ACTIVATION OF DIHYDROFOLIC REDUCTASE BY UREA AND FORMAMIDE

Bernard T. Kaufman

National Institute of Arthritis and Metabolic Diseases
National Institutes of Health
Bethesda 14, Maryland

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During the purification of dihydrofolic reductase from chicken liver utilizing ammonium sulfate precipitation of the enzyme, yields of activity from 150-300% have been consistently recovered prior to the removal of the salt by dialysis. Investigation of this phenomenon revealed that ammonium sulfate as well as a number of apparently unrelated salts caused a 2-3 fold stimulation of enzyme activity when dihydrofolate or folate was used as substrate. A similar observation has been recently reported by Bertino (1962). In view of the apparent lack of specificity and relatively high concentration of salt required for maximum stimulation (0.1-0.2 M) it seemed that this activation of the enzyme may be due to an effect of ionic strength rather than a specific ion requirement for enzyme activity.

Ionic strength effects on enzyme reactions have been discussed in terms of alterations in the activity coefficients of the substrates (Burton & Wilson, 1953) and electrostatic effects on the conformation of the protein molecule (Tanford, 1961). In order to evaluate the possibility that a conformational change in the enzyme protein is responsible for this increased rate of reduction of dihydrofolic acid, the activity of the enzyme was measured in the presence of compounds known to alter the secondary or tertiary structure of proteins in aqueous solution.

Dihydrofolic reductase was purified approximately 100-fold from a high speed supernatent extract of chicken liver by protamine treatment, ammonium sulfate fractionation, calcium phosphate gel adsorption and elution, ethanol fractionation and chromatography on DEAE-cellulose columns. The initial rate

of the reaction as a function of urea concentration is shown in Figure 1. Increasing concentrations of urea stimulate the reaction with maximum stimulation occurring at approximately 4 M. Concentrations above 4 M result in progressive inhibition. Stimulation of activity from 4-6 fold has been observed. Formamide similarly activates dihydrofolic reductase; however, the maximum effect is approximately 40% less than that observed with urea and occurs at about 2.5 M. In addition, at higher concentrations of formamide, the inhibition is much less pronounced, i.e., less than 15% inhibition of the stimulated rate at 8 M formamide. On the other hand, guanidine hydrochloride exhibits its maximum effect on the rate of reduction of dihydrofolate in the same concentration range observed with inorganic salts, i.e., maximum stimulation at approximately 0.2 M. Increasing the concentration above 0.2 M results in marked inhibition of the activity.

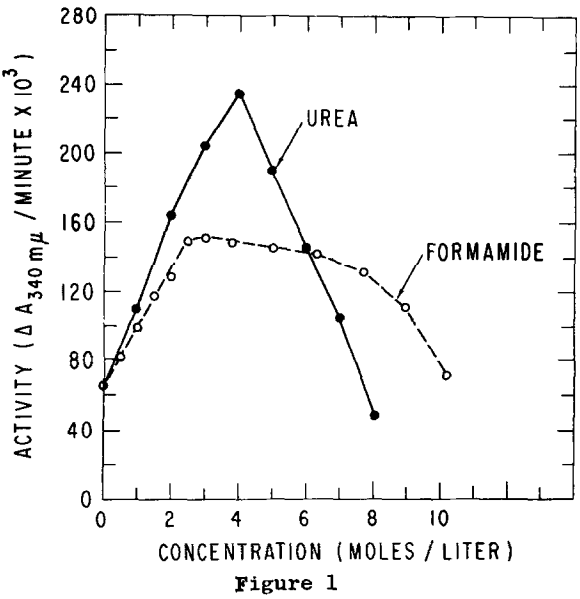

Figure 1

Effect of Urea or Formamide on the Activity of Dihydrofolic Reductase

The reaction mixture, in a volume of 1 ml, contained: potassium phosphate buffer, pH 6, 50 μmoles, 2-mercaptoethanol, 20 μmoles; TPNH, 0.2 μmoles; dihydrofolate, 0.05 μmoles (Futterman, 1957); and 0.02 ml of chicken liver enzyme. Suitable aliquots of formamide (CP) or freshly prepared 10 M urea were added to yield the indicated final concentrations. The reaction was initiated by the addition of enzyme and the rate determined from the decrease in absorbency at 340 mμ measured at 30 second intervals for 2-3 minutes at 26°. The control consisted of a cuvette containing the same reaction mixture minus the substrate, dihydrofolate.

Figure 2 illustrates the rate of the reduction of dihydrofolate in the presence and absence of 4 M urea, as a function of pH. The pH optimum is shifted to a lower value in urea, and below pH 5.5 urea appears to inhibit the reaction. Furthermore, the rate of the reaction in the presence of urea exhibits a marked increase in activity below pH 5.5. This suggests that hydrogen ions as well as salt and urea or formamide may affect the reactivity of this enzyme in a similar manner. It is conceivable that at pH values below 4.5 higher degrees of reactivity may occur; however, due to increased lability of the reduced pyridine nucleotide (TPNH), rate measurements could not be obtained below pH 4.5.

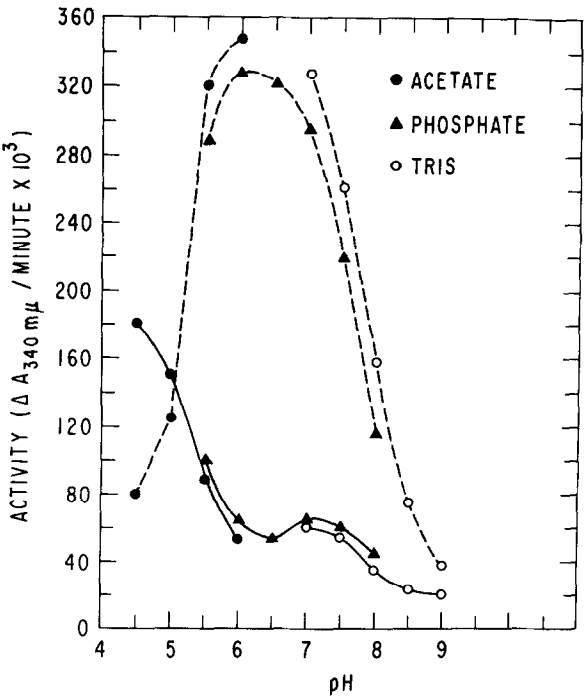

Figure 2

Activity of Dihydrofolic Reductase in the Presence and Absence of 4M Urea as a Function of pH

The reaction mixture was that as described in Figure 1 except 100 μmoles of the appropriate buffer was utilized at the indicated pH in both the experimental and control cuvettes. Dashed lines represent the rate of the reaction in the presence of urea; solid lines—reaction in absence of urea.

Table I shows that the enzyme is relatively unstable in the presence of 4 M urea at 0°. Approximately 90% of the original activity is lost in 60 minutes. However, this inactivation by 4 M urea is prevented by the presence of the substrate, dihydrofolate. No loss in activity was observed for incubation periods up to four hours.

Table I

Stability of Dihydrofolic Reductase in the Presence of Urea

Concentration of Urea molar	Additions	% Original Activity after* 30 min.	60 min.
1	-	100	100
2	-	100	100
3	-	72	60
4	-	40	10
4	dihydrofolate (1 μmole)	100	100

*The incubation mixture, in a volume of 2 ml, consisted of 1 ml of the chicken liver enzyme, suitable aliquots of freshly prepared 10 M urea to yield the indicated final concentrations, and water to the final volume. The mixture was incubated in an ice bath and at the times indicated, 0.05 ml was withdrawn and assayed for enzyme activity as described in Figure 1.

Similar experiments to those described above with dihydrofolate were repeated, using folate as substrate. As has been previously reported (Bertino, 1962), inorganic salts produce approximately the same degree of stimulation with folate as substrate. In the present study it was also observed that 0.2 M ammonium sulfate or a similar concentration of guanidine hydrochloride accelerated the rate of reduction of folate at pH 5. However, the presence of urea or formamide either had no effect on the rate of the reaction at low concentrations (1 M) or resulted in marked inhibition at high concentrations (2-4 M). Similar effects were noted regardless of the buffer or pH.

Thus it would appear that under a variety of conditions, the activity of dihydrofolic reductase is stimulated in a qualitatively similar manner by salts (including guanidine hydrochloride), urea or formamide, and possibly hydrogen ions. Since there is general agreement that urea and formamide alter the secondary and tertiary structure of proteins it is reasonable to suggest that the mode of activation of this enzyme by the above conditions is similarly a result of a change in the configuration or dissociation of the protein molecule. The observation that 4 M urea markedly stimulates the reduction of dihydrofolate, whereas similar concentrations inhibit the reduction of folate, suggests that the mechanism of reduction of these two substrates is different. Current studies are being directed toward obtaining a preparation of enzyme sufficiently purified for optical studies of its sedimentation characteristics.

References

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