Effect of Folic Acid and Analogues on the Dehydrogenase and Isomerase Activities of Liver Alcohol Dehydrogenase*

ROBERT SNIYDER,† WOLFGANG VOGEL, AND MARTIN P. SCHULMAN

From the Department of Pharmacology, University of Illinois College of Medicine, Chicago 12, Illinois

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Several enzymes possess more than one specific function which can be selectively altered by various agents. For example, glutamic dehydrogenase oxidizes both alanine and glutamic acid (1); enzymic activity toward glutamic acid was inhibited by diethylstilbestrol while that toward alanine was stimulated by the same compound.

The present communication describes studies on the alterations of the two activities of liver alcohol dehydrogenase (alcohol: NAD oxidoreductase, EC 1.1.1.1): the dehydrogenation of ethanol to acetaldehyde and the isomerization of several aldehydes to ketones (2). The isomerization proceeds at approximately 8 times the rate of the dehydrogenation (2). While folic acid, aminopterin, and amethopterin inhibited dehydrogenase activity (3), these same compounds stimulated the isomerase activity of the dehydrogenase (4). The inhibitions produced by the folates were different from those produced by 1,10-phenanthroline and 8-hydroxyquinoline, which have been reported by others to inhibit the dehydrogenase by chelation. Kinetic data showed that inhibition by folic acid was complex, which supports the conclusion that inhibition of dehydrogenase activity was not attributable to chelation of enzymic zinc at the active site. The selective alteration of the two enzymic activities of liver alcohol dehydrogenase by the folates, as well as by 1,10-phenanthroline, iodoacetate, and p-mercuribenzoate, suggests that these activities take place on distinctly different sites of the enzyme.

EXPERIMENTAL PROCEDURE

Alcohol dehydrogenase activity was determined at room temperature with a Beckman DU spectrophotometer equipped with a photomultiplier, with the use of cylindrical cuvettes with 5-cm light paths. Unless otherwise specified, the assay system contained 2 × 10⁻⁴ M liver alcohol dehydrogenase, 2.2 × 10⁻⁴ M NAD, 8 × 10⁻³ M ethanol, and 0.1 M sodium pyrophosphate buffer, pH 8.8. Changes in optical density were recorded 15, 30, and 45 seconds after the reaction was started.

Isomerase activity was determined by the method described by van Eys with 3-phosphoglyceraldehyde as the substrate (2). For assay, 5 × 10⁻⁴ M 3-phosphoglyceraldehyde, 3.5 × 10⁻⁴ M NAD, 1.8 × 10⁻⁵ M dehydrogenase, and 0.05 M glycyglycine, pH 7.6, were incubated for 15 minutes at 37°. Dihydroxyacetone phosphate was measured by the method of Bailey (5), by means of its reaction with periodate; formaldehyde was released and measured as a chromogen at 540 μm upon addition of ferricyanide and phenylhydrazine.

Liver alcohol dehydrogenase was obtained from Worthington Biochemical Corporation. The enzyme was monodisperse in the ultracentrifuge and had a Svedberg constant which agreed with a molecular weight of 84,000. Yeast alcohol dehydrogenase was obtained from Nutritional Biochemicals Corporation. Amethopterin, dimethylamethopterin, and dihydroxyfolic acid were kindly supplied by Lederle Laboratories. Either folic acid purified by the method of Johns, Pledgerleith, and Cooper (6) or uochromatographed commercial preparations inhibited the dehydrogenase activity of liver alcohol dehydrogenase in a similar manner. The results reported here were obtained with the latter preparation. Acetaldehyde was freshly distilled.

RESULTS

Inhibition of the alcohol dehydrogenase of liver and yeast by folic acid, amethopterin, and aminopterin are illustrated in Fig. 1 upper and lower, respectively. Inhibition of the liver enzyme commenced at 3.5 × 10⁻⁵ M folates and was completed at 2 × 10⁻⁴ M. Dimethylamethopterin and dihydroxyfolic acid were also inhibitory within this narrow range of concentrations. In contrast, the inhibition of this enzyme by 1,10-phenanthroline reported by Vallee, Williams, and Hoch (7) began at about the same concentration but required more than a 100-fold increase in concentration before inhibition was complete. Yeast alcohol dehydrogenase inhibition by the folates was initiated at 2 × 10⁻⁴ M and was completed at 8 × 10⁻⁴ M. The concentration of 1,10-phenanthroline required for 50% inhibition was 3 × 10⁻³ M; Hoch, Williams, and Vallee (8) reported that 50% inhibition occurred at approximately 10⁻² M.

Preincubation of both enzymes with the folates for 1 hour resulted in the same degree of inhibition seen when the folates were added and readings were recorded as soon as possible (15, 30, and 45 seconds). Inhibition was completely reversed after 24 hours of dialysis at 4° of a solution containing 5 mg of the liver enzyme per ml, 2 × 10⁻³ M folinic acid, and 0.1 M potassium phosphate buffer, pH 7. By using the dilution method of Vallee et al. (7), it was found that the inhibitions produced by each of the folates were reversed when the reaction mixtures were diluted approximately 7-fold. Thus the inhibitions were both reversible and instantaneous under the conditions employed.

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† Department of Pharmacology, Jefferson Medical College, Philadelphia, Pennsylvania.

1 Dimethylamethopterin, 4-dimethylamino-4-deoxy-N10-methylpteroylglutamic acid; dihydroxyfolic acid, 2-hydroxy-2-deaminopteroylglutamic acid.
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The effect of folic acid on the reaction rate of the liver enzyme in the presence of different concentrations of the substrates, NAD, NADH, ethanol, and acetaldehyde is depicted as Lineweaver-Burk plots (Fig. 3). The equations for the straight lines were calculated by the method of least squares. The intercepts and the slopes of the lines increased as the folic acid concentration was increased. When these lines were extended beyond the

**Fig. 1.** Upper, instantaneous inhibition of liver alcohol dehydrogenase by folic acid (X), amethopterin (○), aminopterin (△), and 1,10-phenanthroline (+) at room temperature. Lower, the same plot for yeast alcohol dehydrogenase. The assay system contained 0.015 M sodium pyrophosphate buffer, pH 8.8, 0.1 M NAD, 0.1 M ethanol, and 2 × 10⁻⁴ M yeast alcohol dehydrogenase.

**Fig. 2.** The effect of pH on the inhibition of liver alcohol dehydrogenase by folic acid (X) and 8-hydroxyquinoline (○). Phosphate, 0.1 M, was used as buffer.

The inhibition of liver alcohol dehydrogenase by the chelator 1,10-phenanthroline was reversed by zinc ions, or prevented by addition of zinc ions with 1,10-phenanthroline (9). In contrast, the inhibition of this enzyme by the folates was neither reversed nor prevented in analogous experiments.

The inhibition of the liver enzyme by folic acid was further compared with that produced by another chelator, 8-hydroxyquinoline, at different pH values (Fig. 2). Although the value of \( v_0 \), the rate of the control reaction, was pH-dependent as anticipated from the variation of enzyme activity with pH (10), relative enzymic activity decreased from pH 6 to 10 with 8-hydroxyquinoline but not with folic acid. Inhibition by aminopterin or amethopterin was also independent of pH.

**Fig. 3.** Lineweaver-Burk plots of the inhibition of liver alcohol dehydrogenase by folic acid. Reactions were carried out in 0.02 M phosphate, pH 7.15. Each plot is the average of at least six experiments.

**Fig. 4.** Plot of reciprocal velocity versus inhibitor concentration for the inhibition of liver alcohol dehydrogenase by folic acid (X), amethopterin (○), and aminopterin (△).
The relationship between reciprocal velocity and inhibitor concentration (Dixon plots) exhibited deviations from linearity (Fig. 4). Ebersole, Guttentag, and Wilson (11) suggested that when such curves are upward more than 1 inhibitor molecule may be involved in the reaction. On the assumption that the method of Johnson, Eyring, and Williams (12) applies to complex inhibitions, the number of inhibitor molecules reacting per active site was estimated (Fig. 5). Either for the reduction of acetaldehyde or for the oxidation of ethanol, it appeared that 5 to 6 molecules of folic acid per active site were involved in the inhibition.

Table I summarizes data on the effect of folic acid, aminopterin, 1,10-phenanthroline, iodoacetate, and p-mercuribenzoate on the isomerase activity of the liver enzyme. The untreated enzyme converted 2.5 μmoles of 3-phosphoglyceraldehyde to dihydroxyacetone phosphate. When the concentration of folic acid or aminopterin was increased to $5 \times 10^{-3}\text{ M}$, isomerase activity was stimulated 400 and 350%, respectively. 1,10-Phenanthroline had no effect on isomerase activity but inhibited dehydrogenase activity. At $0.1\text{ M}$ iodoacetate, dehydrogenase activity was not altered but isomerase activity was inhibited to 80% of the control value. $p$-Mercuribenzoate at $6 \times 10^{-4}\text{ M}$ did not affect dehydrogenase activity but lowered isomerase activity to 80% of the control value; when $p$-mercuribenzoate was increased to $6 \times 10^{-4}\text{ M}$, dehydrogenase activity was abolished but only small further decreases in the isomerase activity were noted.

**DISCUSSION**

The possibility that folic acid inhibited enzymes by a chelation mechanism (13) was suggested by the report that the vitamin was a potent zinc chelator (14), as well as by the finding that metal chelators such as 1,10-phenanthroline inhibited alcohol dehydrogenase by chelating enzymic zinc (15). This was rejected for the following reasons. (a) Inhibition of dehydrogenase activity by folic acid was neither reversed nor prevented by the addition of zinc as anticipated from analogous experiments with the enzyme and 1,10-phenanthroline (16). (b) Inhibition of dehydrogenase activity by folic acid was pH-independent. In order to chelate either folic acid or 8-hydroxyquinoline with free zinc ions, the hydroxyl group peri to the tertiary heterocyclic nitrogen atom must be ionized. Since the $pK_a$ of the hydroxyl group of 8-hydroxyquinoline is 9.9, more ionized groups were available for chelation at higher pH values; consequently the enzyme was progressively inhibited by 8-hydroxyquinoline as the pH was increased. If folic acid ($pK_a$ of hydroxyl is 8.3) inhibited by chelation as did 8-hydroxyquinoline, inhibition should have been greater at higher pH values; this was not the case. (c) 4-Aminopteridines (aminopterin, amethopterin) and dimethylamethopterin, which have little or no chelating potentiality (14), were as effective as folic acid in inhibiting alcohol dehydrogenase.

Kinetic studies showed (a) that the inhibition by folic acid was not of the simple competitive or noncompetitive type which is usually associated with direct interaction of the inhibitor with the active site, and (b) that as many as 6 molecules of folate may be involved per active site. It is difficult to visualize a mechanism in which multiple folates are bound at the same enzymic locus. The results rather indicate that the folates inhibited by reacting with the apoenzyme at several folate-sensitive loci that were not identical with the zinc-containing active site for alcohol oxidation.

Since the isomerase and dehydrogenase activities of alcohol dehydrogenase were altered independently by the folates, 1,10-

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**TABLE I**

Effects of several compounds on dehydrogenase and isomerase activities of liver alcohol dehydrogenase

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>Alcohol dehydrogenase activity (%/u)</th>
<th>Isomerase activity</th>
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<tbody>
<tr>
<td>None</td>
<td></td>
<td>1.00</td>
<td>2.5</td>
</tr>
<tr>
<td>Folic acid</td>
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<tr>
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<td>0</td>
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<td></td>
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<td>Iodoacetate</td>
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<tr>
<td></td>
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* $v_i$, rate of the inhibited reaction; $v_c$, rate of the control reaction.
† Expressed as micromoles of dihydroxyacetone phosphate formed in 15 minutes.
phenanthroline, iodoacetate, and p-mercuribenzoate, it appears that the two enzymic activities take place at different locations on the enzyme surface. The inhibition of one enzymic activity by folic acid and the stimulation of the other activity by the same compound is reminiscent of the action of diethylstilbestrol on glutamic dehydrogenase. It is not clear how the alterations of the two enzymic activities of glutamic dehydrogenase may be related to a control mechanism. It is equally difficult to attach biological significance to the alteration of the two activities of alcohol dehydrogenase, but it is obvious that this enzyme, like several others, contains loci other than the active site at which reactions occur that modify enzymic activity.

REFERENCE

SUMMARY
1. Liver and yeast alcohol dehydrogenases were inhibited by folic acid, aminopterin, and amethopterin. Inhibition of the dehydrogenase activity of the liver enzyme by folic acid and analogues was instantaneous, was reversed by dilution or dialysis, was independent of pH, and was not reversed or prevented by addition of zinc ions. Lineweaver-Burk and Dixon plots indicated a complex inhibition with multiple folic acid molecules participating in the reaction.
2. The isomerase activity of the liver enzyme was stimulated 400 and 350% by folic acid and aminopterin, respectively. 1,10-Phenanthroline, iodoacetate, and p-mercuribenzoate also altered its dehydrogenase and isomerase activities independently.
3. It is concluded that folic acid did not inhibit liver alcohol dehydrogenase by chelation of enzymic zinc and that the two activities took place on different sites of the enzyme.

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Effect of Folic Acid and Analogues on the Dehydrogenase and Isomerase Activities of Liver Alcohol Dehydrogenase
Robert Snyder, Wolfgang Vogel and Martin P. Schulman


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