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TREATMENT OF SICKLE CELL ANEMIA

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ABSTRACT OF THE DISCLOSURE

It has been discovered that cyanate, such as a 0.01–1.0M potassium cyanate solution, is useful to prevent the sickling of the red blood cells of sickle cell anemia patients. It has also been discovered that red blood cells when taken from sickle cell anemia patients and treated *in vitro* with a cyanate, such as a sodium cyanate solution, evidence an increased survival or prolonged life when returned to the patient.

The invention described herein was made in the course of work under a grant or award from the Department of Health, Education, and Welfare.

Sickle cell anemia is caused by a hereditary defect in hemoglobin. Sickle cell disease is the generic term applied to disorders characterized by human red blood cells containing an abnormal hemoglobin designated hemoglobin S. Under certain conditions of reduced oxygen tension or pressure red cells containing hemoglobin S tend to change from normal disc-shape into a crescent shape, hence the name of the disease, sickle cell disease. Sickling of red blood cells tends to clog narrow capillaries and cause blood clots giving rise to symptoms such as severe, almost crippling, pain in the affected parts of the body.

A description of sickle cell disease, including the genetic mechanism and clinical manifestation, is to be found in the publication the red cell by John W. Harris and Robert W. Kellermeyer, published by Harvard University Press, Cambridge, Mass. (1970), pages 168–202. The disclosures of the above-identified publication are herein incorporated and made part of this disclosure.

A treatment of a patient in sickle cell anemia crisis or distress has been proposed, see Nalbandian et al. *Ann. Intern. Med. 72*, 795 (1970). This treatment involves the use of urea solution to alleviate the symptoms. Urea has also been used to diagnose the disease. Unfortunately, the use of a urea solution (usually a high concentration of urea, about 1M) involves risk to the patient. The treatment of a patient in sickle cell anemia crisis with a urea solution is based on a theory of the sickling process proposed by M. Murayama, see *Science, 153*, 145 (1966), see also Perutz et al. *Nature, 219*, 902 (1968). The disclosures of these publications are also herein incorporated and made part of this disclosure.

Hemoglobin S which is present in the red blood cells of humans suffering from sickle cell disease and which gives rise to sickle cell anemia crisis differs from normal hemoglobin in that at only one site of the β chain of the hemoglobin which contains 146 amino acids, a valine replaces the glutamic acid of normal hemoglobin. Valine has an isopropyl side chain and according to Murayama this encourages the formation of hydrophobic bonds between the chains at low oxygen concentration. Bonded chains stick together, forming filaments which eventually thicken and force the cell to assume a sickle shape. It has been proposed that the urea treatment tends to reverse sickling by breaking these hydrophobic bonds.

It is an object of this invention to provide materials useful in the treatment of sickle cell anemia, particularly for the treatment of a patient undergoing sickle cell anemia crisis.

It is another object of this invention to provide a treatment of a patient suffering from sickle cell anemia.

Still another object of this invention is to provide materials useful for and a treatment of human red blood cells containing hemoglobin S so as to substantially irreversibly inhibit the sickling of the resulting treated cells.

How these and other objects of this invention are achieved will become apparent in the light of the accompanying disclosure. In at least one embodiment of the practice of this invention at least one of the foregoing objects will be achieved.

It has been discovered that red blood cells, particularly red blood cells obtained from a patient suffering from sickle cell anemia or disease, i.e. red blood cells which contain hemoglobin S, when treated with cyanate, such as an aqueous or saline cyanate solution, e.g. an aqueous or saline potassium cyanate solution at a concentration in the range from about 0.001M–0.005M to about 0.10M, such as a concentration in the range 0.01M–0.075M, are substantially irreversibly inhibited from sickling.

For example, in accordance with one embodiment of the practice of this invention, in contrast to the high concentration of urea (1M) needed to prevent reversibly the *in vitro* sickling of 80% of hemoglobin S-containing red blood cells, an aqueous cyanate solution, such as aqueous potassium cyanate at a concentration in the range 0.01M–0.1M, irreversibly inhibits sickling to the same extent. The amount of sickling appears to be a function of the amount of cyanate incorporated into the acid precipitable protein (0.1 to 1.0 mol of cyanate per mol of hemoglobin).

When the practices of this invention are carried out employing a radioactive carbon [^{14}C]-cyanate most of the radioactivity is accounted for by carbamylation of the NH_2-terminal valine residues of hemoglobin. There is no detectable carbamylation of the lysine or cysteine residues. Hence, the reactive species HN=C=O (isocyanic acid) may be an analog of O=C=O since both compounds bind to the same valine residues of hemoglobin (Hb). It was noted that deoxygenated hemoglobin S (HbS)-containing sickled cells also incorporated [^{14}C]-cyanate but the sickling is not reversed. Upon oxygenation the normal morphology of 75% of these cells is assumed and upon subsequent deoxygenation these cells remain normal. An aqueous cyanate solution in accordance with this invention, such as an aqueous potassium cyanate solution at a concentration of 5 mM., has also been found to be an effective inhibitor of the gelling of deoxyhemoglobin S.

It has been reported that cyanate is in equilibrium with urea in aqueous solutions, see Walker et al., J. Chem. Soc. *67*, 746 (1895) and the equation:

$$NH_2-\overset{\overset{O}{\|}}{C}-NH_2 \rightleftharpoons NH_4^+ + {}^-NCO$$

It has also been reported by Stark et al., see J. Biol. Chem., 235, 3177 (1960), that cyanate carbamylates the ϵ-NH_2 groups of lysine residues and the NH_2-terminal residues of proteins, see the equation:

$$R-NH_2 + NH=C=O \longrightarrow R-\overset{\overset{H}{|}}{N}-\overset{\overset{O}{\|}}{C}-NH_2$$

As indicated hereinabove it has been discovered that an aqueous cyanate solution, e.g. potassium cyanate in aqueous solution, reacts with red blood cells from sickle cell anemia patients RBC (S/S) to prevent sickling and with isolated hemoglobin S to prevent gel formation.

The following is descriptive of a practice of this invention. Blood from sickle cell anemia patients was drawn into tubes containing either heparin or EDTA. The RBC (S/S) were washed three times with Dulbecco's phosphate-

buffered saline, pH 7.40, (PBS), see Dulbecco et al., J. Expt. Med., *99*, 167 (1954), and suspended in PBS to a final hemoglobin concentration of 130 mg. per ml. (2 μmols per ml.). The experiments were always conducted within 24 hours after the blood was drawn. In order to ensure complete oxygenation the suspension of cells was bubbled gently with air for a few minutes.

The morphology of RBC (S/S) depends on the oxygen saturation of HbS, see Hahn et al., Arch. Intern. Med., *39*, 233 (1927). When fully oxygenated most RBC (S/S) have normal morphology; upon deoxygenation these cells sickle.

The following procedure was used to determine the amount of sickling. The cell suspension was diluted tenfold with PBS; the solution was deoxygenated by evacuation at 30 mm. Hg with a water aspirator for seven minutes at 37° C. The evacuated tubes were then incubated an additional five minutes at 37° and the cells were fixed by rapid dilution with buffered formalin. These conditions of deoxygenation regularly produced abnormal morphology in 80–90% of the cells. The cells were counted by two observers using a Wild phase microscope (×600).

After incubation of RBC (S/S) with [^{14}C]-cyanate, the amount of radioactivity incorporated into the protein was determined after precipitation of the cells with cold 5% TCA (trichloracetic acid). The precipitate was collected on a 0.45 μmillipore filter and washed 4 times with 10 ml. of 5% TCA. The filter and the protein were placed in a sample solubilizer (NCS, Amersham/Searle) and the samples in scintillation fluid were counted in a Packard scintillation counter Model 3380.

The site of carbamylation in the RBC was determined as follows. The cells that had been incubated with cyanate (about 50 mg. of Hb) were precipitated and washed four times with 5 ml. of cold 5% TCA. After removal of the TCA by extraction with diethyl ether, the protein was first dried at 40° and then was dissolved in 1–2 ml. of 50% acetic acid. A portion of this solution was heated in 6 N HCl at 110° for 22 hours, see Stark et al., J. Biol. Chem., *238*, 214 (1963); amino acid analysis of the hydrolysate was carried out as described by Spackman et al. Anal. Chem. *30*, 1190 (1958). The amount of homocitrulline present in this hydrolysate is a measure of carbamylation at lysine residues. Another portion of the dissolved protein was treated with an equal volume of concentrated HCl for 1 hour at 100°. By this procedure the carbamylated NH_2-terminal residues are converted to hydantoins. The subsequent identification of the hydantoins was carried out as described by Stark et al. hereinabove.

Cell lysates were perpared after washing the cells three times with PBS; the washed cells were then lysed by the addition of an equal volume of water. The stroma were removed by centrifugation at 10,000x*g* for ten minutes at 4°. The lysates were concentrated by vacuum dialysis at 4° to a hemoglobin concentration of 150–200 mg. per ml. The HbS was stored at 4° in the deoxygenated state.

Gelation of deoxy HbS at 37° was judged to be complete when the solution did not flow upon inversion of the tube.

The cyanate, i.e. potassium cyanate, at low concentration inhibits the sickling of RBC (S/S). The morphology of deoxygenated RBC (S/S) without added cyanate is abnormal, whereas the proportion of normal deoxygenated cell is increased in the cyanate-treated sample.

Urea at high concentration also inhibits the sickling of RBC (S/S) but differs from potassium cyanate in that its effect is reversible, see Table 1 herein. The cells that were both incubated and deoxygenated in 1M urea are inhibited from sickling (Expt. 2); however, the effect of the 1M urea is abolished if the cell suspension is diluted to a final concentration of 0.1M urea before deoxygenation (Expt. 4). Potassium cyanate (Expt. 6) also inhibits sickling to the same degree as 1M urea but this inhibition is not affected by dilution. In fact, when the cyanate-treated cells are dialyzed or washed, see Table 2 herein, the sickling is still inhibited.

TABLE 1

Effect of urea and potassium cyanate on sickling of deoxygenated S/S RBC*

Experiment	Addition	Concentration during Incubation (M)	Concentration during Deoxygenation (M)	Normal deoxygenated cells (percent)
1				17
2	Urea	1.0	1.0	69
3	do	(+)	1.0	70
4	do	1.0	0.1	21
5	do	0.1	0.1	22
6	Potassium cyanate	0.1	0.01	72
7	do	0.01	0.001	34

* An oxygenated sample had 93% normal cells.
\+ The cells in Experiment 3 were deoxygenated immediately.

NOTE.—Oxygenated S/S RBC (2 μmols HbS/ml.) were incubated for hour at 37°. The cells were then diluted into the appropriate buffer and deoxygenated as described herein.

TABLE 2

The effect of dialysis and washing on the sickling of cyanate-treated RBC *

Experiment	Cyanate (M)	Dialysis	Normal deoxygenated cells (percent)
1			17
2		(+)	14
3	0.05		62
4	0.05	(+)	63
		Washing	
5			16
6	0.04		57
7	0.04	(+)	59

* An oxygenated sample had 89% normal cells.

NOTE.—Oxygenated S/S RBC were incubated for 1 hour at 37°. In Experiments 2 and 4 the cell suspensions were dialyzed for 20 hours against 500 volumes of PBS at 4°. In Experiment 7 the cells were washed three times with 22 volumes of PBS. The cells were diluted with PBS and deoxygenated as described herein.

The increase in the proportion of morphologically normal deoxygenated RBC S/S is directly related to the amount of cyanate incorporated. This was shown either by incubation of the cells for a fixed time with increasing potassium cyanate concentrations or by incubation of the cells at a fixed concentration of potassium cyanate as a function of time. Dose response curves for the RBC from six other patients were determined and similar relationships were observed; however, the amount of incorporated cyanate necessary to prevent 50% of the cells from sickling varies from 0.1 to 1 mol of cyanate per mol of hemoglobin.

When deoxygenated sickled RBC (S/S) are incubated with 0.1M potassium [^{14}C]-cyanate, see Table 3 herein, the same amount of radioactivity is incorporated as in the oxygenated cells but the sickling is not reversed. When such cells are oxygenated normal morphology is restored. Upon subsequent deoxygenation the cyanate-treated cells no longer sickle.

TABLE 3

The effect of potassium cyanate on deoxygenated sickle cells

	Percent normal cells	
	Without cyanate	With cyanate
First deoxygenation	14	11
Reoxygenation	65	65
Second deoxygenation	19	61

NOTE.—The cells (0.5 ml.) were placed in a tube with or without potassium [^{14}C]-cyanate (0.1 M) in the side arm; an oxygenated sample had 88% normal cells. After evacuation as described herein the contents of the side arm were mixed with the cells and the solution was then incubated for 1 hour at 37°. A portion was fixed with formalin before and after oxygenation. The cells were deoxygenated again after dilution with 9 volumes of PBS and a portion was fixed with formalin. The deoxygenated cells incorporated 1.80 carbamyl groups per hemoglobin molecule; oxygenated cells incorporated 1.68 carbamyl groups per hemoglobin molecule.

Disc gel electrophoresis, see Davis, Ann. N.Y. Acad. Sci., *121*, 404 (1964), of RBC (S/S) protein from cells that had been incubated with potassium [^{14}C]-cyanate showed that all of the radioactivity was in the HbS band.

The site of carbamylation on HbS was determined by amino acid analyses of hydrolysates of the sample containing 1.6 mols of cyanate per mol of hemoglobin. Alkaline hydrolysis of the isolated hydantoin fraction yielded only valine (1.4 mols per mol of hemoglobin). Homocitrulline was absent in an acid hydrolysate of another portion of the sample; this indicates that there is no detectable carbamylation of the lysine residues. In another experiment cells (from another patient) that were incubated with 0.01M potassium [^{14}C]-cyanate incorporated 1.5 mols of cyanate per mol of hemoglobin. Isolation of the hydantoin fraction yielded 1.2 mols of valine per mol of hemoglobin. Since CO_2 also reacts with the NH_2-terminal residues of Hb to form carbamino compounds, see Rossi-Bernardi et al., J. Physiol., *189*, 1 (1967) and Kilmartin et al., Nature, *222*, 1243 (1969), the reactive tautomer of cyanate, HN=C=O (isocyanic acid), may be considered to be an analog of O=C=O.

In contrast to deoxyhemoglobin A, deoxyhemoglobin S has the unusual property of reversible gel formation at 37°, see Harris, Proc. Soc. Expt. Biol. Med., *75*, 197 (1950) and Allison, Biochem., J., *65*, 212 (1957); the gel redissolves at 0°, see Murayama, J. Biol. Chem., *228*, 231 (1957). This property was used to test the relative efficacy of potassium cyanate and urea on the inhibition of gel formation. The results in Table 4 herein indicate that the gelation of HbS can be prevented by potassium cyanate at a concentration $\frac{1}{100}$ that of urea. The concentration of potassium cyanate needed to prevent gel formation varies with the preparation of HbS; another preparation required about 2.5 mM. potassium cyanate to inhibit gel formation.

TABLE 4

The effect of potassium cyanate and urea on the gelling of hemoglobin S

Addition	Concentration (mM.)	Gelling
	----------	+
Potassium cyanate	1	−
Urea	10	+
Do	50	+
Do	100	−

NOTE.—Oxygenated hemoglobin S (200 mg./ml.; 0.2 ml.) was incubated with potassium cyanate or with urea for 1 hour at 37°. The contents of the tubes were gassed with 90% N_2–10% CO_2 for 5 minutes at 0°. The tubes were incubated at 37°; after three hours the presence or absence of a gel was determined.

The effect of potassium cyanate on deoxygenated hemoglobin S was also tested. An evacuated tube contained a gel of deoxygenated hemoglobin S and potassium cyanate in the side arm. The gel was liquefied by lowering the temperature to 0° and the cyanate and the protein solution were then mixed; the incubation at 37° was resumed immediately. Gel formation was prevented at a concentration of 20 mM. potassium cyanate.

Studies to determine the effect of cyanate on various RBC functions were carried out. Preliminary experiments indicate that cyanate-treated RBC (S/S) (1 carbamyl group per hemoglobin molecule) have a normal capacity for binding and releasing O_2. Horse Hb containing four carbamyl groups on the NH_2-terminal valine residue also has a nearly normal oxygen dissociation curve, see Kilmartin et al., Nature, *222*, 1243 (1969). Another important function of Hb is the transport of CO_2 as carbamino groups on the NH_2-terminal valine residues of the protein, see Rossi-Bernardi et al., J. Physiol, *189*, 1 (1967). Tests were carried out to determine whether blocking of some of the NH_2-terminal valine residues is detrimental *in vivo*. Mice were injected only daily with a sublethal dose of potassium cyanate (28 μmols per mouse) without any apparent ill effects. After 20 injections, the mouse RBC contained an average of 1.6 carbamyl groups per hemoglobin molecule. Thus the presence of several carbamyl groups on hemoglobin does not seem to interfere seriously with the physiology of the red blood cells. This result indicates that cyanate could be used to prevent RBC (E/S) sickling *in vivo*.

In the tests reported herein as illustrative of the practices of this invention potassium cyanate was employed. The tautomeric structure of potassium cyanate is indicated by the equation:

$$\underset{\text{isocyanate}}{(K^+)(^-N{=}C{=}O)} \rightleftharpoons \underset{\text{cyanate}}{(N{\equiv}C{-}O^-)(K^+)}$$

Other cyanates, including the organic isocyanates, also useful in the practices of this invention, are sodium cyanate and ammonium cyanate and the like and other physiologically acceptable or compatible cyanate-producing compounds.

Although the pharmacology of cyanate, specifically sodium cyanate, has been previously studied, see F. Schutz, *Nature, 155,* 759 (1945) and Millington and Schutz Experientia, *5,* 133 (1949), these investigators nowhere disclosed the usefulness of cyanate in the treatment of sickle cell disease.

The tests reported herein and in the article entiled "Potassium Cyanate as an Inhibitor of the Sickling of Erythrocytes *In Vitro*" by Anthony Cerami and James M. Manning, Proc. Nat. Acad. Sci., U.S.A., Vol. 68, No. 6, pp. 1180–1183, June 1971, the disclosure of which are herein incorporated and made part of this disclosure, clearly demonstrate the advantages of the practices of this invention, i.e. a treatment employing a cyanate, such as an aqueous cyanate solution, e.g. an aqueous sodium cyanate or potassium cyanate solution, over a treatment employing a urea solution to prevent sickling of RBC (S/S). The tests demonstrate that the sickling of RBC (S/S) was blocked by aqueous 0.005M cyanate solution and that the effects are irreversible. The tests further demonstrate that the reaction of RBC (S/S) with cyanate occurred whether the cells were oxygenated or deoxygenated.

Additional tests which were carried out on seven patients suffering from sickle cell disease demonstrated that the survival time or life of the red blood cells taken from these patients is substantially increased when treated with cyanate in accordance with this invention. In these tests the red blood cells were treated with sodium cyanate in saline solution, the concentration of sodium cyanate being about 0.05M. The resulting cyanate-treated cells were labeled with sodium 51-chromate. The cyanate-treated, 51-chromate labeled cells were then returned to the patients. The results of these tests indicate that in the seven patients tested the mean 50% survival of the sickle cells was increased from 9.9 days for those cells not treated with sodium cyanate but chromium-51 labeled, to 20.7 days (normal 25–35 days) for those cells treated *in vitro* with sodium cyanate, labeled with chromium-51 and returned to the patient. These results provide evidence that the anti-sickling effect of cyanate observed *in vitro* is retained *in vivo*.

Further, in the above-desecribed additional tests a high level of carbamylation (approximately 4 mols of cyanate incorporated per mol of hemoglobin tetramer) was employed in order to insure an observable *in vivo* effect. A lower level of carbamylation would appear to be equally as effective in prolonging the apparent life span of the sickle cells since a maximum of 50–80% of the erythrocytes or cells from the patients studied were prevented from sickling *in vitro* after incorporation of 0.1 to 1.0 carbamyl group per hemoglobin molecule. Since erythrocytes or cells from normal individuals having a normal life span when transfused to patients with a sickle cell disease, the increased life span of the cyanate-treated sickle cells indicated by the observed results of the above-described tests reflects those cells which do not sickle in the circulation. Therefore, accordingly, the anti-sickling effect of cyanate observed *in vitro* appeared to be retained in the patient.

Although emphasis in this disclosure has been directed to the treatment of persons suffering from sickle cell disease, beneficial results are also obtainable in the treatment

of those persons suffering from the so-called sickle cell trait.

As indicated hereinabove, in the practice of this invention red blood cells from a sickle cell anemia patient may be withdrawn from the patient and treated with a cyanate solution and then re-introduced into the patient. In such an exchange-replacement therapy procedure a unit of the patient's blood would be removed into a bag containing acidi-citrate-dextrose (ACD). The blood would then be centrifuged and the plasma returned to the patient. The recovered red cells would be incubated with an aqueous solution of sodium cyanate (0.001M–0.01M) at about 37° C. for about 1–2 hours. The cells would then be washed to remove any unreacted cyanate and the resulting carbamylated cells would then be re-introduced or re-infused into the patient.

The intraveous introduction of a cyanate solution, e.g. a sterile physiologically acceptable, pyrogen-free sodium cyanate solution, the sodium cyanate being present at a concentration of about 0.1M, would also appear to yield beneficial results and inhibit sickling of the red blood cells in the patient.

Additionally, in accordance with embodiments of the practices of this invention, beneficial results would be obtainable by administering a cyanate or a cyanate-producing compound to a patient via other routes for eventual introduction into the blood stream. Various methods and techniques might be employed for the administration of a cyanate to a sickle cell anemia patient being treated. It would appear that oral administration of a cyanate, such as sodium cyanate, in an amount of about 10 mg. to about 10 grams per patient per day would yield effective results. In addition to oral administration the cyanate may be administered through a natural body opening, such as by the rectum.

In one particularly useful embodiment of this invention the cyanate or cyanate-producing compound could be administered through a composition, such as a suppository composition. Various materials are useful in the preparation of such compositions. In addition to the cyanate or cyanate-producing compound the composition would als include the conventional well-known carriers or bases, such as theobroma oil (cocoa butter), gelatin, glycerinated gelatin, hydrogenated vegetable oils and mixtures of polyethylene glycols of various molecular weights and fatty acid esters of polyethylene glycol, the above-mentioned bases or carriers having compositions so as to melt or disintegrate at about body temperature or when introduced into the body to release the cyanate component therein.

In accordance with another embodiment of the practice of this invention the cyanate could be administered orally to the patient by addition of a cyanate compound or cyanate-producing compound by way of a suitable beverage, such as a fruit drink or artificially flavored aqueous beverage and the like.

When such materials, such as the above-mentioned compositions, particularly the suppository compositions, are employed, the cyanate component is usually present therein in a minor amount. For example, in a suppository the amount of cyanate would be present therein in a minor amount by weight of the composition. When the cyanate is administered orally it may be embodied in suitable tablet form, such as a cyanate-containing tablet provided with an enteric coating, the cyanate being present in a major or minor amount. If desired, the cyanate may be incorporated in a suitable beverage or drink by direct addition thereto or in admixture therewith.

As will be apparent to those skilled in the art in the light of the foregoing disclosure, many modifications, alterations and substitutions are possible in the practice of this invention without departing from the spirit or scope thereof.

We claim:

1. A method of preventing the sickling of red blood cells in a sickle cell anemia patient comprising contacting the blood of said patient with an effective but non-toxic amount of a cyanate selected from the group consisting of sodium, potassium and ammonium cyanate.

2. The method of Claim **1** wherein sodium cyanate is employed.

3. The method of Claim **1** wherein potassium cyanate is employed.

4. The method of Claim **1** wherein ammonium cyanate is employed.

5. The method of Claim **1** wherein the blood is contacted with the cyanate by the exchange replacement therapy procedure.

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