Interaction of Organic Mercurial Compounds with Dihydrofolate Reductase from Ehrlich Ascites Carcinoma Cells*

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ABSTRACT: Stimulation of the Ehrlich ascites cell enzyme by organic mercurial compounds occurs as a result of the formation of an isolable complex between organic mercurial and enzyme, presumably through a mercaptide linkage. Whereas an excess of a thiol such as 2-mercaptoethanol will reverse this stimulation, with certain buffers (phosphate, citrate) the presence of a small amount of the thiol (10^{-3} M) was necessary to produce activation.

These apparently contradictory results have been found to be the result of both an inhibitory and stimulatory effect of mercurials on this enzyme activity; the former effect may be abolished by treatment with 10^{-3} M mercaptoethanol, 5×10^{-3} M EDTA, or passage of the enzyme-*p*-mercuribenzoate complex through a Sephadex G-25 column. The *p*-mercuribenzoate enzyme complex obtained after gel filtration was not stable at 23°, but inactivation was prevented by either reduced nicotinamide–adenine dinucleotide phosphate or dihydrofolate. That an alteration in tertiary structure occurs upon mercurial binding, which may explain the stimulation, is suggested by: (1) the observed increase in sensitivity of the complex to denaturants and heat, (2) the change in substrate specificity, and (3) the observation that an activated enzyme, with similar substrate specificity to that of the mercurated enzyme, can be formed in the presence of thiourea or guanidine-HCl.

The enzyme FH_2[1] reductase has been reported to be stimulated by cations (Bertino, 1962), urea, guanidine-HCl, and formamide (Kaufman, 1963), and by certain aliphatic amines (Misra and Adamson, 1963). Recently the stimulation of this enzyme activity by organic mercurials has been reported, in independent studies, by Kaufman (1964), who used chicken liver as an enzyme source, and by Perkins and Bertino (1964), who used an enzyme from Ehrlich ascites cells. Further studies concerning the properties of the *p*-mercuribenzoate-treated ascites cell enzyme will be presented. Evidence has been obtained which indicates that the active site of the reductase enzyme undergoes a catalytically favorable alteration as a result of stimulation by *p*-mercuribenzoate and/or urealike compounds.

Methods

Materials. The preparation of FH_2, FH_2Cl_2, and N^{10}-CH_3-FH_2 is described in the accompanying paper (Bertino *et al.*, 1965), as are the sources of NADH, $NADP^+$, NADPH, and 2-mercaptoethanol. CH_3-HgBr and CH_3HgI were gifts from Dr. S. H. Chu; PHgAc was purchased from Eastman Organic Chemicals; urea, thiourea, and L-cysteine were obtained from Fisher Scientific Co., and guanidine-HCl was purchased from Nutritional Biochemicals Corp.

Enzyme Preparation. FH_2 reductase from Ehrlich ascites cells was prepared as described in the accompanying paper. Sephadex enzyme refers to enzyme obtained from the Sephadex G-75 purification step; specific activity 7–15 μmoles/hour per mg. Hydroxylapatite enzyme refers to enzyme obtained from that step of purification; specific activity 50–150 μmoles/hour per mg. FH_2 reductase from chicken liver was partially purified by following the procedure used for the ascites cell enzyme. The enzyme activity eluted from Sephadex G-75 was used in the studies described.

Preparation of the Enzyme-p-Mercuribenzoate Complex. The Sephadex ascites cell enzyme was diluted with Tris-HCl, *p*H 7.5, 0.05 M, which was 0.1 M KCl to give an activity of approximately 0.050 absorbancy unit/min per 0.1 ml of enzyme solution. A mixture of enzyme, NADPH (0.1 mg/ml), and 5×10^{-4} M *p*-mercuribenzoate was made at 4°. The solution was allowed to come to room temperature and the activity of 0.1-ml aliquots of the mixture was assayed both in the presence and absence of additional *p*-mercuribenzoate. When the two rates were the same, i.e., approximately 0.250 absorbancy unit/min, 3 ml of the solution was then passed

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[1] Abbreviations used in this work: FH_2, dihydrofolate; FH_2Cl_2, 3′,5′-dichlorodihydrofolate; N^{10}-CH_3-FH_2, N^{10}-methyldihydrofolate; PHgAc, phenylmercuric acetate; CH_3HgBr, methylmercuric bromide; CH_3HgI, methylmercuric iodide; NADH, reduced nicotinamide–adenine dinucleotide; $NADP^+$ and NADPH, oxidized and reduced forms, respectively, of nicotinamide–adenine dinucleotide phosphate.

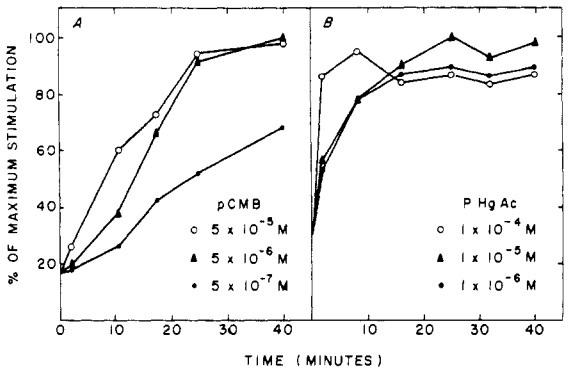

FIGURE 1: The effect of preincubation of FH_2 reductase with *p*-mercuribenzoate (*p*CMB) and PHgAc on enzyme activity. The incubation mixture contained potassium phosphate buffer, *p*H 6.5, 300 μmoles; KCl, 600 μmoles; NADPH, 0.30 μmole; enzyme from hydroxylapatite purification (specific activity 75 μmoles/hr per mg); and distilled water to 6.0 ml. The preincubation was carried out at 23°. Aliquots (0.95 ml) were removed at the times indicated, and 0.07 μmole of FH_2 containing 1.0 μmole of mercaptoethanol, in a volume of 0.05 ml, was added to start the reaction. The ordinate indicates the percentage of the maximum stimulation obtained for each mercurial at the time indicated.

through a Sephadex G-25 column, 2.4 × 12 cm, previously cooled to 5–10° and eluted with Tris-KCl (*p*H 7.5) at 5–10°. The enzyme activity was eluted from the column with the protein peak at a time when unbound *p*-mercuribenzoate and NADPH were still retained on the column. Recovery of enzyme activity was 85–100%.

Assay Procedure. Enzyme assays were performed at 23° in a final volume of 1.0 ml, in 1-cm quartz cuvets, with a Gilford recorder attached to a Beckman DU spectrophotometer. The automatic cuvet changer allowed assays to be recorded at 15-second intervals. The time between the initiation of the reaction and the start of the recording was usually less than 10 seconds. The details of the assay procedures are described in the legends of the various figures and tables.

Results

Specificity of Mercurial Activation. A comparison of the effectiveness of various organic mercurials in stimulating FH_2 reductase is presented in Table I. The mercurials were compared under conditions that gave instantaneous, maximum stimulation. The observation that CH_3HgBr and CH_3HgI actually caused *inhibition* of this enzyme activity suggested the presence of inhibitory sites for mercurial binding; indeed, as will be shown in a later section, *p*-mercuribenzoate can produce inhibition as well as stimulation, depending upon the assay conditions employed. Maximum stimulation was obtained with lower concentrations of the mercurials when a time of preincubation with the enzyme was allowed (Figure 1).

TABLE I: Effect of Organic Mercurials on FH_2 Reductase from Ehrlich Ascites Carcinoma Cells.[a]

Organic Mercurial	Molar Concentration (M)	Enzyme Activity (ΔA 340 mμ/ 4 minutes)
None		0.275
CH_3HgBr	5×10^{-5}	0.240
CH_3HgI	1×10^{-4}	0.200
PHgAc	1×10^{-4}	0.800
p-Mercuribenzoate	5×10^{-4}	1.380
p-Mercurisulfonate	5×10^{-4}	0.660

[a] The assay mixture contained potassium phosphate buffer, *p*H 6.5, 50 μmoles; KCl, 100 μmoles; NADPH, 0.05 μmole; FH_2, 0.07 μmole; 2-mercaptoethanol, 1 μmole, mercurial as indicated; and Sephadex enzyme, 0.1 ml. The reaction was initiated by adding FH_2 containing the mercaptoethanol.

Although PHgAc does not produce the degree of stimulation of the ascites cell enzyme as does *p*-mercuribenzoate (Table I), it is apparent from Figure 1B that the time necessary to produce its maximum effect is less than that for *p*-mercuribenzoate at comparable concentrations. It may also be seen from Figure 1B that the same degree of stimulation is produced over a 100-fold range in concentration, suggesting that PHgAc is not a less effective stimulator because of lack of binding, but more probably because of its lesser efficacy once bound. Similar results were obtained with *p*-mercurisulfonate.

The Effect of Thiols on p-Mercuribenzoate Stimulation. Under certain conditions the stimulation of the ascites cell enzyme by *p*-mercuribenzoate was found actually to *depend* on the presence of the small amount of mercaptoethanol (0.001 M) normally included in the assay mixture owing to its presence in the FH_2 solution (see the legend of Figure 1). When FH_2, kept under a nitrogen atmosphere rather than in 0.02 M mercaptoethanol, was used as the substrate for a reaction carried out in phosphate or citrate buffer, *p*-mercuribenzoate failed to stimulate the enzyme (Table II). Further experimental results illustrating this point are shown in Figure 2. Even though reaction mixtures (2) and (3) contained 5×10^{-4} M *p*-mercuribenzoate, the rate of reaction for the first 2 minutes was not appreciably faster than that catalyzed by the untreated enzyme (reaction 1). Upon the addition of either mercaptoethanol or cysteine the rate immediately increased to that of reaction (4), which included 1.0 μmole of mercaptoethanol from the start of the assay.

Separation of the Stimulatory from the Inhibitory Effects of p-Mercuribenzoate by Gel Filtration. Treatment of enzyme with *p*-mercuribenzoate in the presence

TABLE II: Dependence of Stimulation by *p*-Mercuribenzoate of the Ascites Cell Enzyme on Mercaptoethanol: A Comparison of Buffers.[a]

Concentration of Mercaptoethanol in Assay (μmoles/ml)	*p*-Mercuribenzoate	Enzyme Activity (ΔA 340 mμ/4 minutes) Tris-HCl *p*H 7.0	Citrate *p*H 6.0	Phosphate *p*H 7.0
0	−	0.103	0.096	0.136
1.0	−	0.095	0.097	0.125
0	+	0.266	0.093	0.104
0.2	+	0.310	0.120	0.123
0.5	+	0.450	0.495	0.510
1.0	+	0.432	0.452	0.441

[a] Assay mixtures contained Sephadex enzyme, 0.1 ml; buffer, 50 μmoles; *p*-mercuribenzoate, 5×10^{-4} M where indicated; NADPH, 0.05 μmole; FH_2, 0.07 μmole (kept under nitrogen); and mercaptoethanol as indicated. FH_2 was added last to start the reaction.

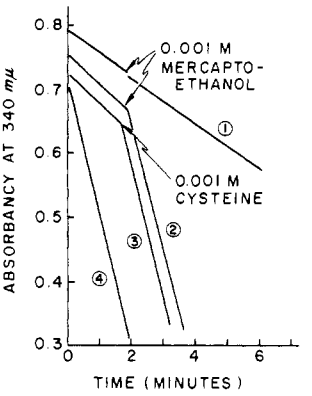

FIGURE 2: Dependence of the stimulation of FH_2 reductase by *p*-mercuribenzoate upon either mercaptoethanol or cysteine. This figure is a reproduction of a continuous recording produced on the Gilford recorder. Cuvet (1) contained the same components as indicated in the legend of Table I, except that FH_2, without mercaptoethanol, was kept under nitrogen and KCl was omitted. Cuvets (2) and (3) were identical to (1), except that the FH_2 (0.05 ml) was 0.02 M with respect to mercaptoethanol. At the times indicated, the following additions were made (0.05 ml): (1) 1.0 μmole of mercaptoethanol, (2) 1.0 μmole of mercaptoethanol, or (3) 1.0 μmole of L-cysteine.

of phosphate buffer and subsequent gel filtration through a Sephadex G-25 column resulted in the isolation of a *p*-mercuribenzoate-enzyme complex with a 4-fold increase in total activity, even when assayed in the absence of mercaptoethanol. In this instance, addition of exogenous *p*-mercuribenzoate to the assay mixture in increasing concentration (5×10^{-7} to 5×10^{-4} M) resulted in increasing *inhibition* of FH_2 reductase activity. This inhibition by exogenous *p*-mercuribenzoate could then be reversed by 5×10^{-3} M EDTA, as well as with 5×10^{-4} M mercaptoethanol. These findings lead us to suggest that under certain conditions *p*-mercuribenzoate may also have a direct inhibitory effect on the enzyme; however an interaction between *p*-mercuribenzoate and FH_2 may also be of importance.[2] It should be emphasized that mercaptoethanol at concentrations that instantly reversed the inhibitory effects of *p*-mercuribenzoate had no effect on either the untreated enzyme or on the stimulatory action of *p*-mercuribenzoate, even with preincubation for up to 2 hours (curve 1, Figure 3). Likewise, EDTA (5×10^{-3} M) did not affect the stimulatory action of *p*-mercuribenzoate. When FH_2 was not present, however, the stimulatory effect could be reversed rapidly by mercaptoethanol (0.001 M). This protection by FH_2 is discussed in a later section.

[2] We have observed that *p*-mercuribenzoate catalyzes the formation from FH_2 of a yellow compound (λ_{max} 425 mμ) in phosphate buffer but not in Tris-HCl. This *p*-mercuribenzoate-catalyzed reaction does not occur in phosphate buffer if either 5×10^{-3} M EDTA or 1×10^{-3} M mercaptoethanol is included. The effect of the reaction product on the enzymic reaction is currently being investigated.

Stability of Enzyme-p-Mercuribenzoate Complex. Kaufman (1964) reported that the enzyme-methylmercury complex prepared from chicken liver FH_2 reductase was stable to repeated freezing and thawing, $(NH_4)_2SO_4$ fractionation, and passage through Sephadex G-25. In contrast, we have found the ascites cell enzyme–*p*-mercuribenzoate complex to be unstable. As shown in Figure 3, the enzyme-*p*-mercuribenzoate complex prepared as described under Methods lost 60% of its activity when kept at 23° for 30 minutes. Since the untreated enzyme is more stable to heat inactivation in the presence of FH_2 or NADPH (Bertino *et al.*, 1965), the effect of these compounds on the stability of the enzyme-*p*-mercuribenzoate complex was investigated. Complete protection was afforded by either FH_2 or NADPH for up to 2 hours (Figuer 4). Although NADH serves only poorly as a cofactor for FH_2 reductase, it does inhibit the NADPH-mediated reaction[3] and thus probably binds at the same site as does NADPH, therefore explaining its activity as a weak protective agent. $NADP^+$ was a more effective inhibitor of the reductive reaction and prevented the breakdown of the complex to an even more significant degree. The loss of activity observed when no protective agent was present was not simply the result of dissociation of the complex, since additional *p*-mercuribenzoate failed to restore activity to the initial value. Although the nature of this inactivation of the complex is not entirely clear, the increased sensitivity of the complex to

[3] Unpublished observations.

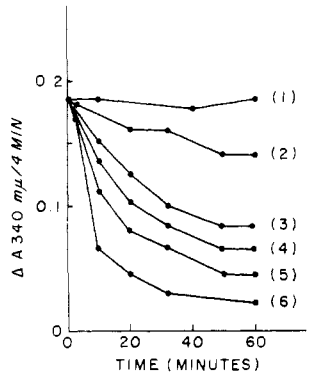

FIGURE 3: Prevention by FH_2 of the reversal of *p*-mercuribenzoate stimulation by mercaptoethanol. The enzyme-*p*-mercuribenzoate complex was prepared as described under Methods. Preincubation mixtures (1) through (5) contained: enzyme-*p*-mercuribenzoate complex, 0.90 ml; FH_2, 0.05 ml (0.07 μmole); mercaptoethanol was added in a 0.05-ml volume to give the following concentrations: (1) 0.001 M, (2) 0.002 M, (3) 0.006 M, (4) 0.011 M, (5) 0.021 M, and (6) same as 1, but 0.05 ml of water was added instead of FH_2. At the times indicated, 0.10-ml aliquots were removed and added to 0.90 ml of the assay mixture described for Figure 4. The rate of the reaction is expressed as the decrease in absorbancy at 340 mμ produced during a period of 4 minutes.

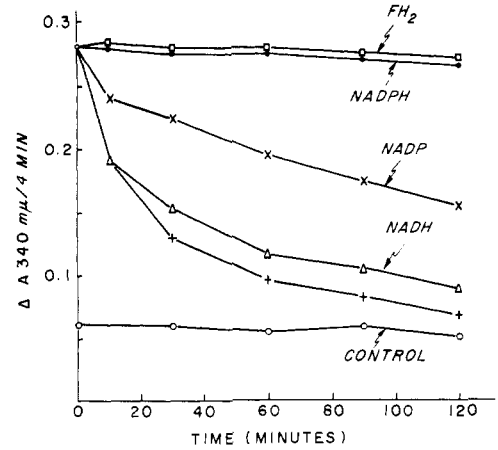

FIGURE 4: Prevention of inactivation of the enzyme-*p*-mercuribenzoate complex by FH_2 or cofactors. The enzyme-*p*-mercuribenzoate complex was prepared as described under Methods. Enzyme-*p*-mercuribenzoate complex, 0.95 ml, and 0.05 ml (0.05 μmole) of either NADPH, $NADP^+$, NADH, or FH_2 were preincubated at 23°. At the times indicated, a 0.10-ml aliquot was removed and added to 0.90 ml of a solution containing: Tris-maleate buffer, *p*H 6.4, 50 μmoles; KCl, 100 μmoles; NADPH, 0.05 μmole; FH_2, 0.07 μmole; and mercaptoethanol, 1.0 μmole. The rate of the reaction is expressed as the decrease in absorbancy at 340 mμ that occurred during a period of 4 minutes. The control contained untreated enzyme and was incubated under the same conditions as have been described.

inactivation by urealike compounds (Figure 5) indicates that the mercurated enzyme is more prone to denaturation than is the normal enzyme.

The presence of FH_2 not only prevented inactivation of the complex but also blocked the reversal of stimulation by high concentrations of mercaptoethanol. This aspect of the protection is demonstrated by the experiment shown in Figure 3. The loss of activity of the enzyme-*p*-mercuribenzoate complex was greatly accelerated in the presence of 0.001 M mercaptoethanol (curve 6). In the presence of FH_2, however, no loss of activity was observed with 0.001 M mercaptoethanol (curve 1). In the presence of 0.006 M mercaptoethanol and excess FH_2 (curve 3) activity was still 50% of the initial value after 1 hour, and more than 0.020 M mercaptoethanol was necessary to overcome the protective effect of FH_2 (curve 5). Saturating levels of NADPH produced only slight protection. The presence of the substrate apparently alters the availability of the bound mercurial for exchange with the thiol.

*p*H *Optimum of Reaction Catalyzed by the Enzyme-p-Mercuribenzoate Complex.* As reported earlier (Perkins and Bertino, 1964), the stimulation of the ascites cell enzyme caused by *p*-mercuribenzoate occurred over a wide *p*H range when the complex was formed in the assay mixture. This procedure did not differentiate between the effect of *p*H on the formation of the enzyme-*p*-mercuribenzoate complex and the effect on the catalytic activity of the isolated complex. Accordingly, the complex was prepared and isolated, as described under Methods, and the effect of *p*H on catalytic activity was determined. The results were in agreement with our previous observations; the same per cent stimulation was observed over the *p*H range 5.0–7.5.

Evidence against Subunit Formation. Since it is likely that one molecule of amethopterin tightly binds to one active site of FH_2 reductase (Werkheiser, 1961; Bertino *et al.*, 1964) an equimolar amount of amethopterin added to enzyme in either the presence or the absence of *p*-mercuribenzoate should produce less percentage inhibition if, as its mechanism of action, *p*-mercuribenzoate produces additional active sites. Data have been presented (Perkins and Bertino, 1964) to show that the same percentage of inhibition is produced by amethopterin in either the presence or the absence of *p*-mercuribenzoate.

Direct evidence against the stimulation of FH_2 reductase by mercurial compounds being a result of a dissociation of the enzyme into subunits was presented by Kaufman (1964), who was unable to demonstrate a difference in the molecular weight of the nonmercurated and mercurated chicken liver enzyme as determined by sedimentation rates in a sucrose gradient. In order to study the possibility that the ascites cell enzyme undergoes dissociation upon treatment with organic mercurials, we have utilized another method, namely, gel filtration on Sephadex (Whitaker, 1963), to determine

the molecular weight of the enzyme and its mercurated derivative.

The details of the method are reported in the accompanying paper; in essence, a comparison was made of the elution volumes of dilute solutions of enzyme and complex under identical conditions. NADPH, 0.0001 M, was added to the elution solvent to stabilize the complex, as well as the normal enzyme. The two forms of the enzyme eluted from the column in essentially the same volume, 86–87 ml. The ratio of elution to void volume when plotted on the standard curve gave a molecular weight of 20,200 for both forms of the enzyme.

Substrate Specificity. Since both the functional capacity and the substrate affinity of an enzyme are dependent on the structure of the protein molecule, if FH_2 reductase is altered structurally by *p*-mercuribenzoate then it is possible that the complex would exhibit an altered substrate specificity. Various analogs of FH_2 that serve as substrates for the reductase from ascites cells have been investigated (Bertino *et al.*, 1965); in Table III two of these, FH_2Cl_2 and N^{10}-CH_3-FH_2, are compared with FH_2 as substrates for the enzyme and the enzyme-*p*-mercuribenzoate complex. The data indicate that the above-mentioned possibility was realized. Although both analogs were reduced by the nonmercurated enzyme at a faster rate than was FH_2, when either *p*-mercuribenzoate or PHgAc was added the rate of reduction of FH_2Cl_2 was actually inhibited, while that of N^{10}-CH_3-FH_2 was increased by only 45%. The rates given are apparent maximal velocities, since additional substrate did not increase the reaction rate. When preformed complex was utilized, as opposed to generating the complex in the assay mixture, as described in Table III, the results were the same.

TABLE III: Effect of Organic Mercurials on FH_2 Reductase: A Comparison of Three Substrates.[a]

Organic Mercurial	Enzyme Activity (ΔA 340 mμ/4 minutes) FH_2	FH_2Cl_2	$N^{10}CH_3$-FH_2
None	0.160	0.416	0.460
p-Mercuribenzoate, 5×10^{-4} M	0.720	0.334	0.664
PHgAc, 1×10^{-4} M	0.470	0.316	0.558
CH_3HgBr, 5×10^{-5} M	0.150	0.324	0.342

[a] The assay mixtures were similar to those described in the legend of Table I, except that Tris-maleate buffer, *p*H 6.4, was used and KCl was omitted; the respective mercurial compounds were included at the concentrations indicated. The reaction was initiated by the addition of substrate, approximately 0.07 μmole, containing 2 μmoles of mercaptoethanol.

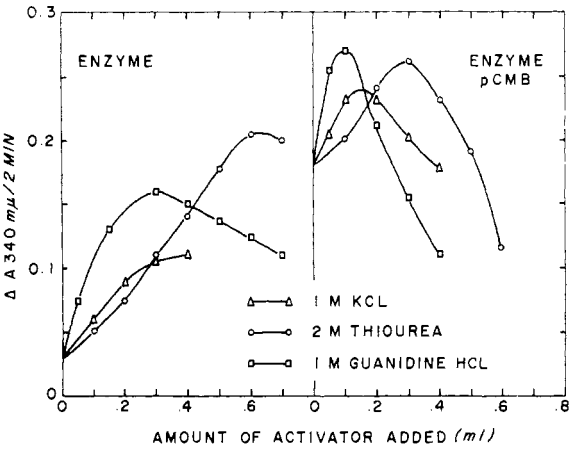

FIGURE 5: A comparison of the effect of KCl, thiourea, and guanidine-HCl on the normal enzyme and the enzyme-*p*-mercuribenzoate complex. The assay mixture was the same as in Figure 4, except that KCl was added only as indicated in the figure. In this experiment the enzyme was added last to initiate the reaction. The abscissa indicates the ml of each stimulator substance added from stock solutions of the indicated concentrations.

Effects of KCl, *Guanidine*-HCl, *and Thiourea.* Table IV shows the results of a comparison of the rate of reduction of three substrate molecules in the presence of increasing concentrations of KCl, thiourea, and guanidine-HCl. In general, those substances that stimulated the reduction of FH_2 caused an inhibition of the reduction of FH_2Cl_2 and only slight stimulation of the rate of reduction of N^{10}-CH_3-FH_2. This result is similar to those obtained when the enzyme was stimulated by mercurial compounds (Table III).

In Figure 5 a comparison is made of the effect of increasing the concentration of either KCl, guanidine-HCl, or thiourea on the rate of reduction of FH_2 by the nonmercurated enzyme and the preformed enzyme-*p*-mercuribenzoate complex. The nonmercurated enzyme was stimulated up to 700% of control rates, while the maximum stimulation of the complex was 50%. The optimal concentration for maximum stimulation of the complex also was lower than that necessary for the nonmercurated enzyme. As shown previously (Perkins and Bertino, 1964), the enzyme-*p*-mercuribenzoate complex is apparently more susceptible than the normal enzyme to inactivation by urea; in this case other denaturing agents, namely, thiourea and guanidine-HCl, produced a comparable effect.

Discussion

The dissociation of enzymes into subunits by *p*-mercuribenzoate has been reported to occur with muscle phosphorylase (Madsen and Cori, 1956) and with yeast alcohol dehydrogenase (Snodgrass *et al.*, 1960). Schachman (1963) postulated that the dissociation

TABLE IV: Effect of KCl, Guanidine-HCl, and Thiourea on FH_2 Reductase: A Comparison of Three Substrates.[a]

Additions	Molar Concentration (M)	Enzyme Activity (ΔA 340 mμ/4 minutes) FH_2	FH_2Cl_2	$N^{10}CH_3$-FH_2
None		0.090	0.358	0.340
KCl	0.1	0.160	0.321	0.336
KCl	0.2	0.230	0.300	0.360
KCl	0.4	0.270	0.214	0.338
Thiourea	0.4	0.228	0.356	0.556
Thiourea	0.8	0.420	0.338	0.608
Thiourea	1.0	0.540	0.242	0.558
Guanidine-HCl	0.05	0.180	0.344	0.360
Guanidine-HCl	0.10	0.300	0.320	0.420
Guanidine-HCl	0.20	0.435	0.234	0.362
Guanidine-HCl	0.30	0.480	0.154	0.286

[a] The assay mixtures contained: Tris-maleate buffer, pH 6.4, 50 μmoles; NADPH, 0.05 μmole; substrate, 0.07 μmole; Sephadex enzyme, 0.1 ml; and the respective stimulators were included at the concentrations indicated. The enzyme was added last to initiate the reaction.

of proteins into subunits by *p*-mercuribenzoate was at least a two-step process: (1) the formation of mercaptide linkages with certain sulfhydryl groups, and (2) an indirect steric effect caused by the bulky benzoic acid group interfering with amino acid side-chain interactions. Li *et al.* (1962) determined the optical rotatory dispersion spectrum of liver alcohol dehydrogenase during inactivation by *p*-mercuribenzoate and interpreted the data as indicating that *p*-mercuribenzoate causes conformational changes in the enzyme that are concomitant with inactivation. These observations of Li *et al.* provide direct evidence that *p*-mercuribenzoate can cause conformational changes resembling protein denaturation, as well as subunit dissociation. The phenomenon of stimulation of FH_2 reductase may well be an example of such a conformational change, which in this case is catalytically favorable. In support of this type of stimulation phenomenon, Maxwell (1962), who studied the effect of steroids and urea on the reaction catalyzed by rabbit liver aldehyde dehydrogenase, came to the conclusion that both types of agents exert their stimulatory effect by causing structural alteration of the enzyme without changing the molecular weight.

The existence of a stable complex between CH_3-HgBr and FH_2 reductase from chicken liver has been described by Kaufman (1964) and, in the presence of substrate, a stable complex between the ascites cell enzyme and *p*-mercuribenzoate has been described in this report. A mercurated enzyme has not been demonstrated directly, but its existence is supported strongly by indirect experimentation; namely, the observation that stimulation increases upon preincubation with low concentrations of *p*-mercuribenzoate, the isolation of an activated enzyme free of unbound *p*-mercuribenzoate, and the reversal of stimulation by treatment of the isolated complex with sufficient mercaptoethanol.

The molecular weight approximations of both the untreated and *p*-mercuribenzoate-treated enzyme from chicken liver (Kaufman, 1964) and from ascites cells support the concept that the stimulation of FH_2 reductase is brought about by conformational changes in the enzyme molecule rather than by association or dissociation of enzyme subunits. The actual number of active sites is apparently the same in both the normal and the complexed enzyme, as indicated previously (Perkins and Bertino, 1964).

The hypothesis that stimulation of FH_2 reductase by *p*-mercuribenzoate occurs as a result of a conformational change in the enzyme is supported by three lines of evidence: (1) The *p*-mercuribenzoate-treated enzyme has altered stability. As demonstrated in Figure 4, the enzyme-*p*-mercuribenzoate complex is much less stable at room temperature when compared with the untreated enzyme. The instability of the enzyme-*p*-mercuribenzoate complex might well be attributed to alterations in the "cooperative" of chemical binding forces known to maintain protein structure (see, e.g., Schachman, 1963). Schachman has suggested that the sheer bulk of the ring portion of the bound *p*-mercuribenzoate molecule could cause distortion of the ordered structure of the enzyme. The alteration in structure caused by the binding of *p*-mercuribenzoate apparently is not drastic, however, since the binding of either substrate or cofactor is sufficient to prevent the rapid loss of activity of the complex seen at 23°. The data expressed in Figure 5 indicate that the enzyme-*p*-mercuribenzoate complex likewise is more susceptible to inactivation by thiourea and guanidine-HCl than is the untreated enzyme. Since denaturation by these urealike compounds depends on the strength of the forces maintaining normal protein structure that must be overcome, one would expect that a protein, partially denatured in one manner, would be more sensitive to a second denaturing agent. (2) The *p*-mercuribenzoate-treated enzyme exhibits an altered substrate specificity (Table III) which may reflect structural alterations in the active site of the enzyme. No information is available to differentiate between an alteration in the active site caused by the interaction of *p*-mercuribenzoate at or near this site or an allosteric effect resulting from an interaction at a point other than the active site. (3) The enzyme can be stimulated by urealike compounds (Figure 5), and the stimulated enzyme exhibits a substrate specificity similar to that of the *p*-mercuribenzoate-treated enzyme (Table IV). Further similarity between the activated states of the enzyme caused by urealike compounds and *p*-mercuribenzoate is the observation that both forms of the stimulated enzyme are unstable at room

temperature and both are stabilized in the presence of FH_2.

That it is an alteration in the tertiary structure of the enzyme that causes stimulation is supported further by the order of activity of the three agents, guanidine-HCl, thiourea, and urea. Gordon and Jencks (1963) have shown that the order of reactivity of these compounds as protein-denaturing agents is the order mentioned. We have observed maximum stimulation of the Ehrlich ascites cell enzyme at 0.30 M guanidine-HCl, 1.2 M thiourea, and 3.5 M urea.

The observation that *p*-mercuribenzoate probably has two effects, stimulation and inhibition, which show different susceptibilities to reversal by mercaptoethanol, has cleared up much of the early confusion about the interrelationship of *p*-mercuribenzoate and the thiol. Since the stimulatory effect is reversible at high concentrations of mercaptoethanol but not EDTA, and is maintained after passage over Sephadex or dialysis, we have suggested that this effect is mediated by binding of *p*-mercuribenzoate through mercaptide linkage. On the other hand, the inhibitory effect is apparent only in certain buffers, is reversed by EDTA as well as by low concentrations of mercaptoethanol, and is effectively reversed by passage of the mercurial-treated enzyme over Sephadex. These findings are characteristic of a loose association between *p*-mercuribenzoate and the enzyme, but the exact nature of this inhibitory effect is still under investigation.

Of interest is the prevention by FH_2 of the inactivation of the enzyme-*p*-mercuribenzoate complex by 2-mercaptoethanol. Two possible explanations are: (1) the binding of FH_2 interferes sterically with the approach of mercaptoethanol to bound *p*-mercuribenzoate; and (2) the binding of the substrate causes a further structural alteration in the enzyme that causes the bound *p*-mercuribenzoate to become less accessible to attack by the thiol. The present data do not allow us to decide between the two alternative mechanisms; however, we favor the latter since both the stabilizing (Figure 4) and protective (Figure 3) effects of FH_2 might be explained if an altered structure results from its binding. The theory that conformational changes can be induced in enzymes by their substrates has been emphasized by Koshland (1963), and Jencks (1963) has recently reviewed the evidence in support of this hypothesis.

The studies of stimulation by organic mercurials and by other compounds have been carried out at saturating levels of FH_2 and NADPH in all experiments, and further increase in substrate concentration did not alter the initial rate of reaction. Thus, the explanation for increased rate must reside in an increased efficiency of product formation, since an increase in affinity alone cannot explain the observations. In fact, preliminary kinetic studies indicate that the K_m value for FH_2 is larger for the enzyme-*p*-mercuribenzoate complex than for the nonmercurated enzyme.

Although FH_2 reductase from both chicken liver (Kaufman, 1964) and Ehrlich ascites cells is stimulated by organic mercurials, important differences have become apparent. Based on a comparison of the results presented by Kaufman (1964) and on studies with the chicken liver enzyme in our laboratory several differences were found. (1) The mercurial specificity for maximum stimulation is different; e.g., the most effective stimulant of the chicken liver enzyme is CH_3HgBr. (2) The presence of 0.001 M mercaptoethanol reverses within minutes the stimulation of the chicken liver enzyme by CH_3HgBr of *p*-mercuribenzoate even in the presence of substrate. The *p*-mercuribenzoate-treated ascites cell enzyme is stable for at least 2 hours under the same conditions. (3) We have observed stimulation of the chicken liver enzyme under conditions such as described in Table II and Figure 2 that do not result in stimulation of the ascites cell enzyme. In spite of these differences, however, the mechanism of stimulation is probably basically the same, i.e., a change in conformation of the enzyme, since both enzymes are stimulated to a similar degree by urealike compounds.

Work now in progress concerns a detailed kinetic study of the phenomenon. Attempts are being made to obtain a pure enzyme in order to facilitate further studies of the interaction of these stimulatory substances with the enzyme.

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