Fractionation Studies of Copper in Erythrocytes from Normal, Sickle Cell Anemia, and Hemoglobin C Disease[1] (34762)

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Increased amounts of copper have been demonstrated in a variety of pathological conditions. Elevated serum copper is associated with acute and chronic infections (1), pregnancy (2), various malignancies including Hodgkin's disease, acute and chronic leukemias (3), and with malignant tumors (4). However, no cellular relationships with copper have been reported in these disorders. Extensive copper deposits in tissues have been demonstrated in Wilson's disease, particularly localized in the mitochondria and lysosomes of neural and liver tissue (5, 6). In sickle cell anemia and sickle cell trait, copper-containing granules were demonstrated cytochemically in every erythrocyte examined (7). The role of these copper-containing granules in sickling red cells is not known.

The purpose of this study was to fractionate by ultracentrifugation crude homogenates of erythrocytes from patients with sickle cell anemia (Hb SS), sickle cell trait (Hb AS), hemoglobin C disease (Hb AC and SC), and from normal individuals and to measure the copper content of the fractions obtained. By this means it was anticipated that a better understanding of the physicochemical nature of copper in these cells might evolve.

Materials and Methods. Blood samples were obtained from 16 patients with sickle cell anemia (Hb SS), 30 with sickle cell trait (Hb AS), 14 with hemoglobin C trait (Hb AC), 5 with hemoglobin SC disease (Hb SC), and 40 normal individuals with no known prior history of hematologic disease. Patients were made available through the various hospitals and the County Health Unit in the Temple area; from Bastrop, Texas; and from Tuskegee Institute, Alabama. Samples were mixed with sufficient ethylenediaminetetraacetic acid (EDTA), disodium salt, anticoagulant and were kept refrigerated prior to processing.

Following removal of the plasma and buffy coat by aspiration, the erythrocytes were washed three times with an excess of physiologic saline. Quantitative hemoglobin electrophoretic analysis (GelmanSepraphore III, cellulose polyacetate) was performed on each sample. For fractionation studies, known amounts (wt, vol, and total hemoglobin content) of saline-washed packed erythrocytes were hemolyzed by the addition of 2 vol of deionized water/g of cells followed by several cycles of freezing and thawing. Sample volumes were adjusted to 10.0 ml with water and were centrifuged for 2 hr at 40,000*g* in the Beckman model L2 preparative ultracentrifuge. The supernates were removed, the pellets were resuspended in water, and the centrifugation step was repeated for 1 hr. The supernatant washes were removed and the pellets were resuspended in measured volumes (2 to 4 ml) of 2 *N* ammonium hydroxide to obtain finely divided suspensions. These suspensions and the supernates and washes from ultracentrifugation were assayed for copper content by atomic absorption spectrophotometry.

Results. Increased amounts of copper were

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TABLE I. Copper Content of Erythrocyte Lysate Fractions Obtained by Ultracentrifugation: Comparison of Normal Controls (Hb AA) with Various Hemoglobinopathies.

Hemoglobin type	% Abnormal hemoglobins		Cu (μg/100 g of cells)			Cu (μg/100 g of hemoglobin)
	S	C	Pellet	Supernate	Total[c]	Pellet
AA (41)[a]	0	0	18.7 ± 0.7[b]	106.2 ± 3.8	124.9 ± 3.8	56.4 ± 2.2
SC (5)	51.0 ± 1.5	47.0 ± 1.6	26.8 ± 4.5	125.4 ± 14.0	152.2 ± 11.1	79.4 ± 13.7
AC (14)	0	45.6 ± 3.6	26.1 ± 2.5	122.4 ± 8.2	148.4 ± 8.4	86.1 ± 8.5
AS (30)	38.8 ± 1.4	0	29.0 ± 2.0	117.4 ± 7.0	146.3 ± 7.6	98.9 ± 7.5
SS (16)	95.9 ± 0.8	0	43.8 ± 4.5	138.6 ± 6.7	179.1 ± 9.1	119.1 ± 13.6

[a] Values in parentheses represent numbers of samples.

[b] Means and standard errors. Any two means *not* bracketed by the same vertical line are significantly different.

[c] Not analyzed for mean significant differences.

found associated with the ultracentrifugally isolated pellets from red cells containing hemoglobin S as compared to normal erythrocyte pellets (Table I). Results are expressed as micrograms of copper per 100 g of packed cells and per 100 g of hemoglobin. The standard errors of the means (SEM) are included with the values. Duncan's Multiple Range Test (8), as extended and applied by Kramer (9) to groups of unequal size, was used to test the significant of group mean differences. As noted by Kramer, this test is a conservative one. Therefore, it is not highly sensitive in excluding small differences between sample means. Further, the evaluation of specific significance and prediction levels is quite difficult and impractical; therefore, the best estimates of probabilities have been assigned at $p = .05$, or less. As indicated in Table I, group AA (pellet) was significantly lower than groups AS (pellet) and SS (pellet), but was not distinguishable from groups SC (pellet) or AC (pellet). Group AA (supernate) was significantly lower than group SS (supernate), but other group differences were not remarkable. Results of pellet copper based on hemoglobin content indicate group AA to be significantly lower than groups AS and SS, while the other groups did not differ.

A frequency distribution plot of erythrocyte particulate (pellet) copper in relation to hemoglobin type is presented in Fig. 1. The majority of group Hb AA subjects fell within the range of 0.11 to 0.20 μg/g of packed cells; groups HB AS and Hb AC fell within the range of 0.21 to 0.30; and group Hb SS at the $>$ .40 level. Group Hb SC contained too few samples for demonstration of a trend.

Discussion. Previous results by cytochemical methods indicated a striking difference in copper content of sickling erythrocytes as compared to normal red blood cells (7). The evidence suggests the conclusion that copper deposits in these cells are localized in "particulate" arrays or are contained in specific "granules" of the cytoplasm. Electron microscopic evidence (to be published) has shown that sickle cells contain lysosome-like organelles that have a unit membrane, and possess acid phosphatase activity. Preliminary evidence indicates that these organelles also contain deposits of copper. The present results demonstrate higher total quantities of erythrocyte copper to be present in sickle cell anemia, sickle cell trait, and in hemoglobin C disease as compared to normal controls. Due in part, at least, to the insensitivity of the statistical analysis employed, the latter groups containing hemoglobin C were not significantly different from the normal controls.

A considerable portion of the total copper from these cells is associated with an ultracentrifugally sedimentable fraction of freeze–thawed lysates. The average copper concentration of the sedimentable or "pellet" fraction of erythrocytes from patients of group Hb SS was approximately twice that of the mean of similarly prepared fractions from normal control erythrocytes (Hb AA).

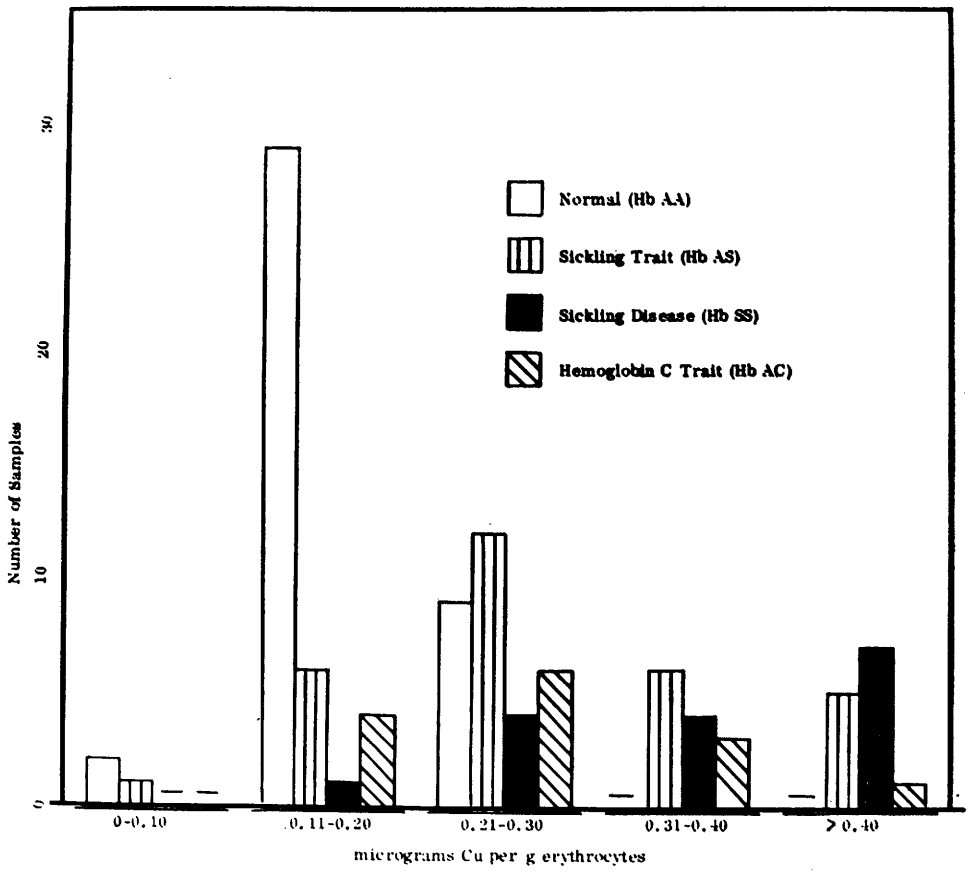

FIG. 1. Frequency distribution of copper in pellets obtained by ultracentrifugation of erythrocyte lysates. Ranges are μg of Cu/g of packed cells as related to hemoglobin type.

These fractions from sickle cell trait (Hb AS) and hemoglobin C disease (Hb AC) were approximately 1.5 times the copper concentration in normal cells. Thus, the results obtained are consistent with those obtained with cytochemical technics although the differences are less pronounced. The total copper (pellet and supernate) detected in normal red blood cells corresponds with data published (10). As shown in Table I, there is a significant copper increase in the pellet fraction of "S" hemoglobinopathies.

Abnormal metal deposits have been reported in several diseases: increased amounts of metal deposits are known to be in the mitochondria and, in later stages, in lysosomes, *i.e.*, iron as ferritin in the mitochondria in Cooley's disease (11); copper in the neural and liver tissue in mitochondria and lysosomes in Wilson's disease (5, 6). The significance of increased copper in sickling erythrocytes in unknown.

Summary. Quantitative copper determinations of erythrocyte fractions obtained by ultracentrifugation of erythrocyte homogenates from 105 persons were made by atomic absorption spectrophotometry. Elevated copper levels were demonstrated in the particulate fraction of erythrocytes of 16 patients with sickle cell anemia (Hb SS), 30 patients with sickle cell trait (Hb AS), 14 patients with hemoglobin C trait (Hb AC), and 5 patients with hemoglobin SC, as compared to similar fractions from 40 normal persons (Hb AA). Erythrocytes from sickle cell anemia patients contained sedimentable copper levels approximately twice that of normal controls. The sedimentable copper

values from sickle cell trait were 1.5 times that of normal cells. The significance of these findings is discussed.

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