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Effect of 4-hydroxypyrazole on tryptophan and formate metabolism in isolated rat liver cells

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1. 4-Hydroxypyrazole inhibits flux through tryptophan 2,3-dioxygenase in cells. The inhibition is apparently non-competitive with $K_i = 0.15$ mM. 2. Hydroxypyrazole inhibits the oxidation of formate to CO_2 in liver cells. 3. Glycollate, which generates H_2O_2, stimulates formate oxidation. This process is inhibited by 4-hydroxypyrazole. 4. Methionine stimulates formate oxidation in cells and this stimulation is insensitive to 4-hydroxypyrazole. 5. It is concluded that, in freshly isolated liver cells, formate oxidation proceeds by a pathway involving catalase. *In vivo*, or when methionine is added to cell incubations, the pathway of oxidation involves tetrahydrofolate, and is insensitive to catalase inhibitors. 6. Methionine at physiological concentrations inhibits the activity of tryptophan 2,3-dioxygenase in isolated liver cells.

Pyrazole is well-known as an inhibitor of alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase; EC 1.1.1.1) (Theorell & Yonetani, 1963). More recently, however, it has been shown *in vivo* to inhibit the two haem-containing enzymes catalase (H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6) and tryptophan 2,3-dioxygenase [L-tryptophan:oxygen 2,3-oxidoreductase (decyclizing); EC 1.13.11.11] (Lieber *et al.*, 1970; Rouach *et al.*, 1976). Inhibition of tryptophan 2,3-dioxygenase occurs also *in vitro*, but only at concentrations well in excess of those effective *in vivo* (Rouach *et al.*, 1976). This is explained by experiments showing that pyrazole is metabolized by a cytochrome *P*-450-dependent system to 4-hydroxypyrazole (Deis *et al.*, 1977*a*) and that this metabolite is *in vitro* a potent inhibitor of catalase (Deis *et al.*, 1977*b*; MacDonald & Pispa, 1980) and tryptophan 2,3-dioxygenase (Rouach *et al.*, 1979).

Fig. 1 shows the pathways of formate, tryptophan and histidine catabolism.

Formate toxicity varies between species (Gilger & Potts, 1955; McMartin *et al.*, 1975, 1977; Palese & Tephly, 1975); this variation correlates with the relative ability to metabolize this acid through the tetrahydrofolate pathway. Peroxidative oxidation mediated by catalase can also occur (Chance, 1950), but is reported to be absent from monkeys (McMartin *et al.*, 1977) and active in rats only in folate-deficient states (Palese & Tephly, 1975).

The tetrahydrofolate pathway is less active in isolated rat liver cells than *in vivo* (Krebs *et al.*, 1976; Billings & Tephly, 1979). Activity is increased to the physiological value by addition of methionine (Krebs *et al.*, 1976; Billings & Tephly, 1979; Cook & Pogson, 1981). The stimulatory effect of methionine has been explained in terms of the 'methyl-trap' hypothesis (Herbert & Das, 1976). This recognizes the stimulatory effect of *S*-adenosylmethionine on methionine synthetase (N^5-methyltetrahydrofolate:homocysteine methyltransferase; EC 2.1.1.13) (Loughlin *et al.*, 1964; Taylor & Weissbach, 1967; Mangum *et al.*, 1972) and the inhibition by the same metabolite of the physiologically-irreversible step catalysed by N^5N^{10}-methylenetetrahydrofolate reductase [5-methyltetrahydrofolate:(acceptor) oxidoreductase; EC 1.1.99.15] (Kutzbach & Stokstad, 1967, 1971). When methionine is absent, the concentration of *S*-adenosylmethionine is correspondingly very low, so that the reductase is active in promoting accumulation of N^5-methyltetrahydrofolate at the expense of free tetrahydrofolate. The decrease in tetrahydrofolate is associated with decreased one-carbon flux through the folate pool. In the presence of methionine, *S*-adenosylmethionine is formed, the reductase is inhibited and methionine synthetase activated. Consequentially, the concentration of tetrahydrofolate rises to stimulate formate metabolism. Recent work has shown that methionine synthetase may be more important than the reductase in mediating these effects of methionine (Billings *et al.*, 1981). The effect of the 'methyl trap', in the shape of an accumulation of N^5-methyltetrahydrofolate, has been seen in cases of vitamin B_{12} deficiency, and this is reversed by administration of methionine (Noronha & Silverman, 1962; Thenen

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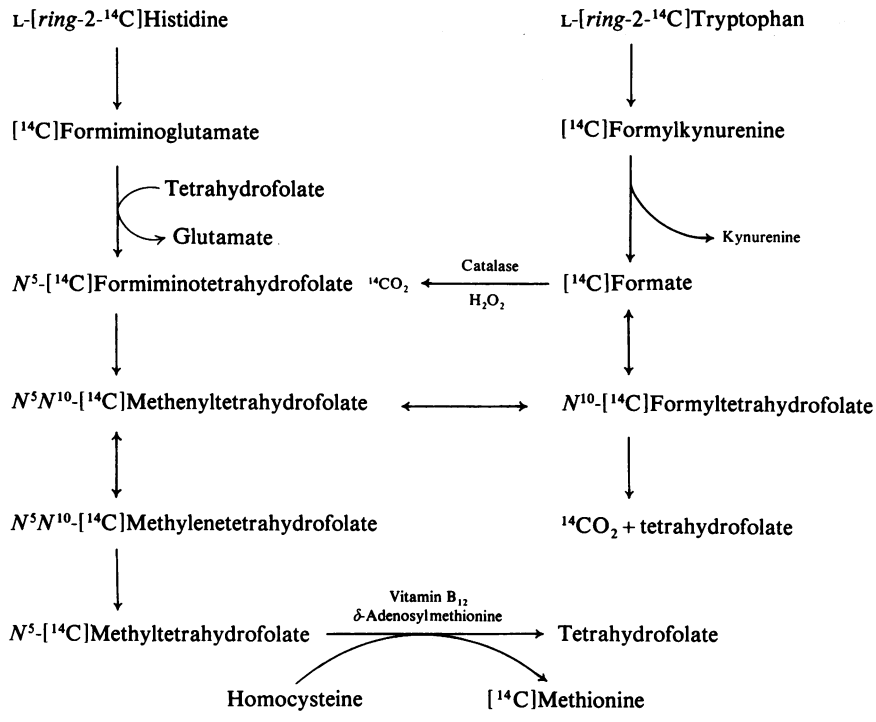

Fig. 1. *Metabolic fates of* ^{14}C *radioactivity originating from* L-[*ring*-2-^{14}C]*histidine and* L-[*ring*-2-^{14}C]*tryptophan*

et al., 1970; Vidal & Stokstad, 1974; Davidson *et al.*, 1975; Shin *et al.*, 1975; Chiao & Stokstad, 1977).

In the present paper we report the effects of 4-hydroxypyrazole on flux through tryptophan 2,3-dioxygenase and on formate metabolism and its interaction with methionine.

Materials and methods

Animals

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

Materials

L-Methionine, L-tryptophan, L-lactic acid, pyruvic acid and glycollic acid were from Sigma. 4-Hydroxypyrazole was a gift from Lilly Research Laboratories (Indianapolis, IN, U.S.A.). Norit GSX was from Clydesdale Co., Glasgow, Scotland, U.K. Bovine serum albumin (fraction V), obtained from International Enzymes, Windsor, Berks., U.K., was freed from fatty acids and other impurities as described by Chen (1967). L-[*ring*-2-^{14}C]Tryptophan (sp. radioactivity 32.5Ci/mol) was obtained from CEA, Gif-Sur-Yvette, France, and was purified by the method of Stewart & Doherty (1973). Sodium [^{14}C]formate (sp. radioactivity 39Ci/mol) was from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals, of the purest grade available, were from standard suppliers.

Preparation of isolated rat liver cells

Isolated rat liver cells were prepared as described by Elliott *et al.* (1976). Viability of cell preparations was assessed by standard criteria (Smith *et al.*, 1978).

Incubation conditions for measurement of L-*tryptophan 2,3-dioxygenase*

The methods were as described previously (Smith & Pogson, 1980, 1981; Smith *et al.*, 1980). Specific additions and treatments of incubations are described in the text. Incubation conditions and measurement of $^{14}CO_2$ production from [^{14}C]formate were similar to those for estimating $^{14}CO_2$ production from L-[*ring*-2-^{14}C]tryptophan.

Results and discussion

The effect of 4-hydroxypyrazole on tryptophan 2,3-dioxygenase in cells

Tryptophan 2,3-dioxygenase converts L-[*ring*-2-^{14}C]tryptophan into *N*-[^{14}C]formyl-L-kynurenine.

Formamidase (aryl-formylamine amidohydrolase; EC 3.5.1.9) removes the formyl group as [^{14}C]-formate. Formate may enter the one-carbon pool and act as a precursor of other cellular constituents or be oxidized to CO_2. In liver cells, there is no evidence of any appreciable formation of ^{14}C-labelled nucleotides or protein (Smith & Pogson, 1980). Charcoal treatment removes all aromatic compounds but leaves formate (and serine) in the supernatant. The activity of tryptophan 2,3-dioxygenase is obtained by adding the counts obtained in the supernatant to those in CO_2; the ratio of radioactivity counts in CO_2 to radioactivity counts in supernatant (non-aromatic product) is a measure of formate oxidation (see also Smith & Pogson, 1980; Smith *et al.*, 1980).

4-Hydroxypyrazole is a potent inhibitor of tryptophan 2,3-dioxygenase activity in rat liver cells (Table 1). Flux is inhibited by 50% at an inhibitor concentration of 0.15 mM. A given concentration of 4-hydroxypyrazole produced a similar percentage inhibition over a wide range of tryptophan concentrations. Double-reciprocal plots of data from experiments in which both substrate and inhibitor concentrations were varied resulted in a series of straight lines. These intercepted the *x*-axis at the same point (unaltered K_m), but caused an increase in the value of the intercept on the *y*-axis with increasing inhibitor concentration (decreased $V_{max.}$). These types of plot are consistent with 4-hydroxypyrazole being a non-competitive or irreversible inhibitor of tryptophan 2,3-dioxygenase. Rouach *et al.* (1979) found the inhibition in cell-free extracts was of the competitive type. This divergence between results may be explained by the known interaction of the inhibitor with haem enzymes, and by the fact that the assay medium used by Rouach *et al.* (1979) contained excess haemin.

Table 1. *The effect of 4-hydroxypyrazole on flux through tryptophan 2,3-dioxygenase in isolated rat liver cells*
Hepatocytes were incubated in Krebs–Henseleit bicarbonate buffer supplemented with 2% (w/v) bovine serum albumin. After 10 min 20 μl of 1 M-lactate/1 M-pyruvate (9:1, v/v) was added, along with 4-hydroxypyrazole to the appropriate concentration. After a further 20 min 20 μl of 0.1 mM-L-[*ring*-2-^{14}C]tryptophan was added (sp. radioactivity 0.05 Ci/mol). The final volume of incubations was 2.0 ml. Incubations were terminated 60 min after the addition of tryptophan. Results for tryptophan 2,3-dioxygenase flux are expressed as nmol of $^{14}CO_2$ plus nmol of ^{14}C-labelled non-charcoal precipitable material formed · (mg dry wt. cells)$^{-1}$ · h^{-1} ± S.E.M. of three independent observations.

[4-Hydroxypyrazole] (mM)	Tryptophan 2,3-dioxygenase flux (nmol · mg^{-1} · h^{-1})
0	4.22 ± 0.49
0.025	3.46 ± 0.19
0.050	2.98 ± 0.15
0.075	2.70 ± 0.15
0.10	2.49 ± 0.13
0.25	1.65 ± 0.09
0.50	1.60 ± 0.17

Time courses of flux through tryptophan 2,3-dioxygenase were linear, and thus hepatocytes do not lose haem during incubation. *In vivo*, the pool of free haem is very small (Granick *et al.*, 1975; Badawy, 1978), and thus similar effects to those observed in hepatocytes on tryptophan 2,3-dioxygenase may occur.

We confirmed, as predicted from previous observations (Rouach *et al.*, 1976), that pyrazole itself had no discernible effect on tryptophan 1,2-dioxygenase flux in whole cells. Any 4-hydroxypyrazole formed would, of course, be substantially diluted in the incubation medium.

The effects of 4-hydroxypyrazole on metabolism of formate

During studies on the effect of 4-hydroxypyrazole on tryptophan 2,3-dioxygenase activity, we noticed that the inhibitor substantially decreased the ratio of $^{14}CO_2$ to ^{14}C-labelled non-aromatic products produced from L-[*ring*-2-^{14}C]tryptophan (Table 2). This effect is not secondary to the interaction with dioxygenase, since decreasing flux through the dioxygenase by decreasing tryptophan concentration increased the ratio of $^{14}CO_2$ to ^{14}C-labelled non-aromatic products.

A direct demonstration of the inhibition of formate oxidation by 4-hydroxypyrazole is shown in Fig. 2. The range of effective concentrations is similar to that producing changes in the $^{14}CO_2$/^{14}C-labelled-non-aromatic-product ratio as described above. The rate of formate oxidation in isolated liver cells, at 10^{-4} M substrate, was 3.76 ± 0.26 nmol · (mg dry wt.)$^{-1}$ · h^{-1} (mean ± S.E.M.; three independent observations), a value similar to that expected from the data of Krebs *et al.* (1976). Half-maximal inhibition was produced by 0.13 mM-4-hydroxypyrazole. The extent of inhibition (as a percentage) by 4-hydroxypyrazole at any one concentration was the same over a range of formate concentrations, consistent with the inhibition being irreversible (MacDonald & Pispa, 1980).

The simplest explanation of these findings is that, in isolated liver cells incubated without methionine, formate is oxidized to CO_2 by reaction with a catalase–H_2O_2 complex (Chance, 1950). Catalase is known to be sensitive to 4-hydroxypyrazole (Dies *et al.*, 1977*b*; MacDonald & Pispa, 1980). A similar pathway has also been proposed for folate-deficient rats *in vivo* (Palese & Tephly, 1975).

Glycollate is a stimulator of the formation of the

Table 2. *The effect of 4-hydroxypyrazole, methionine and glycollate on the $^{14}CO_2/^{14}C$-labelled non-aromatic product ratio during the metabolism of 0.1 mM-L-[ring-2-^{14}C]tryptophan*

Incubation conditions were as described in Table 1. 4-Hydroxypyrazole, methionine and glycollate were added with the 1 M-lactate/1 M-pyruvate. Results are means ± S.E.M. of three independent observations.

[4-Hydroxypyrazole] (mM)	$^{14}CO_2/^{14}C$-labelled-non-aromatic-products ratio		
	Control	Plus 0.2 mM-methionine	Plus 0.5 mM-glycollate
0	1.66 ± 0.16	6.74 ± 0.32	3.05 ± 0.49
0.025	1.05 ± 0.12	6.54 ± 0.50	1.99 ± 0.26
0.050	0.88 ± 0.07	6.88 ± 0.19	1.34 ± 0.17
0.075	0.64 ± 0.04	6.69 ± 0.60	1.27 ± 0.10
0.10	0.55 ± 0.07	6.12 ± 0.67	1.08 ± 0.12
0.20	0.45 ± 0.02	6.43 ± 0.17	0.79 ± 0.04

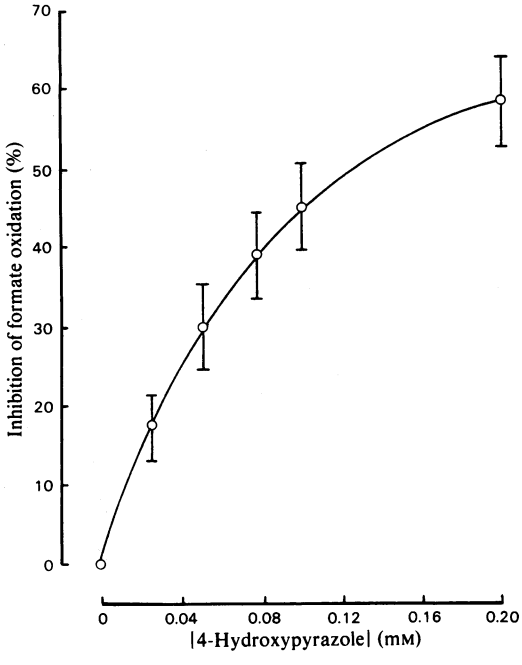

Fig. 2. The effect of 4-hydroxypyrazole on $^{14}CO_2$ production from 0.1 mM-[^{14}C]formate

Incubation conditions were as described in Table 1. 4-Hydroxypyrazole was added at the same time as 1 M-lactate/1 M-pyruvate. [^{14}C]Formate (20 µl; 10 mM; sp. radioactivity 0.05 Ci/mol) was added 20 min later. Results are means ± S.E.M. for three independent observations.

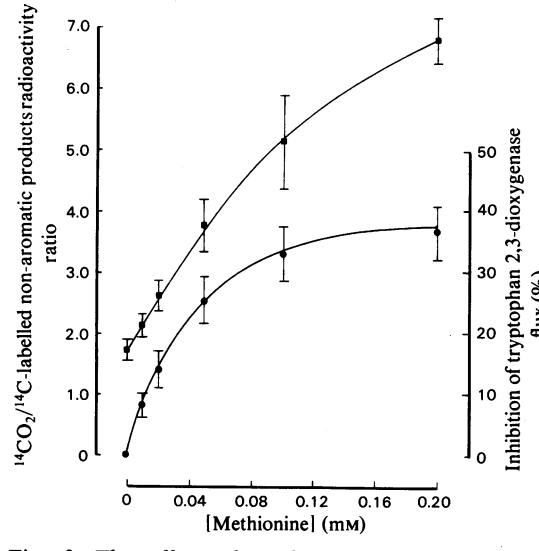

Fig. 3. *The effect of methionine on the $^{14}CO_2$ to [^{14}C]-labelled non-aromatic products ratio, and flux through tryptophan 2,3-dioxygenase*

Incubation conditions are as described in Table 1. Methionine was added with 1 M-lactate/1 M-pyruvate. Each point is the mean ± S.E.M. of three independent observations. ■, $^{14}CO_2/[^{14}C]$-labelled non-aromatic products. ●, % inhibition of tryptophan 2,3-dioxygenase flux.

catalase–H_2O_2 complex (Kun *et al.*, 1954) through its oxidation by glycollate oxidase (Lias & Richardson, 1973), a peroxisomal enzyme (McGroarty *et al.*, 1974). It is metabolized rapidly by isolated liver cells (Harris *et al.*, 1982). The ratio of $^{14}CO_2$ to ^{14}C-labelled non-aromatic products in incubations with [^{14}C]tryptophan increased from 1.66 ± 0.16 in control experiments to 6.23 ± 0.66 in the presence of 1 mM-glycollate (means ± S.E.M. of three separate observations). In Table 2 data show that 4-hydroxypyrazole is a potent inhibitor of formate oxidation in the presence of glycollate, although ratios are larger than when the inhibitor alone is present. This is probably because of the greater activity of the remaining active catalase in the presence of glycollate and inhibitor.

At physiological concentrations of L-tryptophan [total 0.1 mM in the presence of 2% (w/v) bovine serum albumin], methionine substantially increases the ratio $^{14}CO_2/^{14}C$-labelled non-aromatic products with maximal effect at concentrations in the physiological range (Fig. 3). This is attributable to stimulation of the tetrahydrofolate-dependent pathway for formate oxidation. In confirmation of this, 4-hydroxypyrazole did not decrease the methionine-stimulated ratio (Table 2).

Fig. 3 also shows the inhibition of flux through tryptophan 2,3-dioxygenase by methionine. Smith & Pogson (1980) have shown that incorporation of [^{14}C]formate (derived from labelled tryptophan) into proteins, nucleotides and nucleic acids is negligibly small. In the presence of methionine, however, it is conceivable that the extent of labelling of such products (which are not counted in the assay procedures used) is increased. It has been shown elsewhere, however, that the maximum rates of conversion of formate into purine base (in the presence of methionine) are much lower than that necessary for this explanation to have validity (Des Rosiers *et al.*, 1980).

The inhibition of dioxygenase activity by methionine is, at maximum, only partial, and the physiological significance of this effect is at present not clear.

4-Hydroxypyrazole has been reported to inhibit, in addition to catalase and tryptophan 2,3-dioxygenase, an as yet unidentified step in the formation of $^{14}CO_2$ from L-[*ring*-2-^{14}C]histidine (Cook & Pogson, 1981). In view of the possible formation of this inhibitor by microsomal hydroxylation of pyrazole (Dies *et al.*, 1977*a*; Harris *et al.*, 1982), this latter compound should be used with a caution as a 'specific' inhibitor in metabolic studies in which hydroxylation could occur.

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