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Research Article

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The Effects of Cyanate In Vitro on Red Blood Cell Metabolism and Function in Sickle Cell Anemia

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ABSTRACT Cyanate, which is in equilibrium with urea, combines with the α -amino group of the amino-terminal valine of hemoglobin in an irreversible, specific carbamylation reaction. Partial carbamylation (0.72 residues/hemoglobin tetramer) as determined by cyanate- ^{14}C incorporation or hydantoin analysis diminishes the in vitro sickling phenomenon. Since cyanate may react not only with hemoglobin but also with functional groups of other red blood cell proteins, the in vitro effect of cyanate was studied on sickle cells. Cells were incubated with 10 mM KCl (control) or 10 mM KNCO (carbamylation) for 1 hr, washed, and resuspended in autologous plasma. Glycolysis, ATP and 2,3-diphosphoglyceric acid (DPG) stability, auto-hemolysis, and osmotic fragility were not affected by carbamylation. Potassium loss in carbamylated cells (2.8 mmol/liter) was less than in control cells (9.0 mmol/liter). Pyruvate kinase activity of carbamylated cells was decreased ($\sim 25\%$) but the activities of other glycolytic enzymes were similar to those of control cells. Oxygen affinity of carbamylated sickle, normal, and DPG-depleted normal cells increased, and was a sensitive index of the degree and duration of reaction with cyanate. The reactivity of carbamylated cells to DPG was similar to control cells. DPG-depleted carbamylated cells regenerated DPG and increased the P_{50} when incubated with pyruvate, inosine, and phosphate. The Bohr effect of normal and of sickle cells was not affected ($\Delta \log P_{50}/\Delta \text{pH} = -0.48$ and -0.53 , respectively) after carbamylation. The reserve buffering capacity of plasma offset the slightly diminished ($\sim 15\%$) CO_2 capacity of carbamylated cells so that whole blood CO_2 capacity, pH, and P_{CO_2} were normal. These studies provide further support for the potential clinical use of

cyanate in treating and preventing the anemia and painful crises of sickle cell disease.

INTRODUCTION

Recent investigations have suggested that intravenous urea in an invert sugar solution may be useful in the treatment and prevention of painful vaso-occlusive crises in patients with sickle cell anemia (1, 2). The proposed mechanism of action of this protein denaturant was the disruption of those hydrophobic bonds which may be involved in the polymerization and gelation of the hemoglobin S (Hb S)¹ molecule (3, 4). Previous in vitro studies have indicated that concentrations of 1–8 M are required to unfold a protein (5). Since the maximum concentration of urea achieved in treated patients was approximately 30–50 mM, and since urea is rapidly cleared by the body, an alternative explanation has been presented recently to explain the reported salutary effect of urea—that cyanate, which is in equilibrium with urea (6) (Eq. 1) might be the active agent (7).



¹ *Abbreviations used in this paper:* ACD, acid-citrate-dextrose medium; DHAP, dihydroxyacetone phosphate; DPG, 2,3-diphosphoglyceric acid; FDP, fructose diphosphate; F6P, fructose-6-phosphate; G3P, glyceraldehyde 3-phosphate; G3PD, glyceraldehyde 3-phosphate dehydrogenase; G6P, glucose 6-phosphate; G6PD, glucose 6-phosphate dehydrogenase; GSH-Red, glutathione reductase; GSH-PX, glutathione peroxidase; Hb, hemoglobin; HK, hexokinase; P_{50} , pressure of oxygen in mm Hg at which the blood is half saturated; PBS, phosphate-buffered saline; PCA, perchloric acid; PEP, phosphoenolpyruvic acid; PFK, phosphofructokinase; 2PG, 2-phosphoglyceric acid; 3PG, 3-phosphoglyceric acid; PGK, phosphoglycerokinase; PK, pyruvate kinase; RBC, red blood cell; SS, sickle cell disease.

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Cerami and Manning (7) studied the effect of cyanate on the sickling phenomenon and found that, in contrast to the high concentration of urea (1 M) required to prevent *in vitro* sickling, 10–100 mM potassium cyanate (KNCO) significantly inhibited sickling. They described an irreversible, specific carbamylation reaction in which cyanate reacted with the α -amino group of the NH_2 -terminal valine residue of Hb S.



Gillette, Manning, and Cerami (8) have shown that the autologous survival of sickle cells treated *in vitro* with cyanate is prolonged toward the normal range.

These studies (7, 8) suggested that cyanate may be efficacious in preventing or treating sickle cell crises. Cyanate is known to react with functional groups of proteins (9–13). Since cyanate may react with other intracellular or membrane enzymes and proteins, we investigated the *in vitro* effect of cyanate on metabolism and function in the erythrocytes of patients with sickle cell anemia.

METHODS

Subjects. Blood from patients with electrophoretically proven sickle cell anemia was used for the studies of erythrocyte metabolism and hemoglobin function. The reticulocyte counts ranged between 10 and 20%, and none of the subjects had received a transfusion within 12 months.

Metabolic studies. Venous blood, anticoagulated with heparin was centrifuged at 1200 *g* in an International PR6 centrifuge (International Equipment Co., Needham Heights, Mass.) at 4°C. Plasma was removed, the cells were divided into equal portions, and resuspended in an equal volume of Dulbecco's phosphate-buffered saline (14) pH 7.4 (PBS) containing 10 mM glucose and either 10 mM KCl (control) or 10 mM KNCO (carbamyated). The cell suspension was incubated for 1 hr at 37°C in a shaking metabolic incubator after which the cells were washed twice in PBS and resuspended in leukocyte and platelet-free autologous plasma. Glucose was added to a final concentration of 10 mM and the suspensions were incubated for 15 min at 37°C and shaken at 90 oscillations per min in room air. The 15 min sample (zero time) and 3-hr samples were deproteinized in 6% perchloric acid (PCA) and neutralized with 5 M K_2CO_3 . The pH remained constant (pH 7.4 ± 0.1) throughout the incubation period. Glucose utilization, lactate production, and ATP were measured by previously published methods (15), and 2,3-diphosphoglycerate (DPG) was assayed by the method of Rose and Liebowitz (16). The concentrations of glycolytic intermediates and the adenine nucleotides, AMP and ADP, were determined by the method of Minakami, Suzuki, Saito, and Goshikawa (17). Intracellular sodium and potassium concentrations were measured by flame photometry (18). Glutathione (GSH) and GSH stability of the control and carbamyated cells were determined by the method of Beutler, Duron, and Kelly (19).

A second sample of blood was defibrinated with glass beads and using sterile technique, cells were separated, incubated with either KCl or KNCO, and washed as described above. Cells were resuspended in the autologous,

defibrinated plasma and measurements of osmotic fragility of fresh and incubated cells and of autohemolysis with the addition of saline, glucose, or ATP (pH 7.0) were performed (20). The activities of the following enzymes in hemolysates of control and carbamyated cells were measured by previously published methods (21): hexokinase (HK), phosphofructokinase (PFK), glyceraldehyde 3-phosphate dehydrogenase (G3PD), phosphoglycerokinase (PGK), pyruvate kinase (PK) with 0.4 mM and 2.0 mM phosphoenolpyruvate (PEP) and with fructose diphosphate (FDP), glucose 6-phosphate dehydrogenase (G6PD), GSH reductase and GSH peroxidase. A Gilford 2400 automatic recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio.) was used for these assays which were performed in duplicate at 37°C. Methemoglobin was determined by the method of Beutler and Baluda (22) and was less than 1% in all samples after incubation.

Function studies. Heparinized blood was centrifuged as described above, the plasma set aside, and the cells resuspended in PBS containing the designated concentration of either KNCO or KCl. Unless otherwise stated the cells were incubated at 37°C for 1 hr. After the incubation the cells were washed three times with PBS to remove unreacted cyanate. The cells were finally resuspended in autologous plasma or in a 25 mM NaHCO_3 + 117 mM NaCl solution. The pressure of oxygen in millimeters of Hg at which the blood was half saturated (the P_{50}) was measured by the mixing technique of Edwards and Martin (23). Oxygen saturation was measured in an Instrumentation Laboratory Co-Oximeter (Instrumentation Laboratory Inc., Watertown, Mass.) previously calibrated by the manometric method of van Slyke & Neill (24). Measurements of P_{O_2} , P_{CO_2} , and pH were performed with the Instrumentation Laboratory model 313 Blood Gas Analyzer. For calculation of the P_{50} , the P_{O_2} values were corrected to pH 7.40 using the Severinghaus nomogram (25).

To evaluate the effect of DPG on the oxygen affinity of carbamyated and control cells, DPG was added to a syringe of whole blood at 50% oxygen saturation to a final concentration of 1.25 mM. The P_{50} was determined, the syringe was sealed, and the blood was hemolyzed by freezing and thawing, after which the P_{50} was again determined. The purity of DPG was established by chromatography, electrophoresis, phosphate analysis (26), and enzymatic assay (16). Carbamyated and control cells partially depleted of DPG after storage in ACD (1.1 mM DPG) were suspended in saline containing 10 mEq each of inosine, pyruvate, and phosphate (pH 7.4) and incubated in room air at 37°C for 3 hr (27). Portions were analyzed for P_{50} , DPG, and ATP at 0 and 3 hr.

The Bohr effect of control and carbamyated cells suspended either in 0.015 M Tris in saline (pH 7.0, 7.4, 7.8) and tonometered with CO_2 -free gases, or in saline with bicarbonate (6.25, 25, 62.5 mEq) and tonometered with gases containing 5.6% CO_2 was calculated after measurement of the P_{50} by the mixing technique.

CO_2 transport at 10, 50, and 90% oxygen saturations was assessed by comparing the whole blood CO_2 and pH, and plasma CO_2 of control and carbamyated blood tonometered to either normal or elevated P_{CO_2} 's. CO_2 was determined by a modification of the Natelson microgasometric method (28). Red cell ΔpH (plasma pH – red cell pH) was measured at 10, 50, and 90% saturation (29, 30).

Statistical analysis was performed by Student's *t* test for small samples.

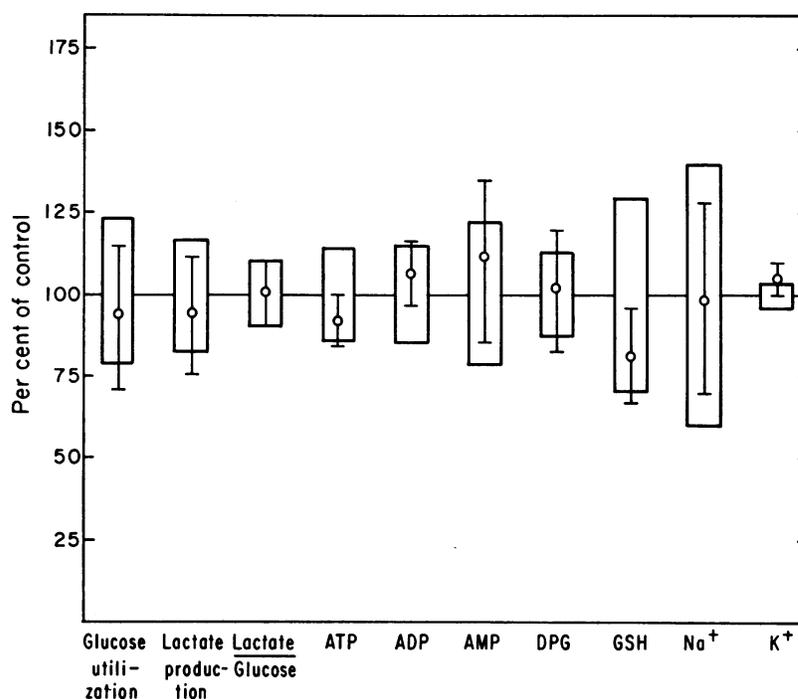


FIGURE 1 The effect of cyanate on glycolysis and intracellular metabolites. Mean (○) and standard deviation (I) of values on sickle cell blood from six patients after incubation with 10 mM KNCO for 1 hr at 37°C are expressed as per cent of control (treated with KCl 10 mM × 1 hr). Standard deviation of the control value is denoted as the open bar. None of these differences were statistically significant including the decrease in GSH ($0.1 > P > 0.05$).

RESULTS

Glycolysis and intracellular metabolites. No significant differences were observed in glucose utilization, lactate production, or the concentrations and stability of ATP, ADP, AMP, DPG, and intracellular sodium (Na⁺) and potassium (K⁺) in control and carbamylated cells (Fig. 1). The lactate:glucose ratios approached the theoretical value of 2:1 in both the control and carbamylated samples. During the 3 hr incubation, the mean loss of K⁺ from carbamylated cells was 2.8 mmoles/liter RBC compared to a mean loss of 9.0 mmoles/liter of control cells. Control cells lost 3.0 mmoles of Na⁺/liter whereas carbamylated cells lost 0.6 mmoles/liter. Autohemolysis and the osmotic fragility of fresh and incubated blood were unaffected by treatment with cyanate.

Glycolytic enzyme activity and glycolytic intermediates. The ratios (carbamylated:control) of activity of the following glycolytic enzymes were slightly altered: G6PD, GSH reductase, GSH peroxidase, HK, PFK, G3PD, and PGK (Fig. 2). Pyruvate kinase was decreased by 21–29% when assayed at either high or

low concentrations of PEP or in the presence of FDP. Although the concentration of GSH in carbamylated cells was lower than that of control cells, the difference was not statistically significant ($0.1 > P > 0.05$). After incubation with acetylphenylhydrazine, the GSH content of carbamylated blood rose 29% whereas a 4% increase was noted in control cells similarly exposed to acetylphenylhydrazine. The concentrations of glycolytic intermediates in the carbamylated cells were not significantly different from those of control cells except for increased levels of F6P and 2PG and decreased levels of pyruvate (Fig. 3).

Oxygen affinity. The oxygen affinity of sickle, normal, and DPG-depleted (< 0.03 mM) cells increased after carbamylation (Fig. 4). The increase in oxygen affinity was irreversible and was not changed by washing the cells free of cyanate after incubation. The decrease in P_{50} was not due to a loss of DPG or ATP (Fig. 1). The shape and slope of the oxyhemoglobin dissociation curve was not affected by carbamylation.

Oxygen affinity is a sensitive index of the degree of carbamylation (Fig. 5). Incubation of sickle cells with 10 mM KNCO for 1 hr resulted in the incorporation of

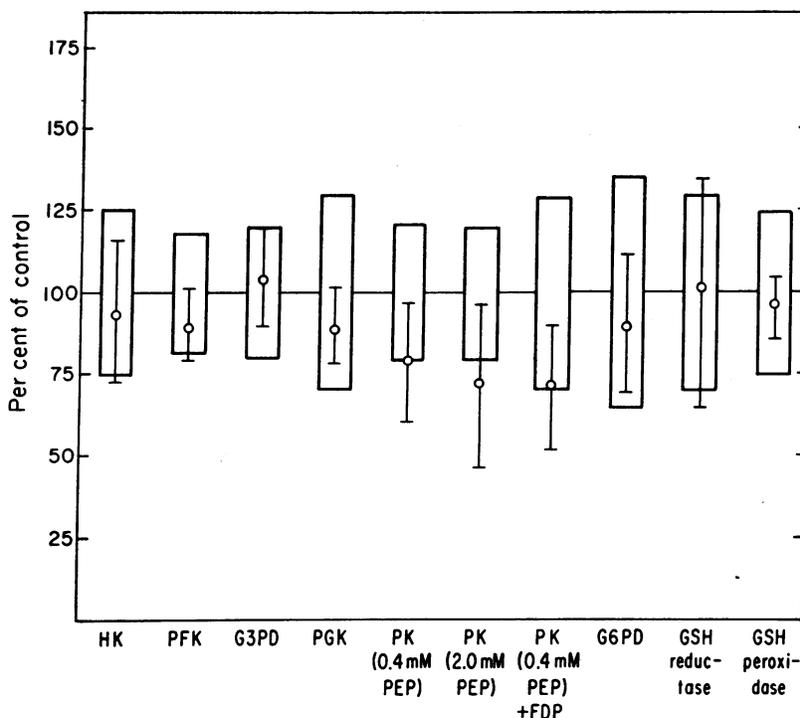


FIGURE 2 The effect of cyanate on glycolytic enzymes. Mean (○) and standard deviation (I) of enzymatic activity of sickle cell blood from six patients after incubation with 10 mM KNCO for 1 hr at 37°C expressed as per cent of control (treated with KCl 10 mM × 1 hr). Standard deviation of the control values are denoted as the open bar. The depression in PK activity was not significant with low substrate concentration ($P = 0.10$) or with low substrate concentration with FDP ($0.10 > P > 0.05$) or high substrate ($0.10 > P > 0.05$).

0.72 moles of cyanate/mole Hb tetramer and a decrease of P_{50} from 31 to 27 mm Hg (Fig. 5). After incubation with 100 mM KNCO the hemoglobin contained 3.9 carbamyl groups per Hb tetramer and the P_{50} had decreased to 19.5 mm Hg. Similar degrees of carbamylation result after incubation with cyanate in autologous plasma in vitro. Oxygen affinity is also a function of the duration of incubation with 10 mM KNCO (Fig. 6).

The response of carbamylated cells to DPG was similar to control cells in three experiments (Table I). The P_{50} of Hb in cells decreases on hemolysis (31-33). The addition of DPG to the hemolysate resulted in an increase in the P_{50} . The addition of DPG to the impenetrable whole cells had a negligible effect on the oxygen affinity. The hemolysate of the cell suspension to which DPG had been added did not exhibit a fall in P_{50} .

The response of DPG-depleted normal cells to incubation with phosphate, inosine, and pyruvate is recorded in Table II. An increase in P_{50} was noted in DPG-depleted cells after carbamylation and was maintained over a 4 hr period. The P_{50} and the concentrations of ATP and DPG were increased similarly in control and

carbamylated cells. These results indicate that carbamylated cells are able to synthesize ATP and DPG.

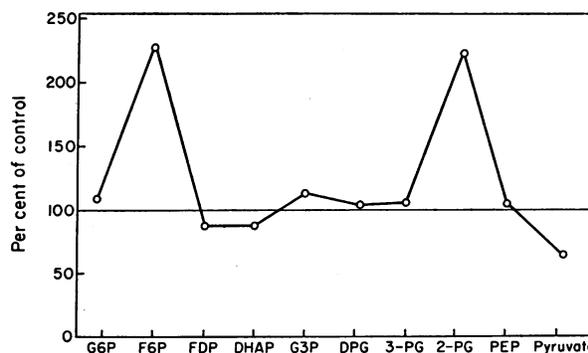


FIGURE 3 The effect of cyanate on glycolytic intermediates. The mean (○) of glycolytic intermediates of sickle cell blood from six patients after carbamylation with 10 mM KNCO and incubation for 3 hr at 37°C is expressed as per cent of control. (The standard deviation of the intermediates expressed as a fraction of the mean are as follows: G6P, ± 0.36 ; F6P, ± 1.04 ; FDP, ± 0.65 ; G3PD, ± 0.70 ; DHAP, ± 0.32 ; 2PG, ± 1.05 ; 3PG, ± 0.70 ; PEP, ± 0.45 ; PYR, ± 0.21).

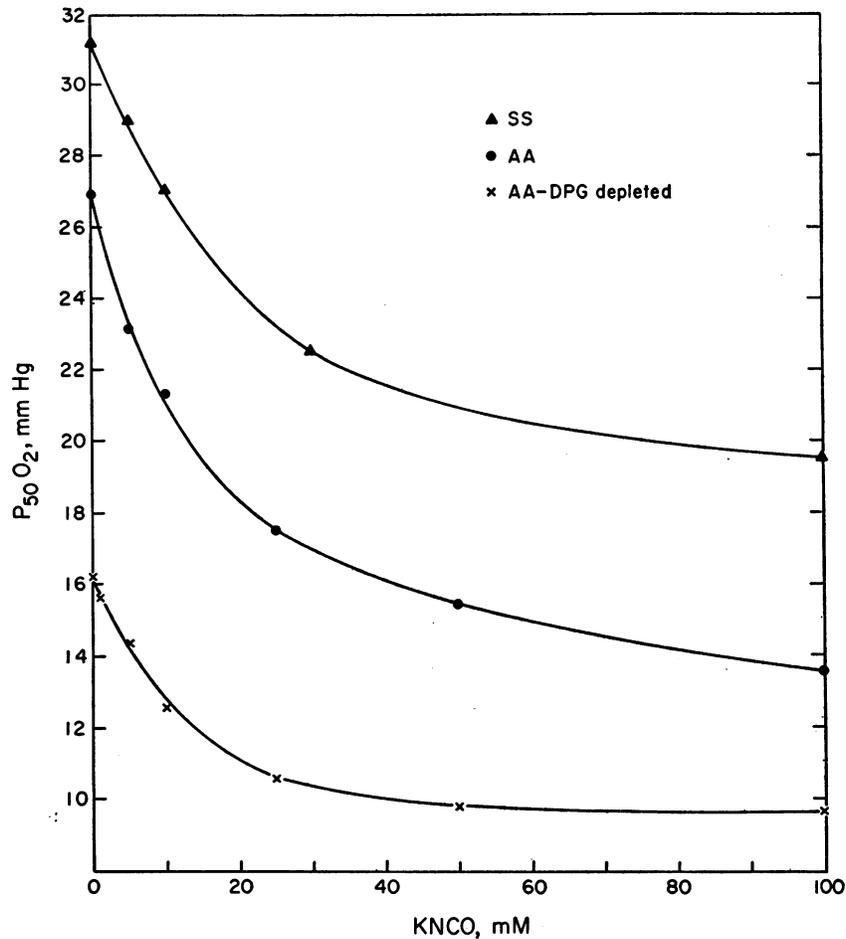


FIGURE 4 Effect of cyanate on oxygen affinity. Sickie, normal, and normal cells depleted of DPG were incubated for 1 hr at 37°C with the indicated concentrations of KNCO. The oxygen affinity, expressed as P_{50} , was then determined after the cells were washed and resuspended in autologous plasma. The standard deviation was 0.2 mm Hg.

The Bohr effect of intact sickle and normal cells suspended in Tris buffer was not altered by carbamylation (Fig. 7). Similar results were obtained in bicarbonate buffer.

TABLE I
The Effect of DPG on P_{50} *

Hb type	Carbamylation	Cells	Cells +DPG	Hemolyzate	Hemolyzate +DPG
AA	-	25.6	26.9	20.3	27.5
AA	+	21.2	21.8	20.0	25.5
SS	-	32.7	32.8	24.4	29.8
SS	+	28.7	28.9	21.6	26.5
DPG-depleted AA	-	15.9	16.2	12.3	17.1
DPG-depleted AA	+	12.5	12.5	8.3	12.0

* P_{50} is the P_{O_2} at 50% saturation, pH7.4, and temperature 37°C.

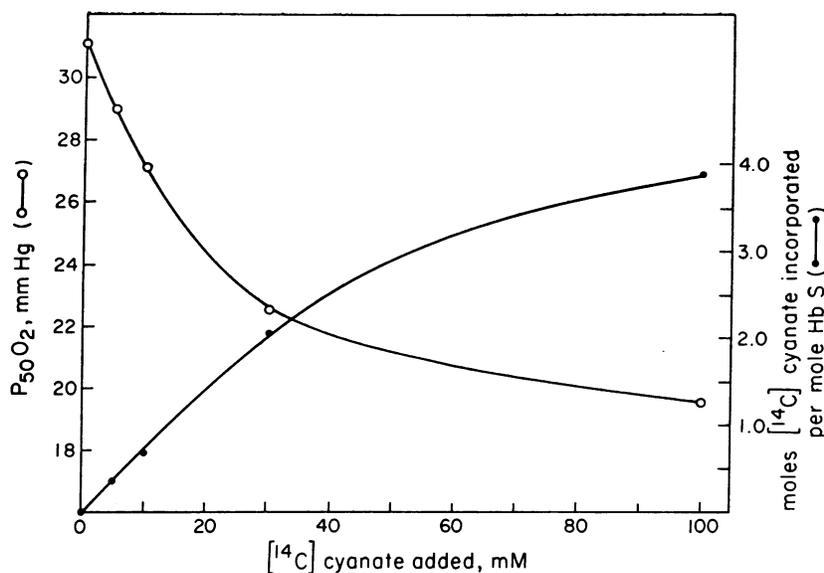


FIGURE 5 Oxygen affinity as a function of carbamylation. The P_{50} of the cyanate-treated cells and the amount of $[^{14}\text{C}]$ -cyanate incorporated into acid precipitable protein are plotted as a function of the concentration of cyanate during the 1-hr incubation at 37°C .

CO₂ transport. The whole blood CO_2 content and pH of sickle and normal blood tonometered to the same P_{CO_2} did not change after carbamylation (Table III). A redistribution of CO_2 with a 15–20% decrease in red cell CO_2 and compensatory increase in plasma CO_2 was regularly noted. The reserve buffer capacity of plasma is sufficient to prevent a difference in blood pH after carbamylation even after tonometry with elevated P_{CO_2} , e.g., 60 mm Hg. A slight elevation in whole blood pH after carbamylation (0.02 pH U) was noted. The RBC ΔpH was not changed significantly after carbamylation (Table III).

DISCUSSION

The reaction of cyanate with Hb S has been shown to inhibit sickling *in vitro* (7), and to prolong erythrocyte survival *in vivo* (8). However, similar carbamylation reactions could conceivably occur with other cellular enzymes and proteins, and alter their physiologic function. The results of the metabolic studies indicate that carbamylation causes a slight but insignificant inhibition of overall erythrocyte glycolysis.

Assays of glycolytic enzymes under ideal conditions may not reflect the true status of intracellular glycolysis but our results indicate that there is no significant reduction of the major rate-limiting (34) enzymes of the Embden-Meyerhof pathway. The observations of (a) a slight reduction in the activities of PFK and PK, (b) the elevation of FDP and 2PG and (c) the decrease in pyruvate, suggest altered metabolic activity

or so-called “cross-over points” (35, 36) at the PFK, enolase, and perhaps the PK steps.

The concentrations of ATP and DPG, which regulate oxygen affinity (32–34) and modulate glycolytic activity (34, 35, 37) are stable after carbamylation. The normal concentrations of intracellular Na^+ and K^+ , the normal osmotic fragility curves, and the normal patterns of autohemolysis suggest that cation-pumping mechanisms are not affected by cyanate and that the integrity of membrane proteins is maintained. In fact, K^+ loss from carbamylated oxygenated sickle cells was less than that observed in control cells. This is of interest since Tosteson, Carlsen, and Dunham (38), and later Mentzer, August, and Nathan (39) observed an excessive rate of K^+ loss from deoxygenated sickle cells which the latter investigators correlated with the clinical severity of the disease.

Although the concentration of GSH was lower in carbamylated sickle cells, the activities of GSH per-

TABLE II
Incubation with Inosine, Pyruvate, and Phosphate

	Preincubation		Postincubation	
	CI	NCO	CI	NCO
P_{50} in mm Hg	18.2	14.8	28.8	24.7
DPG, $\mu\text{moles/ml}$ RBC	1.23	1.13	3.56	3.37
ATP, $\mu\text{moles/ml}$ RBC	1.37	1.40	1.43	1.44

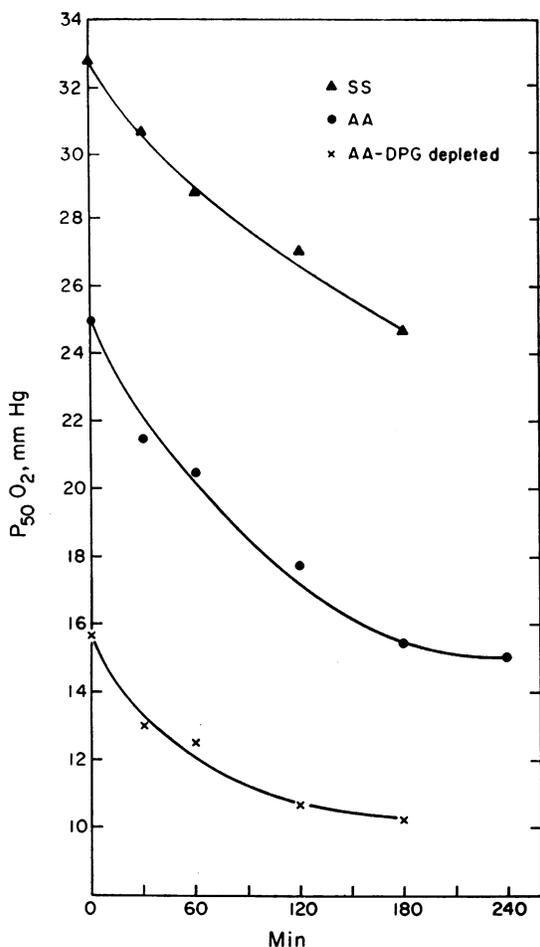


FIGURE 6 Oxygen affinity as a function of duration of incubation with cyanate. Sickie, normal, and DPG-depleted normal cells were incubated at 37°C for the indicated time with 10 mM KNCO. The cells were washed, resuspended in autologous plasma, and P_{50} determined. Cells incubated with 10 mM KCl decreased their P_{50} less than 1 mm after a 4 hr incubation.

oxidase and GSH reductase were normal. Furthermore, the elevated levels of GSH occurring in the presence of acetylphenylhydrazine suggest that cyanate does not inhibit the mechanisms available to regenerate GSH.

The irreversible carbamylation of the NH_2 -terminal valine residues of both α - and β -chains (unpublished observation) of Hb might have resulted in an erythrocyte with an altered physiology since these amino groups have been implicated to be directly involved in several important hemoglobin functions. These functions are (a) the binding of CO_2 as carbamino groups (40), (b) the Bohr effect (40, 44), (c) a binding site for DPG (41, 42), and (d) the formation of a salt bridge which stabilizes the deoxy- conformation of hemoglobin (42). In this present communication, we have attempted

to assess these functions after carbamylation at levels necessary to inhibit sickling in vitro. (~ 1 mole NCO incorporated/mole Hb tetramer.)

It is estimated (43) that 30% of the expired CO_2 is carried as carbamino groups. The carbamylation of all four of the amino-terminal valine residues of horse hemoglobin has been reported by Kilmartin and Rossi-Bernardi (40) to inhibit completely the formation of carbamino compounds with CO_2 . Under our experimental conditions the carbamylation of one of the four NH_2 terminal valine residues of both α - and β -chains of hemoglobin does not lead to a lowered pH of the blood (Table III). The slight reduction in red cell CO_2 levels appears to be compensated for by an increase in the amount of plasma bicarbonate. Approximately 25% of the Bohr effect of isolated horse hemoglobin has been shown to be due to the protonation of the NH_2 -terminal valine residues of the α -chain (40). The oxygen affinity of cells which have been carbamylated at one of the four NH_2 terminal valines of hemoglobin respond to changes in pH as do control cells (Fig. 7).

Organic phosphates have been demonstrated to have an effect on the oxygen affinity of isolated hemoglobin and intact erythrocytes. The amino group of the terminal

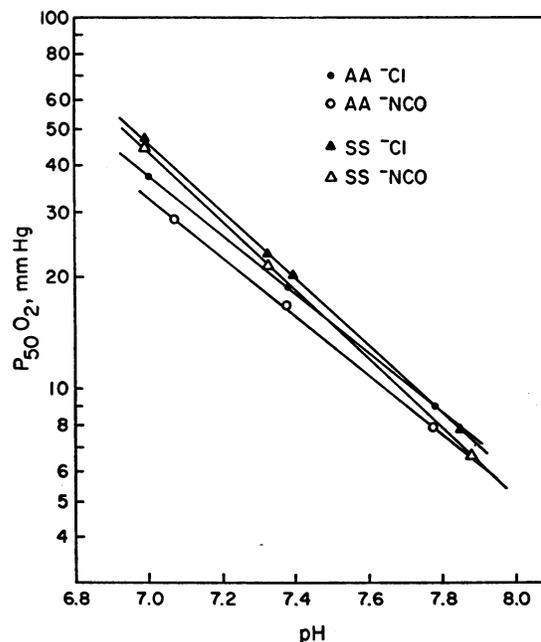


FIGURE 7 The effect of pH on oxygen affinity after carbamylation. Normal and sickle erythrocytes were incubated with either 10 mM KNCO or 10 mM KCl at 37°C. After 1 hr the cells were washed and suspended in a solution containing 0.15 M NaCl and 0.015 M Tris-HCl buffer. The P_{50} at 50% saturation and the pH were measured. The $(\Delta \log P_{50})/\Delta \text{pH}$ (Bohr effect) of normal (-0.48) and sickle (-0.53) cells remained in the normal range (-0.50) after carbamylation.

TABLE III
Blood* CO₂ Content after Tonometry

	CI	NCO	CI	NCO	CI	NCO	CI	NCO
O ₂ % saturation	>90	>90	<10	<10	50	50	50	50
CO ₂ % in gas mixture	5.6	5.6	6.8	6.8	6.2	6.2	8.5	8.5
PCO ₂ mm Hg	40.1	40.2	49.0	49.0	45.4	44.8	58.5	58.6
pH	7.26	7.28	7.26	7.27	7.23	7.26	7.20	7.21
Total CO ₂ mM	16.79	16.94	17.69	18.09	18.18	18.23	19.19	19.11
Plasma CO ₂ mM	18.08	19.17	17.66	20.10	17.65	19.20	18.79	19.40
RBC CO ₂ mM†	5.94	5.44	7.09	6.03	7.59	6.70	7.92	7.47
RBC ΔpH	0.370	0.350	0.175	0.170	0.220	0.200	0.200	0.180

* Hematocrit = 40.

† Calculated value.

valine residue of the β -chain has been proposed to be a site of DPG binding on the hemoglobin molecule (41, 42). The oxygen affinities of the hemolysates of carbamylated normal, sickle, and DPG-depleted cells decrease to the same extent as control hemolysates when DPG is added. In addition, the carbamylation of DPG-depleted cells does not interfere with the synthesis of DPG and the concomitant increase in P_{50} when the cells are incubated with phosphate, inosine, and pyruvate. Thus, this level of carbamylation does not interfere with the effect of DPG on the oxygen affinity.

The most noteworthy effect of the carbamylation of normal, sickle, and DPG-depleted erythrocytes is the increase in the oxygen affinity of the cells (Fig. 4). Since the levels of DPG and ATP are not affected by carbamylation (Fig. 1), the increased oxygen affinity may be the result of a stabilization of the oxy-conformation of the hemoglobin molecule. Perutz et al. (44) have recently presented evidence from X-ray crystallographic studies that the amino-terminal valine residues form intramolecular salt bridges when hemoglobin is in the deoxy-conformation. The presence of the uncharged carbamyl group on the amino-terminal valine residue may interfere with this salt bridge and stabilize the oxy-conformation.

The stabilization of the oxy-conformation by carbamylation may also explain the antisickling effect of cyanate. Since carbamylated deoxygenated Hb S may remain in the oxy-conformation, these molecules may interfere with the aggregation of other noncarbamylated Hb S molecules. This hypothesis is consistent with the finding that cells from some patients require as little as 0.1 carbamyl group per hemoglobin tetramer to inhibit sickling (7).

The increased oxygen affinity of carbamylated sickle cells could conceivably lead to decreased oxygen delivery and tissue hypoxia. However, since carbamylation decreases sickling and prolongs the life-span of sickle cells, the red blood cell mass should increase and in-

crease oxygen delivery. In addition the cells should be able to increase their DPG concentration and counteract the effects of an increased oxygen affinity. These findings provide further support for the potential clinical use of cyanate in treating and preventing painful vaso-occlusive crises of sickle cell anemia.

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