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INDUCTION OF CONFORMATIONAL CHANGES IN BOVINE LIVER GLUTAMATE DEHYDROGENASE BY A STEROID ANALOGUE. I

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SUMMARY

In the presence of bovine liver glutamate dehydrogenase (L-glutamate : $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3), diethylstilboestrol induced a biphasic enhancement of NADH fluorescence and of the fluorescence of the probe 1-anilinonaphthalene-8-sulphonate (ANS). The previous paper (Yeates, R. A. (1973) Biochim. Biophys. Acta 334, 45–57) described the biphasic inhibition of the enzyme by diethylstilboestrol. In all three cases a rapid phase was followed by a slow phase which took several minutes to develop. Evidence is presented that the same transition gave rise to the slow phase of the three phenomena.

The extent of the ANS fluorescence enhancement was increased by NADH, by NAD^+ (particularly in the presence of α-ketoglutarate), by α-ketoglutarate in the presence of NAD^+ or NADH and by pyruvate in the presence of NADH. L-Glutamate, glutarate and NH_4Cl had no effect.

The rapid enhancement of probe fluorescence derived from an increase in probe binding with no change in the quantum yield of bound probe. The slow enhancement corresponded to an increase in probe binding, counteracted by a decrease in the quantum yield, an increase in the measured lifetime and an increase in the radiative lifetime of the fluorescence of the bound probe.

A good correlation existed between the factors which increased the diethylstilboestrol-induced enhancement of probe fluorescence and factors which increased the diethylstilboestrol-induced disaggregation of the enzyme.

An extension of the model put forward in the preceding paper (Yeates, R. A. (1973) Biochim. Biophys. Acta 334, 45–57) is proposed. This involves the induction of a biphasic conformational change in the enzyme by diethylstilboestrol. The results are explained in terms of the properties of the two conformations formed. Evidence is presented that disaggregation of the enzyme follows the rapidly induced conformational transition.

A comparison is made between the two allosteric inhibitors of the enzyme,

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Abbreviation: ANS, 1-anilinonaphthalene-8-sulphonate.

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GTP and diethylstilboestrol. It is shown that the binding sites for the two inhibitors and the conformations they induce in the presence of NADH are distinct.

INTRODUCTION

The preceding paper [1] has described the biphasic inhibition of glutamate dehydrogenase (L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3) by the oestrogen analogue diethylstilboestrol. The results were interpreted in terms of the induction of a biphasic conformational change in the enzyme by diethylstilboestrol. The interaction was further investigated by studying the diethylstilboestrol-induced enhancement of the fluorescence of NADH [2] and of the probe 1-anilinonaphthalene-8-sulphonate (ANS) [3, 4] in the presence of the enzyme. The interaction of glutamate dehydrogenase with the two allosteric inhibitors, GTP and diethylstilboestrol, was compared.

Glutamate dehydrogenase undergoes a concentration-dependent aggregation (e.g. ref. 5). Diethylstilboestrol alone slightly disaggregates the enzyme. This effect is greatly enhanced by NADH and, to a lesser extent, by NAD^+ [6, 7]. It is considered [8] that conformations of the enzyme of low glutamate and high alanine dehydrogenase activity possess a low tendency to associate. The relationship of the diethylstilboestrol interaction to the degree of enzyme aggregation was further investigated.

MATERIALS AND METHODS

Except where mentioned all the experiments were carried out at 15 °C in 0.1 M sodium phosphate, pH 7.7. Boehringer supplied NAD^+. ANS was purified by recrystallization of the magnesium salt [4]. British Drug Houses supplied sodium L-glutamate and glutaric acid.

Diethylstilboestrol concentrations were kept below the saturation limits of 30 μM in water and 60 μM in 1 mg/ml glutamate dehydrogenase.

In the presence of α-ketoglutarate, NADH and enzyme traces of ammonia slowly react, giving NAD^+ and L-glutamate. This effect was negligible within the times of the experiments with diethylstilboestrol.

Fluorescence intensities were measured on a Hitachi–Perkin–Elmer MPF-2A spectrofluorimeter. When it was desired to follow rapid changes in fluorescence intensity at a fixed wavelength, small volumes of solutions were passed into the fluorescence cell on the flattened end of a glass rod.

In all fluorescence experiments the absorbance of the chromophore at the exciting wavelength was kept below 0.1. NADH fluorescence was excited at 380 nm and observed at 470 nm. ANS fluorescence was excited at 410 nm and observed at 550 nm. At these wavelengths ANS fluorescence could be separated from that of NADH. Under the conditions used the intensity of fluorescence was proportional to concentration for both fluorophores in the absence of enzyme.

First-order rate constants for diethylstilboestrol-induced rises in fluorescence were derived from the gradients of plots of $\log (F\infty)/(F(\infty) - F(t))$ against time. $F(t)$ is the rise in fluorescence, measured from an arbitrary zero at time t; $F(\infty)$ is the rise at infinite time, calculated by extrapolation. $F(\infty)$ values could also effectively be

measured directly for rapid rises in fluorescence. Rate constants derived were accurate to about 15%.

Fluorescence lifetimes were determined on a TRW 75A decay time fluorimeter. This used a TRW 31A nanosecond spectral source with a nitrogen lamp (operating at 5 kcycles/s; pulse duration 20 ns), and was linked to a Tektronix Type dual beam oscilloscope with a type ISI sampling attachment. The lamp shape was determined using a scattering sample. The signal was fed to the sampling beam of the oscilloscope. The main beams of the oscilloscope carried the computer simulated traces which were then fitted to the lamp shape trace. The sample decay trace was then displaced, and the computer simulated decay trace fitted to it, the amount of adjustment from the lamp shape being read out as a fluorescence lifetime. For ANS fluorescence an excitation filter with maximum band-pass at 350 nm and an emission filter with cut-off above 550 nm were used.

Qualitative light scattering measurements were carried out on the Hitachi–Perkin–Elmer fluorimeter.

Ultracentrifugation experiments were carried out on a Spinco model E analytical ultracentrifuge with schlieren optics.

Other materials and methods used were as described in the preceding paper [1].

RESULTS

ANS fluorescence enhancement

The enhancement of ANS fluorescence induced by diethylstilboestrol in the presence of glutamate dehydrogenase was biphasic (Fig. 1a). The fast phase was followed by a small slow phase which took several minutes to develop.

The addition of NADH to a mixture of diethylstilboestrol, ANS and glutamate dehydrogenase caused a greatly increased enhancement of probe fluorescence (Fig. 1b). Under most conditions this was all of the slow type. Analogously diethylstilboestrol caused a greater enhancement of probe fluorescence in the presence of NADH.

On adding α-ketoglutarate to a mixture of diethylstilboestrol, NADH, ANS and glutamate dehydrogenase a biphasic enhancement of probe fluorescence was observed (Fig. 1c). In the absence of NADH no effect occurred. The possibility was considered that α-ketoglutarate acted simply by increasing the binding of NADH to the enzyme. A mixture of diethylstilboestrol, ANS and glutamate dehydrogenase was saturated with NADH, so that the addition of further coenzyme caused no enhancement. α-Ketoglutarate still enhanced probe fluorescence (Table I). It was deduced that α-ketoglutarate had a direct effect on the system.

The slow phase of the fluorescence enhancements could be analysed as a first-order change. NADH increased the rate of the slow enhancement (Table II). α-Ketoglutarate, in the presence of NADH, behaved similarly (Table III). Correspondingly, the rate of the slow enhancement induced by adding NADH to a mixture of diethylstilboestrol, probe and enzyme was slower than that induced by the subsequent addition of α-ketoglutarate.

NH_4Cl, L-glutamate and the competitive inhibitor glutarate had no effect on the rate or extent of the diethylstilboestrol-induced enhancement of probe fluorescence.

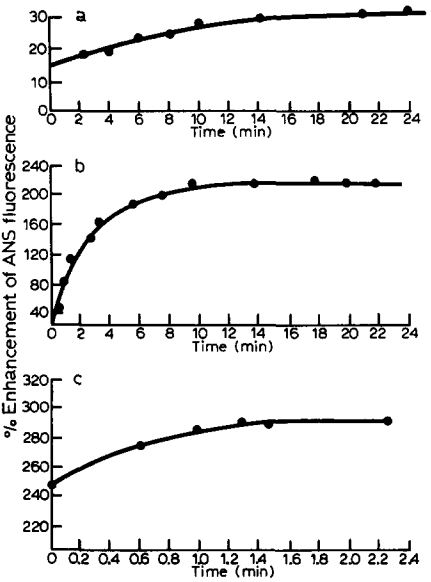

Fig. 1. Successive enhancement of ANS fluorescence by the addition of diethylstilboestrol (a), then NADH (b), then α-ketoglutarate (c) to glutamate dehydrogenase. 30 μM diethylstilboestrol, 40 μM NADH and 0.1 mM α-ketoglutarate were added successively to a mixture of 24 μM ANS and 0.44 mg/ml enzyme.

Concentrations of NAD^+ up to a saturating level (1.4 mM) caused only very slight enhancement of probe fluorescence when added to a mixture of diethylstilboestrol, probe and enzyme. Subsequent addition of α-ketoglutarate caused a large fluorescent enhancement of the slow type (Fig. 2). Addition of low concentrations of NAD^+ to a mixture of α-ketoglutarate, diethylstilboestrol, probe and enzyme caused a biphasic enhancement of probe fluorescence (Fig. 3).

Pyruvate is a poor substrate of glutamate dehydrogenase. The addition of pyruvate to a mixture of NADH, diethylstilboestrol, probe and enzyme produced a slow enhancement of probe fluorescence, even when the enzyme was presaturated

TABLE I

ENHANCEMENT OF ANS FLUORESCENCE BY α-KETOGLUTARATE IN THE PRESENCE OF SATURATING CONCENTRATIONS OF NADH

Sample	Fluorescence enhancement (%)
0.90 mg/ml enzyme, 40 μM ANS, 55 μM diethylstilboestrol	0.0
+50 μM NADH	196
+50 μM NADH (100 μM total)	210
+50 μM NADH (150 μM total)	210
+0.4 mM α-ketoglutarate	270

TABLE II

ACCELERATION OF SLOW PHASE OF ANS FLUORESCENCE ENHANCEMENT BY NADH

The rate of the slow enhancement of ANS fluorescence was measured when 30 μM diethylstilboestrol was added to a mixture of NADH, 50 μM ANS and 0.1 mg/ml glutamate dehydrogenase at 25 °C.

NADH concentration (μM)	Rate of enhancement (min^{-1})
0	0.17
7	1.00
28	1.19
42	1.82

TABLE III

ACCELERATION OF SLOW PHASE OF ANS FLUORESCENCE ENHANCEMENT BY α-KETOGLUTARATE

The rate of the slow enhancement of ANS fluorescence was measured when 30 μM diethylstilboestrol was added to a mixture of ANS, α-ketoglutarate, 26 μM NADH and 0.76 mg/ml glutamate dehydrogenase.

ANS (μM)	α-Ketoglutarate (mM)	Rate of enhancement (min^{-1})
25	0.28	1.6
25	0.00	0.5
43	0.28	1.6
43	0.00	0.5
86	0.28	1.3
86	0.00	0.7

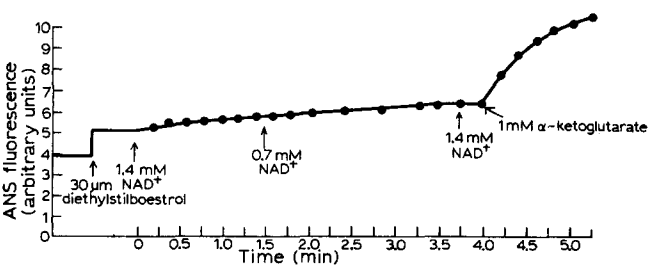

Fig. 2. Enhancement of ANS fluorescence by α-ketoglutarate in the presence of diethylstilboestrol and NAD^+. Initially 1.1 mg/ml enzyme, 40 μM ANS.

with NADH (Fig. 4). No enhancement occurred in the absence of NADH. The pyruvate-induced enhancement was faster than the preceding NADH enhancement.

Correlation of kinetic and fluorescence results

The kinetic studies showed that for the maximal progressive diethylstilboestrol inhibition the presence of α-ketoglutarate and NADH was necessary. The same was

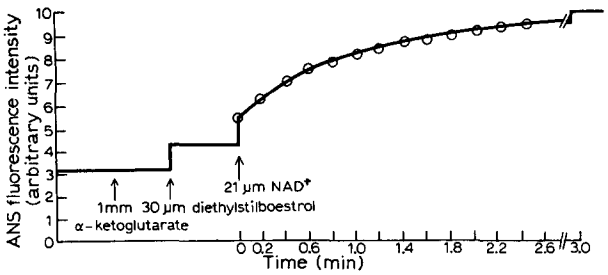

Fig. 3. Enhancement of ANS fluorescence by NAD^+ in the presence of α-ketoglutarate. Initially 74 μM ANS, 0.36 mg/ml enzyme.

found for the maximal enhancement of probe fluorescence by the inhibitor. Also the rates of these two processes were measured on equivalent enzyme samples (Table IV). Values obtained for the rate constants were only approximate. This was because the fluorimeter had to be used at very high sensitivity, with resulting noise. The kinetic

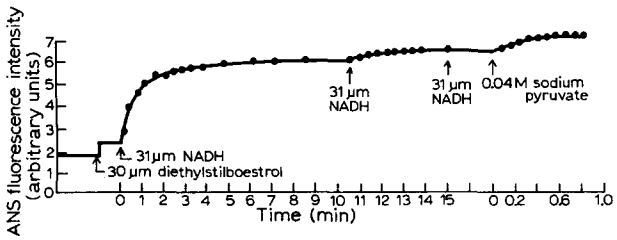

Fig. 4. Enhancement of ANS fluorescence by pyruvate in the presence of diethylstilboestrol and saturating concentrations of NADH. Initially 0.62 mg/ml enzyme, 41 μM ANS.

measurements were impeded by the high values of the enzyme activity necessarily used. A significant fraction of progressive inhibition was over before it could be followed. In spite of these difficulties the values obtained for the rates of the two processes were similar. For these reasons it was considered probable that the two slow processes corresponded to the same molecular event.

TABLE IV

COMPARISON OF THE RATES OF THE PROGRESSIVE INHIBITION AND THE SLOW ANS FLUORESCENCE ENHANCEMENT BY DIETHYLSTILBOESTROL

The mixture used contained glutamate dehydrogenase, 90 μM NADH, 30 μM diethylstilboestrol, 67 μM ANS, 0.05 M NH_4Cl, 50 μM EDTA and 5 mM α-ketoglutarate. The reaction and the fluorescence enhancement were started by the addition of NADH.

Rate of slow fluorescence enhancement (min^{-1})	Rate of progressive inhibition (min^{-1})	Enzyme concentration (mg/ml)
1.8	2.4	0.017
3.8	2.8	0.029
4.4	3.8	0.034
5.2	5.5	0.043

Properties of the enzymic states induced by diethylstilboestrol

The fluorescence of ANS was measured in the presence of glutamate dehydrogenase. Diethylstilboestrol was then added and the fluorescence remeasured. Before the small slow phase of the inhibitor-induced enhancement had significantly developed, NADH was added. The slow enhancement induced was allowed to reach a limit and the fluorescence remeasured. α-Ketoglutarate was then added and the fluorescence rise produced allowed to reach a limit. The total fluorescence was then remeasured. In this way the fast enhancement produced by diethylstilboestrol could be separated from the slow one and the α-ketoglutarate effect studied. The four successive fluorescence signals were measured at a series of enzyme concentrations. A double reciprocal extrapolation was then carried out to distinguish changes in binding from changes in the quantum yield of the fluorescence of bound probe (Fig. 5). Within experimental error the fast phase induced by diethylstilboestrol alone derived solely from an increase in binding, there was no change in the quantum yield of bound probe. The addition of NADH gave rise to two opposing effects, an increase in binding and a small (17%) decrease in the quantum yield. The addition of α-ketoglutarate increased binding and decreased the quantum yield yet further (37% relative to the mixture of enzyme and probe).

From Fig. 5 it would be predicted that at enzyme concentrations of about 6 mg/ml the addition of α-ketoglutarate to a mixture of NADH, diethylstilboestrol,

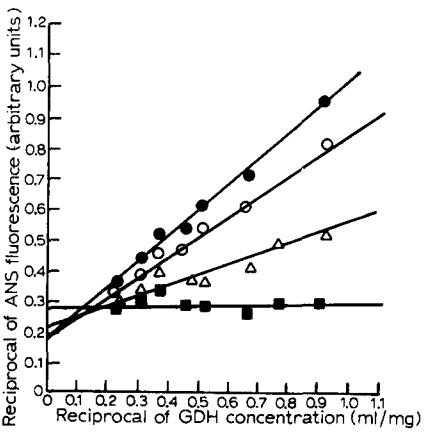

Fig. 5. Quantum yield changes of bound ANS. Double reciprocal plot of fluorescence against enzyme concentration. ●, enzyme, 24 μM ANS; ○, enzyme, 24 μM ANS, 30 μM diethylstilboestrol; △, enzyme, 24 μM ANS, 30 μM diethylstilboestrol, 40 μM NADH; ■, enzyme, 24 μM ANS, 30 μM diethylstilboestrol, 40 μM NADH, 0.1 mM α-ketoglutarate.

ANS and enzyme would give rise to quenching of probe fluorescence. This was in fact observed. A blank in the absence of probe showed that this quenching was not due to chemical consumption of NADH or the quenching of bound coenzyme fluorescence by α-ketoglutarate. At intermediate enzyme concentrations (about 1.5 mg/ml) addition of α-ketoglutarate to a mixture of diethylstilboestrol, NADH, probe and enzyme caused a rapid rise in ANS fluorescence, followed by a slow drop to a limit

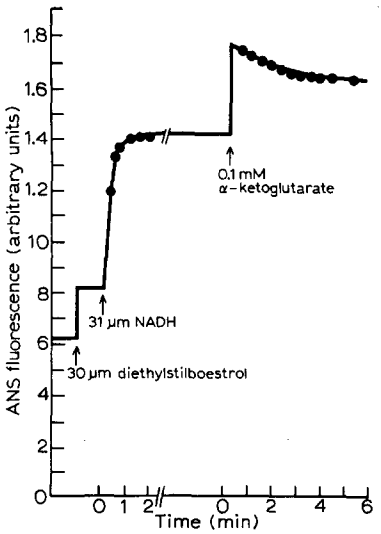

Fig. 6. Slow quenching of ANS fluorescence by α-ketoglutarate at high enzyme concentrations. Initially 1.55 mg/ml enzyme, 41 μM ANS.

(Fig. 6). A blank in the absence of probe showed that this effect was not connected with NADH fluorescence.

Changes in the environment of bound ANS during the diethylstilboestrol–enzyme interaction were further investigated by the measurement of probe fluorescence lifetimes. Experiments were carried out in the presence of α-ketoglutarate; this quenched the fluorescence of bound NADH and hence almost totally removed the contribution of the coenzyme to the signal recorded. The lifetimes measured were a mean for free and bound ANS. The addition of diethylstilboestrol to a mixture of α-ketoglutarate, probe and enzyme caused an increase in the fluorescence lifetime. The addition of NADH caused a further increase. At high enzyme concentrations the diethylstilboestrol-induced increase was lessened, suggesting that it partly derived from increased probe binding. The NADH-induced increase was not significantly altered. Extrapolation to infinite enzyme concentration gave values for changes in lifetime of the fluorescence of bound probe (Fig. 7). Under the conditions used the

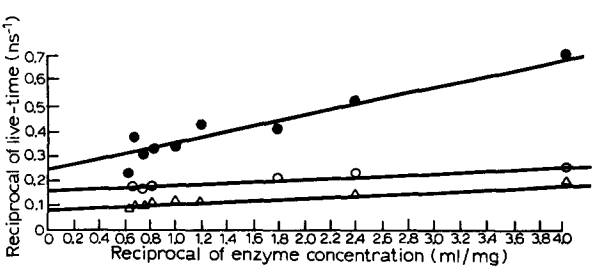

Fig. 7. Double-reciprocal plot of measured fluorescence lifetime against enzyme concentration. ○, enzyme, 36 μM ANS, 1.5 mM α-ketoglutarate; ○, 30 μM diethylstilboestrol added; △, 14 μM NADH also added.

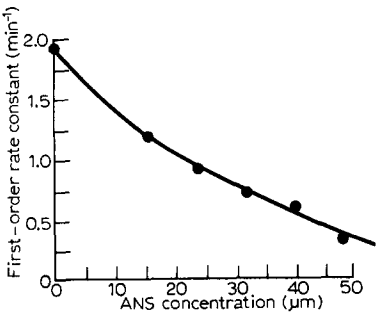

Fig. 8. Deceleration of progressive inhibition by ANS. 30 μM diethylstilboestrol, 0.018 mg/ml enzyme.

addition of diethylstilboestrol increased this from 4 to 6 ns; the subsequent addition of NADH caused a further increase to 12 ns.

Effect of ANS on the diethylstilboestrol–glutamate dehydrogenase interaction

NADH fluorescence studies. It was desirable that ANS, as a fluorescence probe, should not interfere with the change it detected. The effect of increasing probe concentration on the slow kinetic and fluorescence changes was therefore examined. ANS decreased the rate of the slow kinetic change (Fig. 8). The probe promptly inhibited the enzyme in the presence of diethylstilboestrol. It had little effect on the percentage of the activity lost during the progressive inhibition (Table V).

TABLE V

EFFECT OF ANS ON THE PROGRESSIVE INHIBITION BY DIETHYLSTILBOESTROL

ANS concentration (μM)	Initial inhibition (%)	Final inhibition (%)	Loss of activity during progressive inhibition (%)
8	19	29	47
16	24	29	53
23	38	31	49
32	41	53	43
40	43	58	60
48	53	68	50

Under most conditions ANS had no effect on the rate of the slow fluorescence change (Fig. 9). This is probably because the probe to enzyme ratio is lower in the fluorescence than in the kinetic experiments.

To avoid interference of the probe with the phenomena studied the enhancement of NADH fluorescence by diethylstilboestrol was investigated. This enhancement was also found to be biphasic, a small fast phase was followed by a larger rise to a limit (Fig. 10). The slow rise could be analysed as a first-order change in the manner used for the ANS enhancement.

NADH increased the rate of the slow change detected by both ANS and NADH (Fig. 11, Table II). The rate measured by the two fluorophores was the same within experimental error (Fig. 9). It was deduced that the two probes detected the same slow change.

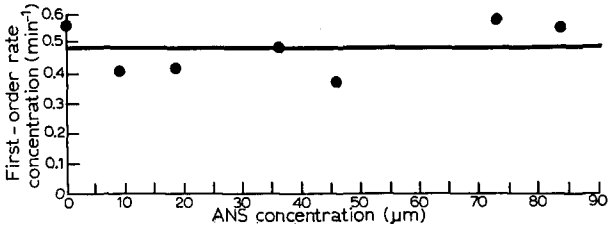

Fig. 9. Effect of ANS on the rate of the slow fluorescence enhancement. The point at zero ANS concentration corresponds to the rate of the slow NADH fluorescence rise. 0.56 mg/ml enzyme, 24 μM NADH, 43 μM diethylstilboestrol.

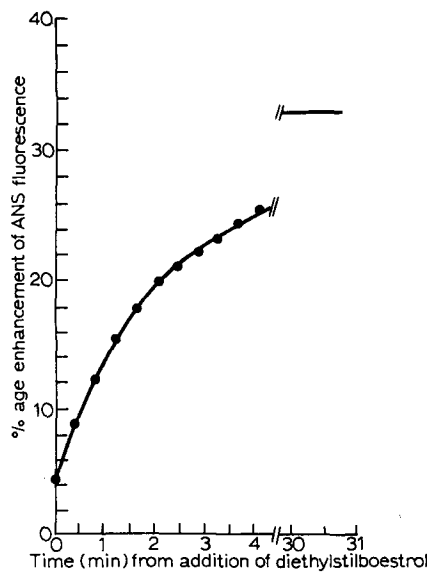

Fig. 10. Biphasic enhancement of NADH fluorescence by diethylstilboestrol. 21 μM NADH, 30 μM diethylstilboestrol, 0.62 mg/ml enzyme. First-order rate constant is 0.31 min^{-1}.

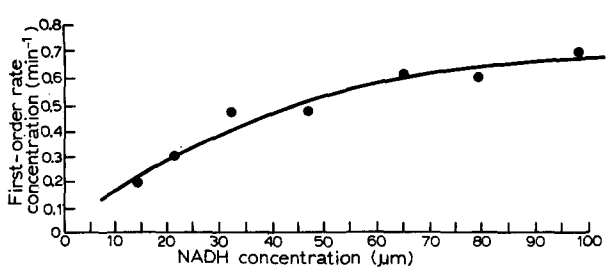

Fig. 11. NADH dependence of the rate of slow diethylstilboestrol-induced enhancement of NADH fluorescence. 30 μM diethylstilboestrol, 0.62 mg/ml enzyme.

Relationship of the aggregation of the enzyme to its interaction with diethylstilboestrol

Churchich and Wold [9] found that 10% dioxan disaggregated glutamate dehydrogenase to the hexamer. In the presence of 10% dioxan a biphasic enhancement of ANS fluorescence was induced by diethylstilboestrol (Table VI). Ultracentrifugation confirmed that the enzyme was disaggregated under these conditions.

TABLE VI

EFFECT OF DIOXAN ON THE ANS FLUORESCENCE ENHANCEMENT

	ANS fluorescence intensity (arbitrary units)	
	No dioxan	10% dioxan
1.33 mg/ml enzyme, 36 μM ANS, 30 μM NADH, 30 μM diethylstilboestrol	1.3	1.6
Size of fast phase	1.1	0.3
Size of slow phase	1.4	1.0
Rate constant of slow phase (min^{-1})	1.1	0.1

The effect of diethylstilboestrol on the aggregation–disaggregation equilibrium was investigated under conditions where significant aggregation occurred. It was predicted that factors which enhanced ANS fluorescence should disaggregate the enzyme. This was found to be the case. Thus α-ketoglutarate disaggregated glutamate dehydrogenase in the presence of diethylstilboestrol and NAD^+ or NADH but not otherwise (Table VII).

TABLE VII

DISAGGREGATION OF GLUTAMATE DEHYDROGENASE BY α-KETOGLUTARATE IN THE PRESENCE OF DIETHYLSTILBOESTROL AND NAD^+ OR NADH

Sample	$s_{20,w}$ value	Approximate height ratio of fast to slow sedimenting peak
1.39 mg/ml enzyme, 38 μM NADH, 60 μM diethylstilboestrol	19	One peak
1.39 mg/ml enzyme, 38 μM NADH, 60 μM diethylstilboestrol, 1 mM α-ketoglutarate	16, 11	1.3
0.87 mg/ml enzyme, 27 μM NAD, 33 μM diethylstilboestrol	22	One peak
0.87 mg/ml enzyme, 27 μM NAD, 33 μM diethylstilboestrol, 10^{-4} M α-ketoglutarate	22, 11	1.3

The rate of the slow diethylstilboestrol–enzyme interaction, detected by NADH fluorescence, was independent of enzyme concentration (Fig. 12).

Qualitative light-scattering experiments showed that the inhibitor-induced disaggregation had both fast and slow components. The slow phase occurred at roughly the same rate as the slow rise in ANS fluorescence in an equivalent sample.

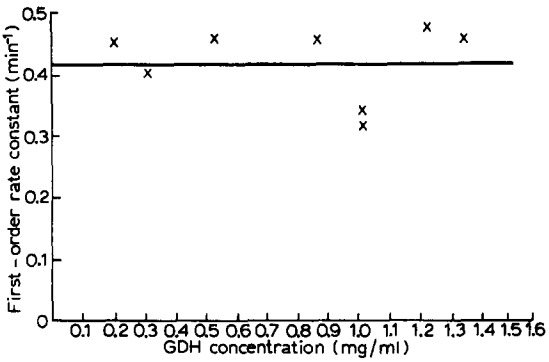

Fig. 12. Dependence of the rate of the slow diethylstilboestrol-induced enhancement of NADH fluorescence on enzyme concentration. 30 μM diethylstilboestrol, 45 μM NADH.

Comparison of diethylstilboestrol and GTP

GTP alone did not alter the fluorescence of ANS bound to glutamate dehydrogenase. Diethylstilboestrol alone enhanced probe fluorescence. The addition of GTP to a mixture of diethylstilboestrol, ANS and enzyme resulted in a rapid enhancement of probe fluorescence (Fig. 13). If the two effectors possessed overlapping or identical sites GTP would be expected to quench the fluorescence by displacing diethylstilboestrol.

The conformation induced in glutamate dehydrogenase by the GTP/NADH couple is detected by the enhancement of ANS fluorescence. The enzyme was put totally in the GTP/NADH conformation by saturation with NADH in the presence

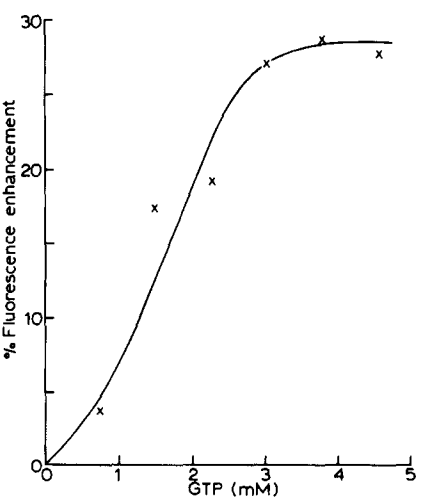

Fig. 13. ANS evidence for GTP–diethylstilboestrol interaction. 0.50 mg/ml enzyme, 36 μM ANS, 30 μM diethylstilboestrol and GTP added. Percentage fluorescence enhancement against GTP concentration.

of GTP. Addition of diethylstilboestrol caused a further, slow enhancement of probe fluorescence. The diethylstilboestrol-induced enhancement was much slower and smaller than in the absence of GTP (Table VIII). Some NADH fluorescence detected under these conditions has been corrected for.

TABLE VIII

COMPARISON OF CONFORMATIONS INDUCED BY GTP AND DIETHYLSTILBOESTROL IN THE PRESENCE OF NADH

Sample	ANS fluorescence (arbitrary units)
Expt 1	
0.62 mg/ml enzyme, 41 μM ANS, 55 μM GTP	3.7
Saturated with 0.25 mM NADH	7.5 (total signal)
30 μM diethylstilboestrol added, enhancement only of the slow type, $k = 1.3\ min^{-1}$	9.3 (total signal)
Diethylstilboestrol-induced enhancement	1.8
Total enhancement	5.6
Expt 2	
0.62 mg/ml enzyme, 41 μM ANS, 0.25 mM NADH	3.5
30 μM diethylstilboestrol added (fast phase)	7.1 (total signal)
(fast and slow phases)	9.8 (total signal)
Slow phase, $k = 3.3\ min^{-1}$	
Diethylstilboestrol-induced slow enhancement	2.6
Total enhancement	6.3

DISCUSSION

The interaction of diethylstilboestrol with glutamate dehydrogenase was resolved into two phases. A fast phase was followed by a slower phase with a half-life of the order of a minute. These could be observed in the biphasic inhibition of glutamate dehydrogenase activity, the biphasic enhancement of the intrinsic fluorescence of NADH and the biphasic enhancement of the extrinsic fluorescence of ANS by diethylstilboestrol. Without further resolution of the rates of the processes involved it cannot be established that the rapid interaction recorded by the three methods corresponds to the same molecular event. For example, the rapid inhibition of enzymic activity could occur before, during or after the rapid enhancement of probe fluorescence. It appears, however, that the same slow process is recorded by the three methods.

A model for the diethylstilboestrol–glutamate dehydrogenase interaction

In the previous paper [1] the inhibition of glutamate dehydrogenase activity by diethylstilboestrol was explained in terms of a biphasic conformational change in the enzyme. If R represents the initial conformation of the enzyme, T that rapidly induced by the inhibitor and X that slowly induced, the interaction may be considered in terms of the model:

$$R \overset{\text{fast}}{\dashrightarrow} T \overset{\text{slow}}{\dashrightarrow} X$$

The biphasic inhibition was observable with highly dilute enzyme solutions, when effectively no agrreagate was present. For this reason the possibility that the slow interaction corresponded to disaggregation of the enzyme could be eliminated. Similarly the slow fluorescence enhancement was observable when virtually no aggregate was present, either because of the dilution of the enzyme, or the presence of 10% dioxan [9] or the presence of GTP and NADH [4].

Diethylstilboestrol alone enhanced the fluorescence of enzyme-bound ANS and disaggregated the enzyme. The successive addition of NADH altered neither the enhancement nor the disaggregation. NAD^+ slightly increased the effector-induced disaggregation and enhancement of ANS fluorescence. The presence of α-ketoglutarate greatly increased both NAD^+ effects.

This striking parallel between the enhancement of fluorescence and disaggregation suggests that enzyme in the T or X conformation dissociates. Two possibilities may be schematically represented as follows:

$$R_n \xrightarrow[\text{fast}]{} T_n \xrightarrow[\text{fast}]{} nT \xrightarrow[\text{slow}]{} nX \qquad (1)$$

$$R_n \xrightarrow[\text{fast}]{} T_n \xrightarrow[\text{slow}]{} X_n \xrightarrow[\text{fast}]{} nX \qquad (2)$$

(subscript n = degree of aggregation of the hexamer)

In the first case disaggregation occurs after induction of the T, in the second after induction of the X conformation. Both are evidently oversimplifications. Scheme 1 is favoured as it predicts that the rapidly induced T state should give rise to some rapid disaggregation of the enzyme. This was observed.

The slow disaggregation was found to occur at approximately the same rate as the slow probe fluorescence enhancement in the same sample. This is compatible with either scheme, as in both the isomerization controls the rate of the disaggregation.

The rate of the slow isomerization (measured with NADH fluorescence) was independent of enzyme concentration and hence of the initial degree of enzyme aggregation. This result is more readily explained by Scheme 1 than by Scheme 2.

It seems therefore that the inhibitor–enzyme interaction is best represented by Scheme 1:

$$R_n \xrightarrow[\text{diethylstilboestrol}]{\text{fast}} T_n \xrightarrow[\text{fast}]{} nT \xrightarrow[\text{slow}]{} nX$$

This model has similarities to others proposed for the interaction of glutamate dehydrogenase with GTP and diethylstilboestrol. Yielding et al. [10] proposed that diethylstilboestrol and GTP induced a conformational change in glutamate dehydrogenase. They have not done experiments to decide whether this occurs before or after inhibitor-induced disaggregation of the enzyme. They do not distinguish between the action of the two inhibitors.

The enhancement of ANS fluorescence by the GTP/NADH couple is biphasic, with the first-order rate constants of 60 and 1.5 s^{-1} [11]. Jallon et al. [11] consider that the faster of these processes corresponds to disaggregation of the enzyme and the slower to a conformational change in the hexamer. They claim that GTP-induced

inhibition of the enzyme occurs before either of these processes, with a rate constant of greater than 500 s^{-1}. Their model is similar to Scheme 2 above.

Frieden and Colman [8] have studied the binding of GTP to glutamate dehydrogenase at different enzyme concentrations. They found that GTP binds more tightly to dilute, dissociated enzyme than to the associated form. This may be because the concentration-dependent aggregation shifts the conformational equilibria away from forms that bind GTP tightly. It is likely that similar results would be obtained for diethylstilboestrol. However, direct binding studies would be technically difficult, because of the limited solubility of the hormone analogue.

Properties of the model

The enhancement of probe fluorescence by various substrates may be described in terms of the model. Thus if a substrate in the presence of diethylstilboestrol produces only slow enhancement of ANS fluorescence it binds preferentially to the slowly formed X conformation. There is no difference between its binding to the R and T conformations. In this class are NAD^+, NADH, α-ketoglutarate in the presence of NAD and pyruvate in the presence of NADH.

If a substrate produces a biphasic enhancement of probe fluorescence it must bind to the rapidly formed T conformation in preference to the R conformation. In this class are α-ketoglutarate in the presence of NADH and NAD^+ in the presence of α-ketoglutarate. The slow phase of the enhancement may result from perturbation of the conformational equilibria and no statement can be made about substrate binding to the X state.

If a substrate produces no enhancement of probe fluorescence in the presence of diethylstilboestrol it must bind to the R in preference to the T and X states. In this class are the substrates α-ketoglutarate and pyruvate in the absence of coenzyme and L-glutamate in the presence or absence of coenzyme.

The fluorescence binding studies carried out show that there is a rapid effector-induced increase in the binding of the extrinsic probe, but no change in the quantum yield of the bound probe. Thus the T conformation binds the probe more strongly than the R conformation, but with no change in the quantum yield. There is a further increase in probe binding during the slow transition induced by adding NADH. The addition of NADH decreases the concentration of the R and T states and increases the concentration of the X state. Therefore the increase in binding does not necessarily mean that the X state binds to the probe to a greater entent than the T state. It does follow, however, that the X state binds more probe than the R state. The T to X isomerization brings about quenching of fluorescence of bound probe.

The R to T isomerization involves little or no change in fluorescence lifetime. The lifetime of the fluorescence of bound probe increased from 4 to 6 ns on the addition of effector to a probe enzyme mixture. The slight increase may indicate the formation of a low concentration of the X state. The lifetime measurements were at room temperature, where the magnitude of the slow phase induced by effector alone is relatively greater than at 15 °C. The further addition of NADH caused the lifetime to increase to 12 ns.

Thus probe bound to the X state has a lower quantum yield but a longer fluorescence lifetime than probe bound to the R or T states. The measured fluorescence lifetime is related to the quantum yield and the radiative lifetime as follows:

$$\text{Quantum yield} = \frac{\text{measured lifetime } (\tau)}{\text{radiative lifetime } (\tau_0)}$$

It follows that the T to X isomerization brings about an increase in the radiative lifetime of the probe. This must derive from changes in the geometry of the probe in the ground and/or in the first excited singlet state.

NADH greatly enhances the rate of denaturation of the enzyme in the presence of diethylstilboestrol [12]. This observation suggests that the X state is more rapidly denatured than the R or T isomers.

Table IX lists some of the properties of the T and X states compared with each other and with the R state. The results of the preceding paper [1] are included.

TABLE IX

PROPERTIES OF THE T AND X CONFORMATIONS

Property	T state	X state
Glutamate dehydrogenase activity	Less than R	Less than R
Alanine dehydrogenase activity	Greater than R	Less than T
NAD^+ binding	Same as R	Greater than R
α-Ketoglutarate binding	Less than R	Less than R
α-Ketoglutarate binding in the presence of NADH	Greater than R	Uncertain
α-Ketoglutarate binding in the presence of NAD^+	Same as R	Greater than R
Pyruvate binding	Less than R	Less than R
Pyruvate binding in the presence of NADH	Same as R	Greater than R
L-Glutamate binding	Less than R	Less than R
ANS binding	Greater than R	Greater than R
Bound ANS quantum yield	Same as R	Less than R
Bound ANS measured lifetime	Same as R	Greater than R
Bound ANS radiative lifetime	Same as R	Greater than R
Degree of disaggregation	Greater than R	Greater than R
Rate of denaturation	Not very different from R	Greater than R

Comparison of diethylstilboestrol and GTP

In the absence of substrates and coenzymes diethylstilboestrol, but not GTP, enhances the fluorescence of enzyme-bound ANS [3]. This is good evidence that the two effectors alone induce different conformations in the enzyme. The experiment of Fig. 13 shows that they bind to distinct, but interacting sites.

The enhancement of enzyme-bound ANS fluorescence by the GTP/NADH couple has been extensively studied [11, 13]. The couple brings about an increase in the fluorescence lifetime of the bound probe [13]. The experiment summarized in Table VIII shows that in the presence of NADH the two effectors induce different conformations.

Brocklehurst et al. [14] found that α-ketoglutarate increases the cooperativity of the GTP/NADH transition detected by the extrinsic probe. At intermediate

points in the transition this corresponds to an α-ketoglutarate-induced enhancement of probe fluorescence similar to that described here for diethylstilboestrol. L-Glutamate has no effect in either system.

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