Tryptophan and glucose metabolism in rat liver cells

The effects of DL-6-chlorotryptophan, 4-chloro-3-hydroxyanthranilate and pyrazinamide

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1. Liver cells pre-incubated with 1 mM-DL-6-chlorotryptophan are less sensitive to tryptophan-mediated inhibition of gluconeogenesis; this effect is apparent both at physiological (0.1 mM) and higher (0.5 mM) concentrations of tryptophan.
2. 4-Chloro-3-hydroxyanthranilate (1–100 μ M) has effects similar to those of DL-6-chlorotryptophan.
3. The effects of both compounds are consistent with a decrease in quinolinate formation, a consequence of inhibition of 3-hydroxyanthranilate oxidase.
4. Pyrazinamide (0.25–5.0 mM) significantly decreased flux through the glutarate pathway and potentiated tryptophan-mediated inhibition of gluconeogenesis; these changes were apparent at physiological concentrations of tryptophan.
5. The effects of pyrazinamide are consistent with an increase in quinolinate formation resulting from inhibition of picolinate carboxylase.

Tryptophan inhibits hepatic gluconeogenesis *in vivo* (Foster *et al.*, 1966; Ray *et al.*, 1966; McDaniel *et al.*, 1973), in perfused rat liver (Veneziale *et al.*, 1967; Alvarez & Ray, 1974) and in isolated rat liver cells (Elliott *et al.*, 1976a; Smith *et al.*, 1978, 1979; Munoz-Clares *et al.*, 1981). The inhibition occurs secondarily to the formation of quinolinate, which blocks the reaction catalysed by phosphoenolpyruvate carboxykinase [GTP:oxalacetate carboxylase (transphosphorylating); EC 4.1.1.32] (Snoke *et al.*, 1971; McDaniel *et al.*, 1972). Half-maximal inhibition occurs at 0.1 mM-tryptophan (Smith *et al.*, 1978); this concentration is within the physiological range (Knott & Curzon, 1972; Knott *et al.*, 1977), and hence tryptophan may be important in modulating gluconeogenic flux.

Liver cells isolated from diabetic rats synthesize less quinolinate from tryptophan because of increased flux through the glutarate pathway (Smith & Pogson, 1981a); this is a result of increased picolinate carboxylase [(3'-oxoprop-2'-enyl)-2-aminobut-2-enedioate carboxylase; EC 4.1.1.45] activity (Mehler *et al.*, 1957). Gluconeogenesis in such cells is less sensitive to tryptophan (Smith *et al.*, 1978), although sensitivity to quinolinate remains

unaltered (Elliott *et al.*, 1977). The sensitivity of glucose synthesis to tryptophan in liver cells isolated from different species is consistent with their relative ability to produce quinolinate (Muñoz-Clares *et al.*, 1981).

With these factors in mind, the effects of various drugs, known to alter hepatic tryptophan metabolism, on tryptophan-mediated inhibition of gluconeogenesis were examined. 6-Chlorotryptophan is metabolized to 4-chloro-3-hydroxyanthranilate, a potent irreversible inhibitor of 3-hydroxyanthranilate oxidase (3-hydroxyanthranilate: oxygen oxidoreductase; EC 1.10.3.5) (Parli *et al.*, 1980). Incubation of liver cells with either DL-6-chlorotryptophan or 4-chloro-3-hydroxyanthranilate should decrease quinolinate synthesis, and hence decrease the sensitivity of gluconeogenesis to tryptophan. Pyrazinamide is metabolized to a potent inhibitor of picolinate carboxylase (Nasu *et al.*, 1981); this should increase quinolinate synthesis, and hence potentiate the effect of tryptophan. Results are presented that are consistent with these predictions.

Materials and methods

Animals

Male Sprague–Dawley rats (200–250 g) were used throughout. All animals were deprived of food for 48 h before preparation of liver cells.

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Materials

L-Tryptophan, L-lactic acid, pyruvic acid and pyrazinamide were from Sigma. 4-Chloro-3-hydroxyanthranilate and DL-6-chlorotryptophan were gifts from Lilly Research Laboratories (Indianapolis, IN, U.S.A.). Norit GSX was from Clydesdale Co., Glasgow, Scotland, U.K. Bovine serum albumin (fraction V), obtained from International Enzymes, Windsor, Berks., U.K., was freed from fatty acids and other impurities as described by Chen (1967). L-[ring-2- 14 C]Tryptophan (CEA, Gif-sur-Yvette, France, through Fluorochem, Glossop, U.K.), L-[carboxy- 14 C]tryptophan and L-[benzene-ring-U- 14 C]tryptophan (Amersham International, Amersham, Bucks., U.K.) were purified by the method of Stewart & Doherty (1973). All other chemicals, of the purest grade available, were from standard suppliers.

Preparation of isolated rat liver cells

Isolated rat liver cells were prepared as described by Elliott *et al.* (1976b). Viability of cell preparations was assessed by measurement of ATP contents (Dickson & Pogson, 1977).

Measurement of tryptophan metabolism

The methods were described previously (Smith & Pogson, 1980, 1981b; Smith *et al.*, 1980). Specific additions and treatments of incubations are described in the text.

For experiments with DL-6-chlorotryptophan, isolated cells prepared from a single animal were split into two equal batches. Both were incubated in 30 ml of bicarbonate buffer (Krebs & Henseleit, 1932), supplemented with lactate/pyruvate (final combined concentration 10 mM; 9:1, v/v), contained in 250 ml conical flasks. DL-6-Chlorotryptophan (final concentration 1 mM) was added to one of the incubations; both were incubated at 37°C for 1 h (WTR-1 orbital shaker; Infors AG; 130 cycles/min). The two batches were washed three times in 30 ml of bicarbonate buffer, and were then resuspended in the appropriate volume of buffer supplemented with 2% (w/v) bovine serum albumin. Dry-weight determinations were performed on both cell suspensions. The cells were then incubated as described above.

Measurement of ATP and glucose

Liver cells were incubated as described by Smith *et al.* (1978). ATP and glucose were assayed in neutralized acid extracts by the methods of Stanley & Williams (1969) and Bergmeyer *et al.* (1974) respectively.

Results and discussion

Tryptophan metabolism

L-[ring-2- 14 C]-, L-[carboxy- 14 C]- and L-[benzene-ring-U- 14 C]-tryptophan were used to estimate the

flux through tryptophan 2,3-dioxygenase [l-tryptophan: oxygen 2,3-oxidoreductase (decyclizing); EC 1.13.11.11], kynureninase (L-kynurenine hydrolase; EC 3.7.1.3) and the glutarate pathway respectively. The rationale and development of these assay procedures have been described and discussed previously (Smith & Pogson, 1980, 1981b; Smith *et al.*, 1980).

DL-6-Chlorotryptophan and 4-chloro-3-hydroxyanthranilate

Incubation of liver cells for 1 h with 1 mM DL-6-chlorotryptophan did not significantly alter the cellular ATP content (8.80 ± 0.19 and 9.17 ± 0.90 nmol/mg dry wt. for control and DL-6-chlorotryptophan-treated cells respectively; means $\pm$ S.D. of triplicate incubations from a single experiment). The basal rate of gluconeogenesis was not altered by similar treatment of cells (Table 1). By these criteria, liver cell metabolic integrity was not affected by DL-6-chlorotryptophan.

The data in Table 1 show that DL-6-chlorotryptophan decreased the sensitivity of gluconeogenesis to 0.1–0.4 mM L-tryptophan, although tryptophan metabolism was apparently not changed.

Concentrations between 1 μ M- and 100 μ M-4-chloro-3-hydroxyanthranilate have no effect on cellular ATP content or glucose synthesis (Table 2). At 1 mM, cell ATP was greatly decreased (9.8 ± 1.2 and 1.1 ± 0.7 nmol/mg dry wt. for control and treated cells respectively; means $\pm$ S.D. of triplicate incubations in a single experiment). Glucose synthesis and tryptophan metabolism were completely abolished.

Table 2 shows that 4-chloro-3-hydroxyanthranilate antagonized the inhibition of gluconeogenesis by tryptophan. This effect was only apparent at 50 and 100 μ M; the latter concentration also inhibited fluxes through tryptophan 2,3-dioxygenase, kynureninase and the glutarate pathway. At a range of tryptophan concentrations (0.1–0.4 mM), 50 μ M-4-chloro-3-hydroxyanthranilate significantly decreased the effects on gluconeogenesis, although tryptophan metabolism was not altered (Table 3).

The results with both inhibitors are consistent with predictions based on the premise that 3-hydroxyanthranilate oxidase is the only enzyme primarily affected. Under these conditions, the fluxes through tryptophan 2,3-dioxygenase and kynureninase should be unchanged. Although the glutarate pathway is subsequent to the point of inhibition, the flux through this pathway, which accounts in any case for only 10% or less of the total tryptophan catabolism, is largely limited by the activity of picolinate carboxylase and not by provision of substrate. Appreciable inhibition of [benzene-ring-U- 14 C]tryptophan oxidation should therefore only be seen when 3-hydroxyanthranilate oxidase activity is very substantially diminished (see Table 2).

Table 1. *The effects of tryptophan concentration and DL-6-chlorotryptophan on gluconeogenesis and tryptophan metabolism*

Liver cells were pre-incubated for 1 h with or without 1 mM-DL-6-chlorotryptophan. Cells were incubated for 20 min before the addition of lactate/pyruvate (final combined concentration 10 mM; 9:1, v/v), with or without L-tryptophan (final concentration as shown). Glucose was assayed in samples after 60 min incubation. Alternatively, cells were incubated for 10 min before the addition of lactate/pyruvate, and a further 10 min before addition of radiolabelled tryptophan (sp. radioactivity 0.03 Ci/mol). The release of radiolabel was measured after a further 60 min. Results are means $\pm$ S.E.M. from three independent observations; significances between means were assessed with the paired *t* test. *P* (versus the corresponding controls): * <0.05 .

[Tryptophan] (mM)	Activity or content (nmol/mg dry wt. per h)			
	Tryptophan 2,3-dioxygenase flux	Kynureninase flux	Glutarate pathway flux	Glucose synthesis
Without DL-6-chlorotryptophan	—	—	—	211 $\pm$ 12
0.1	7.09 $\pm$ 0.11	7.35 $\pm$ 0.33	0.90 $\pm$ 0.02	160 $\pm$ 7
0.2	16.60 $\pm$ 0.08	17.73 $\pm$ 0.25	1.44 $\pm$ 0.08	127 $\pm$ 6
0.4	26.25 $\pm$ 0.84	26.52 $\pm$ 0.57	1.91 $\pm$ 0.14	117 $\pm$ 6
+ 1 mM-DL-6-Chlorotryptophan	—	—	—	215 $\pm$ 3
0.1	6.74 $\pm$ 0.07	7.43 $\pm$ 0.22	0.78 $\pm$ 0.05	186 $\pm$ 5*
0.2	14.70 $\pm$ 0.48	15.39 $\pm$ 0.69	1.39 $\pm$ 0.04	166 $\pm$ 10*
0.4	23.84 $\pm$ 0.72	25.55 $\pm$ 0.44	1.82 $\pm$ 0.08	149 $\pm$ 9*

Table 2. *The effects of 4-chloro-3-hydroxyanthranilate on tryptophan and gluconeogenesis*

Liver cells were incubated with or without 4-chloro-3-hydroxyanthranilate for 20 min before the addition of lactate/pyruvate (final combined concentration 10 mM; 9:1, v/v) with or without 0.5 mM-L-tryptophan. Glucose was assayed in samples incubated for a further 60 min. Alternatively, lactate/pyruvate was added after 10 min, and radiolabelled L-tryptophan (sp. radioactivity 0.03 Ci/mol; final concentration 0.5 mM) after a further 10 min. Release of radiolabel was measured 60 min later. Results are means $\pm$ S.E.M. from three independent observations; significances between means were assessed by using the paired *t* test. *P* (versus the corresponding controls): * <0.05 , ** <0.01 .

[4-Chloro-3-hydroxy-anthranilate] (μ M)	Cellular ATP (nmol/mg dry wt.)	Activity or content (nmol/mg dry wt. per h)				
		Tryptophan 2,3-dioxygenase flux	Kynureninase flux	Glutarate pathway flux	Glucose synthesis	Glucose synthesis (+0.5 mM-tryptophan)
—	8.21 $\pm$ 0.50	25.4 $\pm$ 3.1	16.9 $\pm$ 2.2	1.74 $\pm$ 0.18	241 $\pm$ 16	112 $\pm$ 6
1	8.14 $\pm$ 0.58	26.3 $\pm$ 4.0	15.0 $\pm$ 2.2	1.70 $\pm$ 0.21	238 $\pm$ 16	114 $\pm$ 5
10	8.35 $\pm$ 0.29	25.7 $\pm$ 3.5	14.7 $\pm$ 2.6	1.70 $\pm$ 0.26	239 $\pm$ 16	115 $\pm$ 6
25	8.24 $\pm$ 0.44	25.4 $\pm$ 3.5	14.5 $\pm$ 2.3	1.64 $\pm$ 0.21	244 $\pm$ 18	119 $\pm$ 3
50	7.94 $\pm$ 0.27	25.2 $\pm$ 3.4	13.4 $\pm$ 2.3	1.54 $\pm$ 0.24	241 $\pm$ 20	127 $\pm$ 5**
100	7.81 $\pm$ 0.19	22.6 $\pm$ 2.8*	12.7 $\pm$ 1.4*	1.20 $\pm$ 0.15**	256 $\pm$ 8	145 $\pm$ 17*

In the absence of either drug, the difference between the flux rates with [carboxy- 14 C]tryptophan and [benzene-ring- 14 C]tryptophan is accounted for by quinolinate accumulation (Smith *et al.*, 1980). Inhibition of 3-hydroxyanthranilate oxidase would be expected to increase the formation of xanthurenic and kynurenic acids and to decrease that of quinolinate; this would in turn decrease the degree of inhibition of gluconeogenesis (as observed). With higher effective concentrations of the inhibitor (Table 2), some inhibition of other enzymes of tryptophan catabolism was seen, an effect attributable to the known actions of the accumulated by-products (Wagner, 1964; Hanks *et al.*, 1972; Madras & Sourkes, 1968).

In vitro, 4-chloro-3-hydroxyanthranilate is an effective inhibitor of 3-hydroxyanthranilate oxidase at 10 nM, and completely abolishes the activity at 0.5 μ M (Parli *et al.*, 1980). Concentrations used in the present study (1–100 μ M) should completely block the oxidase. Nevertheless, metabolism through the glutarate pathway was not affected. Liver cells may thus either have limited permeability to, or may contain molecules able to bind, the inhibitor.

Pyrazinamide

At concentrations of 0.25 to 5.0 mM, pyrazinamide had no effect on the ATP content of isolated liver cells; at 2.5 and 5.0 mM it did, however, significantly decrease glucose synthesis (Table 4).

Table 3. *The effects of tryptophan concentration and 4-chloro-3-hydroxyanthranilate on tryptophan metabolism and gluconeogenesis*

Liver cells were incubated with or without 4-chloro-3-hydroxyanthranilate (final concentration 50 μ M) for 20 min before the addition of lactate/pyruvate (final combined concentration 10 mM, 9:1, v/v) with or without L-tryptophan. Glucose was assayed in samples after incubation for a further 60 min. Alternatively, lactate/pyruvate was added after 10 min, and radiolabelled L-tryptophan (sp. radioactivity 0.03 Ci/mol), after a further 10 min. Release of radiolabel was measured 60 min later. Results are means $\pm$ S.E.M. from three independent observations; significances between means were assessed by using the paired *t* test. *P* (versus the corresponding controls): * <0.05.

[Tryptophan] (mM)	Activity or content (nmol/mg dry wt. per h)			
	Tryptophan 2,3-dioxygenase flux	Kyureninase flux	Glutarate pathway flux	Glucose synthesis
Without 4-chloro-3-hydroxyanthranilate				
0	—	—	—	210 $\pm$ 3
0.1	5.40 $\pm$ 0.47	5.26 $\pm$ 0.59	0.33 $\pm$ 0.04	169 $\pm$ 5
0.2	11.87 $\pm$ 1.04	11.08 $\pm$ 1.05	1.32 $\pm$ 0.25	129 $\pm$ 3
0.4	18.60 $\pm$ 2.21	15.81 $\pm$ 0.63	1.75 $\pm$ 0.31	103 $\pm$ 6
+ 50 μ M-4-Chloro-3-hydroxyanthranilate				
0	—	—	—	202 $\pm$ 4
0.1	5.09 $\pm$ 0.38	4.59 $\pm$ 0.24	0.25 $\pm$ 0.06	191 $\pm$ 6*
0.2	10.84 $\pm$ 0.76	9.70 $\pm$ 0.78	1.25 $\pm$ 0.11	145 $\pm$ 5*
0.4	18.67 $\pm$ 0.50	15.90 $\pm$ 1.25	1.55 $\pm$ 0.09	127 $\pm$ 2*

Table 4. *The effects of pyrazinamide on tryptophan metabolism and gluconeogenesis*

Liver cells were incubated for 1 h with or without pyrazinamide. Lactate/pyruvate (final combined concentration 10 mM; 9:1, v/v) with or without 0.5 mM-tryptophan was then added; glucose was assayed in samples incubated for a further 60 min. Alternatively, lactate/pyruvate was added after 50 min incubation, and radiolabelled tryptophan (sp. radioactivity 0.03 Ci/mol; final concentration 0.5 mM) after a further 10 min. Release of radiolabel was measured 60 min later. Results are means $\pm$ S.E.M. from three independent observations; significances between means were assessed with the paired *t* test. *P* (versus the corresponding controls): * <0.05; ** <0.01.

[Pyrazinamide] (mM)	Cellular ATP (nmol/mg dry wt.)	Activity or content (nmol/mg dry wt. per h)				
		Tryptophan 2,3-dioxygenase flux	Kynureninase flux	Glutarate pathway flux	Glucose synthesis	Glucose synthesis (+0.5 mM-tryptophan)
—	9.0 $\pm$ 0.1	22.7 $\pm$ 1.6	13.8 $\pm$ 1.0	1.27 $\pm$ 0.19	248 $\pm$ 8	122 $\pm$ 3
0.25	9.1 $\pm$ 0.1	22.1 $\pm$ 1.5	12.8 $\pm$ 0.4	0.88 $\pm$ 0.16**	248 $\pm$ 6	110 $\pm$ 6*
0.50	8.9 $\pm$ 0.1	22.2 $\pm$ 1.9	12.2 $\pm$ 0.7	0.87 $\pm$ 0.16**	246 $\pm$ 7	104 $\pm$ 3**
1.0	8.8 $\pm$ 0.2	21.6 $\pm$ 1.5	12.1 $\pm$ 0.8	0.81 $\pm$ 0.13**	247 $\pm$ 12	96 $\pm$ 4*
2.5	8.9 $\pm$ 0.2	20.3 $\pm$ 1.2*	11.4 $\pm$ 0.5	0.91 $\pm$ 0.09*	216 $\pm$ 7*	92 $\pm$ 3**
5.0	9.2 $\pm$ 0.3	19.7 $\pm$ 1.1*	11.2 $\pm$ 0.6	1.10 $\pm$ 0.19*	205 $\pm$ 2*	88 $\pm$ 2**

Pyrazinamide (0.25–5.0 mM) significantly decreased flux through the glutarate pathway and potentiated the effect of 0.5 mM-tryptophan on gluconeogenesis (Table 4). Maximum inhibition of the flux through the glutarate pathway (31%) was observed at 1 mM-pyrazinamide. The maximum effect on tryptophan-mediated inhibition of gluconeogenesis is lower (21%), and reflected the relatively low flux through the glutarate pathway. Pyrazinamide at 2.5 and 5.0 mM also significantly decreased flux through tryptophan 2,3-dioxygenase; no parallel change in kynureninase activity was seen (Table 4).

At 0.1 to 0.4 mM-tryptophan, 1 mM-pyrazinamide

significantly altered both flux through the glutarate pathway and tryptophan-mediated inhibition of gluconeogenesis, although neither the flux through tryptophan 2,3-dioxygenase nor that through kynureninase were affected (Table 5).

These effects of pyrazinamide indicate an increase in quinolinate formation, presumably a result of decreased picolinate carboxylase activity. This enzyme is known to control the flux through the glutarate and the NAD(P)-biosynthetic pathways (Nishizuka & Hayaishi, 1963; Mehler *et al.*, 1964; Ichiyama *et al.*, 1965; Ikeda *et al.*, 1965).

The effects of DL-6-chlorotryptophan, 4-chloro-

Table 5. *The effects of tryptophan concentration and pyrazinamide on tryptophan metabolism and gluconeogenesis* Liver cells were incubated for 1 h with or without 1 mM-pyrazinamide. Lactate/pyruvate (final combined concentration 10 mM; 9:1, v/v) and L-tryptophan were then added; glucose was assayed in samples incubated for a further 60 min. Alternatively, lactate/pyruvate was added after 50 min incubation, and radiolabelled tryptophan (sp. radioactivity 0.03 Ci/mol) after a further 10 min. Release of radiolabel was measured 60 min later. Results are means $\pm$ S.E.M. for three independent observations; significances between means were assessed with the paired *t* test. *P* (versus the corresponding controls): * <0.05; ** <0.01.

[Tryptophan] (mM)	Activity or content (nmol/mg dry wt. per h)			
	Tryptophan 2,3-dioxygenase flux	Kynureninase flux	Glutarate pathway flux	Glucose synthesis
Without pyrazinamide				210 $\pm$ 9
0.1	5.25 $\pm$ 0.31	6.38 $\pm$ 0.33	0.44 $\pm$ 0.06	148 $\pm$ 11
0.2	13.35 $\pm$ 0.81	12.80 $\pm$ 0.76	1.31 $\pm$ 0.36	117 $\pm$ 4
0.4	21.37 $\pm$ 0.64	21.26 $\pm$ 1.15	2.69 $\pm$ 0.17	115 $\pm$ 8
+ 1 mM-Pyrazinamide				211 $\pm$ 10
0.1	6.19 $\pm$ 0.08	6.04 $\pm$ 0.34	0.22 $\pm$ 0.01**	117 $\pm$ 3*
0.2	11.67 $\pm$ 0.32	12.26 $\pm$ 0.51	0.66 $\pm$ 0.02**	96 $\pm$ 10*
0.4	20.62 $\pm$ 0.88	19.76 $\pm$ 1.02	1.70 $\pm$ 0.14**	71 $\pm$ 1**

3-hydroxyanthranilate and pyrazinamide on tryptophan metabolism and glucose synthesis in liver cells are consistent with observations *in vivo* and with isolated enzymes *in vitro* (Parli *et al.*, 1980; Nasu *et al.*, 1981); the specificity of the drugs is confirmed. These results complement previous studies in this laboratory (Smith *et al.*, 1978; Muñoz-Clares *et al.*, 1981), and demonstrate that tryptophan-mediated inhibition of gluconeogenesis is affected by factors that alter the metabolism of this amino acid.

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