Inhibition of Hemoglobin Synthesis by Cyanate In Vitro

By Blanche P. Alter, Yuet Wai Kan, and David G. Nathan

Cyanate inhibits sickling and prolongs red cell survival in sickle cell anemia. However, cyanate markedly inhibits hemoglobin synthesis in vitro. Incorporation of radioactive amino acid into hemoglobin by human sickle reticulocytes or bone marrow and by rabbit reticulocytes (whole cell or cell-free lysate) was inhibited by as little as 2 mM cyanate and abolished by 50 mM. Both alpha- and beta-S chains were equally affected. The inhibition was only partially reversible by washing the cells after exposure to cyanate. Transport of radioactive amino acid into the cell was not impaired, and free intracellular amino acid was not carbamylated. Aminocyclation of transfer RNA was not inhibited; the acylated amino acid was not carbamylated. Examination of polysome patterns by sucrose density gradient centrifugation revealed degradation of polysomes to monosomes, suggesting inhibition of initiation of protein synthesis by cyanate. In a cell-free lysate, cyanate prevented hemin stimulation of initiation. We conclude that cyanate profoundly inhibits initiation of hemoglobin synthesis in vitro.

CYANATE PREVENTS in vitro sickling and prolongs the survival of sickle erythrocytes. Since cyanate carbamylates many proteins, abnormalities in red cell function following exposure to the chemical might be anticipated. In fact, it has already been shown that cyanate impairs the activity of certain red cell enzymes. In this communication, we present evidence that the concentrations of cyanate which inhibit in vitro sickling also depress hemoglobin synthesis in human reticulocytes and erythroblasts and in rabbit reticulocytes. Present evidence indicates that this toxic effect of cyanate is primarily due to interference by the drug with the initiation of hemoglobin synthesis.

MATERIALS AND METHODS

Reagents

Sodium cyanate was obtained from Pfaltz and Bauer, Flushing, N.Y. The commercial preparation was used directly, or recrystallized from ethanol and water. The fresh or recrystallized cyanate was dissolved to 0.5 M in either normal saline, or in Krebs-Ringer phosphate buffer pH 7.4 (KRP). 14C-leucine, 250 mCi/mmole, and 14C-lysine, 220 mCi/mmole, were purchased from New England Nuclear Corp., Boston, Mass. 35S-methionine, 23 Ci/mmole, was purchased from Amersham/Searle Corporation, Arlington Heights, Ill. Adenosine triphosphate (ATP), guanosine triphosphate (GTP), and creatine phosphokinase were obtained from Sigma Chemical Corp., St. Louis, Mo., and...
creatinine phosphate from Calbiochem, Los Angeles, Calif. Cycloheximide was obtained from Nutritional Biochemicals, Cleveland, Ohio, anisomycin from the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md., and rabbit liver transfer RNA from General Biochemical, Chagrin Falls, Ohio.

**Inhibition of Hemoglobin Synthesis**

The effect of cyanate on protein synthesis was determined in heparinized samples of peripheral blood (reticulocyte count 10%-25%) and bone marrow of patients with sickle cell anemia, bone marrow from other disorders, reticulocytes from hereditary spherocytosis, and in the reticulocyte-rich blood from phenylhydrazine-treated rabbits.

Incubations were performed in plasma or in KRP. For the latter, cells were thrice washed in KRP, then resuspended to the original hematocrit of 25% in the buffer, to which were added 2 mg/ml glucose, 30 µg/ml FeSO₄ · 7H₂O, and 19 essential amino acids minus the radioactive amino acid, to a final concentration of 0.25 mM. The mixture (usually 1 or 2 ml) was then incubated with vigorous shaking in room air at 37°C. Three microcuries of the radioactive amino acid per milliliter of incubation was added at varying times. Sodium cyanate, final concentrations of 1-100 mM, was added at variable times in relation to the labeled amino acid.

Protein synthesis was stopped by transferring 20-µl aliquots of the incubation mixtures into 1 ml of 0.1 N NaOH. This was incubated at 37°C for 15 min, precipitated with 5% trichloroacetic acid (TCA), collected on glass fiber filters (Reeve Angel, Clifton, N.J.), washed five times with 5% TCA, dried, and counted in a toluene scintillation fluid containing 0.5 g/100 ml, 2,5 diphenyloxazole (PPO) and 0.03 g/100 ml 1,4-bis {2 (5-phenyloxazolyl)}-benzene (POPOP). The counting was performed in a Packard Model 3375 liquid scintillation counter (Packard Instrument Company, Downers Grove, III.).

To evaluate the effect of cyanate on relative alpha- and beta-S globin synthesis, some incubations were terminated by dilution of the cells with ice-cold isotonic saline. The cells were thrice washed, lysed according to Lingrel and Borsook, and globin prepared by acid-acetone precipitation. The globin chains were then separated on carboxymethylcellulose columns in 8 M urea using phosphate buffer pH 6.7 of increasing ionic strength.

**Reversibility of Cyanate Inhibition**

One-milliliter aliquots of reticulocyte-rich blood were preincubated with varying concentrations (0-100 mM) of cyanate for 30 min, thrice washed in ice-cold saline, and resuspended to hematocrit 25% in autologous plasma without cyanate. For comparison, blood was preincubated without cyanate, washed, and resuspended in plasma containing 0-100 mM cyanate. ¹⁴C-leucine was added, incubations performed for 1 hr, and hemoglobin synthesis evaluated as described above.

**Amino Acid Transport**

One-milliliter aliquots of sickle reticulocyte-rich blood (hematocrit 25%) were incubated for 2 hr with ¹⁴C-leucine or lysine, in the presence or absence of 50 mM cyanate. The cells were washed three times in isotonic saline and hemolyzed. Equal amounts of the hemolysates were passed over Sephadex G-25 columns. 0.1-ml aliquots of the fractions were bleached with 0.5 ml of 30% H₂O₂, and counted in 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.).

**Amino Acid Carbamylation**

The effect of cyanate on carbamylation of intracellular amino acid was evaluated by pooling the radioactive fractions from the inclusion volumes of the above Sephadex columns. The samples were lyophilized and iontophoresed at 2.5 kV for 45 min in a pH 1.9 formic acid buffer, using a Varsol-cooled high-voltage electrophoresis apparatus (Gilson Medical Electronics, Model D, Middleton, Wis.). Radioactive areas were determined by radioautography with Kodak film RP/R54 (Eastman Kodak Company, Rochester, N.Y.), or the iontophoresis strips were cut into 4-inch samples and counted in toluene-PPO-POPOP. The positions of carbamylated amino acids were determined by parallel iontophoresis of a 10 : 1 mixture of cyanate with amino acid which had been incubated at 37°C for 1 hr.
Aminoacylation of Transfer RNA

The effect of cyanate on the aminoacylation of transfer RNA was evaluated by the use of rabbit liver transfer RNA and rabbit reticulocyte ribosome-free 1:1 lysate. 10, 20 or 50 mM cyanate was added to the lysate for a 10-min preincubation, or to the final incubation mixture. The reaction mixture consisted of 0.05 M Tris buffer pH 7.5, 0.01 M magnesium acetate, 0.001 M dithiothreitol, 0.001 M ATP, 0.005 mM of 19 amino acids minus leucine, 1 µCi 14C-leucine, 6 µg transfer RNA, and 10 µl lysate, in a final volume of 100 µl. Incubation was performed at 37°C for 15 min, then stopped by the addition of 1 ml of cold 5% TCA followed by filtration on Millipore filters (Millipore Filter Corp., Bedford, Mass.), which were dried and counted in toluene-PPO-POPOP. In some experiments, to determine the nature of the amino acid attached to the tRNA, the incubations were stopped by the addition of 1 ml of 0.01 M ethylenediamine tetraacetic acid (EDTA). The charged tRNA was extracted in equal volumes of phenol and dissolved in 2% sodium acetate, lyophilized, and hydrolyzed with 0.1 M NaOH at 37°C for 30 min. Carbamylation of the amino acid released from the charged tRNA was assessed by iontophoresis at 2.5 kV for 45 min, in a pH 4.7 pyridine : acetic acid : water 5 : 5 : 390 buffer.

Translation

Rabbit reticulocytes were washed and incubated with 35S-methionine as previously described.10 The cells were washed in ice-cold isotonic KRP and resuspended in KRP to a hematocrit of 25%. Two milligrams per milliliter glucose, 30 µg/ml FeSO4·7H2O, and 19 essential amino acids minus methionine, at a final concentration of 5 mM were added. Final volume was 2 ml. After preincubation with 1-20 mM cyanate at 37°C for 10 min, 35S-methionine was added. Five-microliter samples were removed at various times up to 30 min to assess uptake of 35S-methionine into protein, as described above.

At 12 min, most of the sample was removed into ice-cold isotonic saline which contained 50 µg/ml cycloheximide. The cells were thrice washed in this saline, and lysed with 3 volumes of a solution which contained 1 mM magnesium acetate, 0.1 mM EDTA, 50 µg/ml cycloheximide, and 0.2 mg/ml polyvinylsulfate. The membranes were removed by centrifugation for 10 min at 17,000 g; 0.75 ml of the lysate was layered atop a 36-ml 15%-50% linear sucrose density gradient. The gradient was centrifuged at 26,500 rpm in an SW 27 rotor for 6 hr at 4°C, in a Beckman Model L2-65B ultracentrifuge (Beckman Instruments Co., Palo Alto, Calif.). The gradients were pumped from the bottom at 3 ml/min, through a 5-mm quartz flow cell, in a Gilford Model 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 260 nm. One-milliliter fractions were collected, and 0.2-ml aliquots were added to 1 ml of water and counted in 10 ml Aquasol.

In some experiments, after 5 min of preincubation, 35S-methionine was added for 5 min, followed by 4 × 10⁻⁷ M anisomycin or 10 µg/ml cycloheximide for 5 min to prevent chain elongation and polysome run-off,10 followed again by 20 mM cyanate for 15 min. The polysome patterns were then examined as above.

Inhibition of Protein Synthesis in Cell-Free Lysates

Blood was obtained from a phenylhydrazine-treated anemic rabbit. The cells were washed three times in 0.14 M NaCl, 0.005 M KCl, 0.0015 M magnesium acetate, and then lysed with 1 volume of deionized water. The membranes were removed by centrifugation at 17,000 g for 15 min. The final incubation mixture contained in a total volume of 90 µl the following ingredients in the indicated final concentrations: 1 mM ATP, 0.2 mM GTP, 11 mM creatine phosphate, 5 enzyme units of creatine phosphokinase, 5 mM mercaptoethanol, 12 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) pH 7.0, 55 mM KCl, 2 mM magnesium acetate, 0.1 mM of 20 amino acids, 60 µl lysate, 0.5 µCi 14C-leucine. The incubation mixtures also contained either no hemin, or 3 × 10⁻⁵ M hemin, or hemin with or without 10, 50, or 100 mM cyanate. The reactions were incubated at 26°C or 36°C, and frequent samples of 5 µl were precipitated in TCA and counted as described above.

To determine whether the cyanate effect was on ribosomes or supernatant, two aliquots of rabbit reticulocytes were incubated at 37°C for 15 min, one in the presence of 50 mM cyanate, and one without cyanate. The cells were washed and lysed as above, and membranes removed. Five milli-
RESULTS

Inhibition of Hemoglobin Synthesis

Inhibition of hemoglobin synthesis in sickle reticulocytes increased with increasing concentrations of cyanate (Fig. 1). In a representative experiment, 5 mM cyanate decreased protein synthesis by 40%, 50 mM by 96%. In this experiment, the pH of the control incubation dropped from 7.6 to 7.2, while the pH in the 50 mM cyanate incubation fell similarly, from 7.7 to 7.5. The only protein synthesized at the higher concentration of cyanate appeared to be nonglobin, and the inhibition of alpha- and beta-S synthesis was equal (Table 1). When 50 mM cyanate was added to an aliquot of cells 20 min after exposure to \(^{14}\)C-leucine and during the linear phase of protein synthesis,

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**Fig. 1.** Sickle reticulocytes—inhibition of incorporation of \(^{14}\)C-leucine into acid-precipitable protein in the presence of increasing concentrations of cyanate. Incubation performed in KRP, at hematocrit 25%, incubation volume 2 ml. Ordinate represents units of 10,000 counts per min per 20-\(\mu\)l aliquot sampled.

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**Fig. 2.** Sickle bone marrow—effect of addition of 50 mM cyanate on the rate of incorporation of \(^{14}\)C-leucine into acid-precipitable protein. Incubation performed on whole uncentrifuged bone marrow, 1.0 ml for each. Cyanate added at time marked by arrow. Ordinate represents thousands of counts per min per 20-\(\mu\)l aliquot sampled.
INHIBITION OF HEMOGLOBIN SYNTHESIS

Table 1. Effect of Cyanate on Globin* Synthesis†

<table>
<thead>
<tr>
<th>mM Cyanate</th>
<th>Beta-S %</th>
<th>Alpha %</th>
<th>Nonglobin %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>45</td>
<td>45</td>
<td>55</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>1</td>
<td>25</td>
</tr>
</tbody>
</table>

*Globin chains isolated by CM-cellulose chromatography.
†Cells incubated in plasma, at hematocrit 25%, for 2 hr without or with cyanate. The results are expressed as per cent of the protein specific activity observed at 0 cyanate concentration.

Table 2. Protein Synthesis in Reticulocytes, Marrow, and Reticulocyte Cell-Free System in the Presence of Cyanate*

<table>
<thead>
<tr>
<th>System</th>
<th>Concentration of Cyanate, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>(A) Sickle whole blood</td>
<td></td>
</tr>
<tr>
<td></td>
<td>74%</td>
</tr>
<tr>
<td></td>
<td>70%</td>
</tr>
<tr>
<td>Mean</td>
<td>85%</td>
</tr>
<tr>
<td>(B) Sickle cells washed, resuspended in plasma</td>
<td></td>
</tr>
<tr>
<td></td>
<td>44%</td>
</tr>
<tr>
<td>Mean</td>
<td>35%</td>
</tr>
<tr>
<td>(C) Sickle cells in KRP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>61%</td>
</tr>
<tr>
<td>(D) Hereditary spherocytosis, whole blood</td>
<td></td>
</tr>
<tr>
<td></td>
<td>52%</td>
</tr>
<tr>
<td>(E) Rabbit reticulocytes in KRP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>83%</td>
</tr>
<tr>
<td>(F) Bone Marrow:</td>
<td></td>
</tr>
<tr>
<td>Sickle cell disease</td>
<td></td>
</tr>
<tr>
<td>Thalassemia intermedia</td>
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</tr>
<tr>
<td>Acute myelogenous leukemia, 1</td>
<td></td>
</tr>
<tr>
<td>2†</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>Acute myelogenous leukemia</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td>(G) Rabbit, cell-free</td>
<td></td>
</tr>
<tr>
<td></td>
<td>34%</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
</tr>
</tbody>
</table>

*Expressed as per cent of 14C-leucine incorporation into acid-precipitable material in the absence of cyanate. All incubations performed at hematocrit 25%, except bone marrows which were done as obtained. For all except G, cells were preincubated with cyanate for 10 min.
†Repeat study on same patient, 2 wk later.
Table 3. Reversibility of Cyanate Inhibition of Protein Synthesis

<table>
<thead>
<tr>
<th>mM Cyanate</th>
<th>Cyanate Present</th>
<th>Cyanate Washed Away</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>31</td>
<td>66</td>
</tr>
<tr>
<td>50</td>
<td>8</td>
<td>38</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>13</td>
</tr>
</tbody>
</table>

*Cyanate present throughout 1-hr incubation with 14C-leucine.
†Reticulocyte-rich blood preincubated with cyanate for 30 min, washed three times in saline, re-suspended to hematocrit 25% in autologous plasma, and incubated with 14C-leucine for 1 hr. Results expressed as per cent of counts per min per 20 μl observed at 0 cyanate concentration.

synthesis ceased completely within 10 min in sickle reticulocytes (data not shown) and 15 min in sickle bone marrow (Fig. 2).

Table 2 represents a summary of the effect of varying concentrations of cyanate on protein synthesis in different reticulocyte or marrow systems. In all of the systems except the cell-free ones, whole cells were incubated with cyanate for 10 min before 14C-leucine was added. The extent of inhibition of protein synthesis by cyanate varied from one system to another, but within each system the inhibition was proportional to the concentration of cyanate in the incubate.

Reversibility of Cyanate Inhibition

When cells were preincubated with cyanate and then thrice washed prior to incubation with labeled amino acid, inhibition of protein synthesis remained evident (Table 3).
Table 4. Carbamylation of Amino Acid

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Counts per Minute*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Noncarbamylated</td>
</tr>
<tr>
<td>(A) Intracellular lysine</td>
<td>9453</td>
</tr>
<tr>
<td>(B) Leucine on tRNA</td>
<td>621</td>
</tr>
</tbody>
</table>

*Total counts per minute in the region of the electrophoresis paper comprising the designated amino acid.

(A) designates electrophoresis of the small molecular weight peak from the Sephadex G-25 column elution of lysate following incubation of whole sickle blood with 50 mM cyanate for 2 hr.

(B) designates electrophoresis of amino acid released from tRNA by alkali hydrolysis. Rabbit liver tRNA was charged with 14C-leucine in the presence of cyanate.

After preincubation with 100 mM cyanate, less than 15% of the synthetic capacity was regained, and after 20 mM cyanate 66% of protein synthesis was regained.

**Amino Acid Transport**

As shown in Fig. 3, the quantity of amino acid transported into the red cells, but not incorporated into hemoglobin, was essentially unchanged by incubation with 50 mM cyanate, although protein synthesis was severely impaired.

**Amino Acid Carbamylation**

Iontophoresis of the intracellular amino acid peak eluted from the Sephadex G-25 columns above demonstrated that over 80% of the amino acid was not carbamylated (Table 4).

**Aminoacylation of Transfer RNA**

Preincubation of the rabbit lysate with cyanate, or addition of cyanate to the final incubation mixture, did not interfere with the charging of rabbit liver transfer RNA (Table 5). Iontophoresis revealed that the amino acid which was attached to the transfer RNA was not carbamylated (Table 4).

**Translation**

The pattern of the polysomes separated by sucrose density gradient centrifugation from rabbit reticulocytes undergoing active protein synthesis is presented in Fig. 4A. As shown by the radioactivity peaks, most of the protein was synthesized on the heavier polysomes, consisting of four to seven ribosomes. Incubation of reticulocytes with as little as 1-2 mM cyanate caused some degradation of the polysomes. Both the

Table 5. Incorporation of 14C-leucine into Transfer RNA Following Incubation With Cyanate

<table>
<thead>
<tr>
<th>Concentration of Cyanate, mM</th>
<th>Counts per minute per 6 µg transfer RNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1583</td>
</tr>
<tr>
<td>10</td>
<td>2546</td>
</tr>
<tr>
<td>20</td>
<td>3461</td>
</tr>
<tr>
<td>50</td>
<td>5431</td>
</tr>
</tbody>
</table>

*Rabbit lysate was incubated with cyanate for 10 min, followed by 15-min incubation with tRNA. Counts per minute represent incorporation of 14C-leucine into cold TCA-precipitable material.
optical density and radioactivity showed a decline in the number of large polysomes, and an increase in disomes and monosomes (data not shown). Twenty millimolar cyanate led to almost complete breakdown of polysomes to disomes and monosomes (Fig. 4B). Incubation of reticulocytes with $4 \times 10^{-7} M$ anisomycin led to an accumulation of large polysomes, with the appearance of optical density and radioactivity in the region of octasomes and septasomes (Fig. 4C). Addition of cyanate to cells pre-incubated with anisomycin did not lead to destruction of the large polysomes (Fig. 4D). Thus, anisomycin prevented the previously demonstrated breakdown of polysomes to monosomes in the presence of cyanate. The effect of cycloheximide (not shown) was similar to that of anisomycin.

**Inhibition of Protein Synthesis in Cell-Free Lysates**

The incorporation of radioactive amino acid into protein by a cell-free lysate at 26°C was linear for 5 min, and then essentially ceased in the absence of hemin (Figs. 5A and 5B). In the presence of $3 \times 10^{-5} M$ hemin, linearity persisted for over 45 min. This stimulatory effect of hemin was reduced when 50 mM cyanate was also present in the reaction mixture. When 100 mM cyanate was added, protein synthesis was inhibited to a level below that of control. At this temperature both concentrations of cyanate diminished the initial rate of protein synthesis to less than the initial rate of the control or of the hemin-stimulated system (Fig. 5A). This effect of cyanate occurred in the presence or absence of hemin (Fig. 5B). At 36°C cyanate did not significantly alter the initial rate of accumulation of radioactive leucine into protein. However marked deviation from hemin-stimulated linearity occurred after 3 min in the
INHIBITION OF HEMOGLOBIN SYNTHESIS

Fig. 5. Whole lysate—rate of incorporation of 14C-leucine into acid precipitable protein. Ordinate represents thousands of counts per min per 5 µl aliquot. Incubation volume 90 µl. (A) Effect of 50 and 100 mM cyanate on hemin stimulation of protein synthesis at 26°C. (B) Effect of 50 mM cyanate on protein synthesis at 26°C in the presence and absence of hemin. (C) Effect of 10 and 50 mM cyanate on hemin stimulation of protein synthesis at 36°C. (D) Effect of 50 mM sodium chloride and cyanate on hemin stimulation of protein synthesis at 36°C.

The results of recombination of supernatant and ribosomes from cells preincubated with and without cyanate are shown in Fig. 6. When supernatant and ribosomes from cells preincubated without cyanate were recombined, the rate of protein synthesis was linear for 5 min, after which the slope changed but continued to rise for at least 45 min. The cyanate-treated supernatant and ribosomes when recombined had lower initial and subsequent rates of synthesis. The combinations of supernatant from normal cells and ribosomes from cyanate-treated cells, and vice versa, demonstrated intermediate rates of amino acid incorporation.

Fig. 6. Recombination of supernatant and ribosomes of cells incubated with (CNO⁻) and without (norm.) 50 mM cyanate. Rate of incorporation of 14C-leucine in the presence of hemin into acid-precipitable protein by the recombinations designated in the figure. Ordinate represents thousands of counts per min per 5-µl aliquot. Incubation volume 60 µl.
DISCUSSION

The efficacy of cyanate in the inhibition of in vitro sickling, and in the prolongation of sickle erythrocyte survival has led to investigations of its use in the treatment of patients with sickle cell disease. Since cyanate induces carbamylation of a wide variety of proteins, investigations of its toxicity have been pursued in several laboratories. Mild hemolysis induced by cyanate has been reported by Diederich and co-workers, while others have demonstrated a slight decrease in the activity of certain of the enzymes of the glycolytic pathway in carbamylated sickle erythrocytes. We report here a hitherto undescribed effect of cyanate, i.e., marked interference with hemoglobin synthesis in vitro.

Cyanate interfered with hemoglobin synthesis in all of the systems examined: Reticulocytes in sickle cell disease, hereditary spherocytosis, and phenylhydrazine-treated rabbits; bone marrow in several disorders; lysates of rabbit reticulocytes; and recombined rabbit lysates and ribosomes. Whereas the effect of cyanate was variable from one system to another, inhibition of protein synthesis was proportional to cyanate concentration within a given system. As little as 1 mM cyanate had some effect on protein synthesis in the intact cell (Table 2). This concentration is considerably less than the concentration necessary to produce inhibition of sickling in vitro.

The kinetics of cyanate inhibition revealed a constant time requirement of approximately 10-15 min before cyanate concentrations of less than 20 mM produced inhibition. The inhibition then tended to be progressive. We assume that a critical degree of carbamylation was required to achieve irreversible inhibition. Part of the inhibition was reversible, and this reversible component may have been due to rapid but reversible sulfhydryl blockade rather than irreversible amino group carbamylation by cyanate.

Investigation of the effects of cyanate on the various stages involved in protein synthesis was performed. Cyanate does not interfere with red cell glucose transport or lactate production, and we did not detect interference by cyanate with amino acid transport into red cells (Fig. 3). In addition, there was a large amount of radioactive amino acid inside the cell which was free of carbamylation.

Aminoacylation of transfer RNA actually increased with increasing concentrations of cyanate (Table 5). Cyanate may have caused miscoding of leucine onto a transfer RNA species which normally does not accept leucine. On the other hand, cyanate may have increased the amount of leucine accepted by leucine specific RNA, perhaps by the improvement of a previously inefficient reaction or by decreasing the turnover of tRNA-leu in the system.

At 37°C the defect in protein synthesis due to cyanate appears to be predominantly at the level of initiation of translation. Chemical agents which have been demonstrated to inhibit initiation more than elongation, such as sodium fluoride, pactamycin, and aurintricarboxylic acid lead to degradation of polyribosomes into monosomes. By contrast, drugs which interfere with chain elongation more than initiation, such as cycloheximide and anisomycin cause an accumulation of heavier polyribosomes. When the two types of compounds are combined, impairment of chain completion predominates, preventing the monosome accumulation which is caused by inhibition of initiation.

Cyanate causes polysome patterns that resemble those produced by inhibitors of initiation. Monosomes accumulate to an extent dependent on the dose of cyanate.
INHIBITION OF HEMOGLOBIN SYNTHESIS

used. Complete inhibition of protein synthesis by cyanate is accompanied by nearly complete degradation of polysomes to monosomes. Pretreatment of cells with anisomycin or cycloheximide, both of which inhibit chain completion, prevents this accumulation of monosomes. These polysome patterns suggest that at 37°C cyanate inhibits initiation more than it interferes with elongation.

The results of cyanate admixture with a reticulocyte cell-free system also indicated that initiation of hemoglobin synthesis is impaired by the drug. Several investigators have shown that hemin stimulates protein synthesis in unfractionated cell-free systems and that it stabilizes polysomes. We have confirmed the stimulatory effect of hemin and have also demonstrated the prevention of the hemin effect by cyanate. The use of 100 mM cyanate in the cell-free system with hemin reduced protein synthesis to a level less than in the control lysate without hemin, perhaps due to interference with the small amount of initiation which occurs in a hemin-free system.

At 26°C the effect of cyanate on protein synthesis was the same in the presence or absence of hemin; it impaired the immediate rate of uptake of labeled leucine into protein, a phase of the synthesis curve which predominantly represents chain elongation. It also inhibited initiation as demonstrated by the continual change in the slope of the synthetic curve. At 36°C, a temperature at which chain completion is much more rapid, the effect of cyanate was most marked on the initiation of new chains. Thus the data derived from polysome stability and cell-free studies indicate that cyanate inhibits initiation more than chain elongation at physiologic temperature.

Examination of supernatant and ribosomes from cells which had been incubated with 50 mM cyanate revealed a detrimental effect on both components. The impairment of ribosome function by cyanate might be due to carbamylation of ribosomal proteins. The effect on the supernatant might be due to residual cyanate which was incompletely washed away or to carbamylation of supernatant proteins.

In this communication we present evidence that cyanate inhibits hemoglobin synthesis in vitro in concentrations which are less than those required to prevent sickling in vitro, and which are much less than the concentrations used to date to demonstrate increased sickle red cell longevity. The relevance of these in vitro findings to the clinical use of cyanate is not established and will require examination of the effects of cyanate on protein synthesis in vivo. A recent report concerning a deleterious effect of cyanate on learning processes in rodents suggests that nonhematopoietic protein function or synthesis might be impaired by this agent. It is conceivable that the protein synthetic machinery in cells may be spared during progressive carbamylation of red cells by oral ingestion of cyanate. If so, the findings reported here may relate only to sudden high-dosage administration of cyanate to experimental animals and not to the therapeutic situation in man.

ADDENDUM

A recent report by Habib, M., Watson, V., and Schwartz, E. (Blood 41:635, 1973) has also shown suppression of hemoglobin synthesis by cyanate in vitro.

ACKNOWLEDGMENTS

We thank Dr. Harvey Lodish for the generous gifts of cycloheximide, anisomycin, and rabbit liver transfer RNA, and for his stimulating discussions. We are grateful to Dr. Anthony Cerami for kindly providing recrystallized sodium cyanate.
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