ADVANCES IN ENZYMEOLOGY
AND RELATED AREAS OF MOLECULAR BIOLOGY

Founded by F. F. NORD

Edited by ALTON MEISTER
CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK

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ADVANCES IN ENZYMEOLOGY
AND RELATED AREAS OF
MOLECULAR BIOLOGY

Volume 40
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This volume of Advances in Enzymology is dedicated to the memory of Friedrich F. Nord, who founded the series in 1941 and edited the first 34 volumes. Nord was Professor of Organic Chemistry and Enzymology at Fordham University. He was an international authority on the biosynthesis and the degradation of lignin. His work included important contributions to the metabolism and enzymology of molds, and key discoveries on the biochemistry of wood. Nord published more than 400 scientific papers and wrote three books. Fordham University honored him by establishing the F. F. Nord Lectures in Biochemistry. His contributions to science are continued, not only through his numerous doctoral students, many of whom are now noted scientists, but also through the publications that he founded—the Archives of Biochemistry and Biophysics and the Advances in Enzymology.

Alton Meister
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I. Introduction

Sickle-cell anemia, first described by Herrick in 1910 (1), is a genetic disease that results in the synthesis of an abnormal hemoglobin molecule (2,3). The substitution of a valine residue for a glutamic acid residue at the sixth position of the \( \beta \)-chains of hemoglobin S (4) leads to profound changes in the solubility of the deoxygenated protein (5). Erythrocytes from individuals with sickle-cell disease do not retain their biconcave discoid shape on partial deoxygenation (6); such cells appear in the peripheral circulation in a variety of distorted forms, including the sickle-shaped cell. The clinical manifestation of sickle-cell
disease occurs when these sickled cells occlude the capillaries, thus depriving the tissues of their necessary supply of oxygen.

The reasons for the striking difference between the properties of deoxyhemoglobins A and S, where only 2 of the 574 amino acid residues of the tetrameric molecule have been altered, are not understood. Several proposals suggesting that complementary stacking of tetramers of deoxyhemoglobin S within the red cell results in cell sickling have been made (7,8), but definitive experimental data supporting these proposals are not available. Insight into the three-dimensional structure of deoxyhemoglobin S by X-ray diffraction techniques has been delayed by difficulty in obtaining material suitable for study, but recent studies (9,10) offer some hope that the structure of deoxyhemoglobin S may soon be solved.

Cyanate prevents the sickling of cells from patients with sickle-cell disease by carbamylating hemoglobin S at its NH2-terminal valine residues (11). This chapter describes the properties of carbamylated hemoglobin S: the specificity of cyanate for the NH2-terminal residues of hemoglobin, the biological functions of the red cell after treatment with cyanate in vitro, and the physiological properties of the carbamylated red cell in vivo.

II. Effects of Cyanate on the Solubility of Deoxyhemoglobin S and the Sickling of Erythrocytes

Deoxyhemoglobin S is about 100 times less soluble than deoxyhemoglobin A in concentrated phosphate buffers (5). Allison (12) has shown that the viscosity of isolated deoxyhemoglobin S, at a concentration approaching that in the intact erythrocyte, is so much greater than that of deoxyhemoglobin A that deoxyhemoglobin S forms a gel under these conditions (Fig. 1). This and other similar studies have formed the basis for the hypothesis that the abnormal form of the deoxygenated S/S cell (Fig. 2) can be traced directly to the insolubility of the abnormal protein that forms gellike aggregates within the deoxygenated red cell.

The proposal (8) that the deoxygenated hemoglobin S tetramer contains an additional hydrophobic bond between the valine residue at the NH2-terminus of the β-chain and the valine residue at the sixth position of the β-chain of hemoglobin S led to the clinical trials of large amounts of urea as a treatment for sickle-cell disease (13). The rationale for the clinical use of urea (prevention of the formation of
PROPERTIES OF CARBAMYLATED HEMOGLOBIN S

Fig. 1. Hemoglobin S (200 mg/ml) was deoxygenated by equilibration with a mixture of 90% N₂ and 10% CO₂ for 5 min at 0°C. The tube was incubated at 37°C, and after 3 hr the presence or absence of a gel was determined. From Cerami and Manning (11).

The formation of cyanate in urea solutions and the potential reactivity of cyanate with functional groups of proteins (15), which prompted us to investigate the possible role of cyanate as an inhibitor of red-cell sickling, has been the main stimulus for our studies (11). We found that incubation of oxyhemoglobin S in vitro with low concentrations of cyanate inhibited the subsequent gelling of the protein on deoxygenation (Fig. 3) and prevented the sickling of 60–80% of the deoxygenated red cells in vitro (Fig. 4). Thus carbamylation of sickle-cell erythrocytes results in preservation on deoxygenation of normal cell form in most of the cells, approaching that found in a population of oxygenated sickle cells (Fig. 5).

From a comparison of the relative effects of cyanate and urea in vitro (11) we concluded that about 10–100 times more urea than cyanate is necessary to prevent both sickling of red cells and gelling of isolated deoxyhemoglobin S (Tables I and II). In addition, carbamylation of hemoglobin with cyanate is a time-dependent, irreversible reaction, whereas the effect of urea of these cells is immediate and completely
Fig. 2. A suspension of sickle-cell erythrocytes in phosphate-buffered saline solution was deoxygenated by evacuation at 30 mm Hg with a water aspirator for 7 min at 37°C. After an additional 5 min at 37°C the cells were fixed rapidly by dilution with buffered formalin (11). The micrographs were taken by Dr. James Jamieson of Rockefeller University with a Zeiss microscope with Nomarski differential interference contrast optics (×800). From Cerami and Manning (11).

reversible. Thus the mechanism of the inhibition of red-cell sickling in vitro is different for both compounds, and we chose to investigate in detail the mechanism by which the carbamylation of hemoglobin S by cyanate prevents the gelling of the deoxygenated protein and the subsequent sickling of the red cell.

III. Carbamylation of Hemoglobin by Cyanate

A. SPECIFICITY OF CYANATE FOR THE NH₂-TERMINAL RESIDUES OF HEMOGLOBIN

The reactive tautomer of cyanate is isocyanic acid, HN==C==O (16), and the electrophilic carbon atom of this compound can undergo nucleophilic attack by several functional groups of proteins. Stark and
Smyth (17–20) made a comprehensive study of the carbamylation of the functional groups of amino acid residues in proteins. The sulfhydryl groups of cysteine residues react with cyanate, as does the phenolic oxygen of tyrosine residues, the imidazole nitrogen of histidine residues, and the carboxyl groups of aspartic and glutamic acid residues. However, at pH 7.4 with low concentrations of reactants, the equilibria of these reactions are not in favor of the product, but rather toward the free functional group of the amino acid residue. In general, only the amino groups of proteins are irreversibly carbamylated. [The hydroxyl group of a serine residue at the active site of chymotrypsin is irreversibly carbamylated, leading to inactivation of the enzyme, but this is the only reported example of the irreversible carbamylation of hydroxyl groups (21).]

Stark (16) and Smyth (19) have carried out extensive studies on the mechanism of the carbamylation of NH₂-groups, and their results indicate that it is the unprotonated form of the NH₂-group that is carbamyl-
Fig. 4. Oxygenated sickle-cell erythrocytes were treated with 30 mM KNCO for 1 hr at 37°C and then deoxygenated as described in Fig. 2. From Cerami and Manning (11).

Fig. 5. Oxygenated sickle-cell erythrocytes. From Cerami and Manning (11).
**TABLE I**

Effect of Urea and KNCO on the Sickling of Deoxygenated Erythrocytes*.b

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Compound</th>
<th>Concentration during incubation (M)</th>
<th>Concentration during deoxygenation (M)</th>
<th>Normal deoxygenated cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>Urea</td>
<td>1.0</td>
<td>1.0</td>
<td>69</td>
</tr>
<tr>
<td>3</td>
<td>Urea</td>
<td>—b</td>
<td>1.0</td>
<td>70</td>
</tr>
<tr>
<td>4</td>
<td>Urea</td>
<td>1.0</td>
<td>0.1</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>Urea</td>
<td>0.1</td>
<td>0.1</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>KNCO</td>
<td>0.1</td>
<td>0.01</td>
<td>72</td>
</tr>
<tr>
<td>7</td>
<td>KNCO</td>
<td>0.01</td>
<td>0.001</td>
<td>34</td>
</tr>
</tbody>
</table>

*a From Cerami and Manning (11).

b Oxygenated erythrocytes (2 μmoles hemoglobin S per milliliter) were incubated for 1 hr at 37°C. The cells were then diluted into phosphate-buffered saline solution and deoxygenated by evacuation at 30 mm Hg at 37°C. An oxygenated sample had 93% normal cells.

c The cells in experiment 3 were deoxygenated immediately.

**TABLE II**

Effect of Urea and KNCO on the Gelling of Deoxyhemoglobin S*.b

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>Gelling</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>KNCO</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>Urea</td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>Urea</td>
<td>50</td>
<td>+</td>
</tr>
<tr>
<td>Urea</td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>

*a From Cerami and Manning (11).

b Oxygenated hemoglobin S (200 mg/ml; 0.2 ml) was incubated with KNCO or with urea for 1 hr at 37°C. The contents of the tubes were gassed with a mixture of 90% N₂ and 10% CO₂ for 5 min at 0°C. The tubes were incubated at 37°C; after 3 hr the presence or absence of a gel was determined.
lated by isocyanic acid (Diagram I). It follows then that the lower the $pK_a$ of the NH$_2$-group, the greater will be its rate of carbamylation.

\[
\begin{align*}
\text{O} = \text{C} = \text{O} & \quad + \quad \text{H}_2\text{N}-\text{R} \\
\downarrow & \quad \downarrow \\
\text{HNN} = \text{C} = \text{O} & \quad + \quad \text{H}_2\text{N}-\text{R} \\
\text{H} & \quad \text{H} \\
\text{OOC} = \text{N} = \text{R} + \text{H}^+ & \quad \text{H}_2\text{N} = \text{C} = \text{N} = \text{R} + \text{H} \\
\text{H} & \quad \text{O}
\end{align*}
\]

Diagram I

The $pK_a$ of the NH$_2$-terminal valine residues of the α-chain of hemoglobin A has been reported to be 6.7 (22). This is an unusually low value for the $pK_a$ of the NH$_2$-terminal residue of a protein; the reported values have been in the range 7.5–8.0. Thus, at a physiological pH of 7.4, about 10 times as many of the NH$_2$-terminal groups of hemoglobin would be in the unprotonated form compared with the NH$_2$-terminal residues of a protein, where such groups had a $pK_a$ of 7.7. It is the anomalously low $pK_a$ of the NH$_2$-terminal amino groups of hemoglobin that confers on this protein its special affinity for carbon dioxide and, as will be discussed below, for cyanate as well. The ε-NH$_2$-groups of lysine residues have $pK_a$ values in the range 9–10, so that at physiological pH practically all of these NH$_2$-groups would be in the protonated form.

The incorporation of $^{14}$C]cyanate into hemoglobin S parallels the number of cells that maintain their normal discoid form on deoxygenation. This relationship was found to be a function of the concentration of cyanate (Fig. 6) as well as the extent of time that cyanate was in contact with the cells (Fig. 7), but the amount of carbamylation necessary to prevent 50% of the cells from sickling varied with the cells from different patients (11); this phenomenon is still under study. Once cyanate had been incorporated into hemoglobin in the red cell, it was not removable by extensive washing or dialysis (11). These results led us to consider the irreversible carbamylation of amino groups as being responsible for the antisickling property of cyanate. Indeed, we were able to show (Table III) that at relatively low levels of car- bamylation, 80–90% of the $^{14}$C]cyanate incorporated into the cells could be accounted for by carbamylation of the NH$_2$-terminal valine
Fig. 6. The effect of KNCO on deoxygenated sickle-cell erythrocytes. Suspensions of oxygenated erythrocytes (0.5 ml) were incubated at 37°C with the designated amount of $[^{14}\text{C}]$KNCO (3.5 x $10^4$ dpm/µmole). At the end of 1 hr, aliquots were removed for deoxygenation and determination of radioactivity. The percentage of normal oxygenated cells was the same (80%) in the presence or absence of KNCO; the remaining 20% of these cells are irreversibly sickled; that is, they are of abnormal form after oxygenation. On deoxygenation, 17% of the cells are normal in form. From Cerami and Manning (11).

### TABLE III
The Site of Carbamylation of Hemoglobin S by Cyanate*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$[^{14}\text{C}]$KNCO incorporated (mole/mole HbS)</th>
<th>Carbamylation (mole/mole HbS) at NH$_2$-terminal valine</th>
<th>ε-NH$_2$ of lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.6</td>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>1.2</td>
<td>0</td>
</tr>
</tbody>
</table>

* From Cerami and Manning (11).
Fig. 7. The kinetics of carbamylation and the increase in normal deoxygenated sickle-cell erythrocytes. Suspensions of oxygenated erythrocytes (2.0 ml) were incubated at 37°C with 0.01 M [14C]KNCO (3.1 × 10^4 dpm/μmole). At the indicated times aliquots were removed for deoxygenation and determination of radioactivity. From Cerami and Manning (11).

residues of hemoglobin S and that there was no detectable carbamylation of the ε-NH₂-groups of lysine residues.

B. KINETIC STUDIES ON THE CARBAMYLATION OF HEMOGLOBIN S

The difference in the rates of carbamylation of the amino groups of the NH₂-terminal valine residues of hemoglobin S and ε-NH₂-groups of lysine residues is clearly shown by the results of the experiment described in Figure 8. In vitro carbamylation of isolated oxyhemoglobin at pH 7.4 and 37°C with fairly high concentrations of cyanate reveals a distinct triphasic rate profile when examined over an extensive incubation period (23). The initial phase of the reaction can be ascribed almost predominantly to carbamylation of the NH₂-terminal valine residues of the α- and β-chains of hemoglobin up to a level of about 1.5 carbamyl groups per hemoglobin tetramer. The third phase of the reaction represents carbamylation of the ε-NH₂-groups of lysine residues up to a point where nearly half the total protein NH₂-groups have been carbamylated. The intermediate stage of the reaction repre-
Fig. 8. Carbamylation of the $\alpha$- and $\epsilon$-NH$_2$-groups of hemoglobin S. Oxyhemoglobin S (final concentration 0.48 mM) was mixed with $[^{14}C]$NaNCO (final concentration 0.19 M) at pH 7.4 and 37°C. Portions of the reaction mixture were removed at the indicated intervals and placed into cold 5% trichloroacetic acid. The amount of radioactivity was determined after oxidation of the samples. From Lee and Manning (23).

....

sents carbamylation of two to three NH$_2$-terminal valine residues and nonspecific carbamylation of a few of the lysine residues. Autoradiography of a tryptic digest of oxyhemoglobin containing five $[^{14}C]$carbamyl groups per hemoglobin tetramer revealed only two radioactive peptides, whose amino acid compositions indicated that they were derived from the NH$_2$-terminal segments of the $\alpha$- and $\beta$-chains of the protein (23). Hence the lysine residues that are carbamylated are distributed randomly throughout the hemoglobin molecule. If one takes into account the fact that there are 44 lysine residues and 4 NH$_2$-terminal valine residues in hemoglobin, then it can be calculated from the first phase of the reaction described in Figure 8 that the NH$_2$-terminal valine residues of hemoglobin S are carbamylated 50–100 times more rapidly than the $\epsilon$-NH$_2$-groups of lysine residues. This result is in close agreement with what one would have predicted about the relative rates of carbamylation from knowledge of the $pK_a$ values of the two
types of NH₂-group. The fact that one can achieve this specificity in vitro with whole cells (11) at 1-10 mM concentrations of cyanate is probably an indication that the intracellular concentration of cyanate never reaches a level at which lysine residues can be significantly carbamylated.

The specificity of the carbamylation of the NH₂-terminal valine residues of hemoglobin with cyanate can also be demonstrated in vivo with experimental animals (Table IV). For a single dose of [¹⁴C]cyanate injected intraperitoneally into a mouse, the most extensive carbamylation takes place with the hemoglobin in the red cell; the total serum proteins are carbamylated about one-fifteenth the extent of total hemoglobin in vivo. The preferential carbamylation of the NH₂-terminal residues of hemoglobin is undoubtedly due to the anomalously low pKₐ of these residues in the hemoglobin tetramer; the serum proteins have pKₐ values that do not favor extensive carbamylation in vivo.

Njikam et al. (24), in studies with whole blood (S/S) in vitro, have found that with oxygenated erythrocytes both the α- and β-chains of hemoglobin are carbamylated to nearly the same extent. With deoxyhemoglobin, however, the α-chain was carbamylated 1.7 times more than the β-chain in vitro. When the blood samples from patients on oral cyanate therapy were examined for the distribution of carbamyl

<table>
<thead>
<tr>
<th>Organ</th>
<th>Percentage of Injected Dose</th>
<th>Percentage of Injected Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>0.08</td>
<td>Bones</td>
</tr>
<tr>
<td>Lungs</td>
<td>0.05</td>
<td>Muscle</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.06</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.01</td>
<td>Serum proteins</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.05</td>
<td>Subtotal</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>0.17</td>
<td>Urine</td>
</tr>
<tr>
<td>Skin</td>
<td>0.8</td>
<td>Expired as ¹⁴CO₂</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total recovery</td>
</tr>
</tbody>
</table>

* From Cerami et al. (39).

b Female mouse (B₄D₄) injected with 10 μmoles of [¹⁴C]NaNCO.
TABLE V

Distribution of Carbamyl Groups on the NH₂-Terminal Residues of Hemoglobin
After Carbamylation in Vitro and in Vivo

<table>
<thead>
<tr>
<th>Percentage of O₂ saturation</th>
<th>Ratio of α/β chain carbamylation</th>
<th>In vitro</th>
<th>In vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>95</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.7</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>

* From Njikam et al. (24).

groups between the hemoglobin chains, Njikam et al. concluded that most, if not all, of the carbamylation that occurred in vivo was with deoxyhemoglobin (Table V).

C. ANALOGY OF ISOCYANATE AND CARBON DIOXIDE

The reactive species of cyanate, isocyanic acid (HN==C==O), bears a close chemical similarity to the structure of carbon dioxide (O==C==O). As shown in Diagram I, both compounds can undergo nucleophilic attack by an unprotonated amino group. The work of Rossi-Bernardi and Roughton (25) has suggested that from 30 to 50% of the blood carbon dioxide is carried in the form of carbamino compounds on the NH₂-terminal residues of hemoglobin. From our studies it has been clear that cyanate has a specificity for these same NH₂-terminal residues of hemoglobin in that these residues are carbamylated preferentially both in vitro and in vivo compared with the other NH₂-groups of hemoglobin. These experimental findings led us to the consideration of cyanate as a structural analog of carbon dioxide (11). However, there are three notable differences in these reactions. First, whereas carbamino formation is a reversible chemical reaction because of the relative ease of carbon dioxide regeneration, the carbamylation of amino groups with cyanate is an irreversible reaction. Indeed the stability of the carbamyl bond is the basis of a method developed by Stark and Smyth (26) for the quantitative determination of the NH₂-terminal residues of a protein with cyanate. The second essential difference in the product of these reactions is that carbamino formation results in the formation of a negatively charged product, whereas carbamylation yields an uncharged derivative. The third difference, as
discussed below, is that the carbamino derivative of hemoglobin has a lowered affinity for oxygen, whereas carbamylated hemoglobin has an increased oxygen affinity (27,28).

Cyanate preferentially carbamylates deoxyhemoglobin compared with liganded hemoglobin (Fig. 9). Since carbon dioxide has been shown to bind preferentially to the deoxy form of hemoglobin (29), these results provide direct experimental support for the suggestion that cyanate and carbon dioxide are indeed structural analogs. In the

![Fig. 9. Carbamylation rates of liganded hemoglobin and deoxyhemoglobin S. Inhibition of carbamylation by CO2. From Lee and Manning (23).](image-url)
presence of increasing amounts of carbon dioxide as bicarbonate, carbamylation of deoxyhemoglobin is decreased much more than that of liganded hemoglobin (Fig. 9). Thus carbon dioxide competes more effectively with the carbamylation of deoxyhemoglobin than it does with liganded hemoglobin—further evidence of the chemical analogy of isocyanate and carbon dioxide.

IV. Biological Functions of Erythrocytes Partially Carbamylated in Vitro

The relative specificity of cyanate for the NH$_2$-terminal amino groups of hemoglobin S described in Section III.A has raised two main questions concerning the possible deleterious physiological effects of cyanate on erythrocyte function and metabolism:

1. How are the three main functions of the NH$_2$-terminal amino groups of hemoglobin affected by partial carbamylation (i.e., capacity to carry CO$_2$, binding of 2,3-diphosphoglycerate, and uptake of Bohr protons on deoxygenation)?

2. What is the effect of cyanate on the levels of essential metabolites and enzymes within the erythrocyte?

We have carried out studies designed to answer these questions. The in vitro studies were done under conditions (1-h incubation with 10 mM cyanate at pH 7.4 and 37°C) that yielded 0.7-1.0 carbamyl group per hemoglobin tetramer. These conditions had been shown to be effective in the inhibition of sickling for 60-80% of the erythrocytes in vitro at 6 mm of O$_2$ tension (11). Thus an average of three NH$_2$-terminal valine residues per hemoglobin tetramer would remain free for maintaining the essential functions of hemoglobin.

The carbon dioxide–cyanate analogy described in the preceding section led to the question of whether the partially carbamylated erythrocyte would retain its capacity to transport carbon dioxide. As shown in Table VI, there is a slightly decreased capacity of the partially carbamylated red cell to carry carbon dioxide, but the plasma can compensate for this decrease by carrying more than its usual load of bicarbonate. Thus the ability of whole blood to carry carbon dioxide in vitro (either as CO$_2$ or bicarbonate) is not reduced by partial carbamylation.
Since the studies of Bunn and Briehl (30), of Perutz (31), and of Arnone (32), have shown that the positively charged NH$_2$-terminal residues of the $\beta$-chain of deoxyhemoglobin are the primary binding site for 2,3-diphosphoglycerate, we thought that it was important to investigate whether carbamylation of about 25% of the NH$_2$-terminal residues would lead to a significant decrease in the binding of 2,3-diphosphoglycerate. As shown in Table VII, the reduction in the oxygen affinity of hemoglobin mediated by 2,3-diphosphoglycerate is not significantly reduced by partial carbamylation. When inosine,

**TABLE VI**

Carbon Dioxide Capacity of Blood After Carbamylation*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>After carbamylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{CO_2}$</td>
<td>45.4</td>
<td>44.8</td>
</tr>
<tr>
<td>pH</td>
<td>7.23</td>
<td>7.26</td>
</tr>
<tr>
<td>Red cell CO$_2$, mM</td>
<td>7.59</td>
<td>6.70</td>
</tr>
<tr>
<td>Plasma CO$_2$, mM</td>
<td>17.65</td>
<td>19.20</td>
</tr>
<tr>
<td>Total CO$_2$, mM</td>
<td>18.18</td>
<td>18.23</td>
</tr>
</tbody>
</table>

* From de Furia et al. (28).

**Values were determined at 50% oxygen saturation.**

**TABLE VII**

Synthesis of 2,3-Diphosphoglycerate and Its Binding to Hemoglobin After Carbamylation*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Cyanate-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{H_2}O$, mm Hg</td>
<td>18.2</td>
<td>14.8</td>
</tr>
<tr>
<td>DPG, $\mu$moles/ml cells</td>
<td>1.23</td>
<td>1.13</td>
</tr>
<tr>
<td>ATP, $\mu$moles/ml cells</td>
<td>1.37</td>
<td>1.40</td>
</tr>
</tbody>
</table>

* From de Furia et al. (28).
pyruvate, and phosphate are added to an in vitro suspension of partially carbamylated erythrocytes, there is a net synthesis of 2,3-diphosphoglycerate and a concomitant increase in $P_{50}$, the pressure of $O_2$ (in mm Hg) necessary to maintain the hemoglobin at half-saturation with oxygen. We conclude then that the synthesis and function of this important red-cell metabolite are not affected by low levels of carbamylation.

Kilmartin and Rossi-Bernardi (27) had shown that horse hemoglobin with its four NH$_2$-terminal residues carbamylated was able to take up about 75% of the normal complement of Bohr protons on deoxygenation. We would expect that the carbamylation of only one of the NH$_2$-terminal residues of hemoglobin would result in a decrease of about 6% of the Bohr protons at most. Such a decrease in the Bohr effect is within the limits of precision of the procedures that we used, and we found, as shown in Figure 10, that the Bohr effect of sickle-cell erythrocytes is not demonstrably reduced by partial carbamylation. It would appear that the level of carbamylation necessary to inhibit erythrocyte sickling in vitro does not result in a decreased ability of hemoglobin to carry out its other vital functions.

The effect of cyanate on red-cell metabolism and function was also evaluated. As shown in Figure 11, there was no decrease in the levels of several key erythrocyte metabolites after incubation in vitro with levels of cyanate sufficient to achieve in vitro 60–80% inhibition of red-cell sickling. Most of the enzymes of the Embden–Meyerhof pathways were not affected after exposure of the erythrocyte to cyanate (Fig. 12). There is, however, a slight decrease in the levels of pyruvate kinase. The level of the important red-cell enzyme glucose-6-phosphate dehydrogenase is not significantly affected at this level of carbamylation.

The most significant change in erythrocyte function after treatment with cyanate is the increase in the oxygen affinity of the cells. Fresh, untreated erythrocytes from sickle-cell-anemia patients have a decreased oxygen affinity, with $P_{50}$ values reported in the range 30–35 (33). As shown in Figure 13, with increasing concentrations of cyanate in vitro, the $P_{50}$ value decreases (i.e., the oxygen affinity increases) concomitant with the increased levels of hemoglobin carbamylation. The conditions necessary to prevent 60–80% of the cells from sickling in vitro are precisely those that result in a decrease in the $P_{50}$ value from 31 to a normal range of 26–27 (28), corresponding to the incorporation of 0.7 carbamyl group per hemoglobin tetramer.
Fig. 10. The effect of pH on oxygen affinity after carbamylation. Normal and sickle erythrocytes were incubated with either 10 mM KNCO or 10 mM KCl at 37°C. After 1 hr the cells were washed and suspended in a solution containing 0.15 M NaCl and 0.015 M tris–HCl buffer. The $P_{O_2}$ at 50% saturation and the pH were measured. The $(\Delta \log P_{O_2})/\Delta \text{pH}$ (Bohr effect) of normal (−0.48) and sickle (−0.53) cells remained in the normal range (−0.50) after carbamylation. From de Furia et al. (28).

V. Mechanism of the Antisickling Effect of Cyanate

The salient features of the carbamylation of hemoglobin S are (1) the inability of the uncharged carbamyl group of the NH$_2$-terminus of the polypeptide chains to form salt linkages either positively charged (−NH$_3^+$) or negatively charged after carbamino formation (−HNCOO$^-$), and (2) the increased oxygen affinity of the carbamylated protein.

A schematic representation of the oxyhemoglobin and deoxyhemoglobin tetramers is shown in Diagram II. The presence of aggregates of