ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF MOLECULAR BIOLOGY

Volume 40

ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF MOLECULAR BIOLOGY

Founded by F. F. NORD

Edited by ALTON MEISTER

CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK

VOLUME 40

AN INTERSCIENCE ® PUBLICATION

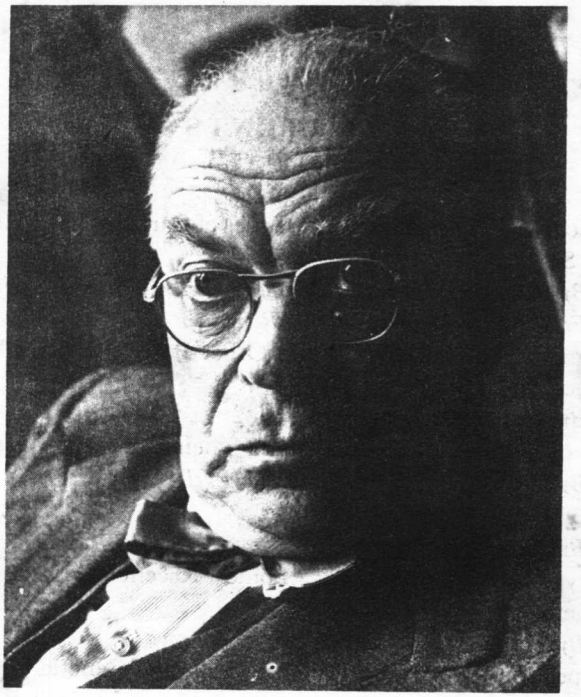
JOHN WILEY & SONS
New York • London • Sydney • Toronto

An Interscience® Publication

Library of Congress Catalog Card Number: 41-9213

ISBN 0-471-59175-0

Printed in the United States of America.



F. F. NORD
1889–1973

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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BIOCHEMICAL AND PHYSIOLOGICAL PROPERTIES OF CARBAMYLATED HEMOGLOBIN S

By JAMES M. MANNING, ANTHONY CERAMI, PETER N. GILLETTE, FRANK G. DE FURIA,* and DENIS R. MILLER, *New York*

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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 β-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

* Deceased.

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 NH_2-terminal valine residues (11). This chapter describes the properties of carbamylated hemoglobin S: the specificity of cyanate for the NH_2-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 NH_2-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

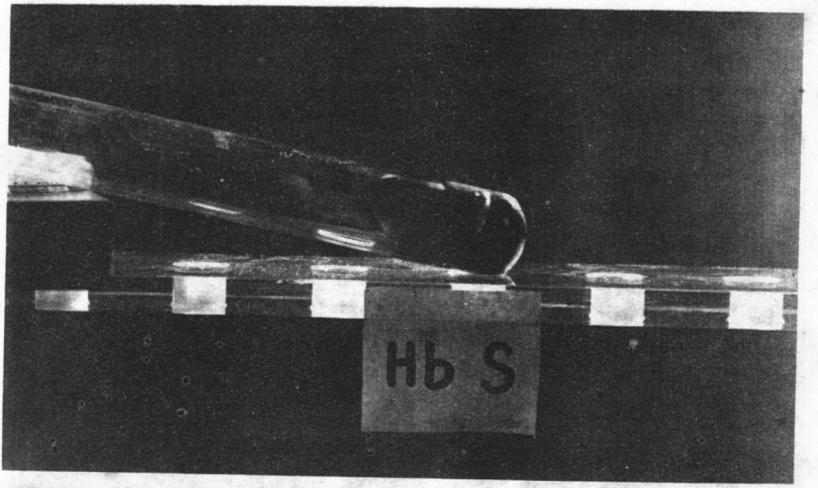

Fig. 1. Hemoglobin S (200 mg/ml) was deoxygenated by equilibration with a mixture of 90% N_2 and 10% CO_2 for 5 min at 0°C. The tube was incubated at 37°C, and after 3 hr the presence or absence of a gel was determined. From Cerami and Manning (11).

the hydrophobic bond peculiar to deoxyhemoglobin S) has not been borne out by the trials reported to date (14).

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

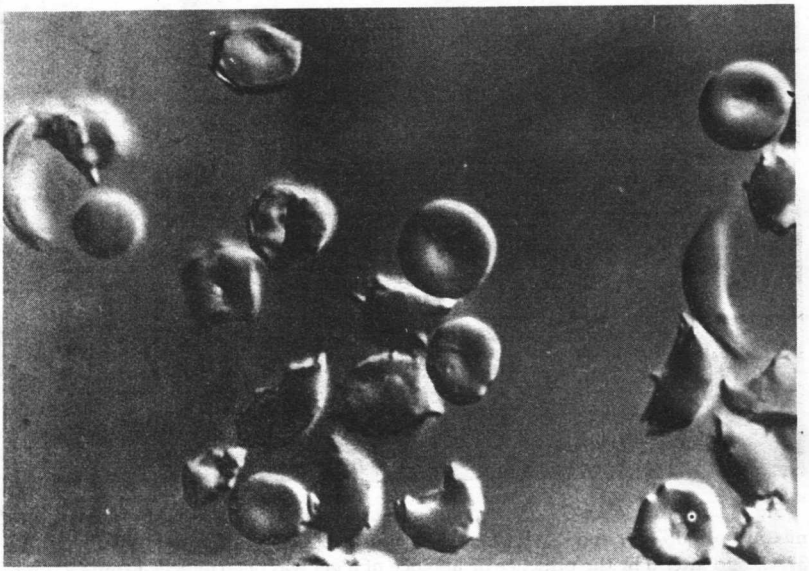

Fig. 2. A suspension of sickle-cell erythrocytes in phosphate-buffered saline solution was deoxygenated by evacuation at 30 mm Hg with a water aspirator for 7 min at 37°C. After an additional 5 min at 37°C the cells were fixed rapidly by dilution with buffered formalin (11). The micrographs were taken 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 NH_2-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

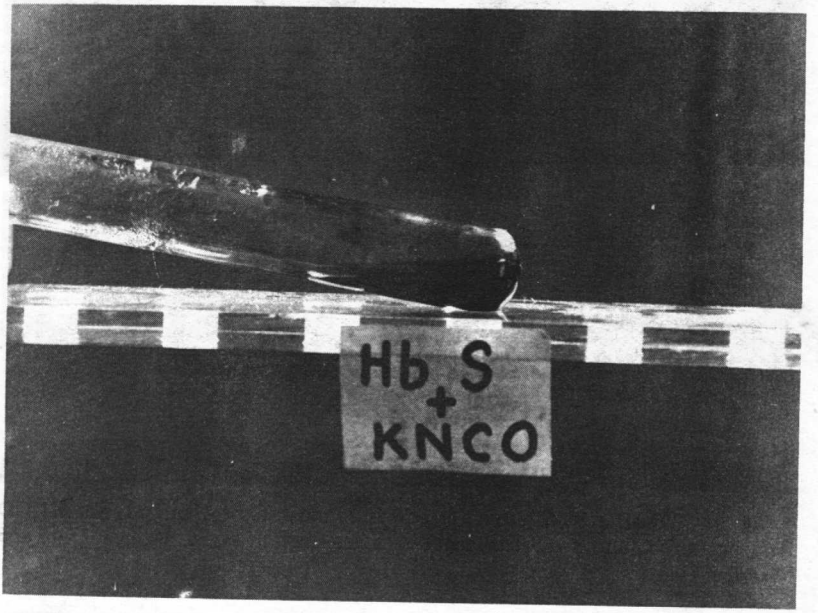

Fig. 3. Oxyhemoglobin S was incubated with 1 m*M* KNCO for 1 hr at 37°C and then deoxygenated as described in Fig. 1. From Cerami and Manning (11).

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 NH_2-groups, and their results indicate that it is the unprotonated form of the NH_2-group that is carbamy-

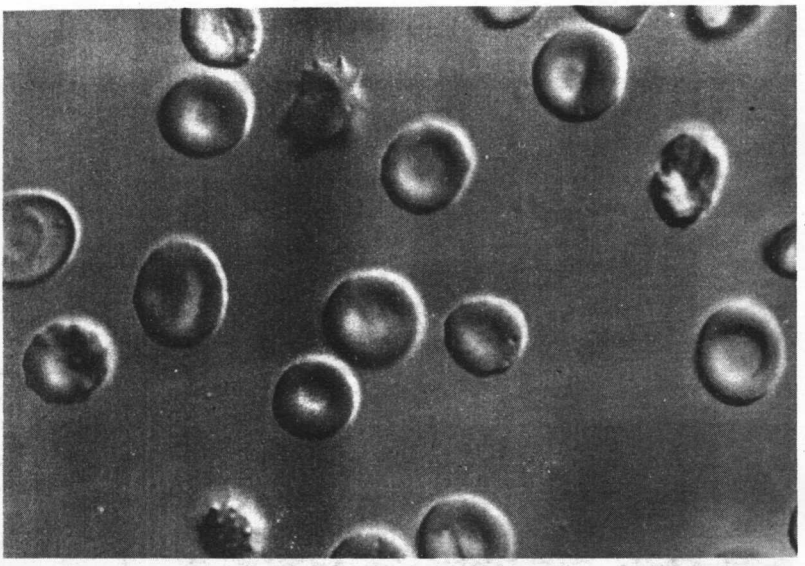

Fig. 4. Oxygenated sickle-cell erythrocytes were treated with 30 m*M* KNCO for 1 hr at 37°C and then deoxygenated as described in Fig. 2. From Cerami and Manning (11).

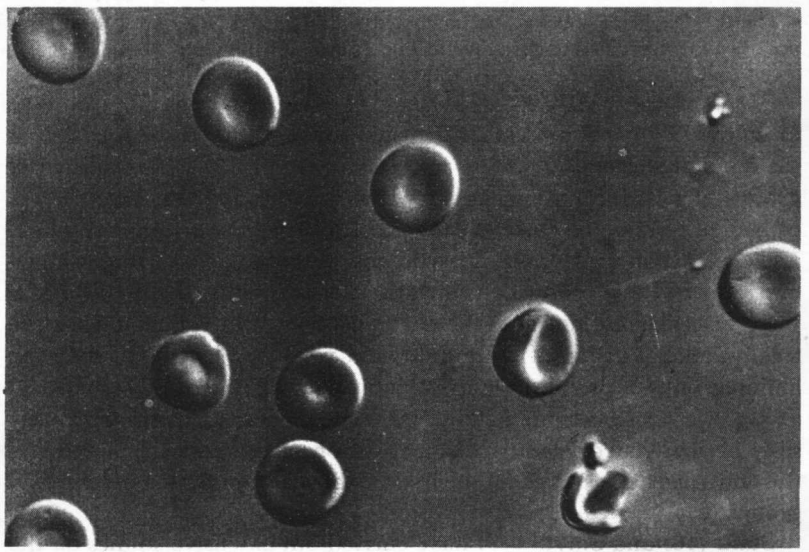

Fig. 5. Oxygenated sickle-cell erythrocytes. From Cerami and Manning (11).

TABLE I

Effect of Urea and KNCO on the Sickling of Deoxygenated Erythrocytes[a,b]

Experiment	Compound	Concentration during incubation (M)	Concentration during deoxygenation (M)	Normal deoxygenated cells (%)
1	—	—	—	17
2	Urea	1.0	1.0	69
3	Urea	—[c]	1.0	70
4	Urea	1.0	0.1	21
5	Urea	0.1	0.1	22
6	KNCO	0.1	0.01	72
7	KNCO	0.01	0.001	34

[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[a,b]

Compound	Concentration (mM)	Gelling
—	—	+
KNCO	1	−
Urea	10	+
Urea	50	+
Urea	100	−

[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% N_2 and 10% CO_2 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.

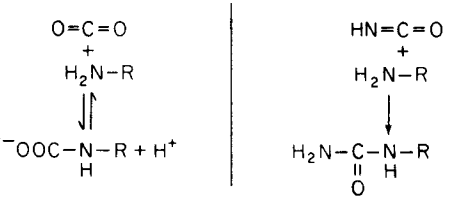

Diagram I

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 carbamylation, 80–90% of the [^{14}C]cyanate incorporated into the cells could be accounted for by carbamylation of the NH_2-terminal valine

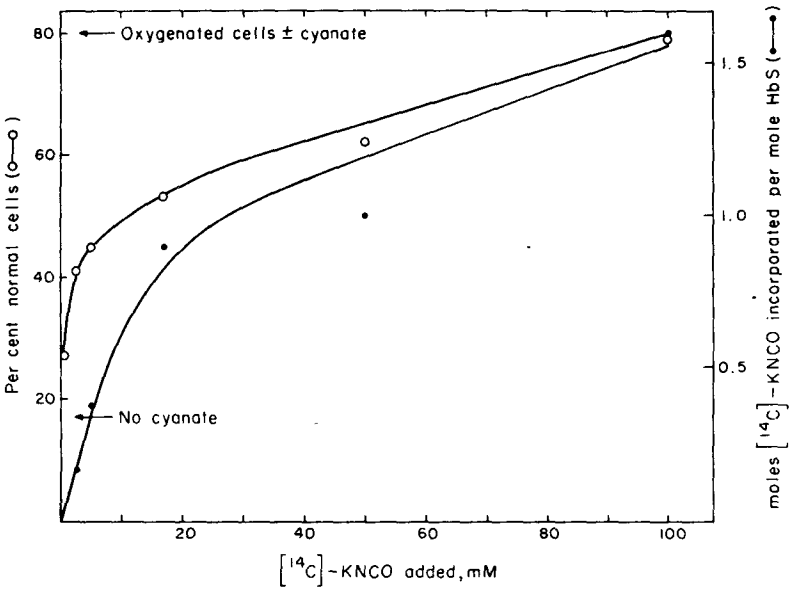

Fig. 6. The effect of KNCO on deoxygenated sickle-cell erythrocytes. Suspensions of oxygenated erythrocytes (0.5 ml) were incubated at 37°C with the designated amount of [^{14}C]KNCO (3.5×10^4 dpm/μmole). At the end of 1 hr, aliquots were removed for deoxygenation and determination of radioactivity. 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[a]

Experiment	[^{14}C]KNCO incorporated (mole/mole HbS)	Carbamylation (mole/mole HbS) at	
		NH_2-terminal valine	ϵ-NH_2 of lysine
1	1.6	1.4	0
2	1.5	1.2	0

[a] From Cerami and Manning (11).

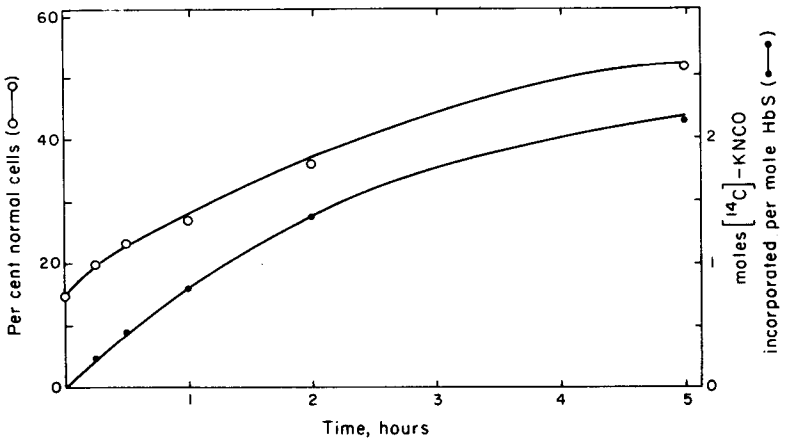

Fig. 7. The kinetics of carbamylation and the increase in normal deoxygenated sickle-cell erythrocytes. Suspensions of oxygenated erythrocytes (2.0 ml) were incubated at 37°C with 0.01 *M* [^{14}C]KNCO (3.1×10^4 dpm/μmole). At the indicated times aliquots were removed for deoxygenation and determination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

residues of hemoglobin S and that there was no detectable carbamylation of the ϵ-NH_2-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 NH_2-terminal valine residues of hemoglobin S and ϵ-NH_2-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 NH_2-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-NH_2-groups of lysine residues up to a point where nearly half the total protein NH_2-groups have been carbamylated. The intermediate stage of the reaction repre-

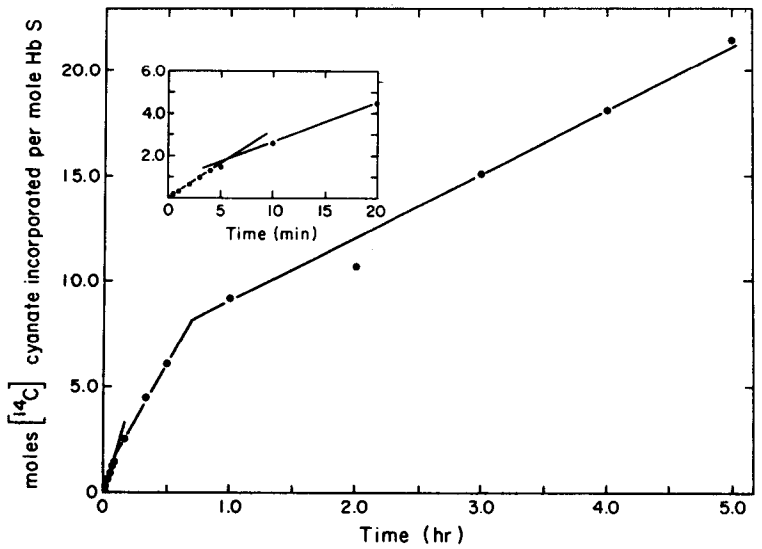

Fig. 8. Carbamylation of the α- and ϵ-NH_2-groups of hemoglobin S. Oxyhemoglobin S (final concentration 0.48 mM) was mixed with [^{14}C]NaNCO (final concentration 0.19 M) at pH 7.4 and 37°C. Portions of the reaction mixture were removed at the indicated intervals and placed into cold 5% trichloroacetic 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 α- and β-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 ϵ-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 NH_2-group. The fact that one can achieve this specificity *in vitro* with whole cells (11) at 1–10 m*M* 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 NH_2-terminal valine residues of hemoglobin with cyanate can also be demonstrated *in vivo* with experimental animals (Table IV). For a single dose of [^{14}C]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 NH_2-terminal residues of hemoglobin is undoubtedly due to the anomalously low pK_a of these residues in the hemoglobin tetramer; the serum proteins have pK_a 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 IV

Extent of Carbamylation in Mice *in Vivo*[a,b]

Organ	Percentage of Injected Dose	Organ	Percentage of Injected Dose
Heart	0.08	Bones	3.5
Lungs	0.05	Muscle	2.1
Stomach	0.06	Red blood cells	7.5
Spleen	0.01	Serum proteins	0.5
Kidneys	0.05	Subtotal	13.6
Intestine	0.4		
Liver	0.7		
Brain	0.17	Urine	7.0
Skin	0.8	Expired as $^{14}CO_2$	72.2
Subtotal	2.3	Total recovery	95.1

[a] From Cerami et al. (39).

[b] Female mouse (B_6D_2) injected with 10 μmoles of [^{14}C]NaNCO.