Biochemistry

Volume 9, Number 25 December 8, 1970

Inhibition of Peptide Chain Initiation in *Escherichia coli* by Hydroxylamine. Reaction of Hydroxylamine with Folate Coenzymes*

P. F. Nixon† and J. R. Bertino‡

ABSTRACT: Previous studies of the means by which hydroxylamine prevents the initiation of peptide synthesis by *Escherichia coli* extracts have suggested that hydroxylamine interferes with the metabolism of folate coenzymes so as to prevent the formylation of methionyl-tRNA by 10-formyltetrahydrofolate. The possible reactions of hydroxylamine with folate coenzymes and with folate-dependent enzymes have now been examined. Hydroxylamine was found to react with 5,10-methylenetetrahydrofolate but not with other folate coenzymes. One product of the reaction of hydroxylamine with 5,10-methylenetetrahydrofolate was tetrahydrofolate and therefore the other was presumably formaldoxime. Hydroxylamine did not form inhibitory complexes with folate-dependent enzymes. It is concluded that the reaction of hydroxylamine with 5,10-methylenetetrahydrofolate is able to deplete the total intracellular pool of one-carbon folate adducts, including 10-formyltetrahydrofolate, and so to prevent the initiation of peptide synthesis by extracts of such cells.

Hydroxylamine has several biological effects, one of which is the inhibition of protein biosynthesis (Beguin and Kepes, 1964). Klein *et al.* (1970) have shown that relatively low concentrations (less than millimolar) of hydroxylamine inhibit protein synthesis of *Escherichia coli* cells by blocking the initiation of peptide chains without affecting chain elongation. This effect of hydroxylamine was shown to be mediated by depletion of the intracellular pool of 10-CHO-FH_4,[1] in turn preventing the enzymic synthesis of formylmethionyl-tRNA (Dickerman *et al.*, 1967) which is necessary for peptide chain initiation of *E. coli* (Lengyel, 1967).

This communication describes data which indicate that this effect of hydroxylamine results from the reaction of hydroxylamine with 5,10-CH_2-FH_4 to remove its HCHO adduct, as has been described by Osborn *et al.* (1960). Since the various folate coenzymes are in equilibrium by enzyme-mediated reactions (Figure 1), removal of the one-carbon adduct of 5,10-CH_2-FH_4 will result in depletion of the intracellular pool of 10-CHO-FH_4. No evidence could be obtained for the direct reaction of hydroxylamine with any other of the principal folate coenzymes, including 10-CHO-FH_4; nor for the formation of significant inhibitory hydroxylamine complexes with those enzymes responsible for the biosynthesis or interconversion of folate coenzymes; nor for inhibition of 10-formyltetrahydrofolate–methionyl-tRNA transformylase.

Materials and Methods

Folic acid, tetrahydrofolic acid, $NADP^+$, NADPH, glucose 6-phosphate, adenosine triphosphate, 5′-deoxyuridine monophosphate, and glucose 6-phosphate dehydrogenase were obtained from the Sigma Chemical Co., St. Louis; Ca-5-CHO-FH_4 (leucovorin) from Lederle. A stock 0.5 M solution of hydroxylamine-HCl was prepared each day at the experimental pH value. Dihydrofolic acid was prepared by the method of Futterman (1957) as modified by Blakley (1960a,b). The method of Gupta and Huennekens (1967) was used for

* From the Departments of Pharmacology and Medicine, Yale University, New Haven, Connecticut. *Received June 5, 1970.* Supported by Research Grant CA-08010.

† Merck, Sharp and Dohme International Fellow in Clinical Pharmacology.

‡ Research Career Development awardee of the National Cancer Institute (Grant 5-K3-CA-8853).

[1] Abbreviations used are as follows: 10-CHO-FH_4, 10-formyltetrahydrofolate; FH_4, tetrahydrofolate; 5-CHO-FH_4, 5-formyltetrahydrofolate; 5-CH_3-FH_4, 5-methyltetrahydrofolate; 5,10-CH_2-FH_4, 5,10-methylenetetrahydrofolate; 5,10-CH-FH_4, 5,10-methenyltetrahydrofolate; dUMP, 5′-deoxyuridine monophosphate; dTMP, thymidine monophosphate.

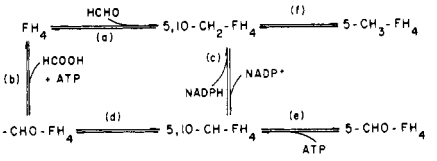

FIGURE 1: The interconversions of some one-carbon adducts of tetrahydrofolate, catalyzed by the following enzymes: (b) 10-formyltetrahydrofolate synthetase (formate:tetrahydrofolate ligase, EC 6.3.4.3); (c) 5,10-methylenetetrahydrofolate dehydrogenase (5,10-methylenetetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.5); (d) 5,10-methenyltetrahydrofolate cyclohedrolase (5,10-methenyltetrahydrofolate 5-hydrolase (decyclizing), EC 3.5.4.9); (e) 5-formyltetrahydrofolate cyclodehydrase; and (f) 5,10-methylenetetrahydrofolate reductase.

the preparation of 5-CH_3-FH_4 which was chromatographed by elution from DEAE-Sephadex (Nixon and Bertino, 1970).

The method of Uyeda and Rabinowitz (1967) was used for the preparation of 5,10-CH_2-FH_4 which was used in spectral studies and for the assay of 5,10-methylenetetrahydrofolate dehydrogenase activity. For the assay of thymidylate synthetase activity, 5,10-CH_2-FH_4 was prepared in the presence of excess HCHO. The enzymically active diastereoisomer of 5,10-CH_2-FH_4 was prepared by a method similar to that of Blakley (1963). Typically, 5 μmoles of dihydrofolate was incubated at 37° for 20 min with 0.5 μmole of NADPH, 20 μmoles of glucose 6-phosphate, 10 units of glucose 6-phosphate dehydrogenase, 10 μmoles of HCHO, 200 μmoles of mercaptoethanol, and 2 units of purified L1210 dihydrofolate reductase (Perkins *et al.*, 1967) in 2.0 ml of 100 mM Tris buffer (pH 7.5). The incubation mixture was placed on a column of DEAE-cellulose (0.8 × 10 cm); some contaminating materials were eluted by mM ammonium bicarbonate (pH 9.5) and the *d,*L-5,10-CH_2-FH_4 was eluted by 100 mM ammonium bicarbonate (pH 9.5). The active fractions were stored frozen in the presence of 200 mM mercaptoethanol.

Preparations of 5,10-CH-FH_4 and of 10-CHO-FH_4 in 50 mM mercaptoethanol at pH 1.0 and 7.5, respectively, were made from 5-CHO-FH_4 as described by May *et al.* (1951).

Spectral Changes with Hydroxylamine. Spectra of solutions of reduced folate coenzymes, at a concentration in the range 20–50 μM, were recorded by use of a Cary 15 recording spectrophotometer and a reference consisting of buffer and mercaptoethanol, each at the same concentration as in the sample solution. Spectra were recorded at 25° over the range 240–400 mμ, and they were rerecorded at timed intervals after the addition of hydroxylamine. Immediately following completion of the recordings the pH of the solutions were measured by means of a glass electrode.

Enzyme Studies. The enzymes 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methyltetrahydrofolate cyclohydrolase, 5-formyltetrahydrofolate cyclodehydrase, and 10-formyltetrahydrofolate synthetase were all obtained from *Clostridium cylindrosporum.* Lyophilized cells, obtained from Worthington Biochemicals, were extracted into maleate buffer containing mercaptoethanol according to the method of Rabinowitz and Pricer (1962). The suspension was centrifuged for 15 min at 144,000*g* and the supernatant was used as the source of the above enzymes.

The enzymes 10-formyltetrahydrofolate–methionyl-tRNA transformylase and thymidylate synthetase were purified, respectively, from *Escherichia coli* B (Dickerman *et al.*, 1967) and from T2 phage-infected *E. coli* B (G. F. Maley and F. Maley, unpublished data).

All enzyme assays were carried out at 25° by means of the Gilford recording spectrophotometer. Dihydrofolate reductase was prepared and assayed as previously described (Perkins *et al.*, 1967). Formyltetrahydrofolate cyclodehydrase (folinic isomerase) was assayed by the method of Kay *et al.* (1960).

The assay of 10-formyltetrahydrofolate synthetase depended on the spectral difference between FH_4 and 10-CHO-FH_4 at 305 mμ. Standard reaction mixtures contained 0.5 mM *d,l,*L-FH_4, 50 mM ammonium formate, 2 mM adenosine triphosphate, 10 mM $MgCl_2$, 100 mM mercaptoethanol, 100 mM Tris (pH 7.5), and enzyme in a total volume of 1.0 ml. The reaction was initiated by final addition of enzyme and the rate of decrease of the absorbance, at 305 mμ, was measured. The molar extinction coefficient for the reaction was not determined, but the initial velocity of the absorbance change was proportional to enzyme concentration and was linear for the first 2 min under the conditions adopted.

Thymidylate synthetase was assayed by a modification of the method of Wahba and Friedkin (1961). Standard reaction mixtures contained a total of 9 mM HCHO, 0.1 mM *d,l,*L-FH_4, 50 mM mercaptoethanol, 5 mM sodium ascorbate, 2.5 mM magnesium chloride, 1 mM 5′-deoxyuridine monophosphate, 100 mM sodium phosphate (pH 6.7), and enzyme in a final volume of 1.0 ml. The reaction mixture, completed except for deoxyuridine monophosphate, was incubated and the "blank" absorbance increase at 338 mμ, presumably due to endogenous deoxyuridine, was recorded for 10 min, in which period its rate became negligible. At that time any inhibitor was added and the reaction was initiated by addition of deoxyuridine.

Methylenetetrahydrofolate dehydrogenase was assayed by the method of Uyeda and Rabinowitz (1967). For measurement of the enzymic reaction in the reverse direction, the substrates 5,10-CH_2-FH_4 and $NADP^+$ were replaced by 0.03 mM 5,10-CH-FH_4 and by 0.08 mM NADPH, respectively. However, the enzyme preparation also contained 5,10-methenyltetrahydrofolate cyclohydrolase. Therefore, the measured initial velocities in the reverse direction represented the sum of the activities of both these enzymes. The two activities were differentiated by omission of the NADPH; under this condition cyclohydrolase activity was measured alone.

Results

Spectral Changes with Hydroxylamine. Solutions of FH_4 and of 10-CHO-FH_4, each with 50 mM mercaptoethanol, and of dihydrofolate, 5-CHO-FH_4 and 5-CH_3-FH_4, each with 10 mM mercaptoethanol, were each incubated at pH 7.5 in 100 mM Tris buffer with 10 mM hydroxylamine and the spectra recorded. In no case was there a change of spectrum within a period of 40 min. Similarly, there was no change in the spectrum of a solution of 5,10-CH-FH_4 incubated with 10 mM hydroxylamine at pH 1.0 with 50 mM mercaptoethanol.

When solutions of 5,10-CH_2-FH_4 were incubated with hydroxylamine, there was a rapid change in the absorbance spectrum which was dependent on time, pH, and the relative concentrations of hydroxylamine and HCHO. The absorbance spectrum shifted from that of 5,10-CH_2-FH_4, characterized by an absorbance maximum at 294 mμ to that of FH_4,

TABLE I: Inhibition of Methylenetetrahydrofolate Dehydrogenase by Hydroxylamine.[a]

Concn of 5,10-CH_2-FH_4 (mM)	Concn of $NADP^+$ (mM)	Concn of Hydroxylamine (mM)	Initial Velocity (ΔA/min)
0.5	0.4	0	0.140
0.5	0.4	3	0.110
0.5	0.4	7	0.068
0.5	0.4	10	0.017
0.5	0.4	15	0.006
1.5	0.4	0	0.140
1.5	0.4	10	0.070
0.5	1.6	10	0.022
0.5	2.8	0	0.140
0.5	2.8	10	0.028

[a] Reaction mixtures of 1 ml contained 30 mM potassium maleate (pH 7.0), 56 mM mercaptoethanol, the indicated concentrations of $NADP^+$, 5,10-CH_2-FH_4, and hydroxylamine, and 5 μl of a preparation of methylenetetrahydrofolate dehydrogenase. The initial rate of increase in absorbance at 356 mμ was recorded (ΔA/min).

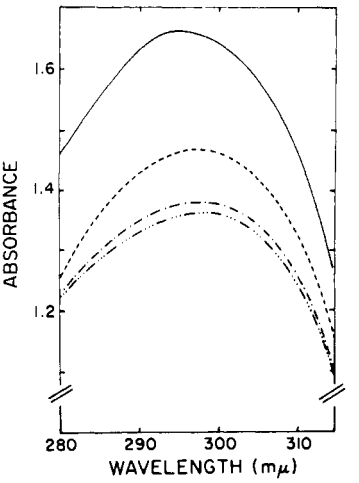

FIGURE 2: The change in spectrum of a solution of 5,10-CH_2FH_4 following addtion of hydroxylamine. Hydroxylamine, 10 mM at pH 7.2, was added to the reaction mixture which contained 50 μm 5,10-CH_2-FH_4, 50 mM mercaptoethanol, 100 mM Tris buffer (pH 7.2), and 2.5 mM formaldehyde. The spectra were recorded prior to the addition of hydroxylamine (——) and at 2 min (------), 5 min (-·-·-·-), 10 min and 15 min (-·-·-·-·-·-·-) after the addition of hydroxylamine.

characterized by an absorbance maximum at 298 mμ and a lower extinction coefficient (Blakley, 1960a,b). The spectral change is illustrated in Figure 2. At pH 7.2, in the presence of 2.5 mM HCHO, the spectral shift was complete in 5 min if the hydroxylamine concentration was 20 mM. At 10 and 5 mM concentrations of hydroxylamine, the spectral shifts were complete at 10 and 15 min, respectively. At 1 mM concentration of hydroxylamine there was no spectral change observed within 30 min.

Following the addition of 10 mM hydroxylamine to a solution containing 10 mM HCHO and 30 μM 5,10-CH_2-FH_4 at pH 7.2, the spectrum was allowed to shift from that having an absorbance maximum at 294 mμ to that having an absorbance maximum at 298 mμ. To this solution was then added further HCHO, to a total concentration of 20 mM, which resulted in reversal of the spectral change to that having an absorbance maximum at 294 mμ. Still further addition of hydroxylamine, to a total concentration of 20 mM again altered the spectrum to that having an absorbance maximum at 298 mμ. When 10 mM hydroxylamine was added to a solution containing 2.5 mM HCHO and 30 μM 5,10-CH_2-FH_4 at pH 8.1 in Tris, the absorbance maximum of the solution shifted from 294 to 296 mμ in 30 min, indicating that the reaction was considerably slower at the higher pH value. In the absence of hydroxylamine there was no change in the absorbance spectrum of 5,10-CH_2-FH_4 under any of the test conditions.

Inhibition of Enzymic Reactions. METHYLENETETRAHYDROFOLATE DEHYDROGENASE. Since spectral studies demonstrated the expected reaction of hydroxylamine with 5,10-CH_2-FH_4, but not with any other folate coenzyme tested, hydroxylamine was tested as an inhibitor of two enzymes dependent on 5,10-CH_2-FH_4, namely methylenetetrahydrofolate dehydrogenase and thymidylate synthetase.

Hydroxylamine apparently inhibited methylenetetrahydrofolate dehydrogenase, the enzyme which catalyzes the reaction shown in eq 1.

$$5,10\text{-}CH_2\text{-}FH_4 + NADP^+ \rightleftharpoons 5,10\text{-}CH\text{-}FH_4 + NADPH \quad (1)$$

The inhibition occurred only if hydroxylamine were present in the assay mixture at concentrations equal to or greater than those of the substrate 5,10-CH_2-FH_4. The extent of inhibition was dependent on the concentrations of both the inhibitor and 5,10-CH_2-FH_4, but not in a manner consistent with the formation of any enzyme–hydroxylamine complex. Representative results are illustrated in Table I. For example, the reaction rate was inhibited to 12% of the control rate by 10 mM hydroxylamine if the concentration of 5,10-CH_2-FH_4 was 1.5 mM, although both these concentrations are well above the K_m value (0.052 mM) for this substrate (Uyeda and Rabinowitz, 1967). To a smuch smaller extent, the apparent inhibition by hydroxylamine was decreased by increasing the concentration of $NADP^+$. In the presence of 2.8 mM $NADP^+$ the inhibition by this concentration of hydroxylamine was to 20% of the control rate.

The initial velocity of 5,10-methenyltetrahydrofolate cyclohydrolase was not affected by hydroxylamine at concentrations up to 10 mM. When the combined activities of 5,10-methylenetetrahydrofolate dehydrogenase, assayed in the reverse direction, and of the cyclohydrolase were measured together, there was no inhibition of the summed velocities by 10 mM hydroxylamine. Therefore, 10 mM hydroxylamine does not inhibit 5,10-methylenetetrahydrofolate dehydrogenase when its activity is measured in the reverse direction (*i.e.*, by use of 5,10-CH-FH_4 as substrate) although it does apparently inhibit the enzyme reaction velocity measured in the forward direction according to eq 1. This observation, together with that of the reversal of hydroxylamine inhibition by small increments of 5,10-CH_2-FH_4 over concentrations which are already saturating, excludes the possibility that hydroxylamine is forming an inhibitory enzyme–hydroxylamine complex. Although

TABLE II: Inhibition of Thymidylate Synthetase by Hydroxylamine.[a]

Concn of dUMP (mM)	Concn of 5,10-CH_2-FH_4 (mM)	Concn of Free HCHO (mM)	Concn of Hydroxylamine (mM)	Initial Velocity (ΔA/min)
0	0.1	9	0	0.0005
1.0	0.1	9	0	0.0400
1.0	0.1	9	10	0.0305
1.0	0.1	42	10	0.0382
1.0	0.1	9	25	0.0005
1.0	0.1	15	25	0.0006
1.0	0.1	42	25	0.0441
1.0	0.1	42	150	0.0023
10.0	0.1	9	25	0.0001
1.0	2.5	7.5	25	0.002

[a] The activity of 50 μl of a preparation of thymidylate synthetase was assayed as described under Methods. The rate of increase of absorbance at 338 mμ was recorded.

the results of Table I can be plotted in the manner of a pseudo-irreversible (tight-binding) inhibitor (Werkheiser, 1960), the concentrations of hydroxylamine used were far too high for it to be regarded as a tight-binding enzyme inhibitor. Since sufficient HCHO reversed the effect of hydroxylamine, the latter cannot be considered as an inactivator of the methylenetetrahydrofolate dehydrogenase. The results are consistent with depletion of the substrate 5,10-CH_2-FH_4, by its chemical reaction with hydroxylamine, to nonsaturating levels at which the reaction rate becomes dependent on the substrate concentration. Presumably hydroxylamine did not affect the initial velocity of the reverse reaction since 5,10-CH_2-FH_4 was then a reaction product.

THYMIDYLATE SYNTHETASE. The reaction of hydroxylamine with 5-10-CH_2-FH_4 was also studied by means of thymidylate synthetase, for which 5,10-CH_2-FH_4 is a substrate (eq 2).

$$5,10\text{-}CH_2\text{-}FH_4 + dUMP \rightleftharpoons FH_2 + dTMP \quad (2)$$

For these studies, 5,10-CH_2-FH_4 was prepared in the presence of excess HCHO, and the total concentrations of 5,10-CH_2-FH_4 and free HCHO were varied independently. The results illustrated in Table II showed that hydroxylamine inhibited the enzyme reaction velocity provided that it was present in excess of free HCHO. The inhibition could be reversed by a sufficient increase of the concentration of HCHO, but not by a moderate increase of the concentrations of either 5,10-CH_2-FH_4 or of deoxyuridine. Since the enzymically active substrate is 5,10-CH_2-FH_4, but not HCHO, it is most likely that the observed inhibition was the result of removal of the HCHO group from 5,10-CH_2-FH_4, and that the extent of this chemical reaction wad dependent on the relative concentrations of hydroxylamine, free HCHO, and 5,10-CH_2-FH_4.

IDENTIFICATION OF FH_4 AS A PRODUCT OF THE REACTION. The products of the reaction of hydroxylamine with 5,10-CH_2-FH_4 might be expected to be formaldoxime and free FH_4, according to eq 3. That one product of this reaction is indeed

$$5,10\text{-}CH_2\text{-}FH_4 + H_2NOH \rightleftharpoons CH_2{:}NOH + FH_4 \quad (3)$$

FH_4 was shown by substituting 5,10-CH_2-FH_4 and hydroxylamine, together, for FH_4 as a required substrate of the 10-formyltetrahydrofolate synthetase reaction (eq 4).

$$HCOOH + FH_4 + ATP \rightleftharpoons 10\text{-}CHO\text{-}FH_4 + ADP + P_i \quad (4)$$

In order to ensure that substrate activity derived from 5,10-CH_2-FH_4 was not due to contaminating FH_4, or to a difference between the diastereoisomers of 5,10-CH_2-FH_4, all but the first three results shown in Table III were obtained by use of chromatographically purified enzymically active diastereoisomer *d*,L-5,10-CH_2-FH_4, prepared as described under Methods.

The addition of hydroxylamine to the standard assay mixture, in which the substrate is FH_4, did not affect the reaction rate (Table III). 5,10-CH_2-FH_4 was effective alone as a replacement for FH_4. This observation demonstrated the ease with which the reaction between HCHO and FH_4, to form 5,10-CH_2-FH_4 (eq 5), can be displaced in the reverse direction. In this experiment the reaction (eq 5) was reversed by

$$HCHO + FH_4 \rightleftharpoons 5,10\text{-}CH_2\text{-}FH_4 + H_2O \quad (5)$$

TABLE III: Identification of FH_4 as a Product of the Reaction of Hydroxylamine with 5,10-CH_2-FH_4, by Its Activity as a Substrate for 10-Formyltetrahydrofolate Synthetase.[a]

Substrate Tested	Concn of Substrate (mM)	Concn of HCHO (mM)	Concn of Hydroxylamine (mM)	Initial Velocity (ΔA/min)
d,*l*,L-FH_4	0.05	0	0	0.069
d,*l*,L-FH_4	0.05	0	20	0.068
d,*l*,L-5,10-CH_2-FH_4	0.05	0	0	0.070
d,L-5,10-CH_2-FH_4	0.05	0	0	0.090
d,L-5,10-CH_2-FH_4	0.06	0.1	0	0.051
d,L-5,10-CH_2-FH_4	0.06	10	0	0.019
d,L-5,10-CH_2FH_4	0.6	40	0	0.004
d,L-5,10-CH_2-FH_4	0.06	0	20	0.143
d,L-5,10-CH_2-FH_4	0.06	0.1	10	0.157
d,L-5,10-CH_2-FH_4	0.06	10	5	0.033
d,L-5,10-CH_2-FH_4	0.06	10	10	0.115
d,L-5,10-CH_2-FH_4	0.06	40	10	0.007
d,L-5,10-CH_2-FH_4	0.06	40	20	0.020
d,L-5,10-CH_2-FH_4	0.06	40	30	0.042
d,L-5,10-CH_2-FH_4	0.06	10	5	0.001[d]
d,L-5,10-CH_2-FH_4	0.06	10	5	0[e]

[a] The activity of 50 μl of a preparation of 10-formyltetrahydrofolate synthetase was assayed as described under Methods. The rate of decrease of the absorbance at 305 mμ was recorded. [b] ATP was omitted from the reaction mixture. [c] $HCOONH_4$ was omitted from the reaction mixture.

removal of FH_4, required to form 10-CHO-FH_4 according to the reaction represented by eq 4.

The addition of HCHO to the mixture would displace to the right the reaction represented by eq 5 and so render 5,10-CH_2-FH_4 unavailable as a source of FH_4. Such results were obtained (Table III). On the other hand, addition of hydroxylamine to 5,10-CH_2-FH_4 resulted in increased activity of the formyltetrahydrofolate synthetase system, suggesting that hydroxylamine increased the availability of the substrate FH_4, from 5,10-CH_2-FH_4. It is clear, too, that subsaturating substrate concentrations were used in these assays. When HCHO and hydroxylamine were both added to the 10-formyltetrahydrofolate synthetase system, the availability of 5,10-CH_2-FH_4 to behave as a substrate depended on the relative molar proportions of each. That the measured activity, for which 5,10-CH_2-FH_4 provided substrate, was indeed formyltetrahydrofolate synthetase was confirmed by omission, in turn, of ATP and of formate. In each case there was no change of absorbance.

FORMYLTETRAHYDROFOLATE–METHIONYL-tRNA TRANSFORMYLASE. The activity of a purified preparation of the transformylase was not affected by hydroxylamine at concentrations of 3.6 and 36 mM. Therefore the *in vivo* effect of hydroxylamine on the initiation of protein synthesis by extracts of *E. coli* must be at a step leading to the synthesis of 10-CHO-FH_4 for this reaction.

FORMYLTETRAHYDROFOLATE CYCLODEHYDRASE. Hydroxylamine at concentrations up to 40 mM did not affect the activity of 5-formyltetrahydrofolate cyclodehydrase.

DIHYDROFOLATE REDUCTASE. Hydroxylamine did not appreciably inhibit the activity of the purified preparation of dihydrofolate reductase provided that the hydroxylamine and enzyme were preincubated in the presence of one or other substrate. If hydroxylamine was preincubated for 5 min with the preparation of dihydrofolate reductase in the absence of any substrate, then the enzyme was inhibited; the extent of inhibition was 25% by 20 mM hydroxylamine and 40% by 40 mM hydroxylamine.

Discussion

The reaction of hydroxylamine with HCHO, to form formaldoxime, has long been known and has been the basis of a volumetric method for the determination of HCHO since 1895 (Cambier and Brochet; Walker, 1964). Osborn *et al.* (1960) demonstrated the ready decomposition of 5,10-CH_2-FH_4 by hydroxylamine at neutral pH values, and used that observation in support of their assignment of the structure 5,10-CH_2-FH_4 to "active formaldehyde." Since the reaction of hydroxylamine with formic acid (Miolati, 1892) requires much more vigorous conditions than does its facile reaction with HCHO, one would not expect hydroxylamine to react with 10-CHO-FH_4, or 5-CHO-FH_4, or 5,10-CH-FH_4 nearly as readily as with 5,10-CH_2-FH_4. Conversely, the methylene adduct to FH_4 would be a better leaving group than those of the former compounds.

These expectations were confirmed by the results obtained, which clearly showed that hydroxylamine reacts readily at neutral pH with 5,10-CH_2-FH_4, to remove the HCHO adduct and to generate FH_4. On the other hand, hydroxylamine neither reacted with other folate coenzymes tested nor did it form any inhibitory complex with any of the tested enzymes, including the 10-formyltetrahydrofolate–methionyl-tRNA transformylase. Hydroxylamine affected the activity of 5, 10-CH_2-FH_4-dependent enzymes only by reducing the substrate to the point where it became rate limiting. Hydroxylamine also affected the activity of dihydrofolate reductase, but this effect required higher concentrations of hydroxylamine than in the case of 5,10-CH_2-FH_4-dependent enzymes, and was dependent on preincubation in the absence of substrates.

Although the overall equilibrium constant for the reaction of HCHO and FH_4 (eq 5) favors the forward reaction ($K_{eq} = 3.2 \times 10^4\ M^{-1}$; Blakley, 1960a,b), the reaction can be rapidly reversed by removal of either FH_4 or HCHO from the system. That this is so was demonstrated by the activity of 5,10-CH_2-FH_4 as substrate for formyltetrahydrofolate synthetase, even in the absence of hydroxylamine.

Under appropriately extreme conditions, hydroxylamine is known to react with other cell components, including NAD^+ (Burton and Kaplan, 1954). Enzymic mechanisms for detoxifying hydroxylamine also exist (*e.g.*, Bernheim and Hochstein, 1968; Nakagawa *et al.*, 1961). Although the hydroxylamine inhibition of methylenetetrahydrofolate dehydrogenase was partly reversed by increasing the concentration of $NADP^+$ (see Results), the extent of this reversal was small when compared with that resulting from increase of the concentration of 5,10-CH_2-FH_4. It appears probable, therefore, that the intracellular effects of relatively low concentrations of hydroxylamine are predominantly the result of its reaction with 5,10-CH_2-FH_4.

Klein *et al.* (1970) have shown that the effect of *in vivo* hydroxylamine on the *in vitro* initiation of peptide synthesis by extracts of *E. coli* can be reversed by addition of 5-CHO-FH_4. It is probable that the effect of hydroxylamine on initiation of peptide synthesis by extracts of *E. coli* can be explained by our results as follows. When cells are grown in the presence of hydroxylamine its reaction with 5,10-CH_2-FH_4 depletes the cell of this one-carbon tetrahydrofolate adduct. Since the various intracellular concentrations of one-carbon adducts of FH_4 are interdependent by means of the enzymic pathways listed in Figure 1, the depletion is not restricted to 5,10-CH_2-FH_4 but also includes those adducts at the oxidation level of formate, including 10-CHO-FH_4. Therefore, the formylation of methionyl-tRNA is prevented and the initiation of the synthesis of peptides is inhibited.

Since the addition of hydroxylamine to the growth medium of cells results in depletion of one-carbon tetrahydrofolate adducts, it should result in inhibition of other folate-dependent reactions additional to the initiation of peptide synthesis. In a nondividing population of mammalian cells, in which the initiation of protein biosynthesis is not dependent on 10-CHO-FH_4, hydroxylamine might provide a means for derepressing the biosynthesis of folate-dependent enzymes, particularly those such as L-serine–tetrahydrofolate 5,10-hydroxymethyltransferase (EC 2.1.2.1), by which one-carbon adducts of tetrahydrofolate are provided. It would be of value to obtain evidence as to whether the enzymes of folate metabolism are responsive to such control mechanisms (Bertino and Hillcoat, 1968).

Acknowledgments

We gladly thank Dr. P. Lengyel for interesting us in this

problem, and the following for their contributions to the work described. Dr. H. W. Dickerman kindly prepared and assayed the formyltetrahydrofolate–methionyl-tRNA transformylase. Drs. F. and G. Maley kindly provided the thymidylate synthetase. Mrs. Arlene Cashmore assisted with the spectral studies.

References

Beguin, S., and Kepes, A. (1964), *C. R. Acad. Sci. Ser. A 258*, 2690.

Bernheim, M. L. C., and Hochstein, P. (1968), *Arch. Biochem. Biophys. 124*, 436.

Bertino, J. R., and Hillcoat, B. L. (1968), *Advan. Enzyme Reg. 6*, 335.

Blakley, R. L. (1960a), *Biochem. J. 74*, 71.

Blakley, R. L. (1960b), *Nature (London) 188*, 231.

Blakley, R. L. (1963), *J. Biol. Chem. 238*, 2113.

Burton, R. M., and Kaplan, N. O. (1954), *J. Biol. Chem. 211*, 447.

Cambier, R., and Brochet, A. (1895), *C. R. Acad. Sci. Ser. A 120*, 449.

Dickerman, H. W., Steers, E., Redfield, B. G., and Weissbach, H. (1967), *J. Biol. Chem. 242*, 1522.

Futterman, S. (1957), *J. Biol. Chem. 228*, 1031.

Gupta, V. S., and Huennekens, F. M. (1967), *Arch. Biochem. Biophys. 120*, 712.

Kay, L. D., Osborn, M. J., Hatefi, Y., and Huennekens, F. M. (1960), *J. Biol. Chem. 235*, 195.

Klein, A., Eisenstadt, A., Eisenstadt, J., and Lengyel, P. (1970), *Biochemistry 9*, 4542.

Lengyel, P. (1967), *in* Molecular Genetics, Part II, Taylor, J. H., Ed., New York, N. Y., Academic, p 194.

May, M., Bardos, T. J., Barger, F. L., Lansford, M., Ravel, J. M., Sutherland, G. L., and Shive, W. (1951), *J. Amer. Chem. Soc. 73*, 3067.

Miolati, A. (1892), *Chem. Ber. 25*, 699.

Nakagawa, H., Suzuki, F., and Takeda, Y. (1961), *J. Biochem. (Tokyo) 49*, 191.

Nixon, P. F., and Bertino, J. R. (1970), *in* Vitamins and Coenzymes. A Volume of Methods in Enzymology, McCormick, D. G., and Wright, L. D., Ed., New York, N. Y., Academic (in press).

Osborn, M. J., Talbert, P. T., and Huennekens, F. M. (1960), *J. Amer. Chem. Soc. 82*, 4921.

Perkins, J. P., Hillcoat, B. L., and Bertino, J. R. (1967), *J. Biol. Chem. 242*, 4771.

Rabinowitz, J. C., and Pricer, W. E. (1962), *J. Biol. Chem. 237*, 2898.

Uyeda, K., and Rabinowitz, J. C. (1967), *J. Biol. Chem. 242*, 4378.

Vonderschmitt, D. J., Vitols, K. S., Huennekens, F. M., and Scrimgeour, K. G. (1967), *Arch. Biochem. Biophys. 122*, 488.

Wahba, A. J., and Friedkin, M. (1961), *J. Biol. Chem. 236*, PC11.

Walker, J. F. (1964), Formaldehyde, New York, N. Y., Reinhold, p 240.

Werkheiser, W. C. (1960), *J. Biol. Chem. 236*, 888.