# Inhibition of kynureninase (L-kynurenine hydrolase, *EC* 3.7.1.3) by oestrone sulphate: an alternative explanation for abnormal results of tryptophan load tests in women receiving oestrogenic steroids

BY DAVID A. BENDER AND DAVID WYNICK

*Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London W1P 7PN*

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1. A partial purification of kynureninase (L-kynurenine hydrolase, *EC* 3.7.1.3) from rat liver and a total resolution of the apoenzyme have been achieved. The hypothesis that conjugates of oestrogenic steroids compete with pyridoxal phosphate for the cofactor binding site of the enzyme, and so disturb tryptophan metabolism, leading to apparent vitamin $B_6$ deficiency, has been tested.

2. Kynureninase from rat liver was partially purified, and the cofactor-free apoenzyme was prepared. Oestrone sulphate inhibited the enzyme uncompetitively with respect to pyridoxal phosphate, and competitively with respect to kynurenine, with a mean (±SE) inhibitor constant ($K_i$) of 82±6 μM.

3. The addition of a saturating concentration of pyridoxal phosphate to unfractionated liver homogenates led to an approximately fivefold increase in kynureninase activity, indicating the presence of a relatively large amount of apo-kynureninase in the tissue.

4. It is suggested that the abnormal results of tryptophan load tests in women receiving oestrogens are the result of inhibition of kynureninase by oestrogen conjugates, and that there is no evidence for oestrogen-induced vitamin $B_6$ deficiency in such cases.

There are many reports showing abnormal tryptophan metabolism in women receiving oestrogenic steroids either as oral contraceptives or as menopausal hormone replacement therapy. Women treated with these steroids show greatly increased excretion of xanthurenic acid, kynurenic acid and kynurenine after administration of an oral dose of 2 g tryptophan, compared with the normal response to such a tryptophan load (Rose & Braidman, 1971). The tryptophan load test is an accepted test of vitamin $B_6$ nutritional status (Coursin, 1964), and the results in women receiving oral contraceptives have been widely interpreted as indicative of some extent of drug-induced vitamin $B_6$ deficiency or depletion, a view which is supported by the finding that administration of supplementary vitamin $B_6$ (in amounts considerably in excess of the usual daily intake) restores tryptophan metabolism to normal (Rose & Adams, 1972).

It has been assumed that this apparent depletion of vitamin $B_6$ is the result of competition between conjugates of the steroids and pyridoxal phosphate, the metabolically-active form of the vitamin, for the cofactor binding sites of vitamin $B_6$-dependent enzymes. Mason & Gullekson (1960) showed that a number of oestrogen sulphates inhibited kynurenine aminotransferase (L-kynurenine: 2-oxo-glutarate aminotransferase (cyclizing), *EC* 2.6.1.7), although they did not demonstrate the mechanism of this inhibition. Similar inhibition of kynureninase (L-kynurenine hydrolase, *EC* 3.7.1.3) would account for the disturbances of tryptophan metabolism already discussed.

Although the disturbances of tryptophan metabolism in response to oestrogen administration have been attributed to vitamin $B_6$ depletion, other indices of vitamin $B_6$ status are normal in women with abnormal tryptophan metabolism, which suggests that some other mechanism must be involved. Leklem *et al.* (1975) showed that methionine metabolism was unaffected, despite the involvement of two pyridoxal phosphate dependent steps in its metabolism. Studies on plasma and erythrocyte aminotransferases in women receiving oral

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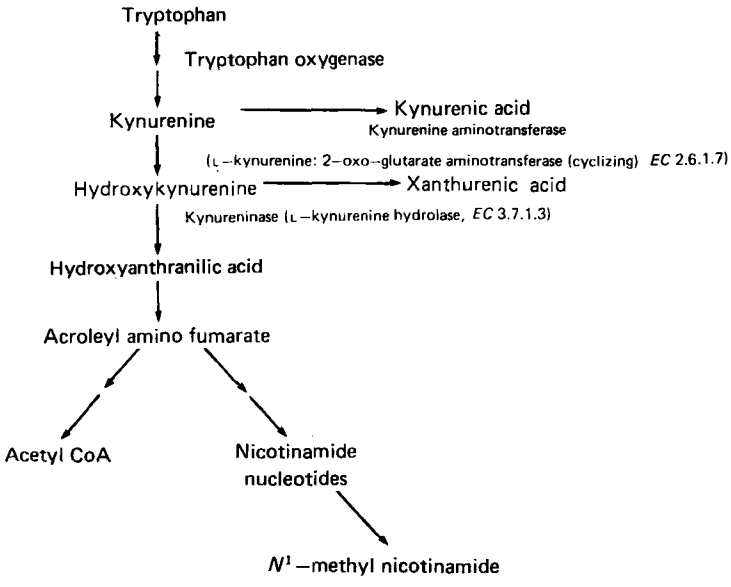


Fig. 1. The oxidative metabolism of tryptophan.

contraceptives have produced conflicting results with respect to the saturation of the enzymes with pyridoxal phosphate (Aly *et al.* 1971; Brown *et al.* 1975; Leklem *et al.* 1975). Plasma concentrations of pyridoxal phosphate and urinary excretion of 4-pyridoxic acid, the principal metabolite of the vitamin, were also unaffected by oral contraceptives (Brown *et al.* 1975; Leklem *et al.* 1975).

Rose & Braidman (1971) showed that women receiving contraceptive steroids excreted more $N^1$-methyl nicotinamide, an endproduct of tryptophan oxidative metabolism (Fig. 1), than did non-users of contraceptives. This has been attributed to increased oxidation of tryptophan as a result of induction of the first and rate-limiting enzyme of the pathway, tryptophan oxygenase (L-tryptophan: oxygen oxido-reductase (decyclizing) *EC* 1.13.11.11); such induction of tryptophan oxygenase by steroids is well established (Schimke *et al.* 1965). However it is difficult to attribute the increased formation of the endproduct of the pathway to an increase in the activity of tryptophan oxygenase if kynureninase, one of the intermediate enzymes, is inhibited by competition with its cofactor.

In the present study, the interaction between apo-kynureninase and pyridoxal phosphate has been studied using a partially-purified preparation of the enzyme from rat liver. Oestrone sulphate was chosen as a readily available model oestrogen conjugate which has been used both as a contraceptive agent and in menopausal hormone-replacement therapy. Its effects on the apoenzyme–coenzyme interaction have been assessed in order to test the hypothesis that oestrogen conjugates lead to vitamin $B_6$ deficiency by competing with the coenzyme.

## METHODS

Female Wistar rats, bred in the Courtauld Institute, were used for this study. Animals weighing between 400 and 500 g were killed by cervical dislocation, the livers were dissected out rapidly, frozen and stored at −20° until required. Liver was homogenized while still frozen in 2 ml 0·15 M-sodium chloride/g tissue, and the homogenate centrifuged at 9000 *g* for 30 min to remove nuclei, cell debris and mitochondria. Pyridoxal phosphate was added

Table 1. *Purification of kynureninase (L-kynurenine hydrolase; EC 3.7.1.3) from rat liver*

| Preparation | Percentage recovery | Specific activity (μmol anthranilic acid/min per mg protein) | Relative activity |
|---|---|---|---|
| Liver homogenate | (100) | 11·9 | (1·0) |
| 9000 *g* supernatant fraction | 100 | 15·5 | 1·4 |
| Heat denaturation supernatant fraction | 64·4 | 22·8 | 1·9 |
| Ammonium sulphate pellet | 21·1 | 494·7 | 41·6 |

to the supernatant fraction to bring the final concentration to 1 mM, and the preparation heated at 60° for 5 min, cooled in ice and centrifuged at 9000 *g* for 30 min to remove denatured protein. Ice-cold saturated ammonium sulphate solution was added to the resulting supernatant fraction. The fraction precipitating between 45–55% saturation was collected and dissolved in a volume of 0·2 M-Tris-acetate, pH 8·4, containing 0·5 mM-2-mercaptoethanol equivalent to one-quarter of the original homogenate volume. Phenylhydrazine hydrochloride was then added to a final concentration of 1 mM and the mixture incubated at 30° for 10 min, cooled in ice and dialysed against four changes of the same buffer at 4° to remove the free cofactor.

Kynureninase activity was determined by the method described previously (Bender & Smith, 1978). The enzyme preparation was pre-incubated at pH 8·4 for 10 min at 30° together with pyridoxal phosphate and any inhibitors, then the reaction was initiated by the addition of kynurenine. The anthranilic acid formed was measured fluorimetrically.

The concentration of pyridoxal phosphate in the livers used for preparation of kynureninase was determined by the following modification of the method of Adams (1979). In order to assess the amount of tissue pyridoxal phosphate that was freely diffusible, one sample of a homogenate of liver in 5 ml water/g was dialysed at 4° against four changes of distilled water, while the other was stored at 4° without dialysis, and was subsequently diluted to the same extent as the dialysed sample had been diluted by endosmosis. Portions of the homogenate (200 μl) were mixed with 200 μl trichloroacetic acid (100 g/l), together with 100 μl water or 2 μM-pyridoxal phosphate as an internal standard, and heated at 50° for 15 min. After cooling to room temperature, 140 μl 3·3 M-dipotassium hydrogen phosphate and 25 μl 60 mM-sodium cyanide were added, and the samples were heated to 50° for a further 25 min. Then 70 μl 28% orthophosphoric acid (280 g/l) and 1 ml 2 M-sodium acetate were added and the fluorescence due to the pyridoxal phosphate–cyanide adduct was measured (excitation 310 nm, emission 418 nm).

## RESULTS

The final enzyme preparation used for kinetic and inhibition studies was a 41-fold enrichment compared with the initial liver homogenate, and had a specific activity of 495 μmol anthranilic acid formed/min per mg protein. The recovery of activity and enrichment at each stage are shown in Table 1.

Dialysis of the ammonium sulphate precipitated preparation against 0·2 M-Tris-acetate, pH 8·4, led to insignificant loss of enzymic activity, indicating that the majority of the cofactor was tightly enzyme-bound. Reaction with phenylhydrazine followed by dialysis led

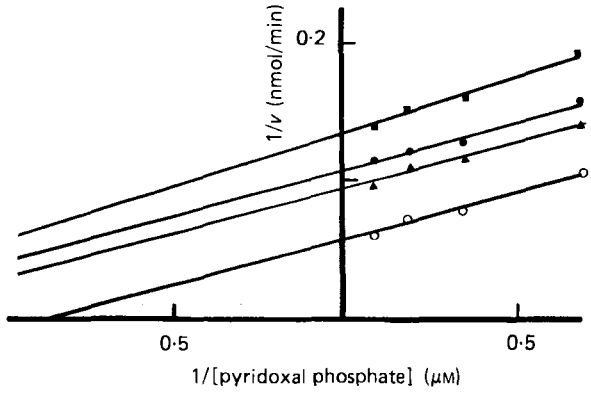


Fig. 2. Inhibition of kynureninase (L-kynurenine hydrolase; *EC* 3.7.1.3) by oestrone sulphate in the presence of a saturating concentration of kynurenine (1 mM) and varying concentrations of pyridoxal phosphate. (○—○), No addition; (▲—▲), 0·085 mM-oestrone sulphate; (●—●), 0·17 mM-oestrone sulphate; (■—■), 0·34 mM-oestrone sulphate; *v*, rate of action.

to a reduction to 0·8% of the initial activity, and addition of pyridoxal phosphate restored 97% of the catalytic activity. Phenylhydrazine has been shown previously to be a potent inhibitor of kynureninase; the phenylhydrazone of pyridoxal phosphate has no catalytic activity, and does not form a Schiff base with the enzyme, so that it is readily removed by dialysis (Bender & Smith, 1978).

The apo-enzyme had a Michaelis constant ($K_m$) for L-kynurenine of 0·12±0·01 mM in the presence of 50 μM-pyridoxal phosphate, and an apparent $K_m$ of 1·0±0·04 μM for pyridoxal phosphate in the presence of 10 mM-L-kynurenine. These results are in close agreement with those reported by other workers (Soda & Tanizawa, 1979).

Addition of pyridoxal phosphate to a final concentration of 1 mM led to a 5-fold increase in the kynureninase activity of the unfractionated liver homogenate, indicating the presence of a considerable amount of apo-kynureninase in the liver under normal circumstances. The total concentration of pyridoxal phosphate in the livers used for preparation of the enzyme was 5·3±0·2 nmol/g, of this 3·0±0·1 nmol/g was tightly protein-bound, and was not removed by extensive dialysis.

Oestrone sulphate inhibited the enzyme. As can be seen from Fig. 2, this inhibition was primarily uncompetitive with respect to pyridoxal phosphate, in the presence of a saturating concentration of kynurenine. Fig. 3 shows that the inhibition was primarily competitive with respect to kynurenine when the enzyme had been pre-incubated with a saturating concentration of pyridoxal phosphate. The Dixon plots shown in Fig. 4 are again indicative of primarily competitive inhibition with respect to kynurenine, and give a value of 82±6 μM for the inhibitor constant ($K_i$) of oestrone sulphate.

## DISCUSSION

The present study was designed to investigate the mechanism by which oestrogenic steroids lead to apparent vitamin $B_6$ deficiency, and in particular to test the hypothesis that metabolites of the steroids inhibit kynureninase by competing with the cofactor, pyridoxal phosphate, for the catalytic site (Mason & Gullekson, 1960).

The results reported here are not compatible with this hypothesis, as they show uncompetitive inhibition of kynureninase by oestrone sulphate with respect to the cofactor, and competitive inhibition with respect to the substrate.

These results support a possible alternative explanation of the disturbances of tryptophan

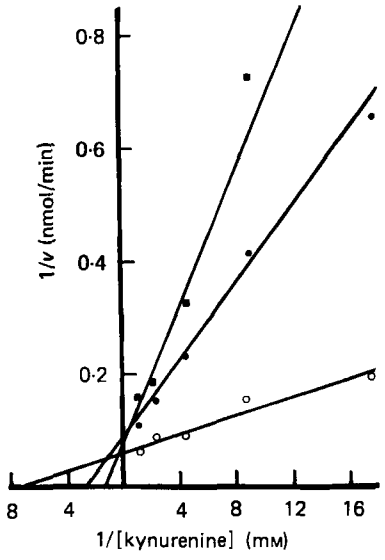


Fig. 3. Inhibition of kynureninase (L-kynurenine hydrolase; *EC* 3.7.1.3) by oestrone sulphate in the presence of a saturating concentration (50 $\mu$M) of pyridoxal phosphate and varying concentrations of kynurenine. (○—○), No addition; (●—●), 0·17 mM-oestrone sulphate; (■—■), 0·34 mM-oestrone sulphate; *v*, rate of reaction.

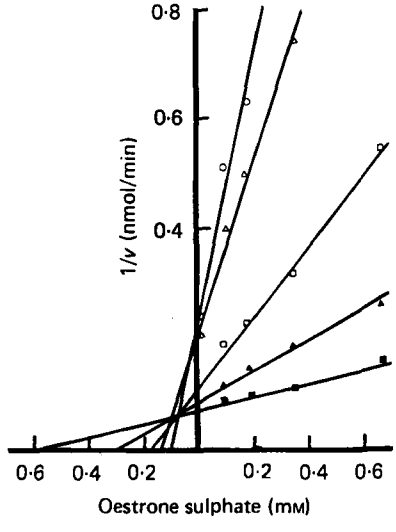


Fig. 4. Dixon plot of the inhibition of kynureninase (L-kynurenine hydrolase; *EC* 3.7.1.3) by oestrone sulphate in the presence of a saturating concentration of pyridoxal phosphate (50 $\mu$M) and varying concentrations of kynurenine. (○—○), 0·057 mM-kynurenine; (△—△), 0·11 mM-kynurenine; (□—□), 0·22 mM-kynurenine; (▲—▲), 0·46 mM-kynurenine; (■—■), 0·91 mM-kynurenine; *v*, rate of reaction.

metabolism that have been observed in women receiving oestrogenic steroids. Inhibition of kynureninase by metabolites of the steroids would result in an increase in the size of the liver pool of kynurenine, and hence an increase in the urinary excretion of kynurenine, even without the additional stress of a tryptophan load, as has been reported in women receiving oestrone sulphate preparations as menopausal hormone replacement therapy (Bender *et al.*

1981). At the same time, the increased concentration of kynurenine in the liver would result in increased formation of xanthurenic and kynurenic acids, by the action of kynurenine aminotransferase, an enzyme that normally has little activity, because of its relatively high $K_m$ (of the order of 1·8 mM; Ueno *et al.* 1963). However, since the inhibition of kynureninase by oestrone sulphate is competitive with respect to the substrate, this increase in the liver concentration of kynurenine would be expected to lead to relief of the inhibition, and hence restoration of normal flux of metabolites through the oxidative pathway (shown in Fig. 1), albeit with an increased pool of kynurenine (and presumably also of hydroxy-kynurenine). The result of this would be normal synthesis of nicotinamide nucleotides, and hence normal excretion of $N^1$-methyl nicotinamide. Induction of the first enzyme of the pathway, tryptophan oxygenase, by steroids (Schimke *et al.* 1965) would lead to an increased flux of metabolites through the pathway, and hence to increased excretion of $N^1$-methyl nicotinamide, as has been reported (Rose & Braidman, 1971).

The finding that tryptophan metabolism could be restored to normal in women receiving oral contraceptives by administration of supplements of vitamin $B_6$ has been considered to be evidence of a deficiency of the vitamin caused by the drugs (Rose & Adams, 1972). However, the finding reported here of a considerable excess of apo-kynureninase in the liver of the rat under normal conditions might permit an alternative explanation of the effect of supplementary vitamin $B_6$; it could lead to activation of the normally-inactive apoenzyme to the holoenzyme, and so mask the inhibition of kynureninase by metabolites of the steroids.

The concentration of pyridoxal phosphate in the liver samples that was removable by dialysis, and hence which might be considered to be available for interchange between enzymes, and for activation of apo-kynureninase, was found to be $2{\cdot}3 \pm 0{\cdot}01$ nmol/g of tissue; approximately twice the apparent $K_m$ of kynureninase for its cofactor ($1{\cdot}0 \pm 0{\cdot}04$ $\mu$M). Since it is to be expected that a number of apoenzymes in the liver would compete with one another for this free cofactor, it is not surprising that there is at least some free apo-kynureninase in the liver. Preliminary studies with the partially-purified preparation of kynureninase used here showed that a threshold concentration of pyridoxal phosphate was required for activation of apo-kynureninase; apparent as an extent of sigmoidicity of the graph of pyridoxal phosphate concentration *v.* kynureninase activity. This presumably reflects the presence of other enzymes in the preparation with a greater affinity for the cofactor than that of kynureninase.

The $K_i$ of oestrone sulphate, $82 \pm 6$ $\mu$M, is relatively high, although it might reasonably be expected that such a concentration could be achieved in the livers of women receiving oestrone sulphate preparations. Oestrone sulphate is not a significant human metabolite; however, it is a conveniently-available model compound, and preliminary experiments suggest that oestrone glucuronide, which is a human metabolite, inhibits kynureninase in a similar manner to that reported here for oestrone sulphate, and is a more potent inhibitor. Unconjugated oestrone was not inhibitory.

The results reported here do not support the accepted hypothesis for the action of oestrogenic steroids in leading to vitamin $B_6$ deficiency, but suggest that the effects on tryptophan metabolism which have been interpreted as indicative of vitamin $B_6$ deficiency are in fact due to a direct effect of oestrogen metabolites on kynureninase, one of the enzymes of tryptophan metabolism. It is therefore suggested that oestrogens do not lead to vitamin $B_6$ depletion, and that administration of vitamin $B_6$ supplements to women receiving oral contraceptives or hormone replacement therapy is probably inappropriate.

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