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The Action of Sulphate and Phosphate Esters of Oestrogens on the Reconstitution of two Pyridoxal 5-Phosphate-Dependent Enzymes

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Mason & Gullekson (1959, 1960) discovered the inhibition of kynurenine aminotransferase (L-kynurenine-2-oxoglutarate aminotransferase, EC 2.6.1.7) and phosphorylase α by sulphate esters of stilboestrol [3,4-di-(*p*-hydroxyphenyl)hex-3-ene], oestradiol (oestra-1,3,5-triene-3,17- β -diol) and oestrone (3-hydroxyoestra-1,3,5-trien-17-one), whereas the unconjugated forms were inactive. The authors suggested that the inhibition was due to a competitive mechanism between the sulphate esters and the coenzyme (pyridoxal 5-phosphate).

The kinetics of the inhibition by oestradiol disulphate and stilboestrol disulphate of aspartate aminotransferase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) formation from apoenzyme and pyridoxal phosphate was previously studied to contribute further to the understanding of the mechanism of the inhibition (Scardi, Magno & Scarano, 1960).

The purpose of the present work was to investigate whether or not sulphate esterification is necessary to inhibit the reconstitution of pyridoxal phosphate-dependent enzymes, and whether or not there is a specificity of oestrogen esters, both sulphate and phosphate, on the kynurenine aminotransferase. The phosphate esters were chosen because of their solubility and of their possible existence *in vivo*. Aspartate aminotransferase was used because of its role in intermediary metabolism and because its enzymic assay affords a ready method for kinetic analysis of the inhibition.

MATERIALS AND METHODS

Chemicals. Oestradiol disulphate (sodium salt) was prepared by Dr P. de Ruggieri (Ormonoterapia Richter S.p.A., Milan, Italy). The compound examined by paper ionophoresis appeared to be 95% pure (the remaining 5% being monosulphates), though the actual purity might be higher because hydrolysis during ionophoresis cannot be excluded. Oestradiol diphosphate, oestradiol 3-phosphate, oestradiol 17-phosphate and stilboestrol diphosphate were given by AB Leo (Halsingborg, Sweden); the preparation and purity of these compounds have been reported by Fernö, Fex, Högberg, Linderot, Veige & Diczfalusy (1958) and Diczfalusy, Fernö, Fex, Högberg & Kneip (1959) respectively. Dehydroepiandrosterone (3 β -hydroxyandrost-5-en-17-one) sulphate as described by D'Alò (1956) was given by Recordati S.p.A. (Milan, Italy). Pyridoxal 5-phosphate, aspartic acid and α -oxoglutaric acid were products of Hoffmann-LaRoche (Basle, Switzerland), and kynurenine sulphate was obtained from California Corp. for Biochemical Research, Los Angeles, U.S.A.

Enzymes and activity determinations. Kynurenine aminotransferase from rat kidney was prepared and assayed as described by Mason (1957). Aspartate aminotransferase was prepared from pig heart by the method of O'Kane & Gunsalus (1947) and freed from pyridoxal phosphate as reported by Scardi *et al.* (1960). The activity was measured spectrophotometrically, according to Cammarata & Cohen (1951), with a Beckman spectrophotometer model DU equipped with thermal spacers and a circulating constant-temperature bath. Protein concentration was calculated from the extinctions at 280 and 260 m μ , according to Kalckar (1947).

The experiments with aspartate aminotransferase were carried out by preincubating apoenzyme with different concentrations of inhibitors and pyridoxal phosphate at

36°; after 15 min., unless otherwise stated, portions were assayed for activity.

The experiments with kynurenine aminotransferase were performed as described by Mason & Gullekson (1960), the concentration of pyridoxal phosphate in the incubation mixture being 0.12 mM.

RESULTS

In Fig. 1 are plotted, according to Dixon (1953), the experimental data of the inhibition of aspartate-aminotransferase reconstitution by the four phosphate esters. The K_i values are 0.425, 0.12, 0.075 and 0.015 mM for oestradiol 3-phosphate, stilboestrol diphosphate, oestradiol diphosphate and oestradiol 17-phosphate respectively.

The inhibitory action of oestradiol disulphate and oestradiol diphosphate on the reconstitution of kynurenine aminotransferase is shown in Fig. 2. Oestradiol diphosphate and stilboestrol diphosphate are more effective inhibitors than the corresponding sulphate esters; the K_i value of both oestradiol disulphate and stilboestrol disulphate is

0.15 $\pm$ 0.05 mM (Scardi *et al.* 1960). These data suggest that the phosphate esters are generally more effective inhibitors than the corresponding sulphate derivatives; however, further experiments are needed to substantiate this point.

Fig. 3 shows that a prolongation of the incubation time of aspartate apoaminotransferase, pyridoxal phosphate and inhibitor removes the inhibition almost completely. The remaining inhibition is probably due to denaturation of the apoenzyme since further addition of excess of pyridoxal phosphate when the asymptote was reached gave no increase of activity. Further, Fig. 3 shows that the holoenzyme is neither competitively inhibited nor denaturated by the oestrogen ester even at high concentration and long incubation time.

Dehydroepiandrosterone sulphate, a non-oestrogenic steroid, was completely without effect on the reconstitution of kynurenine aminotransferase and of aspartate aminotransferase up to a concentration of 2 mM.

Fig. 1. Dixon (1953) plots of the inhibition of aspartate-aminotransferase reconstitution by: A, oestradiol 17-phosphate; B, oestradiol 3-phosphate; C, oestradiol diphosphate; D, stilboestrol diphosphate. The enzymic activity is expressed as μ moles of oxaloacetic acid formed/min./mg. of protein at 36° under the conditions described by Cammarata & Cohen (1951). The concentration of pyridoxal 5-phosphate in the preincubation mixture was 0.02 mM (○) and 0.04 mM (●) respectively.

Fig. 2. Inhibition of kynurenine-aminotransferase reconstitution by oestradiol diphosphate (O) and oestradiol disulphate (Δ). The temperature was 36°. The 100% activity corresponds to 360 μ m-moles of kynurenic acid formed/hr./mg. of protein under the conditions described by Mason (1957).

Fig. 3. Effect of preincubation time at 36° on aspartate holo- and apo-aminotransferase in the presence of oestradiol diphosphate. O, Holoenzyme with no oestradiol diphosphate; ●, holoenzyme + 0.16 mM-oestradiol diphosphate; Δ, apoenzyme + 0.02 mM-pyridoxal 5-phosphate + 0.08 mM-oestradiol diphosphate. The enzymic activity is expressed as μ moles of oxaloacetic acid formed/min./mg. of protein at 36° under the conditions described by Cammarata & Cohen (1951).

DISCUSSION

The kinetic experiments on aspartate aminotransferase with both sulphate (Scardi *et al.* 1960) and phosphate esters of oestrogens demonstrate that the inhibition involves competition between pyridoxal phosphate and the oestrogen ester for

the apoenzyme. No inhibitory effect can be shown on the holoenzyme. The mechanism of the inhibition may be explained by the following reactions:

where apoE, I, apoE-I, P and holoE denote apoenzyme, oestrogen ester, inactive enzyme, pyridoxal phosphate and holoenzyme respectively. In the scheme the first reaction is assumed to be readily reversible and fast, and the second one practically irreversible. The essential irreversibility of the second reaction must be assumed since the oestrogen esters have no effect on the transamination reaction and act only on the formation of the holoenzyme during the preincubation period. The enzymic assay of the aspartate aminotransferase measures only the amount of the holoenzyme formed during the preincubation time and is not influenced by the components of the preincubation mixture. Fig. 3 shows that the inhibition can be removed by prolonging the incubation time during the reconstitution reaction. By stopping the reconstitution reaction after 15 min. the inhibition was readily demonstrable and the experimental data fitted a Dixon (1953) plot.

In every case tested, the phosphate esters are more effective inhibitors than the corresponding sulphate derivatives.

The position of the phosphate groups rather than their number is important in determining the extent of the inhibitory power. Phosphate esterification at C-17 yields the most active compound. The same rule may apply to sulphate esters, although this has not been studied.

These experiments, particularly those with stilboestrol disulphate (Scardi *et al.* 1960), stilboestrol diphosphate and dehydroepiandrosterone sulphate, demonstrate that the inhibitory effect depends both on the oestrogenic and the anionic character of the molecule.

The phosphate and the sulphate esters of oestrogens are ten times as inhibitory on kynurenine-aminotransferase reconstitution as on aspartate-aminotransferase reconstitution; this, however, may not indicate a specific physiological action on the kynurenine enzyme.

Only the sulphate esters of the oestrogens have been shown to be present in organisms; however, the phosphate esters may also be present in cells and play a specific role on the physiological action of these hormones.

SUMMARY

1. The phosphate esters of oestradiol and stilboestrol compete with pyridoxal 5-phosphate for the apoenzyme in the reconstitution reaction of two pyridoxal phosphate-dependent enzymes, namely

kynurenine aminotransferase and aspartate aminotransferase.

2. The esters, however, are without action on the enzymic transamination itself.

3. The phosphate esters are compared with the sulphate esters as inhibitors of the combination of apoaminotransferase with coenzyme.

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