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The metabolism of L-tryptophan by isolated rat liver cells

Quantification of the relative importance of, and the effect of nutritional status on, the individual pathways of tryptophan metabolism

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1. The metabolism of L-tryptophan by liver cells prepared from fed and 48h-starved rats was studied. Methods are described, with the use of L-[*ring*-2-^{14}C], L-[*carboxy*-^{14}C]- and L-[*benzene-ring*-U-^{14}C]-tryptophan, for the simultaneous determination of tryptophan 2,3-dioxygenase and kynureninase activities and of the oxidation of tryptophan to CO_2 and non-aromatic intermediates of the kynurenine–glutarate pathway. 2. At physiological concentrations (0.1mM), tryptophan was oxidized by tryptophan 2,3-dioxygenase at comparable rates in liver cells from both fed and starved rats. Kynureninase activity of hepatocytes from starved rats was 50% greater than that of cells from fed rats. About 10% of the tryptophan metabolized by tryptophan 2,3-dioxygenase was degraded completely to CO_2. 3. In the presence of 0.5mM-L-tryptophan, tryptophan 2,3-dioxygenase and kynureninase activities increased 5–6-fold. Liver cells from starved rats oxidized tryptophan at about twice the rate of these from fed rats. Degradation of tryptophan to non-aromatic intermediates of the glutarate pathway and CO_2 was increased only 3-fold, suggesting an accumulation of aromatic intermediates of the kynurenine pathway. 4. Rates of metabolism with 2.5mM-L-tryptophan were not significantly different from those obtained with 0.5mM-tryptophan. 5. Rates of synthesis of quinolinic acid from 0.5mM-L-tryptophan, determined either by direct quantification or indirectly from rates of radioisotope release from L-[*carboxy*-^{14}C]- and [*benzene-ring*-U-^{14}C]tryptophan, were essentially similar. 6. At all three concentrations examined, tryptophan was degraded exclusively through kynurenine; there was no evidence of formation of either indol-3-ylacetic acid or 5-hydroxyindol-3-ylacetic acid.

In the mammal, four separate pathways exist for the metabolism of L-tryptophan (see Scheme 1). These are: (i) oxidation by tryptophan 2,3-dioxygenase [L-tryptophan–O_2 2,3-oxidoreductase (decyclizing), EC 1.13.11.11] to kynurenine and subsequently to acetyl-CoA (the 'glutarate' pathway) or nicotinamide nucleotides (the 'NAD' pathway); (ii) transamination to yield indol-3-ylpyruvic acid, which may then be oxidized to indol-3-ylacetic acid; (iii) decarboxylation to yield the neurotransmitter tryptamine (Saavedra & Axelrod, 1973; Young *et al.*, 1980); (iv) by tryptophan hydroxylase [L-tryptophan,tetrahydropteridine–O_2 oxidoreductase (5-hydroxylating), EC 1.14.16.4] to 5-hydroxy-L-tryptophan and thence to 5-hydroxytryptamine (the '5-hydroxyindole' pathway).

It is now well established that quantitatively by far the most important route of tryptophan metabolism is oxidation by the kynurenine pathway located in the liver (Young *et al.*, 1978). Several methods have been used to investigate the control of tryptophan oxidation by the kynurenine pathway in intact isolated liver preparations. These include measurements of rates of appearance of specific metabolites such as kynurenine (Green *et al.*, 1976) or of removal of added tryptophan (Kim & Miller, 1969; Ng *et al.*, 1970). The disadvantages of these methods have been discussed elsewhere (Smith & Pogson, 1980).

More extensive information about the regulation of carbon flux through different sections of the

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kynurenine pathway has been derived from experiments with the use of specifically radiolabelled tryptophan (Altman & Gerber, 1967; Ng *et al.*, 1970). However, interpretation of these radioisotope data is complicated because, in most instances, a racemic mixture of D- and L-isomers of tryptophan was used. The rate of uptake and subsequent metabolism of D-tryptophan by rat liver is different from that of the L-isomer (Ng *et al.*, 1970; Rodden & Berg, 1974; Chen *et al.*, 1974). Hence the metabolic profile derived from a small pool (of high specific radioactivity) of D-tryptophan will be superimposed on that of the natural isomer.

In the series of experiments described in the present paper, we have examined the influences of starvation and tryptophan concentration on the activities of tryptophan 2,3-dioxygenase and kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) and on the rate of oxidation of tryptophan to CO_2 and intermediates of the 'glutarate' pathway in isolated rat liver cells. The flux of carbon through specific enzymes and portions of the kynurenine pathway was determined from rates of radioisotope release by liver cells incubated with four radioisomers of L-tryptophan.

Studies with cell-free extracts have shown that, in addition to enzymes required for the kynurenine pathway, the liver possesses the necessary enzymic complement for the catabolism of tryptophan by each of the other three pathways (Weissbach *et al.*, 1959; Civen & Knox, 1959; Renson *et al.*, 1966). It is not known to what extent, if any, tryptophan is catabolized by these alternative pathways in the intact liver cell. This possibility was also investigated in the present study.

Materials and methods

Animals

Male Sprague–Dawley CSE/ASH-strain rats (Charles River U.K. Ltd., Manston, Kent, U.K.; inbred in this laboratory), weighing 200–250g, were used throughout. Animals were fed *ad libitum* (no. 1 maintenance diet; Cooper Nutritional Products, Witham, Essex, U.K.), or were deprived of food for 48h before cell preparation.

Chemicals

DL-[*ring*-2-^{14}C]Tryptophan (specific radioactivity 30Ci/mol) was obtained from Schwarz–Mann, Orangeburg, NY, U.S.A. L-[5-^{3}H)Tryptophan (specific radioactivity 30Ci/mmol), DL-[*benzene-ring*-U-^{14}C]tryptophan (specific radioactivity 45Ci/mol), DL-[*carboxy*-^{14}C]tryptophan (specific radioactivity 50Ci/mol) and other radiochemicals came from The Radiochemical Centre, Amersham, Bucks., U.K.

Radiolabelled DL-tryptophan was resolved optically, and L-[5-^{3}H]tryptophan repurified, by affinity chromatography on bovine serum albumin–Sepharose 4B as described by Stewart & Doherty (1973). The sources of all other materials are detailed in Smith & Pogson (1980).

Liver-cell preparation

Isolated liver cells were prepared from fed or starved rats by the procedure detailed elsewhere (Elliott *et al.*, 1976). The metabolic integrity of cell preparations was assessed on the basis of gluconeogenic capacity (cells from starved rats; Smith *et al.*, 1978) or cellular ATP content (fed rats; Dickson & Pogson, 1977).

Cell incubation conditions and assay procedures

(*i*) *Measurement of radioisotope release from radioisomers of* L-*tryptophan.* The incubation conditions for determining rates of $^{14}CO_2$ production from ^{14}C-labelled tryptophan radioisomers were identical with those described previously (Smith & Pogson, 1980).

The radioactivity of non-aromatic metabolites of tryptophan was measured as follows. Precipitated protein was removed by centrifugation (1200***g*** for 5min), and 1ml portions of supernatant were treated with 0.2ml of a 50mg/ml aqueous suspension of Norit-GSX. After thorough mixing, the charcoal was removed by centrifugation (10000***g*** for 2min); 0.5ml of each supernatant was counted for ^{14}C or ^{3}H radioactivity in PCS scintillator 'cocktail'. Under these conditions, binding of unmetabolized radiolabelled L-tryptophan is quantitative (Smith & Pogson, 1980). Preliminary experiments (results not shown) indicated that there is no detectable binding of the major non-aromatic products of tryptophan catabolism ([^{14}C]formate and [^{14}C]alanine) by charcoal. To determine non-enzymic radioisotope release, appropriate control incubations in which tryptophan was added after $HClO_4$ were included for each radioisomer used. Results presented in the text have been corrected for non-enzymic radioisotope release.

(*ii*) *Incorporation of radioactivity from* L-*tryptophan into protein.* Samples of cell suspensions incubated with either L-[5-^{3}H]tryptophan or L-[*ring*-2-^{14}C]tryptophan as described above were analysed for incorporation of radioactivity into trichloroacetic acid-precipitable material by the filter-disc method of Mans & Novelli (1961).

(*iii*) *Determination of* ^{14}*C incorporation from* L-[*ring-2-*^{14}*C]tryptophan into adenine nucleotides.* To 0.4ml portions of neutralized acid extracts of liver cells incubated with L-[*ring*-2-^{14}C]tryptophan were added: 2 units (μmol/min) of adenylate kinase, 2 units (μmol/min) of pyruvate kinase and 150nmol of phosphoenolpyruvate, followed after 10min by 100nmol of ATP (final volume of mixture 0.6ml).

Then 30min later the mixtures were applied to columns (4cm × 0.4cm) of Amberlite CG-400 (formate form) equilibrated with 10mM-HCl. The columns were eluted with 30ml of 10mM-HCl, to remove all ^{14}C-labelled tryptophan, followed by 10ml of 1M-HCl. Samples of the 1M-HCl eluates were counted for radioactivity in PCS scintillator 'cocktail'.

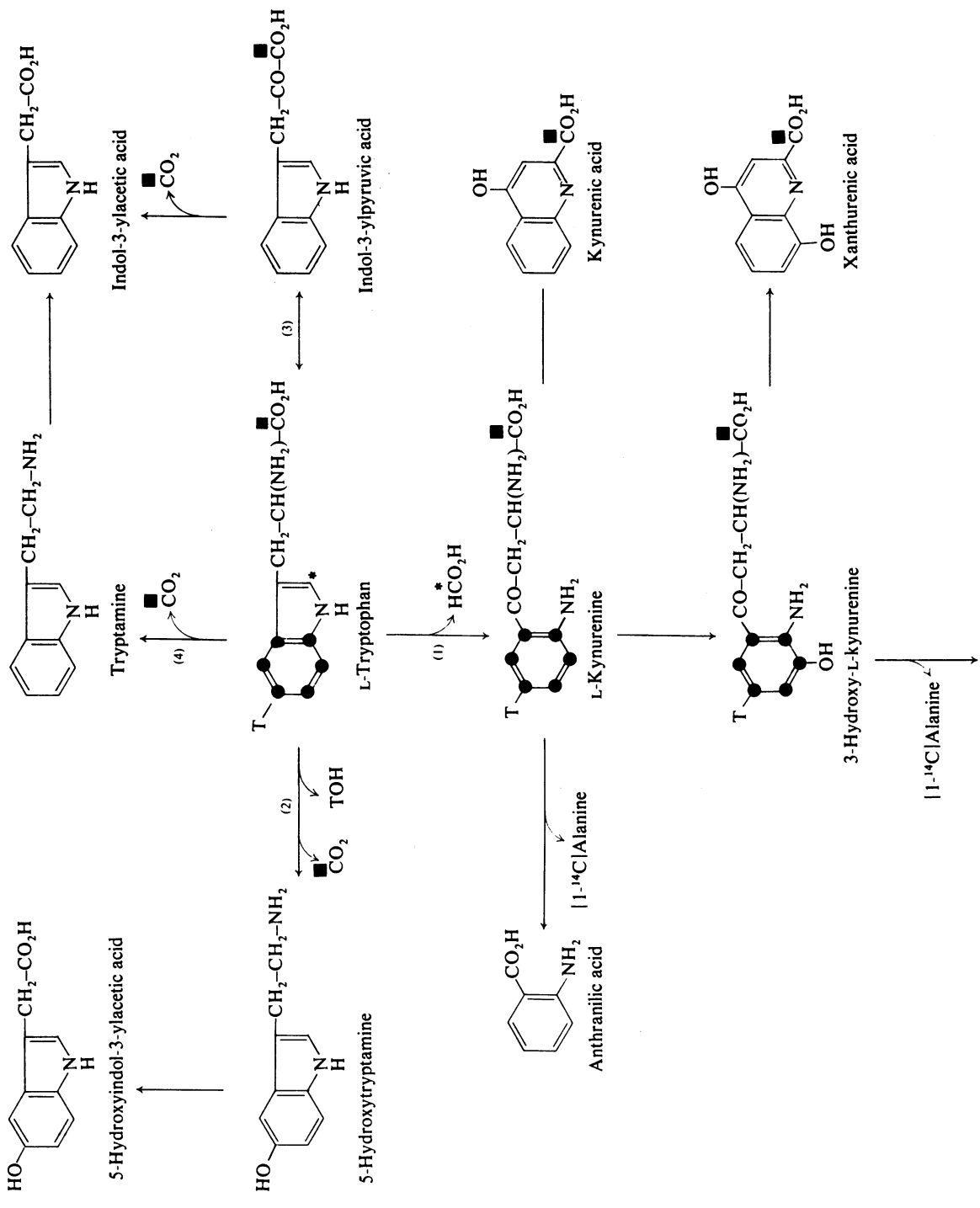
(*iv*) *Procedure for measurement of quinolinic acid synthesis by hepatocyte suspensions.* Portions (5ml) of cell suspensions from starved rats (containing 80–100mg dry wt. of tissue) were incubated in 10ml of buffer [as in (*i*) above] in 100ml silicone-treated glass conical flasks, stoppered with rubber bungs, for 10min before addition of pyruvate (final concn. 10mM); 20min later L-tryptophan (final concn. 0.5mM) was added. The flasks were shaken at 100 oscillations/min for a further 1h, after which time 1.5ml of 2M-$HClO_4$ was added. Incubations were performed in triplicate. In control incubations tryptophan was added after $HClO_4$. Precipitated protein was removed by centrifugation (1200*g* for 5min) and 13ml of supernatant was neutralized with 0.5M-triethanolamine/HCl, pH 7.4, containing 2M-KOH. After removal of $KClO_4$ by centrifugation (1200*g* for 5min) at 4°C, samples were stored at −20°C for subsequent determination of quinolinic acid.

Quinolinic acid was isolated from cell extracts by a combination of ion-exchange chromatography and charcoal-adsorption methods.

Neutralized acid cell extracts were adjusted to approx. pH 1.5 with conc. HCl and applied to columns (10cm × 0.6cm) of Amberlite CG-120 (H^+ form; 100–200 mesh). The columns were washed with 10ml of water. Under these conditions tryptophan is quantitatively adsorbed, but quinolinate remains unbound. After adjustment of the pH to 4.0 with NaOH, the pooled samples were applied to columns (10cm × 0.6cm) of Amberlite CG-400 (formate form; 200–400 mesh). The columns were washed with 10ml of 50mM-HCl. Quinolinate was eluted from the anion-exchange resin with 10ml of 1M-HCl and extracted from these acid solutions by adsorption on Norit A charcoal (1mg/ml final concn.). After centrifugation (1200*g* for 10min) the HCl was removed by aspiration and the charcoal pellets were washed with 5ml of water. The pellets were resuspended in 2ml of ethanol/ammonia (sp.gr. 0.880)/water (10:1:9, by vol.) and transferred to small glass columns, each containing a 5mm bed of washed Celite. The columns were washed with a further 2ml of ethanol/ammonia/water. The two fractions were combined and evaporated to dryness under N_2 at 60–70°C. The residues were dissolved in 1ml of acetone/water (1:1, v/v); 0.75ml of each was transferred to 1ml Reactivials (Pierce Chemical Co.) and evaporated to dryness with dry N_2 at 60°C. The overall recovery of quinolinic acid {determined by addition to each sample of 2nmol of [6-^{14}C]quinolinic acid (sp. radioactivity 61Ci/mol)} was 34 ± 2% (mean ± S.D., 18 determinations). Values of quinolinate content of hepatocyte incubations quoted in the Results section have been appropriately corrected.

For g.l.c. analysis, the trimethylsilyl derivative of quinolinic acid was prepared by addition of 0.02ml of *NO*-bis(trimethylsilyl)acetamide to each vial. To ensure complete derivative formation, samples were left overnight at 18–20°C before analysis in a Pye Unicam model 204 Gas–Liquid Chromatograph equipped with a flame ionization detector. Chromatographic separation was effected on 3% OV-17 on Diatomite CLQ (100–120 mesh) contained in glass columns (2m long × 4mm internal diam.). Preliminary experiments revealed that the greatest resolution was achieved with a simple temperature programme. Samples (1–2μl) containing trimethylsilylquinolinate were injected on to the column at 150°C. Isothermal conditions were maintained for 5min, after which time the temperature was increased by 12°C/min up to 230°C, at which it was maintained for a final 5min period. The N_2 carrier gas flow rate was 60ml/min and the detector temperature was 250°C. Base-line drift resulting from liquid-phase 'bleed' from the column as the temperature increased was minimized by using an equivalent balance column. Under these conditions the retention time for trimethylsilylquinolinic acid was between 12min 12s and 12min 15s. Authenticity of quinolinic acid in samples was verified by internal standardization. With this method, as little as 0.2nmol of quinolinic acid may be conveniently and reliably determined.

(*v*) *Measurement of indole/5-hydroxyindole synthesis.* Portions (1ml) of liver cell suspensions, containing 16–20mg dry wt. of tissue, were incubated in 4ml of buffer [as in (*i*) above] in 50ml silicone-treated Erlenmeyer flasks for 10min before addition of pyruvate (final concn. 10mM). Incubations, in triplicate, were shaken at 100 oscillations/min at 37°C under an atmosphere of O_2/CO_2 (19:1). At 20min after pyruvate addition, L-tryptophan (final concn. 2.5mM) was added. Incubations were terminated 1h after tryptophan addition with 0.5ml of 2M-$HClO_4$. In control incubations tryptophan was added after $HClO_4$. After removal of precipitated protein by centrifugation (1200*g* for 5min), 4ml of each supernatant was transferred to 20ml glass scintillation vials containing 2nmol of [2-^{14}C]indol-3-ylacetic acid (specific radioactivity 40Ci/mol) and extracted with 3vol. of diethyl ether. The ether phase was removed and evaporated at 40°C under a current of N_2. Recovery of labelled indol-3-ylacetate was 97 ± 3% (mean ± S.D., 18 determinations). Residues were redissolved in 0.1ml


5-Hydroxyindol-3-ylacetic acid
5-Hydroxytryptamine
Tryptamine
Indol-3-ylacetic acid
5-Hydroxytryptophan
L-Tryptophan
Indol-3-ylpyruvic acid
Anthranilic acid
L-Kynurenine
Kynurenic acid
3-Hydroxy-L-kynurenine
Xanthurenic acid
[1-14C]Alanine
[1-14C]Alanine
(1)
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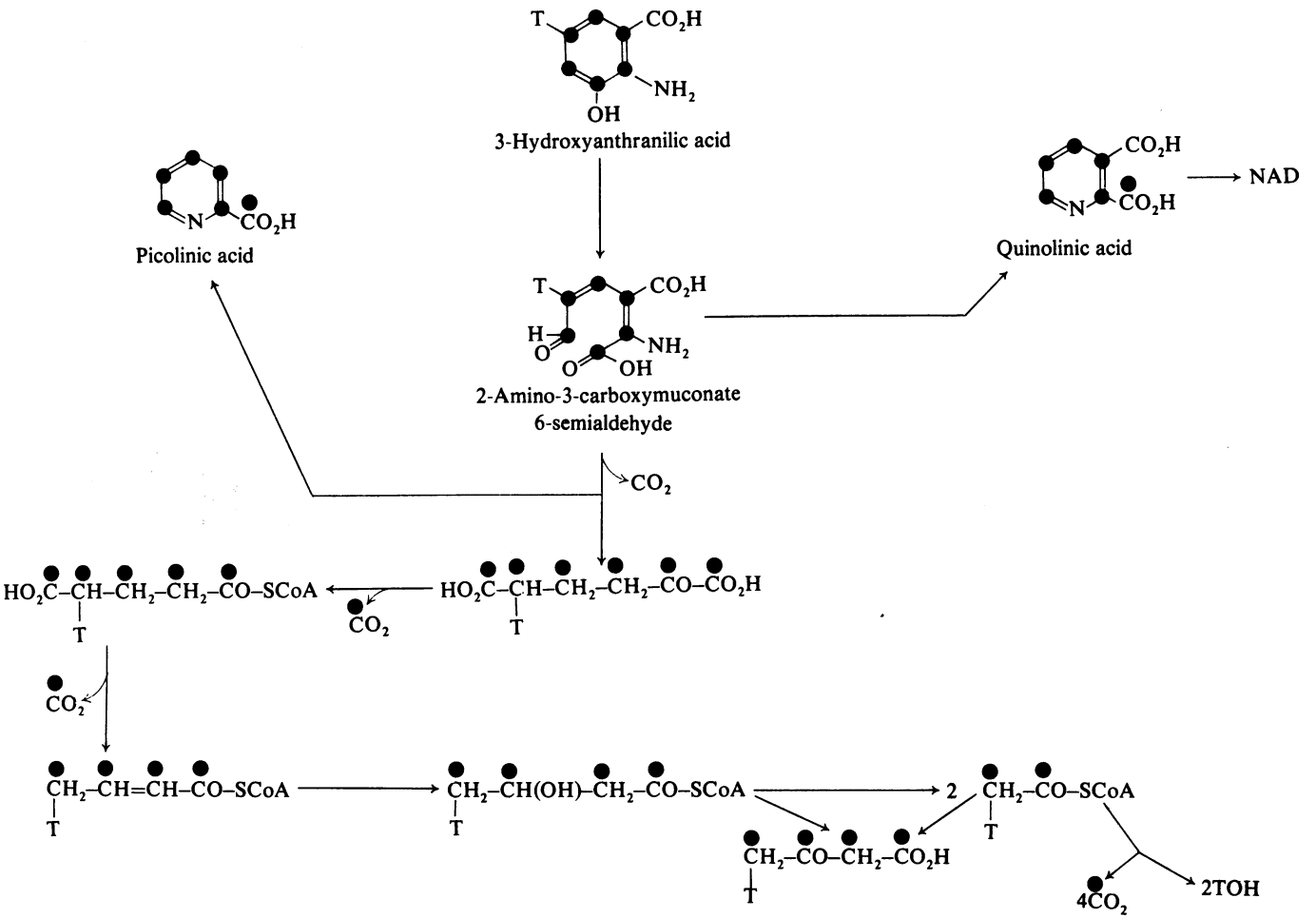
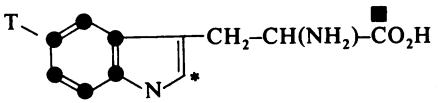


Scheme 1. *Metabolic fate of radioisotope from specifically radiolabelled* L-*tryptophan*
Some of the intermediate steps involved in each pathway have been omitted. Key to radioisotope nomenclature:



*, L-[*ring*-2-^{14}C]tryptophan; T, L-[5-^{3}H]tryptophan; ●, L-[*benzene-ring*-U-^{14}C]tryptophan; ■, L-[*carboxy*-^{14}C]-tryptophan. The metabolic pathways are: (1) kynurenine–glutarate pathway; (2) 5-hydroxytryptamine pathway; (3) indole pathway; (4) tryptamine pathway.

of methanol, samples of which were assayed for indol-3-ylacetate or 5-hydroxyindol-3-ylacetate content by a modification of the fluorimetric method of Stoessl & Venis (1970).

Portions (0.02ml) of the methanolic solutions were transferred to acid-washed glass tubes and evaporated to dryness under a current of N_2 at 0°C. Then 0.1ml of an ice-cold mixture of acetic anhydride/trifluoroacetic acid (1:1, v/v) was added and the tubes were incubated at 0°C for 15min. After addition of 2.5ml of 0.25M-sodium phosphate buffer, pH11.5, and thorough mixing, the fluorescence of each sample was determined with a Perkin–Elmer PE 1000 fluorimeter (excitation wavelength 460nm, emission wavelength 500nm). The endogenous fluorescence of each cell extract was determined in blank incubations in which sample was added after sodium phosphate buffer.

Expression of results

Rates of hepatocyte tryptophan metabolism determined by radioisotope-release methods are expressed as nmol of tryptophan/h per mg dry wt. of cells. The rates of metabolism of radiolabelled 0.1mM-, 0.5mM- and 2.5mM-tryptophan were determined simultaneously with each cell batch. All measured rates of radioisotope release were linear with time for periods up to 2h. To make possible direct comparisons between cells prepared from fed or starved rats, differences in the lipid and glycogen content must be taken into account; rates of tryptophan catabolism are also given as nmol of tryptophan/h per mg of cell DNA. The DNA content, determined by the method of Burton (1956), of liver cells isolated from fed and 48h-starved rats was $16.09 \pm 0.37\mu g$ of DNA/mg dry wt. of cells (mean ± S.E.M., six determinations) and $19.70 \pm 0.73\mu g$/mg dry wt. of cells (mean ± S.E.M., seven determinations) respectively. For ^{14}C-labelled tryptophan, values of 'total' radioisotope release quoted in the text consist of two terms, the one derived from quantification of metabolic $^{14}CO_2$, the other derived from radioactivity determinations of charcoal-treated acid cell extracts. Because charcoal specifically binds aromatic compounds, this latter term is a measure of the formation of non-aromatic metabolites from tryptophan.

Results and discussion

Rationale of radioisomer selection for the determination of tryptophan catabolism in isolated liver cells

In the experiments described, four radioisomers of L-tryptophan were used to measure the relative contributions of specific pathways to the overall pattern of hepatocyte tryptophan metabolism. Scheme 1 outlines the possible enzymic steps at which radioisotope may be released from L-[*ring*-2-^{14}C]-, L-[*carboxy*-^{14}C]-, L-[*benzene-ring*-U-^{14}C]- and L-[5-^{3}H]-tryptophan.

^{14}C from L-[*ring*-2-^{14}C]tryptophan can only be released during catabolism of tryptophan by the 'kynurenine–glutarate' pathway. After oxidative cleavage of the pyrrole moiety by tryptophan 2,3-dioxygenase, the C-2 appears in the formyl group of *N*-formyl-L-kynurenine. This compound is rapidly deformylated by a high-activity formamidase (EC 3.5.1.9) (Mehler & Knox, 1950). Formate may be directly converted into CO_2, or enter the C_1 pool, from which it may participate in C_1-acceptor reactions, e.g. for purine biosynthesis (Letter *et al.*, 1973), exchange with C_1 donors, such as serine, or, again, oxidation to CO_2 (Krebs *et al.*, 1976). To determine the total flux of tryptophan through tryptophan 2,3-dioxygenase, the following measurements were made: $^{14}CO_2$, [^{14}C]formate, ^{14}C-labelled aliphatic amino acid production and incorporation of ^{14}C into protein and purine nucleotides.

^{14}C from L-[*carboxy*-^{14}C]tryptophan may be eliminated at several stages of tryptophan metabolism: (i) as [1-^{14}C]alanine by the action of kynureninase on L-kynurenine or 3-hydroxy-L-kynurenine (McDermott *et al.*, 1973); (ii) as $^{14}CO_2$ by decarboxylation of tryptophan to tryptamine (Weissbach *et al.*, 1959); rapid conversion of [1-^{14}C]alanine into [1-^{14}C]pyruvate by high alanine aminotransferase activity in liver and the subsequent oxidative decarboxylation of pyruvate would lead to the appearance of label as $^{14}CO_2$; (iii) as $^{14}CO_2$ by oxidation of indol-3-ylpyruvate to indol-3-ylacetate after transamination of tryptophan with 2-oxoglutarate (Civen & Knox, 1959); (iv) as $^{14}CO_2$ by decarboxylation of 5-hydroxytryptophan to 5-hydroxytryptamine after metabolism of tryptophan by tryptophan hydroxylase.

Total radioisotope release from L-[*carboxy*-^{14}C]-tryptophan therefore represents the sum of kynureninase flux and indole synthesis.

Labilization of ^{14}C from L-[*benzene-ring*-U-^{14}C]-tryptophan occurs only during degradation of tryptophan along the 'kynurenine–glutarate' pathway. After cleavage by 3-hydroxyanthranilate 3,4-dioxygenase [3-hydroxyanthranilate–O_2 3,4-oxidoreductase (decyclizing), EC 1.13.11.6], the benzene-ring moiety is converted into 2 molecules of acetyl-CoA, with concomitant loss of one-third of the original radioactivity as $^{14}CO_2$. Further $^{14}CO_2$ may be produced from oxidation of [^{14}C]acetyl-CoA, or alternatively label may be incorporated into ketone bodies or lipid. Measurement of $^{14}CO_2$ production and the non-charcoal-precipitable (i.e. non-aromatic) radioactivity content of cell extracts provides an index of the rate at which tryptophan is catabolized to aliphatic metabolites of the glutarate pathway.

Enzymic release of ^{3}H from L-[5-^{3}H]tryptophan

may occur during metabolism of tryptophan by two different pathways: (i) after hydroxylation by tryptophan hydroxylase at position 5 of the benzene ring, to give 5-hydroxy-L-tryptophan, the immediate precursor of 5-hydroxytryptamine; ^3H is incorporated into water (but see Renson *et al.*, 1966); (ii) after oxidation of tryptophan by the 'kynurenine–glutarate' pathway, in a manner analogous to ^{14}C release from [*benzene-ring*-U-^{14}C]tryptophan; ^3H is incorporated into water, ketone bodies or lipid; measurement of the ^3H content of charcoal-treated cell extracts represents the sum of the rates of 5-hydroxylation and of the oxidation of the benzene-ring portion of tryptophan to aliphatic metabolites.

Metabolism of 0.1 mM-L-tryptophan by isolated liver cells

The rates of radioisotope release from radiolabelled 0.1 mM-L-tryptophan by hepatocytes prepared from fed and starved rats are shown in Table 1. Release of [^{14}C]formate from L-[*ring*-2-^{14}C]-tryptophan is an index of tryptophan 2,3-dioxygenase activity (Smith & Pogson, 1980). Although the greater fraction of [^{14}C]formate was oxidized to $^{14}CO_2$, a significant proportion of the radioactivity remained in charcoal-treated cell extracts. Incomplete oxidation of [^{14}C]formate to $^{14}CO_2$ by hepatocytes is analogous to the situation *in vivo*, in which only 50% of injected [^{14}C]formate appears as respiratory $^{14}CO_2$ (Young & Sourkes, 1975). There was no detectable incorporation of label from 0.1 mM-L-[*ring*-2-^{14}C]tryptophan into adenine nucleotides. These findings contrast with the studies *in vivo* by Letter *et al.* (1973), who found that 10% of the label had been incorporated into the adenine nucleotide pool 2h after administration of DL-[*ring*-2-^{14}C]tryptophan to rats.

Similarly, we could not detect any incorporation into protein of label from L-[*ring*-2-^{14}C]tryptophan. This indicates that there is no significant incorporation of either tryptophan itself (confirmed with L-[5-^3H]tryptophan) or ^{14}C-labelled amino acids, the label of which is derived indirectly from tryptophan via C_1-transfer reactions, into protein. An earlier study, with the perfused rat liver, claimed that only 10% of 0.1 mM-DL-[*ring*-2-^{14}C]tryptophan added to the perfusate appears as metabolic CO_2; the greater proportion is recovered as protein-bound serine and alanine (Altman & Gerber, 1967). The reason for this discrepancy is unclear, particularly as, in the earlier study, the livers, from starved animals, were perfused with an insulin-free medium, conditions that favour proteolysis rather than protein synthesis.

Under the conditions used in the present experiments, therefore, total hepatocyte tryptophan 2,3-dioxygenase flux is given by the sum of $^{14}CO_2$ and non-charcoal-precipitable radioactivity (representing the sum of [^{14}C]formate and ^{14}C-labelled aliphatic amino acids) production from L-[*ring*-2-^{14}C]tryptophan. There was no significant dif-

Table 1. *Metabolism of 0.1 mM-L-tryptophan by liver cells isolated from fed or starved rats*

Liver cells were preincubated for 10min before addition of pyruvate (10mM). Radiolabelled L-tryptophan was added after a further 20min. Rates of radioisotope release were determined between 30 and 90min after addition of tryptophan (the sp. radioactivities of ^{14}C- and ^3H-labelled tryptophan radioisomers were 0.25 and 0.50 μCi/μmol respectively). Results are expressed as means ± S.E.M. for either four (fed) or three (starved) separate experiments. *$P < 0.01$ versus corresponding value of fed cells (Student's *t* test).

		Tryptophan metabolized			
		Liver cells from starved rats		Liver cells from fed rats	
Tryptophan radioisomer	Metabolite fraction	(nmol/h per mg dry wt. of cells)	(nmol/h per mg of cell DNA)	(nmol/h per mg dry wt. of cells)	(nmol/h per mg of cell DNA)
L-[*ring*-2-^{14}C] (tryptophan 2,3-dioxygenase flux)	$^{14}CO_2$	2.40 ± 0.20	122 ± 10	2.02 ± 0.14	126 ± 9
	^{14}C-labelled non-aromatic products	1.60 ± 0.50	81 ± 25	0.90 ± 0.11	56 ± 7
	Total	4.10 ± 0.40	208 ± 26	2.71 ± 0.36	170 ± 20
L-[*carboxy*-^{14}C] (kynureninase flux)	$^{14}CO_2$	1.20 ± 0.10	61 ± 5	0.60 ± 0.07	37 ± 4
	^{14}C-labelled non-aromatic products	1.70 ± 0.13	86 ± 7	0.97 ± 0.08	60 ± 4
	Total	2.90 ± 0.16*	147 ± 8	1.60 ± 0.13	99 ± 8
L-[*benzene-ring*-U-^{14}C]	$^{14}CO_2$	0.40 ± 0.10	20 ± 5	0.35 ± 0.10	22 ± 6
	^{14}C-labelled non-aromatic products	0.50 ± 0.05	25 ± 3	0.3 ± 0.07	21 ± 4
	Total	0.90 ± 0.10	46 ± 5	0.70 ± 0.09	44 ± 6
L-[5-^3H]	Total ^3H release	1.30 ± 0.19	66 ± 10	0.70 ± 0.17	44 ± 10

ference between the rates of oxidation of 0.1 mM-L-[*ring*-2-^{14}C]tryptophan by cells from fed or starved rats (Table 1).

In hepatocytes from both fed and starved rats, total radioisotope release from L-[*carboxy*-^{14}C]-tryptophan was less than that from L-[*ring*-2-^{14}C]-tryptophan. Non-charcoal-precipitable radioactivity in extracts of liver cells incubated with [*carboxy*-^{14}C]tryptophan can only be derived from cleavage of [1-^{14}C]alanine by kynureninase from L-kynurenine or 3-hydroxy-L-kynurenine. However, $^{14}CO_2$ may be released at two different steps in indole synthesis (see Scheme 1) or, alternatively, from [1-^{14}C]alanine oxidation. In a parallel series of experiments, total rates of radioisotope release ($^{14}CO_2$ plus non-charcoal-precipitable radioactivity) from 0.1 mM-L-[*methylene*-^{14}C]- and L-[*carboxy*-^{14}C]-tryptophan by hepatocytes isolated from starved rats were equal (S. A. Smith, unpublished work). Formation of metabolic $^{14}CO_2$ from L-[*methylene*-^{14}C]tryptophan can occur only indirectly as a consequence of [3-^{14}C]alanine oxidation; this is not feasible during indole synthesis. It is therefore unlikely that indole synthesis occurs to any appreciable extent under these conditions. Total radioisotope release from L-[*carboxy*-^{14}C]tryptophan thus provides a reliable index of kynureninase flux.

It should be noted that, although the greater portion of L-kynurenine is normally hydroxylated to 3-hydroxy-L-kynurenine, the preferred substrate of kynureninase (De Castro *et al.*, 1956; McDermott *et al.*, 1973), it is not possible, without quantification of anthranilate, to determine the relative proportions of [1-^{14}C]alanine derived from L-kynurenine or 3-hydroxy-L-kynurenine.

The difference in rates of radioisotope release from 0.1 mM-L-[*ring*-2-^{14}C]- and L-[*carboxy*-^{14}C]-tryptophan (about 1.2 nmol/h per mg dry wt. of cells) must be accounted for by the accumulation of intermediates between the tryptophan 2,3-dioxygenase and kynureninase steps. Livers taken from fed rats and perfused with medium containing 0.1 mM-L-tryptophan release kynurenine into the perfusate at rates comparable with those quoted above (Green *et al.*, 1976). In addition, L-kynurenine and 3-hydroxy-L-kynurenine, together with their respective transamination products kynurenic acid and xanthurenic acid, are normal constituents of urine of many mammalian species (Yeh & Brown, 1977).

Kynureninase flux measured with [*carboxy*-^{14}C]-tryptophan was greater in cell preparations from starved rats than in those derived from fed rats. This may be a reflection of differences in kynureninase activity itself, or of altered disposition of L-kynurenine and/or 3-hydroxy-L-kynurenine by alternative pathways. To our knowledge, there have been no reports of the effect of starvation either on hepatic kynureninase activity or on synthesis of kynurenic acid or xanthurenic acid in the rat.

$^{14}CO_2$ production from L-[*benzene-ring*-U-^{14}C]-tryptophan is an index of the rate at which the benzene-ring moiety of tryptophan is oxidized completely to CO_2. Table 1 shows that only 10% of the tryptophan oxidized by tryptophan 2,3-dioxygenase was completely degraded to CO_2. However, quantification only of $^{14}CO_2$ may be an unreliable estimate of tryptophan-benzene-ring oxidation, because changes in $^{14}CO_2$ release may be a reflection of changes in the pool size or disposition of acetyl-CoA rather than of changes in flux through the glutarate section of the kynurenine–glutarate pathway. Measurement of $^{14}CO_2$ and non-charcoal-precipitable radioactivity (i.e. aliphatic intermediates derived from 3-hydroxyanthranilic acid) enables a more reliable estimate of flux through the glutarate part of the 'kynurenine–glutarate' pathway to be made. Only 50% of the total radioisotope released from [*benzene-ring*-U-^{14}C]tryptophan appeared as metabolic $^{14}CO_2$; the remainder accumulated as aliphatic intermediates (and compounds derived thereform) of the glutarate pathway.

In incubations of liver cells from both fed and starved rats, rates of release of ^{3}H from 0.1 mM-L-[5-^{3}H]tryptophan were similar to rates of conversion of L-[*benzene-ring*-U-^{14}C]tryptophan into $^{14}CO_2$ and ^{14}C-labelled non-aromatic products (Table 1). This indicates that at low, physiological, concentrations of tryptophan there is no detectable hydroxylation at position 5 of the benzene ring. Similar results were obtained when acidified samples were heated for extended periods, a procedure that ensures total release of ^{3}H after tryptophan hydroxylation (see Renson *et al.*, 1966). This confirms earlier findings in which no evidence was found of accumulation of 5-hydroxytryptamine or 5-hydroxyindol-3-ylacetic acid by isolated rat livers perfused with 0.1 mM-L-tryptophan (Green *et al.*, 1976).

Metabolism of 0.5 mM-L-tryptophan

When the initial concentration of tryptophan in the incubation medium was increased from 0.1 to 0.5 mM, the flux through tryptophan 2,3-dioxygenase was increased 5–6 fold (Table 2). In contrast with incubations with 0.1 mM-tryptophan, in which the rates were approximately equal, liver cells from starved rats oxidized tryptophan at about twice the rate of those from fed rats. In addition, there was a considerable difference in the distribution of ^{14}C radioactivity between the $^{14}CO_2$ and [^{14}C]formate fractions; this emphasizes the necessity of quantifying all possible metabolic fates of formate, rather than $^{14}CO_2$ alone. There was no detectable incorporation of ^{14}C from 0.5 mM-L-[*ring*-2-^{14}C]tryptophan into either protein or adenine nucleotides.

Table 2. *Metabolism of 0.5 mM-L-tryptophan by liver cells isolated from fed or 48 h-starved rats*
Liver cells were preincubated for 10 min before addition of pyruvate (10 mM). Radiolabelled L-tryptophan was added after a further 20 min. Rates of radioisotope release were determined between 30 and 90 min after addition of tryptophan (the sp. radioactivities of ^{14}C- and ^{3}H-labelled tryptophan radioisomers were 0.05 and 0.50 μCi/μmol respectively). Results are expressed as means ± S.E.M. for either four (fed animals) or three (starved animals) individual experiments. *$P < 0.01$ versus corresponding value for fed cells (Student's t test).

		Tryptophan metabolized			
		Liver cells from starved rats		Liver cells from fed rats	
Tryptophan radioisomer	Metabolite fraction	(nmol/h per mg dry wt. of cells)	(nmol/h per mg of cell DNA)	(nmol/h per mg dry wt. of cells)	(nmol/h per mg of cell DNA)
L-[*ring*-2-^{14}C] (tryptophan 2,3-dioxygenase)	$^{14}CO_2$	7.00 ± 0.40	355 ± 20	8.23 ± 0.41	511 ± 25
	^{14}C-labelled non-aromatic products	18.70 ± 1.00	949 ± 50	4.02 ± 0.58	250 ± 36
	Total	25.60 ± 1.61*	1300 ± 82*	12.31 ± 1.20	764 ± 75
L-[*carboxy*-^{14}C] (kynureninase)	$^{14}CO_2$	2.90 ± 0.21	147 ± 11	2.10 ± 0.33	131 ± 19
	^{14}C-labelled non-aromatic products	13.30 ± 0.90	675 ± 46	6.68 ± 0.32	415 ± 20
	Total	16.10 ± 1.38*	817 ± 70*	8.80 ± 0.70	547 ± 44
L-[*benzene-ring*-U-^{14}C]	$^{14}CO_2$	1.60 ± 0.10	81 ± 5	1.10 ± 0.23	68 ± 14
	^{14}C-labelled non-aromatic products	0.90 ± 0.23	46 ± 12	1.15 ± 0.47	71 ± 29
	Total	2.50 ± 0.19	127 ± 10	2.30 ± 0.90	143 ± 56
L-[5-^{3}H]	Total ^{3}H release	2.20 ± 0.49	137 ± 35	3.10 ± 0.50	193 ± 31

As noted for tryptophan 2,3-dioxygenase, raising the tryptophan concentration from 0.1 mM to 0.5 mM produced a 5–6-fold increase in kynureninase flux (total radioisotope release from L-[*carboxy*-^{14}C]-tryptophan). Kynureninase activity in hepatocytes from starved rats was about 50% greater than in those from fed rats; this may be related to the higher tryptophan 2,3-dioxygenase activity and hence availability of L-kynurenine in the starved state.

Although a 5-fold increase in tryptophan concentration produced equivalent changes in the activities of tryptophan 2,3-dioxygenase and kynureninase (expressed on a percentage basis), these changes must be accompanied, by virtue of the greatly increased carbon flux, by a greater accumulation of intermediates between the two enzymic steps. For example, the differences between the rates of radioisotope release from L-[*ring*-2-^{14}C]tryptophan and L-[*carboxy*-^{14}C]tryptophan by hepatocytes from starved rats incubated with radiolabelled 0.1 mM- or 0.5 mM-tryptophan were 1.2 and 9.5 nmol/h per mg dry wt. of cells respectively.

These observations are consistent with the earlier findings by Green *et al.* (1976), who showed that L-kynurenine output of the perfused rat liver is increased markedly when the perfusate tryptophan concentration is raised above 0.1 mM. Similarly, urinary excretion of L-kynurenine, kynurenic acid and xanthurenic acid is increased by tryptophan loading of the intact animal (Wolf, 1974; Yeh & Brown, 1977).

Unlike the 5–6-fold increases observed with L-[*ring*-2-^{14}C]- and L-[*carboxy*-^{14}C]tryptophan, total radioisotope release from 0.5 mM-L-[*benzene-ring*-U-^{14}C]tryptophan was increased only 3-fold. In addition, there was no difference in the rates at which cells from fed and starved rats oxidized the benzene-ring moiety of tryptophan (Table 2). Rates of ^{3}H release from 0.5 mM-L-[5-^{3}H]tryptophan were comparable with those of total ^{14}C release from L-[*benzene-ring*-U-^{14}C]tryptophan; this indicates that metabolism via the 5-hydroxytryptamine pathway does not occur at this concentration of tryptophan.

Metabolism of 2.5 mM-L-tryptophan

At 1–1½h after the administration of L-tryptophan to rats at doses that alter the activities of several hepatic enzymes (Smith *et al.*, 1979) and that produce hypoglycaemia (Smith & Pogson, 1977) the plasma tryptophan concentration reaches 2–3 mM. We therefore performed a series of experiments to investigate the capacity of liver cells to metabolize a large tryptophan load.

Tryptophan 2,3-dioxygenase activities of hepatocytes from fed or starved rats incubated with 2.5 mM-L-tryptophan were not significantly greater than those observed with 0.5 mM-tryptophan (Table 3). Rates of radioisotope release from 2.5 mM-L-[*ring*-2-^{14}C]tryptophan were linear with time for up to 2 h; there was therefore no evidence for enhancement of dioxygenase activity, which has

Table 3. *Metabolism of 2.5 mM-L-tryptophan by liver cells isolated from fed or 48 h-starved rats*

Liver cells were preincubated for 10 min before addition of pyruvate (10 mM). Radiolabelled L-tryptophan was added after a further 20 min. Rates of radioisotope release were determined between 30 and 90 min after addition of tryptophan (the sp. radioactivities of ^{14}C- and ^{3}H-labelled tryptophan radioisomers were 0.025 and 0.1 μCi/μmol respectively). Results are expressed as means ± S.E.M. for either four (fed rats) or three (starved rats) individual experiments.

		Tryptophan metabolized			
		Liver cells from starved rats		Liver cells from fed rats	
Tryptophan radioisomer	Metabolite fraction	(nmol/h per mg dry wt. of cells)	(nmol/h per mg of cell DNA)	(nmol/h per mg dry wt. of cells)	(nmol/h per mg of cell DNA)
L-[*ring*-2-^{14}C] (tryptophan 2,3-dioxygenase flux)	$^{14}CO_2$	7.40 ± 0.20	376 ± 10	8.70 ± 0.90	541 ± 56
	^{14}C-labelled non-aromatic products	23.60 ± 3.61	1198 ± 183	10.60 ± 2.00	659 ± 124
	Total	28.80 ± 2.80	146 ± 142	19.00 ± 3.00	1180 ± 188
L-[*carboxy*-^{14}C] (kynureninase flux)	$^{14}CO_2$	4.20 ± 0.50	213 ± 25	2.10 ± 0.53	131 ± 33
	^{14}C-labelled non-aromatic products	10.60 ± 1.41	538 ± 72	7.10 ± 1.24	441 ± 77
	Total	14.90 ± 2.42	756 ± 123	9.20 ± 1.60	571 ± 99
L-[*benzene-ring*-^{14}C]	$^{14}CO_2$	2.40 ± 0.42	122 ± 21	1.41 ± 0.60	87 ± 37
	^{14}C-labelled non-aromatic products	Not detectable	—	Not detectable	—
	Total	2.40 ± 0.42	122 ± 21	1.41 ± 0.60	87 ± 37
L-[5-^{3}H]	Total ^{3}H release	10.00 ± 2.71	508 ± 138	6.30 ± 1.20	392 ± 75

been measured in cell-free extracts of livers perfused with medium containing high concentrations of tryptophan (Kim & Miller, 1969; Green *et al.*, 1976). Again there was no detectable incorporation of label from L-[*ring*-2-^{14}C]tryptophan into adenine nucleotides or protein.

Similarly, in the presence of 2.5 mM-tryptophan, rates of kynurenine metabolism, determined from rates of total radioisotope release from L-[*carboxy*-^{14}C]tryptophan, and metabolism through the glutarate pathway ([*benzene-ring*-U-^{14}C]tryptophan) were comparable with those obtained with 0.5 mM-tryptophan. These observations are consistent with the view that, at extracellular concentrations of tryptophan of 0.5 mM and above, flux through the kynurenine pathway is maximal.

In contrast with incubations with 0.1 mM- and 0.5 mM-tryptophan, in which rates of radioisotope release from [*benzene-ring*-U-^{14}C]- and [5-^{3}H]-tryptophan were equal, rates of ^{3}H release from 2.5 mM-L-[5-^{3}H]tryptophan were 4 times greater than total ^{14}C release from 2.5 mM-L-[*benzene-ring*-U-^{14}C]tryptophan. This difference was manifest in cell preparations from both fed and 48 h-starved rats. ^{3}H may be labilized from [5-^{3}H]tryptophan during or after oxidation of the amino acid by the kynurenine–glutarate pathway (cf. [*benzene-ring*-U-^{14}C]tryptophan), or alternatively may become incorporated into water after 5-hydroxytryptamine synthesis (Scheme 1). The possibility that the liver metabolizes high concentrations of tryptophan by both the kynurenine and 5-hydroxyindole pathways was investigated by analysis for 5-hydroxyindol-3-ylacetic acid content of diethyl ether extracts of liver cells incubated for 1 h with unlabelled 2.5 mM-tryptophan. With a fluorimetric assay method (with a sensitivity of 100 pmol), the end product of 5-hydroxytryptamine oxidation, 5-hydroxyindol-3-ylacetic acid, was undetectable.

This finding is in agreement with that of Green *et al.* (1976), who found no evidence for synthesis of either 5-hydroxytryptamine or 5-hydroxyindol-3-ylacetic acid by rat livers perfused with 1 mM-tryptophan. The assay method used for determination of 5-hydroxyindol-3-ylacetic acid cannot distinguish between indol-3-ylacetic acid and 5-hydroxyindol-3-ylacetic acid (Stoessl & Venis, 1970); since there was no measurable fluorescence increase over control tissue samples (no added tryptophan), it is concluded that hepatocytes metabolize tryptophan only by the kynurenine pathway and not by the indole or 5-hydroxyindole pathways. The failure to detect any indol-3-ylacetic acid accumulation lends further suport to the view that enzymic release of radioisotope from [*carboxy*-^{14}C]tryptophan occurs exclusively at the kynureninase step.

In the absence of metabolism of tryptophan by the 5-hydroxyindole pathway, the disparity between rates of metabolism of 2.5 mM-[5-^{3}H]- and [*benzene-*

ring-^{14}C]-tryptophan is difficult to explain. The absence of incorporation of label from [*benzene-ring*-U-^{14}C]tryptophan into non-charcoal-precipitable radioactivity (see Table 3) must contribute, in part at least, to this discrepancy. It is possible that at high intracellular concentrations of the amino acid, ^{14}C derived from the benzene ring of tryptophan is diverted into compounds that adsorb on charcoal. However, if this were the case, these compounds must be derived from a precursor pool from which hydrogen, originally in the 5-position of the benzene ring, has already been, or can readily be, eliminated. Alternatively, ^{3}H may be labilized from [5-^{3}H]-tryptophan by a novel metabolic route. It is unlikely that the difference in rates of radioisotope release from 2.5mM-[5-^{3}H]- and [*benzene-ring*-U-^{14}C]-tryptophan arise as an artifact of the assay procedures, because rates of radioisotope release from these two radioisomers at tryptophan concentrations of 0.1 and 0.5mM were equal.

Quinolinic acid formation by liver cells

Tryptophan is a potent inhibitor of gluconeogenesis in isolated rat liver cells; glucose synthesis from lactate is inhibited by about 50% in the presence of 0.1mM-tryptophan (Smith *et al.*, 1978). Studies with a range of gluconeogenic substrates and metabolites of tryptophan have indicated that the action of the amino acid is dependent on the inhibition by quinolinic acid of phosphoenolpyruvate carboxykinase (Veneziale *et al.*, 1967; Smith *et al.*, 1978). Quinolinic acid itself, however, is only inhibitory at concentrations in excess of 1mM, an observation that cannot be attributed to poor cellular penetration (Elliott *et al.*, 1977). If quinolinate alone is responsible for the inhibition by tryptophan of gluconeogenesis, then its rate of synthesis from tryptophan must be sufficiently rapid to attain intracellular concentrations greater than 1mM.

Formation of quinolinic acid is non-enzymic; it occurs by spontaneous cyclization of 2-amino-3-carboxymuconate 6-semialdehyde, a labile intermediate produced by the action of 3-hydroxyanthranilate 3,4-dioxygenase on 3-hydroxyanthranilate (Bokman & Schweigert, 1951). Alternatively, the unstable intermediate may be oxidized enzymically to acetyl-CoA and CO_2 (see Scheme 1). Theoretically, therefore, maximal rates of quinolinic acid formation by liver cell suspensions can be determined from differences between the rates of 3-hydroxyanthranilate formation and those of oxidation of 3-hydroxyanthranilate to aliphatic compounds of the glutarate pathway and CO_2. Rates of 3-hydroxyanthranilate formation and oxidation may be estimated from measurements of radioisotope release from [*carboxy*-^{14}C]tryptophan (kynureninase activity) and [*benzene-ring*-U-^{14}C]-tryptophan respectively. The rate of maximal quinolinate accumulation by liver cells from starved rats incubated with 0.5mM-tryptophan calculated from the differences in rates of total radioisotope release from [*carboxy*-^{14}C]- and [*benzene-ring*-U-^{14}C]-tryptophan was 13.60 ± 1.48nmol of quinolinate/h per mg dry wt. of cells (mean ± S.E.M., three experiments; values taken from Table 2).

Estimation of quinolinic acid synthesis by this method is dependent on three assumptions: (i) release of [^{14}C]alanine from [*carboxy*-^{14}C]tryptophan occurs exclusively by the action of kynureninase on 3-hydroxy-L-kynurenine, i.e. anthranilic acid formation is negligible; (ii) 3-hydroxyanthranilic acid does not accumulate, i.e. 3-hydroxyanthranilate 3,4-dioxygenase activity is much greater than that of kynureninase; (iii) picolinic acid formation is negligible.

The validity of these assumptions was resolved by direct quantification of the quinolinic acid formation by liver cell suspensions incubated for 1h with unlabelled 0.5mM-tryptophan.

Currently available methods for the isolation of quinolinic acid from animal tissues and fluids include charcoal adsorption (McDaniel *et al.*, 1972*a*) and ion-exchange procedures (Toseland, 1969). Subsequently, quinolinate may be quantified colorimetrically (McDaniel *et al.*, 1972*b*) or by g.l.c. (Toseland, 1969; Altschuler & Gold, 1971). For isolation of quinolinic acid from hepatocyte incubations, we used a combination of ion-exchange and charcoal-adsorption methods; the latter step was included to avoid practical difficulties encountered in the freeze-drying of strongly acidic solutions. However, commercial supplies of Norit A charcoal contain impurities that are eluted by ethanol/ammonia/water mixtures and that may interfere with the subsequent determination of quinolinate (S. A. Smith, unpublished work). It is therefore recommended that before use the charcoal is purified by thorough washing with ethanol/ammonia (sp.gr. 0.880)/water (10:1:9, by vol.). Because of the small amounts of quinolinate involved, these methods require use of large quantities of biological material; this necessitated incubations containing up to 100mg dry wt. of tissue.

Quinolinic acid was measured by g.l.c. analysis, because this method is considerably more sensitive than the colorimetric procedure of McDaniel *et al.* (1972*b*).

There was no significant difference between rates of quinolinic acid formation derived either from radioisotope data or by direct measurement. Quinolinate production (estimated by g.l.c. analysis of extracts of liver cells from starved rats incubated for 1h with 0.5mM-tryptophan) was 11.50 ± 2.90nmol of quinolinate/mg dry wt. of cells (mean ± S.E.M. for three separate cell batches). The

endogenous rate of quinolinate production (no added tryptophan) was 0.14 ± 0.05 (mean $\pm$ S.E.M., three determinations) nmol of quinolinate/h per mg dry wt. of cells.

The equivalence of the two methods therefore substantiates the assumptions made in the calculation of quinolinate synthesis from radioisotopic data. In the rat the urinary excretion of anthranilate and picolinate (whose formation would lead to an overestimation of quinolinate accumulation) is negligible compared with that of quinolinate (Yeh & Brown, 1977). The hepatic activity of 3-hydroxyanthranilate 3,4-dioxygenase is about 100 times greater that that of kynureninase (Ikeda *et al.*, 1965). Similarly, further metabolism of quinolinate to intermediates of the NAD pathway does not occur to any measurable extent, otherwise the measured quinolinate accumulation would be less than that predicted from radioisotopic data; this hypothesis has been validated experimentally (Elliott *et al.*, 1977).

The measured rate of synthesis of quinolinate from 0.5 mM-tryptophan was about 12 nmol/h per mg dry wt. of cells. Assuming a dry-weight/wet-weight conversion factor of 3.7 (Krebs *et al.*, 1974) and that 1 g of cells contains 0.7 ml of intracellular water (Elliott *et al.*, 1977), this would yield an intracellular quinolinate concentration of 4.4 mM. This concentration is greater than that required (0.1 or 0.5 mM, depending on whether ferrous quinolinate or free quinolinate is the inhibitory species) for half-maximal inhibition of phosphoenolpyruvate carboxykinase *in vitro* (McDaniel *et al.*, 1972*b*). The liver-cell membrane is relatively permeable to extracellular quinolinate (Elliott *et al.*, 1977). Since the kinetics of efflux of endogenous quinolinate from the hepatocyte are unknown, it is not possible without direct measurement of intracellular quinolinate to determine whether these intracellular concentrations are indeed achieved. Nevertheless, it is probable that synthesis and accumulation of quinolinate from low concentrations of tryptophan occur at rates sufficiently rapid to inhibit gluconeogenesis.

General discussion

Determination of enzymic flux from rates of metabolism of radiolabelled substrates will accurately reflect net carbon-flux rates only if the specific radioactivities of the intracellular and extracellular pools are equal and are not significantly decreased by dilution with endogenous unlabelled substrate. We have demonstrated previously that, under the experimental conditions described, changes in the extracellular tryptophan concentration are accompanied by equivalent changes in the intracellular concentration of the amino acid (Smith & Pogson, 1980). Over the range of tryptophan concentrations used, radiolabelled tryptophan equilibrates rapidly (in less than 10 min) between the extracellular and intracellular compartments. In addition, the rapid rate of turnover of the intracellular tryptophan pool (about 1 min) implies that dilution of the specific radioactivity of the intracellular pool of radiolabelled tryptophan with unlabelled tryptophan is unlikely to be a source of error.

From the patterns of radioisotope release from the four radioisomers of tryptophan used in the experiments described in this paper, it is clear that tryptophan is catabolized by isolated rat liver-cell preparations exclusively by the kynurenine–glutarate pathway. Although cell-free extracts of liver are capable of hydroxylating high concentrations of tryptophan (a reaction catalysed by phenylalanine hydroxylase; Renson *et al.*,1966), this is unlikely to be a physiologically important pathway of tryptophan catabolism in the liver, there being no evidence of 5-hydroxyindol-3-ylacetate formation by liver cells incubated with 2.5 mM-tryptophan. Similarly, despite the presence of the necessary enzymic complement for the oxidation of tryptophan to indol-3-ylacetic acid (Weissbach *et al.*, 1959; Chen *et al.*, 1974), metabolism of the amino acid by this route does not occur in intact liver cell preparations. Indol-3-ylacetic acid was not detectable in liver cell suspensions incubated with tryptophan at concentrations up to 2.5 mM. It is unlikely therefore that indol-3-ylacetic acid, a normal metabolite in urine (Chen *et al.*, 1974), is produced by the liver of the intact animal. The gut microflora probably play a key role in the production of indol-3-ylacetic acid from dietary tryptophan; normal rats excrete considerably more indol-3-ylacetic acid after administration of an L-tryptophan load than do germ-free rats (Chen *et al.*, 1974).

Flux through tryptophan 2,3-dioxygenase will be determined both by availability of tryptophan from the incubation medium and by the prevailing cellular activity of tryptophan 2,3-dioxygenase. The K_m for tryptophan of tryptophan 2,3-dioxygenase (from measurements *in vitro*) is about 0.1 mM (Feigelson & Greengard, 1962). On simple kinetic considerations one would therefore predict that, on raising the substrate concentration from a physiological value of 0.1 to 0.5 mM, tryptophan 2,3-dioxygenase flux would increase. This was indeed found to be the case; a 5-fold increase in substrate concentration produced a 3- and 6-fold increase in dioxygenase flux in liver cells from fed and starved rats respectively (Tables 1 and 2). At concentrations of tryptophan of 0.5 mM and above, the enzyme is virtually substrate-saturated; a rise in tryptophan concentration to 2.5 mM elicited no further increase in the rate of tryptophan oxidation. The activity of tryptophan 2,3-dioxygenase is regulated by the interconversion between inactive apoenzyme and

catalytically active holoenzyme forms (Knox & Piras, 1967). Conjugation of apoenzyme with its haem cofactor to form the holoenzyme is stimulated by tryptophan. Linearity of rates of radioisotope release from L-[*ring*-2-^{14}C]tryptophan with time suggests that in liver cell preparations tryptophan 2,3-dioxygenase exists entirely as the holoenzyme or that rapid activation of the apoenzyme occurs.

Tryptophan 2,3-dioxygenase activity is also enhanced by corticosteroids (Schimke *et al.*, 1965) and by direct substrate stabilization (Li & Knox, 1972). Although a substrate-mediated mechanism may be of importance in the regulation of hepatic tryptophan 2,3-dioxygenase in the intact animal, we could find no evidence of this mechanism being operative in isolated liver cells; linear rates of oxidation of L-[*ring*-2-^{14}C]tryptophan were obtained in every experiment.

Availability of tryptophan to the hepatocyte will also influence directly the rate of tryptophan catabolism; this will be of particular importance at low substrate concentrations, in the region of the K_m value for tryptophan of tryptophan 2,3-dioxygenase. Of the plasma amino acids, tryptophan alone exists in equilibrium between a free and an albumin-bound pool; 80–90% is normally bound to albumin, whereas the remainder circulates in free solution (McMenamy *et al.*, 1961). The rate of oxidation of low concentrations of tryptophan is known to be entirely dependent on tryptophan availability; this is determined by the concentration of, and extent of tryptophan binding to, the albumin of the incubation medium (Smith & Pogson, 1980). In our experiments, liver cells were incubated in a medium containing 2% (w/v) defatted albumin; this is about half of the normal physiological value (Yuwiler *et al.*, 1977). Hence some caution should be exercised in extrapolating rates of tryptophan oxidation measured in these experiments to those that occur *in vivo*. Another factor that might influence rates *in vivo* is the provision of other substrates. Pyruvate was used in all the experiments described here; preliminary studies with a range of alternative substrates, both singly and in mixtures (e.g. lactate and amino acids), showed that tryptophan metabolism was, perhaps surprisingly, unaffected by these variations.

To our knowledge this is the first detailed investigation that provides quantitative and simultaneous measurements of carbon fluxes through different portions of the kynurenine pathway of L-tryptophan metabolism in isolated intact liver preparations. The procedures described will be of use for determining the influence of hormones, pharmacological agents and other factors on the oxidation of tryptophan by the liver.

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