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EFFECT OF PHOSPHATE ESTERS OF OESTRADIOL AND OESTRONE ON THE RECONSTITUTION OF ASPARTATE AMINOTRANSFERASE

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SUMMARY

Phosphate esters of oestradiol and oestrone inhibit the *in vitro* reconstitution of the aspartate aminotransferase. The inhibition is due to an interaction of the phosphate esters and the apoenzyme with formation of an inactive complex. By comparing the dissociation constant of these complexes and that of the complexes formed by the apoenzyme and the phosphate esters of non-oestrogenic steroids, it appears that the inhibitory effect is specific for oestrogen phosphates and can be related to structural features of their molecule.

INTRODUCTION

Sulphate and phosphate esters of oestradiol and stilboestrol were reported to inhibit specifically the *in vitro* reconstitution, i.e. the reactivation of apoenzyme with coenzyme, of two pyridoxal 5′-phosphate-dependent enzymes, namely kynurenine aminotransferase (EC 2.6.1.7) and aspartate aminotransferase (EC 2.6.1.1), without otherwise influencing the catalytic activity of the reconstituted holoenzymes [1, 2]. The inhibitory effect of the oestrogen esters was ascribed to a competition with the coenzyme for the combination with the apoenzyme [2]. Starting from this assumption, a possible physiological role of the oestrogens, consisting in controlling through their esters the tissue level of active aminotransferases, was also suggested [2]. Subsequent experiments on the isolated rat heart, in which the level of active aspartate aminotransferase, alanine aminotransferase (EC 2.6.1.2) and tyrosine aminotransferase (EC 2.6.1.5) was readily depressed upon perfusion with oestrogen esters, seemed to substantiate the suggested role of the oestrogens [3].

Further research demonstrated the existence of a correlation between the oestrogenic activity of some stilboestrol analogues [4] and the inhibitory activity of their phosphate esters on the reconstitution of the aspartate aminotransferase [5]. As a consequence of this and of new facts recently acquired in our laboratory during a kinetic study of the interaction between these esters and pure aspartate apo-aminotransferase [6]. It became of interest to reinvestigate the role of the phosphate esters of natural oestrogens (sulphate esters being much less active inhibitors) with special regard to their specificity and mode of action.

Abbreviations—in this paper, oestrogen esters refer to phosphates and sulphates of oestrone, oestradiol and stilboestrol; phosphate esters to phosphates of both oestrogenic and non-oestrogenic steroids.

EXPERIMENTAL

Materials

The apoenzyme preparation of aspartate aminotransferase, [7], retained 7–8% of original activity due to the presence of some unresolved holoenzyme. The apoenzyme concentration was measured spectrophotometrically at 278 nm by using an extinction coefficient $E^{1\%}_{1cm}$ of 14·40 and a minimum molecular weight of 47,000. The presence of 0·1 mM glutathione permitted storage of the apoenzyme solution at +5°C for several days, without affecting the reconstitution reaction with the coenzyme.

Pyridoxal 5′-phosphate was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Oestradiol 3-phosphate, oestradiol 17-phosphate, oestradiol 3,17-diphosphate and oestrone 3-phosphate were from AB Leo, Halsingborg, Sweden. Except for cortisol 21-phosphate, which was a Sigma product, the phosphate esters of the non-oestrogenic steroids were samples obtained from Prof. V. Botte, Institute of Histology and Embryology, Faculty of Science, University of Naples.

Methods

The reconstitution rate of aspartate aminotransferase was determined by measuring the activity of the

holoenzyme formed on incubation of apoenzyme with a 20-fold excess of coenzyme at room temperature for 7 s, as described by Gianfreda *et al.*[8]. A sample of 25 μl containing 0·1 nm of apoenzyme in 0·1 M Tris-HCl buffer, pH 8·3, with or without the phosphate ester under examination was withdrawn from a larger volume (100–200 μl) after a minimum incubation time of 3 min at 22 ± 1 C and added to 2 μl of a solution containing 2 nm of pyridoxal 5′-phosphate on the bent end of a small plastic spatula. After exactly 7 s, the reconstitution reaction was arrested by dipping the end of the spatula into a quartz cuvette containing 2·7 ml of the reagent mixture for the spectrophotometric assay of the aspartate aminotransferase activity according to Jenkins *et al.*[9]. Under these conditions, the activity measured represents the amount of apoenzyme reactivated (or holoenzyme formed) in 7 s, providing an indication on the velocity of reconstitution. All measurements were carried out in a Beckman DB spectrophotometer recording with a thermostatically-controlled cell-holder.

RESULTS

In previous work [2] the inhibitory effect of oestrogen esters on the reconstitution of aspartate aminotransferase was ascribed to a competition between esters and pyridoxal 5′-phosphate for the apoenzyme. This interpretation was based on the fact that the inhibition was overrun by prolonging the incubation time, i.e. the apoenzyme was almost completely reactivated by the coenzyme [2]. In the present investigation the inhibition of the reconstitution process has been reinterpreted on the basis of the following considerations. Phosphate esters inhibit the reconstitution by depressing its normal rate, without affecting the catalytic activity of the holoenzyme; in other words, phosphate esters do not displace the coenzyme from the holoenzyme, irrespective of their concentration (as compared to that of the coenzyme) and their affinity for the apoenzyme. Therefore, the inhibition cannot be considered as fully reversible and competitive, in contrast to what was seen [2]. It should be assumed that the phosphate ester, I, interacts with the apoenzyme, A, yielding an inactive complex, AI, the interaction occurring near or at the active site and thus inhibiting the binding of the coenzyme, P. The interaction proceeds rapidly to an equilibrium (equation 1), in contrast to the reconstitution, which is an irreversible and comparatively slow process (Eq. 2).

$$A + I \rightleftarrows AI \quad (1)$$

$$A + P \xrightarrow{k} AP. \quad (2)$$

Provided the coenzyme is in excess with respect to the apoenzyme, the reconstitution rate in the absence of phosphate esters is proportional to the concentration of apoenzyme.

$$v = k'(A_0) \quad (3)$$

where k' is a pseudo-first order rate constant given by $k(P)$; in the presence of phosphate esters the reconstitution rate is proportional to the concentration of the free apoenzyme,

$$v_i = k'(A) \quad (4)$$

where $(A) = (A_0) - (AI)$. It can be easily demonstrated that the rate ratio of uninhibited and inhibited reconstitution is given by the following equation:

$$v/v_i = 1 + (I)/K_i \quad (5)$$

where (I) is the phosphate ester concentration and K_i the dissociation constant of the inactive complex AI, i.e. $K_i = (A)(I)/(AI)$.

A plot of v/v_i against (I) will give a straight line which cuts the base-line at a value of $-(I)$ equal to K_i. The K_i values obtained from the plots of Fig. 1 are 0·167, 0·212, 0·337 and 0·640 mM respectively for oestradiol 17-phosphate, oestradiol 3,17-phosphate, oestradiol 3-phosphate and oestrone 3-phosphate.

Owing to the low inhibitory effect of the phosphate esters of the non-oestrogenic steroids examined, the corresponding K_i values were calculated by plotting the relative reconstitution rate, v_i/v, against the logarithm of the reciprocal of the concentration of phosphate ester, log $1/(I)$, and extrapolating the resulting curve to $v_i/v = 0{\cdot}5$*. The following approximate values were obtained: 2 mM for androsterone 3-phosphate and epiandrosterone 3-phosphate, 10 mM for testosterone 17-phosphate and cortisol 21-phosphate, 25 mM for dehydroepiandrosterone 3-phosphate. By the same procedure a K_i value of 1·25 mM for divalent phosphate ion, HPO_4^{2-}, was obtained.

DISCUSSION

Although the order of inhibitory effectiveness of the three oestradiol phosphates previously reported [2] has been confirmed in the present investigation, the K_i values of these esters have not been found in accord with those reported above. This was to be expected, if one considers the markedly different conditions under which the previous and present experiments were carried out. Besides differences in temperature, pH, buffer composition†, apoenzyme purity and rate measurement methods,‡ two different criteria were followed for

* Putting $v_i = 0{\cdot}5$ in Eq. 5, it can be seen that K_i equals $(I)_{0.5}$, i.e. the concentration of the phosphate ester producing 50% decrease of the reconstitution rate.

† In the previous investigation [2] phosphate buffer was used since at that time it was not yet known that the reconstitution is depressed by phosphate ions[10].

‡ It should be noted that the method previously used [2] did not allow measurement of the initial velocity of reconstitution.

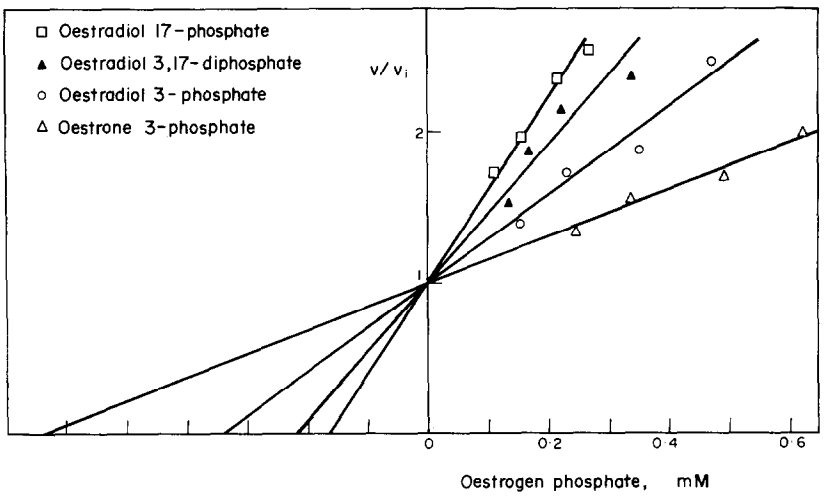

Fig. 1. Inhibitory effect of oestrogen phosphates on the *in vitro* reconstitution of aspartate aminotransferase. Extrapolation of the linear plots to $v/v_i = 0$ gives the K_i values for each oestrogen phosphate. Each point is the average of three determinations. For details see the text.

evaluating K_i. In the earlier investigation [2] oestrogen phosphates were considered as competitive inhibitors of the reconstitution and consequently the K_i were obtained by plotting data according to Dixon for pure competitive enzyme inhibition[11]; in the present investigation the K_i values have been obtained graphically according to Eq. 5, formally identical to that for non-competitive enzyme inhibition.

The peculiar inhibitory effect of oestrogen phosphates on the reconstitution of aspartate aminotransferase is confirmed by comparison with the phosphate esters of the androgenic and adrenocortical steroids;* these esters show a negligible inhibitory effect, in fact lower than that of the phosphate ion, which in turn is a weak inhibitor with respect to oestrogen phosphates. This peculiarity of oestrogen phosphates should be related to structural features that differentiate oestrogenic from non-oestrogenic steroids, such as the aromatic character of ring *A* and the presence of a phenolic hydroxyl group at c–3 (cf. K_i values of oestrone 3-phosphate vs androsterone 3-phosphate, and oestradiol 17-phosphate vs testosterone 17-phosphate).

As previously observed [2], the position appears to be more important than the number of anionic groups in determining the inhibitory efficiency: efficiency is at its maximum when the 17-hydroxyl group is esterified and at its minimum when this group is free or replaced by an oxygen function and the phenolic 3-hydroxyl group is esterified.

All these facts seem to indicate that the formation of an inactive "apoenzyme–phosphate ester" complex is the result of the electrostatical attraction between the phosphate group of the ester and an oppositely charged group of the apoenzyme, at or near the active site; however the stability of such a complex depends on the extent of the interaction between the nonpolar portion of the ester and the apoenzyme, an interaction that in turn depends on the stereochemical feature of the steroid nucleus and on the nature of nuclear substituents.

In conclusion, the results of the present investigation are a further confirmation of the existence of a correlation between the oestrogenic activity of a compound and the inhibitory activity of its phosphate esters(s) on the reconstitution of aspartate aminotransferase and very likely of other pyridoxal 5′-phosphate-dependent enzymes.

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* The specific inhibitory effect of oestrogen esters on the reconstitution of aspartate aminotransferase was based on comparison with only two esters of non-oestrogenic steroids, namely deidroepiandrosterone 3-sulphate [2] and betamethasone 21-phosphate [3].

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