Borate and Molybdate Inhibition of Catechol Estrogen and Pyrocatechol Methylation by Catechol-O-Methyltransferase

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ABSTRACT

The possibility that boron and molybdenum anions can influence sex steroid metabolism by forming complexes with catechol estrogens has been studied in vitro. The formation of 2-methoxyestrone (2-OHE_1 2-Me) from 2-hydroxyestrone (2-OHE_1) by catechol-O-methyltransferase (COMT) was followed by measuring the transfer of the radiolabeled methyl group from S-adenosylmethionine. In the presence of both sodium tetraborate and sodium molybdate using a phosphate buffer medium, the formation of 2-OHE_1 2-Me decreased as the anion:2-OHE_1 molar ratio was increased. However, the reverse effect was observed when using a tris buffer medium and further investigation showed that phosphate and sulphate also enhanced COMT activity in a tris buffer medium. Boric acid affinity medium, used as a substitute for borate salt, also showed a negative relationship with enzyme activity in a phosphate buffer medium, and inhibition of methylation was more marked than with the free anion. Erythrocytes contain appreciable amounts of COMT, which is mostly responsible for the rapid O-methylation of catechol estrogens in blood. The methylation of a simple catechol compound, 1,2-dihydroxybenzene (pyrocatechol) was therefore studied using rat red blood cell lysates. Methylation was inhibited in a concentration-related manner by borate, as found in the studies of 2-OHE_1. It is possible that high dietary intakes of boron or molybdenum could regulate the rate of catabolism, or even the metabolic fate of the major estrogens.

INTRODUCTION

Boron has many important biochemical roles in plant growth and development [1] and its apparent lack of essentiality in animals is therefore surprising. However, recent evidence suggests that postmenopausal women receiving a dietary supplement of 3 mg boron.d^{-1} after lengthy acclimation to a low-boron diet show increased plasma testosterone and estradiol-17β concentrations and decreased urinary calcium

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excretion, particularly when dietary magnesium is low [2]. An interaction of boron compounds with steroid metabolism, possibly involving hydroxylation reactions, has been suggested [3].

The biochemical properties of boron which underlie its essential role in plants and its toxicity in plants and animals have been studied extensively [4]. The effect of boron is mainly attributed to the affinity of borate ions for polyhydroxyl groups on biologically important molecules, with disturbance of enzymatic reactions and even the fate of many intermediates in a variety of metabolic pathways. The toxicity of boron has been attributed to similar complex formation with biologically active molecules such as riboflavin. Indeed, the deleterious effects of boron can be ameliorated by the administration of polyhydroxyl compounds [5].

Other anions such as molybdate form complexes with hydroxylated compounds [6], and both molybdate and borate complex across the cis vicinal hydroxyl groups of catechol estrogens at alkaline pH. It has been suggested (W. R. Humphries, personal communication) that such an interaction in vivo could interfere with estrogen metabolism and explain the reduced circulating levels of lutenizing hormone in cattle on high molybdenum diets [7] and elevated levels of plasma estradiol and testosterone in postmenopausal women taking boron supplements [2]. In order to investigate this hypothesis, we have studied the influence of borate and molybdate anions on the methylation of the catechol estrogen, 2-hydroxyestrone (2-OHE$_1$) by catechol-O-methyltransferase (COMT). Since erythrocytes contain considerable amounts of COMT, which is responsible for the rapid methylation of blood catechol estrogens, we have also studied the methylation of a simple catechol compound, pyrocatechol or 1,2-dihydroxybenzene (DHB), by rat erythrocyte COMT.

MATERIALS AND METHODS

Purified COMT Assays Using Phosphate Buffer

The methylation of 2-OHE$_1$ was studied in vitro using the method of Zürcher and Da Prada [8], with some modifications. Unless otherwise specified, all biochemicals were obtained from Sigma Chemical Co. (Poole, U.K.) and chemicals, which were of AnalaR, AristaR, or Scintran grade, were purchased from BDH (Poole, U.K.). A 33.3 mM solution of 2-OHE$_1$ in 67% ethanol was prepared, diluted as appropriate, and added to an equal volume of a 1% solution of fatty acid-free bovine serum albumin (BSA). The BSA acted as a carrier for the nonpolar catechol estrogen in the aqueous environment of the incubation medium. 90μl aliquots of this solution were added to 20 ml scintillation vials followed by 45 or 90 μl of 0.1 M potassium phosphate buffer, pH 7.6, or the buffered test anion (see below). The resulting solutions were allowed to stand at room temperature for 10 min and 20μl of an aqueous 5.5 mM solution of [^{3}H]S-adenosyl methionine (SAM: specific activity 15 Ci.mmol^{-1} (Amersham International, U.K.) diluted to 3.64 Ci.mol^{-1} with unlabeled SAM) was added. Following addition of 20 μl of 100 mM $MgCl_2$, the solution was made up to a final volume of 500 μl with 0.1 M phosphate buffer.

The enzyme reaction was started by adding 50 units of purified porcine COMT (specific activity 2000 U.mg protein^{-1}) in 100 μl of potassium phosphate buffer, pH 7.6, containing 4.7 U of adenosine deaminase (ADA) and the solutions were incubated in a water bath at 37°C for 30 min. The reaction was terminated by placing the vials on ice and adding 500 μl of 1.0 M HCl.

Control samples contained no borate or molybdate and blanks were prepared by placing samples identical to the controls on ice and adding the HCl prior to the enzyme. In one series of test assays, the catechol estrogen substrate was maintained at a concentration of 2.5 mM (1.5 μmoles/assay vial) and buffered sodium tetraborate and sodium molybdate were added to give a range of boron and molybdenum concentrations from 2.5–160 mM (anion:2-OHE_1 molar ratios of 1:1 to 64:1). Two replicates were used for each incubation condition.

In a second study, sodium tetraborate was replaced by boric acid affinity medium (Sigma Chemical Co.). The boron atoms in this gel are bonded to an inert polymer but they retain two reactive hydroxyl ligands which can complex with the cis vicinal hydroxyls of compounds in solution. The dry gel was prepared as recommended by the manufacturer and was allowed to hydrate overnight in the phosphate incubation buffer. The gel was drained of buffer on filter paper and weighed quantities were transferred to the incubation medium before commencing the assay. The molarity of boric acid groups on the gel were derived from the binding capacity, and quantities of gel used were similar on a boron molar basis to that of sodium tetraborate.

To investigate the influence of borate and molybdate on the kinetics of 2-OHE_1 methylation by COMT, the estrogen substrate concentration was varied over the range of 0.00625–0.1 mM while maintaining the test anion concentration at 72.7 mM. The incubation time was 20 min because this was shown in preliminary studies to be within the linear initial rate of the reaction.

Phosphate buffer is commonly used for the determination of COMT activity because it is a medium of physiological relevance. However, phosphate also has potential to form chemical complexes with components in the incubation medium. A further study was therefore carried out to assess the effect of various anions, including phosphate, sulphate, and nitrate, on COMT activity using a 0.1 M tris-HCl buffer at pH 7.6 instead of the 0.1 M phosphate buffer. All anions were added as the sodium salts which were prepared by adding sodium hydroxide to appropriate acid solutions to achieve a pH of 7.6. The anion concentration in the incubation media was 72.7 mM.

Rat Erythrocyte COMT Assay

A blood sample was obtained by cardiac puncture from an adult male rat (Rowett Hooded Lister) under ether anesthesia. One milliliter of the heparinized blood was diluted with 10 ml of saline and then centrifuged at $1000 \times g$ for 10 min. The supernatant and white cells were discarded and the pellet was washed with saline and recentrifuged. The cell pellet was lysed in 2 ml of a 0.2% solution of Triton X-100 containing 0.002% dithiothreitol. The assay procedure using phosphate buffer was identical to that described above except that 100 μl of red blood cell (RBC) lysate replaced the addition of purified COMT. Adenosine deaminase was added separately to the incubation medium prior to the addition of the RBC lysate. The incubation time was 45 min.

Measurement of Product Formation

To each sample vial was added 10 ml of scintillation fluid, 0.5% butyl-PBD in 2:8 toluene:n-hexane, and the contents were shaken vigorously for 1 min. This extracted the tritiated product, 2-OHE_1 2-[^{3}H]Me or [^{3}H]guaiacol, into the scintillation fluid and the unmetabolized [^{3}H]SAM remained in the lower, aqueous phase Radioactivity was measured directly using a Packard Tricarb 1900CA scintillation counter. In

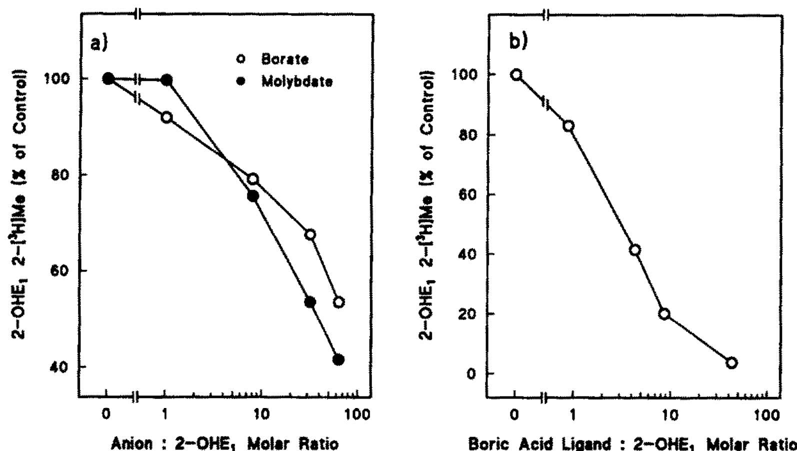

FIGURE 1. The influence of (a) sodium tetraborate and sodium molybdate and (b) boric acid affinity medium on the formation of 2-[^{3}H]methoxyestrogen (2-OHE$_1$ 2-[^{2}H]Me) from 2-hydroxyestrone and S-adenosyl-[^{3}H]methionine by purified porcine catechol-O-methyltransferase in phosphate incubation buffer. Values are presented as a percent of the control which contained no binding ligand (molar ratio 0) and gave radioactivity of 81700 DPM for (a) and 46800 DPM for (b). The estrogen concentration was 2.5 mM.

the case of the more concentrated molybdate and RBC lysate samples, the color in the aqueous phase resulted in some quenching and the scintillation fluid was therefore removed and counted separately. Blank solutions gave counts of about 2000 DPM and the counting efficiency was approximately 49%.

RESULTS AND DISCUSSION

This study was designed to test the hypothesis that complex formation between molybdate or borate and catechol estrogens could result in reduced methylation of the hormone, with subsequent disturbances in its metabolism. When this hypothesis was tested by incubating mixtures of the anion, hormone, SAM, cofactor, and COMT enzyme in phosphate buffer at pH 7.6, there was indeed significant inhibition of methylation (Fig. 1). Moreover, methylation was almost totally inhibited when boron was added to the system in the form of boric acid affinity medium at a B:estrogen molar ratio of 43:1. Anionic borate, molybdate, and the affinity boric acid medium at a mineral:estrogen molar ratio of 8:1, reduced methylation to only 79%, 76%, and 20%, respectively, of that in the control.

On varying the estrogen substrate concentration at fixed levels of borate and molybdate, the oxyanions had a marked influence on the reaction kinetics. Both anions increased the slope of the double reciprocal plot of substrate concentration versus velocity and also increased the K_m from the control value of 7.3×10^{-5} M, suggesting a competitive type of inhibition (Fig. 2). Classically, this would indicate that the inhibitor competes with the substrate for the active site of the enzyme. However, an interaction of inhibitor with substrate which prevents the formation of an enzyme-substrate complex also shows competative inhibition kinetics [16]. In this instance, the competition of oxyanions and 2-OHE$_1$ for the enzyme active site is possible since its activity is dependent on a thiol group [15] and thiols may react with

oxyanions. More probable is an interaction of the borate and molybdate with the estrogen 3'hydroxyl group which is the site of methylation by COMT. The boric acid affinity medium has a high specificity for polyhydroxyl compounds and methylation inhibition found in this study was most likely caused by sequestration of 2-OHE$_1$ substrate. Therefore, as regards free anions, the inhibition is probably explained by the formation of borate and molybdate anion complexes with the coplanar 2- and 2-hydroxyl ligands which constitute the estrogen catechol group. This form of inhibition is concordant with borate-mediated regulation of enzyme reactions in plant metabolism [4]. Apparent differences in V_{max} values derived from all three reciprocal plots (Fig. 2) suggest additional influences of the the anions on rate limiting reactions and may be related to the stimulatory effects of the anions on enzyme activity discussed below. As compared to the control incubations (absence of borate and molybdate), the V_{max} was decreased in the presence of borate but increased in the presence of molybdate.

Dissociation equilibria for various catechol compounds have been studied with both molybdate and borate. ^{11}B nuclear magnetic resonance studies of DHB and another catechol compound, L-dopa, show that the borate-catechol complex is in dynamic equilibrium and speciation and stability constants are influenced by pH and the molar ratio of the reacting ligands [9]. They found that alkaline conditions favor complex formation between borate and catechol groups and that a large proportion of catechol was in the form of a complex at pH 7.6 and a molar ratio of 1:1. The kinetics of molybdate complex formation with DHB have also been studied [6], and as shown for borate, speciation is related to changes in the molar ratio of reacting ligands and in the pH.

In all studies with 2-OHE$_1$, the steroid was bound to BSA to maintain solubility but this did not appear to impede the O-methylation by COMT. The immediate formation of a colored complex with molybdate confirmed that anions were free to associate with the estrogen catechol group in the presence of BSA. However, recent attempts to determine the dissociation constant of the catechol-molybdate complex suggest that the affinity of the catechol group for molybdate increases with increasing albumin concentration (A. Weir, personal communication). It is possible that certain anions such as phosphate and indeed, molybdate itself, affect the association of catechol estrogens with albumin, thus modifying their availability for methylation. This could explain the increase in estrogen methylation found on addition of certain anions (see below).

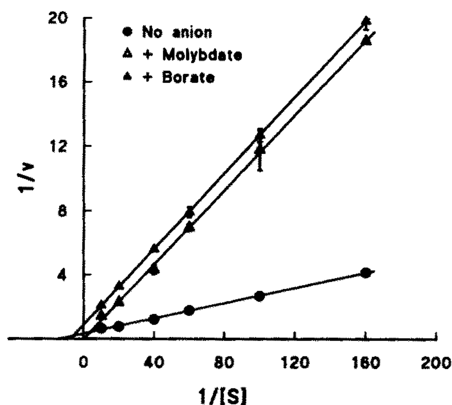

FIGURE 2. Lineweaver-Burk double reciprocal plots of methylation rate versus the concentration of 2-OHE$_1$ (0.00625–0.1 mM) in the presence of 72.7 mM borate ($y = 0.118x + 0.874$), 72.7 mM molybdate ($y = 0.116x - 0.029$) and in the absence of borate and molybdate ($y = 0.024x + 0.331$).

Over 80% of catechol estrogens in plasma are transported bound to albumin [10] and the present results indicate that in this respect an anion interaction in vivo is plausible. Even accounting for possible interactions with other polyhydroxyl compounds, high circulating levels of boron may retard the rate of catechol estrogen methylation which, under normal conditions, is known to be rapid in blood [11]. Methylation of 1,2-dihydroxybenzene by rat erythrocyte COMT was markedly inhibited by the addition of sodium tetraborate as can be seen from the relationship in Figure 3. As shown for 2-OHE_1, the inhibition of methylation was directly related to the anion:catechol molar ratio and the formation of [^{3}H]guaiacol was only 40% of that found in the control at a molar ratio of 8:1. Inhibition was all but complete at the highest ratio of 64:1. This indicates that inhibition of catechol methylation by borate within the erythrocyte in vivo is possible.

In these investigations, reactions were carried out in phosphate buffer because it has physiological relevance and has been used previously in the study of COMT activity. However, interactions can occur between molybdate and phosphate to form phosphomolybdate complexes and these are the basis of colorimetric assays for phosphate. To test whether such a reaction occurs at alkaline pH, absorption spectra of the DHB-molybdate complex in the absence or presence of phosphate (1:1 molar ratio) were compared using 0.1 M tris HCl buffer at pH 7.6. The absorption spectra (not shown) showed no change in absorbance over the range of 200–500 nm and no wavelength shift in absorption maxima on addition of phosphate. Increasing the phosphate:molybdate or nitrate:molybdate molar ratios from 1:5 to 2:1 had no influence on absorbance at 400 nm due to the molybdate-catechol complex. It can be concluded that phosphate does not react with either molybdate or the catechol group of DHB in conditions similar to those of the enzyme assay.

Nevertheless, systematic study of the effects of phosphate and of other buffers or anions revealed that they did have a significant influence on the rate of methylation of 2-OHE_1. Thus COMT activity was 2.5 times greater in 0.1 M phosphate buffer than in 0.1 M tris buffer, both at pH 7.6, and increased in relation to increasing phosphate concentration (Table 1). This enhancement in COMT activity was also found, to a lesser extent, when other anions such as sulphate, molybdate, and borate were added to the tris buffer (Table 1). Chloride and nitrate had no effect. This stimulation in COMT activity by molybdate and borate in tris buffer is therefore the opposite of the inhibition observed in phosphate buffer.

As stated above, there was no evidence of reactions between phosphate and either molybdate or the catechol groups in conditions similar to those of the enzyme assay.

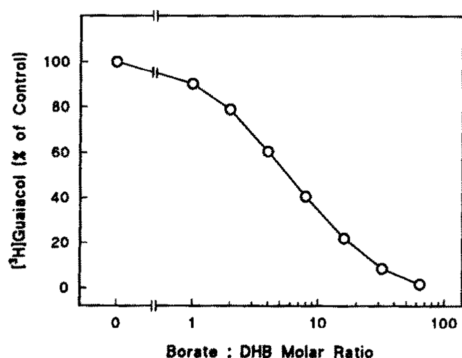

FIGURE 3. The influence of sodium tetraborate on the formation of [^{3}H]guaiacol from 1,2-dihydroxybenzene (DHB) and S-adenosyl-[^{3}H]methionine by rat red blood cell lysate. Values are presented as a percent of the control which contained no binding ligand (molar ratio 0) and gave a radioactive count of 69800 DPM. The DHB concentration was 2.5 mM.

TABLE 1. The Influence of Different Buffers and Anions on COMT Activity (Production of 2-[^{3}H]Methoxyestrogen) at pH 7.6

Phosphate Buffer	Anion Added	Activity (DPM)	
0.05 M	None	90482 ± 55	
0.1 M	None	111009 ± 2048	
0.5 M	None	140727 ± 354	
Tris Buffer	**Anion Added**	**Activity (DPM)**	**Activity Relative to Control (%)**
	None (control)	45544 ± 365	100
	Nitrate	43963 ± 571	96.5
	Sulphate	71268 ± 561	156.5
0.1 M	Phosphate	97758 ± 134	214.6
	Borate	54468 ± 966	119.6
	Molybdate	77153 ± 911	169.4
	Chloride	46667 ± 535	102.5

The 2-OHE$_1$ level was 2.5 mM and additional anions were added to a concentration of 72.7 mM.

Since the enzyme reaction rate was greatly affected by changing the phosphate buffer concentration in the absence of tris, it seems that changes in the methylation rate have more to do with the phosphate concentration than the presence of tris. Chemical precipitation of Mg, the cofactor for COMT activity, as the phosphate salt is an unlikely explanation since the enzyme activity actually increased with increasing phosphate levels, moreover, sodium sulphate also enhanced activity and Mg sulphate is water soluble. No evidence of precipitate formation was found in any of the enzyme assays.

By a process of elimination, it appears that certain anions have a direct effect either on COMT or on the cosubstrate SAM, resulting in an increase in enzyme activity. A third possibility may relate to the association of the estrogen with albumin which was discussed above. Since molybdate and phosphate on their own enhanced COMT activity in tris buffer but the addition of molybdate to phosphate buffer inhibited activity compared to the buffer alone, molybdate anions may have an additional and independent inhibitory effect on COMT activity.

Molybdate is used to stabilize steroid hormone receptors in receptor binding studies in vitro [12], but until recently there was no evidence that it influenced estrogen metabolism in vivo. However, it has now been shown that high dietary intakes of molybdate reduce circulating levels of lutenizing hormone in cattle [7] and that injection of female rats with the molybdate complex of 2-OHE$_1$ diminishes the estrogenic response, as indicated by the changes in ovarian and uterine weight after a subsequent injection of estradiol (W. R. Humphries, personal communication). It is suggested that a reduction in the O-methylation of the catechol estrogen due to complexation with molybdate could enhance competition between 2-OHE$_1$ (or the complex) and estradiol for sites on estrogen receptors.

On forming a complex with borate anions, the catechol estrogens are transformed from their naturally hydrophobic state to become polar and soluble in an aqueous medium [13]. The polarity of conjugated steroids is a major factor enhancing their excretion [14] and complex formation of borate and other anions with the catechol

estrogens may modify the rate and route of steriod excretion. It may also modify the form in which they are excreted since the formation of a complex is likely to inhibit conjugation of the molecule at the 3-hydroxyl position. The influence of borates on the metabolic fate of estrogens is under investigation.

In conclusion, the incubation buffer used for the study of COMT activity has a major influence on this enzyme under our assay conditions, which has significant implications for the analysis of COMT in biological samples. Both borate and molybdate anions inhibit, in vitro, the 2-hydroxyl O-methylation of 2-OHE_1 by the enzyme COMT in a phosphate buffer medium. A similar effect of boric acid affinity gel suggests that boron inhibits the enzyme reaction through association with the 2- and 3-hydroxyl ligands that constitute the estrogen catechol group. The physiological significance of these interactions is still unknown but the results suggest that both borate and molybdate anions could influence estrogen metabolism.

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