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

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SUMMARY

Glutamate dehydrogenase (L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.2) catalyses the reductive amination of α-ketoglutarate. It was shown that under certain conditions the inhibition of this reaction by diethylstilboestrol was biphasic. The first phase was rapid; the second developed within a few minutes. The progressive character of the inhibition could be removed by prior incubation of the enzyme with a mixture of diethylstilboestrol, NADH and α-ketoglutarate. Thus the progressive inhibition was related to the action of diethylstilboestrol on the α-ketoglutarate–NADH–glutamate dehydrogenase complex.

Diethylstilboestrol activated a minor activity of glutamate dehydrogenase, the reductive amination of pyruvate. During a kinetic run this activation decreased. It is suggested that in the presence of diethylstilboestrol equilibrium between three conformational isomers of glutamate dehydrogenase is slowly attained. These isomers possess different specific activities for the two reactions studied.

The percentage inhibition of glutamate dehydrogenase activity by diethylstilboestrol dropped during modification of the enzyme with the active site-directed irreversible inhibitor 4-iodoacetamidosalicylic acid. This suggests the involvement of protein subunit interaction in diethylstilboestrol inhibition. The curves of inhibition against time of modification were compared for the two inhibitors GTP and diethylstilboestrol.

INTRODUCTION

The activity of bovine liver glutamate dehydrogenase (L-Glutamate: $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.2) is affected by a large number of small molecules, such as purine nucleotides [1], oestrogens [2] ,and the oestrogen analogues clomiphene [3] and diethylstilboestrol [2]. The GTP binding site is separate from the active site [4].

GTP and diethylstilboestrol both inhibit the glutamate dehydrogenase activity

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and, under most conditions, activate the minor alanine dehydrogenase activity of the enzyme. The inhibition of the glutamate dehydrogenase activity by rotenone is time dependent [5].

The diethylstilboestrol–glutamate dehydrogenase interaction was investigated in detail and comparison made with the better studied GTP–glutamate dehydrogenase interaction. This paper contains kinetic studies of the diethylstilboestrol interaction, which was found to be biphasic. The following paper includes fluorescence studies of the conformational isomers of glutamate dehydrogenase induced by diethylstilboestrol.

MATERIALS AND METHODS

Glutamate dehydrogenase was obtained from Boehringer und Soehne GmbH, as a glycerol solution. The enzyme was freed of glycerol by dialysis against buffer. Any denatured protein was removed by centrifugation. Glutamate dehydrogenase concentrations were determined from the extinction at 280 nm, using the value of 0.93 $cm^2 \cdot mg^{-1}$ for the extinction coefficient [6].

GTP and diethylstilboestrol were obtained from Sigma; NADH and α-ketoglutaric acid from Boehringer; 4-iodoacetamidosalicylic acid and sodium pyruvate from Koch–Light; NH_4Cl from Harrington Brothers; EDTA from British Drug Houses. The diethylstilboestrol purity was stated to be at least equal to that of a United States Pharmacopeia Reference Grade Sample.

All experiments were carried out in 0.1 M sodium phosphate buffer, pH 7.7. NADH solutions were made up in 0.1 M Tris–HCl buffer, pH 9.0, and concentrations were determined from the extinction at 340 nm. Stock solutions of diethylstilboestrol were made up by weight in ethanol.

Enzyme activity measurements were carried out by following changes in NADH absorption, using a Hilger–Gilford kinetic spectrophotometer. Glutamate dehydrogenase activity measurements were in the direction of α-ketoglutarate reduction using an assay mixture containing 5 mM α-ketoglutarate, 50 mM NH_4Cl, 90 μM NADH and 50 μM EDTA.

Alanine dehydrogenase activity measurements were in the direction of pyruvate reduction, using an assay mixture containing 0.04 M pyruvate, 0.15 M NH_4Cl, 90 μM NADH and 80 μM EDTA. Except when otherwise stated, assays were started by the addition of enzyme. For experiments at 15 °C all samples were allowed to equilibrate in the thermostatted cell compartments for 15 min before the reaction was initiated.

The irreversible modification of glutamate dehydrogenase was studied by incubating the enzyme with 4-iodoacetamidosalicylic acid, an active site-directed irreversible inhibitor [7, 8]. Aliquots were taken out and the activity measured. The 4-iodoacetamidosalicylic acid concentration in the assay mixture was too low for reversible inhibition to result. In experiments in which a direct comparison between GTP and diethylstilboestrol was desired both inhibitors were dissolved in an ethanol–water (1:1, v/v) mixture.

RESULTS

Biphasic inhibition of glutamate dehydrogenase activity by diethylstilboestrol

At 15 °C diethylstilboestrol inhibition of α-ketoglutarate reduction had both prompt and progressive components. Importantly the kinetic plots of NADH concentration against time of reaction became linear before sufficient substrate had been consumed to produce a further decrease in reaction velocity (Fig. 1). At 25 °C these plots were only slightly curved and at 32 °C they became strictly linear. An abrupt increase in initial inhibition over this temperature range was observed, which was consistent with earlier observations.

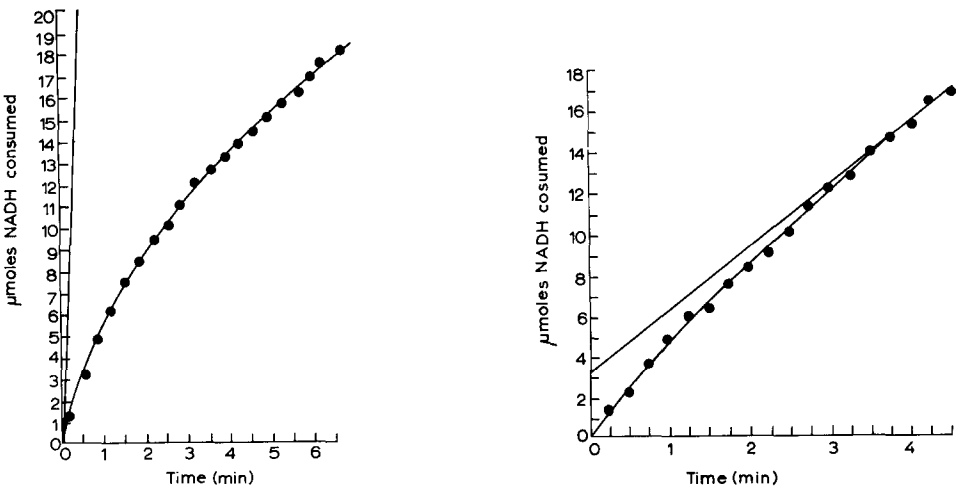

Fig. 1. Biphasic inhibition of glutamate dehydrogenase by diethylstilboestrol. 4 μM diethylstilboestrol, 0.015 mg/ml enzyme. The rate constant for progressive inhibition is 0.95 min^{-1}. The straight line corresponds to the activity in the absence of inhibitor.

Fig. 2. Abolition of the progressive inhibition. 0.03 mg/ml enzyme, 8 μM diethylstilboestrol. Reaction started with enzyme (●). Compared with linear NADH consumption after prior incubation of enzyme with diethylstilboestrol, NADH and α-ketoglutarate and reaction started by the addition of NH_4Cl (and EDTA) (continuous line).

When glutamate dehydrogenase was preincubated with diethylstilboestrol and the reaction started by the addition of a small volume of assay mixture the kinetic plots remained curved. If NADH was included in the preincubation mixture and the reaction started by addition of the remaining components of the assay mixture it was found that the curvature of the kinetic plots was reduced. However even after 14 h preincubation it was not totally eliminated. When glutamate dehydrogenase was incubated for 10 min with diethylstilboestrol, NADH and α-ketoglutarate and the reaction started by the addition of NH_4Cl (and EDTA) the resulting kinetic plots were strictly linear (Fig. 2). The gradient was equal to the final slope obtained when the reaction was started by the addition of enzyme.

It is possible for contaminating traces of ammonia to react with α-ketoglutarate and NADH in the presence of glutamate dehydrogenase to form NAD^+ and L-glutamate. However examination of NADH fluorescence intensity showed that this

effect resulted in the consumption of less than 1% of the NADH present during the experiment. Preincubation of the enzyme with diethylstilboestrol, NADH and NH_4Cl or diethylstilboestrol, α-ketoglutarate and NH_4Cl or diethylstilboestrol and α-ketoglutarate did not remove the curvature.

The curved kinetic plots could be analysed in terms of an exponential change using the method described in Appendix 1. Curved plots suitable for the derivation of first-order rate constants could only be obtained under rather closely defined conditions. Firstly, the diethylstilboestrol concentration had to be sufficiently high (> 3 μM) to give rise to significant curvature. Secondly, the rate of the inhibition process had to be adjusted so that it was slow enough to follow, but not so slow that it was still continuing while a secondary curvature developed through lack of substrate. Controls in the absence of diethylstilboestrol showed that the kinetic plots were strictly linear for only the first 24 μmoles NADH consumed. At 15 °C it was found that the inhibition was completed within the prescribed limit if the inhibitor to enzyme ratio was adjusted so that 24 μmoles NADH was consumed in about 5 min. A very high inhibitor to enzyme ratio gave rise to impractically slow kinetic runs. Typical experiments used $5 \cdot 10^{-3}$ mg/ml enzyme and 4–9 μM diethylstilboestrol. This resulted in 60–90% initial inhibition, rising to a final inhibition between 75 and 95%. At temperatures much above 15 °C the progressive inhibition was too fast to follow. At 10 °C it did not occur within the prescribed limit. Therefore further experiments were carried out at 15 °C. The result of the analysis of one such kinetic run is shown in Fig. 3. In this way first-order rate constants for the slow inhibition process could be calculated to an accuracy of about 10%.

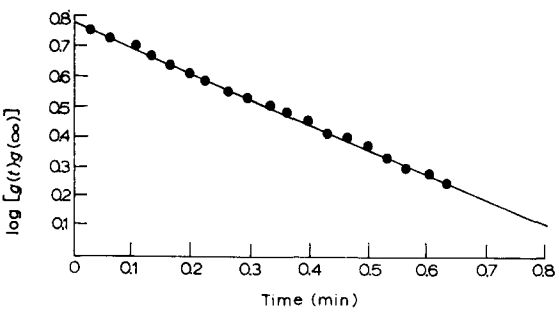

Fig. 3. Analysis of progressive inhibition by diethylstilboestrol (see Appendix 1). 0.018 mg/ml enzyme, 30 μM diethylstilboestrol. Rate constant, 1.93 min^{-1}.

The inhibition was also studied by incubating glutamate dehydrogenase with diethylstilboestrol, taking out aliquots and measuring the activity after dilution. If the incubation was carried out in the presence of NADH, but not otherwise, partial inhibition of the enzyme was observed (Table I). Within the error of these measurements (about 10%) inclusion of α-ketoglutarate in the inhibition mixture caused no increase in inhibition. Under the conditions obtained after dilution direct inhibition by diethylstilboestrol in the assay mixture was negligible. It was also shown that incubation of NADH with diethylstilboestrol in the absence of enzyme did not increase the potency of the inhibitor.

The activity of the enzyme partially inhibited in this manner increased during

TABLE I

PARTIAL INHIBITION OF GLUTAMATE DEHYDROGENASE ON INCUBATION WITH DIETHYLSTILBOESTROL AND NADH

The procedure and conditions used were as described in the text. The incubations were at 15 °C.

Glutamate dehydrogenase (mg/ml)	Diethyl-stilboestrol (μM)	NADH (μM)	α-Keto glutarate (mM)	Percentage loss in activity
0.20	40	100	0	17 (mean of two)
0.20	40	0	0	0
0.20	0	100	0	0
0.11	40	25	0	24
0.11	40	25	5	24
0.11	40	230	0	48
0.11	40	230	3.6	45

assay, giving rise to slight upward curvature of the kinetic plots. This suggested that the inhibition was reversible and this was confirmed by the recovery of glutamate dehydrogenase activity after removal of diethylstilboestrol by dialysis. Glutamate dehydrogenase (0.31 mg/ml) was incubated for 15 min at 15 °C with 30 μM diethylstilboestrol and 37 μM NADH, and the activity measured (mean of six readings taken). After correction for dilution during dialysis, the activity was found to be equal (100 $\pm$ 3%) to that of a control in which ethanol replaced diethylstilboestrol.

Biphasic effect of diethylstilboestrol on alanine dehydrogenase activity

The time course of the diethylstilboestrol activation of the alanine dehydrogenase activity of the enzyme was investigated. At low diethylstilboestrol concentrations alanine dehydrogenase assays were slightly, but reproducibly, curved in the direction of decreasing activity (Fig. 4). Controls in the absence of diethylstilboestrol were linear. At higher diethylstilboestrol concentrations the assays were again linear and the initial activation of the enzymic activity was less (Table II).

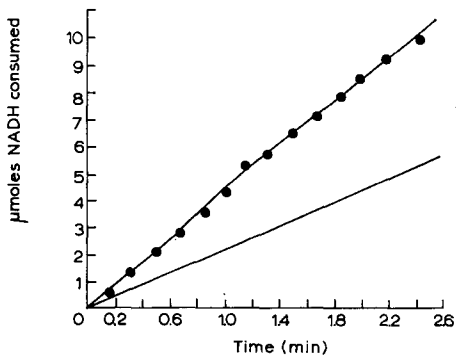

Fig. 4. Decreased activation of the alanine dehydrogenase reaction by diethylstilboestrol during a kinetic run (●) 0.03 mg/ml enzyme, 3 μM diethylstilboestrol. The straight line corresponds to the activity in the absence of stilboestrol.

TABLE II

EFFECT OF DIETHYLSTILBOESTROL ON ALANINE DEHYDROGENASE ACTIVITY

Assays were at 15 °C using 0.03 mg/ml glutamate dehydrogenase. Other conditions were as described in the text. In the absence of diethylstilboestrol the activity measured was unchanged during the consumption of 16 μmoles NADH.

Diethylstilboestrol (μM)	Percentage activation	Percentage activation after consumption of 16 μmoles NADH
0	0	0
3	91	52
6	89	64
12	48	34
24	39	39

Chemical modification with 4-iodoacetamidosalicylic acid

Glutamate dehydrogenase was incubated with 4-iodoacetamidosalicylic acid. The glutamate dehydrogenase activity of partially inactivated samples of the enzyme was then measured in the presence of diethylstilboestrol. The percentage inhibition was found to drop during inactivation. For this reason the activity in the presence of diethylstilboestrol first rose then dropped during the modification process (Fig. 5). This was true both for the initial and for the final diethylstilboestrol inhibition. As a result the assays became nearly linear during modification.

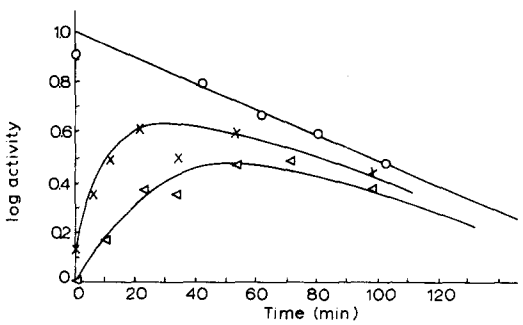

Fig. 5. Reduction of curvature of assays with diethylstilboestrol during chemical modification. 0.02 mg/ml enzyme incubated with 1.4 mM 4-iodoacetamidosalicylic acid and assayed in the presence of 4.0 μM diethylstilboestrol. ×, initial activity; △, final activity; ○, assay without inhibitor.

GTP and diethylstilboestrol concentrations were adjusted so that the percentage initial inhibition by the two compounds of the glutamate dehydrogenase activity of the unmodified enzyme was the same. During modification the curves of activity against time were superimposable for the two inhibitors (Fig. 6).

Rotenone progressively inhibited the oxidation of α-ketoglutarate. Similarly to diethylstilboestrol, rotenone inhibition dropped during 4-iodoacetamidosalicylic acid modification and the curvature of the assays was reduced (Fig. 7). If the diethylstilboestrol concentration was adjusted so that the initial response of the unmodified

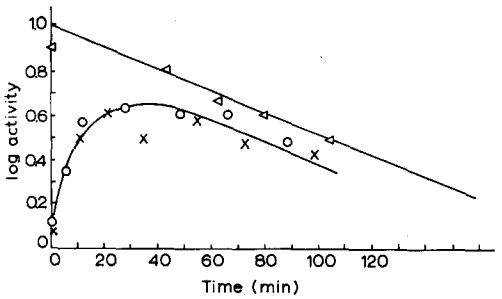

Fig. 6. Comparison of the GTP inhibition with the initial diethylstilboestrol inhibition during modification of the enzyme with 4-iodoacetamidosalicylic acid. 0.02 mg/ml enzyme incubated with 1.5 mM 4-iodoacetamidosalicylic acid. Assay in the presence of 4.0 μM diethylstilboestrol (×) or 10 μM GTP (○). Assay with neither (△).

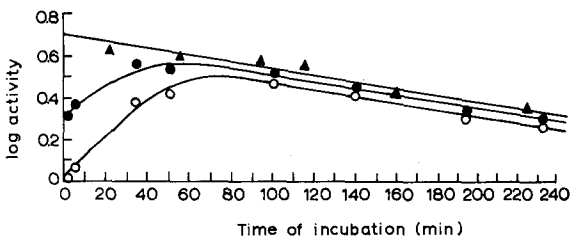

Fig. 7. Reduction of curvature of assays with rotenone during chemical modification. 0.01 mg/ml enzyme incubated with rotenone during chemical modification. 0.01 mg/ml enzyme incubated with 1.4 mM 4-iodoacetamidosalicylic acid, assayed in the presence of 20 μM rotenone. ○, initial activity; ●, final activity; ▲, blank without inhibitor.

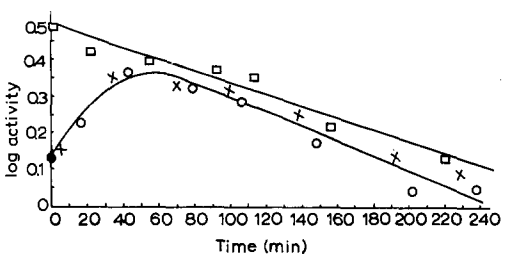

Fig. 8. Comparison of the initial inhibitions by diethylstilboestrol and rotenone during modification with 4-iodoacetamidosalicylic acid. 0.01 mg/ml enzyme incubated with 1.4 mM iodoacetamidosalicylic acid. Assay in the presence of 3.7 μM diethylstilboestrol (×), or 20 μM rotenone (○). Blank (□).

enzyme to the two inhibitors was the same, superimposable curves of activity against time were obtained during modification (Fig. 8).

DISCUSSION

The possibility was considered that the progressive inhibition of glutamate dehydrogenase by diethylstilboestrol was the result of an increase in the K_m for one or more of the substates, brought about by the inhibitor. As a result of this the

enzyme would no longer be saturated by the substrate concentrations in the assay mixture and the rate of NADH consumption would fall continuously. In fact the rate of NADH consumption fell to a constant level. A diethylstilboestrol induced increase in K_m can therefore be eliminated.

It was also considered possible that the progressive inhibition resulted from the formation of the products of the reaction, NAD^+ and L-glutamate. However, this too would give rise to a continuous drop in reaction velocity with time, and can be eliminated.

The progressive character of the diethylstilboestrol inhibition was removed by prior incubation of the enzyme with inhibitor, NADH and α-ketoglutarate. This relates the curvature of the kinetic plots to the action of diethylstilboestrol on the α-ketoglutarate–NADH–enzyme complex to produce an inhibited form of the enzyme.

Possibilities for the rate determining step in the slow inhibitor–enzyme interaction included enzyme-catalyzed chemical modification of the inhibitor, inhibitor binding to the enzyme and conformational isomerization of the enzyme. The observation that the diethylstilboestrol induced inhibition of the enzyme is slowly reversible on dilution makes chemical modification of the inhibitor an unlikely possibility.

The slow interaction could not be identified with the slow disaggregation of the enzyme as it was observable when no aggregate was present. Rogers et al. [9], working with pyrophosphate buffer at pH 8.5 at 4 and 24 °C, found by a molecular sieve technique that there was no change in the Stokes radius of the enzyme in the 0.00046–0.117 mg/ml concentration range. It was deduced that only glutamate dehydrogenase hexamer was present under these conditions. Biphasic inhibition of the enzyme by diethylstilboestrol was observed with enzyme concentrations down to 0.003 mg/ml.

It seems likely that the slow interaction is too slow to be ascribed to inhibitor binding [10]. To explain the biphasic interaction it would be necessary to postulate the presence of two different classes of inhibitor binding sites of different inhibitory characteristics and to which the inhibitor bound at different rates. A more probable explanation is that the inhibitor induces a slow conformational change in the enzyme.

As diethylstilboestrol does not resemble the substrates of the enzyme, but rapidly inhibits it, this probably takes place through a rapidly induced conformational change. Further evidence for this is the observation that, unlike any of the substrates of the enzyme, diethylstilboestrol enhances the fluorescence of the probe, 1-anilinonaphthalene-8-sulphonate [11]. This enhancement is rapid. It follows that the inhibitor induces two conformations in the enzyme, one rapidly and one slowly. As these are observable on a homogeneous enzyme population it follows that they must be induced sequentially. Therefore the diethylstilboestrol–glutamate dehydrogenase interaction may be described in terms of the following model:

$$R \xrightarrow[\text{fast}]{\text{inhibitor}} T \xrightarrow[\text{slow}]{\text{NADH, } \alpha\text{-ketoglutarate, inhibitor}} X$$

R represents the conformation initially present; T is the rapidly induced and X is the slowly induced conformation. The equilibrium concentration of the conformations is attained in the presence of inhibitor, NADH and α-ketoglutarate.

The rapid inhibition of glutamate dehydrogenase activity by diethylstilboestrol shows that the T state has a lower specific activity than the R state. During the T to X conversion progressive inhibition occurs. During this conversion the concentration of the R and T state drops and the concentration of the X state rises. It is assumed that there is initial equilibrium between the R and T states and that finally equilibrium between the three states is established. It may then be shown that the progressive inhibition is explicable if the X state has a lower specific activity for the glutamate dehydrogenase reaction than the R state (Appendix 2). No statement about the relative activities of the T and X states may be made.

Diethylstilboestrol rapidly activated the alanine dehydrogenase activity of the enzyme. This shows that the T state has greater alanine dehydrogenase activity than the R state. From the progressive drop in activation it may be shown (Appendix 2) that the alanine dehydrogenase activity of the X state is lower than that of the T state. No statement about the relative activities of the R and X states may be made. It has been shown that in 0.2 M phosphate at pH 9.0, diethylstilboestrol inhibits alanine dehydrogenase activity [12]. Possibly under these conditions the equilibrium between the conformations is different from that in 0.1 M phosphate, pH 7.7. In the latter conditions the author found that at high diethylstilboestrol concentrations the kinetic plots were again linear and the initial activation was decreased. This may stem from an increase in the rate of the T to X isomerization with diethylstilboestrol concentration.

Butow [5] found that glutamate dehydrogenase was progressively inhibited by rotenone. He explained his results in terms of the production of a rotenone-sensitive form of the enzyme during turnover. His results are also explicable in terms of an interaction analogous to that of the enzyme with diethylstilboestrol. Shemisa and Fahien [13] found biphasic inhibition of glutamate dehydrogenase by the drugs perphenazine and haloperidol. They explained their results in terms of the rapid formation of an inhibitor–enzyme complex which slowly isomerized to another complex. This is probably similar to the diethylstilboestrol–glutamate dehydrogenase interaction. Engel [14] found that under certain conditions (pH 7.0 phosphate, 2 μM NAD, 1 mM GTP) the GTP inhibition of the oxidation of glutamate took up to 30 s to develop. There was no initial inhibition. Frieden [15] has considered the kinetic consequences of the slow response of enzymes to effectors and has listed some examples. There appears to be no other case known where a soluble enzyme interacts with an allosteric effector as slowly as glutamate dehydrogenase does with diethylstilboestrol and similar hydrophobic compounds (see ref. 16).

The drop in percentage inhibition by GTP observed during chemical modification of the enzyme with 4-iodoacetamidosalicylic acid has been interpreted as being due to the abolition of subunit interactions by the modifying agent [8]. If this is correct it follows from the similar results obtained with diethylstilboestrol that the rapid and slow actions of this inhibitor also involve subunit interaction. This strengthens the conclusion that diethylstilboestrol action involves conformational isomerizations. The similar results obtained for rotenone suggest that this inhibitor behaves analogously. The comparisons carried out suggest that the conformation induced in the enzyme by GTP involves similar subunit interaction to those initially induced by diethylstilboestrol and rotenone. There appear to be qualitative differences in the effect of modification on the states initially and finally induced by diethylstilboestrol.

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APPENDIX I

Treatment of progressive inhibition data (cf. ref. 15)

Consider the case where NADH is consumed by glutamate dehydrogenase, a fraction of which undergoes a first-order inhibition process. If the observed NADH concentration is $g(t)$ at time t and $g(0)$ at some arbitrary time zero, it may be seen that

$$g(t) = g(0) - mt - f(t) \tag{1}$$

where the mt term represents the linear consumption of NADH by non-inhibited enzyme (within the range of adequate substrate concentration); m is a constant. $f(t)$ corresponds to the NADH consumed by enzyme that is being inhibited.
Then

$$\frac{\mathrm{df}(t)}{\mathrm{d}t} = K \cdot [\mathrm{E}] \tag{2}$$

where K is a constant within the range of adequate substrate concentration and [E] is the remaining concentration of the enzyme that is undergoing inhibition. Then

$$[\mathrm{E}] = [\mathrm{E}]_0 \cdot \mathrm{e}^{-kt} \tag{3}$$

where $[\mathrm{E}]_0$ is the value of [E] at zero time and k is the first-order rate constant of the inhibition process. Hence

$$\frac{\mathrm{df}(t)}{\mathrm{d}t} = K \cdot [\mathrm{E}]_0 \cdot \mathrm{e}^{-kt} \tag{4}$$

Integrating and putting $\mathrm{f}(t) = 0$ at $t = 0$,

$$\mathrm{f}(t) = \frac{K \cdot [\mathrm{E}]_0}{k}(1 - \mathrm{e}^{-kt}) \qquad \text{and} \tag{5}$$

$$g(t) = g(\mathrm{O}) - mt - \frac{K \cdot [\mathrm{E}]_0}{k}(1 - \mathrm{e}^{-kt}) \tag{6}$$

When the inhibition has occurred (effectively at $t = \infty$),

$$g(\infty) = g(0) - mt - K \cdot [\mathrm{E}]_0/k \tag{7}$$

Hence

$$g(t) - g(\infty) = \frac{K \cdot (\mathrm{E})_0 \, \mathrm{e}^{-kt}}{k} \tag{8}$$

$$\log(g(t) - g(\infty)) = \log(K \cdot [\mathrm{E}]_0/k) - kt/2.3 \tag{9}$$

$(g(t) - g(\infty))$ may be obtained by extrapolating the linear portion of the kinetic curves and measuring the difference between this and the original curved portion. A plot of the log of this function against time gives k. This model is also applicable if there is a separate rapid phase of the inhibition.

APPENDIX 2

Specific activities of three enzyme conformations

Let the total activity of the enzyme be given by

$$A = \varepsilon_R \cdot [R] + \varepsilon_T \cdot [T] + \varepsilon_X \cdot [X] \tag{1}$$

where ε_R, ε_T, ε_X and [R], [T] and [X] represent the specific activities and concentration of the R, T and X conformations, respectively. Initially only the R and T forms are present. Assuming them to be in equilibrium, let

$$[R] = K \cdot [T] \tag{2}$$

where K is an equilibrium constant. Then the total enzyme concentration, [E], is given by

$$[E] = [R] + [T] \tag{3}$$

From Eqns 2 and 3 it follows that

$$[R] = \frac{K \cdot [E]}{1 + K} \tag{4}$$

and

$$[T] = \frac{[E]}{1 + K} \tag{5}$$

Hence initially

$$\frac{A_1}{[E]} = \frac{K}{1 + K} \times \varepsilon_R + \frac{1}{1 + K} \times \varepsilon_T \tag{6}$$

Finally, the three conformations are in equilibrium and from similar consideration to the above it follows that the final activity A_2 is given by

$$\frac{A_2}{[E]} = \frac{K}{1 + K + K'} \times \varepsilon_R + \frac{1}{1 + K + K'} \times \varepsilon_T + \frac{K'}{1 + K + K'} \times \varepsilon_X \tag{7}$$

where $K' = [T]/[X]$ defines the T–X equilibrium. If the activity measured decreases with time

$$\frac{A_1}{[E]} - \frac{A_2}{[E]} > 0 \tag{8}$$

An example of this is the effect of diethylstilboestrol on the glutamate and alanine dehydrogenase activities of the enzyme

From the Eqns 6 and 7 simplification leads to

$$K \cdot \varepsilon_R + \varepsilon_T > (1 + K)\varepsilon_X \quad (9)$$

Let $\varepsilon_T = f \cdot \varepsilon_R$ (10)

Hence $(f + K)\,\varepsilon_R > (1 + K)\varepsilon_X$ (11)

For glutamate dehydrogenase inhibition

$\varepsilon_R > \varepsilon_T$ and $f < 1$ (13), (14)

and for Eqn 11 to be true

$$\varepsilon_R > \varepsilon_X \quad (15)$$

For alanine dehydrogenase activation

$$\varepsilon_T > \varepsilon_X \quad (16)$$

and no conclusion follows from inequality Eqn 11, Putting

$$\varepsilon_R = g \cdot \varepsilon_T \quad (17)$$

From Eqn 9

$$\varepsilon_T (1 + gK) > (1 + K)\varepsilon_X \quad (18)$$

For glutamate dehydrogenase inhibition

$$g > 1 \quad (19)$$

and no conclusion follows from Eqn 18. For alanine dehydrogenase activation

$$g < 1 \quad (20)$$

and hence

$$\varepsilon_T > \varepsilon_X$$

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