Evidence for the Chemical Interaction between 2-Mercaptoethanol and Tetrahydrofolate*

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SUMMARY

Evidence is presented that tetrahydrofolate reacts chemically with 2-mercaptoethanol. The resulting product is more stable than tetrahydrofolate itself. The rate of decomposition of this derivative has been investigated. The possible sequences of interaction of tetrahydrofolate with 2-mercaptoethanol are discussed.

Ascorbate, 2-mercaptoethanol, and 2,3-dimercaptopropanol (1-3) are used as protective agents to stabilize solutions of tetrahydrofolate and its derivatives. The protective action of these compounds has been thought to be due exclusively to their reducing properties.

The spontaneous decomposition of H4-folate to the diazotizable amine, p-aminobenzoylglutamate, has been used to measure quantitatively the reduction of folate with folate reductase (4-6). This method has been criticized as not being quantitative (7). It has been found in this laboratory that when no mercaptoethanol is used during the enzymatic reduction of folate, the amount of diazotizable amine formed is quantitative.

On the other hand, when samples of H4-folate prepared by different procedures in the presence of mercaptoethanol were assayed for the content of diazotizable amine, in no case was there a quantitative release of ABG.1 The purpose of the investigation reported here was to clarify this discrepancy. Evidence for a chemical interaction between H4-folate and 2-mercaptoethanol is presented. This interaction has a somewhat stabilizing effect on H4-folate. It will also be shown that the rate of spontaneous decomposition of H4-folate to the diazotizable amine is much slower in the case of H4-folate-mercaptoethanol complex.

METHODS

Preparation of H4-folate—Two methods were used to prepare this compound: (a) reduction of folate with dithionite (8) and (b) enzymatic reduction of dihydrofolate (9). In each case the product was isolated from the reaction mixture by means of chromatography on DEAE-cellulose as described below. In one experiment, the original procedure of Silverman and Noronha (8) for reduction of folate by dithionite was modified by omitting the ascorbate and carrying out the reaction in 0.15 M phosphate buffer, pH 6.1.

The enzymatic H4-folate was prepared by incubating 80 mg of dihydrofolate with 180 mg of TPNH and 1.2 × 10^{-3} mole of folate reductase from cultured cells of Sarcoma 180 subline AT in 400 ml of 0.05 M solution of sodium citrate at 37°C (10). 2-Mercaptoethanol at the concentration of 0.1% was present in the incubation medium. The final pH was 7.5. The progress of the reaction was followed by measuring the change in optical density at 340 nm. After the reaction was complete, additional mercaptoethanol (4 ml) was added and the solution was applied to the chromatographic column.

Chromatographic Procedures—The column for chromatography was prepared in the following way: 8.0 g of DEAE-cellulose were suspended in 0.1 M NaOH and the slurry was poured into a chromatographic column (inside diameter, 22 mm). The cellulose was compressed by applying slight pressure, to a height of 13 to 14 cm and washed with water until the effluent was no longer alkaline. Subsequently, 500 ml of 1.0 M acetate buffer, pH 6, were passed through the column. After the unbound acetate was washed out with 500 ml of a 1% solution of 2-mercaptoethanol, the column was ready for use. A column of this size can handle up to 80 mg of H4-folate. The linear gradient elution was carried out with 500 ml of 1% aqueous solution of 2-mercaptoethanol in the mixing flask and 500 ml of 0.75 M acetic acid containing 1% 2-mercaptoethanol in the reservoir. The first 150 ml of the effluent were discarded. Subsequently, fractions of 8 ml were collected, 0.1 ml of each fraction was diluted with 1 ml of water, and the optical density at 280 nm was determined in a Beckman model DU spectrophotometer.

In the case of the enzymatic preparation there was only one peak, that of H4-folate, in the effluent. In the case of the chemical preparation, the peak of H4-folate was preceded by that of ascorbate. Since there was some overlapping of the peaks, the ultraviolet spectra of the first few fractions of H4-folate peak were recorded, and those fractions which produced distorted spectra of H4-folate were not used. The fractions containing p-aminobenzoylglutamate (11). The assumption has been made that 1 mole of aminopterin is bound per 1 mole of enzyme.

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1 The abbreviation used is: ABG, p-aminobenzoylglutamate.
Interaction between 2-Mercaptoethanol and Tetrahydrofolate

Folate, $2.9 \times 10^{-5} \text{ M}$, TPNH, $2.45 \times 10^{-4} \text{ M}$, partially purified folate reductase from cultured Sarcoma 180 AT, and 2-mercaptoethanol as indicated were incubated in $5 \times 10^{-2} \text{ M}$ citrate buffer in Beckman cuvettes at room temperature. The final volume was $1.02 \text{ ml}$. Folate was omitted from the control cuvette. The change of absorbance at 340 nm was recorded every 5 min. After $22.0 \times 10^{-3}$ from the control cuvette. The change of absorbance at 340 nm was recorded every 5 min. After

Effect of 2-mercaptoethanol on release of p-aminoxyenylglutamate

TABLE I

<table>
<thead>
<tr>
<th>pH</th>
<th>2-Mercaptoethanol</th>
<th>Folate reduced</th>
<th>ABG released</th>
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</thead>
<tbody>
<tr>
<td>5.5</td>
<td>$5.6 \times 10^{-2}$</td>
<td>$7.9 \times 10^{-6}$</td>
<td>$7.4 \times 10^{-6}$</td>
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<tr>
<td>5.5</td>
<td>$9.8 \times 10^{-2}$</td>
<td>$8.8 \times 10^{-6}$</td>
<td>$4.4 \times 10^{-6}$</td>
</tr>
<tr>
<td>5.5</td>
<td>$14.0 \times 10^{-2}$</td>
<td>$8.5 \times 10^{-6}$</td>
<td>$2.9 \times 10^{-6}$</td>
</tr>
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<td>5.0</td>
<td>$14.0 \times 10^{-2}$</td>
<td>$9.3 \times 10^{-6}$</td>
<td>$3.9 \times 10^{-6}$</td>
</tr>
<tr>
<td>6.0</td>
<td>$14.0 \times 10^{-2}$</td>
<td>$8.6 \times 10^{-6}$</td>
<td>$3.8 \times 10^{-6}$</td>
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</tbody>
</table>

Effect of 2-mercaptoethanol on Bratton-Marshall reaction (16)

TABLE II

<table>
<thead>
<tr>
<th>2-Mercaptoethanol</th>
<th>Absorbance at 560 nm</th>
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<tr>
<td>None</td>
<td>$0.468$</td>
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<tr>
<td>$5.6 \times 10^{-3}$</td>
<td>$0.478$</td>
</tr>
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<td>$11.2 \times 10^{-3}$</td>
<td>$0.157$</td>
</tr>
<tr>
<td>$16.8 \times 10^{-3}$</td>
<td>$0.090$</td>
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<tr>
<td>$22.4 \times 10^{-3}$</td>
<td>$0.083$</td>
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<tr>
<td>$28.0 \times 10^{-3}$</td>
<td>$0.072$</td>
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RESULTS

Preliminary Observations—When a solution of folic acid was incubated with folate reductase (Sarcoma 180 AT) and about an 80-fold excess of TPNH, pH 5.5, at room temperature for 45 min, quantitative conversion of folate to H$_4$-folate took place. The concentration of folate before the incubation ($1.28 \times 10^{-5} \text{ M}$) was determined by treatment of the solution with zinc and HCl followed by the determination of diazotizable amine (14). The

<table>
<thead>
<tr>
<th>H$_4$-folate</th>
<th>H$_4$-folate- HS(CH$_2$)OH</th>
<th>H$_4$-folate- HS(CH$_2$)OH-2H$_2$O</th>
<th>Chemical$^a$</th>
<th>Enzymatic$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
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<tr>
<td>Carbon</td>
<td>51.2</td>
<td>48.2</td>
<td>46.7</td>
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<td>Hydrogen</td>
<td>9.17</td>
<td>8.34</td>
<td>6.72</td>
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<td>Nitrogen</td>
<td>22.0</td>
<td>18.7</td>
<td>18.1</td>
<td>17.5</td>
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<tr>
<td>Sulfur</td>
<td>6.12</td>
<td>5.8</td>
<td>5.7</td>
<td>4.90 ± 1.00</td>
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</tbody>
</table>

$^a$ Mean values of eight analyses performed on five different samples.

$^b$ Mean values of three analyses performed on two different samples.
concentration of NH₄-folate at the end of the incubation (1.26 × 10⁻⁵ M) was determined by the diazotizable amine reaction as described elsewhere (4). It appears that under these conditions the release of ABG from NH₄-folate was quantitative.

Folate was incubated with folate reductase and TPNH in the absence or presence of varying amounts of mercaptoethanol (Table I). In each case, TPNH was the limiting factor and the reaction was carried out to the complete exhaustion of this compound. The amount of folate reduced was calculated from the change of absorbance at 340 nm by using a molar extinction coefficient of 18.3 × 10⁻³ (2 × 6.2 × 10⁻⁴ for the oxidation of 2 moles of TPNH to 2 moles of TPN + 5.9 × 10⁻⁶ for the reduction of folate to tetrahydrofolate). After the reaction was completed, the concentration of free ABG was determined by the Bratton-Marshall reaction (15). This last reaction was carried out as described elsewhere (4) except that the concentration of NaNO₃ reagent was increased to 2.5% and that of ammonium sulfamate reagent to 12.5%. Mercaptoethanol reacts with nitrate and thus interferes with the diazotization merely by using up NO₃⁻ ions. Table II shows that this interference could be overcome by using high concentrations of NaNO₃ and ammonium sulfamate (2.5 and 12.5%, respectively). Mercaptoethanol had no effect on the rate of reduction of folate, and in each case the reaction proceeded to complete exhaustion of TPNH. The amount of diazotizable amine released from NH₄-folate was, however, inversely proportional to the concentration of mercaptoethanol. This indicates that some chemical interaction between NH₄-folate and mercaptoethanol took place.

Analysis of NH₄-folate Preparations—In Table III are presented the results of the elemental analysis of the enzymatically and chemically prepared NH₄-folate. In each case approximately 1 atom of sulfur is present per mole of NH₄-folate. Although the nitrogen and sulfur values are somewhat low, the analytical data agree best with the formulation: NH₄-folate .HS(CH₃)₂OH.H₂O, which indicates a molecular weight of 541. However, the nitroprusside test and the amperometric silver titration for free —SH groups both gave negative results.

Several freshly prepared samples of NH₄-folate were analyzed for the content of NH₄-folate (see "Methods"). The results of these analyses are summarized in Table IV. Using the molecular weight of 541 to calculate the number of moles of the NH₄-folate, all freshly prepared (1 day old), twice purified, synthetic
samples appeared to be nearly 100% pure. The purity of the one enzymatic sample was about 90%. The ultraviolet spectra of both the chemical and the enzymatic preparations at pH 7.3 in the presence of 0.1% mercaptoethanol had maxima at 297 m\(\mu\) as reported by others (16) for \(\text{H}_4\)-folate. Assuming the molecular weight of 541, the molar extinction coefficient at this wave length was estimated at about \(27 \times 10^3\).

**Stability of \(\text{H}_4\)-Folate Preparations**—The dry samples of \(\text{H}_4\)-folate, if kept in air, deteriorated by 20 to 25% in the first 2 or 3 days after preparation, but subsequently little or no decomposition occurred (Table IV). This may have been due to the decomposition of the \(\text{H}_4\)-folate on the surface of the particles only.

Fig. 1 shows the elution pattern from the analytical column (see “Methods”) of a 3-day-old preparation of \(\text{H}_4\)-folate, the purity of which was determined to be 76.5%. It can be seen that the decomposition products were ABG, dihydrofolate, and folate, in addition to some unidentified pteridines. Except for ABG, no quantitative determination of each of the components has been performed. The amount of ABG was about 10% of that of \(\text{H}_4\)-folate.

The rates of decomposition of both preparations in the absence of 2-mercaptoethanol were compared in (a) 1.0 m HCl and (b) phosphate buffer of pH 7.7. In the former case the formation of free diazotizable amine, in the latter the change in the ultraviolet spectrum, was followed. In each preparation the decomposition in HCl followed first order kinetics (Fig. 2), with a half life of about 45 min. The amount of the diazotizable amine released after 3 to 3\(\frac{1}{2}\) hours amounted to nearly 100% of that theoretically available.

The decomposition at pH 7.7 led, in each preparation, to the formation of dihydrofolate as evidenced by the change in the ultraviolet spectrum. The time required for the shift of the peak of \(\text{H}_4\)-folate (295 m\(\mu\)) to that of dihydrofolate (280 m\(\mu\)) was about 20 to 30 min. The decomposition at pH 7.7 followed neither first nor second order kinetics.

**Discussion**

The data presented above leave no doubt that 2-mercaptoethanol reacts with \(\text{H}_4\)-folate. The likelihood of simple contamination of the preparations of \(\text{H}_4\)-folate with mercaptoethanol is excluded by the following observations. There is always 1 atom of sulfur per mole of \(\text{H}_4\)-folate, there are no free --SH groups, and there is no reason to expect that such preparations containing only 1 mole of mercaptoethanol per mole of \(\text{H}_4\)-folate in the form of a contamination should be any more stable than pure \(\text{H}_4\)-folate.

Osborn and Huennekens (2) reported that \(\text{H}_4\)-folate in solution at pH 7.5, unprotected by mercaptoethanol, was oxidized to dihydrofolate in 9 min. This compound was prepared by catalytic hydrogenation of folate and no mercaptoethanol was used during the isolation procedure. Under similar conditions, it takes 20 to 30 min to oxidize preparations of \(\text{H}_4\)-folate which have been exposed to mercaptoethanol. Also, the decomposition in HCl of the enzymatically prepared \(\text{H}_4\)-folate which has never been exposed to mercaptoethanol is almost instantaneous, as indicated by the formation of a diazotizable amine (4, 5). This is not the case with the preparations described above.

The relative stability of the complex indicates that the most likely point of attachment of mercaptoethanol to \(\text{H}_4\)-folate is nitrogen atom 5. It is known that substitution of hydrogen on nitrogen atom 5 of \(\text{H}_4\)-folate by formate (1, 17), formaldehyde (3, 18), or methyl (19) yields compounds which are considerably more stable than unsubstituted \(\text{H}_4\)-folate. Also, no interaction between 7,8-dihydrofolate and mercaptoethanol could be detected.

Preliminary experiments performed with the oxidized and reduced forms of cysteine in place of mercaptoethanol revealed that only the reduced form interacts with \(\text{H}_4\)-folate. This implies that the free --SH group is involved in the binding.

In Fig. 3 is presented the suggested sequence of events which takes place when \(\text{H}_4\)-folate is dissolved in mercaptoethanol solution. If the first step is indeed oxidation similar to that of two --SH groups forming --S-- bond, then this reaction is most likely irreversible. Also the fact that no free --SH groups could be detected in the solutions of \(\text{H}_4\)-folate-mercaptoethanol complex indicates irreversibility of this reaction. As can be concluded from the experiments presented in the preceding sections, the stability of the mercaptoethanol-\(\text{H}_4\)-folate complex is much lower than that of other \(N^5\)-substituted derivatives of \(\text{H}_4\)-folate. In addition, Kaufman (20) observed that \(\text{H}_4\)-folate can stimulate aerobic oxidation of mercaptoethanol. Thus, it can be postulated that the mercaptoethanol-\(\text{H}_4\)-folate complex decomposes to an unstable form of dihydrofolate and mercaptoethanol. Formation of such unstable dihydropteridine during the oxidation of 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine was demonstrated by Kaufman (20). Mercaptoethanol in turn reduces this form of dihydrofolate to \(\text{H}_4\)-folate, itself being oxidized to a disulfide. Since no free mercaptoethanol occurs in the solutions of \(\text{H}_4\)-folate-mercaptoethanol complex, it must be assumed that the first step in the decomposition of \(\text{H}_4\)-folate-mercaptoethanol complex, i.e. the formation of the unstable form of dihydrofolate and mercaptoethanol, is the rate-limiting reaction. The possible biological significance of the ability of \(\text{H}_4\)-folate to form complexes with thiols deserves investigation.

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**References**

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