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THE METABOLIC FATE OF [^{3}H]ESTRADIOL IN RELATION TO DIETARY INTAKE OF BORON IN OVARIECTOMIZED RATS

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Summary—It has been reported that boron (B) deprivation reversibly lowers plasma estradiol levels in postmenopausal women. In order to establish whether this reflects disturbances in the estrogen catabolic pathway and in particular in catechol estrogen metabolism, the influence of dietary B on the catabolism of [^{3}H]estradiol-17β has been studied in ovariectomized rats. Rats were given diets containing <0.1 or 40 mg B.kg^{-1}, ovariectomized and then infused with [^{3}H]estradiol-17β using osmotic pumps. Analysis of urine samples for conjugated, catechol and non-catechol estrogens did not reveal any effects of B on the recovery or the metabolic fate of tritium from the infused estradiol. These results do not therefore support the proposal that B influences estrogen catabolism by interacting with catechol estrogens.

INTRODUCTION

Ovarian estradiol secretion declines rapidly at the menopause with deleterious consequences for the maintenance of bone mass. Hormone replacement therapy can reduce postmenopausal bone loss but is unsuitable for some individuals [1]. As there is a need for more natural methods of controlling bone loss, it is of interest that elevated dietary boron (B) intakes can have beneficial effects on bone, mineral and bone-related hormone levels in animals and humans [2, 3].

Although B is essential for the growth and development of plants, it has until recently been regarded as non-essential in animals. However Nielsen *et al.* [4] showed that increasing dietary intake of B from 0.25 to 3.25 mg.d^{-1} significantly increased plasma estradiol and testosterone concentrations and reduced urinary calcium excretion in postmenopausal women, especially when magnesium (Mg) intake was marginally adequate (200 mg.d^{-1}). B may therefore be an important nutritional factor determining the incidence of osteoporosis and supplements containing B are now sold as remedies for this condition. B is found mainly in foods of plant origin, particularly fruit and vegetable products [5], and so individuals at risk would be those consuming diets deficient in these foods.

It has been suggested (W. R. Humphries, personal communication) that borates could perturb estrogen catabolism by complex formation with catechol estrogens in an analogous way to the suggested mechanism for effects of molybdate on fertility in cattle [6]. In accord with this view, sodium tetraborate inhibited the methylation of 2-hydroxyestrone by catechol-*O*-methyltransferase *in vitro*, possibly by competing with the enzyme for the substrate by forming a complex across the 2′ and 3′ carbon hydroxyl groups [7]. Unlike the free catechol estrogen, such borate complexes are polar and therefore hydrophillic [8, 9]. These studies raise the possibility that borate could affect estrogen catabolism by (1) inhibiting methylation and thereby increasing major estrogen levels through negative feedback of the estrogen metabolic pathway or (2) diverting the pathway for estrogen excretion due to changes in the polarity of catechol estrogens. Borate complexation may also inhibit catechol estrogen conjugation at the 3′ carbon hydroxyl group.

A study was therefore designed to establish the effects of dietary B on the metabolic fate of exogenous radiolabelled estradiol in rats. The administration of radiolabelled estrogens has been used extensively to trace their metabolic fate [10], and more recently, to study the influence of dietary factors such as fibre on estrogen excretion [11]. A novel chemical extraction procedure was developed to quantify the distribution of radioactivity between conjugated, catechol and non-catechol urinary estrogen fractions.

EXPERIMENTAL

Animals

Two groups of 5 female rats (Hooded Lister, Rowett strain) aged 5 months and weighing about 260 g were individually housed in grid-base plastic cages. They were given a casein/sucrose/arachis oil based semi-synthetic diet [12] containing recommended levels of vitamins, trace elements and minerals but only 100 mg $Mg.kg^{-1}$. Molybdate and vanadate were excluded as they have some similar chemical properties to borate, the test anion. This Mg-deficient diet contained <0.1 mg $B.kg^{-1}$ and the distilled water, which was supplied *ad libitum*, contained 0.3 μg $B.l^{-1}$. B was analysed by inductively coupled plasma emission spectroscopy and diet samples were prepared for analysis in nitric acid using a sealed vessel microwave digestion technique [13]. The rats were maintained at 20°C with a 12 h light/dark cycle.

Infusion solution

Estradiol secretion rate by the ovaries of adult female rats is about 3 $ng.h^{-1}$ at di-estrus, increasing to just over 12 $ng.h^{-1}$ at pro-estrus [14]. In an attempt to maintain estradiol at low but physiological levels after ovariectomy, a pump delivery rate of 3 $ng.h^{-1}$ was selected. 37 MBq of [6,7-^{3}H]estradiol (2035 GBq/mmol, Amersham Int., England) in toluene was diluted with 11.39 μg of unlabelled estradiol in ethanol and the solution was evaporated to dryness under nitrogen. The estradiol was re-dissolved in 50 μl of ethanol and diluted with 2.95 ml of 0.1% fatty-acid free rat serum albumin in water (Sigma Chemical Co., England), which was added as a carrier for estradiol. The solution was sterilized by filtration through a 0.2 μM membrane and duplicate 25 μl aliquots were retained for measurement of radioactivity. Alzet osmotic pumps (Model 2002, Alza Corp., Palo Alto, CA, U.S.A.) with a capacity of approx. 220 μl were filled with the estradiol solution under sterile conditions and placed in sterile saline at 37°C for no longer than 2 h before implantation in the rats. At the end of the study, each pump was removed and the residual volume of solution measured.

Study protocol

Seven days after the rats were given the low B diet, one group was given the same diet supplemented with 40 mg $B.kg^{-1}$ (B-supplemented group) while the other continued to receive the unsupplemented diet (B-depleted group). On day 13, bilateral ovariectomies were performed on each rat under ether anaesthesia and the osmotic pump primed with [^{3}H]estradiol was placed subcutaneously in an interscapular position. The animals, were monitored over the next 48-h and were returned to preoperative cage conditions on resuming normal behaviour and feeding. From days 15 to 22, they were individually housed in metabolic cages and 24-h urine samples were collected in receptacles containing 1.5 ml of 1.0 M sodium acetate buffer and 50 mM ascorbic acid, pH 3.0 to preserve urinary catechol estrogens in a reduced and stable form [15]. The urine volume was determined and samples were frozen at -20°C until required.

Separation of urinary estrogen fractions

The urine samples from each rat were pooled, filtered through Whatman No. 1 filter paper, diluted to 105 ml with distilled water and a 35 ml aliquot was passed through a C_{18} Sep-Pak cartridge (Waters, Watford, England) prepared first with methanol and then with 0.1 M sodium acetate buffer pH 4.5. The effluent was collected to monitor radioactivity. After washing the cartridge with excess acetate buffer, estrogens were eluted with 5 ml of methanol. The cartridge was re-equilibrated with acetate buffer and the procedure was repeated twice for the remaining 2×35 ml aliquots. The pooled methanol extracts were dried in screw cap glass tubes under a stream of nitrogen gas, and the free and conjugated estrogens were separated on the basis of their polarity by adding 2.5 ml of ethyl acetate containing 0.07% 2-mercaptoethanol followed by 1 ml of 0.85 M carbonate buffer pH 10.5. The tubes were sealed under nitrogen, shaken for 30 min, and the aqueous and solvent phases transferred to clean tubes. Catechol estrogens were separated from non-catechol estrogens by adding 1 ml of 0.1 M borate buffer, pH 10.5, sealing the tubes under nitrogen and shaking for 30 min. This step was repeated and the pooled aqueous phases containing the catechol estrogens, were acidified with 0.2 ml of 6 M HCl. The catechol estrogens were then back-extracted into 5 ml of ethyl acetate containing 0.07% 2-mercaptoethanol.

The carbonate buffer containing the conjugated estrogens was diluted and passed through a further Sep-Pak cartridge, eluting bound components with methanol and drying extracts

under nitrogen. The dry samples were re-dissolved in 5 ml of hydrolysis buffer which contained 500 U of glucuronidase and 500 U or sulphatase (Sigma) in 0.15 M acetate/0.03 M ascorbic acid buffer, pH 4.1. After incubation at 39°C overnight, the samples were centrifuged at 1000*g* for 30 min and estrogens in the supernatant were again extracted using Sep-Pak cartridges and eluted with methanol. After drying the samples under nitrogen, the free estrogens were extracted and separated as described above.

To validate these methods, diluted non-radioactive urine samples were passed through Sep-Pak cartridges as described above and radiolabelled estrogen standards were added to the methanol elution fractions in order to determine the recovery of radioactivity at each step in the extraction procedure. These tests were carried out 3 times using duplicates of each of the following estrogens: [3,4,6,7-^{3}H]estradiol (3256 GBq/mmol) and [^{3}H]estrone-3-glucuronide (466.2 GBq/mmol) from Amersham and [^{3}H]estriol (3885 GBq/mmol) from New England Nuclear (England). Radiolabelled 2-hydroxyestradiol was prepared from [^{14}C]estradiol (2.072 GBq/mmol, Amersham) using the tyrosinase hydroxylation method of Jellinck and Brown [16] and subsequent purification by Sephadex LH-20 chromatography (column dimensions 0.6 × 15 cm) using a methanol–toluene (15:85, v/v) solvent containing 0.07% mercaptoethanol. The yield of purified catechol estrogen, which eluted well after the unhydroxylated substrate, was approx. 50%. The identity and purity of the product was verified by GC/MS. Tritiated estrone was obtained by hydrolysis of [^{3}H]estrone-3-glucuronide as described above.

The radioactivity of aliquots of each extract throughout the separation procedure was measured using NE265 liquid scintillator (NE Technology Ltd, Edinburgh, Scotland) and a Packard Tricarb 1900CA scintillation counter. Thus the recovery of steroids in each extract fraction could be calculated, taking into account the cumulative removal of samples for radioactivity counting. The counting efficiencies were 45 to 47% for tritium and 90 to 94% for ^{14}C.

RESULTS

Recovery of standard estrogens

Recoveries of tritiated or ^{14}C-labelled standard estrogens at critical steps in the extraction procedure are shown in Table 1. Due to the strong polarity of conjugated estrogens, 96% of estrone-3-glucuronide was recovered in the carbonate buffer fraction whereas >93% of the hydrophobic, major estrogens were found in the ethyl acetate phase. Similarly, 88% of the catechol estrogen 2-hydroxyestradiol was recovered in the organic phase with 52% being transferred back into the aqueous phase during the subsequent extraction with borate buffer. The major estrogens remained in the organic fraction on extraction with borate.

Urinary estrogen excretion in relation to B intake

The osmotic pumps were almost empty when removed from the rats and the total radioactivity dispensed to the B-depleted and -supplemented rats was (mean ± SD) 2.075 ± 0.007 and 2.112 ± 0.004 MBq, respectively. Recovery of tritium in the pooled 7-day urine samples was 2.52 ± 1.15 and 2.93 ± 1.11% in B-depleted and supplemented animals, respectively. This difference was not significant (unpaired *t*-test).

Recovery of radioactivity at each stage in the extraction of urinary estrogens is summarized in Fig. 1 for B-depleted and -supplemented rats and a comparison of the radioactivity distribution in selected urine fractions is presented in Table 2. The largest proportion of tritium was recovered in the conjugated estrogen fraction and the remainder was divided almost equally between the non-catechol and the catechol fractions. Hydrolysis of the glucuronide and sulphate conjugates revealed a slight excess of

Table 1. The recovery of radiolabelled standard estrogens at different stages in the urine separation procedure

Estrogen	Radioactivity added (dpm × 10^{-3})	Carbonate extraction step		Borate extraction step	
		Aqueous	Solvent	Aqueous	Solvent
Estrone	10,000	6.3 ± 0.8	93.7 ± 0.8	1.3 ± 0.8	92.4 ± 0.8
Estradiol	30,000	6.5 ± 0.6	93.5 ± 0.6	4.7 ± 0.6	88.8 ± 0.6
Estriol	36,000	3.3 ± 0.7	96.7 ± 0.7	1.3 ± 1.3	95.4 ± 1.3
Estrone-3-glucuronide	22,000	95.8 ± 0.2	4.2 ± 0.2	0.9 ± 0.1	3.3 ± 0.1
2-Hydroxyestradiol	41.4	11.6 ± 2.5	88.4 ± 2.5	52.1 ± 0.8	36.3 ± 0.8

The standards were added to the Sep-Pak methanol extracts of urine samples and values are expressed as the mean ± SD percentages of the activity in this fraction.

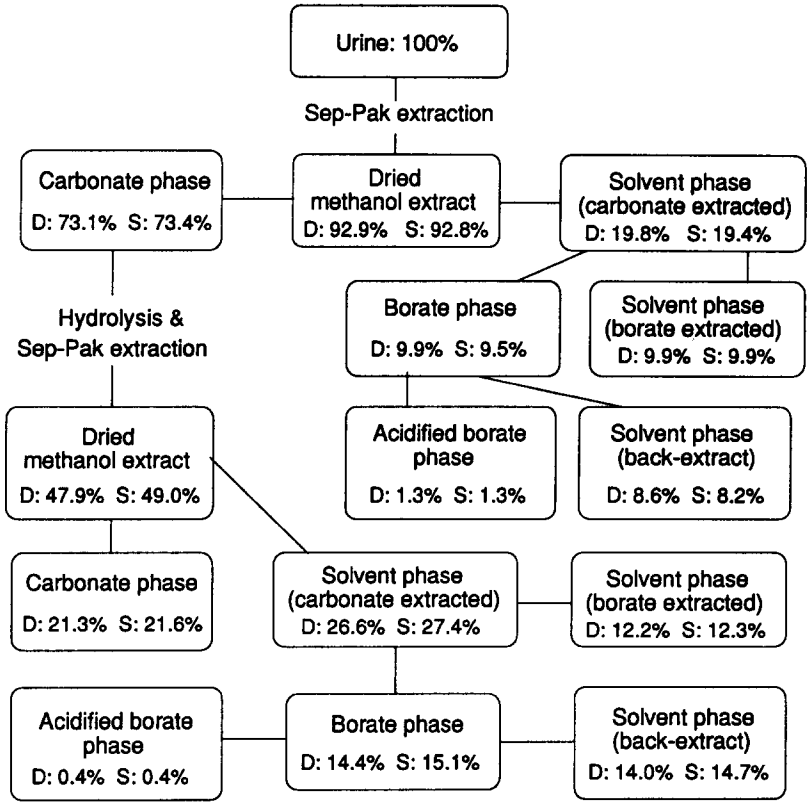

Fig. 1. Proportion of tritium in different fractions of urine from rats fed B-supplemented diets (40 mg $B.kg^{-1}$, S) and B-depleted animals receiving diets containing <0.1 mg $B.kg^{-1}$ (D). The values have been corrected for incomplete recovery of tritium during the extraction procedures and for samples removed to count radioactivity.

catechol over non-catechol estrogens. Dietary B had no significant effect on the distribution of radioactivity between any of the fractions ($P > 0.05$).

DISCUSSION

Radiolabelled steroids have been used extensively to investigate their metabolism *in vivo* and the present work describes a method whereby urinary catechol estrogen excretion can be measured without specialized preparative or assay techniques such as reverse phase HPLC [17]. To minimize problems of transfer of tritium from [^{3}H]estradiol and its metabolites to non-estrogen compounds, the estradiol used in this study was labelled only at carbon 6′ and 7′. Although these sites can be hydroxylated by 6- and 7-hydroxylase enzymes the production of 6- and 7-hydroxylated estrogens is not generally very significant [10]. However, it should not necessarily be assumed that all radioactivity in the urine fractions is associated with estrogens.

The recovery of tritium in the 7-day urine collections (2%) was lower than that reported following i.v. injection of radiolabelled estradiol

Table 2. The relative % distribution of tritium in 3 estrogen fractions from the urine of ovariectomized rats given a B-depleted diet (<0.1 $mg.kg^{-1}$) or a B-supplemented diet (40 $mg.kg^{-1}$) and implanted with osmotic pumps containing [^{3}H]estradiol-17β

		Free estrogens	
Dietary B ($mg.kg^{-1}$)	Conjugated estrogens	Non-catechol	Catechol
<0.1	79.8 ± 3.3	10.8 ± 1.9	9.4 ± 2.3
40	80.1 ± 1.1	10.9 ± 1.7	9.0 ± 1.9
	After hydrolysis		
<0.1	—	37.2 ± 3.3	42.6 ± 3.3
40	—	36.5 ± 3.4	43.6 ± 3.4

Values are the mean ± SD for 5 animals.

(14%) [11]. This suggests that the method of administration effects the rate and route of estrogen excretion. Presumably, a large proportion of the tritium was excreted in the faeces and there may also have been a significant exchange and therefore isotopic dilution from reserves of estrogens in adipose tissue.

The precision and specificity of partition of the conjugated, catechol and non-catechol estrogens between the various extracts were excellent as determined by spiking urine samples with radiolabelled estrogens. The low recovery of 52% for the extraction of the catechol estrogen 2-hydroxy[^{14}C]estradiol into borate buffer reflects the fact that the complex is in dynamic equilibrium and not covalently bound. Repeated extractions with fresh borate improved this recovery but also increased the degree of contamination by non-catechol estrogens (data not shown). A precision of 1.5% RSD on the catechol estrogen recovery with $<5\%$ contamination by the major estrogens was achieved using 2 extractions with borate buffer and so this procedure was adopted for routine use.

Similar degrees of partition and precision were obtained for the conjugated estrogen [^{3}H]estrone-3-glucuronide, with $>95\%$ recovery in the carbonate phase. About 80% of the radioactivity in the urine from rats dosed with [^{3}H]estradiol was recovered in the conjugated form, which is in accord with previous reports on estrogens in urine, bile and faeces [10]. Several significant estrogen components have been identified including the non-catechol, major estrogens estrone, estradiol-17β and estriol and several catechol estrogens, namely 2-hydroxyestrone, 2-hydroxyestradiol-17β and 4-hydroxyestrone [18]. It appears the molar ratio of non-catechol:catechol estrogens in the urine of pre- or postmenopausal women is about 1.5 [18]. However, there was slightly more labelled catechol than non-catechol estrogens in the conjugated fraction of the rat urine. There is normally a significant contribution to total estrogen excretion in the form of methoxyestrogens although these steroids are very insoluble in aqueous media, including carbonate and borate buffers (data not shown), and would contribute to the radioactivity in the solvent extracts containing the major estrogens.

The wide difference in dietary B intake had no effect on the amount or distribution of radioactivity in the urine and therefore, by implication, on the excretion rate and metabolic fate of estradiol. These data do not therefore support the hypothesis that B influences the catabolism of major estrogens [3] or the finding that borate inhibits the *O*-methylation of catechol estrogens *in vitro* [7].

It is possible that 10 days of B deprivation was insufficient to deplete body stores of B. Nielsen suggests that a long preliminary period of B deprivation is essential before the subsequent effects of B supplementation can be demonstrated (see comments, [19]), but there have been no systematic studies on the effect of deprivation period. Indeed there seem to be few ligands in animal cells capable of binding and retaining B compounds and hence they are excreted rapidly [20]. Since bone is turned over relatively slowly, the contribution of B released from this source could prolong the period to depletion equilibrium. However, bone B concentrations in animals with a normal B intake are generally within one order of magnitude of those in other tissues such as liver and kidney [21]. Furthermore, the urinary excretion of B by postmenopausal women decreased rapidly during the first 3 days after they were given a low B diet (0.33 mg $B.d^{-1}$) but remained relatively constant for the following 18 days, suggesting that the amount entering the circulation from bone was relatively insignificant compared to the contribution from dietary sources [13].

It seems unlikely that the effects of B on plasma estradiol levels of postmenopausal women [4] are related to perturbations in estrogen catabolism or excretion. However there remains the possibility that estradiol synthesis is enhanced by B, perhaps by the metabolic interconversion of estrone to estradiol.

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