PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 578–585 (1974)

"Holly Wreath" Morphology of Feline Erythrocytes—The Effects of Cyanate and 4,4′-Dipyridyldisulfide[1] (37855)

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(Introduced by J. M. Fujimoto)

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During the course of work on the domestic cat hemoglobins in this laboratory, some feline erythrocytes were occasionally seen to assume a non-discoidal "holly wreath" or acanthocytic shape under conditions in which normal human cells retain a normal morphology. From analogy with well-studied instances of deformed erythrocytes in human diseases, it appears that at least three mechanisms can cause the abnormal red cell morphology: (i) low hemoglobin solubility; (ii) abnormal erythrocyte membrane; and (iii) abnormal glycolytic enzymes resulting in low levels of high energy phosphate compounds. These mechanisms often do not operate independently, so at least initially all three must be considered. Based on present knowledge, it is impossible to identify the mechanism responsible for cat red cell deformation. Indeed, a rational argument can be advanced in favor of each of the three: (i) Low hemoglobin solubility: Previous work in this laboratory (1) has shown that cat hemoglobin crystallizes in the deoxy-form from erythrocyte hemolysates that are stored in tightly stoppered containers at 4°. Deoxyhemoglobin is formed readily by spontaneous deoxygenation of the hemolysate (2) because of the low oxygen affinity of cat hemoglobin (3), and its crystallization is facilitated presumably because of the lower solubility of the deoxy- compared to the oxy-form of the protein. Inclusion bodies are frequently associated with the occurrence of malformations. Such inclusions have long been recognized in cat erythrocytes (4, 5) although no explanation for their occurrence was offered. Recently, however, Altman, Melby and Squire (6) have described the occurrence of crystalloid bodies in circulating erythrocytes of cats and have suggested that this is due to an intrinsic property of cat hemoglobin. (ii) Abnormal erythrocyte membrane: The unusual transport and permeability properties of the feline erythrocyte membrane may render them particularly susceptible to deformation (7). (iii) Abnormal glycolytic enzymes resulting in low levels of high energy phosphate compounds: Although the glycolytic enzymes of the feline erythrocyte are largely uncharacterized, it has long been known that organic phosphates are present in low concentrations in these cells (8–10). It is possible, therefore, that lacking an adequate reservoir of ATP, cat red cells are unable to maintain a normal discoidal shape *in vitro*.

Evidence is presented here which emphasizes the role of hemoglobin solubility in deformation of cat red cells. Furthermore, the prevention of this deformation by treatment of cat cells *in vitro* with cyanate and 4,4′-dipyridyldisulfide is described and correlated with the effects of modification of hemoglobin with these agents on the properties of the protein.

Materials and Methods. Blood was obtained in heparin from anesthetized domestic cats by cardiac puncture. The blood

[1] This work was supported by grants from the U. S. Public Health Service (AM HE 15770) and the Wisconsin Heart Association.

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was diluted (1:5) with isotonic phosphate buffer (11) immediately after collection for the preparation of slides. Silicone grease was used to seal a drop of diluted blood under a cover slip on a glass slide for microscopic observation of changes in red cell shape with time. In some cases, cells were fixed by adding 2 drops of the blood suspension into 5–10 ml of cold 3.7% formaldehyde in isotonic phosphate buffer (12). Dry smears were prepared after treatment of the blood with buffered sodium dithionite as described by Pauling and Itano (11).

Reaction of 2 ml of blood or washed red cell suspensions with 0.2 ml of 0.2 *M* KCNO in 0.9% NaCl was conducted for 2 hr at 37° in a Dubnoff shaker incubator. Control cells were treated with isotonic saline instead of the KCNO solution. To evaluate hemoglobin modification, 4 ml of cell suspension were reacted with 0.01 *M* cyanate containing 25 μCi ^{14}C-KCNO for 2 hr, washed with isotonic saline, hemolyzed with 2 vol of water, and centrifuged. The hemoglobin solution was then passed through a 0.9 × 10 cm Sephadex G-25 (fine) column to remove unreacted reagent. Peptide mapping of the hemoglobins was carried out as described by Baglioni (13). The labeled peptide maps were allowed to stand in contact with X-ray film for 2–4 wk for radioautography. For reaction with PDS, 1 ml of cell suspension (30% hematocrit) was reacted with 1 ml of 10^{-3} *M* PDS in isotonic buffer for 1 hr at 37°. The suspension was then centrifuged, the supernatant was removed, and the packed erythrocytes were resuspended in isotonic buffer. This procedure was repeated 3× to remove excess reagent. The cells were finally diluted with isotonic phosphate buffer (1:5) for microscopic observation. Photomicrographs were taken with a camera attached to a Leitz phase-contrast microscope.

Oxygen saturation measurements were carried out as described before (3).

Results. Figure 1 shows a photograph of crystals of cat deoxyhemoglobin that formed spontaneously when the cat hemolysate was

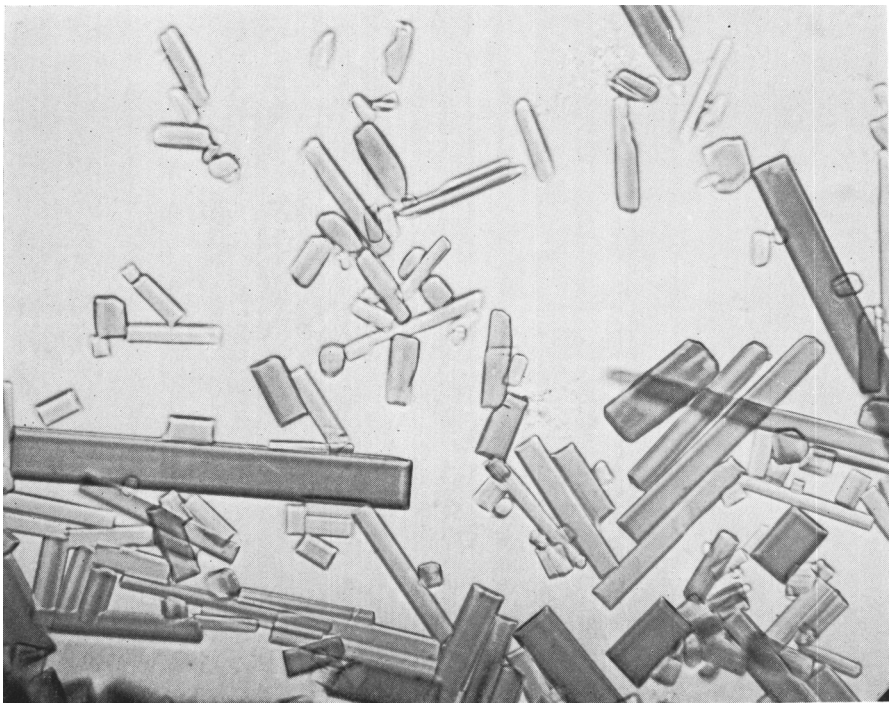
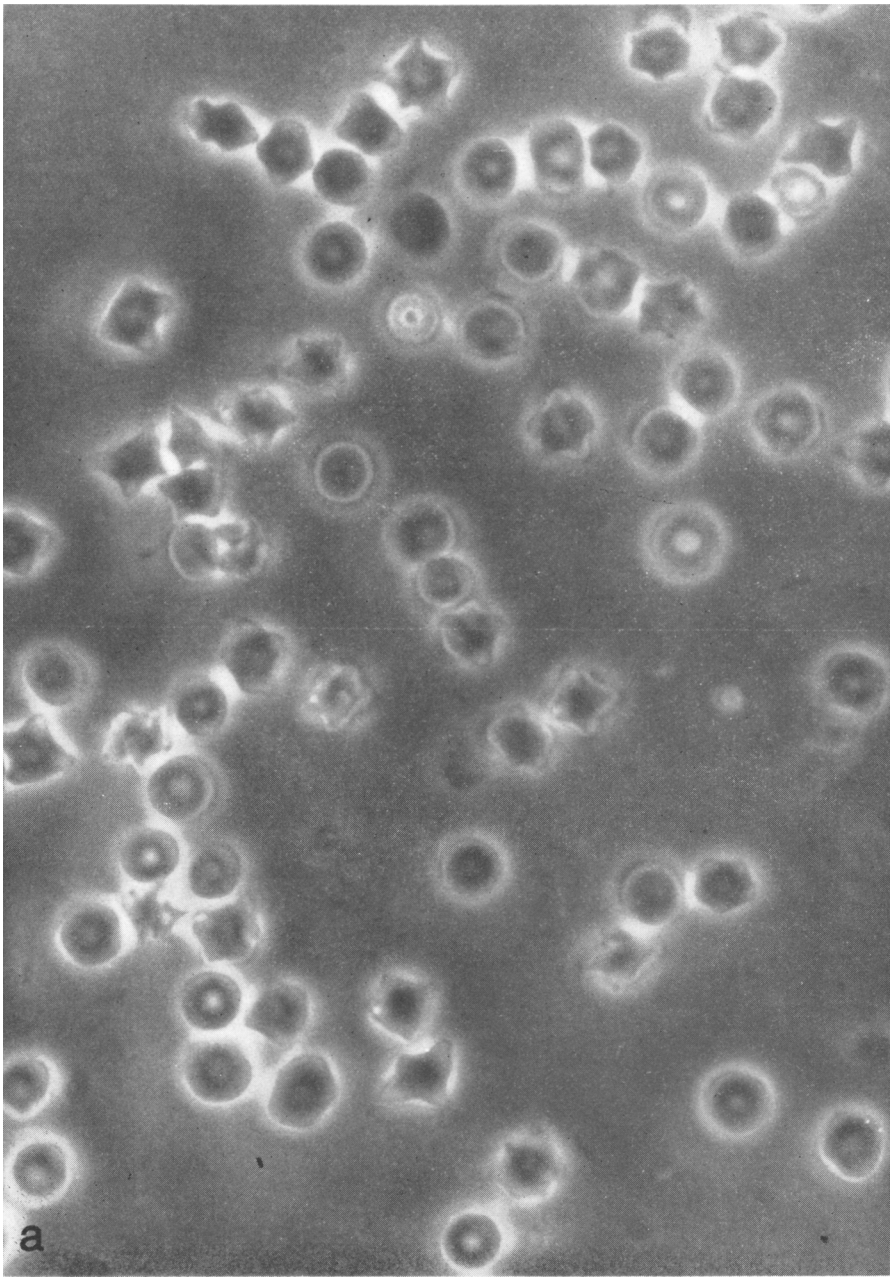
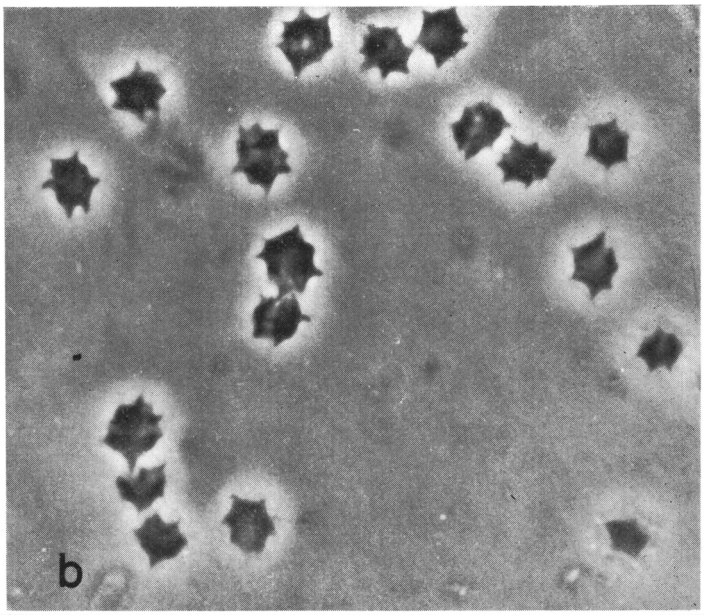


FIG. 1. Cat hemoglobin crystals formed spontaneously from an hemolysate in a stoppered container at 4°.

stored in a stoppered container at 4°. Such crystals are usually seen to form from a 6–15% hemoglobin solution after about 1 wk. Fresh cat blood observed immediately after preparation of the wet smear contains erythrocytes of normal morphology. However, all feline blood suspensions exhibit a relatively rapid spontaneous development of "holly wreath" forms; as soon as 15 min after preparation of the sealed slides, a significant number of morphologically aberrant cells is observed, and the severity of these malformations increases with time. Figure 2a depicts cells that have been sealed in this manner for 26 hr. Although it is not clear from this photograph,





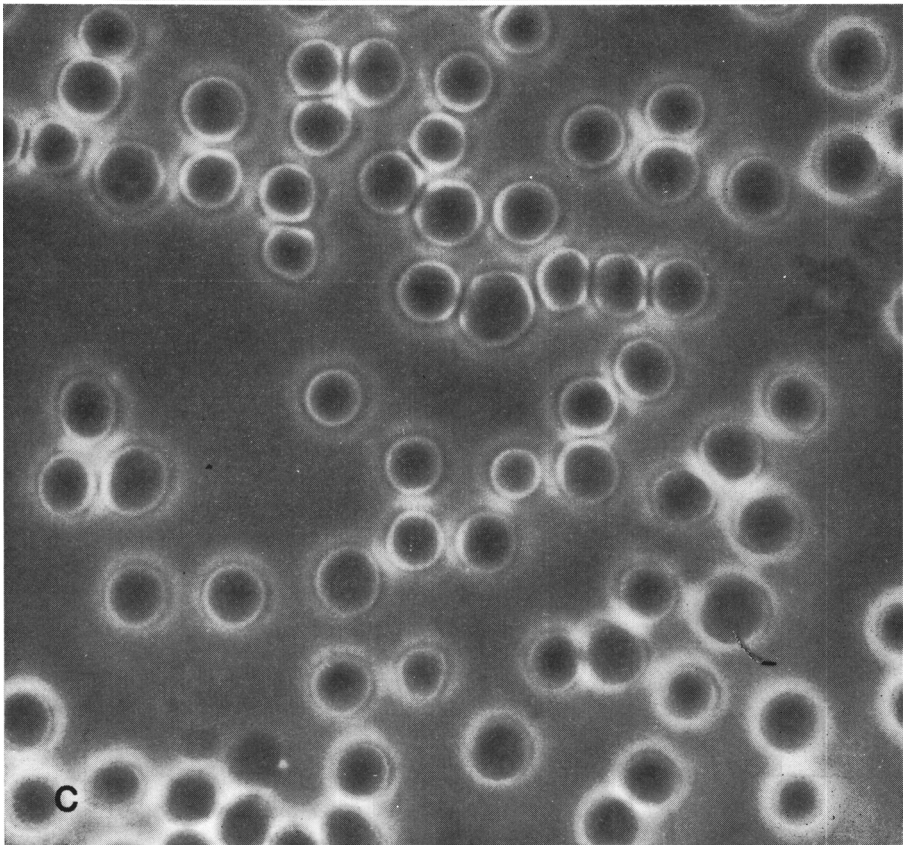


FIG. 2a. Cat erythrocytes photographed 23 hr after preparation of wet smear. (b) Dry smear of cat erythrocytes treated with dithionite as described by Pauling and Itano (11). (c) Cyanate treated cat erythrocytes photographed 26 hr after preparation of a wet mount. The cells were treated with cyanate by mixing equal volumes of cells and 0.2 *M* KCNO (dissolved in isotonic saline) and incubating at 37° for 2 hr. Cells treated with PDS appear similar to those treated with cyanate. Wet mounts of cyanate and PDS treated and control cells were prepared after dilution of cells in the Pauling and Itano buffer.

the formation of inclusion bodies after several hours is invariably observed in all of the cells so treated. To evaluate the role of deoxygenation in this phenomenon, the effect of sodium dithionite, a reducing agent commonly used to remove dissolved oxygen from solutions of hemoglobin, was examined. Deoxygenation with isotonically buffered sodium dithionite results in immediate and complete transformation of the cells from discoidal to the acanthocytic form (Fig. 2b). Normal human or rabbit erythrocytes do not exhibit such a phenomenon, but similar changes are reported to occur when the red cells from sickle cell anemia patients are treated with dithionite (14). The forms of most of these latter cells are "holly wreath" rather than crescentic presumably because the rapidity of deoxygenation does not allow time for the reduced HbS molecules to become oriented in the paracrystalline state (15).

When feline erythrocytes are treated with KCNO, transformation into acanthocytes as well as formation of inclusion bodies are dramatically prevented (Fig. 2c). The normal discoidal shapes are retained even after reaction with sodium dithionite. Modification of the α-NH_2 groups at the amino termini of the α- and β-chains of HbA and of the α-chain of HbB is demonstrated by radioautography of tryptic peptide fingerprints of labeled hemoglobins prepared by reaction with ^{14}C-KCNO (Fig. 3a and b). Three peptides are labeled in HbA and two in HbB. Two of these are found in common in both digests and are identified as carbamylated αT-1 and the undigested αT-1,2 peptides. The locations of these amino terminal peptides on the fingerprints were described earlier (1). The third radioactive peptide in the HbA digest is identified as carbamylated βT-1 from its location on the map. The absence of a correspond-

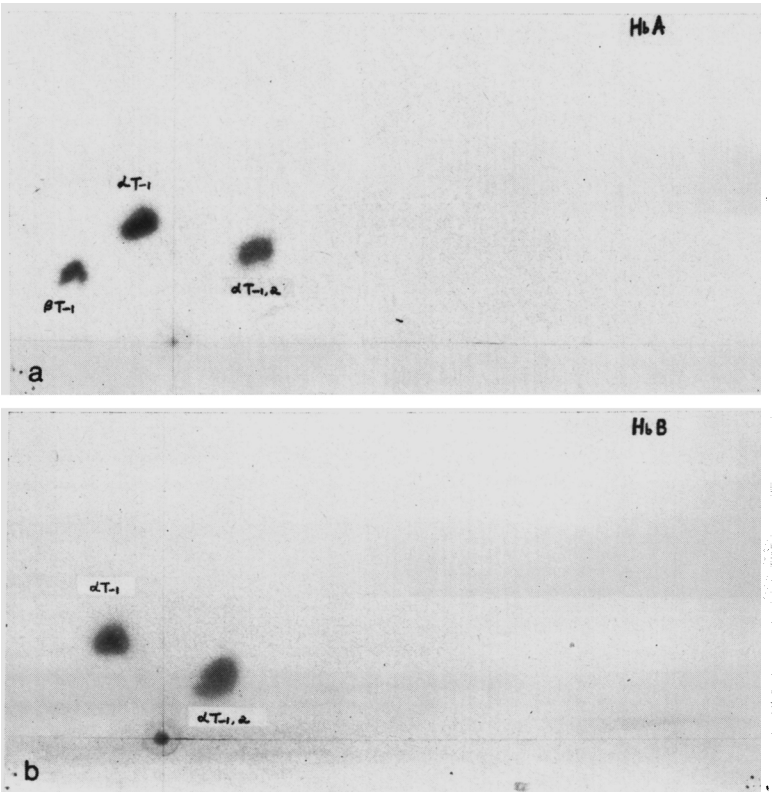


FIG. 3. Autoradiographs of tryptic fingerprints of cat hemoglobins reacted with ^{14}C-KCNO. (a) HbA and (b) HbB.

ing spot in the HbB digest confirms earlier conclusions (16) that HbB is blocked at these amino termini with acetyl groups. Under the conditions employed here, it is unlikely that any other residues in the protein were carbamylated. Additional experiments incidental to these studies have shown that the reactivity of the amino terminal groups is significantly greater in the deoxy- than in the oxy-forms.

The oxygen affinity of cat hemolysates containing carbamylated hemoglobins is markedly increased (Fig. 4). The P_{50} decreases from a value of 25 mm to about 10 mm at pH 7.0 and 20°, and the ability of an organic phosphate (inositol hexaphosphate) to lower the oxygen affinity (17) of the protein is diminished. The latter effect is presumably due to carbamylation of the B-chain amino termini which eliminates the required charged binding sites for organic phosphates (18, 19).

Since the effect of KCNO in preventing cell deformation could be ascribed to a modification of hemoglobin that leads to increased oxygen affinity, it was of interest to examine the effects of another type of modification that is known to cause an increase in hemoglobin oxygen affinity on this process. Sulfhydryl blocking reagents are known to react with intracellular hemoglobin under appropriate conditions without causing significant hemolysis and to thereby cause an increase in oxygen affinity of the protein. Reaction of erythrocytes with the reagent 4,4′-dipyridyldisulfide results in rapid modification of most of the –SH groups of intracellular hemoglobin and like KCNO has a dramatic influence in preventing the deformation of the erythrocyte. Normal discoidal shapes are also retained even after treatment with sodium dithionite.

Discussion. Erythrocytes from goats (20), some deer (21), and sheep (22) also exhibit changes in shape that are linked to the oxygenation–deoxygenation process. However, in contrast to the deformations that are associated with deoxygenation of cells that contain HbS and cat hemoglobin, the development of morphologically abnormal cells in the blood of deer and sheep occurs only when the red cells are fully oxygenated. In the case of the goat, the relationship between oxygenation–deoxygenation and malformation of erythrocytes is as yet unclear.

Cat blood has previously been shown to contain two hemoglobins (A and B) that occur in ratios that range from 9/1 to 1/1 in different individuals (23). The possible involvement of one or both of the cat hemoglobins in this phenomenon is sug-

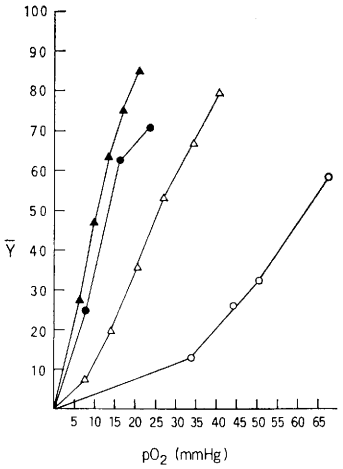


FIG. 4. Oxygen saturation curves of a control and cyanate-treated cat hemolysate with an HbA/HbB ratio of 9/1 in the presence and absence of excess inositol hexaphosphate. (▲) KCNO-treated with no added IHP; (●) KCNO-treated plus IHP; (△) control with no added IHP; (○) control with added IHP.

gested by the series of observations reported here. Preliminary work indicates that the tendency towards "holly wreath" formation is independent of the ratio of HbA/HbB in the blood examined. In retrospect, the behavior of cat erythrocytes is not surprising since both cat hemoglobin A and B exhibit relatively low oxygen affinities (3) and readily co-crystallize as deoxyhemoglobin (1) on spontaneous deoxygenation of the hemolysate. The occurrence of crystalloid bodies associated with the cell malformations, the facilitation of the process with dithionite, and its prevention with KCNO or PDS indicate that the phenomenon is related to deoxygenation of the hemoglobins. Other studies in this laboratory have shown that reaction of cat hemolysates with cyanate or PDS will markedly inhibit the tendency of the hemoglobins to crystallize spontaneously. This is presumably due to increased hemoglobin oxygen affinity that results from modifications with these reagents although it is possible that other changes in the physical properties of the protein may be of greater significance.

"Sickling" of erythrocytes in sickle cell anemia occurs with deoxygenation and can be prevented by reaction of the intact erythrocyte with potassium cyanate (KCNO) or carbamyl phosphate (24, 25). Carbamylation of amino terminal groups of sickle cell hemoglobin (HbS) profoundly increases its oxygen affinity and also inhibits its tendency to gel. "Sickling" of the erythrocyte is presumably inhibited as a result of this modification. These observations suggest some similarities in the properties of the cat hemoglobins and human HbS and that perhaps the mechanism of erythrocyte deformation in the cat resembles that proposed for human erythrocytes with HbS. However, attempts to reverse this process in cat erythrocytes with carbon monoxide or oxygen have given equivocal results. Nevertheless, irreversible sickling in the human is also known to occur and is said to involve alterations in both hemoglobins S and the cell membrane (22). Since it is known that both KCNO and PDS can react with membrane proteins (25, 27), the possibility remains that the observed distortion in deoxygenated cat cells primarily involves the erythrocyte membrane and that the effects of cyanate and PDS stem from modification of membrane components. It is also possible that the feline erythrocyte is prone to deform because of limitations in the amounts of available ATP and 2,3-diphosphoglycerate. Cat erythrocytes are known to contain relatively low amounts of these phosphate compounds and are low in total phosphate concentration as well (8–10). Development of acanthocytes associated with lowered ATP concentrations has been described in human pyruvate kinase deficiency (28). Thus, a combination of factors that is peculiar to the feline erythrocyte system may be responsible for the observed cell deformation. It is of interest that during the preparation of this manuscript Jain and Kono (29) published observations of unusual shapes in feline erythrocytes seen by scanning electron microscopy. Clarification of the mechanism of action by which cyanate and PDS can prevent this deformation clearly requires further investigation.

Summary. Feline erythrocytes develop into "holly wreath" or acanthocytic forms upon deoxygenation. Prior reaction with KCNO or with the sulfhydryl modifying reagent, PDS, prevents the development of abnormal morphology. Cyanate causes irreversible modification of the terminal NH_2-groups and PDS modifies the reactive –SH groups in the hemoglobins to profoundly alter the properties of the feline hemoglobins. A possible analogy with the human "sickle cell" system is indicated.

We thank Mrs. S. L. Tsai, Mrs. Y. P. Huang, and Miss Colleen Counihan for technical assistance. We also thank Mr. Gene Putz for helpful discussions and Mr. Anthony Kuzma for expert help in preparations of the photomicrographs.

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Received Sept. 4, 1973. P.S.E.B.M., 1974, Vol. 145.