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Phenylalanine metabolism in isolated rat liver cells

Effects of glucagon and diabetes

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1. Methods are described for monitoring the metabolic flux through phenylalanine hydroxylase, the tyrosine catabolic pathway and phenylalanine: pyruvate transaminase in isolated liver cell incubations. 2. The relationship between hydroxylase flux and phenylalanine concentration is sigmoidal. 3. Glucagon increases hydroxylase activity at low, near-physiological, substrate concentrations only. The hormone does not affect the rate of formation of phenylpyruvate. 4. Experimental diabetes (for 10 days) increases phenylalanine catabolism, and this is further increased by glucagon. 5. These results are discussed in the light of the known mechanisms for control of phenylalanine hydroxylase activity *in vitro*.

Phenylalanine hydroxylase [L-phenylalanine, tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating); EC 1.14.16.1] catalyses the first, and physiologically irreversible, step in phenylalanine catabolism:

L-Phenylalanine + tetrahydrobiopterin
$+ O_2 \rightarrow$ L-tyrosine + dihydrobiopterin + H_2O

The enzyme has been purified from rat liver and its properties examined in detail *in vitro* (see Goodwin, 1979, for general review). In particular, each subunit of the tetramer exists in both phosphorylated and non-phosphorylated forms, the phosphorylation being catalysed *in vitro* by the cyclic AMP-dependent protein kinase (Abita *et al.*, 1976; Donlon & Kaufman, 1980). *In vivo*, the three isoenzymic forms are explicable on the basis of differing degrees of phosphorylation (Donlon & Kaufman, 1980). It has furthermore been recently shown that exposure to glucagon increases the extent of phosphorylation both in whole animals (Donlon & Kaufman, 1978) and in isolated liver cells (Abita *et al.*, 1980).

In the present paper we report a method for following phenylalanine metabolism in isolated cells. Use of this method reveals that glucagon increases flow through the phenylalanine hydroxylase step at physiologically relevant concentrations of the amino acid substrate, and that this effect is additive with the increased enzyme activity in cells from diabetic rats. The rate of formation of phenylpyruvate and phenyl-lactate is approx. 10% of that of the hydroxylase flux in normal, fed rats, and is more responsive to phenylalanine concentration than to glucagon availability.

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Materials and methods

Animals

Male Sprague–Dawley rats (University of Manchester breeding colony), weighing 180–220g, were used throughout. Animals were fed *ad libitum* (no. 1 maintenance diet; Cooper Nutritional Products, Witham, Essex, U.K.).

Diabetes was induced by the intravenous injection of streptozotocin [60mg/kg body wt., in 0.9% (w/v) NaCl]. Glucosuria was checked with Clinistix (Ames Co., Stoke Poges, Slough, Bucks., U.K.) and diabetes was confirmed by plasma glucose measurement (> 16.5mM; Krebs *et al.*, 1963). Diabetic animals were used 10 days after injection.

Chemicals

Amino acids, *N*-iodosuccinimide, L-lactic acid and glucose oxidase (type II) were from Sigma. Peroxidase was from International Enzymes, Windsor, Berks., U.K., glucagon from Uniscience, Cambridge, U.K., and charcoal (Norit GSX) from Norit Clydesdale Co., Glasgow, Scotland, U.K. Cocktail 'T' scintillation fluid was from Hopkin and Williams, Chadwell Heath, Essex, U.K. Bovine serum albumin (fraction V; Miles Laboratories, Slough, Berks., U.K.) was freed of fatty acids and other materials as

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described by Chen (1967). [1-^{14}C]- and [4-^{3}H]-Phenylalanine (sp. radioactivities 59Ci/mol and 27Ci/mmol respectively) were from The Radiochemical Centre, Amersham, Bucks., U.K., and were freeze-dried before use. Streptozotocin was a gift from Dr. D. C. N. Earl, ICI Pharmaceuticals Division, Macclesfield, Cheshire, U.K. Other chemicals were of the purest grade available from standard suppliers.

Preparation and incubation of cells

Isolated liver cells were prepared from rats essentially by the procedure described previously (Elliott *et al.*, 1976). Metabolic integrity was assessed by measurement of ATP content throughout incubations (Dickson & Pogson, 1977).

Incubation procedures and the measurement of $^{14}CO_2$ release were as described previously (Smith *et al.*, 1978; Smith & Pogson, 1980). Cells were pre-incubated for 20min before addition of lactate/pyruvate (10:1) substrate (final total concentration 10mM). Phenylalanine was added 20min later. All incubations (final volume 2ml) contained $(2.5–3.0)\times 10^5$ c.p.m. of either [^{14}C]- or [^{3}H]-phenylalanine. Where glucagon (from a stock 3mM solution in 3.3mM-HCl; final concentration (10nM) was used, it was added 5min before phenylalanine. All additions were made by injection through the septa. Incubation was terminated with 0.2ml of 2M-$HClO_4$.

In experiments with [4-^{3}H]phenylalanine, 0.2ml of the acidified deproteinized supernatant was treated with 0.2ml of Norit GSX (100mg suspended/ml of water). After centrifugation at 12000*g* for 3min at 4°C, 0.2ml portions of the supernatants were counted for radioactivity. A further 0.15ml of the acidified supernatant was added to 1.0ml of 0.2M-sodium acetate, pH5.5, and 0.2ml of freshly prepared 1% (w/v) *N*-iodosuccinimide. After 5min, the samples were acidified with 0.05ml of 30% (w/v) trichloroacetic acid. This procedure displaces a fixed percentage of ^{3}H at the 3-position (Guroff & Abramowitz, 1967). The resulting $^{3}H_2O$ was separated from excess [4-^{3}H]phenylalanine as follows. Each sample was added to a column of Amberlite CG-120. [H^+ form; 200 mesh; 1.5ml of a 60% (w/v) slurry] covered by 20mg of Norit GSX in a Pasteur pipette plugged with cotton wool. $^{3}H_2O$ was washed through with 1ml of double-distilled water and was counted for radioactivity in scintillation cocktail 'T' (Guroff & Abramowitz, 1967).

After collection of $^{14}CO_2$ in the centre wells after acidification of cell suspensions incubated with [1-^{14}C]phenylalanine, the contents of each vial were centrifuged (3000*g*, 5min, 4°C). Portions (0.5ml) of supernatants were added to 0.5ml columns of Amberlite CG-120 (H^+ form) in Pasteur pipettes. The columns were washed with 0.5ml of double-distilled water and samples of the combined eluates were counted for radioactivity in cocktail 'T'. These eluates contain [1-^{14}C]phenylpyruvate and [1-^{14}C]phenyl-lactate.

Results and discussion

Phenylalanine metabolism has been studied not only *in vivo* in man (Goodwin, 1979; Trefz *et al.*, 1979) and in rats (Milstien & Kaufman, 1975*b*), but also *in vitro* in the perfused rat liver preparation (Youdim *et al.*, 1975; Woods & Youdim, 1977) and in liver slices (Milstien & Kaufman, 1975*a*). The results of such studies have been limited and are, in some cases, of little physiological relevance. All authors agree, however, that the major site of phenylalanine catabolism under physiological conditions is the liver and involves phenylalanine hydroxylase as the first, and probably rate-limiting, step.

Preliminary experiments with isolated liver cells established that: (*a*) isotopic equilibrium is rapidly (<5min) achieved between extracellular and intracellular phenylalanine pools; (*b*) the measured rate of hydroxylation with [4-^{3}H]phenylalanine is unaffected by variations in the sp. radioactivity of the substrate over a 100-fold range, indicating the absence of any isotope effect; (*c*) the disposition of ^{3}H during incubation accords with that predicted by Guroff & Abramowitz (1967), and also tallies with measurements of removal of [1-^{14}C]phenylalanine from the medium (results not shown). Briefly, ^{3}H in [4-^{3}H]phenylalanine migrates to the C-3 position as a result of hydroxylase activity (the 'NIH shift'; Guroff *et al.*, 1967); only 8% is released directly as $^{3}H_2O$. When the resulting [3-^{3}H]tyrosine is further metabolized through homogentisate oxidase [homogentisate:oxygen 1,2-oxidoreductase (decyclizing), EC 1.13.11.5], all ^{3}H appears as $^{3}H_2O$. Label is released from (3-^{3}H)-labelled intermediates between phenylalanine and homogentisate by treatment of cell extracts with *N*-iodosuccinimide. Calculation can thus provide values for both hydroxylase activity and flux through homogentisate.

The time courses of phenylalanine hydroxylation, *p*-hydroxyphenylpyruvate decarboxylation, homogentisate oxidation and formation of phenylalanine transamination products are shown in Fig. 1. These experiments were performed with 50μM-phenylalanine, a concentration slightly lower than that reported for rat plasma (Herbert *et al.*, 1966). Rates for the three steps of phenylalanine oxidation were linear for at least 30min and then decreased. The reasons for this non-linearity are not known. It is possible that some role is played by the change in specific radioactivities brought about by release of unlabelled phenylalanine and tyrosine by proteolysis.

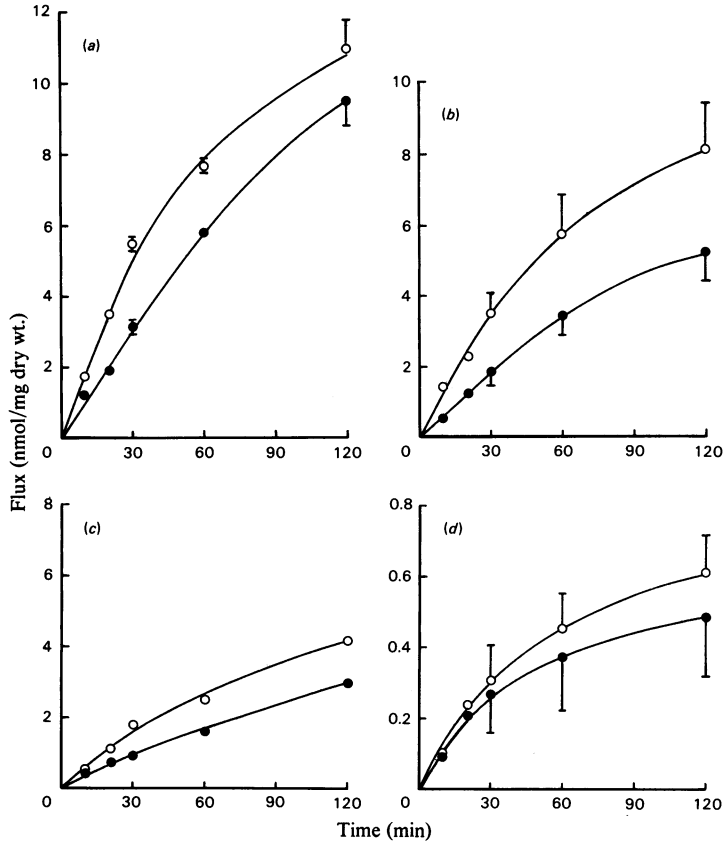

Fig. 1. *Time course of phenylalanine metabolism in isolated liver cells*

The conditions for incubation and methods of calculation were as described in the text. Results are means ± S.E.M. from three independent experiments or single determinations (average of triplicate incubations; 10 and 20 min). In (*c*), standard error bars at 30, 60 and 120 min are within the symbols. ●, Control; ○, plus glucagon (10 nM). Metabolic fluxes: (*a*) Phenylalanine to tyrosine; (*b*) tyrosine to homogentisate; (*c*) homogentisate oxidation; (*d*) phenylalanine transamination.

The hepatocyte breaks down approx. 4% of its protein per hour under conditions comparable with those used in the present paper (Seglen, 1975). The impact of such proteolysis may, however, be lessened if the amino acids so derived do not equilibrate rapidly with the extracellular pool (see Mortimore *et al.*, 1972; Vidrich *et al.*, 1977; Ward & Mortimore, 1978); it should, in any case, be quite small for phenylalanine itself because radiochemical dilution in the relatively large extracellular pool will naturally be rather slow.

The lower rate of tyrosine catabolism (relative to phenylalanine hydroxylation) may be again, in part, attributable to dilution of labelled tyrosine with endogenously-derived amino acid. Measurements of intracellular tyrosine after 5, 10 and 15 min of incubation show that, at 50 μM-phenylalanine, the cellular content is constant at 0.02 ± 0.01 nmol/mg dry wt., and that, at 1 mM-phenylalanine, this rises to 0.06 ± 0.01 nmol/mg dry wt. A proportion of the phenylalanine may, however, appear as tyrosine released into the medium (Woods & Youdim, 1977); the extent of this depends on the activity of tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5), the rate-limiting step for tyrosine breakdown (Dickson *et al.*, 1981). The rate of tyrosine removal is assessed by the release of $^{14}CO_2$ from [1-^{14}C]phenylalanine, because *p*-hydroxyphenylpyruvate oxidase [4-hydroxyphenylpyruvate:oxygen oxidoreductase (hydroxylating, decarboxylating), EC 1.13.11.27] activity parallels that of the transaminase (Dickson *et al.*, 1981).

Accumulation of homogentisate in isolated cell incubations has previously been noted (Jones & Mason, 1978). This is consistent with the observation here that the rates of homogentisate oxidation appear to be less than those of its formation. This does not seem to occur in the perfused liver

preparation (Woods & Youdim, 1977), however, and may therefore reflect the properties of homogentisate oxidase in isolated cells. The specific activity of this enzyme is, nevertheless, similar in hepatocytes and *in vivo* (Jones & Mason, 1978).

The method used here to measure transamination products depends on the retention of label from [1-^{14}C]phenylalanine in compounds lacking the amino function, i.e. phenylpyruvate and phenyllactate. *p*-Hydroxyphenylpyruvate will also be measured, but the concentration of this intermediate remains both very low and constant. *In vivo*, a small but significant proportion of phenylalanine administered appears as phenylacetate or its derivatives (Martin *et al.*, 1979). The pathway by which phenylacetate is formed probably involves phenylethylamine as an intermediate (Edwards & Blau, 1972; Blau, 1979), although formation from phenylpyruvate has also been proposed (Curtius *et al.*, 1972). The rate of phenylethylamine production is, however, relatively low (Haley & Harper, 1978) despite a previous report to the contrary (David *et al.*, 1974); phenylacetate itself is not oxidized in the rat (Haley & Harper, 1978). In addition, it has been shown that very little phenylacetate is formed in the liver *in vivo* (Edwards & Blau, 1972) and in the perfused organ *in vitro* (Blau *et al.*, 1976). It therefore seems reasonable to exclude this compound from our assay procedure (since it will be unlabelled when [1-^{14}C]phenylalanine is used).

Fig. 2 shows a sigmoidal relationship between the concentration of [4-^{3}H]phenylalanine and phenylalanine hydroxylase flux (Hill coefficient = 2.7). This resembles the response previously observed *in vitro* (Fisher & Kaufman, 1973; Dhondt *et al.*, 1978) and indicates that the lipoprotein fractions (Fisher & Kaufman, 1973) and protein stimulators in rat liver (Bessman & Huzino, 1969; Kaufman, 1970) are not effective, at least under the conditions in our experiments. The rate at 1.0mM-phenylalanine is very similar to that reported previously by Woods & Youdim (1977). The apparent K_m for phenylalanine is approx. 0.2mM. This compares with values of 1.5mM (Youdim *et al.*, 1975) and 0.2–0.3mM (Milstien & Kaufman, 1975*a*). The similarity of this value to the K_m of the enzyme with tetrahydrobiopterin as cofactor (Fisher & Kaufman, 1972) supports the view that this latter compound is the naturally-effective cofactor.

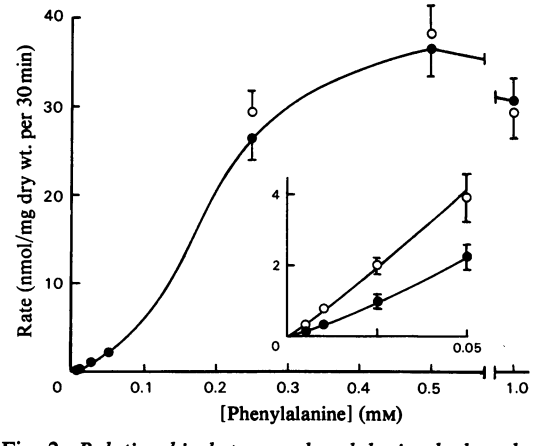

Fig. 2. *Relationship between phenylalanine hydroxylase flux in isolated liver cells and substrate concentration*
The conditions for incubation and methods of calculation were as described in the text. Results are means ± S.E.M. for three independent observations; where not shown, standard error bars are within the symbols. The inset is an enlargement of results at the lowest concentrations. ●, Control; ○, plus glucagon (10nM).

Glucagon (10nM) stimulates hydroxylase activity very markedly at lower, physiological, concentrations of phenylalanine (Fig. 2), but has little effect at higher concentrations. This is consistent with the observation that the enzyme is phosphorylated *in vivo* by a cyclic AMP-dependent protein kinase (Donlon & Kaufman, 1978; Abita *et al.*, 1980), and that such phosphorylation is accompanied by increased activity under physiological conditions within the liver cell.

Brand & Harper (1974*a*) have proposed that glucagon stimulation of flux may be caused primarily by an increase in dihydropteridine reductase (NADPH:6,7-dihydropteridine oxidoreductase, EC 1.6.99.7) activity. The increase in activity of this enzyme is, however, slow (> 1 day); the rapid effects of glucagon in our experiments do not support this suggestion.

The effect of glucagon and experimental diabetes on various parameters are shown in Table 1. Glucagon is without effect at 1mM-phenylalanine, but stimulates flux through all the segments of the catabolic pathway at 50μM-substrate. The flux through phenylalanine:pyruvate transaminase [L-phenylalanine (L-histidine):pyruvate aminotransferase, EC 2.6.1.58] is, however, insensitive to glucagon over the time period studied (see also Fig. 1). Although the activity of the transaminase is increased very markedly by glucagon over several days (Brand & Harper, 1974*b*), an effect associated with an increase in enzyme protein (Shih & Chan, 1979), no parallel increase in flux can be demonstrated (Brand & Harper, 1974*b*).

Experimental diabetes is associated with a significant increase in phenylalanine hydroxylase activity. This is reflected also in increases in flux through *p*-hydrophenylpyruvate and homogentisate. Although, to the authors' knowledge, the effects of diabetes on this system in liver have not previously been studied, the direction of the changes is that

Table 1. *Rates of metabolism through the phenylalanine catabolic pathway in liver cells from fed and diabetic rats: the effect of glucagon*

Rates were measured as described in the text, and are expressed as nmol/mg dry wt. per h (based on initial rates over 30min); means ± S.E.M. are given for the number of independent observations shown. The significance between means was examined by the Student *t* test (I vs. V, II vs. VI) or the paired *t* test (other comparisons); N.S., not significant; vs., versus.

	Normal fed rats				Diabetic rats	
[Phenylalanine] ...	50 μM		1 mM		50 μM	50 μM
Glucagon (10 nM) ...	−	+	−	+	−	+
	I	II	III	IV	V	VI
Metabolic flux						
Phenylalanine → tyrosine	6.0 ± 0.3	10.9 ± 0.2	66.7 ± 13.0	66.0 ± 14.5	12.4 ± 1.9	17.0 ± 2.2
P		(vs. I, <0.005)		(vs. III, N.S.)	(vs. I, <0.05)	(vs. II, <0.05; vs. V, <0.05)
Tyrosine → homogentisate	3.4 ± 0.5	6.5 ± 1.0	33.7 ± 4.1	36.4 ± 4.1	5.5 ± 0.8	7.5 ± 1.0
P		(vs. I, <0.01)		(vs. III, N.S.)	(vs. I, <0.05)	(vs. II, N.S.; vs. V, <0.005)
Homogentisate oxidation	1.8 ± 0.1	3.5 ± 0.2	17.2 ± 1.7	14.6 ± 0.3	3.3 ± 0.4	4.5 ± 0.7
P		(vs. I, <0.01)		(vs. III, N.S.)	(vs. I, <0.01)	(vs. II, N.S.; vs. V, <0.05)
Through phenylalanine: pyruvate transaminase	0.56 ± 0.19	0.62 ± 0.17	7.5 ± 0.2	7.7 ± 0.3	0.40 ± 0.08	0.40 ± 0.08
P		(vs. I, N.S.)		(vs. III, N.S.)	(vs. I, N.S.)	(vs. II, <0.05; vs. V, N.S.)
Number of experiments	4	4	3	3	3	3

expected by analogy with the increase of other enzymes of amino acid metabolism. Paradoxically, insulin has been reported to cause an increase in phenylalanine hydroxylase activity in one liver-derived cell line (Tourian, 1976).

Glucagon increases the activity of the hydroxylase still further in hepatocytes from diabetic rats; this is consistent with the hypothesis that the amount of enzyme protein is increased in such cells.

Changes in the concentration of tetrahydrobiopterin are potentially also significant in the regulation of phenylalanine hydroxylase. Addition of this cofactor to liver slices increases enzyme activity (Milstien & Kaufman, 1975*a*), suggesting that the enzyme may not be fully saturated in this preparation. Although most biopterin in liver is in the fully reduced form (Kaufman, 1964), the concentration is only approx. 13 μM (calculated from the results of Fukushima & Nixon, 1980). The K_m for the cofactor is as yet unresolved, values of 4.5 μM (Kaufman, 1971) and 23 μM (Ayling *et al.*, 1974) having been reported; the concentration of enzyme itself may also influence this parameter (Milstien & Kaufman, 1975*a*). Brand & Harper (1974*a*) were, however, unable to show any change in cofactor availability in glucagon-treated rat liver cytoplasm. It therefore seems improbable that the results of the present study are explicable on the basis of changes in tetrahydrobiopterin content, although we cannot exclude this mechanism.

Another possible alternative is that glucagon might stimulate phenylalanine transport into the cell with a consequent increase in the intracellular concentration of the amino acid, leading secondarily to greater flux through the hydroxylase. It is claimed, however, that rat liver cells possess amino acid transport systems similar to those described for other cells (Le Cam & Freychet, 1977). Phenylalanine is believed to be transported almost exclusively by the L-system (Christensen, 1969) and not to be concentrated against the gradient (Oxender & Christensen, 1963). In addition, we have recently shown that tyrosine is not concentrated in liver cells incubated under similar conditions to those described in the present paper (Dickson *et al.*, 1981). It therefore seems improbable that hormone action is exerted at the level of membrane transport.

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