The role of tryptophan 2,3-dioxygenase in the hormonal control of tryptophan metabolism in isolated rat liver cells

Effects of glucocorticoids and experimental diabetes

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1. The metabolism of L-tryptophan by isolated liver cells prepared from control, adrenalectomized, glucocorticoid-treated, acute-diabetic, chronic-diabetic and insulin-treated chronic-diabetic rats was studied. 2. Liver cells from adrenalectomized rats metabolized tryptophan at rates comparable with the minimum diurnal rates of controls, but different from rates determined for cells from control rats 4 h later. 3. Administration of dexamethasone phosphate increased the activity of tryptophan 2,3-dioxygenase (EC 1.13.11.11) 7–8-fold, and the flux through the kynurenine pathway 3–4-fold, in cells from both control and adrenalectomized rats. Increases in flux through kynureninase (EC 3.7.1.3) and to acetyl-CoA can be explained in terms of increased substrate supply from tryptophan 2,3-dioxygenase. 4. The metabolism of tryptophan was increased 3-fold in liver cells isolated from acutely (3 days) diabetic rats, with a 7–8-fold increase in the maximal activity of tryptophan 2,3-dioxygenase. The oxidation of tryptophan to CO₂ and metabolites of the glutarate pathway increased 4–5-fold, consistent with an increase in picolinate carboxylase (EC 4.1.1.45) activity. 5. Liver cells isolated from chronic (10 days) diabetic rats metabolized tryptophan at rates comparable with those of cells from acutely diabetic rats, but with a 50% decrease in the activity of tryptophan 2,3-dioxygenase. The proportion of flux from tryptophan 2,3-dioxygenase to acetyl-CoA, however, was increased by 50%; this was indicative of further increases in the activity of picolinate carboxylase. 6. Administration of insulin partially reversed the effects of chronic diabetes on the activity of tryptophan 2,3-dioxygenase and flux through the kynurenine pathway, but had no effect on the increased activity of picolinate carboxylase. 7. The role of tryptophan 2,3-dioxygenase in regulating the blood tryptophan concentration is discussed with reference to its sensitivity to the above conditions.

In mammals more than 90% of the total tryptophan is degraded in the liver through the kynureninase pathway (Young *et al.*, 1978). The importance of the regulation of this process is emphasized by the relationship between the plasma tryptophan concentration and the synthesis of 5-hydroxytryptamine in the brain (Curzon, 1979). The first enzyme of tryptophan oxidation, tryptophan 2,3-dioxygenase [L-tryptophan:O₂ 2,3-

oxidoreductase (decyclizing), EC 1.13.11.11], is regulated by glucocorticoids (Knox & Mehler, 1951; Voigt & Sekeris, 1980) and by tryptophan itself (Knox, 1951, 1966), although possibly by different mechanisms (Civen & Knox, 1959). In common with that of tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5), tryptophan 2,3-dioxygenase activity in rat liver exhibits a diurnal rhythm which correlates closely with the concentration of corticosterone in the blood (Wurtman, 1974). Although the sensitivity of total dioxygenase activity to steroid has been well documented (see, e.g., Schimke & Doyle, 1970), there are no reports relating this phenomenon to flux through the pathway under other-

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wise physiological conditions. It has been suggested, on the basis of measurements of maximal activities *in vitro*, that kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) may become a significant factor in overall regulation of the pathway when the dioxygenase concentration is increased by steroid administration (Knox, 1953; Magboul & Bender, 1983). It is far from clear, however, that such measurements *in vitro* can be interpreted directly in the physiological context.

The activities of both tryptophan 2,3-dioxygenase and picolinate carboxylase (aminocarboxymuconate-semialdehyde carboxylase, EC 4.1.1.45) are increased in the livers of chronically diabetic rats (Mehler *et al.*, 1958a; Ikeda *et al.*, 1965). Picolinate carboxylase controls the relative fluxes of tryptophan carbon to nicotinamide coenzymes and to further oxidation (Mehler *et al.*, 1964), and it has been suggested that the increased activity of this enzyme serves to decrease synthesis of nicotinamide coenzymes in diabetes (Mehler *et al.*, 1958a; McDaniel *et al.*, 1973). This increased activity is reversed by insulin, but only after long-term treatment (Sanada *et al.*, 1980).

The effects of diabetes on tryptophan metabolism *in vivo* are known from measurements of metabolites in urine (Kotake & Tani, 1953; Mehler *et al.*, 1958a), but these data include contributions from other tissues (Rose, 1972), so that the specific effects on flux in the liver are not known.

In the present paper we report studies with liver cells exposed to glucocorticoid and from animals rendered both acutely and chronically diabetic with streptozotocin. Correlations are made between flux data in cells and enzyme activities assayed in cell extracts.

Materials and methods

Animals

Male Sprague-Dawley rats (180–220 g) were used throughout, and were given a standard laboratory diet (CRM diet; Labsure, Poole, Dorset, U.K.) *ad libitum*. Bilateral adrenalectomy was performed as described by Smith & Pogson (1977); adrenalectomized rats were given 1% (w/v) NaCl instead of drinking water. Half of the adrenalectomized rats were given dexamethasone phosphate, subcutaneously under diethyl ether anaesthesia, at 10:00 daily (2 mg/kg body wt., in pyrogen-free 0.9% NaCl). Tryptophan 2,3-dioxygenase activity was unaffected by subcutaneous administration of 0.9% NaCl under diethyl ether anaesthesia. Control rats were given dexamethasone phosphate, intraperitoneally, at 07:30 h (3 mg/animal, in 0.9% NaCl) 5 h before liver-cell preparation. Diabetes was induced by intravenous injection into the caudal vein of streptozotocin (80 mg/kg

body wt., in 0.9% pyrogen-free NaCl). Diabetes was confirmed by the appearance of glucose in the urine (Clinistix; Ames Co., Slough, Berks., U.K.). Insulin was administered intraperitoneally twice daily (3 units/animal; Neusulin, Wellcome, Beckenham, Kent, U.K.). Isolated liver cells were prepared from normal fed rats (controls), from rats 5 days after adrenalectomy and from rats 3 days or 10 days after streptozotocin injection.

Chemicals and purification of labelled compounds

All radiochemicals were from Amersham International, Amersham, Bucks., U.K., except for L-[ring-2-¹⁴C]tryptophan, which was from CEA, Gif-sur-Yvette, France, through Fluorochem, Glossop, Derbyshire, U.K. The sources of other reagents were as given previously (Salter *et al.*, 1984).

Radiolabelled L-tryptophan was purified by affinity chromatography (Stewart & Doherty, 1973) as described in detail by Smith (1977).

Preparation and incubation of cells

The procedures were as described previously (Smith & Pogson, 1980; Smith *et al.*, 1980), with the following amendments. (1) Liver-cell preparation started at 12:30 h each day, unless stated. (2) Perfusion medium contained 20 mM-D-glucose. (3) Cells were washed in incubation medium (see below) without addition of albumin. (4) The incubation medium consisted of buffer (Krebs & Henseleit, 1932) containing 2.5 mM-Ca²⁺ and 2% (w/v) bovine serum albumin (defatted as described by Chen, 1967) and supplemented with 5 mM-D-glucose, 5 mM-L-glutamine, 4.5 mM-L-lactate and 0.5 mM-pyruvate. These substrates were added to maintain glycogen stores. The final cell concentration was 2.5–3.5 mg dry wt./ml, final volume 2 ml. (5) Cells were preincubated for 25 min before addition of tryptophan, and incubated for a further 30 min or 90 min before metabolism was terminated with 200 μ l of 20% (v/v) HClO₄.

Flux through the tryptophan catabolic pathway was assessed as described in detail by Smith *et al.* (1980).

Assays

Determinations of the maximal activity of tryptophan 2,3-dioxygenase, in cell extracts, were as described by Metzler *et al.* (1982). Extracts were prepared as previously described (Salter *et al.*, 1984). Cell viability was assessed on each occasion by measurement of ATP content (Stanley & Williams, 1969). All results were obtained with cells with ATP contents more than 8 nmol/mg dry wt. DNA was measured as described by Burton (1956).

Results and discussion

Effect of glucocorticoid

Table 1 shows the effects of variations in glucocorticoid status on tryptophan metabolism in liver cells. Adrenalectomy had no significant effect on either the intracellular degradation of tryptophan or the total activity of tryptophan 2,3-dioxygenase. This lack of effect on flux confirms previous results (Smith & Pogson, 1981). Smith *et al.* (1979), however, reported a decrease in total enzyme activity after adrenalectomy. This discrepancy may be attributed to differences in rats and composition of the diet, but more probably may be explained on the basis that activities reported in that previous paper were not obtained at the low point in the diurnal rhythm. For the present study, experiments were performed deliberately at approximately the lowest point of activity in the diurnal cycle of tryptophan 2,3-dioxygenase, that is, at 12:30h. Changes in activity and flux can therefore be more readily compared with a basal state. When enzyme activity in adrenalectomized and control rats was measured 4h later, control activity was significantly increased (Table 1), but there was no change in the activity from adrenalectomized animals (results not shown). The increase in the glucagon/insulin ratio after adrenalectomy (Van Lan *et al.*, 1974) does not affect the activity of tryptophan 2,3-dioxygenase (Table 1). It has been reported that glucagon induces the dioxygenase in cultured hepatocytes and that this effect is antagonized by insulin (Nakamura *et al.*, 1980). However, if glucocorticoids are permissive for both the long- and short-term actions of glucagon (Krone *et al.*, 1976; Postle & Bloxham, 1982), one would not expect to see any change in activity in adrenalectomized rats. The lack of effect of hypoinsulinaemia (Van Lan *et al.*, 1974) on the activity of the dioxygenase (Table 1) suggests that insulin may only attenuate the activity after its increase by glucagon.

Replacement of glucocorticoid with low doses of dexamethasone phosphate resulted in a 7–8-fold increase in the total activity of tryptophan 2,3-dioxygenase and a 3-fold increase in the flux through the enzyme in cell incubations. Parallel increases in flux through the rest of the pathway were seen, suggesting that the dioxygenase plays a significant role in the regulation of the overall process. A similar increase in the activity of the dioxygenase and flux through the kynurenine pathway was seen when a larger dose of dexamethasone phosphate was given intraperitoneally to control rats 5h before the experiment. Thus the metabolism of tryptophan in cells from control and adrenalectomized rats shows a similar response to glucocorticoid.

Table 1. Effect of the absence, replacement and diurnal increase of glucocorticoid on the metabolism of L-tryptophan by isolated rat liver cells. Conditions for the treatment of animals and the preparation and incubation of liver cells were as described in the Materials and methods section. Results are means $\pm$ S.E.M. from three independent observations (five for cells from control animals). Differences between means were assessed by Student's *t* test: *P* (versus fed controls) * <0.05; ** <0.01; *** <0.001; other differences not significant. Data for control animals are from Salter *et al.* (1984).

Flux	Metabolic flux rates (nmol/h per mg dry wt.)				
	Control	Adrenalectomized	Adrenalectomized + glucocorticoid	Control + glucocorticoid	Control (16:30h)
Through tryptophan 2,3-dioxygenase	2.58 $\pm$ 0.17	2.38 $\pm$ 0.22	8.92 $\pm$ 0.45***	7.75 $\pm$ 0.11***	3.50 $\pm$ 0.10**
Through kynureninase	2.49 $\pm$ 0.14	2.38 $\pm$ 0.29	7.10 $\pm$ 0.29***	7.53 $\pm$ 0.20***	3.46 $\pm$ 0.10**
To acetyl-CoA	0.38 $\pm$ 0.03	0.31 $\pm$ 0.04	1.36 $\pm$ 0.07***	1.14 $\pm$ 0.06***	0.53 $\pm$ 0.04**
Activity of tryptophan 2,3-dioxygenase in cell extracts	13.7 $\pm$ 0.3	12.5 $\pm$ 1.3	101.9 $\pm$ 4.7***	101.3 $\pm$ 7.0***	21.8 $\pm$ 0.4***
Percentage of total activity expressed by flux through dioxygenase	18.8 $\pm$ 0.9	19.5 $\pm$ 1.0	8.8 $\pm$ 0.5***	7.7 $\pm$ 0.1***	16.1 $\pm$ 0.2*
Percentage of flux through dioxygenase to acetyl-CoA	14.5 $\pm$ 0.4	12.9 $\pm$ 1.0	15.3 $\pm$ 1.2	14.7 $\pm$ 1.0	15.1 $\pm$ 0.3

When the control experiment was repeated 4h later (i.e. at 16:30h), the metabolism of tryptophan had increased significantly from values determined at the earlier time. The concentration of circulating blood glucocorticoid should, at this time, be increased from its diurnal minimum (Wurtman & Axelrod, 1967). Thus increases in circulating steroid are associated with increases in both enzyme protein and flux through the pathway, although these increases are not necessarily parallel.

Effect of diabetes and insulin replacement

Acute streptozotocin-induced diabetes increased the metabolism of tryptophan by isolated liver cells 3–4-fold, with an 8-fold increase in tryptophan 2,3-dioxygenase activity (Table 2). This increased synthesis of tryptophan 2,3-dioxygenase may be associated with the large rise in the glucagon/insulin ratio; Nakamura *et al.* (1980) have shown that glucagon is a potent inducer of the synthesis of tryptophan 2,3-dioxygenase in cultured rat liver cells. Steroid is unlikely to be involved in these effects; there are negligible differences in plasma corticosterone concentrations between control and acutely diabetic rats (R. G. Knowles & C. I. Pogson, unpublished work). The ratio of the flux to acetyl-CoA to that through tryptophan 2,3-dioxygenase was increased by 50%. This suggests that the activity of picolinate carboxylase was increased, thereby diverting the tryptophan-derived carbon flux away from nicotinamide synthesis. The flux through kynureninase increased solely as a response to the increased substrate supply from tryptophan 2,3-dioxygenase.

Chronic (10 days) diabetes again was associated with an increase in the metabolism of tryptophan by isolated rat liver cells. The flux through tryptophan 2,3-dioxygenase was decreased only slightly from the values found for cells from acutely diabetic rats; the total activity of tryptophan 2,3-dioxygenase, however, was decreased by 50%.

The proportion of tryptophan metabolized to acetyl-CoA was greater in cells from chronically than from acutely diabetic rats. This is indicative of a further increase in the activity of picolinate carboxylase. This increased activity of picolinate carboxylase did not, however, decrease the absolute flux to the nicotinamide coenzymes (cf. Smith & Pogson, 1981), because the overall flux through the pathway is increased. It is worth noting, nevertheless, that the basal activity of picolinate carboxylase in man (Ikeda *et al.*, 1965) is twice that of the rat, so that defective synthesis of NAD(P) is a possibility in human diabetes.

Daily injection of insulin resulted in a significant decrease in the activity of tryptophan 2,3-dioxy-

Table 2. Effect of acute, chronic and insulin-treated chronic diabetes on the metabolism of L-tryptophan by isolated rat liver cells. Conditions for the treatment of animals and the preparation and incubation of liver cells were as described in the Materials and methods section. Results are means $\pm$ S.E.M. from three independent observations (five for cells from control animals). Differences between means were assessed by Student's *t* test. *P* (versus fed controls) * <0.05 ; ** <0.01 ; *** <0.001 ; *P* (versus insulin-treated chronic diabetics) † <0.001 ; other differences not significant. Data for control animals are from Salter *et al.* (1984).

Flux	Metabolic flux rates (nmol/h per mg dry wt.)			
	Control	Acute diabetes	Chronic diabetes	Chronic diabetes + insulin
Through tryptophan 2,3-dioxygenase	2.58 $\pm$ 0.17	7.91 $\pm$ 0.32***†	6.73 $\pm$ 0.20***†	4.60 $\pm$ 0.17***
Through kynureninase	2.49 $\pm$ 0.14	7.68 $\pm$ 0.32***†	6.63 $\pm$ 0.26***†	4.35 $\pm$ 0.27***
To acetyl-CoA	0.38 $\pm$ 0.03	1.77 $\pm$ 0.03***†	2.25 $\pm$ 0.14***†	1.50 $\pm$ 0.10***
Activity of tryptophan 2,3-dioxygenase in cell extracts	13.7 $\pm$ 0.3	110.4 $\pm$ 6.3***†	57.5 $\pm$ 2.2***†	30.1 $\pm$ 0.03***
Percentage of total activity expressed by flux through dioxygenase	18.8 $\pm$ 0.9	7.2 $\pm$ 0.2***†	11.6 $\pm$ 0.3***†	15.3 $\pm$ 0.05*
Percentage of flux through dioxygenase to acetyl-CoA	14.5 $\pm$ 0.4	22.4 $\pm$ 0.2***†	33.4 $\pm$ 0.5***	32.6 $\pm$ 0.4***

genase and in flux through the whole pathway of metabolism (when compared with measurements in cells from untreated chronically diabetic rats). The activity of picolinate carboxylase, however, was not affected by the administration of insulin, at least as demonstrated by an uncharged ratio of flux through the dioxygenase to flux to acetyl-CoA through the lower part of the pathway (again in comparison with cells from diabetic animals). Mehler *et al.* (1958*b*) have shown that the activity of picolinate carboxylase, which is elevated in diabetes, returns to control values only after 3 weeks of insulin treatment; this may be indicative of a long half-life for picolinate carboxylase in rat liver.

General discussion

The rate-limiting enzyme for 5-hydroxytryptamine synthesis in the brain, tryptophan hydroxylase (L-tryptophan, tetrahydropteridine: oxygen oxidoreductase, EC 1.14.16.4) (Jequir *et al.*, 1967) is thought to be not fully saturated with substrate *in vivo* (Knowles & Pogson, 1984). Thus changes in the tryptophan concentration in the blood may be significant as a determinant of the concentration of 5-hydroxytryptamine in the brain. It is of interest, therefore, that the total concentration of plasma tryptophan is decreased in chronic diabetes in the rat (Crandall & Fernstrom, 1983) and in ketoacidotic diabetes in man (Curzon *et al.*, 1982). Although there is likely to be an increased input of tryptophan in diabetes because of increased muscle proteolysis, there will be a net loss to the body as a result of the increased metabolism of tryptophan by the liver. This increase in metabolism arises from the increase in activity of tryptophan 2,3-dioxygenase and also from an increase in free, and thus available, tryptophan (Fernando *et al.*, 1976; Smith & Pogson, 1980; Curzon *et al.*, 1982). The relative concentration of free tryptophan (as a proportion of the total) is elevated in diabetes, because of the displacement of bound amino acid by free fatty acid (Knott & Curzon, 1972; Smith & Pogson, 1980). The concentration of total plasma tryptophan, however, is unchanged in acute diabetes (Fernando *et al.*, 1976), which may reflect a higher rate of muscle proteolysis in the acutely diabetic rat, compared with the chronically diabetic rat (as the rate of disposal is approximately constant; Table 2).

Crandall & Fernstrom (1983) have shown that administration of insulin to chronically diabetic rats returns the concentrations of tryptophan in blood and brain to control values. This is consistent with the observed effects of insulin on flux through tryptophan 2,3-dioxygenase (Table 2) and with the hypothesis that the regulation of tryptophan 2,3-dioxygenase may be an important factor

in the maintenance of blood tryptophan, and, as a result, of the concentration of 5-hydroxytryptamine in the brain, although a direct relationship between plasma tryptophan and 5-hydroxytryptamine synthesis does not always occur (Trulson & MacKenzie, 1980). Work in our laboratory has shown that the activity of the major regulatory enzyme of tyrosine catabolism in the liver, tyrosine aminotransferase, is also increased in chronic diabetes (and antagonized by insulin administration) (J. C. Stanley, M. J. Fisher & C. I. Pogson, unpublished work). Interestingly, Crandall & Fernstrom (1983) observed changes in plasma tyrosine which paralleled those of tryptophan in the diabetic state, although Curzon *et al.* (1982) saw no change in human ketoacidotic diabetes. The role of both amino acids as precursors of neurotransmitters suggests that significant alterations in central nervous activity may be associated with prolonged diabetes.

It is clear that the basal capacity of the liver to metabolize tryptophan is unaffected by the absence of glucocorticoid. However, larger loads of tryptophan are handled much less effectively by adrenalectomized rats (Smith & Pogson, 1981), presumably because of the inability of these rats to produce glucocorticoid and thus increase tryptophan 2,3-dioxygenase activity. Smith & Pogson (1977) have shown that tryptophan is much more toxic to adrenalectomized rats than to normal rats. The activity of tryptophan 2,3-dioxygenase is clearly very sensitive to the diurnal increases in blood glucocorticoid. The consequent increased

Fig. 1. Relationship for tryptophan 2,3-dioxygenase between the flux as a percentage of total activity and the total activity itself

Flux and activity of tryptophan 2,3-dioxygenase were determined from liver cells isolated from control or experimental rats, as described in the Materials and methods section.

capacity of the liver to degrade tryptophan is required to prevent excessive increases in the amino acid in the blood, especially at times of increased input, such as feeding or increased muscle degradation.

The relationship between the total activity of tryptophan 2,3-dioxygenase and its flux divided by the total activity for control and experimental rats is shown in Fig. 1. It is clear from Fig. 1 that the flux in cells does not increase linearly with the activity in cell extracts (if this were so, the flux/activity ratio would remain constant with increasing activity) and that at high enzyme activity other factors become significant for the control of tryptophan oxidation. We have shown that the transport of tryptophan into liver cells is relatively slow and may become limiting with increased dioxygenase activities (M. Salter & C. I. Pogson, unpublished work).

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