INTERACTION OF Z-4,4-BIS(4-ETHYLPHENYL)-2,3-DIBROMO-2-BUTENOIC ACID WITH AMINOTRANSFERASES: CHANGES IN ABSORPTION AND CIRCULAR DICHROISM SPECTRA

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SUMMARY

The influence of Z-4,4-bis(4-ethylphenyl)-2,3-dibromo-2-butenoic acid, the compound originally synthetized as a cytostatic edikron and showing inhibitory effect on several pyridoxal enzymes, on absorption and circular dichroism spectra of alanine and aspartate aminotransferases (ALT, AST) in the region of coenzyme absorption characteristics was studied. In the case of AST, the compound decreased absorption and CD maxima at 360 nm, which represents the active form of the enzyme, but it did not seem to prevent formation of the pyridoxamine form of the enzyme, produced in the presence of L-aspartate. Edikron caused insignificant spectral changes of ALT, but it partially denatured the enzyme. Circular dichroism measurement of both enzymes uncovered some effects of edikron at 250-300 nm, which suggests conformational changes in the aromatic amino acids of the apoenzymes due to the compound studied.

KEY WORDS

aspartate aminotransferase, alanine aminotransferase, circular dichroism, absorption spectra, Z-4,4-bis(4-ethylphenyl)-2,3-dibromo-2-butenoic acid

INTRODUCTION

Z-4,4-bis(4-ethylphenyl)-2,3-dibromo-2-butenoic acid was synthetized as a cytostatic edikron /1/. The compound has been shown to inhibit a number of enzymes: Inhibition of tetrahydrofolate reductase, folate reductase, formiminotransferase /2/, glucose-6-phosphate dehydrogenase and glycolytic thiol enzymes /3/ seemed to be the most important from the point of view of cytostatic effects of this compound, while the inhibition of several pyridoxal-5'-phosphate enzymes of rat tissues - aromatic amino acid decarboxylase /4, 5/ and alanine and aspartate aminotransferases /6, 7/ - suggested rather unspecific effects of the potential cytostatic. *In vitro* reaction of edikron with cysteine has been demonstrated after several hours of incubation of the compound with the amino acid /8/, but this apparently slow reaction could not explain completely the inhibitory effect of edikron, which seemed to be immediate in the case of aromatic amino acid decarboxylase /8/ as well as aminotransferases /7/.

The presence of pyridoxal-5'-phosphate coenzyme in molecules of enzymes provides them with a characteristic absorption spectrum in the wavelength range 300-500 nm that differs from that of the free pyridoxal-5'-phosphate. These spectral properties have been studied in detail in AST. In general, they are the same regardless of species or cellular fraction (see /9/) and they are illustrated in Figure 1: Free AST shows, depending on pH, absorption peaks at 360 nm (an active, unprotonized form of the coenzyme, prevailing at higher pH values) and/or at 430 nm (an inactive, protonized form, prevailing at lower pH values) /10/. After the reaction of AST with L-aspartate, which is the first substrate of a ping-pong transamination reaction, the pyridoxamine form of the enzyme appears and the original absorption maxima of the AST shift to 325 nm. On the other hand, the presence of only the second substrate (2-oxoglutarate) in AST solution does not produce any substantial change in the coenzyme absorption spectrum.

The absorption spectrum of free ALT /11/ shows maxima at 430 nm (protonized form) and 335 nm (unprotonized form); reaction of the enzyme with L-alanine (pyridoxamine formation) causes their shift to 325 nm.

While the free pyridoxal-5'-phosphate is optically inactive, it shows optical activity when bound to an aminotransferase apoenzyme. This makes it possible to follow circular dichroism (CD)

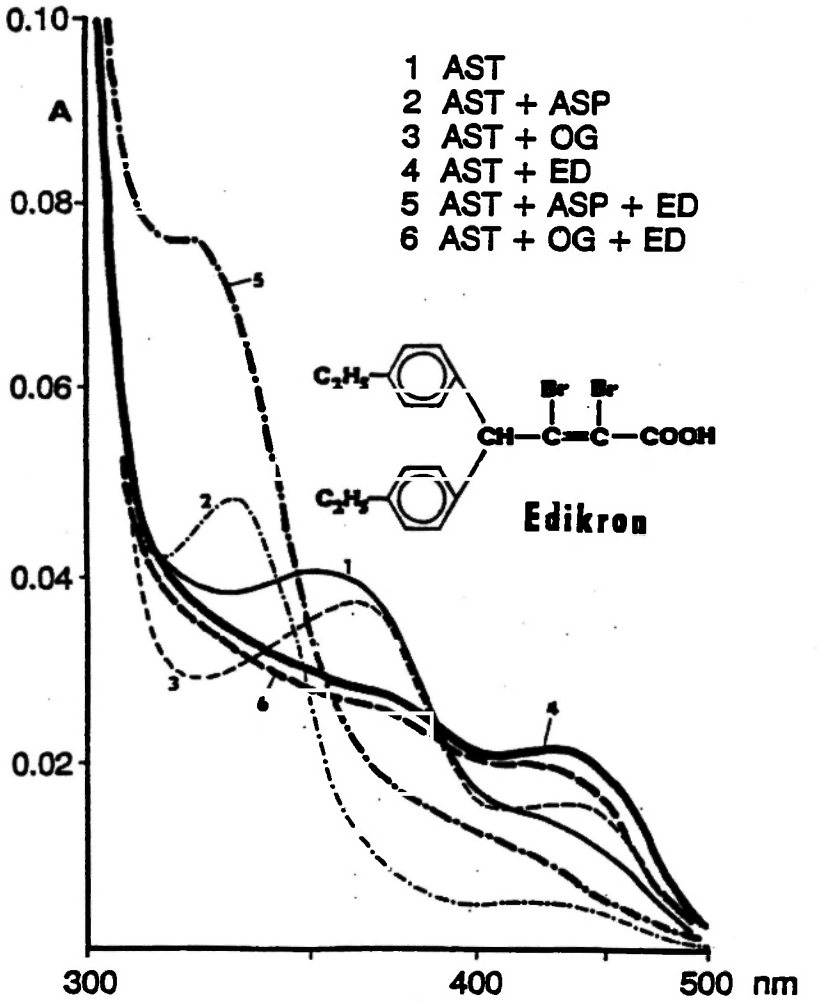

Fig. 1: Absorption spectra of aspartate aminotransferase from porcine heart (AST, 7×10^{-6} M in 0.1 M sodium phosphate buffer, pH 7.4) under the influence of Z-4,4-bis(4-ethylphenyl)2,3-dibromo-2-butenoic acid (Ed, 1×10^{-3} M). Substrate concentrations: L-aspartate (Asp) 2×10^{-2} M, 2-oxoglutarate (oG) 1×10^{-3} M.

spectra, which in the visible region are very similar to the absorption spectra of the coenzyme except that they are "cleaned" of optically inactive elements (see /12, 13/). In addition, CD spectra present their own characteristics related to conformational properties of proteins. In the case of AST such characteristics appear in the range of 250-300 nm, which are close to the coenzyme CD characteristics and represent optical properties of aromatic amino acids /12/.

The present study was carried out to investigate possible influences of Z-4,4-bis(4-ethylphenyl)2,3-dibromo-2-butenoic acid on the absorption and CD spectra of the coenzyme region of aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2). This approach seemed to be promising to bring new data on the character of interactions of the potential cytostatic with pyridoxal-5'-phosphate enzymes.

MATERIALS AND METHODS

Aspartate aminotransferase from porcine heart was supplied by Serva Feinbiochemica (Heidelberg & New York) as a suspension in $(NH_4)_2SO_4$, analytical grade. Declared catalytic activity was 170 U x mg^{-1} of protein, estimated enzyme purity about 50%. The enzyme suspension was centrifuged (15,000 *g*, 20 min), the $(NH_4)_2SO_4$ suspension medium was discarded and the precipitate was dissolved in 0.1 M sodium phosphate buffer, pH 7.4. Final concentration of AST during measurements of spectra was 7×10^{-6} M.

Alanine aminotransferase from porcine heart (Calbiochem; analytical grade, 900 U x mg^{-1} protein, estimated enzyme purity about 25%) was isolated from the suspension in the $(NH_4)_2SO_4$ solution in the same way as AST. Final concentration of the enzyme in 0.1 M sodium phosphate buffer pH 7.4 during spectral measurements was 3×10^{-6} M.

L-Aspartate (in the case of AST) and L-alanine (in the case of ALT) were used at a final concentration of 2.0×10^{-2} M; the final concentration of 2-oxoglutarate during measurements was 1×10^{-3} M. All substrates were of analytical grade and their concentrations corresponded to those used in aminotransferase assays /7/.

Z-4,4-bis(4-ethylphenyl)-2,3-dibromo-2-butenoic acid (edikron) was synthetized by Dr. J. Hartl (Dept. of Pharm. Chemistry, Faculty

of Pharmacy, Hradec Králové). Final concentrations of the compound during spectral measurements were 1.0×10^{-5} M in the case of AST and 3.3×10^{-5} M in that of ALT. In both cases the molar concentrations of the compound exceeded that of aminotransferase and were close to IC_{100} /6/. Solutions containing free enzyme, the enzyme with one of the substrates, and samples with edikron in addition to these components were kept at +4°C for four hours after their preparation and then incubated at 20°C for another two hours before measurements of spectra.

Absorption spectra were measured using a Specord M40 spectrophotometer (Carl Zeiss Jena) in a 0.5 cm quartz cuvette against the respective blank containing all components except the enzyme. Circular dichroism was measured on a Mark 5 dichrograph (Jobin Yvon) in a 0.5 cm quartz cuvette against water. Measurements and interpretations of spectra were simplified by the fact that aminotransferase substrates as well as Z-4,4-bis(4-ethylphenyl)-2,3-dibromo-2-butenoic acid present no absorption and CD characteristics within the wavelength range studied (250-500 nm).

RESULTS AND DISCUSSION

Aspartate aminotransferase

Results concerning absorption spectra of the enzyme are summarized in Figure 1. Enzyme spectra without edikron correspond with similar findings of other authors (see, e.g., /14/ for chicken heart enzyme). In the presence of edikron, the "active" peak (360 nm) of free AST and of the enzyme in the mixture with 2-oxoglutarate is depressed, apparently in favour of the "inactive" band at 430 nm. This shift is in agreement with the formerly found /6/ inhibitory effect of the compound on AST. In the case of the mixture of AST with L-aspartate, addition of edikron does not seem to prevent the enzyme-substrate reaction. This results in disappearance of the original maxima and appearance of the maximum at 325 nm. Nevertheless, in the presence of edikron, this maximum forms a shoulder of a larger peak with its maximum at shorter wavelengths. Moreover, there seems to be an overall slight increase in absorption and suppression

of the peaks in the whole range of measurements in the presence of edikron, which might be the result of an increased turbidity due to partial protein denaturation. The fact that edikron does not prevent spectral changes in AST characteristic of pyridoxamine formation is not in contradiction with the inhibitory effect of the compound since the spectra represent results of incubation for several hours (see Materials and Methods) which would enable the reaction of AST with L-aspartate to complete its course even in the case were it slowed down.

Circular dichroism spectra of AST are presented in Figures 2-3. Figure 2 shows spectra of the free enzyme and of AST in the presence of substrates. The spectra are in general agreement with other authors' findings /12, 14/. In comparison to absorption spectra, optically inactive absorption components are cleaned off and new bands in the UV region appear: at least one positive at 270-280 nm and a negative one at 290-300 nm. The concentration and purity of the enzyme used in this study did not allow us to distinguish three or four finer peaks, described in this region /12/. In the presence of L-aspartate, the coenzyme maxima are distinctly shifted to one positive band at 325-300 nm (pyridoxamine form). Moreover, the positive peak at 270-280 nm and the negative peak at 290-300 nm, both representing aromatic amino acids of the apoenzyme involved in the active site /12/, seem to be increased, which suggests a conformational change of the aromatic component of the apoenzyme after the reaction of AST with L-aspartate.

Figure 3 shows the influence of edikron on circular dichroism of the coenzyme of AST: After addition of the compound to the free enzyme or to its mixture with 2-oxoglutarate, CD spectra confirm in general the findings obtained with the absorption spectra, but additional changes are apparent in both cases at 270-300 nm, suggesting an effect of edikron on the apoenzyme. In the case of the AST-2-oxoglutarate-edikron mixture, both the positive (270-280 nm) and the negative (290-300 nm) peaks are shifted to negative values, while the mixture of AST with edikron shows an increase in the positive band. Comparing Figures 2 and 3, an influence of edikron on the pyridoxamine form of the coenzyme and on the apoenzyme can be observed. The peak at 325-330 nm is decreased, the positive peak at 270-280 nm is slightly increased and the negative peak at 290-300 nm disappears in the presence of edikron.

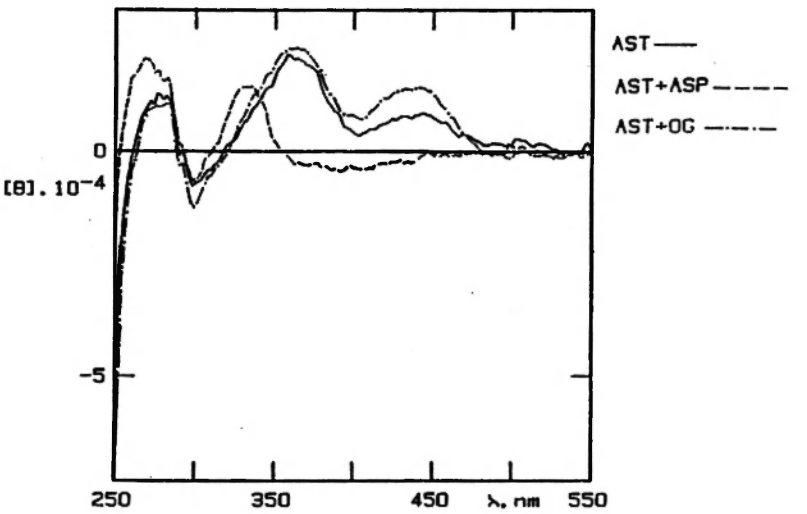

Fig. 2: Circular dichroism of aspartate aminotransferase. For conditions see Fig. 1.

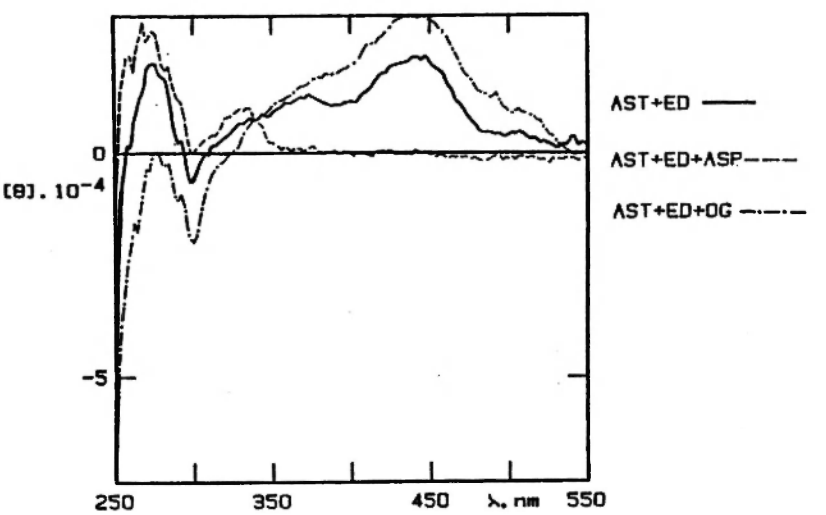

Fig. 3: Circular dichroism of aspartate aminotransferase under the influence of Z-4,4-bis(ethylphenyl)2,3-dibromo-2-butenoic acid (Ed). For conditions see Fig. 1.

Alanine aminotransferase

Edikron does not affect the shape of the spectra of free ALT and of the ALT-2-oxoglutarate mixture (Figure 4). In the case of the mixture of the enzyme with L-alanine, edikron does not seem to prevent pyridoxamine formation since the peak of the pyridoxal form (430 nm) shifts to 325 nm representing the pyridoxamine form of ALT, regardless of the presence or absence of edikron. In each case shown in Figure 4, the presence of edikron elevates the absorption curves. This may be caused by partial denaturation of the enzyme protein by edikron accompanied by an increase in turbidity of the samples. This denaturating effect of edikron on ALT was confirmed in separate experiments, where a 3.7×10^{-4} M concentration of edikron caused opalescence followed by protein precipitation during several hours of incubation of the sample containing ALT, while a 1×10^{-3} M concentration of the compound caused immediate precipitation of ALT. Significant contribution of this denaturation of ALT to the time-dependent gradual increase in irreversible inactivation of the enzyme by edikron /15/ is highly probable.

CD spectra of ALT are presented in Figures 5-7. In all the cases (free ALT and ALT in the presence of one of the two substrates), edikron does not show any significant influence on the spectra of ALT in the region of optical characteristics of pyridoxal-5'-phosphate (300-500 nm). Slight differences may be due to noise at a relatively low concentration and purity of the enzyme. On the other hand, at least in the cases of free ALT and of the enzyme in the presence of 2-oxoglutarate (Figures 5 and 6), edikron seems to affect the ALT spectra slightly at 270-290 nm (see difference spectra), which would suggest, as in the case of AST, changes in the conformation of aromatic amino acid(s) of the apoenzyme. From this point of view, it might be promising to study circular dichroism of both aminotransferases under the influence of edikron in the shorter UV region since this makes it possible to reveal changes in protein secondary structure (see, e.g., /16/). Considering the chemical reactivity of Z-4,4-bis(4-ethylphenyl)-2,3-dibromo-2-butenoic acid /8/ and the findings made in this study, interactions of the compound with proteins resulting in substantial conformational changes are probable.

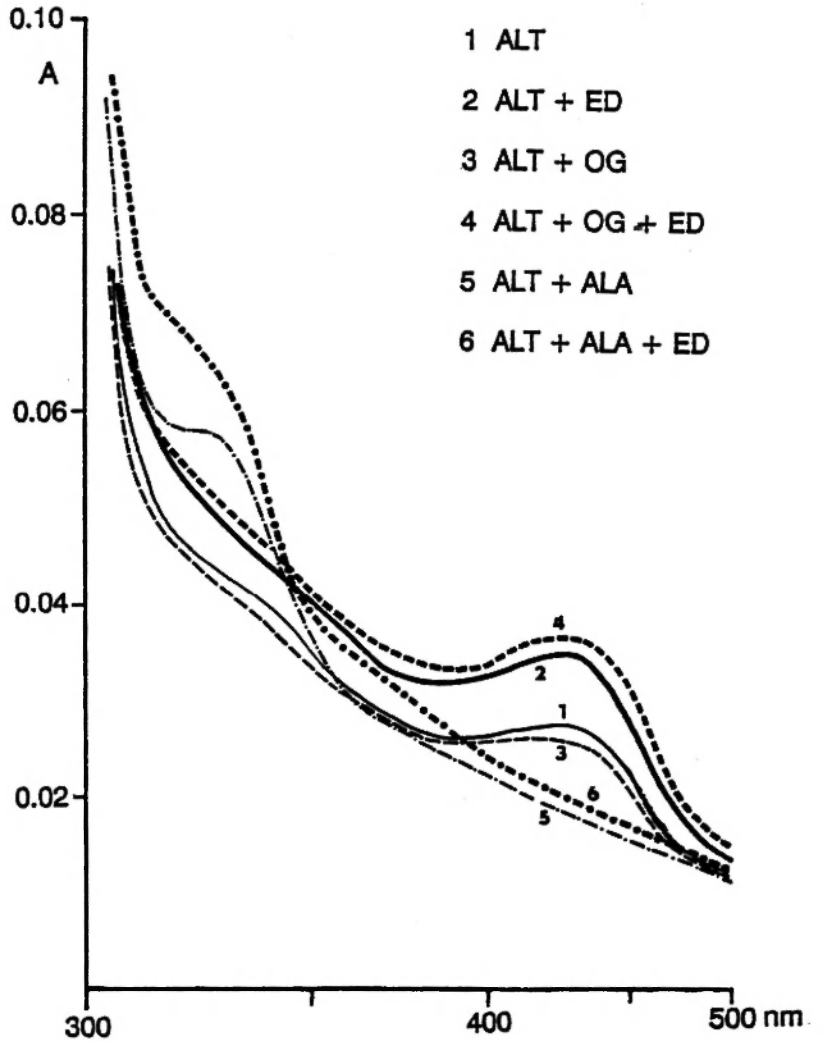

Fig. 4: Absorption spectra of aspartate aminotransferase from porcine heart (ALT, 3 x 10^{-6} M in 0.1 M sodium phosphate buffer, pH 7.4) under the influence of Z-4,4-bis(4-ethylphenyl)2,3-dibromo-2-butenoic acid (Ed, 3.3 x 10^{-5} M). Substrate concentrations: L-alanine (Ala) 2 x 10^{-2} M, 2-oxoglutarate (oG) 1 x 10^{-3} M.

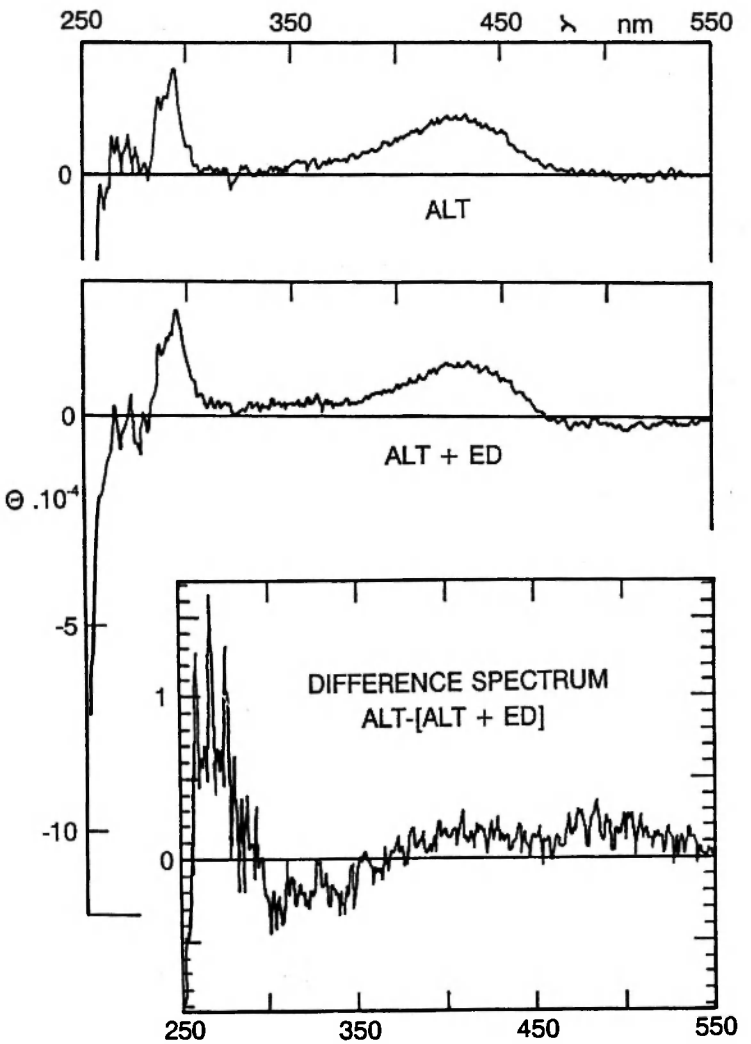

Fig. 5: Circular dichroism of free alanine aminotransferase under the influence of Z-4,5-bis(ethylphenyl)2,3-dibromo-2-butenoic acid (Ed). For conditions see Fig. 4.

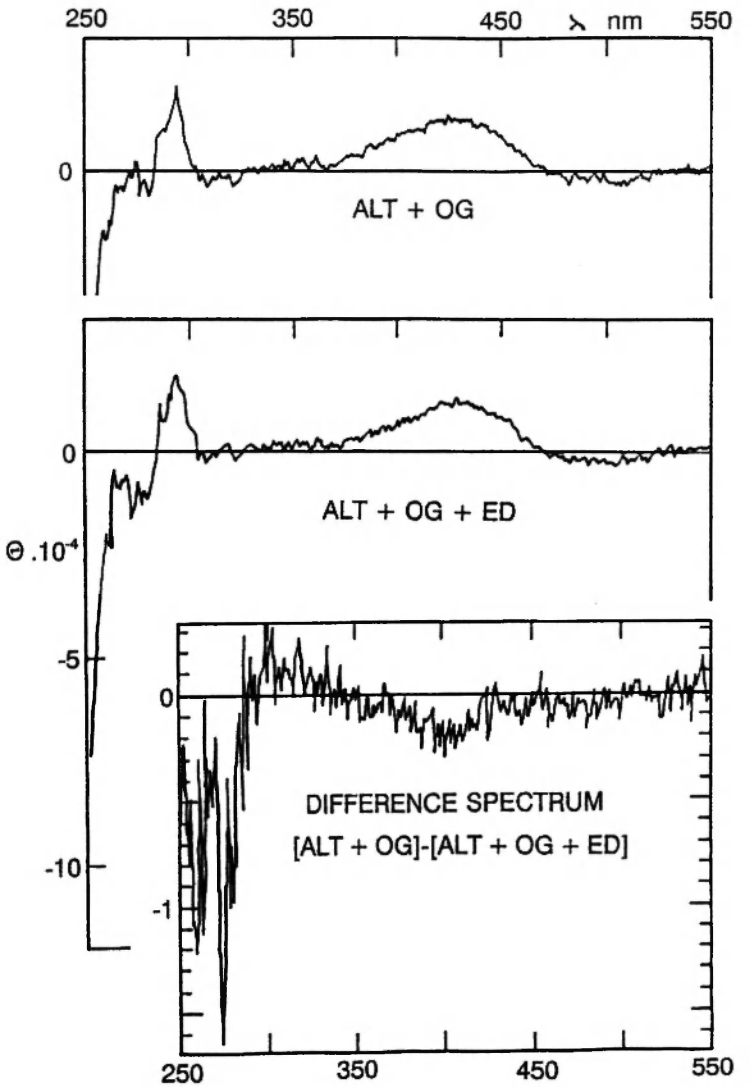

Fig. 6: The influence of Z-4,4-bis(4-ethylphenyl)2,3-dibromo-2-butenoic acid (Ed) on circular dichroism of alanine aminotransferase in the presence of 2-oxoglutarate. For conditions see Fig. 4.

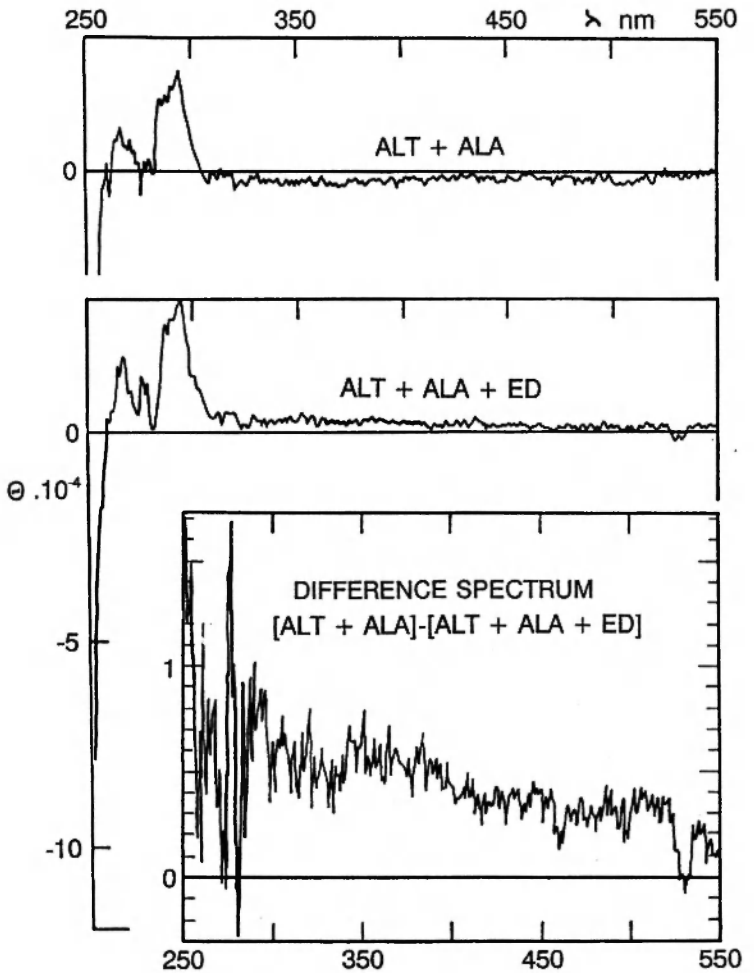

Fig. 7: The influence of Z-4,4-bis(4-ethylphenyl)2,3-dibromo-2-butenoic acid (Ed) on circular dichroism of alanine aminotransferase in the presence of L-alanine. For conditions see Fig. 4.

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