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The Metabolism of L-Tryptophan by Isolated Rat Liver Cells

EFFECT OF ALBUMIN BINDING AND AMINO ACID COMPETITION ON OXIDATION OF TRYPTOPHAN BY TRYPTOPHAN 2,3-DIOXYGENASE

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1. Novel methods, using L-[*ring*-2-^{14}C]tryptophan, are described for the measurement of tryptophan 2,3-dioxygenase activity and tryptophan accumulation in isolated rat liver cells. 2. The effects of bovine serum albumin, non-esterified fatty acids and neutral amino acids on tryptophan oxidation by hepatocytes and on the partition of tryptophan between free and albumin-bound forms were investigated. 3. Oxidation of physiological concentrations (0.1 mM) of tryptophan was inhibited by approx. 50% in the presence of 2% (w/v) bovine serum albumin; no effects were found at tryptophan concentrations of 0.5 mM and above. 4. Increases in free tryptophan concentrations produced by displacement of 0.1 mM-tryptophan from albumin-binding sites by palmitate resulted in increased flux through tryptophan dioxygenase. 5. Addition of a mixture of neutral amino acids, at plasma concentrations, to hepatocyte incubations had no effect on the rate of tryptophan oxidation. 6. It is concluded that alterations in free tryptophan concentrations consequent to changes in albumin binding may be an important factor in regulating tryptophan uptake and catabolism by the liver. The results are briefly discussed with reference to possible consequences on brain tryptophan metabolism.

It is now well established that synthesis of 5-hydroxytryptamine within serotoninergic neurons of the brain is dependent upon the concentration of brain tryptophan. This phenomenon derives from the finding that tryptophan hydroxylase [L-tryptophan tetrahydropteridine–O_2 oxidoreductase (5-hydroxylating), EC 1.14.16.4], the rate-limiting enzyme of the pathway of 5-hydroxytryptamine synthesis, is, under normal physiological conditions, not fully saturated with tryptophan (Friedman *et al.*, 1972). Since all brain tryptophan must be derived from the plasma pool, the mechanisms that govern the availability of blood tryptophan to the brain, and hence 5-hydroxytryptamine synthesis, have been intensively studied. Although there is still controversy over the relative importance of each proposed determinant of brain tryptophan availability, it is clear, at least, that several distinct factors are involved.

Tryptophan uptake by the brain does not occur by a process of simple diffusion; transport of tryptophan and other neutral amino acids is mediated by a saturable carrier system (Blasberg & Lajtha, 1965; Pardridge, 1977). Competition between tryptophan and these neutral amino acids (tyrosine, phenylalanine, leucine, isoleucine and valine) has been proposed as the most important determinant of brain tryptophan concentrations under physiological conditions (Fernstrom & Wurtman, 1972; Wurtman, 1978).

In addition, tryptophan, unlike other amino acids in plasma, exists in equilibrium between a free and an albumin-bound pool; 80–90% is normally bound to albumin, while the remainder circulates in free solution (McMenamy *et al.*, 1961). It has been suggested that only the free tryptophan pool determines the supply of the amino acid to the brain and other tissues (Knott & Curzon, 1972). Hence factors that alter the binding of tryptophan to plasma albumin, such as non-esterified fatty acids (Curzon *et al.*, 1973), clofibrate (Curzon & Knott, 1974), salicylates (McArthur & Dawkins, 1969) and phenothiazones (Bender & Cockcroft, 1977), may indirectly alter the rate of 5-hydroxytryptamine synthesis through changes in free tryptophan concentrations. There is some evidence from recent work, however, that indicates that, in the intact

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animal, both free and albumin-bound tryptophan may be available to the brain (Madras *et al.*, 1974; Yuwiler *et al.*, 1977).

Little attention has been given to the possible consequences on brain 5-hydroxytryptamine metabolism that may result from changes in the utilization of plasma tryptophan by other tissues (for a review, see Badawy, 1977). It is now accepted that, quantitatively, by far the most important route of tryptophan metabolism is oxidation by hepatic tryptophan 2,3-dioxygenase]L-tryptophan–O_2 2,3-oxidoreductase (decyclizing), EC 1.13.11.11] to kynurenine and subsequently to acetyl-CoA or NAD (Young *et al.*, 1978). Thus the plasma concentration of tryptophan will be a reflection of its rate of uptake and catabolism by the liver. Changes in the activity of tryptophan dioxygenase (the rate-limiting enzyme of the kynurenine pathway) may indirectly influence the synthesis of 5-hydroxytryptamine by altering the availability of circulating tryptophan to the brain. The possibility of an inverse relationship between liver tryptophan dioxygenase activity and brain 5-hydroxytryptamine synthesis is supported by the findings that: (i) elevated tryptophan dioxygenase activities (produced by cortisol or acute ethanol administration) lowers both plasma tryptophan and brain 5-hydroxytryptamine turnover (Green & Curzon, 1968; Joseph *et al.*, 1976; Badawy & Evans, 1976); (ii) chronic ethanol administration, which inhibits tryptophan dioxygenase activity, elevates both plasma tryptophan concentrations and brain 5-hydroxytryptamine synthesis (Badawy *et al.*, 1979).

However, no direct measurements have been made of the influence on hepatic tryptophan catabolism of those factors that directly regulate the availability of exogenous tryptophan to the brain. In the present study, we have developed a new method for determining tryptophan dioxygenase flux and have examined the influence of albumin concentration, non-esterified fatty acids and a neutral amino acid mixture on the rate of oxidation of tryptophan in isolated rat hepatocytes. The results are discussed with reference to possible effects on brain tryptophan metabolism.

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. All animals were deprived of food for 48h before preparation of hepatocytes.

Chemicals

L-Tryptophan, inulin, palmitic acid (sodium salt), glucose oxidase (type II), peroxidase and L-lactic acid were from Sigma. Norit GSX was from Norit Clydesdale Co., Glasgow, Scotland, U.K. PCS solubilizer and Cocktail 'T' scintillation fluid were obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. Bovine serum albumin (fraction V; Miles Laboratories, Slough, Berks., U.K.) was freed of fatty acids and other materials by the method of Chen (1967). DL-[*ring*-2-^{14}C]Tryptophan (sp. radioactivity 35Ci/mol) was obtained from Schwarz/Mann, Orangeburg, NY, U.S.A., and was resolved optically by affinity chromatography on bovine serum albumin–Sepharose 4B as described by Stewart & Doherty (1973). [^{3}H]Inulin (sp. radioactivity 300Ci/mol), [^{14}C]formate (sp. radioactivity 50Ci/mol) and L-[5-^{3}H]tryptophan (sp. radioactivity 30Ci/mmol) came from The Radiochemical Centre. L-[5-^{3}H]Tryptophan was purified on bovine serum albumin–Sepharose 4B before use. All other enzymes and biochemicals were the products of Boehringer Corp. (London) Ltd. Other basic chemicals were of the purest grade available from standard suppliers.

Liver cell preparation

Isolated liver cells were prepared from rats essentially by the procedure described earlier (Elliott *et al.*, 1976), except that albumin-free Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) was used throughout the isolation and washing procedure. Metabolic integrity was assessed by standard criteria (Smith *et al.*, 1978).

Cell incubation conditions and assay procedures

(*i*) *Measurement of* L-*tryptophan oxidation.* Portions (0.3ml) of cell suspension (containing 4–6mg dry wt. of tissue) were added to 1.7ml of Krebs–Henseleit bicarbonate buffer, supplemented with the appropriate concentration of bovine serum albumin and gassed with O_2/CO_2 (19:1), in 10ml conical flasks (Kontes Glass Co., Vineland, NJ, U.S.A.). The flasks were closed with rubber stoppers from which were suspended disposable polypropylene centre wells. Incubations, in triplicate, were carried out under an atmosphere of O_2/CO_2 (19:1) at 37°C; the vials were shaken at 100 oscillations/min in Dubnoff-type shaking water baths (Mickle Engineering, Gomshall, Surrey, U.K.). Substrates and L-[*ring*-2-^{14}C]tryptophan were added by syringe injection. Incubations were terminated by injection of 0.2ml of 2M-$HClO_4$ into each flask. 2-Phenethylamine/methanol (1:1, v/v; 0.25ml) was injected into the centre well, which contained a piece of folded filter paper. Both additions were made through a septum in the stopper. All flasks were then shaken at 75 oscillations/min at room temperature (18–20°C) for a further 1h to ensure complete absorption of CO_2. The wells were removed and

added directly to vials containing 5ml of Cocktail 'T' scintillation fluid for counting of ^{14}C radioactivity. Precipitated protein was removed by centrifugation (1200*g*, 5min), and portions of the supernatant were analysed for glucose (Krebs *et al.*, 1963) or [^{14}C]formate content. [^{14}C]Formate release from L-[*ring*-2-^{14}C]tryptophan was determined as follows: 1.0ml samples of acid cell extracts were treated with 0.2ml of a 50mg/ml suspension of Norit GSX charcoal in water. After thorough mixing, the charcoal was removed by centrifugation (10000*g*, 2min), and 0.5ml of the supernatant was counted for [^{14}C]formate radioactivity in PCS scintillator 'cocktail'. Under these experimental conditions, binding of unmetabolized L-[*ring*-2-^{14}C]tryptophan by charcoal was quantitative (determined in preliminary experiments in which [^{14}C]tryptophan was added to acid cell extracts; results not shown). The possibility that formate may bind to charcoal was excluded by determining the recovery of added [^{14}C]formate (10μmol, 0.1μCi) from acid cell extracts treated with charcoal.

(*ii*) *Measurement of* L-[*ring-2-*^{14}C]*tryptophan accumulation by liver cells.* A modification of the method of Joseph *et al.* (1978) was employed. Isolated cells (12–16mg dry wt.) were incubated under an atmosphere of O_2/CO_2 (19:1) in 0.75ml of albumin-free Krebs–Henseleit bicarbonate buffer saturated with O_2/CO_2 (19:1) contained in 50mm × 15mm silicone-treated glass vials. Incubation vessels (37°C) were shaken at 100 oscillations/min for 10min before addition of L-[*ring*-2-^{14}C]tryptophan (40nCi). At intervals after tryptophan addition, duplicate samples (0.25ml) of cell suspension were removed and cells separated from medium by centrifugation through silicone oil (AR200/DC550, 4:1, sp. gravity 1.02) into 0.05ml of 0.6M-$HClO_4$. ^{14}C radioactivity was determined in 0.1ml of the medium fraction with PCS scintillator 'cocktail'. After removal of the remaining medium and silicone oil by aspiration, the cell pellet was resuspended, and precipitated protein removed by centrifugation (12000*g*, 2min). Two 0.02ml samples of supernatant were removed. The first was counted in PCS scintillator 'cocktail' to measure total ^{14}C content (the sum of [^{14}C]tryptophan and [^{14}C]formate). To determine the amount of [^{14}C]formate present in the cell pellet, 0.02ml of an aqueous 10mg/ml suspension of Norit GSX charcoal was added to the second supernatant sample. After thorough mixing, the charcoal was removed by centrifugation (1200*g*, 2min) and the ^{14}C content of the supernatant determined as above. Contamination of the cell pellet with incubation medium was calculated from the 'carry-down' of [^{3}H]inulin (sp. radioactivity, 0.25μCi/mg), which was added to incubations immediately before cell separation. The contamination of cell pellets with radioactivity from adhering medium was less than 5%. Values for tryptophan accumulation quoted in the text have been appropriately corrected.

(*iii*) *Determination of endogenous intracellular tryptophan concentrations.* Samples (1ml) of freshly prepared isolated cell suspensions (containing 15–20mg dry wt. of tissue) were centrifuged at 50*g* for 2min, and 0.5ml of the supernatant was removed and acidified with 0.05ml of 7% (w/v) trichloroacetic acid. The remainder of the medium was removed by aspiration and the cell pellet was deproteinized with 1ml of 7% (w/v) trichloroacetic acid. After centrifugation (10000*g*, 2min), tryptophan was assayed in the medium and cell extracts by the method of Denckla & Dewey (1967) as modified by Bloxam & Warren (1974).

Determination of L-*tryptophan binding to bovine serum albumin*

The binding of L-tryptophan (0.1–2.5mM) to defatted bovine serum albumin was measured by equilibrium dialysis (Lipsett *et al.*, 1973). Portions (2ml) of Krebs–Henseleit bicarbonate buffer (saturated with O_2/CO_2, 19:1) supplemented with defatted bovine serum albumin (1–6%, w/v) and pre-equilibrated with L-tryptophan (containing 10nCi of L-[5-^{3}H]tryptophan) were dialysed against 0.5ml of albumin-free Krebs–Henseleit bicarbonate buffer, with shaking at 37°C under an atmosphere of O_2/CO_2 (19:1), for 3½h. Samples of the stock tryptophan solutions and from the non-protein compartment were counted for ^{3}H radioactivity in PCS scintillator 'cocktail'. Albumin-bound tryptophan was calculated by difference.

In experiments in which the effect of palmitic acid on the binding of tryptophan by albumin was determined, sodium palmitate was added to Krebs–Henseleit bicarbonate buffer containing 2% (w/v) bovine serum albumin pre-equilibrated with 0.1mM-L-tryptophan (containing 10nCi of L-[5-^{3}H]tryptophan) by the method of Garland & Randle (1964). Free and albumin-bound tryptophan were then determined by equilibrium dialysis as described above. All determinations were performed in triplicate; for each, the S.D. values were less than 2% and have been omitted from the text.

Binding ratios have been calculated by assuming a mol.wt. for bovine serum albumin of 69000 (McMenamy & Oncley, 1958).

Results and Discussion

Accumulation of L-*tryptophan by isolated hepatocytes*

Before examining the effects of altered tryptophan availability on the tryptophan dioxygenase activity of liver cells, it was necessary to determine the effect of tryptophan concentration on the

transport and distribution of the amino acid between intracellular and extracellular compartments. Table 1 shows the time course of the intracellular accumulation of [^{14}C]tryptophan after the addition of 0.1mM-, 0.5mM- or 2.5mM-L-[*ring*-2-^{14}C]tryptophan to hepatocyte suspensions. Within 10min of addition of either 0.1mM- or 0.5mM-tryptophan there was equilibration of [^{14}C]tryptophan between the extracellular and intracellular pools. With the highest concentration used (2.5mM) equilibration was incomplete; the intracellular concentration had reached 1.8mM 10min after tryptophan addition.

It is not possible to study the transport of tryptophan by isolated cells under conditions where no net metabolism occurs or where metabolism can be specifically inhibited [as for α-aminoisobutyrate (Fehlmann *et al.*, 1979) and alanine, serine and glutamine (Joseph *et al.*, 1978)], because there are no specific potent inhibitors of tryptophan dioxygenase. For this reason, it is also impossible to measure accumulation over time periods greater than 10min, because a significant proportion of added tryptophan is metabolized. It must be emphasized therefore that the values quoted in Table 1 represent the net accumulation of [^{14}C]tryptophan within the cell and reflect the differences between the rate of transport of the amino acid and its subsequent metabolism.

In this series of experiments, bovine serum albumin was omitted from the incubation medium, since the relatively slow (5min) equilibration of tryptophan with albumin (McMenamy *et al.*, 1961) would seriously distort uptake kinetics.

The kinetics of tryptophan accumulation observed here bear a striking resemblance to those obtained for the uptake of valine by similar liver cell suspensions (Seglen & Solheim, 1978). Hepatocyte valine transport occurs by a high-K_m facilitated diffusion-type system, which works equally well in both directions; there is no evidence for active uptake. Earlier experiments with perfused livers have also shown that under conditions of net tryptophan oxidation there is no net accumulation of the amino acid (Ng *et al.*, 1970; Green *et al.*, 1976). The data presented in Table 1 therefore show that transport of tryptophan occurs at rates that result in rapid equilibration of the intracellular and extracellular pools. Hence it may be predicted that changes in intracellular and extracellular tryptophan concentrations will be co-variant. It is unlikely that membrane transport will be the rate-limiting factor in tryptophan catabolism.

Use of L-[*ring*-2-^{14}C]*tryptophan as a measure of flux through tryptophan dioxygenase*

Several methods have been used to measure tryptophan oxidation by tryptophan dioxygenase in intact isolated liver preparations. These include measurement of rates of removal of added tryptophan from the medium by perfused livers (Ng *et al.*, 1970; Green *et al.*, 1976) or appearance of specific metabolites, such as kynurenine (Green *et al.*, 1976). Both of these methods have inherent disadvantages. First, determination of tryptophan disappearance from the perfusate is tedious, and accurate measurement often is not feasible, particularly when high concentrations of the amino acid are used. Second, estimation of kynurenine production may not be a reliable index of tryptophan dioxygenase flux, since changes in enzyme activity may be masked by parallel changes in kynurenine metabolism.

$^{14}CO_2$ production from DL-[*ring*-2-^{14}C]tryptophan has been employed as a determinant of tryptophan dioxygenase activity in both whole animals (Young *et al.*, 1974; Joseph *et al.*, 1976) and isolated liver preparations (Ng *et al.*, 1970). Tryptophan dioxy-

Table 1. *Accumulation of* L-[*ring*-2-^{14}C]*tryptophan by isolated rat liver cells*

Cells were incubated in albumin-free Krebs–Henseleit bicarbonate buffer for 10min before addition of L-[*ring*-^{14}C]-tryptophan (40nCi). Cells were separated from incubation medium at each time point by centrifugation through silicone oil as described in the Materials and Methods section. All values are corrected for cross-contamination of cells with medium (from [^{3}H]inulin measurements) and are the means of paired observations from two separate batches of cells. These agreed within a range of ±10%. Calculations are based on the assumption that 1g of cells contains 700μl of intracellular water.

Extracellular tryptophan concentration (mM) ...	0.1		0.5		2.5	
Time after tryptophan addition (min)	[^{14}C]Tryptophan in cells (mM)	Concentration ratio (medium/cells)	[^{14}C]Tryptophan in cells (mM)	Concentration ratio (medium/cells)	[^{14}C]Tryptophan in cells (mM)	Concentration ratio (medium/cells)
1	0.049	2.0	0.16	3.1	0.73	3.4
2	0.057	1.8	0.17	2.9	0.99	2.5
5	0.060	1.7	0.31	1.6	1.51	1.7
10	0.089	1.1	0.49	1.0	1.76	1.4

genase catalyses the oxidative cleavage of the pyrrole moiety of tryptophan; the carbon at position 2 appears in the formyl group of *N*-formylkynurenine. *N*-Formylkynurenine is rapidly deformylated by a broad-specificity high-activity formamidase (EC 3.5.1.9) (Mehler & Knox, 1950). CO_2 is released from the subsequent oxidation of formate. Unfortunately, all previous determinations of tryptophan dioxygenase activity made with [*ring*-2-^{14}C]tryptophan have involved the use of a racemic mixture of D- and L-isomers. D-Tryptophan is metabolized by the liver, but its rate of uptake and metabolism is considerably lower than those of the L-isomer (Ng *et al.*, 1970; Rodden & Berg, 1974). Consequently, the metabolic profile of the pool of D-tryptophan (of potentially high specific radioactivity) will be superimposed upon that of the natural isomer. We have eliminated this problem by using only L-[*ring*-2-^{14}C]tryptophan.

In addition, determination only of $^{14}CO_2$ release from L-[*ring*-2-^{14}C]tryptophan will not give a quantitative index of tryptophan dioxygenase activity, since only a fraction of [^{14}C]formate is oxidized to $^{14}CO_2$ (Young & Sourkes, 1975). Hence, to measure total flux through tryptophan dioxygenase it is necessary to determine $^{14}CO_2$ and [^{14}C]formate (plus products derived therefrom). Formate enters the C_1 pool, from which it may participate in C_1-acceptor reactions [e.g. for purine biosynthesis (Letter *et al.*, 1973)], or exchange with C_1 donors (e.g. serine), or be oxidized to CO_2 (Krebs *et al.*, 1976). Under the conditions used in all our experiments we could not detect any incorporation of label from L-[*ring*-2-^{14}C]tryptophan either into protein or adenine nucleotides (S. A. Smith, unpublished work). The rate of ^{14}C release from L-[*ring*-2-^{14}C]tryptophan was linear with time under all conditions, and the values quoted represent the sum of [^{14}C]formate, ^{14}C-labelled aliphatic amino acids (measured in charcoal-treated acid extracts; see above) and $^{14}CO_2$.

Use of L-[*ring*-2-^{14}C]tryptophan to measure total tryptophan dioxygenase flux is only valid if the specific radioactivity of the intracellular tryptophan pool is not significantly diluted by endogenous unlabelled tryptophan. The endogenous tryptophan content of freshly prepared liver cells was 0.058 ± 0.010 nmol of tryptophan/mg dry wt. of cells (three observations; ±S.E.M.). This is equivalent to an intracellular concentration of $23 \pm 4\,\mu$M and is comparable with that measured in livers perfused with a tryptophan-free medium (Green *et al.*, 1976). As discussed by Krebs *et al.* (1976), amino acids are lost from hepatocytes during the isolation procedure; on incubation for 1 h at 37°C in the absence of added substrates, however, the intracellular tryptophan content rose 3-fold to 0.18 ± 0.02 nmol of tryptophan/mg dry wt. of cells (three observations; ±S.E.M.), equivalent to 65 μM, presumably because of proteolysis. In both freshly prepared and incubated hepatocytes there was a concentration gradient of tryptophan between cells and medium (cells/medium ratio about 10:1). In absolute terms, however, because the volume of incubation medium is about 50 times that of the cells, only 20% of total tryptophan is intracellular.

The rate of oxidation of a low physiological concentration (0.1 mM) of L-[*ring*-2-^{14}C]tryptophan in hepatocytes from starved rats (in the absence of added albumin) was 9.4 nmol of tryptophan/h per mg dry wt. of cells (from Fig. 1*b*). This indicates that the turnover of the intracellular tryptophan pool is rapid (about 1 min), which, together with the finding that hepatocytes rapidly accumulate [^{14}C]tryptophan from the incubation medium, suggests that dilution of the pool of [^{14}C]tryptophan with endogenous unlabelled tryptophan is unlikely to be a source of error.

The validity of this method is further supported from parallel experiments in which the tryptophan dioxygenase activity of isolated liver cell suspensions was measured by following the removal of 0.1 mM-L-tryptophan from the incubation medium using a fluorimetric assay for the amino acid. Rates of tryptophan metabolism determined from either radioisotopic or fluorimetric measurements agreed within 5% (results not shown).

Effect of bovine serum albumin concentration on L-tryptophan binding and on the metabolism of tryptophan by isolated rat liver cells

Table 2 shows the distribution of tryptophan between free and albumin-bound pools as a function of both tryptophan and bovine serum albumin concentrations. Some of the data from Table 2 are reproduced in Fig. 1(*a*) for comparative purposes. With 0.1 mM-L-tryptophan, a concentration within the normal physiological range for plasma tryptophan, maximum binding was observed at an albumin concentration of 3%; increasing the albumin concentration to 6% did not significantly increase the amount of tryptophan bound.

Tryptophan binding to albumin is a reversible equilibrium process. On simple mass-action considerations, increasing the albumin concentration should decrease the proportion of free ligand. This discrepancy has been noted previously (Bowman & Lindup, 1978) and may arise from protein–protein interactions that serve to decrease binding-site availability and/or affinity as the albumin concentration increases. Under conditions of maximum binding, 80% of tryptophan is albumin-bound, resulting in a free tryptophan concentration of 0.02 mM. The effect of albumin addition to the incubation medium on the rate of oxidation of 0.1 mM-L-[*ring*-2-^{14}C]tryptophan by isolated liver

Table 2. *Binding of L-tryptophan to bovine serum albumin*

The binding of L-tryptophan to fatty acid-free bovine serum albumin in Krebs–Henseleit bicarbonate buffer (pH 7.4) was determined at 37°C by equilibrium dialysis as described in the Materials and Methods section. Values are the means of three separate determinations; standard errors were within 2% and have not been included.

Bovine serum albumin concentration (%, w/v)	Tryptophan concentration (mM) ... 0.1			0.5			2.5		
	Bound tryptophan (% of total)	Free tryptophan (mM)	Molar ratio bound tryptophan/albumin	Bound tryptophan (% of total)	Free tryptophan (mM)	Molar ratio bound tryptophan/albumin	Bound tryptophan (% of total)	Free tryptophan (mM)	Molar ratio bound tryptophan/albumin
0	0	0.100	—	0	0.50	—	0	2.50	—
1	56	0.044	0.39	26	0.36	0.90	11	2.23	1.90
2	73	0.027	0.25	41	0.28	0.70	14	2.15	1.53
3	79	0.021	0.18	55	0.23	0.65	16	2.10	0.88
4	83	0.016	0.14	65	0.18	0.55	20	2.00	0.88
5	80	0.020	0.11	66	0.17	0.45	28	1.80	0.96
6	80	0.020	0.09	71	0.15	0.40	33	1.68	0.94

Fig. 1. *Binding of* L-*tryptophan to bovine serum albumin* (*a*) *and effect of bovine serum albumin concentration on metabolism of* L-[*ring*-2-^{14}C]*tryptophan by hepatocytes from starved rats* (*b*)

(*a*) Free tryptophan concentrations (expressed as percentages of total tryptophan) measured as functions of bovine serum albumin concentrations have been taken from Table 2. (*b*) Cells were pre-incubated in Krebs–Henseleit bicarbonate buffer containing the appropriate concentration of bovine serum albumin for 10 min before addition of pyruvate (final concentration 10 mM); 20 min later L-[*ring*-2-^{14}C]tryptophan was added. Incubations were stopped 1 h after tryptophan addition. Rates of tryptophan oxidation at each albumin concentration are expressed as percentages of the rate in the absence of added albumin. The rates of metabolism of 0.1 mM, 0.5 mM and 2.5 mM-tryptophan measured in the absence of added albumin were 9.4 ± 0.6, 19.4 ± 3.3 and 31.9 ± 4.9 nmol of tryptophan/h per mg dry wt. of cells respectively and are the means ± S.E.M. from three separate experiments. ●, 0.1 mM-tryptophan; ■, 0.5 mM-tryptophan; ▲, 2.5 mM-tryptophan.

cells is shown in Fig. 1(*b*). In the presence of 1% bovine serum albumin, a 20% ($P < 0.05$) decrease in tryptophan dioxygenase flux was observed; this inhibition rose to 75% with 6% albumin. Hepatocyte preparations metabolized 0.1 mM-tryptophan at

a greater rate (about double) than did perfused liver preparations (Green *et al.*, 1976), although direct comparisons are not possible because of differences in the albumin (and hence free tryptophan) concentration used.

It should be noted, however, that, although changes in free tryptophan concentrations produced as a result of altered albumin binding had marked effects on tryptophan dioxygenase flux, the two phenomena were not simply quantitatively correlated (Figs. 1*a* and 1*b*). For example, the addition of 1% bovine serum albumin decreased the free tryptophan concentration by 55% from 0.1 to 0.45mM, but only inhibited the rate of tryptophan oxidation by about 20%. The K_m for tryptophan of tryptophan dioxygenase (from measurements *in vitro*) is about 0.1mM (Feigelson & Greengard, 1962). Assuming that Michaelis–Menten kinetics obtain, a decrease in intracellular substrate concentration from 0.1 to 0.45mM should, theoretically, decrease enzymic flux by about 40%. The measured decrease was 22%. It is possible that at low external concentrations, tryptophan is concentrated within the cell; from our measurements of accumulation made with a wide range of tryptophan concentrations we could, however, find no evidence of concentrative uptake.

In addition, tryptophan dioxygenase flux changes were observed in the absence of changes in free tryptophan concentrations. When the albumin concentration of the incubation medium was raised from 3 to 6%, there was a progressive decrease in dioxygenase activity from 44 to 23% (expressed as a percentage of the rate of tryptophan oxidation measured in the absence of added albumin), despite the fact that the free tryptophan concentration remained constant at 0.02mM. It is clear therefore that the extracellular free tryptophan concentration is not the sole determinant of availability of tryptophan to hepatocyte tryptophan dioxygenase. One parameter that must be considered is the partition of tryptophan between the two competitive binding sites, i.e. between albumin and the plasma-membrane amino acid carrier. The distribution of tryptophan between these two competing systems is a function of the relative capacity/affinity ratio of each system. This may be calculated from the expression:

$$\text{Relative capacity/affinity ratio} = \frac{\text{binder concentration}}{\text{affinity constant}} \times n$$

where n is the number of tryptophan-binding sites per mol of binder (Pardridge, 1979). For transport of tryptophan across the blood–brain barrier it has clearly been demonstrated that the capacity/affinity ratio of the brain amino acid-transport system is greater than that of plasma albumin; hence large changes in free tryptophan concentrations produced as a result of albumin binding only have minimal effects on brain tryptophan uptake (Pardridge, 1977). Similarly, if the affinity of the liver transport system for tryptophan was high, then the liver cell would be able to withdraw tryptophan from the bound as well as the free pool by mass action and albumin concentration would have only a small influence on tryptophan dioxygenase flux. However, the converse appears to hold; oxidation of 0.1mM-tryptophan by hepatocytes was found to be very sensitive to changes in albumin concentration. This marked difference from the situation observed in the brain probably derives from the different affinities for tryptophan of the two amino acid-transport systems. The K_m for tryptophan of the brain transport system is approx. 0.7mM (Pardridge, 1977), whereas hepatic amino acid transport is mediated by low-affinity systems with K_m in the range 5–50mM (Joseph *et al.*, 1978; Fehlmann *et al.*, 1979).

When the initial concentration of added tryptophan was increased from 0.1 to 0.5mM, changes in albumin concentration had only a small influence on the rate of tryptophan oxidation (Fig. 1*b*). Statistically significant ($P < 0.05$) decreases in flux were only observed at albumin concentrations of 3% and above, despite the fact that under these conditions more than 50% of the tryptophan was albumin-bound (Table 2). Since the K_m of tryptophan dioxygenase for tryptophan is relatively low (0.1mM), it may be predicted that at relatively high substrate concentrations, albumin-binding effects will have only modest influences on net flux rates. This proposal is supported by the results obtained with 2.5mM-tryptophan. The rate of tryptophan oxidation was totally independent of the concentration of added albumin. At high molar ratios of tryptophan to bovine serum albumin the molar ratio of bound tryptophan to bovine serum albumin exceeded 1 (Table 2). It is generally accepted that there is one specific tryptophan-binding site per albumin molecule; binding ratios of greater than 1 have been reported previously and probably represent non-specific interactions between tryptophan and albumin (McMenamy & Oncley, 1958).

Effect of long-chain non-esterified fatty acids on albumin binding and oxidation of L-*tryptophan*

Binding of L-tryptophan and long-chain non-esterified fatty acids to plasma albumin is known to be a competitive process (Curzon *et al.*, 1974). Fatty acids may directly regulate tryptophan availability to the tissues through changes in plasma-free tryptophan concentrations. This has been proposed as an important factor in controlling brain tryptophan concentrations (Curzon & Knott, 1974). Through these changes in albumin binding, fatty

acids may also have an indirect action on brain tryptophan by influencing the rate of hepatic uptake and oxidation of tryptophan. Table 3 depicts the changes in binding of 0.1 mM-tryptophan to 2% (w/v) bovine serum albumin produced by the addition of increasing amounts of palmitate. Significant changes in free and bound tryptophan concentrations only occurred at palmitate concentrations of 0.4 mM and above. With 2.0 mM-palmitate, displacement of tryptophan from albumin was virtually complete; 95% of added tryptophan was present in free solution. As expected, addition of palmitate to hepatocytes incubated in medium containing 2% albumin produced corresponding increases in the rate of oxidation of 0.1 mM-L-[*ring*-2-^{14}C]tryptophan (Fig. 2). Because the rate of fatty acid oxidation by liver cell preparations from starved rats is high (Smith *et al.*, 1978), changes in palmitate concentrations and hence in tryptophan–fatty acid–albumin binding equilibria were minimized by reducing total incubation times to 40 min. Under these conditions, release of ^{14}C from L-[*ring*-2-^{14}C]tryptophan was linear with time. Long-chain fatty acids may have important effects on hepatic tryptophan oxidation in the intact animal, as increases in tryptophan dioxygenase flux were produced by concentrations of fatty acids within the normal physiological range. These vary between 0.2 mM in the fed rat to 1.0 mM or above in the starved and diabetic animal (Curzon *et al.*, 1973; Madras *et al.*, 1974).

Neutral amino acids and hepatic tryptophan oxidation

It has been argued that the major factor controll-

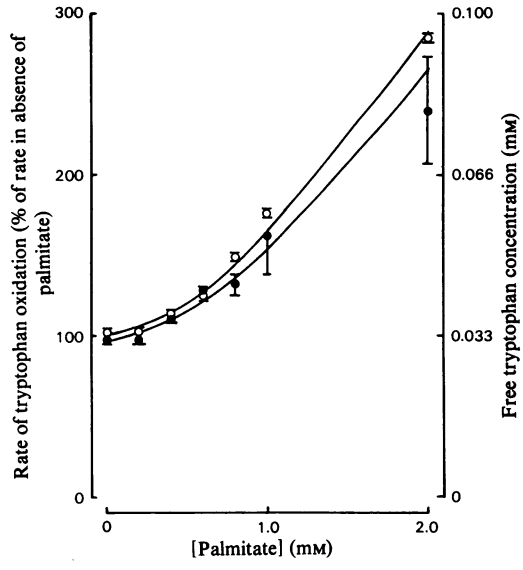

Fig. 2. *Effect of palmitate on albumin binding and metabolism of 0.1 mM-tryptophan by liver cells from starved rats*

Hepatocytes were incubated in Krebs–Henseleit bicarbonate buffer supplemented with 2% (w/v) bovine serum albumin and pre-equilibrated with palmitate for 10 min before addition of L-[*ring*-2-^{14}C]tryptophan (final concn. 0.1 mM). Incubations, in triplicate, were terminated 30 min after tryptophan addition. Rates of tryptophan metabolism are expressed as percentages of the rate in the absence of added palmitate (this rate was 1.49 ± 0.30 nmol of tryptophan/30 min per mg dry wt. of cells), and are the means ± S.E.M. from three separate cell batches. ○, Free tryptophan concentration (mM) (from Table 3); ●, rate of tryptophan oxidation.

Table 3. *Effect of palmitic acid on binding of 0.1 mM-L-tryptophan to 2% bovine serum albumin*

Krebs–Henseleit bicarbonate buffer containing 2% (w/v) fatty acid-free bovine serum albumin saturated with O_2/CO_2 (19:1) was equilibrated with 0.1 mM-L-[5-^{3}H]tryptophan for 20 min before addition of palmitate. Free and albumin-bound tryptophan were then determined by equilibrium dialysis at 37°C as described in the Materials and Methods section. The values are the means of triplicate determinations; individual values agreed within 5%.

Palmitate concentration (mM)	Molar ratio palmitate/albumin	Molar ratio palmitate/tryptophan	Bound tryptophan (% of total)	Free tryptophan concentration (mM)
0	—	—	67	0.033
0.2	0.69	2.0	66	0.034
0.4	1.38	4.0	62	0.038
0.6	2.07	6.0	58	0.042
0.8	2.76	8.0	50	0.050
1.0	3.45	10.0	41	0.059
2.0	6.90	20.0	5	0.095
4.0	13.79	40.0	5	0.095

ing brain tryptophan concentrations is competition between tryptophan and other plasma neutral amino acids for the brain amino acid carrier (Wurtman, 1978). It was of interest therefore to determine whether similar competitive effects influence hepatic tryptophan metabolism. The rate of oxidation of 0.1 mM-tryptophan by liver cell suspensions was determined in the presence or absence of a mixture of neutral amino acids of the following composition: tyrosine, 0.09 mM; phenylalanine, 0.08 mM; leucine, 0.15 mM; isoleucine, 0.13 mM; valine, 0.19 mM. These concentrations represent the normal plasma concentrations of these amino acids (Pardridge, 1977). To eliminate any possible effects of these amino acids on the tryptophan–albumin binding equilibrium, incubations were carried out in albumin-free buffer. Addition of amino acids had no effect; the rates of tryptophan oxidation by hepatocytes incubated with or without the amino acid mixture were 3.12 ± 0.24 nmol of tryptophan/30 min per mg dry wt. of cells and 3.07 ± 0.23 nmol of tryptophan/30 min per mg dry wt. of cells (means $\pm$ S.E.M., $n = 3$) respectively. Thus, in contrast with the brain, uptake and catabolism of tryptophan by the liver is unlikely to be influenced directly by neutral amino acids at normal physiological concentrations. The great sensitivity of tryptophan uptake by the brain to the competitive effects of other plasma amino acids derives from the finding that the K_m of the transport system is approximately equal to the sum of the plasma concentrations of these amino acids (Pardridge & Oldendorf, 1977). If the K_m of the transport system is much greater than plasma amino acid concentrations, which appears to be true for liver amino acid carriers, then no significant competitive effects would be seen until high amino acid concentrations are achieved.

General discussion

Control of plasma tryptophan concentrations and hence availability of the amino acid to the tissues is effected largely by hepatic uptake and subsequent metabolism of tryptophan by the kynurenine pathway. It is considered unlikely that uptake of tryptophan into the liver cell is a limiting factor, since hepatocytes were found to accumulate the amino acid rapidly and the presence of physiological concentrations of other neutral amino acids that may compete for the membrane transport system had no effects on tryptophan oxidation. Thus the major factor determining the availability of plasma tryptophan to extrahepatic tissues is the regulation of the rate at which it is oxidized by tryptophan dioxygenase in the liver.

Elaborate mechanisms exist to control tryptophan dioxygenase activity. These include enzyme induction by corticosteroids and enhancement of enzyme activity by substrate (Schimke *et al.*, 1965). Enzyme-induction phenomena may be important in long-term adaptive changes in tryptophan metabolism, but are probably not involved in the 'acute' regulation of dioxygenase activity. In rat liver homogenates, the enzyme appears to exist in two distinct forms; as a catalytically active haem-containing holoenzyme and as an inactive haem-free apoenzyme (Knox & Piras, 1967). The conjugation of apoenzyme with the haem cofactor *in vitro* is stimulated by tryptophan. The presence of tryptophan dioxygenase in rapidly interconvertible active and inactive forms would obviously confer on the liver a greater flexibility of tryptophan handling. Although administration of haematin to rats, or addition of haematin to liver homogenates increases tryptophan dioxygenase activity (Badawy & Evans, 1975), no direct experimental evidence is available to suggest that tryptophan dioxygenase exists in interconvertible forms in the intact cell. The values obtained for rates of tryptophan oxidation by hepatocyte suspensions in albumin-free media (see the legend to Fig. 1) lie within the theoretical range predicted for a single enzyme species. If tryptophan dioxygenase does exist in interconvertible forms in the intact rat liver cell, then conversion of apoenzyme to the holoenzyme must be rapid, quantitative and occur at very low tryptophan concentrations, since enzymic flux was linear with time at all tryptophan concentrations.

In isolated liver cell suspensions, changes in extracellular free tryptophan concentrations produced by changes in albumin concentration or addition of palmitate have clearly been shown to influence rates of tryptophan oxidation; these effects are manifest at physiological tryptophan concentrations. These results illustrate that, in contrast with brain (Yuwiler *et al.*, 1977), hepatic tryptophan metabolism is acutely sensitive to changes in tryptophan–albumin-binding equilibria. Elevated concentrations of non-esterified fatty acids are found in the plasma of diabetic animals, so that tryptophan is displaced from albumin-binding sites. This may be a contributory factor to the enhanced rate of hepatic tryptophan degradation, characteristic of the diabetic condition (Wolf, 1974). Other compounds that alter plasma fatty acid concentrations or directly displace tryptophan from albumin-binding sites may similarly stimulate hepatic tryptophan oxidation. Again, in contrast with the brain, liver tryptophan metabolism appears to be insensitive to the effects of amino acid competition.

The exquisite sensitivity of tryptophan dioxygenase flux to changes in extracellular free tryptophan concentrations implies that the liver can act as a 'buffer' of plasma tryptophan concentrations. Elevations of plasma free, and hence brain, concentrations of tryptophan and 5-hydroxytryptamine as a result of decreased albumin binding are

counteracted by a concomitant increase in the rate of hepatic tryptophan oxidation. The hypothesis that there exists an hepatic element in the control of brain 5-hydroxytryptamine metabolism is supported by the observation that liver dysfunction is accompanied by large increases in brain 5-hydroxytryptamine concentrations (Bloxam & Curzon, 1978).

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