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Experimental Evidence for a Hydrophobic Active Center of Glutamic-Aspartic Transaminase

Specific Interaction of Holoenzyme and Apoenzyme with Two Fluorescein Derivatives

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1. It has been found that fluorescein dimercuric acetate acts as a potential inhibitor of transaminase. Although the holoenzyme was inhibited also by fluorescein isothiocyanate the latter fluorochrome was found to be a rather specific inhibitor of the apoenzyme. The interactions involved are fast, the induced effect reaching its maximum value in less than 1 min.

2. The isolated fluorescein dimercuric acetate or fluorescein isothiocyanate and transaminase or apotransaminase complexes suggest that the fluorochromes reacts specifically with two active sulfhydryl or amino groups of the enzyme, respectively.

3. The fluorescence intensity of the fluorochromes is substantially quenched during their interaction with transaminase and apotransaminase. A red shift in the absorption spectrum maximum from 498 nm to 512 nm was also observed when fluorescein dimercuric acetate solutions were titrated with transaminase or apotransaminase. The recorded shift for fluorescein isothiocyanate in the fluorescein-isothiocyanate · apotransaminase complex was from 493 nm to 502 nm, respectively. In contrast to the previous spectral changes a blue shift in the spectrum maximum from 498 nm to 478 nm was recorded during the stoichiometric interaction of fluorescein dimercuric acetate with cysteine.

4. Comparative studies concerning the reactivity of fluorescein dimercuric acetate or fluorescein isothiocyanate with transaminase, apotransaminase and cysteine or lysine suggest that the alterations observed in the spectral and fluorescence properties of the fluorochromes during their interaction with transaminase and apotransaminase arose from their complexation with specific sulfhydryl groups of the enzyme. These groups should be located in a critical hydrophobic area of the transaminase molecule since their interaction abolishes the catalytic activity of the enzyme and alters the spectral and fluorescence properties of the fluorochromes in the same way as low polarity solvents. These sulfhydryl and amino groups might also play a functional role since they interact faster with fluorescein dimercuric acetate and fluorescein isothiocyanate than the sulfhydryl group of cysteine or the amino group of lysine.

5. Quantitative parameters of the data obtained by conventional techniques and stopped-flow spectroscopy during the interaction of the enzyme with the fluorochromes are also given.

The active centers of catalytic proteins contain not only particular amino acid residues which function in catalysis but also those which form specific binding sites for substrates and cofactors. It appears that the evolutionary process found it advantageous in some cases to use both protonatable groups of a vitamin and of an amino acid side chain to facilitate catalysis.

Enzyme. Glutamic-aspartic transaminase or L-aspartate 2-oxoglutarate aminotransferase (EC 2.6.1.1).

Glutamic-aspartic transaminase is a vitamin B_6 enzyme; it contains two molecules of pyridoxal phosphate per molecule of protein with a molecular weight of 90000 [1,2]. The enzyme has been found sensitive to sulfhydryl group reagents [3,4] and it has been demonstrated that the reactivity of its sulfhydryl groups is varied [5—7]. An increase in the titratable sulfhydryl groups of the enzyme after its interaction with aspartate was observed spectroscopically [8]. Protection of the catalytic activity

of transaminase against *p*-chloromercuribenzoate by 2-oxoglutarate has been reported [9]; other investigators suggested that lysyl and cysteinyl side chains are possibly involved in coenzyme-apoenzyme binding [10,11]. The spectroscopic and fluorescence-emission characteristics of the coenzyme were extensively used for studies concerning the function and three-dimensional structure of transaminase [12—16]. Fluorescent compounds specifically bound to free active groups of protein molecules have been used to gain information concerning the conformational structure and dynamic of macromolecules]17,18].

In the present work we report some implications of the interaction of lysyl and cysteinyl side chains of transaminase with fluorescein derivatives on the catalytic activity and other physical properties of the holoenzyme, apoenzyme and the fluorochromes. The effect in the absorption spectra and the fluorescence intensity of the fluorescein derivatives during the interactions as well as the stoichiometry of the complexes obtained were used to characterize potential groups and evaluate their reactivity.

EXPERIMENTAL PROCEDURE

Glutamic-aspartic transaminase from pig hearts was prepared by the method of Jenkins *et al.* [10] using succinate and adipate buffer [19]. The method was modified in its last step since it was found that by the substitution of the hydroxyapatite column by an SP-Sephadex C-50 column followed by a DEAE-Sephadex A-50 one, resulted in the isolation of transaminase in better yields and higher purity. When the SP-Sephadex C-50 column (4×60 cm) was eluted with 0.02 M phosphate buffer pH 5.8, transaminase usually appeared in the front of the elution diagram. The eluates containing the enzyme were pooled and after adjustment of their pH to 7.2 were poured on the top of the DEAE-Sephadex A-50 column (4×60 cm); elution was subsequently performed with 0.02 M phosphate buffer pH 7.2. The fractions containing the enzyme were precipitated by ammonium sulfate as before. The enzyme preparations obtained in this way had an absorption ratio at 280/360 nm of about 10 and at 360/330 nm of about 3 at pH 9.0. This corresponds to an enzyme purity of more than 95% which gives a single peak in the sedimentogram during ultracentrifugation. All the experiments were performed with native transaminase, no attempt being made to isolate individual forms of transaminase separately [20],

Apotransaminase was prepared from transaminase with the coenzyme in the pyridoxal phosphate form by precipitation with ammonium sulfate at low pH values according to the method of Wada and Snell [21] and from transaminase with the coenzyme in the pyridoxamine phosphate form according to the method of Scardi *et al.* [22]. All reagents used were commercial chemicals supplied by Calbiochem., BDH Ltd or Pharmacia.

Calculations of transaminase and apotransaminase molarity were based on an enzyme molecular weight of 90000 [23,24]. Transaminase activity was determined by measuring the absorbance at 280 nm of oxaloacetate formed using an automatic Unicam SP 500 spectrophotometer. Kinetic measurements were obtained from a 3-ml reaction mixture containing 100 μmol Tris buffer pH 8.5 and 20 μmol each of L-aspartate and 2-oxoglutarate according to the method of Cammarata *et al.* [25]. Interaction of the enzyme with fluorescein derivatives was followed at three different pH values; the buffer solutions used were phosphate pH 5.5, Tris pH 8.0 and carbonate pH 10.0 of the same molarity (0.05 M); catalytic activity was always estimated at pH 8.5.

Fluorescence intensity measurements were accomplished using the following system. Monochromatic light obtained by passing radiation of a 250 V/650 W Philips PF 810 lamp through a monochromator of a Hilger UV-visible spectrophotometer was directed to a circular cell compartment housing four cells selected by means of a rotating device. Adjacent to the cell compartment and perpendicular to the beam of the monochromatic light was located the window of a photomultiplier tube (XP-1110, Philips, high-voltage maximum, 1,800 V) connected to a microamperometer (SEC-Model, CT 330). Scattered light was prevented from reaching the photomultiplier window by inserting the Ilford filters No 109 or 110, depending on the fluorochrome used, between the cell compartment and the window. Blank experiments were performed using buffer of enzyme solutions without the fluorochromes. Fluorescence spectra of the fluorescein derivatives or of their reaction products with the enzyme were obtained using an Aminco-Bowman recording spectrofluorometer. Absorption spectra were taken by means of a Bosh and Lomb UV-visible recording spectrophotometer. Protein concentration was determined by the method of Lowry *et al.* [26].

Acrylamide disc electrophoresis of fluorescein-derivative · transaminase complexes was performed on a Shanon instrument by use of a reservoir buffer system containing 28.8 g glycine and 6.0 g Tris in 1 l distilled water (pH 8.0). For 16 ml gel solution the following procedure was used. Stock solution of bis-acrylamide (0.8%) and acrylamide (30%) containing potassium ferricyanate (0.015%) was mixed with water, stock buffer (36.3 g Tris, 48.0 ml 1 N hydrochloric acid and 0.46 ml *N,N,N',N'*-tetramethylethylenediamine in 100 ml distilled water) in a ratio of 4.0:2.0:8.0 (v/v/v), respectively. The gel solution was degassed and introduced into the electrophoretic tubes for polymerization. The aliquots of the complexes or of the free reactants in 50 μl 10% sucrose solutions were layered on the top of the

running gel tubes. Electrophoresis was performed at a constant current 5 mA per tube (approximately 100 V) for 1 h. Gels were stained for 5 min with an amido black solution (0.5 g amido black, 50 ml methanol, 540 ml distilled water and 10 ml acetic acid) and destained by extensive washing.

RESULTS

We have selected for our studies fluorescein dimercuric acetate and fluorescein isothiocyanate. The former derivative of fluorescein reacts specifically with sulfhydryl groups of cysteine or cysteinyl residues while the latter reacts with free amino groups of amino acids and proteins [27,28]. The choice originated from previous studies on transaminase [10,11] suggesting that an ε-amino group of a lysine side chain and a sulfhydryl group of a cysteinyl residue of the enzyme are possibly involved in coenzyme-apoenzyme binding or in catalytic action.

INHIBITORY EFFECT OF THE FLUORESCEIN DERIVATIVES ON THE CATALYTIC ACTIVITY OF TRANSAMINASE

The enzyme (2.5 μM) was incubated with different quantities of the fluorescein derivatives ranging from a molar ratio of inhibitor/enzyme of 0.1 to 100. Although various experiments were performed at different pH values and incubation time, enzymatic activity was always determined at pH 8.5. In Fig. 1 it is shown that a molar ratio of fluorescein dimercuric acetate/transaminase of the order of 2 is sufficient to accomplish maximum transaminase inactivation. Although transaminase was also inhibited by fluorescein isothiocyanate its effect was far smaller; a high molar ratio of fluorescein isthiocyanate/transaminase was needed in the reaction system for a substantial inactivation (Fig. 2).

To explore further the nature of the interactions involved a comparative study was performed in which the inhibitory action of the fluorochromes on apotransaminase *versus* holotransaminase was examined. In a series of small tubes containing apotransaminase or transaminase (2.5 μM) fluorescein dimercuric acetate or fluorescein isothiocyanate was added in the optimum inhibitory concentration and the reaction mixtures were allowed to interact for different time intervals. The apotransaminase systems were incubated subsequently for 15 min with stoichiometric amounts of pyridoxal phosphate. A far more pronounced inhibitory effect of the fluorochromes on the apoenzyme was detected in both cases (Fig. 3).

Effect of Substrates

The effect of amino substrates or oxo-acid substrates on the interaction between the enzyme and

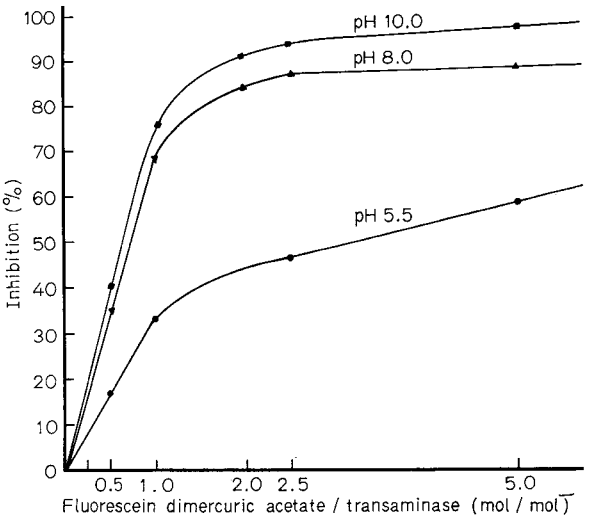

Fig. 1. *Inactivation of transaminase by fluorescein dimercuric acetate as a function of the molar ratio of the reactants.* Reaction systems of 2 ml in final enzyme concentration 2.5 μM were used in the buffers described in the Methods. (■) Carbonate; (▲) Tris; (●) phosphate

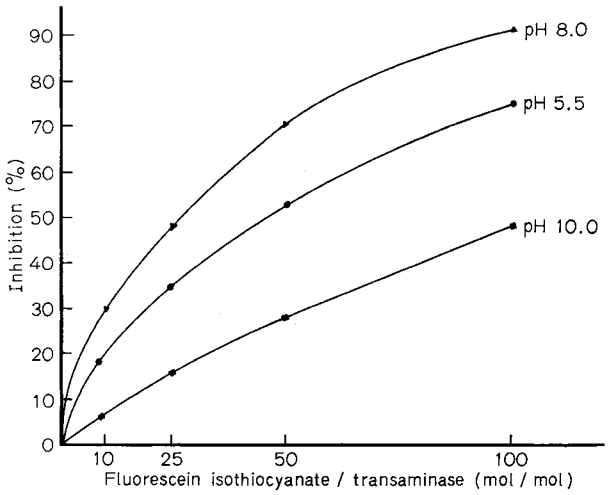

Fig. 2. *Transaminase inactivation by fluorescein isothiocyanate as a function of the molar ratio of the reactants.* Experimental conditions as in Fig. 1. (■) Carbonate; (▲) Tris; (●) phosphate

fluorochromes was tested in a series of experiments. The enzyme or apoenzyme in a final concentration of 2.5 μM was preincubated for 30 min with 2-oxoglutarate or L-aspartate in a final concentration of 5 mM and 10 mM respectively before the addition of the fluorochromes in the systems. The enzymatic activity of the reaction mixtures was determined at different time intervals after the addition of the inhibitors. The results obtained for systems reacted up to 1 h are summarized in the Table 1.

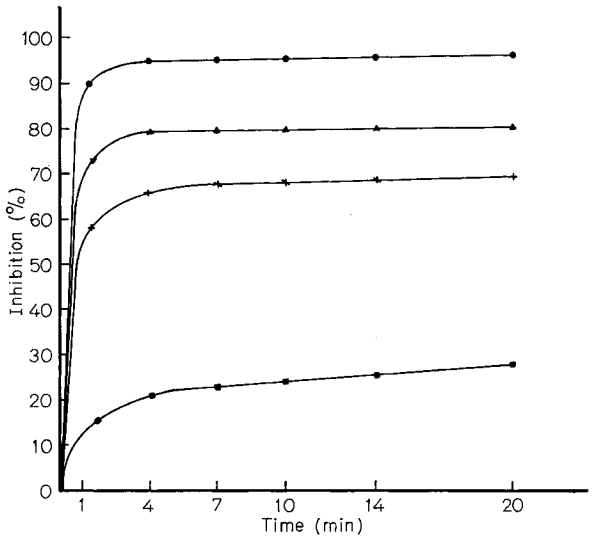

Fig. 3. *Transaminase and apotransaminase inhibition by fluorescein dimercuric acetate and fluorescein isothiocyanate* versus *time*. Reaction systems of 2 ml in 0.05 M carbonate buffer pH 9.2, enzyme or apoenzyme concentration 2.5 μM and molar ratios of fluorescein dimercuric acetate/transaminase or apotransaminase 2 and fluorescein isothiocyanate/transaminase or apotransaminase 10, were used. The reaction mixtures were allowed to interact for different time intervals shown in the figure. The apotransaminase systems were incubated for 15 min with stoichiometric amounts of pyridoxal phosphate prior to their determination of the catalytic activity. (●) Fluorescein-dimercuric-acetate · apotransaminase; (+) fluorescein-dimercuric-acetate · transaminase; (▲) fluorescein-isothiocyanate · apotransaminase; (■) fluorescein-isothiocyanate · transaminase

Table 1. *Effect of amino substrates or oxo-acid substrates on the inhibitory capacity of fluorescein dimercuric acetate or fluorescein isothiocyanate on the enzyme*
Reaction systems of 2 ml 0.05 M carbonate buffer pH 9.2 containing 2.5 μM transaminase and the corresponding quantity of inhibitors to give the molar ratio of fluorescein dimercuric acetate/transaminase or fluorescein isothiocyanate/transaminase indicated in the table were used. Catalytic activity determined as described in Methods

Enzyme system	Inhibition			
	Fluorescein dimercuric acetate/enzyme		Fluorescein isothiocyanate/enzyme	
	1	2	10	100
	%	%	%	%
Transaminase	55	70	30	80
Transaminase in 10 mM aspartate	65	75	45	90
Transaminase in 5 mM 2-oxoglutarate	45	60	25	70
Transaminase in 10 mM aspartate and 5 mM 2-oxoglutarate	70	80	35	80
Apotransaminase	75	90	75	90
Apotransaminase in 5 mM 2-oxoglutarate	60	75	65	75

Effect of Cysteine and Lysine

The observed efficiency of the fluorochromes employed as inhibitors of transaminase suggested that cysteine and lysine residues of the active center of the enzyme could be the sites of reaction of these inhibitors. It appeared then of interest to study the effect of the addition of these amino acids in systems containing the enzymes and fluorescein derivatives. The experiments were carried out in solutions of transaminase of a concentration 2.5 μM and fluorescein dimercuric acetate or fluorescein isothiocyanate at concentrations selected to have a final molar ratio in the system fluorescein dimercuric acetate/transaminase 1 and 2 and fluorescein isothiocyanate/transaminase 10 and 100. Cysteine or lysine was added in the previous systems in three different ways. First, fluorescein dimercuric acetate and cysteine or fluorescein isothiocyanate and lysine were added simultaneously in the enzyme solution; second, cysteine and lysine were added 30 min after the addition of the fluorochrome to the enzyme and third, fluorescein dimercuric acetate and cysteine or fluorescein isothiocyanate and lysine were preincubated for 30 min prior to their addition in the transaminase solution. The molar ratio of amino acids to fluorochromes used in the reaction systems ranged for cysteine/fluorescein dimercuric acetate and lysine/fluorescein isothiocyanate from 2 to 20 and 20 to 100, respectively. The results obtained for systems reacted up to 1 h are presented in Table 2. No protection of the catalytic activity of transaminase from the inhibitory action of fluorescein dimercuric acetate and fluorescein isothiocyanate was observed in the cases where amino acid addition followed or had taken place simultaneously with fluorescein dimercuric acetate or fluorescein isothiocyanate in the systems. A small protective effect of about 20% was found when high concentrations of cysteine and the usual quantity of fluorescein dimercuric acetate were preincubated for 30 min before their addition in the transaminase solution. Comparable results were obtained with lysine and fluorescein isothiocyanate.

THE ISOLATION OF STABLE TRANSAMINASE OR APOTRANSAMINASE COMPLEXES WITH FLUOROCHROMES

The formation of stable complexes between transaminases and the fluorochromes was accomplished through the following procedure. A quantity of the enzyme or apoenzyme (22.5 mg or 0.25 μmol) was mixed with fluorescein dimercuric acetate (2 mg or 2.5 μmol) in 0.05 M carbonate buffer pH 9.2 and allowed to react for 30 min at room temperature and then for 24 h in the cold room; the final volume of the reaction mixture was adjusted to 2 ml. After the reaction was completed the excess quantity of fluor-

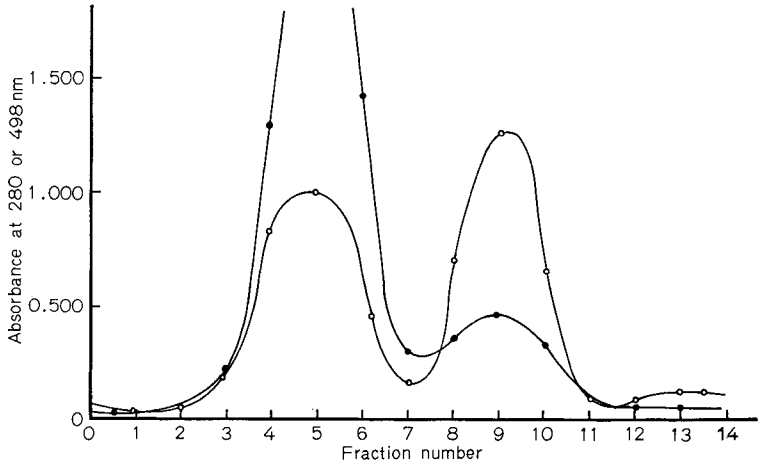

Fig. 4. *Elution diagram of a reaction mixture composed of 0.25 μmol transaminase and 2.5 μmol fluorescein dimercuric acetate in 2 ml 0.05 M carbonate buffer pH 9.2.* Fractionation was performed on a Sephadex G-25 (fine) column (1×25 cm) equilibrated and eluted with the above buffer. Fractions of 3 ml were collected and their absorbance at 280 (●) and 498 nm (○) was measured

Table 2. *Effect of cysteine or lysine on the inhibitory capacity of fluorescein dimercuric acetate or fluorescein isothiocyanate on the enzyme*

Reaction systems of 2 ml 0.05 M carbonate buffer pH 9.2 containing 2.5 μM transaminase and the corresponding quantity of inhibitors to give the molar ratio of fluorescein dimercuric acetate/transaminase or fluorescein isothiocyanate/transaminase indicated in the table. (a) Cysteine or lysine was preincubated for 30 min with the enzyme prior to the addition of fluorescein dimercuric acetate or fluorescein isothiocyanate. (b) Cysteine and lysine were preincubated for 30 min with fluorescein dimercuric acetate and fluorescein isothiocyanate respectively prior to their addition in the enzyme solution

Enzyme system	Inhibition			
	Fluorescein dimercuric acetate/enzyme		Fluorescein isothiocyanate/ enzyme	
	1	2	10	100
	%	%	%	%
Transaminase	55	70	30	80
Transaminase in (a)				
0.02 mM cysteine	50	70		
0.05 mM cysteine	45	75		
0.2 mM cysteine	50	65		
0.2 mM lysine			30	75
0.5 mM lysine			25	80
1.0 mM lysine			20	70
Transaminase in (b)				
0.02 mM cysteine	50	65		
0.05 mM cysteine	45	60		
0.2 mM cysteine	40	60		
0.2 mM lysine			25	65
0.5 mM lysine			20	60
1.0 mM lysine			20	55

escin dimercuric acetate was removed by passing the reaction mixture through a Sephadex G-25 column (1×25 cm) equilibrated and eluted with 0.05 M carbonate buffer pH 9.2. Eluate fractions of 3 ml obtained by means of a fraction collector were used to determine the concentration of the protein and the fluorochrome by measuring the absorbance of the fractions at their absorption maxima, namely 280 and 498 nm, respectively. Two main peaks appeared in the elution diagram (Fig. 4): the first peak was assigned to a partially active transaminase · fluorescein-dimercuric-acetate complex and the second to free fluorescein dimercuric acetate.

Complex Composition

The particular concentration of the enzyme or of the fluorochrome in the complex was calculated from the absorbancies of the eluates of the first peak using the formula: molar ratio of fluorescein dimercuric acetate/transaminase = $1.14/[(0.9\ A_{280}/A_{512})-0.2]$, taking into account that fluorescein dimercuric acetate concentration = $A_{498}\times 13$ mg/l (where A_{498} is the absorbance measured in a 1-cm cell at pH 9.2), A_{280}/A_{498} for fluorescein dimercuric acetate = 0.2, A_{512} (conjugated)/A_{498} (free) = 0.9 for fluorescein dimercuric acetate, transaminase concentration = $A_{280}\times 1.2$ mg/ml (where A_{280} is absorbance measured through a 1-cm cell at pH 9.2), transaminase concentration conjugated to fluorescein dimercuric acetate = $(A_{280}-0.2\ A_{498})\times 1.2$ mg/ml, transaminase molecular weight = 90000 and fluorescein dimercuric acetate molecular weight = 850.

The previous procedure was also used for the isolation of transaminase or apotransaminase and fluorescein isothiocyanate complexes. The elution diagram obtained after passing through a Sephadex G-25 column (1×25 cm) mixtures of the enzyme and fluorescein isothiocyanate, prepared as described above for fluorescein dimercuric acetate, was very

similar to the one shown in the Fig. 4. The particular concentration of transaminase or fluorescein isothiocyanate in the isolated complexes was calculated by using the following formula: molar ratio of fluorescein isothiocyanate/transaminase = $1/[(0.75\ A_{280}/A_{502})-0.38]$, taking into account that fluorescein isothiocyanate concentration = $5\times A_{493}$ mg/l (where A_{493} = absorbance measured in a 1-cm cell at pH 9.2), A_{280}/A_{493} for fluorescein isothiocyanate = 0.38, A_{502} (conjugated)/A_{493} (free) = 0.75 for fluorescein isothiocyanate, transaminase concentration = $1.2\times A_{280}$ mg/ml, transaminase concentration conjugated to fluorescein isothiocyanate = $(A_{280}-0.38\ A_{493})\times 1.2$ mg/ml, transaminase molecular weight = 90000 and fluorescein isothiocyanate molecular weight = 389.

Calculations from triplicate experiments performed as described previously and use of the above formulae gave molar ratios for the fluorescein dimercuric acetate/transaminase complexes ranging from 1.9 to 2.5, while the molar ratio for the fluorescein isothiocyanate/transaminase complexes was ranging from 0.5 to 0.3.

To gain further information about the site of the enzyme in which the fluorochromes are bound, additional experiments were performed using apotransaminase in place of transaminase. Calculations of the molar ratios of the complexes fluorescein dimercuric acetate/apotransaminase or fluorescein isothiocyanate/apotransaminase were made applying the formula used before, slightly modified for the calculation of apoenzyme concentration and assuming identical molecular weights for holotransaminase and apotransaminase. The molar ratio of the reactants in the isolated complexes obtained from duplicate experiments were found to be 2.1 and 2.3 for fluorescein dimercuric acetate/apotransaminase complexes and 1.8 and 2.0 for fluorescein isothiothanate/apotransaminase.

Enzyme Conformation and Complex Formation with Fluorochromes

In another series of experiments an attempt was made to explore whether the three-dimensional structure of transaminase was important for fluorescein dimercuric acetate or fluorescein isothiocyanate and enzyme complexation. Experiments such as the ones described previously using 0.25 μmol transaminase and 2.5 μmol fluorescein dimercuric acetate were performed in the presence and absence of 6 M urea. The fluorescein-dimercuric-acetate · transaminase complexes, obtained after passing the reaction mixtures through two identical Sephadex G-25 columns eluted with 0.05 M carbonate buffer pH 9.2 and the same buffer containing 6 M urea, differed markedly. Duplicate experiments demonstrated a molar ratio fluorescein dimercuric acetate/transaminase of 1 for the complex of the urea system in contrast to the molar ratio 2 for the complex of the control system. Similar experiments were performed using fluorescein isothiocyanate and apotransaminase. The complexes of fluorescein-isothiocyanate · apotransaminase isolated in the presence and absence of urea were found to consist of the same fluorochrome/apoenzyme molar ratio (about 2).

Complex Stability

The stability of the isolated fluorescein-dimercuric-acetate · transaminase or fluorescein-isothiocyanate · apotransaminase complexes was tested by extensive dialysis against 0.1 M cysteine or 0.1 M lysine, respectively. Different experiments were performed in which solutions of the complexes were dialysed against 0.1 M cysteine or lysine in 0.05 M carbonate buffer pH 9.2 for 6, 12, 24 and 48 h in the cold room by stirring gently. Calculations of the molar ratio of the reactants in the complexes after dialysis for 48 h revealed that cysteine was able to remove only one molecule of fluorescein dimercuric acetate from the complex; the molar ratio after dialysis against cysteine was found equal to 1. Comparable results were obtained for the fluorescein-isothiocyanate · apotransaminase system. After dialysis for 48 h against 0.1 M lysine the molar ratio of the reactants in the fluorescein-isothiocyanate · apotransaminase complex was reduced from 2 to 1.

Transaminase or Apotransaminase Polymerization

The possibility of transaminase polymer formation, either through the interaction of each of the two mercuric-reactive sites of fluorescein dimercuric acetate with sulfhydryl groups of two different transaminase molecules or by apotransaminase polymer formation because of the interaction of the —NCS group of fluorescein isothiocyanate with amino groups of two different apotransaminase molecules, was examined by acrylamide disc electrophoresis and column chromatography.

Acrylamide disc electrophoresis was performed as described in the Methods. In many electrophoretic experiments using fluorescein-dimercuric-acetate · transaminase or fluorescein-isothiocyanate · apotransaminase complexes only one band of protein was stained at about the same distance of the gel where free transaminase was moving. Chromatography on Sephadex G-200 columns (2×70 cm) was also used for the same purpose. Quantities of 0.25 μmol of the complexes obtained as described at the beginning of this section were layered on the top of the Sephadex column. Elution was performed using 0.05 M carbonate buffer pH 9.2. One main peak absorbing at 280 nm was detected in the elution diagram, developed by collecting 3-ml fractions, in the same area where free transaminase was eluted.

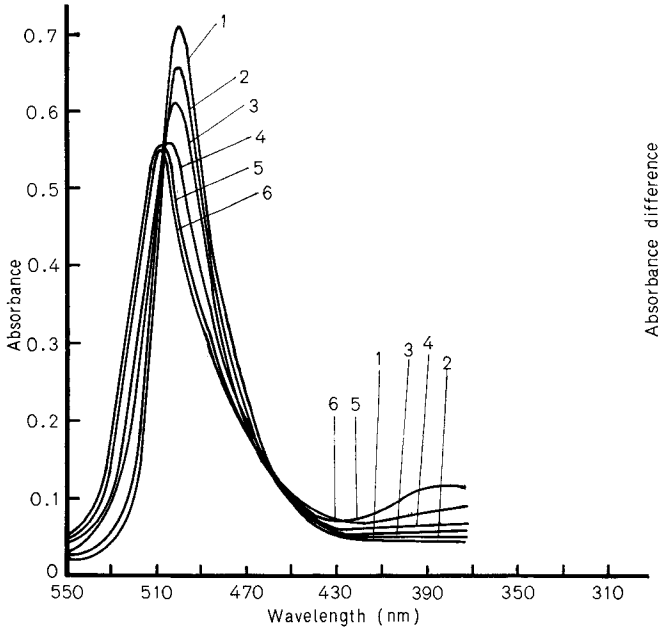

Fig. 5. *Changes in the absorption spectrum of fluorescein dimercuric acetate (1) during its interaction with transaminase in final concentrations 2 μM (2), 5 μM (3), 10 μM (4), 20 μM (5) or 40 μM (6).* Reaction systems of 3 ml containing 10 μM fluorescein dimercuric acetate in 0.05 M carbonate buffer pH 9.2 were used

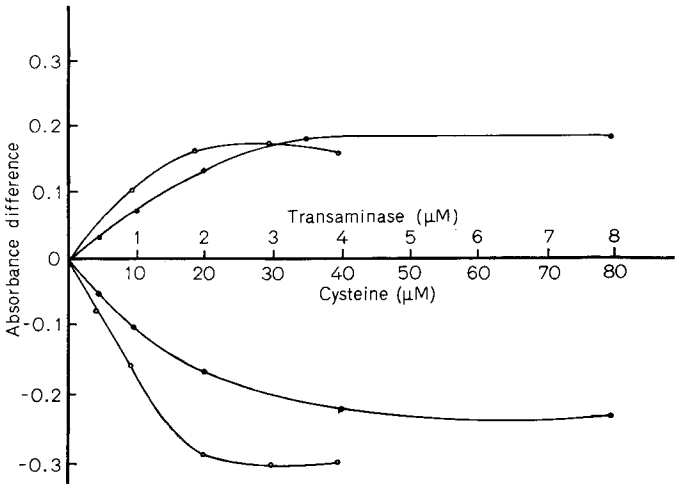

Fig. 6. *Changes in the absorption spectrum of fluorescein dimercuric acetate solutions during their interaction with transaminase (increase at 522 nm, decrease at 498 nm) or cysteine (increase at 478 nm, decrease at 498 nm) at different molar ratios.* Reaction systems of 3 ml containing 10 μM fluorescein dimercuric acetate in 0.05 M carbonate buffer pH 9.2 and the corresponding quantity of transaminase (●) or cysteine (○) were used

SPECTROSCOPIC CHANGES DURING THE INTERACTION OF TRANSAMINASE AND APOTRANSAMINASE WITH FLUOROCHROMES

Spectroscopic studies were carried out by adding increasing quantities of fluorescein dimercuric acetate or fluorescein isothiocyanate ranging from 1 to 100 μM in solutions of the enzyme (100 μM) in 0.05 M carbonate buffer pH 9.2 or increasing quantities of the enzyme ranging from 2 to 40 μM in 10 μM solutions of the fluorochromes. No significant changes were observed on the transaminase spectrum during the former experiments. In contrast, substantial changes in the spectrum of the fluorescein dimercuric acetate were recorded during its titration with transaminase (Fig. 5). The 498 nm absorption maximum of the fluorescein derivative decreases during the addition of the enzyme in the solution of the fluorochrome and eventually a new absorption maximum appears at 512 nm representing a red shift of the spectrum maximum of about 15 nm (Fig. 5). The kinetics of the reaction can be followed either by plotting the decrease in the absorbance at 498 nm or the increase of the absorbance at 522 nm *versus* the molarity of the reactant (Fig. 6). It appeared from these plots that equilibrium of the reaction was reached at a molar ratio of reactants in the solutions fluorescein dimercuric acetate/transaminase 2 to 2.5 (transaminase 5 μM, fluorescein dimercuric acetate 10 μM).

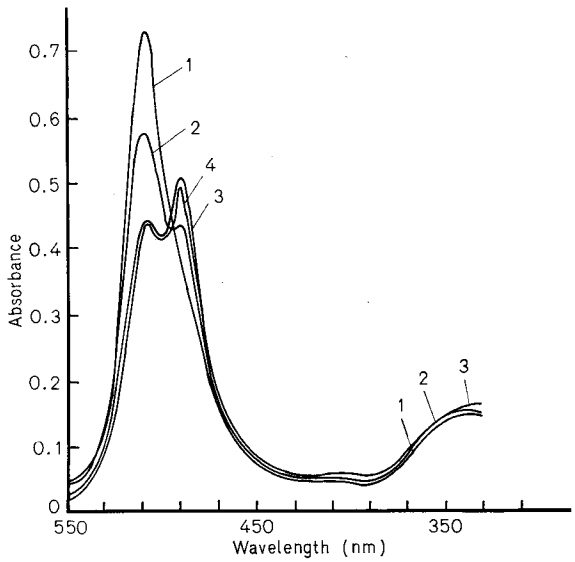

Fig. 7. *Changes in the absorption spectrum of fluorescein dimercuric acetate (1) during its interaction with cysteine in final concentrations 10 μM (2), 20 μM (3) or 40 μM (4).* Reaction systems of 3 ml containing 10 μM fluorescein dimercuric acetate in 0.05 M carbonate buffer pH 9.2 and the corresponding quantity of cysteine were used

The results obtained during the interaction of fluorescein dimercuric acetate with cysteine were substantially different. When increasing concentrations of cysteine ranging from 5 to 100 μM were added in a solution of fluorescein dimercuric acetate (10 μM) in 0.05 M carbonate buffer pH 9.2 the absorption maximum of the solution at 498 nm decre-

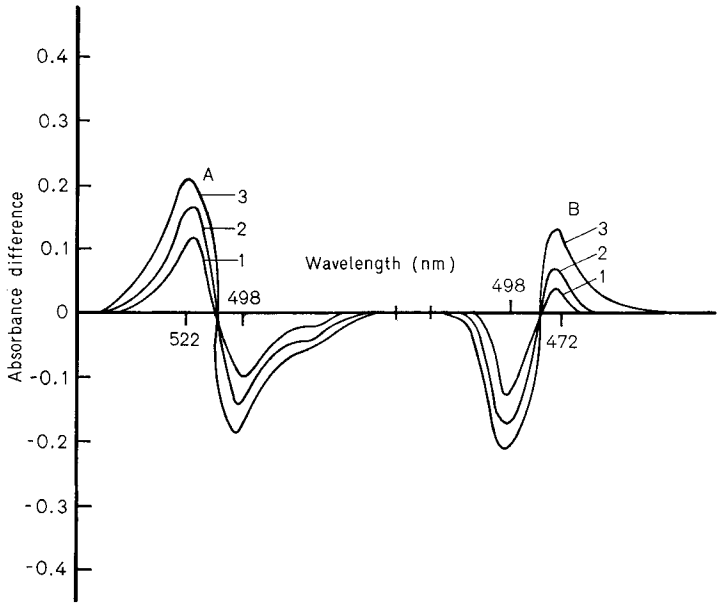

Fig. 8. *Difference spectra of the reaction products of fluorescein-dimercuric-acetate · transaminase (A) or fluorescein-dimercuric-acetate · cysteine (B)* versus *fluorescein dimercuric acetate.* (A, 1) Difference spectra of the reaction system fluorescein dimercuric acetate (10 μM) with transaminase (2 μM) *versus* fluorescein dimercuric acetate (10 μM) in 0.05 M carbonate buffer pH 9.2. (2) Same as (1) but with 5 μM transaminase. (3) Same as (1) but with 10 μM transaminase. (B, 1) Difference spectra of the reaction system fluorescein dimercuric acetate (10 μM) with cysteine (5 μM) *versus* fluorescein dimercuric acetate (10 μM) in 0.05 M carbonate buffer pH 9.2. (2) Same as (1) but with 10 μM cysteine. (3) Same as (1) but with 20 μM. The quantities of transaminase and cysteine used do not interfere with the absorption maximum of fluorescein dimercuric acetate

ased and eventually a new absorption maximum appeared at 478 nm representing a blue shift of about 20 nm (Fig. 7). The described reaction can be followed as before either by plotting the decrease in the absorbance at 498 nm or the increase at 478 nm *versus* the molar ratio of the components of the system (Fig. 6).

Previous experimental studies demonstrated that cysteine provoked a blue shift in the spectrum of fluorescein dimercuric acetate while transaminase under the same conditions induced a red shift. The remarkable properties of this system, shown very distinctly in the difference spectra of fluorescein dimercuric acetate-transaminase and fluorescein dimercuric acetate-cysteine *versus* fluorescein dimercuric acetate of the Fig. 8, were used to test spectroscopically the reactivity of the various sulfhydryl groups of transaminase with fluorescein dimercuric acetate. In a series of experiments fluorescein dimercuric acetate solutions (10 μM) in 0.05 M carbonate buffer pH 9.2 were titrated with different solutions containing the same quantity of transaminase (10 μM) in the same buffer as before and increasing quantities of cysteine ranging from 0.2 to 20 mM. It appeared from the spectroscopic changes of the Fig. 9 that more than 100 times more cysteine than transaminase was needed in order to

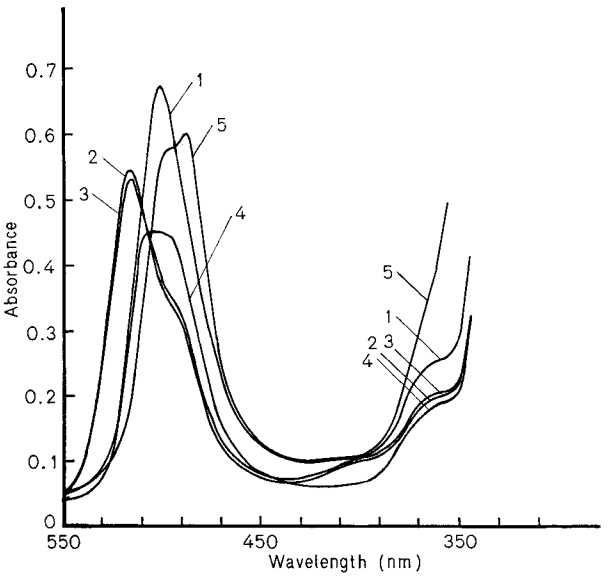

Fig. 9. *Changes in the absorption spectrum of fluorescein dimercuric acetate (1) during its interaction with transaminase in final concentration 10 μM (2) or in 10 μM transaminase and 0.2 mM cysteine (3) or in 10 μM transaminase and 2 mM cysteine (4) or in 10 μM transaminase and 20 mM cysteine (5).* Reaction systems of 3 ml containing 10 μM fluorescein dimercuric acetate in 0.05 M carbonate buffer pH 9.2 were used

neutralize the red shift effect induced in the spectrum maximum of fluorescein dimercuric acetate by the enzyme and provoke the appearance of the blue shift connected with the complexation of cysteine and fluorescein dimercuric acetate.

Experiments of the previous section concerning the formation of fluorescein-dimercuric-acetate · transaminase complexes demonstrated that less fluorescein dimercuric acetate is combined with the enzyme when the reaction takes place in 6 M urea. In the following experiments an attempt was made to demonstrate spectroscopically through titration the observed difference. Solutions of fluorescein dimercuric acetate (10 μM) in 0.05 M carbonate buffer containing 6 M urea were reacted with increasing quantities of transaminase in buffer solutions of 6 M urea. As was revealed from the spectroscopic changes of Fig. 10, transaminase in 6 M urea acts very poorly in inducing the characteristic spectral alterations to fluorescein dimercuric acetate. Apotransaminase was found to be a slightly more potential reagent than transaminase in decreasing the absorbance of the maximum of fluorescein dimercuric acetate at 498 nm.

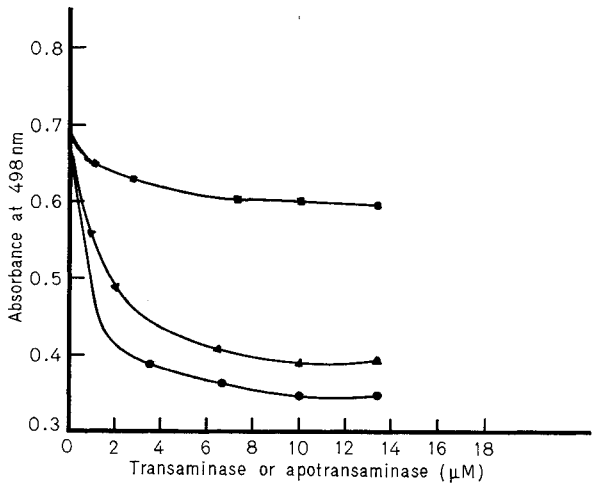

Fig. 10. *Changes in the absorption maximum of fluorescein dimercuric acetate solutions during their interaction with increasing quantities of transaminase (▲), apotransaminase (●) or transaminase in 6-M urea (■).* Reaction systems of 3 ml containing 10 μM fluorescein dimercuric acetate in 0.05 M carbonate buffer pH 9.2 and the quantities of reactants shown in the figure were used

Microenvironment of the Reacting Sulfhydryl Groups

In previous experiments it was found that two particular sulfhydryl groups of transaminase (one per subunit?) were about a 100 times more reactive towards fluorescein dimercuric acetate than the sulhydryl group of cysteine and that the reaction products of fluorescein dimercuric acetate with these two different categories of sulfhydryl groups differ markedly. The differences in the reactivity and spectral behaviour of the systems should reflect the effect of the microenvironment of the sulfhydryl groups of transaminase on fluorescein dimercuric acetate structure and affinity. To test this hypothesis the spectroscopic behaviour of the fluorochrome was tested in a series of solutions of decreasing polarity. Spectra of fluorescein dimercuric acetate were recorded in solutions composed from mixtures in different ratios of 0.05 M carbonate buffer pH 9.2, ethyl alcohol and butyl alcohol. It appeared from the spectroscopic changes of the Fig. 11 that the maximum in the spectrum of fluorescein dimercuric acetate shifts from 498 nm in 0.05 M carbonate buffer

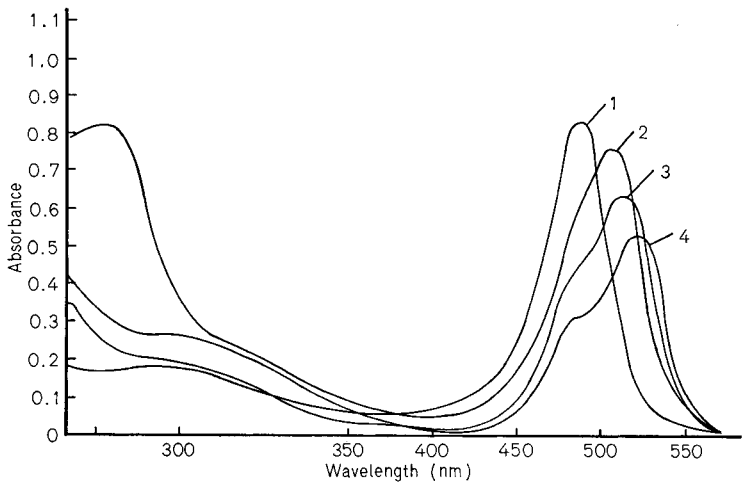

Fig. 11. *Absorption spectra of fluorescein dimercuric acetate in 0.05 M carbonate buffer pH 9.2 (1) or in a 10 μM transaminase solution in the same buffer (2) or in a mixture of 95% ethyl alcohol and 5% carbonate buffer (3) or in a mixture of 95% butyl alcohol, 2% ethyl alcohol and 3% carbonate buffer (4).* Reaction systems of 3 ml containing 10 μM fluorescein dimercuric acetate in the above solutions were used

pH 9.2, to 516 nm in a mixture of 95% ethanol and 5% buffer and finally to 522 nm in a solution composed of 95% *n*-butyl alcohol, 2% ethyl alcohol and 3% carbonate buffer. The observed similarities in the shifts of the spectrum maximum of fluorescein dimercuric acetate during its interaction with transaminase, with those obtained previously in solvents of decreasing polarity, suggest that the microenvironment of the two reacting sulfhydryl groups of transaminase is basically hydrophobic and is expressed roughly with the polarity of a solution composed of 80 to 90% ethanol and 10 to 20% carbonate buffer pH 9.2.

Changes in the Spectrum of Fluorescein Isothiocyanate

The spectroscopic changes of fluorescein isothiocyanate during its interaction with apotransaminase followed the previous pattern. The spectrum maximum of the free fluorochrome at 493 nm was moved to 502 nm in the fluorescein-isothiocyanate · apotransaminase complex, representing a red shift of about 10 nm, comparable with that in 99.2% ethanol (maximum 503 nm) or 98% butanol (maximum 505 nm), No significant spectral alterations were observed during fluorescein-isothiocyanate · transaminase or lysine interaction. The spectroscopic data obtained previously suggest a similarity in the sites of the enzyme which interact with the two fluorochromes.

CHANGES IN THE EMISSION SPECTRA OF FLUORESCEIN DIMERCURIC ACETATE AND FLUORESCEIN ISOTHIOCYANATE DURING THEIR INTERACTION WITH TRANSAMINASE OR APOTRANSAMINASE

The optimal conditions of experimentation for fluorescence intensity measurements were determined by studying the emission of the fluorochromes solutions as a function of their concentration and pH. In Table 3 are summarized the results obtained. It appeared that concentration of the solutions of the fluorescein derivatives of the order 10 to 1 μM and pH around 9.5 constituted the optimal conditions for studies concerning the emission of the fluorochromes when they were excited at 498 nm and 493 nm.

In most of the experiments which follow solutions of 10 μM fluorescein dimercuric acetate in 0.2 M carbonate buffer pH 9.2 were used. In various tubes containing the dimercuric fluorescein derivative, transaminase or apotransaminase was added in final concentrations ranging from 0.5 to 50 μM; the final volume of the reaction mixtures was adjusted to 3 ml. The systems were incubated for 15 min at 37 °C and emission spectra were recorded by exciting with monochromatic light at 498 nm using a Aminco-Bowman recording spectro-fluorimeter. The results

Table 3. *Fluorescence intensity of fluorescein dimeruric acetate and fluorescein isothiocyanate solutions excited at 498 and 493 nm respectively as a function of their concentration or pH*
Reaction systems of 3 ml were used containing the corresponding quantity of fluorescein dimercuric acetate or fluorescein isothiocyanate at the indicated pH values. Different buffers referred in the Methods at a concentration of 0.2 M were used. Arbitrary units of fluorescence intensity were used

Fluorochrome	concn	Fluorescence intensity at pH					
		6	8	9	10	12	14
	μM						
Fluorescein dimercuric acetate	500			13			
	100			15			
	50			16			
	10	3	7	16	16	16	16
	5			14			
	1			7			
Fluorescein isothiocyanate	500			10			
	100			11			
	50			12			
	10	8	9	13	13	13	13
	5			8			
	1			4			

are shown in Fig. 12 where substantial quenching in the intensity of the fluorescence spectra of fluorescein dimercuric acetate is observed. In another series of experiments performed as described previously cysteine to final concentrations ranging from 1 to 100 μM was added to fluorescein dimercuric acetate solutions (10 μM) in 0.2 M carbonate buffer pH 9.2; the recorded spectra are included in Fig. 12. The decrease in the total fluorescence intensity of the fluorescein dimercuric acetate solutions excited at 498 nm as a function of the molar ratio of fluorescein dimercuric acetate to transaminase, apotransaminase or cysteine studied in a different series of experiments is shown in Fig. 13. It appeared both from emission spectra and total fluorescence quenching measurements that the maximum decrease of the fluorescence of fluorescein dimercuric acetate solutions was reached at a molar ratio of fluorescein dimercuric acetate/transaminase, fluorescein dimercuric acetate/apotransaminase or cysteine/fluorescein dimercuric acetate around 2.

Similar experiments were performed using solutions of fluorescein isothiocyanate (10 μM) in 0.2 M crabonate buffer pH 9.2 and concentrations of transaminase, apotransaminase or lysine the same as the ones used for the fluorescein dimercuric acetate-transaminase-cysteine systems. The observed changes in the fluorescence spectra of the solutions of fluorescein isothiocyanate during the interactions are shown in Fig. 14. Apotransaminase was found to be a more potential reactant in quenching the fluorescence spectrum of fluorescein isothiocyanate than holotransaminase. No major changes were observed

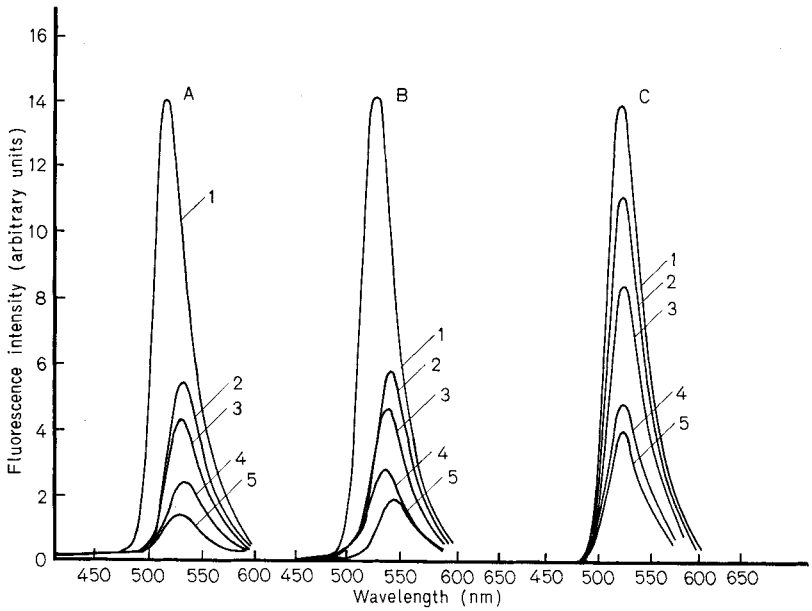

Fig. 12. *Changes in the emission spectra of fluorescein dimercuric acetate (obtained after excitation at 498 nm) during its interaction with transaminase (A), apotransaminase (B) or cysteine (C).* The spectra are uncorrected. Curves (A) represent reaction systems of 3 ml containing 10 μM fluorescein dimercuric acetate in 0.2 M carbonate buffer with transaminase concentrations of 0 (1), 2 μM (2), 5 μM (3), 10 μM (4) or 20 μM (5). Curves (B) represent similar systems as (A) except that apotransaminase was used in place of transaminase. Curves (C) represent reaction systems of 3 ml containing 10 μM fluorescein dimercuric acetate in 0.2 M carbonate buffer pH 9.2 and cysteine concentrations of 0 (1), 5 μM (2), 10 μM (3), 20 μM (4) or 40 μM (5)

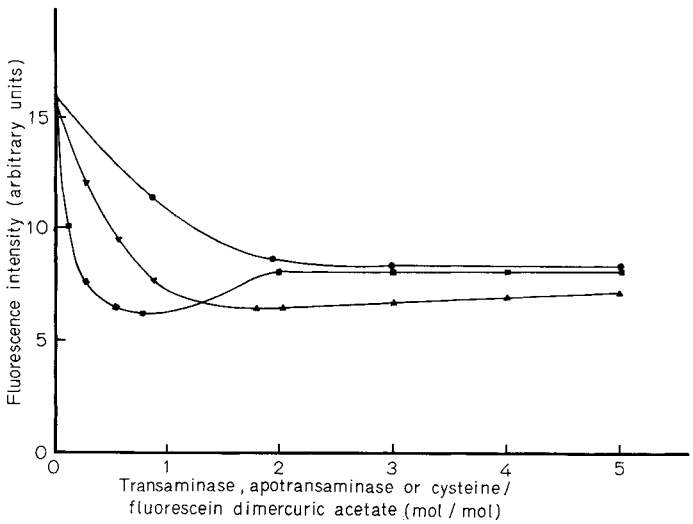

Fig. 13. *Fluorescence quenching of fluorescein dimercuric acetate solutions excited at 498 nm, after their interaction with transaminase (▲), apotransaminase (■) and cysteine (●) at different molar ratios.* Reaction systems of 3 ml containing 10 μM fluorescein dimercuric acetate in 0.2 M carbonate buffer pH 9.2 and the corresponding quantity of transaminase, apotransaminase and cysteine to give the molar ratio indicated in the figure were used

in the emission spectrum of fluorescein isothiocyanate during the addition of lysine to the solutions of the fluorochrome. The previous data were in agreement with the results obtained during the studies of transaminase and apotransaminase inhibition by fluorescein isothiocyanate.

QUANTITATIVE PARAMETERS OF THE DATA OBTAINED

In previous sections it has been shown that fluorochromes inactivate the enzyme with concomitant incorporation of the dyes into the protein molecule. The rate of the reaction of transaminase with fluores-

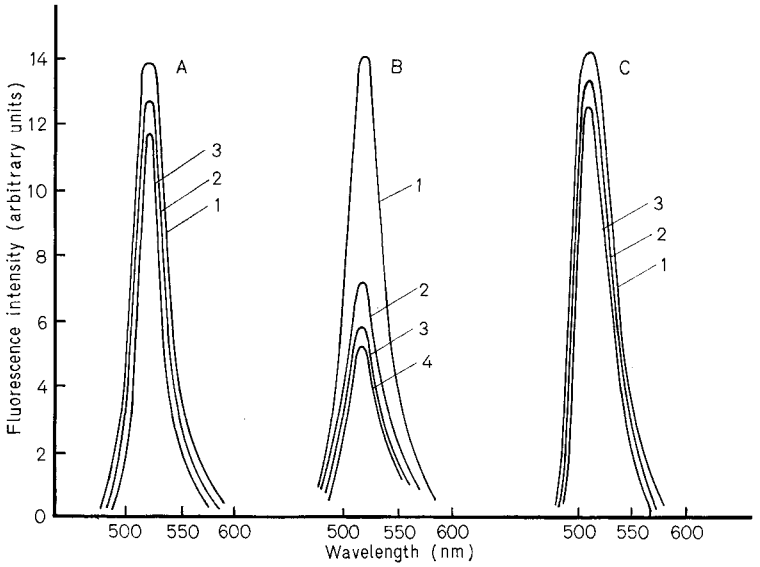

Fig. 14. *Changes in the emission spectra of fluorescein isothiocyanate (obtained by excitation at 493 nm) during its interaction with transaminase (A), apotransaminase (B) and lysine (C).* The spectra are uncorrected. Curves (A) represent reaction systems of 3 ml containing 10 μM fluorescein isothiocyanate in 0.2 M carbonate buffer with transaminase concentrations of 0 (1), 5 μM (2) or 10 μM (3). Curves (B) represent identical systems with (A) except that apotransaminase was used in place of transaminase; system (4) contained 20 μM apotransaminase. Curves (C) represent identical systems with (A) except that lysine was used in place of transaminase

cein dimercuric acetate was too fast to be measured by conventional techniques. Stopped-flow kinetic experiments were conducted on a Durrum-Gibson stopped-flow spectrophotometer. The reaction was followed at 498 and 520 nm corresponding to the maxima of free and transaminase-conjugated fluorescein dimercuric acetate, respectively. Oscilloscope traces of voltage *versus* time were measured directly from polaroid photographs of the oscilloscope screen (Fig. 15).

The special case of the general second-order rate equation $A + B \rightarrow C + D$, when $[A] = [B]$, was used with concentrations of fluorescein dimercuric acetate and transaminase equal to 5 μM to calculate the velocity constant of the second-order reaction. At a given time t the above equation can be written $-d[A]/dt = [A][B]k$ or $-d[A]/dt = [A]^2 k$; integration yields $1/[A]_t - 1/[A]_0 = kt$ where $[A]_t$ and $[A]_0$ are the concentrations of the reactants at the time t and zero, respectively. The stopped flow data of the Fig. 15 A obtained by following the increase of the transmittance (decrease in absorbance) at 498 nm were analyzed by plotting $1/[A]_t$ *versus* t. The velocity constant calculated from the slope of the linear plot was $k = 1.15 \times 10^6\ M^{-1}\ s^{-1}$, with a half-life period of the reaction $t_{1/2} = 170$ ms [30—32]. The isolation method for the determination of the order of a reaction was used to analyze the stopped-flow data of Fig. 15 B obtained by following the decrease in the transmittance (increase of the absorbance) at 520 nm [33]. According to this method for a reaction of n order the velocity is calculated from the equations:

$$-\frac{dc}{dt} = k\,c^n \quad \text{or} \quad \log\left(-\frac{dc}{dt}\right) = \log k + n \log c$$

which can be used in the following way. A plot of c *versus* t is constructed from the data. The slope of the curve dc/dt is measured at several different values of t; the corresponding value of c is read from the plot. The logarithm of $(-dc/dt)$ is then plotted against log c. The slope of the line gives the order of the reaction. It was found that the reaction followed second-order kinetics for a period of 900 ms with $n = 2.1$ and a velocity constant $k = 1.26 \times 10^6\ M^{-1}\ s^{-1}$.

Stopped-flow experiments between fluorescein isothiocyanate and transaminase gave poor photographs which were no suitable for kinetic studies. The parameters of the reaction were determined for this reason, from the data of enzyme inhibition of the first section (Fig. 3). The experimental data were analyzed using the second-order equation:

$$\log -\frac{b\,(a-x)}{a\,(b-x)} = kt\,\frac{(a-b)}{2.303}\,.$$

By plotting $[2.203/(a-b)]$ log $[b\,(a-x)/a\,(b-x)$ *versus* t a linear plot is obtained with a slope equal to the velocity constant [31, 32]. The described procedure gave $k = 330\ M^{-1}\ s^{-1}$ and $t_{1/2} = 85$ s for a reaction system fluorescein isothiocyanate (25 μM) and transaminase (2,5 μM) and a $k = 1160\ M^{-1}\ s^{-1}$

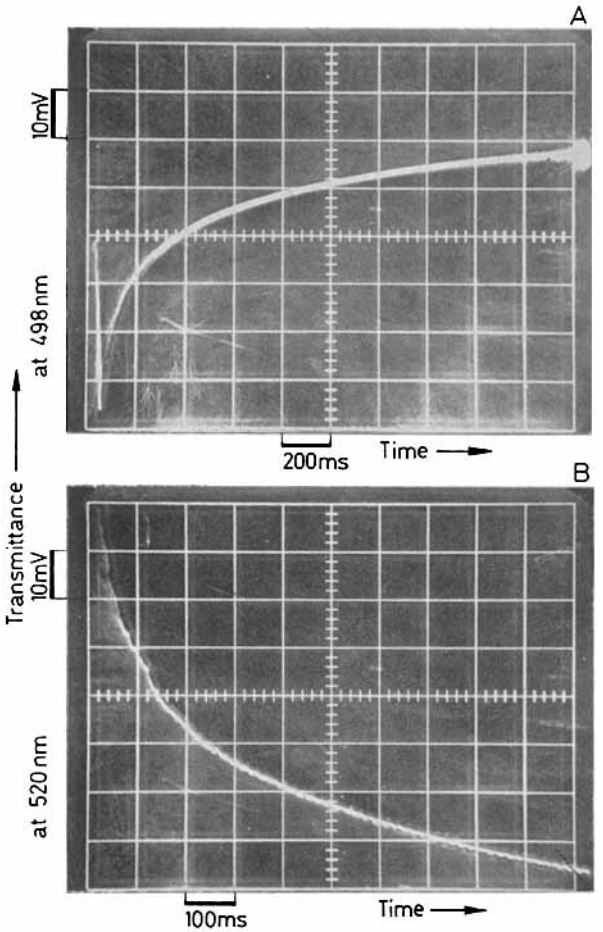

Fig. 15. *Oscilloscope traces of transmittance* versus *time showing the reaction between fluorescein dimercuric acetate and transaminase.* Curve (A). Reaction system containing fluorescein dimercuric acetate and transaminase of the same molarity, 5 μM, in 0.05 M carbonate buffer pH 9.2 at 22 °C. The reaction was followed by recording the increase of the transmittance of the system at 498 nm. Each division of the ordinate equals to 10 mV, while each division of the abscissa is equivalent to 200 ms. Curve (B). Reaction system containing 5 μM fluorescein dimercuric acetate and 10 μM transaminase in 0.05 M carbonate buffer pH 9.2. The reaction was followed by recording the decrease in the transmittance of the system at 520 nm. Each division of the ordinate equals to 10 mV, while each division of the abscissa is equivalent to 100 ms

and $t_{1/2} = 45$ s for a similar system of fluorescein isothiocyanate and apotransaminase.

The dissociation constant (K_d) of the complex transaminase · fluorescein-dimercuric-acetate or transaminase · fluorescein-isothiodcyanate was obtained by treating the spectrophotometric data in the third section of Results according to the method of Jenkins and Taylor [34]. Using the equation

$$\frac{[E]}{\Delta A} = \frac{K_d}{(\varepsilon_1 - \varepsilon_2)\,[I]} + \frac{[E]}{(\varepsilon_1 - \varepsilon_2)\,[I]}$$

and plotting $[E]/\Delta A$ *versus* [E] a linear plot is obtained which intercepts the axis [E] at a point which gives the dissociation constant of the enzyme · inhibitor. The procedure described gave $K_d = 2$ μM for the system transaminase · fluorescein-dimercuric-acetate and $K_d = 50$ μM for the system apotransaminase · fluorescein-isothiocyanate (ε_1 and ε_2 denote the absorption coefficients of fluorescein dimercuric acetate or fluorescein isothiocyanate and their complexes with transaminase or apotransaminase, respectively, while [E] and [I] represent the concentration of enzyme and inhibitor. Equilibrium constants K_i for the fluorochromes were also determined from the data of their inhibition on the catalytic activity of the enzyme. The activity of transaminase was measured in a series of systems containing final concentrations of fluorescein dimercuric acetate 2, 4 or 10 μM, fluorescein isothiocyanate 4, 8 or 12 μM, 2-oxoglutarate 8 mM and final concentrations of aspartate ranging from 0.8 to 16 mM. By plotting $1/v$ *versus* 1/[S] from the data of the different systems, families of linear curves were obtained which demonstrated typical non-competitive plots for the system transaminase · fluorescein-dimercuric-acetate. The plots of the systems of transaminase or apotransaminase with fluorescein isothiocyanate revealed a picture of competitive inhibition. The mean values of the equluibrium constants determined from the Lineweaver-Burk plots [35] which intercept the vertical axis at $(1 + [I]/K_i)/V$ in the non-competitive inhibition and the base line at $1/[K_m\ (1 + [I]/K_i)]$ in the competitive inhibition were found for the transaminase · fluorescein-dimercuric-acetate system $K_i = 4.7$ μM, and for the systems of transaminase or apotransaminase with fluorescein isothiocyanate $K_i = 16$ μM and $K_i = 8.9$ μM, respectively.

The specific activity of the enzyme or of its complexes with fluorescein dimercuric acetate and fluorescein isothiocyanate was found to be 30, 1 and 10 A_{280} units × mg protein^{-1} × min^{-1}, respectively. All-or-none tests were carried out on the two half reactions of transaminase using difference spectroscopy. Experiments were performed by adding increasing concentrations of glutamate or 2-oxoglutarate on substrate amounts of transaminase in the pyridoxal —*P* or pyridoxamine-*P* form in the presence and absence of fluorescein dimercuric acetate or fluorescein isothiocyanate. While no inhibition in the formation of the 333-nm transaminase form was observed during the addition of glutamate to the pyridoxal form of the enzyme in the presence of fluorescein dimercuric acetate, a substantial inhibition (about 40%) was observed in the conversion of the 333-nm form of transaminase to the 362-nm one during the addition of 2-oxoglutarate to the pyridoxamine-*P* form of the enzyme in the presence of the mercuric derivative of fluorescein. The results obtained with fluorescein isothiocyanate were different.

No inhibition in the transformation of the 333-nm form of the enzyme to the 362-nm one was observed during the addition of 2-oxoglutarate in the presence of fluorescein isothiocyanate. In contrast, when high concentrations of fluorescein isothiocyanate were used, the addition of glutamate to the 362-nm form of the enzyme resulted in the appearance of a maximum in the system at 410 nm with a partial inhibition in the formation of the 333-nm form of the enzyme. Both fluorochromes, on the other hand, were found to inhibit the formation of transaminase · *erythro*-3-hydroxyaspartate complex with a maximum at 492 nm or to increase the rate of its dissociation when *erythro*-3-hydroxyaspartate was added to the systems prior to the addition of fluorescein derivatives. Since the interconvertibility of the modified enzyme from the pypidoxal-*P* form to the pyridoxamine-*P* one and *vice versa* is incomplete but not comparable with the observed specific activities, it is rather difficult to ascertain whether the residual activity of the modified transaminase is due to the partial loss of activity of all transaminase molecules or to complete inactivation of some of them.

DISCUSSION

It has been found that glutamic-aspartic transaminase was inhibited strongly by two fluorescein derivatives reacting with sulfhydryl and amino group (Fig. 1, 2 and 3). Apotransaminase was found to be even more sensitive than transaminase towards fluorescein dimercuric acetate and fluorescein isothiocyanate. Since spectroscopic data indicated that fluorochromes are unable to displace pyridoxal-*P* from the active center of the enzyme, the enhanced inhibition of apotransaminase suggests that fluorochromes interfere but do not prevent the coenzyme-apoenzyme binding.

The isolation of stable transaminase or apotransaminase complexes with fluorescein dimercuric acetate or fluorescein isothiocyanate is of particular interest. The molar ratio of the reactants in the isolated complexes was fluorescein dimercuric acetate/transaminase or apotransaminase around 2, fluorescein isothiocyanate/apotransaminase 2, and fluorescein isothiocyanate/transaminase 0.5. The previous data suggest that of the 10 sulfhydryl groups of transaminase [4, 5] only two (that is one per subunit) are able to be complexed in a stable way with fluorescein dimercuric acetate. The possibility of interaction of each of the two mercuric sites of fluorescein dimercuric acetate with sulfhydryl groups of two different transaminase molecules should be excluded since no transaminase polymers were detected by acrylamide gel electrophoresis or chromatography on Sephadex G-200. The same reasoning could be applied for the fluorescein isothiocyanate complexes. Less than one of the available 44 ε-amino groups of the lysine residues of transaminase [3, 4] is reacting with a well-known reagent for amino groups as fluorescein isothiocyanate, while the figure for apotransaminase is two reactive amino groups. There is no evidence whether the observed inertness of $-SH$ and $-NH_2$ groups towards fluorescein dimercuric acetate and fluoresceine isothiocyanate is due to their hydrogen or electrostatic bonding to other groups of the enzyme or because of their location in polar sites not available to the rather non-polar aromatic fluorescein nucleous. The enhanced reactivity of fluorescein isothiocyanate with the amino groups of apoenzyme is possibly due to the fact that these $-NH_2$ groups are located in the non-polar area inside the active center of the enzyme [1]. The non-polar character of this area is also suggested by the similarity in the spectra of fluorescein-isothiocyanate · apotransaminase or fluorescein-dimercuric-acetate · transaminase complexes with those of fluorescein isothiocyanate or fluorescein dimercuric acetate in non-polar solvents (Fig. 11 and Results). It seems reasonable therefore that a multi-nuclear aromatic molecule like fluorescein dimercuric acetate or fluorescein isothiocyanate reaches and complexes more easily with amino or sulfhydryl groups located at the above site than others sitting at the exterior polar part of transaminase. The proximity of fluorescein dimercuric acetate and fluorescein isothiocyanate reactive sites of transaminase with each other and with the active center of the enzyme is suggested by the potential stoichiometric inhibition of transaminase by fluorescein dimercuric acetate and apotransaminase by fluorescein isothiocyanate, by the spectroscopic changes of the fluorochromes as well as by the fluorescence intensity measurements of the Fig. 12, 13 and 14.

Additional evidence for the specific interaction of the fluorochromes with a non-polar reactive site of transaminase arises from the experiments in which fluorescein dimercuric acetate and fluorescein isothiocyanate reacted with transaminase and apotransaminase in the presence of cysteine and lysine (Table 2, Fig. 9). Although cysteine or lysine were present in the systems at concentrations higher than the ones corresponding to cysteine or lysine residues of transaminase, no substantial effect was noted in the characteristic changes arised from the interaction of transaminase and apotransaminase with fluorescein dimercuric acetate and fluorescein isothiocyanate. The red shift in the spectrum of fluorescein dimercuric acetate induced by the enzyme was reversed by the blue shift induced by cysteine (Fig. 5 and 7) only when the concentration of the latter in the system exceeded by more than 50 times that of transaminase (Fig. 9). Even dialysis of the fluorescein-dimercuric-acetate · transaminase or fluorescein-isothiocyanate · apotransaminase complexes against 0.1 M cysteine or

lysine respectively failed to replace completely transaminase or apotransaminase with the amino acids in the complexes.

Maximum fluorescence quenching of fluorescein dimercuric acetate was observed when two molecules of cysteine or one molecule of transaminase per molecule of fluorochrome were added in the system. There are two mercuric-reactive sites for sulfhydryl groups in fluorescein dimercuric acetate. As was demonstrated, these sites are able to interact with two sulfhydryl groups of two cysteine molecules but not with two different sulfhydryl groups of cysteine residues of two transaminase molecules, since no transaminase polymerisation was observed. It appears then that the two active sulfhydryl groups of transaminase react with two different fluorescein dimercuric acetate molecules (Fig. 6, 8, 12 and 13). The possibility of four sulfhydryl groups per transaminase molecule reacting with two molecules of fluorescein dimercuric acetate should not be excluded although such a complexation seems unrealistic since it requires location of cysteine residues in specific places of the enzyme.

Although the presence of amino substrates or oxo-acid substrates or both in the systems of the enzyme was found previously to protect [9] or accelerate [8, 29] the inactivation of transaminase during its treatment with sulfhydryl group reagents, no major changes of the results obtained were noted when transaminase and fluorescein dimercuric acetate reacted in the presence of substrates (Table 1). This is probably due to the non-competitive nature of the reaction and to the very low dissociation constant ($K_d = 2\ \mu M$) of the complex between transaminase and fluorescein dimercuric acetate. Difficult to explain are the findings obtained during the interaction of transaminase and fluorescein isothiocyanate in the presence of substrates (Table 1). No protection of the enzyme from the inhibitory effect of fluorescein isothiocyanate was observed during the presence of substrates though the inhibitor competes with aspartate for the active center of the enzyme. This might be explained by the low equilibrium constant of the transaminase · fluorescein-isothiocyanate system ($K_i = 16\ \mu M$), from the fact that the K_i for the apotransaminase system is even lower (8.9 μM) and from the observation of the all-or-none tests according to which an unidentified complex with a characteristic maximum at 410 nm appears during the interaction of fluorescein isothiocyanate with substrate amounts of the enzyme in the presence of an aminosubstrate.

Considering the fact that the complete primary structure of transaminase is now known [36] and that two particular amino acids Lys-258 and Tyr-40 are situated in the proximity of the active center of the enzyme, one could suggest that the most probable cysteine residues reacting with fluorescein dimercuric acetate might be either Cys-252 or Cys-45. It is rather difficult to suggest the particular amino group responsible for the fluorescein isothiocyanate binding to the enzyme.

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