Effect of Glucagon on Phenylalanine Metabolism and Phenylalanine-Degrading Enzymes in the Rat

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Glucagon administered subcutaneously to rats for 10 days had no significant effect on liver phenylalanine hydroxylase activity, but induced liver dihydropteridine reductase more than twofold. In rats administered a phenylalanine load orally, glucagon treatment stimulated oxidation and depressed urinary phenylalanine excretion. These responses could not be related to an effect of glucagon on hepatic tyrosine- α -oxoglutarate aminotransferase activity. Even in rats with phenylalanine hydroxylase activity depressed to 50% of control values by *p*-chlorophenylalanine administration, glucagon treatment increased the phenylalanine-oxidation rate substantially. Although hepatic phenylalanine-pyruvate aminotransferase was increased tenfold in glucagon-treated rats, glucagon treatment did not increase urinary excretion of phenylalanine transamination products by rats given a phenylalanine load. Glucagon treatment did not affect phenylalanine uptake by the gut or liver, or the liver content of phenylalanine hydroxylase cofactor. It is suggested that dihydropteridine reductase is the rate-limiting enzyme in phenylalanine degradation in the rat, and that glucagon may regulate the rate of oxidative phenylalanine metabolism *in vivo* by promoting indirectly the maintenance of the phenylalanine hydroxylase cofactor in its active, reduced state.

Phenylalanine is converted into tyrosine primarily in the liver by the cytosolic, mixed-function oxidase, phenylalanine hydroxylase, with tetrahydrobiopterin as the immediate electron donor. This cofactor is maintained in its reduced form by a cytosolic, NADH-dependent enzyme (Nielsen *et al.*, 1969), dihydropteridine reductase. Although the enzymology of the mammalian phenylalanine-hydroxylating system has been studied extensively, primarily by Kaufman and his co-workers (Kaufman, 1971), relatively little is known about the physiological regulation of this essential step in aromatic amino acid metabolism.

Administration of cortisol to neonatal rats is reported to induce liver phenylalanine hydroxylase (McGee *et al.*, 1972*a*), but this hormone is a much less effective inducer in adult animals (Freedland, 1963; Chari-Bitron, 1963). Haggerty *et al.* (1973) observed substantial, cortisol-induced increases in the phenylalanine hydroxylase activity of rat hepatoma-cell cultures, but this effect could not be reproduced in intact animals. Tourian (1971) and Sullivan *et al.* (1973) reported that phenylalanine is an allosteric activator of rat liver phenylalanine hydroxylase. However, numerous investigators have found that high-phenylalanine diets result in a depression of phenylalanine hydroxylase activity (Freedland *et al.*, 1964; Woods & McCormick, 1964; Kerr & Waisman,

1967). Fisher & Kaufman (1972) reported that lysophosphatidylcholines were potent activators of phenylalanine hydroxylase *in vitro*; workers from that laboratory also succeeded in purifying a protein from rat liver that stimulates the activity of phenylalanine hydroxylase (Huang *et al.*, 1973). The extent to which these effectors *in vitro* function *in vivo* remains to be determined.

McGee *et al.* (1972*b*) demonstrated that treatment of neonatal rats with sufficient cortisol to induce phenylalanine hydroxylase also promoted phenylalanine removal from the plasma after phenylalanine loading. It has long been known that depressed phenylalanine hydroxylase activity in heterozygote phenylketonuric individuals is associated with decreased ability to dispose of a phenylalanine load, and experimental suppression of this enzyme activity by *p*-chlorophenylalanine treatment impairs tyrosine formation *in vivo* (McGee *et al.*, 1972*b*). In general, however, the functional significance of experimentally produced alterations in the activity of liver phenylalanine hydroxylase has not been assessed by physiological studies *in vivo*. Further, emphasis in investigations of the control of phenylalanine degradation has focused primarily on the hydroxylating enzyme; the possibility that the rate of phenylalanine hydroxylation *in vivo* may be regulated at the level of cofactor regeneration by dihydropteridine reductase has not

been fully explored. In the present study, the possibility of a role for glucagon in the regulation of phenylalanine metabolism was investigated in the rat by examining effects of treatment with this hormone on phenylalanine oxidation *in vivo* and on the activities of the enzymes involved in phenylalanine catabolism.

Materials and Methods

Chemicals

Peroxidase (EC 1.11.1.7, type VI), NADH (disodium salt, grade III), pyridoxal 5-phosphate, DL-*p*-chlorophenylalanine, dithiothreitol, diethyldithiocarbamate (sodium salt), phenazine methosulphate, sodium pyruvate (type II) and α -oxoglutaric acid were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; L-[1- 14 C]phenylalanine (24 Ci/mol, in 1.0M-HCl) was from New England Nuclear Corp., Boston, Mass., U.S.A.; L-phenylalanine was from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; 2-amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4-ol hydrochloride was from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.; glycyl-DL-phenylalanine and ninhydrin were from Pierce Chemical Co., Rockford, Ill., U.S.A.; α -nitroso- β -naphthol was from Matheson, Coleman and Bell, Cincinnati, Ohio, U.S.A. All other chemicals were of reagent grade purity.

Hormonal preparation

Glucagon (combination package, ampoule 668) was obtained from Eli Lilly and Co., Indianapolis, Ind., U.S.A. Solutions for injection were made up to 1.25 mg/ml by using the manufacturer's diluent (containing 1.6% glycerol and 0.2% phenol).

Animals

Male Holtzman albino rats, weighing 80–100g initially, were obtained from the Holtzman Company, Madison, Wis., U.S.A.

Methods

Animal treatments. Rats were housed in individual cages and maintained in a room at 23°C with a 12h light–12h dark cycle. Food and water were available *ad libitum*. Unless otherwise noted, diets consisted of the following in % (w/w): vitamin-free casein, 9; corn oil, 5; mineral mixture (Rogers & Harper, 1965), 5; vitamin mixture (Rogers & Harper, 1965), 0.5; choline chloride, 0.2; L-methionine, 0.3; autoclaved cornstarch, 40; glucose monohydrate, 40. All diets were made up as 3% (w/v) agar gels (Rogers & Harper, 1965). Animals were maintained on this diet for at least 4 days before the start of any experimental treatments. Except where otherwise noted, glucagon was administered by subcutaneous injection at a dosage of 1.25 mg/kg every 12h for 10 days. Control animals received injections of either the diluent or

0.9% NaCl. Injections were given daily at 10:00 and 22:00h. For animals used in metabolism experiments, glucagon injections were discontinued 18h before the start of the experiment, with the objective of eliminating as much as possible any effect on phenylalanine metabolism of tyrosine- α -oxoglutarate aminotransferase induction, since it has been demonstrated that tyrosine oxidation *in vivo* may be increased after induction of this enzyme (Ip & Harper, 1973). Tyrosine- α -oxoglutarate aminotransferase in liver is rapidly induced by glucagon injection and decays to control values within 12h (Civen *et al.*, 1967); *p*-hydroxyphenylpyruvate oxidase, the enzyme ultimately responsible for elimination of phenylalanine carboxyl carbon, is not induced by glucagon treatment (Civen & Brown, 1971; Ip & Harper, 1973). *p*-Chlorophenylalanine (0.11%, w/w) was administered in the diet at the expense of glucose monohydrate (Wapnir *et al.*, 1970); treatment was continued for 1 week.

Phenylalanine oxidation measurements. Phenylalanine oxidation rates *in vivo* were determined by measuring the production of 14 CO₂ from L-[1- 14 C]-phenylalanine. Carboxyl-labelled phenylalanine was used in these studies because the rate of 14 CO₂ production from this compound reflects only the activity of the pathway leading to homogentisic acid formation and not those responsible for the metabolism of the acetoacetate and fumarate ultimately resulting from phenylalanine degradation (Scheme 1).

Rats starved overnight, were force-fed one meal consisting of 3.88 mmol of L-phenylalanine/100g body wt., the phenylalanine was suspended in glass-distilled water at a concentration of 0.91 mmol/ml, to which had been added L-[1- 14 C]phenylalanine to give a final specific radioactivity of 0.147 μ Ci/mmol. Immediately after being force-fed, animals were placed in sealed glass metabolism units which allowed periodic sampling of expired CO₂ and collection of urine. Water was available *ad libitum*. Expired CO₂ was trapped in 15 ml of an ethanolamine-methyl-Cellosolve solution (1:2, v/v), which was changed and sampled hourly for 11h. Radioactivity was determined by counting in a Packard Tri-Carb liquid-scintillation spectrometer samples of vol. 3 ml, to which 15 ml of the organic scintillation fluid of Jaffay & Alvarez (1961) had been added. The total amount of phenylalanine oxidized/h was calculated from the equation:

$$P = \frac{5 \times (C-B) \times 100}{E \times D}$$

where P is the percentage of the phenylalanine load oxidized/h, C is the measured radioactivity (c.p.m.) of the 3 ml sample, B is the blank value, E is the counting efficiency (typically 67%) and D is the amount (d.p.m.) of L-[1- 14 C]phenylalanine radioactivity in

Scheme 1. Pathway of oxidation of L-[1-¹⁴C]phenylalanine in liver

the oral load. At the end of the oxidation experiment, the animals were decapitated and the livers immediately excised and placed in chilled 0.9% NaCl.

Tissue-distribution experiments. Animals starved overnight were loaded orally with L-phenylalanine as described above, except that label was omitted from the force-feeding mixture. Animals were placed in stainless-steel metabolism cages and urine was collected periodically. At desired intervals, animals were anaesthetized with ether, thoracic cavities opened, and blood samples taken by cardiac puncture; plasma was prepared and frozen for later analysis. After exsanguination of the animals, livers were removed and placed in cold 0.9% NaCl. Muscle samples were obtained from the right gastrocnemius muscle and frozen immediately in liquid N₂. The entire gastrointestinal tract was removed and frozen in solid CO₂ for future analysis.

Enzyme assays. Livers were chilled immediately in 0.9% NaCl solution; they were then blotted to remove blood, trimmed and weighed and a 3 g portion was minced and homogenized in 12 ml of chilled 0.25 M-sucrose with a Potter-Elvehjem tissue-grinder tube and a Teflon pestle. Homogenates were centrifuged at 105000g for 1 h in a Beckman L-2 ultracentrifuge. Supernatants were stored frozen below -20°C and enzyme assays performed within 1 week, during which time enzyme activities did not diminish significantly.

Phenylalanine hydroxylase (EC 1.14.16.1) was assayed either at 25°C by the spectrophotometric assay of Ayling *et al.* (1973) or at 37°C by the method of Bublitz (1969), by using a 4 ml reaction volume shaken at 100 cycles/min in a 50 ml beaker to provide adequate oxygenation, and a micro modification of the iodination method of Kaufman (1957) for the determination of tyrosine formed. The synthetic cofactor used in both assays was 2-amino-6,7-dimethyl-5,6,7,8-tetrahydropteridin-4-ol.

Dihydropteridine reductase (EC 1.6.99.7) was assayed at 25°C by the spectrophotometric method of Nielsen *et al.* (1969), but with 3.0 mM-H₂O₂ in a 3.3 ml reaction volume.

Phenylalanine-pyruvate aminotransferase was assayed at 30°C by the method of Spolter & Baldrige (1963) for histidine-pyruvate aminotransferase as modified by Schirmer & Harper (1970), but substituting equimolar L-phenylalanine for L-histidine in the reaction mixture.

Tyrosine- α -oxoglutarate aminotransferase (EC 2.6.1.5) and phenylalanine- α -oxoglutarate aminotransferase were assayed by the colorimetric method of Schepartz (1969). The latter assay was carried out at pH 7.9.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Enzyme units in all cases are expressed as nmol of

substrate transformed/min. Enzyme activities are expressed as units of activity per mg of protein and per 100 g body wt. because, as discussed by Wergedal & Harper (1964), the latter expression takes into consideration effects of treatments on organ size relative to body mass, as well as an enzyme specific activity, and therefore reflects more accurately the capacity of the animal for metabolism of substrates involving the enzyme in question.

Amino acid assays. Plasma samples and 20% (w/v) liver homogenates were treated with one-fifth vol. of 30% (w/v) metaphosphoric acid and centrifuged at 600g for 15 min to remove precipitated protein. Muscle samples frozen in liquid N₂ were first shattered with a tissue crusher of the type described by Stone (1938), then homogenized in 4 vol. (w/v) of glass-distilled water with a Polytron homogenizer. The resulting 20% (w/v) homogenates were deproteinized as above. Whole frozen gastrointestinal tracts were similarly crushed and homogenized, but the deproteinization was effected by the addition to the homogenates of solid trichloroacetic acid to a final concentration of 7.5% (w/v). Urine samples were assayed without deproteinization.

Phenylalanine was determined by a modification (Wong *et al.*, 1964) of the McCaman & Robbins (1962) method, with glycyl-DL-phenylalanine as the peptide reagent.

Tyrosine was determined by the colorimetric method of Udenfriend & Cooper (1952).

Aromatic acid assays. Urine samples were assayed for phenylpyruvic acid content by the method of Schepartz (1969). In rats loaded with carboxyl-labelled phenylalanine, urinary phenyl-lactic acid content could be conveniently measured by extracting the aromatic acids from acidified samples of urine with ethyl acetate as described by Hill *et al.* (1972), and determining the percentage of administered radioactivity appearing in the organic phase. After colorimetric determination of the phenylpyruvic acid content of the urine, the remaining labelled material in the ethyl acetate fraction was considered to be phenyl-lactic acid, since paper radiochromatography of the radioactive urines in propan-2-ol-aq. NH₃-water (8:1:1, by vol.) indicated the only significant labelled components to be phenylalanine, phenylpyruvic acid and phenyl-lactic acid; tyrosine and *p*-hydroxyphenylpyruvic acid were shown to be absent. Similarly, the radioactivity in the aqueous phase of this extraction procedure could be used for determination of urine phenylalanine content of these animals. Radioactivity in 1 ml samples of the aqueous and ethyl acetate fractions was determined by liquid-scintillation spectrometry as described above, by using 15 ml of aqueous (Bruno & Christian, 1961) or organic (Jaffay & Alvarez, 1961) scintillation fluid respectively.

Phenylalanine hydroxylase cofactor determination.

Liver bipterin content was determined by a modification of the method of Guroff *et al.* (1967). Immediately after the animals were killed, their livers were excised and a portion of each was frozen on solid CO₂. A 2g portion of the frozen liver was weighed out, placed in 3.9ml of chilled 0.3M-Tris-HCl buffer, pH7.2, containing 0.01M-dithiothreitol, then finely minced and homogenized with a Potter-Elvehjem

Fig. 1. Rate of expiration of ¹⁴CO₂ from glucagon-treated rats after force-feeding on L-[1-¹⁴C]phenylalanine

Rats were loaded with 3.88mmol of L-[1-¹⁴C]phenylalanine (sp. radioactivity 0.147mCi/mol)/100g body wt. Expired ¹⁴CO₂ was trapped hourly in 15ml of ethanolamine-methyl Cellosolve (1:2, v/v). ●, Untreated controls (*n*=4); □, animals injected immediately after loading with 2.50mg of glucagon/kg (*n*=3); ○, animals injected for 10 days at 12h intervals with 1.25mg of glucagon/kg, injections ceasing 18h before force-feeding (*n*=7). Points represent means ± S.E.M.

apparatus. The supernatant fractions obtained after centrifugation at 15000g for 10min were deproteinized in a 60°C water bath (Guroff *et al.*, 1967). The final preparation was used as the source of cofactor, in place of the 6,7-dimethyltetrahydropterin, in a phenylalanine hydroxylase assay. Source of phenylalanine hydroxylase for the assay was the pooled 105000g supernatant fractions from equal numbers of glucagon-treated and control rats. The assay mixture, after that of Bublit (1969), contained initially the following components (in ml): 25mM-L-phenylalanine, 0.5; 0.3M-Tris-HCl buffer, pH7.2, 0.5; 50mM-dithiothreitol, 0.3; enzyme, 0.2. Assay mixture, contained in 50ml beakers shielded from light, was preincubated in a 37°C shaker for 3 min. The reaction was initiated by the addition of 1.0ml of liver extract, samples (0.5ml) were withdrawn after intervals of 10, 20 and 40min and placed immediately in an equal volume of 12% (w/v) trichloroacetic acid. After centrifugation at 600g for 15min, supernatant fractions were assayed for tyrosine content by a micro modification of the iodination method of Kaufman (1957). Rates of tyrosine production were determined from the calculated slopes of the reaction-kinetics plots, which were linear with time to 40min.

Statistical methods. The significance of the difference between two arithmetic means was evaluated by Student's *t* test.

Results

Effect of glucagon on phenylalanine oxidation

The rate of ¹⁴CO₂ production from an L-[1-¹⁴C]-phenylalanine load in rats treated chronically with glucagon was more than twice that in the untreated group, whereas the rate in rats receiving only one injection of hormone immediately before administration of the phenylalanine load did not differ significantly from that of control animals (Fig. 1).

Effects of glucagon treatment on disposal of the phenylalanine load are summarized in Table 1. Concomitant with an increase of more than 140% in the fraction of the phenylalanine load oxidized by the chronically treated animals, the fractions excreted in

Table 1. Disposal of an oral phenylalanine load in chronically glucagon-treated and control rats

The proportions of the phenylalanine load oxidized and excreted in the urine 10h after force-feeding, by the animals described in Fig. 1, were determined. Values given are means ± S.E.M. (Significance of difference from control is indicated as follows: * *P*<0.05; ** *P*<0.02; *** *P*<0.01; **** *P*<0.0001.)

Treatment	Cumulative % of load oxidized to CO ₂ in 10h	Cumulative % of load excreted in urine after 10h		
		as phenylalanine	as phenylpyruvic acid	as phenyl-lactic acid
None (<i>n</i> = 4)	11.6 ± 2.0	21.5 ± 5.7	14.4 ± 2.0	2.8 ± 1.5
Glucagon (10 days; <i>n</i> = 7)	27.8 ± 4.4***	3.0 ± 0.9**	7.6 ± 1.7*	2.8 ± 0.8

Table 2. *Hepatic phenylalanine hydroxylase and phenylalanine-pyruvate aminotransferase activities of glucagon-treated and control rats*

Livers of the animals described in Fig. 1 were assayed for phenylalanine hydroxylase and aminotransferase activity. Phenylalanine hydroxylase was assayed by the method of Bublitz (1969). Values given are means $\pm$ S.E.M. Significance of difference from control is denoted as in Table 1.

Treatment	Phenylalanine hydroxylase		Phenylalanine-pyruvate aminotransferase	
	(units/mg of protein)	(units/100g body wt.)	(units/mg of protein)	(units/100g body wt.)
None ($n = 4$)	17.6 $\pm$ 1.6	9200 $\pm$ 570	1.6 $\pm$ 0.1	840 $\pm$ 60
Glucagon (10 days, $n = 7$)	16.1 $\pm$ 0.6	10000 $\pm$ 420	15.7 $\pm$ 2.0****	9600 $\pm$ 1100****

Table 3. *Specific activities of liver phenylalanine-degrading enzymes of glucagon-treated and control rats 10h after administration of an oral phenylalanine load*

Values of hepatic enzyme activities at the conclusion of the experiment referred to in Fig. 2 are expressed here on a specific activity basis. Values given are means $\pm$ S.E.M. Significance of difference from control is indicated as in Table 1.

Treatment	Enzyme specific activity (units/mg of protein)				
	Phenylalanine hydroxylase	Dihydropteridine reductase	Tyrosine- α -oxoglutarate aminotransferase	Phenylalanine- α -oxoglutarate aminotransferase	Phenylalanine-pyruvate aminotransferase
None ($n = 4$)	3.22 $\pm$ 0.42	98.8 $\pm$ 4.6	28.3 $\pm$ 3.2	0.71 $\pm$ 0.09	1.3 $\pm$ 0.2
Glucagon (10 days; $n = 7$)	2.73 $\pm$ 0.17	205.2 $\pm$ 9.0***	16.1 $\pm$ 2.5*	0.83 $\pm$ 0.08	13.7 $\pm$ 1.0****

the urine as phenylalanine and phenylpyruvic acid decreased. Urinary phenyl-lactic acid excretion was the same for the control and experimental animals. Although there were substantial differences in the fate of the phenylalanine load between the control and glucagon-treated groups, liver phenylalanine hydroxylase activity was not significantly greater in the hormone-treated rats (Table 2). Also, despite the eightfold induction of liver phenylalanine-pyruvate aminotransferase (Table 3), urinary excretion of phenylpyruvic acid decreased in animals receiving glucagon (Table 1).

Effect of glucagon on amounts of liver enzyme

In a parallel study, activities of hepatic phenylalanine-degrading enzymes and tissue amino acid concentrations were determined in rats injected with glucagon for 10 days as above and appropriate controls loaded orally with 3.88 mmol of unlabelled phenylalanine/100g body wt. The activity of phenylalanine hydroxylase expressed on an activity/body wt. basis was significantly greater in glucagon-treated rats than in control animals (Fig. 2) owing to the increase in liver/body wt. ratio as a result of glucagon treatment. In contrast with the observations of Belzecka *et al.* (1967), phenylalanine loading did not depress phenylalanine hydroxylase activity in either group. The activity of dihydropteridine reductase was increased in glucagon-treated animals to more than twice the control values. This finding appears to

represent a previously unreported response of dihydropteridine reductase to a hormonal effector. Tyrosine- α -oxoglutarate aminotransferase activity of animals receiving glucagon was increased initially despite efforts mentioned above to prevent this from occurring. The specific activity of tyrosine aminotransferase of the control group at 2h was 6.8 $\pm$ 0.2 units/mg of liver protein and of the glucagon treated group was 10.5 $\pm$ 0.9 units/mg of liver protein ($P < 0.05$). The relative difference between the two groups is greater when the activity of this enzyme was expressed as units/100g body wt., as it is in Fig. 3, owing to the larger average relative liver weight of the latter. During the latter part of the experimental period, the activity of this enzyme was significantly lower in glucagon-treated animals than in controls. Induction of tyrosine- α -oxoglutarate aminotransferase has been observed in animals loaded with phenylalanine (Belzecka *et al.*, 1967), but this was evidently suppressed in the animals that received hormone. The activities of the hepatic phenylalanine-degrading enzymes at the termination of this experiment, expressed on a specific activity basis, are given in Table 3.

Of the liver enzymes responsible for oxidizing phenylalanine to homogentisic acid, only dihydropteridine reductase was substantially induced under conditions of glucagon treatment that increased $^{14}\text{CO}_2$ production *in vivo* from L-[1- ^{14}C]phenylalanine. The relationship between the activity of

Fig. 2. Activities of phenylalanine-catabolizing enzymes in glucagon-treated and control rats after oral phenylalanine loading

Rats loaded with 3.88 mmol of L-phenylalanine were killed at the intervals indicated and livers assayed for enzyme activities. (a) Dihydropteridine reductase. (b) Phenylalanine hydroxylase. (c) Tyrosine- α -oxoglutarate aminotransferase. Phenylalanine hydroxylase was assayed by the method of Ayling *et al.* (1973). ●, Untreated controls; ○, animals injected subcutaneously for 10 days at 12 h intervals with 1.25 mg of glucagon/kg; injections were terminated 18 h before force-feeding. Points represent means of four animals $\pm$ S.E.M.

Fig. 3. Relationship between phenylalanine oxidation *in vivo* and liver dihydropteridine reductase activity in glucagon-treated and control rats

Dihydropteridine reductase activities at the $t = 6$ and $t = 10$ h intervals after phenylalanine loading (Fig. 2) were paired with the corresponding phenylalanine oxidation rates taken from Fig. 1 and the correlation between them was determined. ●, Untreated control rats; ○, rats injected for 10 days at 12 h intervals with 1.25 mg of glucagon/kg, injections ceasing 18 h before force-feeding. Values are means $\pm$ S.E.M.

dihydropteridine reductase at the $t = 6$ and $t = 10$ h time-points of this experiment (Fig. 2) and the corresponding rates of phenylalanine oxidation *in vivo* taken from the preceding oxidation experiment (Fig. 1) is indicated in Fig. 3. This suggests that the increased rate of phenylalanine oxidation in the rat treated chronically with glucagon is a consequence of induction of the reductase.

Effect of glucagon on tissue amino acids

Since it has been shown that the rate of amino acid oxidation *in vivo* increases in proportion to substrate availability up to the point of saturation of the enzymes involved (Kim & Miller, 1969), it was important to know the time-course of changes in phenylalanine and tyrosine concentrations in the tissues of these animals subsequent to phenylalanine loading. Fig. 4 shows that animals of both groups were essentially saturated with phenylalanine throughout the experiment. At the $t = 6$ h time-point, tissue and plasma phenylalanine concentrations were significantly lower ($P < 0.05$) in the glucagon-treated animals, in agreement with the observation that the rate of phenylalanine oxidation was higher in these animals. Tyrosine concentrations were, however, not significantly greater in the plasma or tissues of glucagon-treated rats. Particularly striking is the substantial accumulation of tyrosine in the plasma and muscle of both hormone-treated and control animals. The tissue amino acid determinations indicate that the higher rate of phenylalanine oxida-

tion of glucagon-treated rats *in vivo* (Fig. 1) is not the result of a higher concentration of phenylalanine in the liver. Analyses of the phenylalanine content of the gastrointestinal tracts of the animals in this study (Table 4) indicate that the rate of gastric emptying of the phenylalanine load is not increased by the hormone administration, and therefore that this is not a factor in the effect of this treatment on phenylalanine catabolism.

Effect of glucagon on urinary excretion of phenylalanine and phenylpyruvate

The cumulative excretion of phenylalanine in the urine of control rats from the above experiment (Figs. 2 and 4) was over nine times that of the glucagon-treated animals (Table 5), a relation similar to that found in the previous experiment. Excretion of phenylpyruvic acid, after the phenylalanine load, was initially lower in the hormone-treated rats, but the final value at 10 h was not significantly below that for the control rat probably owing to high variability. Nonetheless, it is curious, as noted above, that the excretion of this transamination product of phenylalanine was not increased in rats given glucagon, as measurements of hepatic phenylalanine aminotransferases at intervals during the course of this experiment (Fig. 5) indicate that the theoretical capacity of the livers of glucagon-treated rats to transaminate phenylalanine was significantly increased. Final aminotransferase specific activities are given in Table 2.

Induction kinetics of liver dihydropteridine reductase

Because evidence from the above experiments indicated that the increased rate of phenylalanine metabolism in chronically glucagon-treated rats was associated with liver dihydropteridine reductase induction, the kinetics of the response of this enzyme

Table 4. Effect of chronic glucagon treatment on gastrointestinal emptying of an oral phenylalanine load

Phenylalanine remaining in the gastrointestinal tracts of the animals referred to in Fig. 2 were determined at the indicated intervals after force-feeding. Values are from pooled gastrointestinal tracts of three or four rats.

Time (h)	% of phenylalanine load remaining	
	Controls	Glucagon-treated
0	100	100
2	27.5	27.3
6	16.0	14.6
10	14.4	8.0

Fig. 4. Plasma and tissue concentrations of phenylalanine and tyrosine in glucagon-treated and control rats after oral phenylalanine loading. Phenylalanine and tyrosine contents were measured in (a) plasma, (b) liver and (c) muscle of the rats referred to in Fig. 2. — indicates phenylalanine concentrations; ---- indicates tyrosine concentrations; ●, glucagon-treated; ○, untreated controls. Points are means of four animals $\pm$ S.E.M.

Table 5. Cumulative urinary excretion of phenylalanine and phenylpyruvate acid by glucagon-treated and control rats after phenylalanine loading

Values given are means $\pm$ s.e.m. Significance of differences from control values is indicated as in Table 1. Weights of control and glucagon-treated rats were 138 ± 3 and 100 ± 4 respectively. Final urine volumes for these groups were 5.7 ± 0.7 and 2.2 ± 0.3 respectively.

Time after loading (h)	Phenylalanine excreted (μ mol)		Phenylpyruvic acid excreted (μ mol)	
	Controls	Glucagon-treated	Controls	Glucagon-treated
2	8.2 ± 6.0	0.8 ± 0.2	10.1 ± 2.4	7.6 ± 2.2
6	155 ± 34	$7.2 \pm 2.5^{***}$	202 ± 21	$50.1 \pm 8.1^{***}$
10	190 ± 48	$20.3 \pm 4.8^{***}$	305 ± 23	239 ± 62

Fig. 5. Activities of hepatic phenylalanine-transaminating enzymes in glucagon-treated and control rats after phenylalanine loading

(a) Phenylalanine- α -oxoglutarate aminotransferase, and (b) phenylalanine-pyruvate aminotransferase activities were determined in the rats referred to in Fig. 2. Values given are means of four animals $\pm$ s.e.m.

to glucagon administration were examined. Rats that had received the hormone (1.25 mg/kg) every 12 h for 1, 3, or 10 days, were taken for enzyme measurements 12 h after the last injection. Fig. 6 shows that after 3 days of treatment the specific activity of the enzyme had increased to nearly twice its initial value. When the results are expressed on an enzyme activity/unit body wt. basis, the value at the 10 day time-point is seen to be approximately three times that of the control value.

Effect of glucagon on amount of phenylalanine hydroxylase cofactor

Since glucagon apparently increased the capacity of the liver to regenerate the tetrahydropterin cofactor of phenylalanine hydroxylase, the possibility was considered that this hormone might also have an effect on total liver content of this substance as well. The ability of liver extracts from glucagon-treated and control animals to stimulate tyrosine formation

Fig. 6. Kinetics of liver dihydropteridine reductase induction by glucagon

Rats were injected subcutaneously with 1.25 mg of glucagon/kg at 12 h intervals for 1 day (two injections), 3 days (six injections), or 10 days (20 injections). Animals were killed 12 h after the last injection. ○, Units/mg of protein; □, units/100g body wt.; ●, ■, corresponding untreated control rats and control rats receiving diluent for 10 days respectively. Values given are means of at least five animals ± S.E.M.

Table 6. Effect of chronic glucagon treatment on liver content of phenylalanine hydroxylase cofactor

Liver extracts prepared from rats injected with glucagon (1.25mg/kg for 10 days) and untreated controls were assayed for biopterin content by determining their ability to stimulate tyrosine formation by a crude phenylalanine hydroxylase preparation containing insufficient cofactor. Conditions of the assay are given in the Materials and Methods section. The blank assay contained 1.0ml of 0.3 M-Tris-HCl buffer (pH 7.2) in place of the sample; the control assay contained in place of the sample 0.6 ml of 0.3 M-Tris-HCl buffer and 0.4 ml of 50 mM-dithiothreitol containing 8.6 μmol of the 6,7-dimethyltetrahydropterin used in the phenylalanine hydroxylase assays. Values given are means of four animals ± S.E.M.

Treatment	Tyrosine produced (nmol/min per ml of sample)
None	26.3 ± 1.6
Glucagon (10 days)	28.0 ± 1.2
Blank assay	4.0
Control assay	101

in vitro by a crude phenylalanine hydroxylase preparation was taken as a measure of biopterin content. No significant difference in cofactor activity between the two groups was apparent (Table 6).

Effect of *p*-chlorophenylalanine on stimulation of phenylalanine oxidation by glucagon

To evaluate further the role of dihydropteridine reductase induction in the regulation of phenylalanine catabolism, phenylalanine oxidation *in vivo* was studied as described above in rats simultaneously treated with glucagon to induce dihydropteridine reductase and with *p*-chlorophenylalanine to depress phenylalanine hydroxylase activity. If phenylalanine hydroxylase activity is the rate-limiting factor in phenylalanine degradation, the rate of oxidation *in vivo* of a large phenylalanine load should be proportional to phenylalanine hydroxylase activity under the conditions of this experiment. Administration of 0.11 % *p*-chlorophenylalanine in the diet for 7 days was moderately effective in depressing phenylalanine hydroxylase activity (Table 7), but much less effective than would be expected from the observations of Wapnir *et al.* (1970). The drug had no apparent effect on dihydropteridine reductase activity. In a separate experiment, rats similarly treated with dietary *p*-chlorophenylalanine for 7 days and appropriate controls were found to have liver dihydropteridine reductase activities of 113 ± 6 and 128 ± 8 units/mg of liver protein, respectively. The effect of the drug on the enzymes of hepatic tyrosine oxidation was not determined; however, a significant effect is unlikely, since it has been demonstrated in rats that *p*-chlorophenylalanine administration sufficient to depress phenylalanine hydroxylase activity and elevate plasma phenylalanine concentration substantially had no effect on plasma tyrosine concentration (Lipton *et al.*, 1967; Wapnir *et al.*, 1970). Fig. 7 shows that the rate of phenylalanine oxidation in rats receiving glucagon for 10 days was in general significantly (*P* < 0.01) greater than in untreated control rats, regardless of whether they received dietary *p*-chlorophenylalanine as well. Table 7 shows that although phenylalanine hydroxylase activity was decreased by approximately one-half in the *p*-chlorophenylalanine-treated animals, the total fraction of the phenylalanine load oxidized by this group was significantly greater than that oxidized by control rats with much higher phenylalanine hydroxylase activity.

Discussion

The finding that the rate of phenylalanine degradation was increased in rats treated with glucagon agrees with the known role of this hormone in the regulation of gluconeogenesis from amino acids. Stimulation of gluconeogenesis by glucagon is associated with induction of amino acid-degrading enzymes. Among those shown to be induced by glucagon are: serine dehydratase (Peraino *et al.*, 1966); tyrosine-α-oxoglutarate aminotransferase

Table 7. Effect of glucagon and *p*-chlorophenylalanine treatments on phenylalanine hydroxylase activity and phenylalanine oxidation *in vivo*

Hepatic phenylalanine hydroxylase activity was determined in the rats described in Fig. 7, by the method of Bublitz (1969). The total fraction of the phenylalanine load oxidized to homogentisic acid by these animals was also determined. Values are means $\pm$ S.E.M. Significance of difference from control is indicated as in Table 1.

Treatment	% of phenylalanine load oxidized	Phenylalanine hydroxylase activity	
		(units/mg of protein)	(units/100g body wt.)
None ($n = 7$)	11.4 $\pm$ 1.2	18.0 $\pm$ 1.0	9030 $\pm$ 360
Glucagon (10 days) + <i>p</i> -chlorophenylalanine (7 days; $n = 3$)	18.5 $\pm$ 2.8*	9.0 $\pm$ 0.5***	5380 $\pm$ 160***
Glucagon (10 days; $n = 4$)	32.6 $\pm$ 5.9***	15.9 $\pm$ 1.3†	10300 $\pm$ 860†

† Significant difference from *p*-chlorophenylalanine group, $P < 0.01$.

Fig. 7. Effect of partial inactivation of phenylalanine hydroxylase by *p*-chlorophenylalanine treatment on rate of $^{14}\text{CO}_2$ expiration from glucagon-treated and control rats after force-feeding on L-[1- ^{14}C]phenylalanine

Phenylalanine-oxidation experiment was performed as described in Fig. 1. ●, Untreated controls ($n = 7$); ○, animals injected for 10 days at 12h intervals with 1.25 mg of glucagon/kg, injections terminated 18h before force-feeding ($n = 4$); □, animals similarly injected with glucagon and also fed on 0.11% DL-*p*-chlorophenylalanine in the diet ($n = 3$). Values represent means $\pm$ S.E.M.

(Civen *et al.*, 1967); cystathionine synthetase (Finkelstein, 1967); ornithine- α -oxoglutarate aminotransferase (Peraino *et al.*, 1966); histidase and urocanase (Lee & Harper, 1971); alanine-glyoxylate aminotransferase (Snell, 1971); glutamate-pyruvate aminotransferase (Snell & Walker, 1972). The present study indicates that although phenylalanine hydroxylase was not induced by glucagon the substantial effect of glucagon on phenylalanine oxidation *in vivo* was associated with an approximately threefold increase in hepatic dihydropteridine reductase activity.

Craine *et al.* (1972) suggested that in view of the large excess of dihydropteridine reductase activity over hydroxylase activity observed in these tissues, the rate-limiting step in the biopterin-dependent hydroxylations of tyrosine and tryptophan in adrenal glands and brain respectively, is unlikely to be tetrahydrobiopterin regeneration. On the basis of activity measurements *in vitro* (Table 3), this would appear to be true of hepatic phenylalanine hydroxylation as well. Nevertheless, the observation that phenylalanine oxidation *in vivo* was stimulated by glucagon treatment even when hepatic phenylalanine hydroxylase activity was significantly depressed (Fig. 7) suggests that this enzyme is not rate-limiting in rat liver. Enhancement of phenylalanine oxidation *in vivo* concomitant with dihydropteridine reductase induction is consistent with the hypothesis that phenylalanine catabolism is limited by the rate of tetrahydrobiopterin regeneration, at least under conditions of saturating amounts of phenylalanine.

The absolute values of enzyme activity measurements made *in vitro* cannot be considered to provide a reliable index of the magnitude of the flux of substrate through the various steps in a given pathway. Activity of glutamine synthetase, for example, is very high in liver as measured *in vitro*, however, the perfused organ appears to have little capacity for net

glutamine synthesis (Lund, 1972). Brenneman & Kaufman (1965) have presented evidence suggesting that tetrahydrobiopterin supply may be the limiting factor in phenylalanine hydroxylation in neonatal rats, and studies by Huang & Kaufman (1973) have strongly suggested that the activity of phenylalanine hydroxylase may be significantly greater *in vivo* than *in vitro*, owing to the presence of a 'stimulator' enzyme. The possibility that dihydropteridine reductase is rate-limiting in phenylalanine degradation in liver, as suggested by the present study, thus should not be ruled out by the apparently higher activity of this enzyme as measured *in vitro*.

Under the conditions of the phenylalanine-oxidation experiments described in the present study, the liver phenylalanine-hydroxylating system should be saturated with phenylalanine, since the reported K_m value of the rat liver enzyme for this substrate is 0.3 mM (Fisher & Kaufman, 1972), and the liver phenylalanine concentration is more than 10 times this value throughout the entire course of the experiment (Fig. 4). For an increase in tetrahydrobiopterin reduction to enhance phenylalanine oxidation *in vivo*, however, the initial concentration of this cofactor must not be appreciably greater than the K_m value of phenylalanine hydroxylase, reported to be 4.5 μ M (Kaufman, 1971). The concentration of tetrahydrobiopterin in liver *in vivo* is not known. Kaufman (1964) has suggested that most of the biopterin present in the normal rat liver exists as the active tetrahydro form. If tetrahydrobiopterin is in fact the predominant form of biopterin in normal rat liver, then, as discussed by Craine *et al.* (1972), the hydroxylation of phenylalanine should depend on the NADH-linked regeneration of this cofactor by dihydropteridine reductase. This process could conceivably become limiting when phenylalanine is oxidized at a high rate as in rats heavily loaded with phenylalanine. Rat liver dihydropteridine reductase has a K_m value of 1.0 μ M for quinonoid-dihydrobiopterin (Craine *et al.*, 1972), hence it would be expected to be saturated by this substrate at somewhat lower concentrations than phenylalanine hydroxylase would be by the tetrahydro form. Although this situation would itself facilitate the regeneration of cofactor, induction of dihydropteridine reductase could enhance this capability, especially when reductase substrates were being generated rapidly.

The observed effect of glucagon administration on phenylalanine oxidation is evidently not due to facilitation of tyrosine oxidation. Although tyrosine- α -oxoglutarate aminotransferase, the rate-limiting enzyme of tyrosine oxidation (Ip & Harper, 1973), was increased in the chronic glucagon-treated rats despite discontinuation of hormone injections 18 h before initiating the metabolism experiments to allow time for decay of the activity induced by individual glucagon injections (Civen *et al.*, 1967),

the steep rise in activity which occurred in the control rats after phenylalanine loading was apparently suppressed in the chronically glucagon-treated animals (Fig. 2). Thus fortuitously, tyrosine aminotransferase activity was actually lower in rats receiving the hormone than in the control animals, except at the 2 h time-point, at which time the differences between the rates of phenylalanine oxidation between the two groups were less significant ($P > 0.05$) than during the 4–10 h interval ($P < 0.01$). The explanation for this unexpected finding is not certain; however, Mohrenweiser & Yatwin (1972) have suggested that pancreatic hormones mediate the effects of free amino acids in inducing certain liver enzymes; it is possible that chronic administration of glucagon to rats depresses the response of the pancreas to amino acids in this regard. In any case, it appears very unlikely that an increased rate of tyrosine transamination is responsible for the glucagon-induced stimulation of phenylalanine oxidation *in vivo*, since conditions that are known to increase tyrosine aminotransferase activity and tyrosine oxidation in liver had virtually no effect on the kinetics of $^{14}\text{CO}_2$ production from L-[1- ^{14}C]-phenylalanine (Fig. 1). Also the kinetics of $^{14}\text{CO}_2$ production in these experiments differ qualitatively from those observed in the analogous experiment performed with L-[1- ^{14}C]tyrosine (Ip & Harper, 1973). Therefore it appears that the rate-limiting step in the liberation of carboxyl carbon from phenylalanine occurs at some point before tyrosine transamination.

Measurements of aromatic amino acid concentrations in the plasma and tissue of these animals indicate that peak plasma and tissue phenylalanine concentrations after administration of the phenylalanine load were significantly lower ($P < 0.05$) in the glucagon-treated group (Fig. 4), a finding consistent with the observed stimulation of phenylalanine oxidation. Tissue and plasma tyrosine concentrations were, however, not correspondingly increased in the experimental rats, even though this might be expected when the rate of phenylalanine hydroxylation is increased. If, as appears to be the case, tyrosine transamination is not rate limiting under these conditions, an increased rate of tyrosine formation would probably not be reflected in an increased rate of tyrosine accumulation. Thus measurements of tissue and plasma tyrosine concentrations under these circumstances probably do not reflect reliably the rate of hepatic tyrosine synthesis.

The postulated hormonal modulation of phenylalanine catabolism through control of phenylalanine hydroxylase cofactor regeneration would appear to represent a somewhat unique type of regulatory system. However, the mechanism by which prolonged treatment of rats with glucagon results in an enhanced capacity for phenylalanine degradation may well be more complex than that discussed here.

The lower urinary excretion of phenylalanine after phenylalanine loading of glucagon-treated rats is consistent with increased rate of phenylalanine oxidation in these animals. However, in view of the substantial increase in liver phenylalanine-transaminating capacity resulting from chronic glucagon administration (Fig. 5), the finding that urinary phenylpyruvic and phenyl-lactic acid output in hormone-treated animals (Table 1) was not increased is somewhat unexpected; Fuller *et al.* (1972) have made similar observations. Under analogous conditions, Morris *et al.* (1972) have, however, observed significantly increased urinary imidazolepyruvic acid excretion after an oral histidine load in rats in which hepatic histidine-pyruvate aminotransferase was induced severalfold by glucagon, even though the hormonal treatment used also increased the rate of histidine oxidation *in vivo*. In view of this, it is noteworthy that some investigators have concluded that phenylalanine is transaminated primarily in the muscle (Fellman *et al.*, 1972), a tissue which shrinks in mass during the course of prolonged glucagon treatment (Lee & Harper, 1971). The depression in urinary loss of phenylalanine (more than 85%) in the animals chronically pretreated with glucagon (Tables 1 and 6) is remarkable considering that plasma phenylalanine concentrations of both control and experimental groups after loading are in excess of physiological amounts by at least a factor of 30 (Fig. 4). The slightly lower plasma phenylalanine concentration in the hormone-treated rats would not appear to account for such a dramatic difference in renal phenylalanine excretion. It is possible that plasma phenylalanine concentration is close to the renal threshold or that the hormone exerts an effect directly on the kidney. Effects of glucagon on renal excretion of ions have been reported (Staub *et al.*, 1957; Pullman *et al.*, 1967; Danford, 1970).

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