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Effects of oestrogen administration on vitamin B₆ and tryptophan metabolism in the rat

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1. In order to assess the effects of oestrogens on the metabolism of tryptophan and vitamin B₆, ovariectomized rats have been maintained on diets providing known amounts of tryptophan, nicotinamide and vitamin B₆. They received oestrone sulphate, 210 µg/kg body-wt per d, either incorporated in the diet for 8 weeks, or by daily intraperitoneal injection for periods of 1–3 d.

2. Oestrone sulphate administration caused a slight reduction in the concentration of pyridoxal phosphate in plasma. It had no effect on the concentration of pyridoxal phosphate in liver or kidney, the urinary excretion of 4-pyridoxic acid, the activation of erythrocyte aspartate aminotransferase (L-aspartate:2-oxo-glutarate aminotransferase, EC 2. 6. 1. 1) by incubation with added pyridoxal phosphate, or the activity of pyridoxal oxidase (aldehyde: oxygen oxido-reductase, EC 1. 2. 3. 1) in the liver.

3. Oestrone sulphate administration caused an increase in the urinary excretion of kynurenine and a reduction in the activity of liver kynureninase (L-kynurenine hydrolase, EC 3. 7. 1. 3). It had no effect on the urinary excretion of N¹-methyl nicotinamide or the concentrations of nicotinamide nucleotides in blood, liver or kidney.

4. There was a considerable excess of the apoenzyme of kynureninase in the liver. Incubation of liver homogenates with added pyridoxal phosphate led to a 4- to 5-fold increase in activity.

5. We conclude that there is no evidence of any significant effect of oestrogens on vitamin B₆. It is suggested that abnormalities of tryptophan metabolism in women receiving oestrogens, which have been widely attributed to drug-induced vitamin B₆ depletion, can be accounted for by inhibition of kynureninase by oestrogen metabolites.

It is widely believed that oestrogens, given as oral contraceptives or as menopausal hormone replacement therapy, cause depletion of vitamin B₆. Rose & Braidman (1971) reviewed a number of reports of abnormal tryptophan metabolism, indicative of vitamin B₆ deficiency, in women receiving contraceptive steroids. Rose & Adams (1972) showed that the administration of supplementary vitamin B₆ to such women would restore tryptophan metabolism to normal. Other studies (Brown *et al.* 1975; Leklem *et al.* 1975) have similarly shown impaired tryptophan metabolism in response to oestrogen administration in women consuming controlled diets providing known amounts of vitamin B₆.

All these studies have depended on the use of the tryptophan load test as an indicator of vitamin B₆ status. The enzyme kynureninase (L-kynurenine hydrolase, EC 3. 7. 1. 3) is pyridoxal phosphate dependent, and in deficiency of the vitamin its activity is impaired, leading to increased excretion of kynurenic and xanthurenic acids and kynurenine after administration of a test dose of tryptophan (Coursin, 1964). The use of the tryptophan load test in such cases can be criticized on the grounds that inhibition of kynureninase by oestrogens or their metabolites would give results indistinguishable from vitamin B₆ deficiency. Such inhibition has been demonstrated (Bender & Wynick, 1981).

A number of studies cast further doubt on the interpretation of an abnormal response to a test dose of tryptophan as oestrogen-induced vitamin B₆ deficiency, since other indicators of vitamin B₆ status are unaffected by oestrogen administration. Leklem *et al.* (1975) showed no effect of oestrogens on the metabolism of a test dose of methionine. The plasma concentration of pyridoxal phosphate and urinary excretion of 4-pyridoxic acid are also unaffected by use of either oral contraceptives (Brown *et al.* 1975; Leklem *et al.* 1975)

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or menopausal hormone replacement therapy (Bender *et al.* 1981). The activation of plasma or erythrocyte aspartate or alanine aminotransferase by incubation with added pyridoxal phosphate has yielded conflicting results; some workers have shown effects that suggest vitamin B₆ deficiency in response to oestrogens (Aly *et al.* 1971; Salkeld *et al.* 1973), while others have been unable to demonstrate any effect (Rose & Adams, 1972; Leklem *et al.* 1975; Wien, 1978).

The present study was designed to determine whether there is any depletion of vitamin B₆ in response to oestrogen administration, and to what extent the abnormalities of tryptophan metabolism that have been reported in clinical studies reflect inhibition of kynureninase by oestrogen metabolites. A number of different indicators of the metabolism of tryptophan and vitamin B₆ have been assessed in rats that were maintained on diets providing known amounts of tryptophan, nicotinamide and vitamin B₆. Ovariectomized rats were used so as to avoid complications due to endogenous oestrogen secretion. The results indicate that oestrogens do not cause depletion of vitamin B₆, and that all of the effects on tryptophan metabolism can be accounted for by inhibition of kynureninase.

METHODS

Animals and diets

Female Wistar rats, bred in the Courtauld Institute, were used. They were weaned at 21 d of age onto a diet similar to that described by Bender *et al.* (1982), providing (mg/kg dry matter (DM)): 1300 tryptophan, 2 nicotinamide, 1.2 pyridoxine hydrochloride. All animals were maintained on this diet throughout the experiment.

After 2 weeks, the rats were bilaterally ovariectomized under anaesthesia with Valium (Roche; Diazepam 2.5 mg/kg body-wt (BW), intraperitoneally) and Hypnorm (Janssen; (mg/kg BW) fentanyl citrate 0.315, fluanisone 10, intramuscularly). For the first 3 d after operation they were provided with a solution of oxytetracycline (Pfizer, Terramycin syrup; 2.5 mg/ml) in the drinking water.

Between 3–4 weeks after ovariectomy, animals were treated with oestrone sulphate, 210 µg/kg BW per d in 0.15 M-sodium chloride (saline) by intraperitoneal injection daily for 1–3 d. This is approximately three times the dose of oestrone sulphate that is used clinically as menopausal hormone replacement therapy. Control animals received injections of saline alone. A further group of animals was treated with oestrone sulphate for 8 weeks, beginning 2 weeks after ovariectomy. In this group the hormone was incorporated in the diet at a concentration of 3 mg/kg DM; from the observed consumption of 70 g dietary DM/kg BW per d this would provide the same dose of 210 µg/kg BW per d as in the acute study.

For 24 h before killing, the rats were housed individually in stainless-steel metabolism cages (Acme Metal Co Inc, Chicago, Ill., USA) to permit collection of urine. No preservative was added, and the urine was stored at –20° until it was required. The rats were killed by decapitation, and blood was collected into heparinized beakers on ice. One sample was used immediately for the determination of nicotinamide nucleotides, and the remainder was centrifuged (2000 g, 20 min); plasma was frozen and stored at –20°. Erythrocytes were washed with saline, then lysed in a volume of distilled water equal to the plasma volume; the lysate was frozen and stored at –20°. Liver and kidneys were dissected out and frozen in liquid nitrogen and were used within 2 h, and the remainder was stored at –20°.

Uteri were dissected out and inspected to ensure that those animals that had received oestrone sulphate were indeed adequately oestrogenized, as shown by considerable uterine enlargement compared with the control animals. Any animal in which a partial ovary was visible, indicating inadequate ovariectomy, was discarded.

Analytical methods

The total concentration of nicotinamide nucleotides (a combined estimate of the oxidized and reduced forms of NAD and NADP) in blood, liver and kidney was measured after treatment with or homogenization in dilute hydrogen peroxide solution, by the fluorimetric method that has been described by Bender *et al.* (1982).

The concentration of pyridoxal phosphate in plasma, liver and kidney was determined by the fluorimetric method of Adams (1979), after denaturation of proteins with 110 g trichloroacetic acid/l.

The concentration of tryptophan in plasma was measured by a modification of the norharman fluorimetric method (Denckla & Dewey, 1967), using perchloric acid rather than trichloroacetic acid.

Urine 4-pyridoxic acid was determined by the lactone fluorimetric method of Reddy *et al.* (1958) after ion-exchange chromatography on columns of Dowex 1 (OH⁻) and Dowex 50W (H⁺) resins.

Urine kynurenine was determined by the colorimetric method of Joseph & Risby (1975) after ion-exchange chromatography on columns of Dowex 50W (H⁺) resin as described by Satoh & Price (1958).

Urine *N*¹-methyl nicotinamide was determined by a small-scale modification of the alkali-ketone fluorimetric method of Carpenter & Kodicek (1950), as described previously (Bender, 1980).

The activity of kynureninase in liver was measured by the fluorimetric method of Bender & Smith (1978), both with and without the addition of pyridoxal phosphate to a final concentration of 1 mmol/l in the incubation mixture, in order to assess both the holoenzyme and total (holoenzyme + apoenzyme) content of the tissue.

The activity of pyridoxal oxidase (aldehyde: oxygen oxido-reductase, EC 1.2.3.1) in liver was measured by the following modification of the method of Stanulovic & Chaykin (1971). Liver was homogenized while still frozen in 2 ml saline/g tissue, and the homogenate was centrifuged at 20000 g for 30 min to remove cell debris, nuclei and mitochondria. Samples of the supernatant fraction (1 ml) were incubated at 30° with 1 ml 0.1 M-sodium phosphate, pH 7.0 and 100 μl 10 mM-pyridoxine hydrochloride for 30 min, with vigorous shaking to ensure adequate oxygenation. The reaction was stopped by the addition of 1 ml 1 M-trichloroacetic acid in 5 M-hydrochloric acid, and samples were centrifuged to remove denatured protein (2000 g, 20 min). The amount of 4-pyridoxic acid formed was measured by the lactone fluorimetric method of Reddy *et al.* (1958).

The activity of aspartate aminotransferase (L-aspartate: 2-oxo-glutarate aminotransferase, EC 2.6.1.1) in the erythrocyte lysate was measured both with and without the addition of pyridoxal phosphate to a final concentration of 1 mmol/l, by the following modification of the method of Schuster *et al.* (1978). A 100 μl sample of the lysate was incubated with 100 μl 0.2 M-sodium phosphate, pH 7.5 in which pyridoxal phosphate was dissolved as appropriate, for 10 min at 30°. The reaction was initiated by the addition of 100 μl of a solution of 0.1 M-2-oxo-glutarate and 0.05 M-[2,3-³H]aspartate (0.5 Ci/mol), and allowed to continue for 15 min. The reaction was stopped by immersing the tubes in a boiling water-bath for 2 min; they were then cooled, diluted with 200 μl water and centrifuged at 2000 g for 20 min to remove denatured protein. The substrate was tritiated in those atoms that were lost as water during the transamination reaction, so that measurement of the amount of ³H₂O formed would reflect the activity of the enzyme. Unreacted [³H]aspartate was removed from the reaction mixture by pouring the supernatant fraction onto columns of finely-pulverized Zerolit DMF mixed-bed ion-exchange resin (approximately 300 mg resin per column), followed by washing with 1 ml water. The eluate was then mixed with

Table 1. *The effects on vitamin B₆ and tryptophan metabolism of the administration of oestrone sulphate (210 µg/kg body-weight per d) to rats*
(Mean values and standard deviations; no. of animals in parentheses)

Treatment	Oestrone sulphate administration					
	Control (36)		1-3 d (28)		8 weeks (8)	
	Mean	SD	Mean	SD	Mean	SD
Pyridoxal phosphate						
Plasma (µM)	0.65	0.31	0.52	0.21*	0.48	0.14
Liver (µmol/g)	15.6	4.0	15.6	4.3	15.3	2.3
Kidney (µmol/g)	18.9	6.7	21.9	5.8	16.5	3.3
Activation of erythrocyte aspartate aminotransferase by 1 mM-pyridoxal phosphate (activated: basal)	1.21	0.16	1.15	0.14	1.24	0.15
Urine 4-pyridoxic acid (nmol/24 h)	49.9	15.1	49.0	18.8	51.8	17.4
Liver pyridoxal oxidase (nmol product formed/min per g)	0.115	0.039	0.119	0.035	0.105	0.029
Plasma tryptophan (µM)	59.2	24.1	53.6	25.0	77.2	27.3*
Urine kynurenine (nmol/24 h)	5.9	0.3	7.2	2.2**	7.9	1.1***
Urine N ¹ -methyl nicotinamide (nmol/24 h)	21.9	5.4	23.1	4.6	19.4	2.1
Nicotinamide nucleotides						
Blood (µM)	73.0	12.3	74.3	14.5	81.0	18.4
Liver (µmol/g)	0.42	0.15	0.43	0.20	0.53	0.39
Kidney (µmol/g)	0.77	0.20	0.84	0.35	0.82	0.22
Liver kynureninase (nmol product formed/min per g):						
No added pyridoxal phosphate	5.67	1.15	4.53	0.80***	4.40	0.80***
+ 1 mM-pyridoxal phosphate	21.9	5.4	23.1	4.6	19.4	2.1

Statistical significance of differences by *t* test: * $0.1 \geq P \geq 0.05$, ** $0.005 \geq P \geq 0.001$, *** $P \leq 0.001$.

15 ml scintillator solution (9 g PPO+60 mg POPOP in 600 ml Triton X-100+1800 ml toluene). Radioactivity was measured using a Packard TriCarb liquid-scintillation spectrometer.

RESULTS

Analysis of variance showed no significant effect of the time of treatment with oestrone sulphate for 1, 2 or 3 d, and no significant effect of the age of the rats, on any criteria measured. For this reason, the results in Table 1 show a single group of control animals; those animals that received oestrone sulphate for 1, 2 or 3 d by intraperitoneal injection have similarly been grouped together. Those animals that received oestrone sulphate in the diet for 8 weeks have been considered separately because of the different mode of administration of the hormone.

Short-term treatment with oestrone sulphate led to a slight fall in the plasma concentration of pyridoxal phosphate ($0.1 > P > 0.05$); after treatment for 8 weeks, the concentration was again lower than in control animals, but the difference was not significant by *t* test. Neither group of oestrone sulphate-treated animals showed any significant difference from controls in the concentration of pyridoxal phosphate in liver or kidney, the urinary excretion of 4-pyridoxic acid, the activity of pyridoxal oxidase in the liver or the activation of erythrocyte aspartate aminotransferase by incubation with added pyridoxal phosphate.

Administration of oestrone sulphate for 8 weeks, but not for 1–3 d, led to a slight increase in the concentration of tryptophan in plasma ($0.1 > P > 0.05$), and both short- and long-term administration of the hormone led to an increase in the excretion of kynurenine, and a reduction in the activity of kynureninase in the liver. Addition of pyridoxal phosphate (1 mM) to the incubation mixture led to a 4- to 5-fold increase in the activity of kynureninase, with no difference in the total activity of the enzyme between oestrone sulphate-treated animals and controls. Oestrone sulphate had no effect on the excretion of *N*¹-methyl nicotinamide or on the concentration of nicotinamide nucleotides in blood, liver or kidney.

DISCUSSION

The results reported here cannot be interpreted as providing support for the hypothesis that administration of oestrogens causes depletion or deficiency of vitamin B₆. None of the indicators of vitamin B₆ status assessed showed any effect of the administration of oestrogen sulphate. This agrees with the clinical studies cited previously that oestrogen administration does not affect the plasma concentration of pyridoxal phosphate, urinary excretion of 4-pyridoxic acid or the activation of aspartate aminotransferase by pyridoxal phosphate (Brown *et al.* 1975; Leklem *et al.* 1975; Wien, 1978). It is difficult to explain the increase in the plasma concentration of tryptophan in response to prolonged administration of oestrone sulphate. It is known that tryptophan oxygenase (*L*-tryptophan: oxygen oxidoreductase (decyclizing), EC 1.13.11.11) is induced by oestrogens (Patnaik & Sarangi, 1980), and this would be expected to lead to a reduction in the circulating concentration of tryptophan, rather than an increase. A similar effect of oestrogen administration on plasma tryptophan has been observed in women receiving either ethinyl oestradiol or piperazine oestrone sulphate as menopausal hormone replacement therapy (Bender, Papadaki and Coulson, unpublished observations).

We have reported previously that oestrone sulphate inhibits the enzyme kynureninase (Bender & Wynick, 1981). The finding of reduced activity of the holoenzyme in the livers of oestrone-sulphate treated rats, and increased urinary excretion of kynurenine, suggests that this inhibition, previously demonstrated *in vitro*, is also important *in vivo*. The enzyme kinetic studies (Bender & Wynick, 1981) suggested that the inhibition of kynureninase was competitive with respect to the substrate, and the evidence from the present study indicates a similar mode of inhibition *in vivo*. Although the rats treated with oestrone sulphate excreted more kynurenine than did control animals, there was no reduction in their excretion of *N*¹-methyl nicotinamide, or in the blood and tissue concentrations of nicotinamide nucleotides, as might be expected to follow inhibition of kynureninase. This suggests a competitive mode of inhibition; an accumulation of kynurenine, resulting from inhibition of the enzyme, will lead to some displacement of the inhibitor from the enzyme, and hence will result in more or less normal flux of metabolites through the pathway to NAD (and hence to *N*¹-methyl nicotinamide), but with a larger than normal intracellular pool of kynurenine.

Treatment with oestrone sulphate seems not to affect the apoenzyme of kynureninase; addition of a saturating concentration of pyridoxal phosphate led to a 4- to 5-fold increase in enzyme activity, with no difference between oestrogen-treated and control animals. This could be explained by competition between the hormone or its metabolites and pyridoxal phosphate, as suggested by Mason & Gullekson (1960). However, kinetic studies have shown that the inhibition of kynureninase by oestrone sulphate is uncompetitive with respect to pyridoxal phosphate (Bender & Wynick, 1981). It therefore appears that oestrone sulphate reacts only with the holoenzyme, and not to any significant extent with the apoenzyme.

A number of studies of women receiving oestrogens as oral contraceptive agents have shown that the administration of supplements of vitamin B₆ will restore tryptophan metabolism to normal, and this has been adduced as evidence that the oestrogens cause vitamin B₆ depletion (Rose & Adams, 1972). However, from the results reported here it is possible to advance an alternative explanation; there is a considerable amount of apo-kynureninase in the liver, as shown by the 4- to 5-fold increase in activity on addition of pyridoxal phosphate. Similar activation of a relatively large amount of apo-kynureninase in vivo would explain the effect of supplementary vitamin B₆ in overcoming the effects of oestrogens on tryptophan metabolism, regardless of vitamin B₆ status.

From the results reported here it seems that the disturbances of tryptophan metabolism reported in women receiving oestrogens, which have been attributed to vitamin B₆ depletion, can in fact be accounted for by a direct action of oestrogen metabolites on kynureninase. Although the administration of vitamin B₆ supplements will increase the activity of kynureninase, and hence normalize the metabolism of tryptophan, this does not necessarily show that there is a deficiency of the vitamin.

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