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Inhibition of Peptide-Chain Initiation in *Escherichia coli* by Hydroxylamine and Effects on Ribonucleic Acid Synthesis*

Albrecht Klein,† Audrey Eisenstadt, Jerome Eisenstadt, and Peter Lengyel‡

ABSTRACT: In an *Escherichia coli* extract which was prepared from cells exposed to 10^{-2} M hydroxylamine and was supplemented with 10^{-2} M hydroxylamine, bacteriophage f2 RNA did not promote protein synthesis unless either formyltetrahydrofolate or fMet-tRNA$_f^{Met}$ was added to the reaction mixture. For studies on the nature of the hydroxylamine effect, an extract free of tetrahydrofolate was prepared from cells treated with trimethoprim, an inhibitor of dihydrofolate reductase. In order to make possible protein synthesis directed by f2 RNA in this extract, it had to be supplemented with either fMet-tRNA$_f^{Met}$ or formyltetrahydrofolate. Hydroxylamine (1.5×10^{-2} M) had no effect upon protein synthesis if the extract was supplemented with fMet-tRNA$_f^{Met}$. Hydroxylamine inhibited protein synthesis however if the extract was supplemented with formyltetrahydrofolate. Hydroxylamine blocked peptide-chain initiation but had no effect on peptide-chain elongation. Hydroxylamine was found to form a compound with the formyl residue of formyltetrahydrofolate in a nonenzymic reaction. (The nature of this reaction is described in the accompanying paper by Nixon, P. F., and Bertino, J. R. (1970), *Biochemistry* (in press).) These results indicate that hydroxylamine blocks protein synthesis *in vitro* by causing depletion of the formyltetrahydrofolate pool and thus inhibiting the formation of fMet-tRNA$_f^{Met}$, the initiator of peptide chains. The effect of hydroxylamine on protein and RNA synthesis *in vivo* was tested in an *E. coli* strain (CP 78) in which the RNA synthesis is under "stringent" control of amino acids and in a strain (CP 79) in which the control is "relaxed." Hydroxylamine, 5×10^{-4} M, blocked protein synthesis in both the stringent and the relaxed *E. coli* strain growing in a medium supplemented with twenty amino acids and five purine and pyrimidine bases. The same concentration of hydroxylamine inhibited net RNA synthesis greatly (80%) in the stringent and only slightly (10%) in the relaxed *E. coli* strain. These effects of hydroxylamine upon RNA and protein synthesis are similar to the effects resulting from the removal of a required amino acid from the growth medium. It remains to be seen whether these results reflect a dependence of net RNA synthesis upon fMet-tRNA$_f^{Met}$ in a stringent but not in a relaxed strain or if other phenomena are involved.

Hydroxylamine is a highly reactive nucleophilic reagent. It is a mutagen for bacteria and bacterial viruses, and stops the growth of a number of microorganisms (Borek *et al.*, 1951; Price *et al.*, 1960; Phillips and Brown, 1967). At a concentration of 10^{-3} M it was found to stop the synthesis of DNA, RNA, and protein (Rosenkranz and Bendich, 1964); at lower concentrations it preferentially inhibits protein synthesis (Beguin and Kepes, 1964). Kepes and Beguin (1965) studied the effect of inhibitors on the kinetics of the various phases of β-galactosidase induction. They concluded that hydroxylamine blocked a (at the time unknown) phase of protein synthesis, occurring between the synthesis of mRNA and the growth of the peptide chain, that is, peptide-chain initiation.[1] More recently, insight into the details of initiation was obtained and it thus became feasible to study the mode in which hydroxylamine inhibits. The major, if not the sole, peptide-

* From the Department of Molecular Biophysics and Biochemistry and the Department of Microbiology, New Haven, Connecticut 06520. *Received June 8, 1970.* This study was supported by research grants from the National Institutes of Health (GM 13707 and AM 07189) and by a training fellowship from the Deutsche Forschungsgemeinschaft to A. K.

† Present address: Max Planck Institut für Virusforschung, Tübingen, Germany.

‡ To whom to address correspondence atBo x 1937, Yale Station, New Haven, Conn. 06520.

[1] According to a recent communication, catabolite repression contributes to the effect of hydroxylamine upon protein synthesis in certain *E. coli* strains (Basu *et al.*, 1967).

chain initiator in *Escherichia coli* is fMet-tRNA$_f^{Met}$[2] (for a review, see Lengyel and Söll, 1969). In the last step of the biosynthesis of this compound, 10-CHO-FH$_4$ serves as a formyl donor in the formylation of Met-tRNA$_f^{Met}$ (Marcker, 1965; Dickerman *et al.*, 1967).

In this communication we present results indicating that (a) *in vitro*, hydroxylamine blocks the initiation of peptide chains, but does not effect chain elongation and (b) this is due to the fact that hydroxylamine forms a compound with the formyl residue of 10-CHO-FH$_4$ in a nonenzymic reaction, thus depleting the 10-CHO-FH$_4$ pool and inhibiting the synthesis of the chain initiator fMet-tRNA$_f^{Met}$. The nature of the reaction of hydroxylamine with folate coenzymes is described in the accompanying paper (Nixon and Bertino, 1970).

The major portion, if not all, of the RNA in the cell serves in protein synthesis. In *E. coli* if protein synthesis is inhibited by the lack of a required amino acid, the rate of increase in the amount of RNA, *i.e.*, the rate of net RNA synthesis decreases below 10% of the normal value. Thus, net synthesis is under stringent control of amino acids. (Net RNA synthesis is measured by determining the incorporation of uracil from the medium into RNA; see Nierlich, 1967.) Cells in which this stringent control operates are designated as RC^{str}. Net RNA synthesis is inhibited in RC^{str} cells even in the presence of all amino acids if the charging of a tRNA species is blocked, *e.g.*, at elevated temperatures, in cells with heat-sensitive AA-tRNA synthetases (see Neidhardt, 1966). In certain *E. coli* mutants, net RNA synthesis continues at nearly the normal rate after the removal of a required amino acid. Strains of this type were stated to have a relaxed control of RNA synthesis (RC^{rel}) (Stent and Brenner, 1961; for a review, see Edlin and Broda, 1968).

In this communication we present data indicating that hydroxylamine inhibits net RNA synthesis greatly in RC^{str}, but only slightly in RC^{rel} strains. These results may indicate that net RNA synthesis is inhibited by a block in the formylation of Met-tRNA$_f^{Met}$. Results consistent with this conclusion were obtained previously in studies in which formylation of Met-tRNA$_f^{Met}$ was blocked in consequence of the inhibition of dihydrofolate reductase (Shih *et al.*, 1966). Some of the above data were presented in a preliminary communication (Eisenstadt and Lengyel, 1967).

Experimental Section

Materials

L-[^{14}C]Arginine (specific activity 225 μCi/μmole), L-[^{14}C]-threonine (specific activity 100 μCi/μmole), L-[^{3}H]histidine (specific activity 419 μCi/μmole, L-[^{14}C]phenylalanine (specific activity 360 μCi/μmole), [^{14}C]uracil (specific activity 55 μCi/μmole), and [^{14}C]guanine (specific activity 32 μCi/μmole) were obtained from Schwarz BioResearch Inc., KATP from P-L Biochemicals, NaGTP from Mann Research Laboratories; AICAR, aminopterin, and phosphoenolpyruvate (sodium salt) from Calbiochem, the calcium salt of 5-CHO-FH$_4$ (calcium leucovorin) from Lederle Laboratories, chloramphenicol from Parke, Davis and Co., and poly (U) from Miles Laboratories. Trimethoprim (2,4-diamino-5-[3′4′5′-trimethoxybenzyl]pyrimidine) was donated by Dr. G. H. Hitchings (Burroughs Wellcome Co.). Phosphoenolpyruvate kinase was obtained from Boehringer, recrystallized beef pancreatic deoxyribonuclease and ribonuclease A from Worthington.

The *E. coli* K$_{12}$ strains Cp 78 (arg$^-$his$^-$leu$^-$thr$^-$B$_1^-$RC^{str}) and Cp 79 (arg$^-$his$^-$leu$^-$thr$^-$B$_1^-$RC^{rel}) isolated by Dr. N. Fiil were made available by Drs. G. Edlin and G. Stent (Department of Molecular Biology, University of California at Berkeley). The two strains differ from each other in the control of RNA synthesis by amino acids and are otherwise presumably isogenic. *E. coli* K$_{10}$S$_{26}$, a phosphatase negative strain, was obtained from Dr. A. Garen (Department of Molecular Biophysics and Biochemistry, Yale University).

Methods

The medium used for growing bacteria contained the following components in grams per liter: KH$_2$PO$_4$, 5.4; K$_2$HPO$_4$, 36.6; glucose, 5.0; (NH$_4$)$_2$SO$_4$, 4.0; sodium citrate, 1.0; MgSO$_4$·7H$_2$O, 0.005; vitamin B$_1$, 0.002; each of the 20 L-amino acids, 0.050 (one of the amino acids labeled if indicated); adenine, cytosine, guanine, thymidine, uracil 0.040 (whenever uracil or guanine was labeled, its concentration was 0.010, whereas the concentration of the other unlabeled bases remained 0.040). The cells were incubated in erlenmeyer flasks on a rotary shaker at 37°.

The incorporation of labeled amino acids into protein was determined by the filter paper disc method of Mans and Novelli (1961).

Bacteriophage f2 RNA was prepared by treating purified virus with phenol (Nathans *et al.*, 1962). The method for preparing and discharging tRNA was described earlier (Eisenstadt and Lengyel, 1966). fMet-tRNA$_f^{Met}$ was obtained by charging unfractionated tRNA with methionine and subsequent formylation, with 5-CHO-FH$_4$ serving as the source of formate (Huennekens and Osborn, 1959; Nakamoto and Kolakofsky, 1966). 5-CHO-FH$_4$ is converted in *E. coli* cell extract into 10-CHO-FH$_4$ and thus can substitute for the latter.

Experiments in Vitro. "Hydroxylamine-inhibited S-30 extract" from cells treated with hydroxylamine was prepared in the following way. A culture of *E. coli* K$_{10}$S$_{26}$ grown to an A_{600} of 0.11 was supplemented with 10^{-2} M hydroxylamine and the incubation continued for 30 min. The culture was then cooled to 0° and a cell free extract prepared according to the method of Eisenstadt and Lengyel (1966) with the exception that trimethoprim was omitted and 10^{-3} M hydroxylamine was added to all solutions used in preparing the extract.

In order to decrease the endogenous mRNA content of the hydroxylamine-inhibited S-30 extract it was incubated before using it in the incorporation experiments. One volume of the hydroxylamine-inhibited S-30 extract (1 ml of which contained about 10 mg of total RNA) was diluted to two volumes and was incubated at 37° for 15 min. One milliliter of this incubation mixture contained the following components (in micromoles, unless otherwise indicated): NH$_4$Cl, 75; Tris-HCl (pH 7.8) 50; magnesium acetate, 10; glutathione, 10; hydroxylamine, 10; phosphoenolpyruvate, 5; ATP, 3; GTP, 0.2; 19 amino acids (arginine omitted) each, 0.025; pyruvate kinase, 20 μg.

[2] Abbreviations used are: ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; AICAR, 4-amino-5-imidazolecarboxamide nucleoside; phospho-AICAR, 4-amino-5-imidazolecarboxamide nucleotide; Met-tRNA$_f^{Met}$, methionyl-tRNA the formylatable species; fMet-tRNA$_f^{Met}$, *N*-formylmethionyl-tRNA; 10-CHO-FH$_4$, 10-formyltetrahydrofolate; 5-CHO-FH$_4$, 5-formyltetrahydrofolate.

TABLE I: fMet-tRNA Requirement for Amino Acid Incorporation Directed by f2 RNA in an S-30 Extract from a Culture of *E. coli* ($K_{10}S_{26}$) Treated with Hydroxylamine.[a]

Mg^{2+} Concn (mM)	Additions: f2 RNA (100 μg/ml)	Additions: fMet-$tRNA_f^{Met}$ (775 μg/ml)	Arg Incorpd[b]
5	−	−	6
5	−	+	10
5	+	−	5
5	+	+	181
10	−	−	20
10	−	+	38
10	+	−	289
10	+	+	235

[a] The incubation was performed at 37° for 30 min. The total volume was 0.2 ml. One milliliter of the basic incubation mixture was supplemented with the following components: hydroxylamine, 10 μmoles; L-[^{14}C]arginine, 5.6 μCi; 490 μg of discharged tRNA; and 0.25 ml of incubated hydroxylamine-inhibited S-30 extract. The Mg^{2+} concentration of, and further additions to the reaction mixture are indicated in the table. [b] The amount of arginine incorporated is expressed in micromicromoles per milligram of total RNA in the S-30 extract.

This incubated hydroxylamine-inhibited S-30 extract was used immediately after the incubation in the incorporation experiments.

Trimethoprim-inhibited S-30 extract from cells treated with trimethoprim was prepared as described in a previous communication (Eisenstadt and Lengyel, 1966) with the exception that the cells were grown in the medium described in this paper.

The trimethoprim-inhibited S-30 extract was incubated prior to use in the incorporation experiments in order to decrease amino acid incorporation due to endogenous mRNA. One volume of the trimethoprim-inhibited S-30 extract (1 ml of which contained about 10 mg of total RNA) was diluted to two volumes and was incubated at 37° for 15 min. One milliliter of this incubation mixture contained the following components (in micromoles unless otherwise indicated): NH_4Cl, 75; Tris-HCl buffer, pH 7.8, 50; magnesium acetate, 10; glutathione, 10; phosphoenolpyruvate, 5; ATP, 3; GTP, 0.2; 19 amino acids (threonine omitted), each, 0.025; aminopterin, 20 μg; pyruvate kinase, 20 μg.

This incubated trimethoprim-inhibited S-30 extract was used immediately after the incubation in the incorporation experiments. The RNA content of S-30 extracts was determined according to Brawerman (1963).

Basic Incubation Mixture for Experiments in Vitro. One milliliter of the incubation mixture, in which the experiments *in vitro* were performed, contained the following components (in micromoles unless otherwise indicated): NH_4Cl, 75; Tris-HCl (pH 7.8), 50; glutathione, 10; phosphoenolpyruvate, 5; ATP, 3; GTP, 0.2; each of the 20 amino acids, 0.025 (usually nineteen unlabeled, one labeled). Subsequently a solution of the above composition will be referred to as "basic incubation mixture." Further additions to the basic incubation mixture are indicated in the legends to the tables and figures.

Studies in Vivo. EXPERIMENTS ON THE EFFECT OF 5×10^{-4} M HYDROXYLAMINE ON HISTIDINE, URACIL, AND GUANINE INCORPORATION IN RC^{str} AND RC^{rel} STRAINS. Cells grown overnight were diluted into prewarmed medium to a density of 45 $Klett_{660}$ units and incubated for two generation times. Subsequently, the culture was diluted to a density of 10 $Klett_{660}$ units into a medium containing 2.5 μCi of L-[^3H]histidine/ml and either 0.1 μCi of [^{14}C]uracil/ml or 0.1 μCi of [^{14}C]guanine/ml and further incubated until the density reached 20 $Klett_{660}$ units. The culture was then divided into two parts: one serving as control, the other being supplemented with 5×10^{-4} M hydroxylamine freshly neutralized with Tris base. Both cultures were incubated for one generation. Subsequently each of them was divided further: one part serving as a control, the other being supplemented with 100 μg/ml of chloramphenicol. The four parts were incubated for one generation. The optical density of the cultures was followed. Incorporation of uracil or guanine into cold acid-insoluble product (RNA) and the incorporation of histidine into hot acid-insoluble product (protein) was determined by the filter paper disc method of Mans and Novelli (1961). At the times indicated in the figures, two 0.1-ml aliquots of each of the bacterial cultures were placed on filter paper discs and submerged in cold 10% trichloroacetic acid. Subsequently, one of the disks (prepared for determining radioactivity in protein) was heated in 5% trichloroacetic acid at 80° for 30 min. Under these conditions, the RNA was hydrolyzed. The hydrolysis products were removed from the disk by subsequent washes in 5% trichloroacetic acid. In this way, it was assured that the [^{14}C]uracil or [^{14}C]guanine incorporated into RNA should not interfere with the counting of [^3H]histidine incorporated into protein. The other disk (prepared for determining radioactivity in RNA) was washed three times with ice-cold 5% trichloroacetic acid before counting. Radioactivity in the disks was measured in a Packard TriCarb scintillation counter. Under the conditions used, less than 1% of the counts in the ^3H channel were found in the ^{14}C channel. An appropriate correction was applied in calculating ^{14}C counts.

EXPERIMENTS ON THE EFFECT OF DIFFERENT CONCENTRATIONS OF HYDROXYLAMINE ON THE INCORPORATION OF URACIL INTO RC^{str} CELLS (Figure 5). Cells grown overnight were diluted to a density of 45 $Klett_{660}$ units into prewarmed medium and incubated for two generations. Subsequently, the culture was diluted to a density of 10 $Klett_{660}$ units into a medium containing 0.1 μCi of [^{14}C]uracil/ml and incubated until the density reached 20 $Klett_{660}$ units. The culture was then distributed into five flasks: one served as a control, and various levels of hydroxylamine, freshly neutralized with Tris base, were added to the other four flasks. The rate of uracil incorporation into cold acid-insoluble product was followed as outlined in the previous section.

Results

Studies with E. coli Cell Extract. EXTRACTS OF CELLS TREATED WITH HYDROXYLAMINE. An S-30 extract was prepared from *E. coli* cells, the growth of which was stopped by 10^{-2} M hydroxylamine. The effect of f2 RNA on amino acid incorporation in the extract was assayed in the presence of 10^{-2} M

TABLE II: Effect of Hydroxylamine on Amino Acid Incorporation in an S-30 Extract from a Culture of *E. coli* Treated with Trimethoprim.[a]

	Other Additions				
Messenger	Hydroxylamine (mM)	5-CHO-FH_4 (mM)	fMet-$tRNA_f^{Met}$ (μg/ml)	Amino Acid	Amt Incorpd,[b]
				Arg	14
		0.24		Arg	24
	20	0.24		Arg	21
f2 RNA				Arg	19
(100		0.24		Arg	496
μg/ml)	20	0.24		Arg	104
			70	Arg	265
	20		70	Arg	264
			240	Arg	521
	20		240	Arg	497
Poly (U)				Phe	3260
(50	20			Phe	3160
μg/ml)					

[a] The incubation was performed at 37° for 30 min. The total volume was 0.2 ml. One milliliter of the basic incubation mixture was supplemented with the following components: Magnesium acetate 5 μmoles, 0.10 ml of incubated trimethoprim-inhibited S-30 extract and in the case of reaction mixtures with f2 RNA or no messenger L-[^{14}C]arginine (5.6 μCi) and 19 nonlabeled amino acids and in the case of reaction mixtures with poly (U), L-[^{14}C]phenylalanine (5.6 μCi), and no unlabeled amino acid. [b] The amount of amino acids incorporated is expressed in micromicromoles per milligram of total RNA in the S-30 extract.

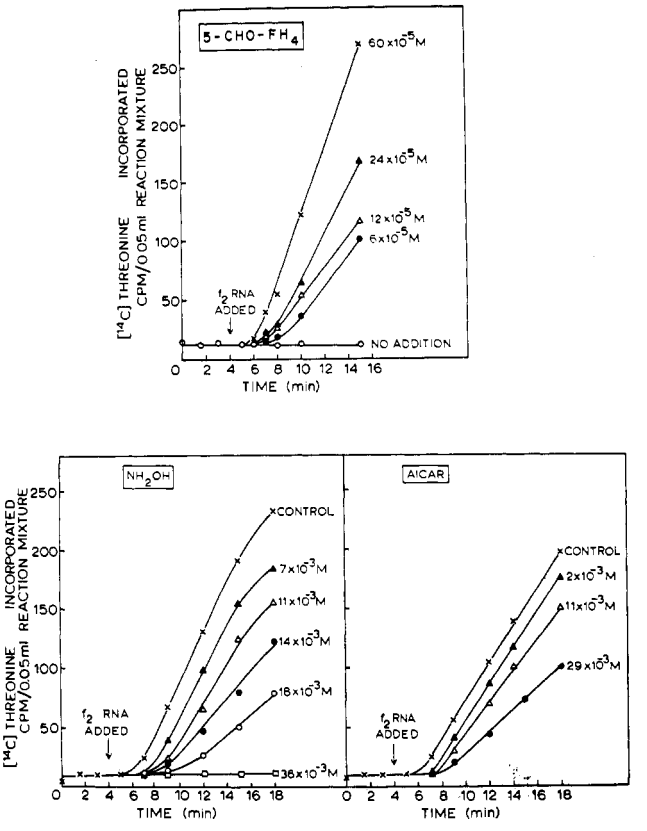

FIGURE 1: Inhibition by hydroxylamine and by AICAR of amino acid incorporation directed by f2 RNA. One milliliter of the basic incubation mixture was supplemented with the following components: magnesium acetate, 5 μmoles; L-[^{14}C]threonine, 2.5 μCi; aminopterin (an inhibitor of dihydrofolate reductase), 20 μg; and incubated trimethoprim-inhibited S-30 extract (see Methods), 0.25 ml. Further components of the reaction mixture are described subsequently. The temperature of incubation was 30°. The amount of L-[^{14}C]threonine incorporated into protein was determined in samples taken at the times indicated in the figure. Top part: dependence of amino acid incorporation directed by f2 RNA on the concentration of 5-CHO-FH_4. Different amounts of 5-CHO-FH_4 (indicated in the figure) were added to four tubes containing reaction mixture; a fifth tube, serving as control, received none. The reaction mixture in each tube was supplemented with 490 μg/ml of discharged tRNA. After 4-min incubation, 100 μg/ml of f2 RNA was added to each tube and the incubation continued. Bottom left: inhibition by hydroxylamine of amino acid incorporation directed by f2 RNA. Different amounts of hydroxylamine (indicated in the figure) were added to five tubes containing reaction mixture; a sixth tube containing reaction mixture served as control and received none. The reaction mixture in each tube was supplemented with 24×10^{-5} M 5-CHO-FH_4. After 2-min incubation, 490 μg/ml of discharged tRNA was added to each (zero time on the figure); 4 min later 100 μg/ml of f2 RNA was added to each and the incubation continued. Bottom right: inhibition by AICAR of amino acid incorporation directed by f2 RNA. Different amounts of AICAR (indicated in the figure) were added to three tubes containing reaction mixture; a fourth tube, containing reaction mixture served as control and received none. The reaction mixture in each tube was supplemented with 24×10^{-5} M 5-CHO-FH_4. After 2-min incubation, 490 μg/ml of discharged tRNA was added to each (zero time on the figure); 4 min later 100 μg/ml of f2 RNA was added to each, and the incubation was continued.

hydroxylamine. If tested at 0.005 M Mg^{2+} the incorporation strictly depended on fMet-$tRNA_f^{Met}$ added to the incubation mixture (Table I). This indicates that the inhibition of protein synthesis results from hydroxylamine making fMet-$tRNA_f^{Met}$ unavailable. At 0.01 M Mg^{2+} f2 RNA promoted amino acid incorporation even without added fMet-$tRNA_f^{Met}$. This result is consistent with earlier observations which showed that, at 0.01 M or higher Mg^{2+}, protein synthesis does not depend on fMet-$tRNA_f^{Met}$ (see also Eisenstadt and Lengyel, 1966).

EXTRACTS FROM CELLS TREATED WITH TRIMETHOPRIM–HYDROXYLAMINE MAKES fMet-tRNA UNAVAILABLE. Whether the formylation of Met-$tRNA_f^{Met}$ was blocked by hydroxylamine was tested in the following way. An S-30 extract, free of fMet-$tRNA_f^{Met}$ and 10-CHO-FH_4, was prepared from *E. coli* cells in which the formation of tetrahydrofolate, a precursor of 10-CHO-FH_4, was blocked. This was accomplished by treating the cells first with trimethoprim, an inhibitor of the enzyme reducing dihydrofolate to tetrahydrofolate, and preparing and storing the extract in the presence of the inhibitor (Eisenstadt and Lengyel, 1966). In this extract amino acid incorporation directed by f2 RNA strictly depended on 5-CHO-FH_4 (which is transformed to 10-CHO-FH_4 in the extract) (Figure 1, top part, and Table II).

In one series of experiments, aliquots of this extract were supplemented with 2.4×10^{-4} M 5-CHO-FH_4 and the effect of various concentrations of hydroxylamine on amino acid in-

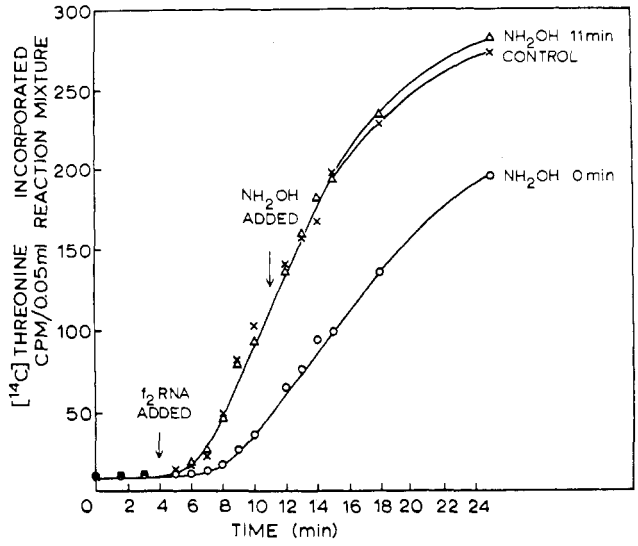

FIGURE 2: Effect of hydroxylamine on peptide-chain initiation and peptide-chain elongation directed by f2 RNA. One milliliter of the basic incubation mixture was supplemented with the following components: magnesium acetate, 5 μmoles; L-[^{14}C]threonine, 2.5 μCi; aminopterin, 20 μg; and incubated trimethoprim-inhibited S-30 extract (see Methods), 0.25 ml. Further components of the reaction mixture are described subseqently. The incubation was performed at 30°. The incorporation of L-[^{14}C]threonine into protein was determined in samples taken at the times indicated. Each of the three tubes containing reaction mixture was supplemented with 2.4×10^{-4} M 5-CHO-FH_4. One of the tubes (O-O, NH_2OH, 0 min) also received 1.25×10^{-2} M hydroxylamine at 0 time. After 2-min incubation each of the three tubes was supplemented with 490 μg/ml of discharged tRNA; after 4 min each of the three tubes was supplemented with 133 μg/ml of f2 RNA; after 11 min 1.25×10^{-2} M hydroxylamine was added to one tube (Δ-Δ, NH_2OH, 11 min). No hydroxylamine was added to the tube serving as control (×-×, control). Samples for determining the amount of threonine incorporated in protein were taken at the times indicated.

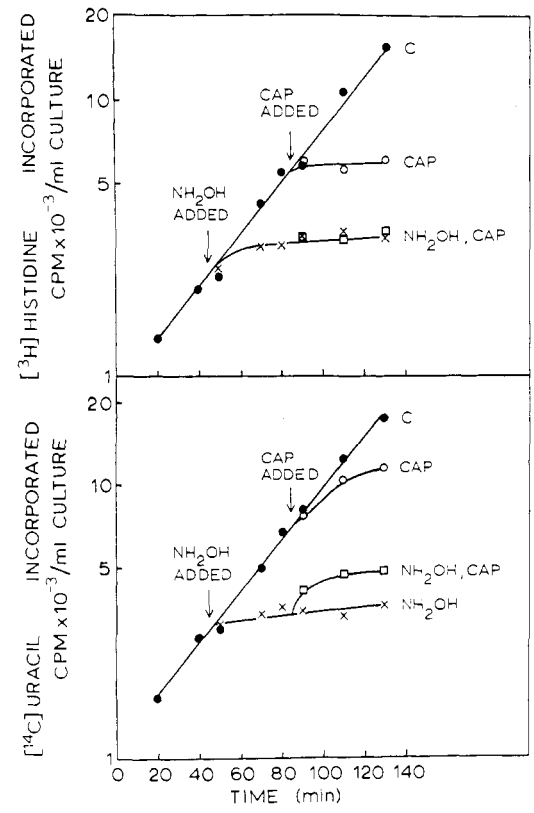

FIGURE 3: Effect of 5×10^{-4} M hydroxylamine and of 100 μg/ml of chloramphenicol on histidine and uracil incorporation in *E. coli* (strain Cp 78) with stringent control of RNA synthesis. Hydroxylamine (NH_2OH) and chloramphenicol (CAP) were added at the times indicated by the arrows. (●-●) Control, (×-×) hydroxylamine added, (○-○) chloramphenicol added, and (□-□) hydroxylamine and chloramphenicol added. Top: incorporation of [^{3}H]histidine into hot acid-insoluble product. Bottom: incorporation of [^{14}C]uracil into cold acid-insoluble product. For experimental details, see Methods.

corporation promoted by f2 RNA was tested (Figure 1, bottom left). It can be seen that 1.4×10^{-2} M hydroxylamine decreased the incorporation by about 50% (see also Table II). A possible way in which hydroxylamine could cause the inhibition was by forming a compound with the formyl residue of 5-CHO-FH_4 and thus making formyl residues for the formylation of Met-$tRNA_f^{Met}$ unavailable.

The following results are consistent with this hypothesis. (a) Hydroxylamine (2×10^{-2} M) inhibits protein synthesis directed by f2 RNA in a system in which fMet-$tRNA_f^{Met}$ is being generated from 5-CHO-FH_4 and Met-$tRNA_f^{Met}$ (Table II and Figure 1 bottom left) but does not inhibit protein synthesis in the presence of added fMet-$tRNA_f^{Met}$ (Table II). (b) Polyphenylalanyl-tRNA synthesis as directed by poly (U) *in vitro* does not depend on fMet-$tRNA_f^{Met}$ (see Eisenstadt and Lengyel, 1966), and hydroxylamine (2×10^{-2} M) does not inhibit polyphenylalanyl-tRNA synthesis (Table II). (c) An acceptor of formyl residues from 10-CHO-FH_4 inhibits protein synthesis directed by f2 RNA similarly to hydroxylamine. Phospho-AICAR, the acceptor in question, is an intermediate in the biosynthesis of purines known to accept a formyl residue from 10-CHO-FH_4 (see the review by Magasanik, 1962). Since phospho-AICAR was not available we examined the effect of AICAR, whose conversion into phospho-AICAR was shown to occur in yeast extract (Greenberg, 1956). Different amounts of AICAR were added to a series of react on mixtures including incubated trimethoprim-inhibited S-30 extract and 2.4×10^{-4} M 5-CHO-FH_4. AICAR (3×10^{-2} M) was found to inhibit amino acid incorporation by about 50% (Figure 1, bottom right).

The formation of a compound including the formyl residue from 10-CHO-FH_4 and hydroxylamine is described in the accompanying paper by Nixon and Bertino (1970).

HYDROXYLAMINE BLOCKS CHAIN INITIATION. If hydroxylamine inhibits protein synthesis by making fMet-$tRNA_f^{Met}$ unavailable, then hydroxylamine should block the initiation of peptide chains without affecting their elongation. This was verified in the following way. The kinetics of amino acid incorporation were followed in three reaction mixtures. fMet-$tRNA_f^{Met}$ was generated in each from 10-CHO-FH_4 and Met-$tRNA_f^{Met}$ (Figure 2). Hydroxylamine was omitted from one reaction mixture; the second was supplemented with hydroxylamine 2 min before the addition of f2 RNA; the third was supplemented with hydroxylamine 11 min after the addition of f2 RNA. The kinetics of amino acid incorporation in the control reaction mixture seem to reflect on several phases in peptide-chain formation. Amino acid incorporation accel-

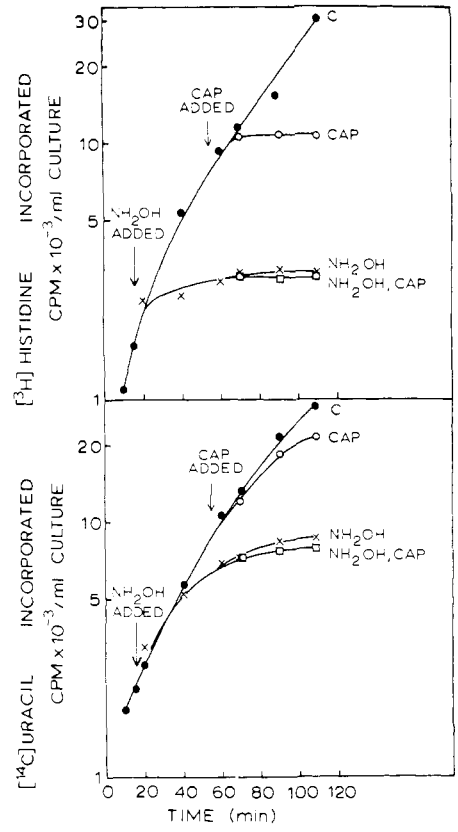

FIGURE 4: Effect of 5×10^{-4} M hydroxylamine and of 100 μg/ml of chloramphenicol on histidine and uracil incorporation in *E. coli* (strain Cp 79) with relaxed control of RNA synthesis. For details, see legend to Figure 3.

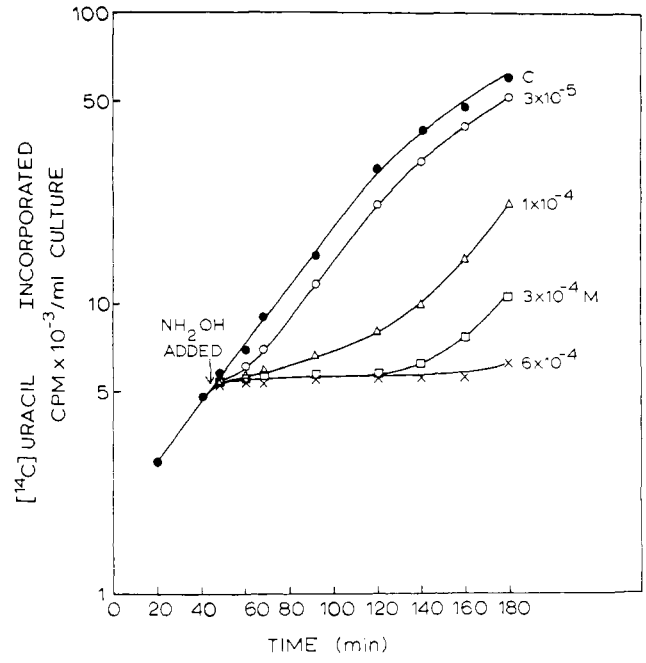

FIGURE 5: Effect of different concentrations of hydroxylamine on the incorporation of uracil in *E. coli* (strain Cp 78) with stringent control of RNA synthesis. Hydroxylamine was added to the cultures at the concentrations shown in the figure at the time indicated by the arrow. C (control) no hydroxylamine added. For experimental details, see Methods.

erates during the first 8 min of the incubation, apparently indicating that translation of new chains is initiated during this phase. It can be seen that hydroxylamine added during this phase (actually at zero time) is inhibitory. Beginning at about 8 min the rate of amino acid incorporation is at first constant, later it decreases. This might indicate that no new peptide chains are initiated in this phase, and that only chains are elongated and later terminated. In this phase hydroxylamine (added at 11 min) is without effect. These results are consistent with the view that the inhibitory effect of hydroxylamine upon protein synthesis is due to its inhibition of initiation.

STUDIES WITH *E. coli* CELLS. The results presented in the previous section indicate that hydroxylamine blocks protein synthesis *in vitro* by depleting the 10-CHO-FH_4 pool. The synthesis of 10-CHO-FH_4 can be inhibited by trimethoprim. We reported earlier that adding trimethoprim to a growing *E. coli* culture results in (a) an over 90% inhibition of both protein and net RNA synthesis in *E. coli* RC^{str} cells, and (b) an over 90% inhibition of protein synthesis, but only a much slighter and more gradual inhibition of net RNA synthesis in *E. coli* RC^{rel} cells (Shih *et al.*, 1966).

It was of some interest to test if the effect of hydroxylamine upon protein and net RNA synthesis *in vivo* was similar to that of trimethoprim.

In addition to being required for formylating Met-$tRNA_f^{Met}$, 10-CHO-FH_4 is also needed for the biosynthesis of several metabolites, including certain amino acids and nucleotides. Since we wanted to test the effect of hydroxylamine specifically as far as possible on peptide-chain initiation (and the consequences thereof) we cultured the cells in a medium containing all twenty common amino acids, and all the five common purines and pyrimidines. The effect of 5×10^{-4} M hydroxylamine on amino acid incorporation into protein and uracil incorporation into RNA in *E. coli* RC^{str} cells is shown in Figure 3, the effect in *E. coli* RC^{rel} cells in Figure 4.

As noted, the incorporation of labeled uracil from the medium into RNA is taken as a measure of net RNA synthesis (see Nierlich, 1967). Protein synthesis is inhibited to the same high extent in both RC^{str} and RC^{rel} cells by hydroxylamine. The degree of inhibition of net RNA synthesis, however, is remarkably different in the two cell types. There is about 80% inhibition of net RNA synthesis in RC^{str} cells and only 10% inhibition in RC^{rel} cells during the first 30 min after adding hydroxylamine. In RC^{str} cells, in which the rate of RNA synthesis is decreased in consequence of either the lack of an amino acid or added trimethoprim, addition of chloramphenicol causes an acceleration of RNA synthesis to almost the normal rate (for review see Neidhardt, 1964; Shih *et al.*, 1966). When 100 μg/ml of chloramphenicol was added to RC^{str} cells 50 min after hydroxylamine addition, the rate of RNA synthesis increased (although it did not reach the rate of the control treated with chloramphenicol alone). As expected, the effect of chloramphenicol on RNA synthesis in RC^{rel} cells was very slight. Chloramphenicol inhibited protein synthesis in both strains and had no effect upon the inhibition of protein synthesis by hydroxylamine in either.

These results show that the effect of 5×10^{-4} M hydroxylamine on protein and net RNA synthesis in both stringent and

relaxed cells is similar to that of trimethoprim. Furthermore, the effect of either trimethoprim or hydroxylamine upon net RNA and protein synthesis in either RC^{str} or RC^{rel} cells is similar to the effect of removing a required amino acid from the medium.

The effect of various concentrations of hydroxylamine upon uracil incorporation into cold acid-insoluble product in *E. coli* RC^{str} cells is shown in Figure 5. It can be seen that at very low concentrations of hydroxylamine the inhibition is transient, and the extent and length of the inhibition of uracil incorporation increases with the concentration of hydroxylamine added.

Discussion

The following results indicate that hydroxylamine (0.02 M) blocks protein synthesis in *E. coli* extracts by making fMet-$tRNA_f^{Met}$ unavailable. Amino acid incorporation directed by f2 RNA depends strictly on added fMet-$tRNA_f^{Met}$ in extracts prepared from cells treated with hydroxylamine and tested in the presence of hydroxylamine. fMet-$tRNA_f^{Met}$ is required in the initiation of the peptide chains which are translated from f2 RNA and hydroxylamine blocks that initiation. fMet-$tRNA_f^{Met}$ is not involved either in the elongation of peptide chains translated from f2 RNA or in the synthesis of polyphenylalanyl-tRNA translated from poly (U) and neither of these processes is affected by hydroxylamine.[3]

That it is the formylation of Met-$tRNA_f^{Met}$ which is blocked by hydroxylamine and that this block is a consequence of hydroxylamine making 10-CHO-FH_4 unavailable is indicated by the following observations. In an extract free of 10-CHO-FH_4, f2 RNA does not promote amino acid incorporation unless the extract is supplemented with either fMet-$tRNA_f^{Met}$ or with 10-CHO-FH_4 or 5-CHO-FH_4 (making the formylation of Met-$tRNA_f^{Met}$ possible). Hydroxylamine causes an over 80% inhibition of amino acid incorporation in the extract supplemented with 10-CHO-FH_4 but has no effect on that in the extract supplemented with fMet-$tRNA_f^{Met}$.

The hypothesis according to which hydroxylamine makes 10-CHO-FH_4 unavailable by forming a compound with the formyl residue of 10-CHO-FH_4 is supported by studies of Nixon and Bertino (1970), described in the accompanying paper. These authors established that hydroxylamine reacts readily with 5,10-methylenetetrahydrofolate (though not with other folate coenzymes) in a nonenzymatic reaction giving rise presumably to formaldoxime (CH_2:NOH) (see also Osborn *et al.*, 1960). Since the various intracellular concentrations of the different one carbon adducts of tetrahydrofolate are interdependent by known enzymic pathways the reaction of hydroxylamine with the 5,10-methylenetetrahydrofolate results in the depletion of the pool of 10-CHO-FH_4.

Hydroxylamine, 5×10^{-4} M, blocks protein synthesis in both RC^{str} and RC^{rel} *E. coli* grown in a medium supplemented with the 20 common amino acids and the 5 common purine and pyrimidine bases. Hydroxylamine in the above concentration, inhibits net RNA synthesis greatly (80%) in RC^{str} *E. coli* and only slightly (10%) in RC^{rel} *E. coli*. It remains to be seen if this effect of hydroxylamine upon net synthesis of RNA in RC^{str} cells reflects a direct or indirect involvement of fMet-$tRNA_f^{Met}$ in the regulation of the synthesis and breakdown of RNA. It is also conceivable that folate coenzymes function in this regulation (in addition to their role in the formation of the purines, pyrimidines, amino acids, and fMet-$tRNA_f^{Met}$) in an as yet unknown fashion and that this hypothetical function of folate coenzymes is blocked by the depletion of the 10-CHO-FH_4 pool by hydroxylamine.

It should be noted that hydroxylamine as well as trimethoprim can apparently serve as tools for distinguishing RC^{str} from RC^{rel} *E. coli* strains even if these are prototrophic.

[3] Rosenkranz and Bendich (1964) compared polyphenylalanyl-tRNA formation directed by poly (U) in extracts from *E. coli* treated with 10^{-3} M hydroxylamine with that in extracts from cells not treated with hydroxylamine. The amount of polyphenylalanyl-tRNA formed in the extracts from hydroxylamine treated cells was less than 20% of that in the controls. It is possible that the discrepancy between the results of Rosenkranz and Bendich and ours is due to differences in the mode of treatment of the cells with hydroxylamine as well as in preparation of the extracts.

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Active Site of Hemerythrin. Iron Electronic States and the Binding of Oxygen*

J. Lyndal York† and Alan J. Bearden

ABSTRACT: Hemerythrin, a molecular oxygen-binding nonheme protein, found in marine organisms, binds a single oxygen molecule per protein subunit containing two Fe atoms.

The nature of the oxygen binding and the electronic state of Fe in oxy, deoxy, and oxidized forms of the protein subunits has been investigated by Mossbauer spectroscopy, magnetic susceptibility measurements, and by chemical means. Oxidized (met) hemerythrin derivatives show a single high-spin Fe(III) ($S = {}^5/_2$) site for the two Fe atoms; these sites are then spin coupled to give molecular diamagnetism. In deoxyhemerythrin the single environment is high-spin Fe(II) ($S = 2$) with no evidence of spin coupling as shown by susceptibility measurements. The oxy form of the protein shows two Fe environments, a result in contrast to a symmetrical oxo bridge or superoxide anion model. The relation of these physical studies to other studies and a discussion of oxygen binding in hemerythrin is given.

Hemerythrin is a nonheme iron protein which serves as a reversible oxygen carrier in the red cells of the brachipods and the sipunculids. The mechanism of reversible oxygen binding is of great interest *vis a vis* the oxygen binding of the heme proteins.

Klotz and coworkers (Klotz and Klotz, 1955; Klotz *et al.*, 1957; Klotz and Keresztes-Nagy, 1963; Keresztes-Nagy and Klotz, 1965; Groskopf *et al.*, 1966; Klapper and Klotz, 1968; Langerman and Klotz, 1969) have characterized the macromolecular properties of the protein.

Hemerythrin can be dissociated into eight subunits by sulfhydryl reagents; each subunit has a molecular weight of 13,500, contains two Fe atoms, and is capable of binding a single molecule of oxygen (Love, 1957; Boeri and Ghiretti-Magaldi, 1957). There is but a single cysteine residue per monomer unit; this residue is involved in subunit binding, not iron binding (Keresztes-Nagy *et al.*, 1965), and there is no "acid-labile" sulfide in contrast to the ferredoxins (Fry and San Pietro, 1962).

The oxidation state of the iron in various forms of hemerythrin has been in dispute as has the mechanism of reversible oxygen binding. Kubo (1953) on the basis of magnetic susceptibility measurements proposed a ferrous state for the iron in the oxygen-bound complex. Other workers have presented data which suggest a ferric–peroxy complex (Klotz and Klotz, 1955; Klotz *et al.*, 1957). These latter workers assayed the content of bivalent iron by color formation with *o*-phenanthroline after liberation of the iron from the protein by acid treatment. No ferrous iron was released from oxygenated hemerythrin. Deoxyhemerythrin was shown to contain 2 moles of ferrous and 0.4 mole of ferric iron per mole of protein. The conclusion was drawn that the ferrous deoxyhemerythrin was converted into a ferric oxyhemerythrin by oxygen binding. The rational and conclusions of these experiments have been challenged (Williams, 1955; Boeri and Ghiretti-Magaldi, 1957) on the basis that rapid oxidation of ferrous ion occurs at pH values below 3 or 4 (Roaf and Smart, 1923). Thus the question of the oxidation state of iron in the oxygenated hemerythrin and the nature of the oxygen iron complex has not been resolved although reasonable answers to several of these objections have been given (Keresztes-Nagy *et al.*, 1965). These workers have more recently presented Mossbauer and circular dichroic spectral data which has been interpreted as supporting the earlier conclusion of a ferric–peroxy complex for the oxygen compound (Garbett *et al.*, 1969; Okamura *et al.*, 1969).

In this paper nuclear γ-ray resonance spectroscopy (Mossbauer effect) and measurements of magnetic susceptibility are employed in order to investigate the active site of hemerythrin. A preliminary abstract of the work has appeared (York and Bearden, 1968).

Experimental Section

Preparation of Hemerythrin and Derivatives. The method of purification of hemerythrin was essentially that previously described (Florkin, 1933; Klotz *et al.*, 1957). The worms were cut; the contents squeezed out and filtered through cheesecloth. The red cells were washed free of white cells and a brown coagulum by centrifugation and resuspended in fresh sea water. To the packed red cells was added an equal

* From the Department of Biochemistry, University of Tennessee Medical Units, Memphis, Tennessee, and the Donner Laboratory, University of California, Berkeley, California. *Received June 22, 1970.*

† Present address: Department of Biochemistry, University of Arkansas School of Medicine, Little Rock, Arkansas. To whom correspondence should be addressed.