STIMULATION OF DIHYDROFOLATE REDUCTASE ACTIVITY BY p-CHLOROMERCURIBENZOATE

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During the investigation of the effects of sulfhydryl inhibitors on a partially purified preparation of dihydrofolate reductase from Ehrlich ascites cells, it was noted that, under certain conditions, this enzyme not only is resistant to inhibition by p-chloromercuribenzoate (pCMB), but a marked increase in the velocity of the reaction that it catalyzes occurs in the presence of this compound. Hellerman, Schellenberg and Reiss (1958) previously have reported an analogous stimulation of L-glutamic acid dehydrogenase by certain organic mercurials; subsequently, several investigations have been concerned with the possible mechanism of this effect (Rogers et al., 1963a; Rogers and Thompson, 1963b). The data reported here on the specificity and nature of the stimulation of dihydrofolate reductase activity by organic mercurials indicates that certain differences exist between the stimulation of this enzyme and that of L-glutamic acid dehydrogenase. Thus, evidence is provided to show that pCMB stimulates dihydrofolate reductase activity by reacting directly with the enzyme, probably via a protein sulfhydryl group; this effect may result in a change in the conformation or configuration of the enzyme.

Soluble lysates prepared by repeated freeze-thawing of suspensions of Ehrlich ascites cells were concentrated 150-fold (specific activity: (34 μmoles/hr/mg) with respect to dihydrofolate reductase activity by acid

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precipitation of inert protein (pH 5.1), fractionation with solid ammonium sulfate (0.55 to 0.85 saturation), and chromatography on DEAE-cellulose. In the studies described below, the highly purified enzyme was used, although pCMB stimulated the enzyme to a similar degree at every step of purification.

The degree of activation of dihydrofolate reductase by organic mercurials depended on the nature of the mercury compound (Table I). Thus, phenylmercuric acetate (PMA) and pCMB produced three-fold and four-fold stimulation of enzyme activity, respectively, while under the same conditions, p-chloromercuric sulfonate (pCMS) and chloromerodrin had less effect. Methyl mercuric iodide and mercuric chloride produced little or no effect. The values in Table I represent the maximum rates obtained with the optimal concentration of the mercurial. Greatest stimulation was produced by concentrations of 10^{-5} to 10^{-4} M; lower or higher concentrations of mercurials resulted in a decrease in facilitating effects. In contrast to the requirements for maximal activation of the glutamic acid dehydrogenase system by organic mercurials, preincubation of these compounds with the dihydrofolate reductase system, for periods up to 30 minutes, did not change their effect. In addition, the mercury compound affecting optimum stimulation is different for the two enzymes.

TABLE I. Stimulation of dihydrofolate reductase activity by organic mercurials.

Mercury compound	Enzyme Activity Δ A 340 mμ/4 min
None	0.065
$HgCl_2$	0.080
CH_3HgI	0.065
Chloromerodrin*	0.100
p-Chloromercuric sulfonate	0.105
phenylmercuric acetate	0.185
pCMB	0.260

*1-[3-(Chloromercuri)-2-methoxypropyl] urea. The reaction mixture, in a final volume of 1.0 ml contained, in order of addition: 0.05 ml of a partially purified enzyme, potassium phosphate buffer, pH 7.0, 50 μmoles; KCl, 50 μmoles; water; mercurial, 0.1 ml; and TPNH, 0.05 μmole. Dihydrofolate, 0.07 μmole, containing 5 μmoles of 2-mercaptoethanol, was added last to start the reaction. The velocity of the reaction was determined by measuring the decrease in absorbancy that occurred at 340 mμ at one-minute intervals for 4 minutes. The concentration of mercurial employed was 5×10^{-4} M, except for PMA, which was used at a concentration of 1×10^{-4} M.

Two non-mercurial sulfydryl reagents, iodoacetamide (IA) and N-ethylmaleimide (NEM), were tested in a similar manner; neither stimulation nor inhibition was observed at a concentration of 10^{-3} M at neutral pH.

Stimulation of enzyme activity by pCMB occurred over a broad pH range between 5.5 and 8.0 with potassium phosphate buffer. Stimulation also was observed with tris, tris-maleate, and pyrophosphate buffers. However, increased buffer concentrations caused a decrease in the stimulation observed.

Since previous experiments indicated that stimulation of this enzyme activity could be produced by certain cations (Bertino, 1962) the effect of increasing salt concentration (KCl and $CaCl_2$) on enzyme activity in the presence or absence of pCMB was investigated. Figures Ia and Ib indicate that stimulation was enhanced in the presence of salts; optimal stimulation occurred with 0.05 M KCl or 0.025 M $CaCl_2$. These results indicate that the effectiveness of the stimulation by pCMB is related to the ionic strength

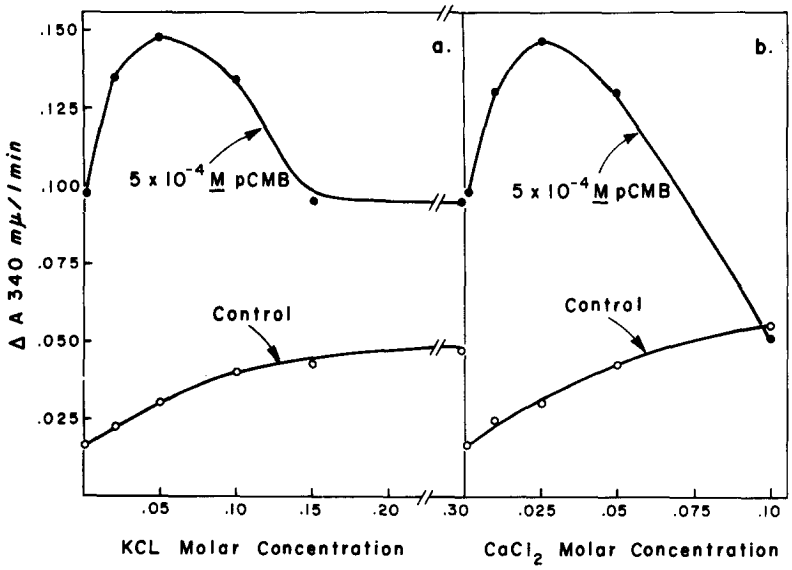

Figure 1. (A) The effect of pCMB on dihydrofolate reductase activity in the presence of various concentrations of KCl. The reaction mixture, in a total volume of 1.0 ml, contained: phosphate buffer, pH 7.0, 50 μmoles; KCl, as indicated; TPNH, 0.05 μmole; 0.05 ml enzyme, and pCMB, 5×10^{-4} M; dihydrofolate, 0.07 μmole, was added last to start the reaction. (B) The reaction mixture and assay were similar to that described for the experiment presented in A, except that $CaCl_2$ replaced KCl, as indicated, and tris buffer, pH 7.0, 50 μmoles, replaced phosphate buffer.

of the assay mixture. Also compatible with this theory is the decreased stimulation apparent at higher buffer concentrations.

The dihydrofolate reductase of Ehrlich ascites cells, like the enzyme from chicken liver (Kaufman, 1963) was stimulated by high concentrations of urea. When pCMB was present in optimum concentration, however, an increase in the concentration of urea did not result in an increase in stimulation, as was the case with the control (Fig. 2b); indeed, when both pCMB and urea are present, a decrease in the velocity of the reaction occurs. This finding could be explained if each of these reagents caused a similar configurational change of the enzyme; however, when either or both of the reagents are added in excess, a decrease in activity results. By contrast, an additive effect is observed when KCl and urea are both present (Fig. 2a). The results are also consistant with the inhibition, by exogenous pCMB, of a functionally important sulfhydryl group newly exposed by the urea denaturation.

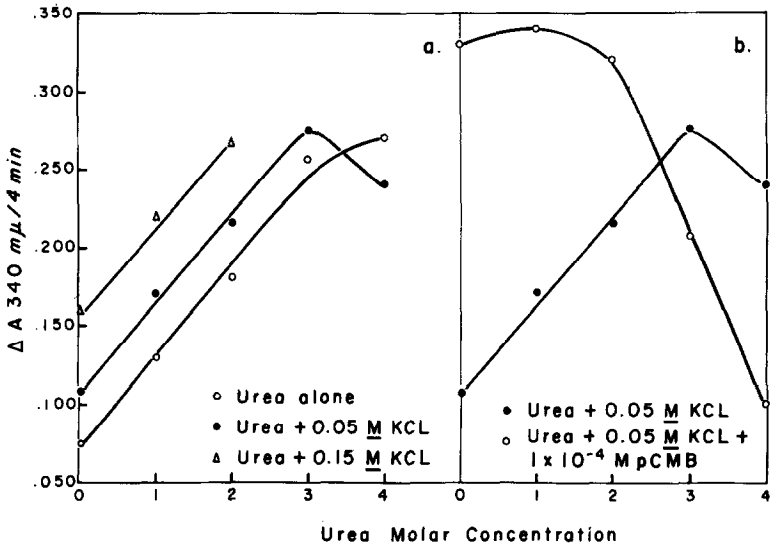

Figure 2. (A) Stimulation of dihydrofolate reductase activity by urea in the presence and absence of KCl. The reaction mixture was similar to that described in Figure 1, except that urea was added in suitable aliquots to give the indicated concentrations. In these experiments the enzyme was added last to initiate the reaction. (B) Stimulation of dihydrofolate reductase activity by urea in the presence and absence of pCMB. The reaction mixture and conditions of the assay were identical to those described in 2A, except that 0.1 ml of enzyme was mixed with an equal volume of 1×10^{-4} M pCMB that was 0.05 M in KCl and this mixture was added to start the reaction.

Although, as previously noted, NEM and IA did not alter dihydrofolate reductase activity, it was of interest to test the ability of these compounds to inhibit the activation produced by the organic mercurials. In a typical experiment, 10^{-3} M NEM caused 50% inhibition of the activation of dihydrofolate reductase produced by 5×10^{-4} M pCMB, but only when NEM was added to the enzyme before the addition of pCMB. Since both pCMB and NEM can bind to sulfhydryl groups, the inhibition produced by NEM is evidence that pCMB produces its effect via sulfhydryl binding. In agreement with these results, 2-mercaptoethanol (0.06 M) completely prevented stimulation by pCMB if added to the reaction mixture before the mercurial. When the same amount was added after the enzyme and pCMB were mixed, no reversal was produced for 4 minutes; however, the velocity of the reaction thereafter slowly decreased to control levels.

Recent reports of the activation of L-glutamic acid dehydrogenase by mercury compounds have suggested that this effect is caused by dissociation of the enzyme molecules into subunits, i.e., by increasing the number of active sites (Rogers et al., 1963a). This possibility was rendered unlikely for the dihydrofolate reductase activation by experiments in which amethopterin was added to mixtures with and without pCMB, at pH 6.0. Since it is likely that one molecule of amethopterin tightly binds to one active center of enzyme (Werkheiser, 1961; Bertino et al., 1964) an equimolar amount of amethopterin should produce less percentage inhibition if, as its mechanism of stimulation, pCMB produces additional active sites. The data in Table II indicate that the same percentage of inhibition was produced by 5×10^{-9} M amethopterin either in the presence of pCMB or in its absence.

Analogous to the results of Kaufman, who found that urea did not stimulate folate reduction by the chicken liver enzyme, we have found that pCMB fails to stimulate the reduction of folate (at pH 6.0) by the Ehrlich ascites enzyme. These findings agree with conclusions reached from kinetic data, i.e., that the mechanisms of reduction of folate and dihydrofolate are different (Bertino et al., 1964).

TABLE II. The effect of amethopterin on the stimulation of dihydrofolate reductase by pCMB.

Amethopterin 5×10^{-9} M	pCMB 5×10^{-4} M	Activity A 340 mμ/4 min	Inhibition %
-	-	0.100	-
+	-	0.050	50
-	+	0.388	-
+	+	0.188	48

The assay mixture was identical to that described in Table I, except that potassium phosphate buffer, pH 6.0, was employed.

Based on the evidence cited, we believe that pCMB reacts with the enzyme, probably via a mercaptide linkage, producing a favorable conformational change in the enzyme that results in a more rapid reduction of dihydrofolate. Further studies of the mechanism of this facilitative effect are in progress.

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