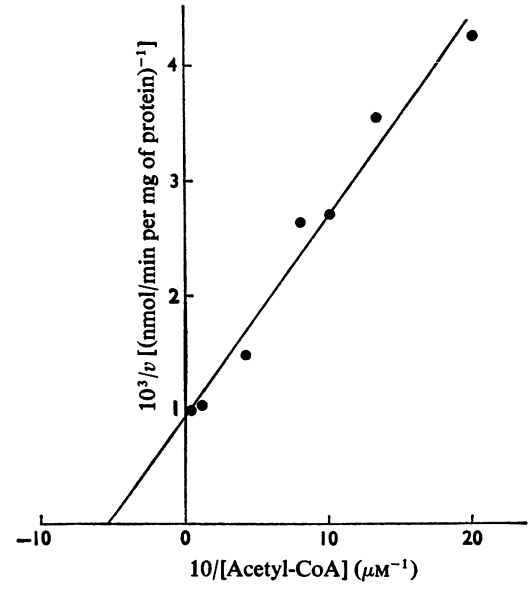

Fig. 4. *K_m for acetyl-CoA of citrate synthase from adult rat brain*

All conditions were as described in Fig. 1 or in the Materials and Methods section, except that the enzyme preparation was derived from adult (9-week-old) rats. The data yield a K_m for acetyl-CoA of 16.6 μM and a $V_{max.}$ of 1070 nmol of acetyl-CoA incorporated/min per mg of mitochondrial protein.

Acetyl-CoA carboxylase activity

The activity of acetyl-CoA carboxylase derived from the cytosol of both 14-day-old and adult rat brains was also studied. Preliminary experiments indicated that the presence of bovine plasma albumin was required for maximum activity of the enzyme from both young and adult rats. A ratio of approx. 2 mg of bovine plasma albumin/mg of cytosolic protein was necessary for optimum activity and was always used in the assays. The bovine plasma albumin used was defatted by acetone washing and contained no significant endogenous citrate as a contaminant (J. M. Land, unpublished work). Further, assay of the acetyl-CoA carboxylase in the presence of twice the normally used quantity of bovine plasma albumin (i.e. 4 mg instead of 2 mg), but in the absence of added citrate, gave the same blank reading, indicating that even if small quantities of citrate were present (undetectable by our assay method) they were not sufficient to cause activation of the enzyme. This requirement for bovine plasma albumin may be because of the presence of fatty acids and their derivatives in the

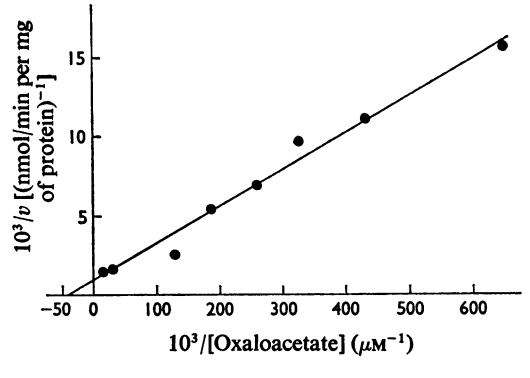

Fig. 5. *K_m for oxaloacetate of citrate synthase from adult rat brain*

All conditions were as described in Fig. 2 or in the Materials and Methods section, except that the enzyme preparation was derived from adult (9-week-old) rats. The data yield a K_m for oxaloacetate of 2.5 μM.

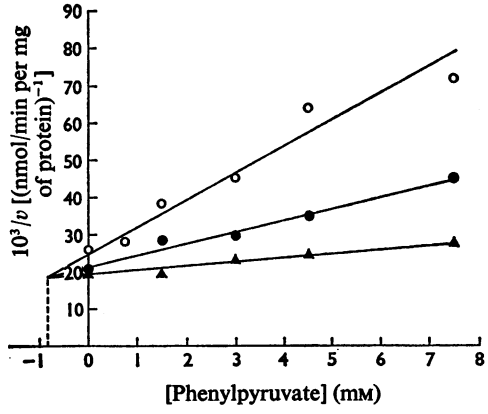

Fig. 6. *Effect of phenylpyruvate on citrate synthase from adult rat brain*

All conditions were as described in Fig. 3 and in the Materials and Methods section, except that the enzyme activity was measured at three different acetyl-CoA concentrations (○, 13.3 μM; ●, 20 μM; ▲, 24 μM) in the presence of five different phenylpyruvate concentrations (0, 1.5, 3, 4.5 and 7.5 mM). The data have been subjected to computer analysis and yield a K_i of phenylpyruvate for citrate synthase of 700 μM.

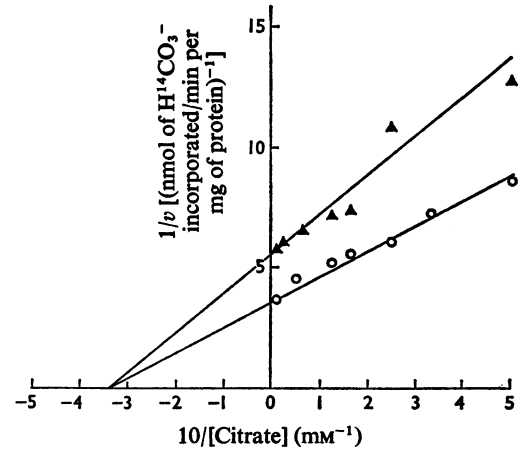

Fig. 7. *Effect of citrate on the activity of acetyl-CoA carboxylase from the brains of young and adult rats*

Acetyl-CoA carboxylase was extracted and assayed at 37°C as outlined in the Materials and Methods section with the exception that the citrate concentrations were varied from 2 to 100 mM (final concentrations). The enzyme activities from the brains of 14-day-old (○) and adult (▲) rats are expressed as nmol of $H^{14}CO_3^-$ incorporated/min per mg of supernatant protein and are plotted as a double-reciprocal plot against citrate concentration. For both 14-day-old and adult brain the results yield a K_a for citrate of 3 mM. The data have been subjected to computer analysis to yield the best-fit straight line and the enzyme parameter.

enzyme preparations, since these compounds are known to be potent inhibitors of acetyl-CoA carboxylase (see Lane & Moss, 1971). In common with other tissues (see Lane & Moss, 1971) the brain acetyl-CoA carboxylase exhibited a requirement for citrate for maximal activity, both the young and adult enzyme exhibiting a K_a for citrate of 3 mM (Fig. 7). Fig. 8 shows the reciprocal plot of the activity of the brain acetyl-CoA carboxylase from 14-day-old rats in the absence and presence of phenylpyruvate (2 or 5 mM final concn.). From the results in the absence of phenylpyruvate a $V_{max.}$ of 0.248 nmol/min per mg of protein and a K_m for acetyl-CoA of 21 μM were obtained. The activities obtained in the presence of phenylpyruvate show that phenylpyruvate acts as a non-competitive inhibitor with a K_i of 10 mM. Similar results for the adult brain acetyl-CoA carboxylase are shown in Fig. 9. In this case the $V_{max.}$ is 0.173 nmol/min per mg of protein and the K_m for acetyl-CoA is 21 μM. Again phenylpyruvate acts as a classical non-competitive inhibitor with a K_i of 10 mM.

Fatty acid synthetase activity

We have previously reported (Land & Clark, 1973*a*) that the fatty acid synthetase derived from the brains of 14-day-old rats, in common with the citrate synthase, was not significantly affected by phenylalanine, phenyl-lactate or *o*-, *m*- or *p*-hydroxyphenylacetate at a final concentration of 2 mM. However, in the presence of 2 mM-phenylpyruvate the fatty acid synthetase was inhibited by 38% (Land & Clark, 1973*a*). Very similar results under the same conditions of substrate and inhibitor concentrations were obtained for the adult fatty acid synthetase, when phenylpyruvate inhibited the enzyme by almost 35%, but the other compounds had no effect (J. M. Land & J. B. Clark, unpublished work). Further studies on the brain fatty acid synthetase from 14-day-old animals indicated a $V_{max.}$ of 1.1 nmol of malonyl-CoA incorporated/min per mg of supernatant protein and a K_m for acetyl-CoA of 7.58 μM (Fig. 10). However, for the adult brain fatty acid synthetase (Fig. 11) the $V_{max.}$ was 0.48 nmol of malonyl-CoA incorporated/min per mg of protein together with a K_m for acetyl-CoA of 4.9 μM.

Fig. 12 is a plot of the reciprocal of the initial velocity of the activity of fatty acid synthesis from

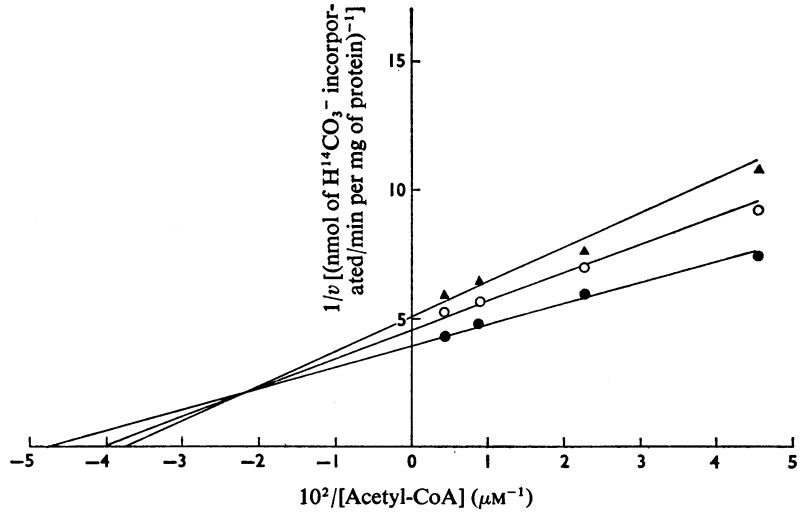

Fig. 8. *Effect of phenylpyruvate on the activity of acetyl-CoA carboxylase from the brain of* 14*-day-old rats*

Acetyl-CoA carboxylase was extracted and assayed at 37°C as outlined in the Materials and Methods section, with the exception that the activity was measured at various acetyl-CoA concentrations in the presence of 0 (●), 2 (○) or 5 (▲) mM-phenylpyruvate (final concentrations). The enzyme activities are expressed as nmol of $H^{14}CO_3^-$ incorporated/min per mg of supernatant protein and plotted as a double-reciprocal plot. The results have been subjected to computer analysis and yield the following parameters: K_m for acetyl-CoA, 21 μM; $V_{max.}$, 0.248 nmol of $H^{14}CO_3^-$ incorporated/min per mg of protein; K_i for phenylpyruvate, 10 mM.

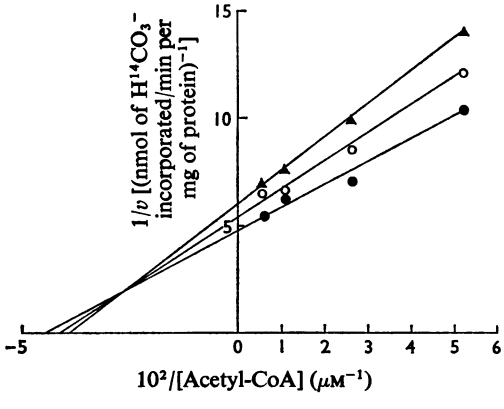

Fig. 9. *Effect of phenylpyruvate on the activity of acetyl-CoA carboxylase from the brain of adult rats*

All conditions were as described in Fig. 8, where the activity was measured at varying acetyl-CoA concentrations in the presence of 0 (●), 2 (○) or 5 (▲) mM-phenylpyruvate (final concentrations). The activities are expressed as nmol of $H^{14}CO_3^-$ incorporated/min per mg of protein and after computer analysis yield the following data: K_m for acetyl-CoA, 21 μM; $V_{max.}$, 0.173 nmol of $H^{14}CO_3^-$ incorporated/min per mg of protein; K_i for phenylpyruvate, 10 mM.

brains of 14-day-old rats against different phenylpyruvate concentrations at three different acetyl-CoA concentrations (6.4, 8.9 and 10.4 μM). From Fig. 12 it may be deduced that phenylpyruvate was acting as a classical competitive inhibitor with respect to acetyl-CoA with a K_i of 250 μM. The low activity of the adult brain fatty acid synthetase made it difficult to measure inhibited activities in the presence of phenylpyruvate, thus rendering it impossible to obtain a K_i value. However, the similarity in the degree of inhibition of the young and adult brain fatty acid synthetase by 2 mM-phenylpyruvate, as previously reported (Land & Clark, 1973*a*), suggests that the enzyme from adult animals will also have a K_i for phenylpyruvate of the same order as the enzyme from young animals.

Discussion

Enzymes of fatty acid synthesis

Tables 1, 2 and 3 present a summary of the currently available kinetic data for the citrate synthase, acetyl-CoA carboxylase and fatty acid synthetase from brain and other tissues. With respect to citrate synthase (Table 1) it is notable that, in terms of mitochondrial enzyme activity, the adult brain has approximately 10 times the potential activity of liver but only two-

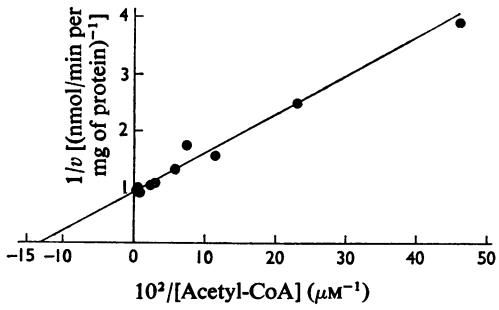

Fig. 10. *K_m for acetyl-CoA of brain fatty acid synthetase from 14-day-old rats*

The fatty acid synthetase was extracted and assayed at 25°C, essentially as described by Saggerson & Greenbaum (1970) as outlined in the Materials and Methods section. The reaction conditions were as described in the Materials and Methods section, except that the acetyl-CoA concentration was varied. The activity of the fatty acid synthetase is expressed as nmol of malonyl-CoA incorporated/min per mg of supernatant protein [assuming 2mol of NADPH oxidized/mol of malonyl-CoA incorporated (Lynen, 1969)]. The results are plotted as a double-reciprocal plot and have been fitted by computer analysis to the best line. The K_m for acetyl-CoA was 7.6μM and the $V_{max.}$ was 1.1nmol of malonyl-CoA incorporated/min per mg of protein.

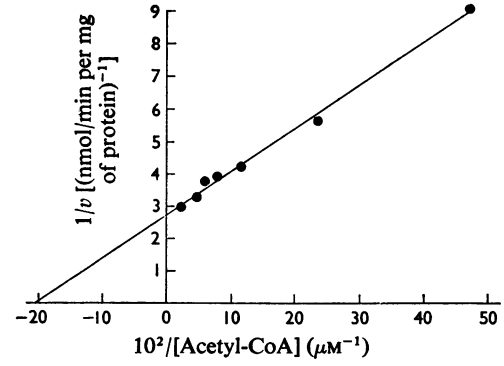

Fig. 11. *K_m for acetyl-CoA of brain fatty acid synthetase from adult rats*

The enzyme was extracted and assayed as described in Fig. 10, except that the enzyme was extracted from the brains of adult rats. The K_m for acetyl-CoA was 4.9μM and the $V_{max.}$ was 0.48nmol of malonyl-CoA incorporated/min per mg of protein.

thirds that of heart. Calculated per gram of tissue, the adult brain citrate synthase has an activity of 3.2 mmol/h. This value is based on a recovery of

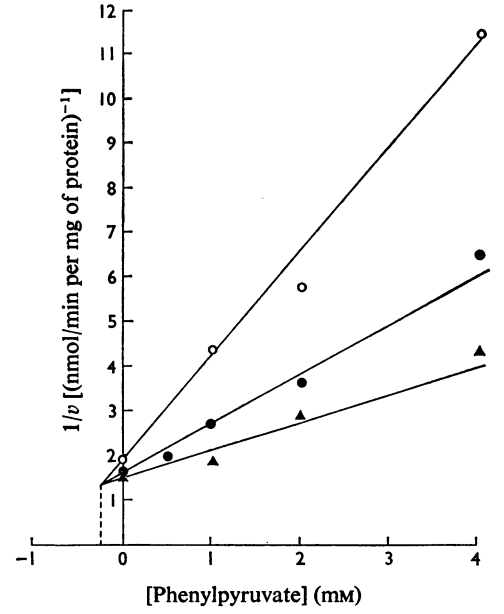

Fig. 12. *Effect of phenylpyruvate on brain fatty acid synthetase from 14-day-old rats*

The enzyme was extracted and assayed as described in Fig. 10 at three different acetyl-CoA concentrations (○, 6.4μM; ●, 8.9μM; ▲, 10.4μM) in the presence of four different concentrations of phenylpyruvate (0, 1, 2 and 4mM final concentrations). The results are plotted on single-reciprocal plots and best-fit straight lines have been determined by computer analysis. Extrapolation yields a K_i of phenylpyruvate for fatty acid synthetase of 250μM.

approx. 3% of the total enzyme present in the homogenate in this particular mitochondrial fraction. Evidence for this is based on distribution studies of succinate dehydrogenase (Clark & Nicklas, 1970) and pyruvate dehydrogenase (J. M. Land & J. B. Clark, unpublished work), and although a 3% recovery may seem low it must be recalled that the mitochondrial population referred to here represents only one of the several heterogeneous brain mitochondrial populations (Blokhuis & Veldstra, 1970; Lai *et al.*, 1973). The whole tissue activity of citrate synthase quoted here is some four times that obtained by Balazs (1965), but it correlates well with other glycolytic and tricarboxylic acid-cycle enzymes in brain (Balazs, 1970) with the notable exception of the pyruvate dehydrogenase, which is some 25 times lower in potential activity (Land & Clark, 1973*b*). Brain acetyl-CoA carboxylase, in common with this enzyme from other tissues, requires citrate as an activator (Table 2). However, in contrast to the rat

Table 1. *Michaelis parameters of citrate synthase*

Tissue	K_m (μM) Oxaloacetate	K_m (μM) Acetyl-CoA	$V_{max.}$ (nmol of acetyl-CoA incorporated/min per mg of protein)	Reference
Liver	2.1	16.5	80–120	Garland *et al.* (1969)
	—	8.8	86	J. M. Land & J. B. Clark, unpublished work
	3.6	2.8	—	Moriyama & Srere (1971)
	5.0	5.8	—	Jangaard *et al.* (1968)
Heart	1.6	8.0	—	Smith & Williamson (1971)
	5.0	4.6	—	Moriyama & Srere (1971)
	2.0	2.7	—	Jangaard *et al.* (1968)
	—	—	1600	Garland *et al.* (1969)
Brain				
(14 days)	2.4	16.9	838	J. M. Land & J. B. Clark, present paper
(adult)	2.5	16.6	1070	J. M. Land & J. B. Clark, present paper

Table 2. *Michaelis parameters of acetyl-CoA carboxylase*

Tissue	K_m for acetyl-CoA (μM)	K_a for citrate (mM)	$V_{max.}$ (nmol of $H^{14}CO_3^-$ incorporated/min per mg of protein)	Reference
Liver				
(rat)	25	4.5	—	Hashimoto & Numa (1971)
(chicken)	16	—	11000	Gregolin *et al.* (1968)
Adipose tissue	20	3–4	—	Moss *et al.* (1972)
	—	—	12000	Kleinschmidt *et al.* (1969)
Mammary gland	50	—	5500	Miller & Levy (1969)
Brain				
(14 days)	21	3	0.248	Present paper
(adult)	21	3	0.173	Present paper

liver, mammary gland and adipose tissue enzymes, which all require a 20–30min preincubation with citrate at 25–38°C before complete activation is attained, the brain enzyme was activated instantaneously. This is a property the brain enzyme possesses in common with the avian liver and bovine adipose tissue acetyl-CoA carboxylases (Lane & Moss, 1971).

The brain appears to have a much less-active fatty acid synthetase than do other tissues (Table 3). The activities reported in this paper on a whole-tissue basis (14-day-old rats, 6.6μmol/h per g; adult rats, 2.9μmol/h per g) compare well with other values reported in the literature for 14-day-old rats [4.4μmol/h per g (Volpe & Kishimoto, 1972)] and for adult rats [2.5μmol/h per g (McIlwain & Bachelard, 1971) and 2.8μmol/h per g (Volpe & Kishimoto, 1972)].

Developmental aspects

It is now reasonably well established that the period of maximum myelin deposition during the development of the rat is 10–14 days after birth (Davison & Dobbing, 1968). It is apparent, however, from a comparison of the citrate synthase, acetyl-CoA carboxylase and fatty acid synthetase from 14-day-old and adult rats (Tables 1, 2 and 3) that very little difference exists between their K_m values. The citrate synthase maximal activity (Table 1), however, increases by 28% from 14 days to adult status (9 weeks). This is of a similar order to that reported for

Table 3. *Michaelis parameters of fatty acid synthetase*

Tissue	K_m for acetyl-CoA (μM)	$V_{max.}$ (nmol of malonyl-CoA incorporated/min per mg of protein)	Reference
Liver	4.4	—	Burton *et al.* (1968)
	—	21	Hsu *et al.* (1965)
Adipose tissue	—	17	Calculated from Saggerson & Greenbaum (1970)
Mammary gland	—	440	Carey *et al.* (1970)
Brain			
(14 days)	7.6	1.1	Present paper
(9 weeks)	4.9	0.48	Present paper
(14 days)	—	0.73	Volpe & Kishimoto (1972)
(6 weeks)	7.0	0.42	Volpe & Kishimoto (1972)

succinate dehydrogenase (Davison & Dobbing, 1968), another mitochondrial enzyme. However, the acetyl-CoA carboxylase $V_{max.}$ falls by 30% and that of the fatty acid synthetase by 56% during the same developmental period (Tables 2 and 3), both these enzymes being cytosolic in origin. The changes in enzyme activities from one cell compartment to another during development may well reflect changes in the metabolic pattern in the young and adult brain. The young developing brain appears to be geared towards a more synthetic pattern than the adult brain, which would be more involved in energy provision and transmitter synthesis. However, in both the young and the adult rat the brain citrate synthase has a $V_{max.}$ almost three orders of magnitude greater than the acetyl-CoA carboxylase and fatty acid synthetase. These two last-named enzymes are in the ratio of 1:3 (adult rats) and 1:4 (young rats), and although acetyl-CoA carboxylase is therefore the effective rate-limiting enzyme in lipogenesis it is possible that under certain conditions fatty acid synthetase may also become controlling (see Lane & Moss, 1971).

Phenylketonuria

The relevance of the inhibition of the brain fatty acid synthetase and citrate synthase by phenylpyruvate reported here to phenylketonuria is complicated by the lack of accurate measurements of cerebral phenylpyruvate concentrations in phenylketonuria. One approach to this problem has been to study hyperphenylalanaemia in rats, which is known to give rise to symptoms of phenylketonuria. Recent studies of this nature (Edwards & Blau, 1972) have indicated that several metabolites of phenylalanine, including phenylpyruvate, may arise in the brain tissue in the presence of high plasma phenylalanine concentrations. Whether these metabolites arise by direct penetration of the blood–brain barrier from the plasma, or by the penetration of phenylalanine and then subsequent metabolism, is as yet not apparent. Indeed, it could be a combination of both mechanisms, since there is evidence for a high-affinity carrier system for phenylalanine across the blood–brain barrier (Oldendorf, 1971), and Edwards & Blau (1972) have provided data suggesting that phenyl-lactate itself may cross the blood–brain barrier. Further complications arise over the development of the blood–brain barrier and its status in young rats (14 days). It appears reasonable, however, to suggest that phenylpyruvate and other metabolites do exist in brain under conditions similar to phenylketonuria, but that the actual concentrations must remain, for the moment at least, a matter of uncertainty.

We have shown previously (Land & Clark, 1973*a*) that of all the commonly occurring metabolites of phenylalanine, phenylpyruvate is the only one that significantly affects the brain citrate synthase and fatty acid synthetase. Other workers have also reported that phenylpyruvate is the most potent inhibitor of all the phenylalanine metabolites, e.g. Shah *et al.* (1969) on inhibition of cholesterol synthesis, Weber *et al.* (1970) on glycolysis, Bowden & McArthur (1972*a*) on pyruvate decarboxylation and Patel (1972) on pyruvate carboxylation. Table 4 indicates, together with values for K_i, the brain enzymes that phenylpyruvate has been reported to inhibit. Of these enzymes the citrate synthase and fatty acid synthetase have K_i values for phenylpyruvate that are an order of magnitude lower than for the other enzymes. Leaving out the uncertainty of the actual cerebral concentrations of phenylpyruvate in phenylketonuria, it is thus obvious that of the enzyme systems currently known to be inhibited by phenylpyruvate fatty acid synthetase will be the primary site of inhibition, followed closely by citrate

Table 4. *Enzymes inhibited by phenylpyruvate*

Enzyme	K_i	Reference
Hexokinase	3–6mM	Weber *et al.* (1970)
6-Phosphogluconate dehydrogenase	3–6mM	Weber *et al.* (1970)
Pyruvate dehydrogenase	6mM	Bowden & McArthur (1972*a*)
Pyruvate carboxylase	—	Patel (1972)
Citrate synthase	700μM	Land & Clark (1973*a*) and present paper
α-Oxoglutarate dehydrogenase	4mM	Bowden & McArthur (1972*b*)
Acetyl-CoA carboxylase	10mM	Present paper
Fatty acid synthetase	250μM	Land & Clark (1973*a*) and present paper

synthase. Other enzyme inhibitions will start to occur only at much higher phenylpyruvate concentrations. Further, of the few reported plasma phenylpyruvate concentrations measured in phenylketonuric subjects (see Jervis, 1952; Knox, 1972; Patel, 1972) it seems unlikely that plasma phenylpyruvate concentrations exceed 0.5mM. This gives support to the hypothesis that inhibition of the fatty acid synthetase and possibly citrate synthase by phenylpyruvate may be considered as a primary metabolic lesion in phenylketonuria. Evidence for this suggestion is also available from studies on more integrated systems. The work of Glazer & Weber (1971) on the effects of phenylpyruvate on [^{14}C]glucose metabolism by brain slices has shown that incorporation of [^{14}C]glucose into lipids is much more sensitive to phenylpyruvate inhibition than is incorporation of [^{14}C]glucose into protein or nucleic acids. Also, Patel (1972) has demonstrated a 60% inhibition of citrate formation by phenylpyruvate in rat brain mitochondria metabolizing pyruvate, a result that could be explained on the basis of inhibition of citrate synthase by phenylpyruvate.

The inhibition of the fatty acid synthetase by phenylpyruvate would lead to a marked decrease in the availability of fatty acids for incorporation into myelin. Also, the inhibition of the citrate synthase would significantly impair lipid biosynthesis, since not only is cytosolic citrate the source of acetyl-CoA for fatty acid synthesis but acetyl-CoA carboxylase itself has a definite requirement for citrate for maximal activity (Table 2). Thus although acetyl-CoA carboxylase itself might not be significantly inhibited directly by phenylpyruvate the effects of phenylpyruvate on the citrate synthase would lead to an effective but indirect inhibition of the acetyl-CoA carboxylase.

These results could therefore explain the incomplete myelination that is characteristic of phenylketonuria, and the presence of phenylpyruvate during the developing period critical to the myelination procedure (10–14 days in rats) might be expected to lead to defective structural features in the nervous system. Whether such an effect would also explain the defective functional features in the nervous system and the mental retardation associated with phenylketonuria would be a subject of speculation. However, if the evidence presented here on the effects of phenylpyruvate on the rat brain citrate synthase and fatty acid synthetase, and in the preceding paper (Land & Clark, 1973*b*) on the pigeon liver arylamine acetyltransferase, are representative of a general inhibition by phenylpyruvate of acetyl-group metabolism, then it might be predicted that the enzyme responsible for acetylcholine formation (choline acetyltransferase, EC 2.3.1.6) would also be inhibited. Such an effect would undoubtedly lead to a serious mental impairment and warrants further investigation.

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References

Balazs, R. (1965) *J. Neurochem.* **12**, 63–76
Balazs, R. (1970) *Handb. Neurochem.* **3**, 1–36
Blokhuis, G. G. D. & Veldstra, H. (1970) *FEBS Lett.* **11**, 197–199
Bowden, J. A. & McArthur, C. L. (1972*a*) *Nature (London) New Biol.* **235**, 230
Bowden, J. A. & McArthur, C. L. (1972*b*) *Abstr. Fed. Amer. Soc. Exp. Biol.* no. 1566
Burton, D. N., Haavik, A. G. & Porter, J. W. (1968) *Arch. Biochem. Biophys.* **126**, 141–154
Carey, E. M., Dils, R. & Hansen, H. J. M. (1970) *Biochem. J.* **117**, 633–635
Clark, J. B. & Nicklas, W. J. (1970) *J. Biol. Chem.* **245**, 4724–4731
Coore, H. G., Denton, R. M., Martin, B. R. & Randle, P. J. (1971) *Biochem. J.* **125**, 115–127
Davison, A. N. & Dobbing, J. (1968) in *Applied Neurochemistry* (Davison, A. N. & Dobbing, J., eds.), pp. 253–286, Blackwell, Oxford
Edwards, D. J. & Blau, K. (1972) *Biochem. J.* **130**, 495–503
Garland, P. B., Shepherd, D., Nicholls, D. G., Yates, D. W. & Light, P. A. (1969) in *Citric Acid Cycle* (Lowenstein, J. M., ed.), pp. 163–212, Dekker, New York
Glazer, R. I. & Weber, G. (1971) *J. Neurochem.* **18**, 2371–2382
Goldstein, F. B. (1961) *J. Biol. Chem.* **236**, 2656–2661

Gregolin, C., Ryder, E. & Lane, M. D. (1968) *J. Biol. Chem.* **243**, 4227–4235
Hashimoto, T. & Numa, S. (1971) *Eur. J. Biochem.* **18**, 319–331
Hsu, R. Y., Wasson, G. & Porter, J. W. (1965) *J. Biol. Chem.* **240**, 3736–3746
Jangaard, N. O., Unkeless, J. & Atkinson, D. E. (1968) *Biochim. Biophys. Acta* **151**, 225–235
Jervis, G. A. (1952) *Proc. Soc. Exp. Biol. Med.* **81**, 715–720
Jervis, G. A. (1953) *Proc. Soc. Exp. Biol. Med.* **82**, 514–515
Kleinschmidt, A. K., Moss, J. & Lane, M. D. (1969) *Science* **166**, 1276–1278
Knox, W. E. (1972) in *Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B. & Fredrickson, D. S., eds.) 3rd edn., pp. 266–295, McGraw-Hill, New York
Lai, J. C. K., Land, J. M. & Clark, J. B. (1973) *Abstr. Int. Congr. Neurochem. 4th*, in the press
Lan, J. M. & Clark, J. B. (1972) *Abstr. FEBS Meet. 8th*, Abstr. no. 1103
Land, J. M. & Clark, J. B. (1973*a*) *Biochem. Soc. Trans.* **1**, 463–466
Land, J. M. & Clark, J. B. (1973*b*) *Biochem. J.* **134**, 539–544
Lane, M. D. & Moss, J. (1971) *Metab. Pathways* 3rd edn., **5**, 23–54
Lynen, F. (1969) *Methods Enzymol.* **14**, 17–33
McIlwain, H. & Bachelard, H. S. (1971) *Biochemistry and the Central Nervous System*, 4th edn., pp. 98–150, Churchill-Livingstone, London
Miller, A. L. & Levy, H. R. (1969) *J. Biol. Chem.* **244**, 2334–2342
Moriyama, T. & Srere, P. A. (1971) *J. Biol. Chem.* **246**, 3217–3223
Moss, J., Yamagishi, M., Kleinschmidt, A. K. & Lane, M. D. (1972) *Biochemistry* **11**, 3779–3786
Ochoa, S. (1955) *Methods Enzymol.* **1**, 688–694
Oldendorf, W. H. (1971) *Amer. J. Physiol.* **221**, 1629–1639
Patel, M. S. (1972) *Biochem. J.* **128**, 677–684
Saggerson, E. D. & Greenbaum, A. L. (1970) *Biochem. J.* **119**, 221–242
Shah, S. N., Peterson, N. A. & McKean, C. M. (1969) *Biochim. Biophys. Acta* **187**, 236–242
Shah, S. N., Peterson, N. A. & McKean, C. M. (1970) *J. Neurochem.* **17**, 279–284
Smith, C. M. & Williamson, J. R. (1971) *FEBS Lett.* **18**, 35–38
Volpe, J. J. & Kishimoto, Y. (1972) *J. Neurochem.* **19**, 737–753
Weber, G., Glazer, R. I. & Ross, R. A. (1970) *Advan. Enzyme Regul.* **8**, 13–36