Membrane protein carboxymethylation was determined in intact normal, reversibly sickled, and irreversibly sickled erythrocytes after incubation with l-[methyl-\(^{3}H\)]methionine. Irreversibly sickled cells incorporated approximately three times more [\(^{3}H\)]methyl groups into membrane components compared to fresh uninfected normal cells. The extent of methyl esterification of membrane proteins as determined by the quantification of alkali-labile methyl groups showed, in contrast, that 90% of total [\(^{3}H\)]methyl incorporation by normal cells was alkali-labile compared to 88% for reversibly sickled cells and 70% for irreversibly sickled cells. In irreversibly sickled cells, protein methylase II substrates (periodic acid Schiff I and II and band 4.5) are methylated as has been observed for normal cells; however, substantially more methyl incorporation into these substrates, and into low molecular weight membrane protein components occurs in the irreversibly sickled cell. The radioactivity distribution profile revealed that irreversibly sickled cells incorporated 2-fold more cycloleucine-sensitive [\(^{3}H\)]methyl groups into periodic acid Schiff I plus II (glycoporphin A) compared to normal or reversibly sickled cells. This increased incorporation of [\(^{3}H\)]methyl groups into membrane components is not due to altered protein methylase II activity or endogenous levels of S-adenosylmethionine. Results also show that the most dense subpopulation of normal erythrocytes incorporate 3-fold more [\(^{3}H\)]methyl groups into the membrane compared to least dense, younger, red cell fractions. These data suggest similarities between aged normal cells and irreversibly sickled cells, and indicate that in irreversibly sickled cells, the organization of membrane protein components contiguous to the cytoplasm and presumably to protein methylase II, is different from normal cells resulting in the increased availability of methyl acceptor sites.

Post-translational methylation and phosphorylation of proteins are widely occurring biochemical reactions in both eukaryotes and prokaryotes (1–4). Recent studies have demonstrated that membrane proteins and phospholipids of erythrocytes undergo specific posttranslational modifications. These covalent modifications have been shown to affect structure and function of cell membrane (5–9). For example, studies have shown that erythrocyte membrane proteins undergo phosphorylation-dephosphorylation reactions which have been implicated to play a role in the regulation of cell shape and deformability (6, 7). It has also been demonstrated that methylation of erythrocyte membrane phospholipids results in an increase in membrane fluidity with a concomitant increase in \(Ca^{2+}\)-ATPase activity (8, 9). Carboxymethylation in vivo of erythrocyte membrane protein has been studied in human erythrocytes (10). It was shown that esterification of free carboxyl groups of erythrocyte membrane proteins was catalyzed by endogenous S-adenosylmethionine:protein O-methyltransferase (EC 2.1.1.24, protein methylase II) using S-adenosyl-L-methionine as the methyl donor (10). As a result of carboxymethylation, the negative surface charges on the membrane protein contiguous to the cytoplasm become neutralized. This transitory alteration of the surface charge may therefore affect the dynamic properties of erythrocyte membranes.

It has been observed that erythrocytes in the circulation of patients with sickle cell anemia have membrane abnormalities as reflected in altered permeability (11), elevated calcium content (12), and increased rigidity (13). These abnormalities are predominantly observed in the erythrocyte subpopulation referred to as irreversibly sickled cells (ISC). These irreversibly sickled cells constitute 2–30% of cells in the oxygenated blood of sickle cell patients. Since these cells do not undergo rapid shape change as do normal cells, ISC have been considered likely candidates which may initiate microvascular occlusions (14–16). Recent studies of Hebbel et al. (17) showed that erythrocytes from sickle cell patients adhered to cultured endothelial cells, unlike normal red cells, as a result of an aberration of normal topographic distribution of these charged groups on the membrane surface. These and other studies (18–20) have suggested the existence of distinct differences in the organization of membrane proteins in sickle erythrocytes compared to normal cells. The present study was undertaken to further define these phenomena.

The purpose of this investigation, therefore, is to determine if the altered structure and function of irreversibly sickled cells are correlated with an impaired ability of these cells to carry out posttranslational modification of membrane constituents as a result of the interaction of hemoglobin S with the membrane. The specific process which is the focus of this investigation was methyl esterification of erythrocyte membrane proteins catalyzed by endogenous protein methylase II. The results presented in this communication show that fresh in vivo irreversibly sickled cells incorporate approximately 3-fold more [\(^{3}H\)]methyl groups into the membrane, compared to normal cells, and that the distribution of alkali-stable and alkali-labile methyl groups incorporated into membrane polypeptides of irreversibly sickled cells is altered compared to normal erythrocytes.

The abbreviations used are: ISC, irreversibly sickled cells; RSC, reversibly sickled cells; AA, normal cell; PAS, periodic acid Schiff stain; SDS, sodium dodecyl sulfate, AdoMet, S-adenosylmethionine.
**Experimental Procedures**

**Materials**—S-Adenosyl-l-[methyl-3H]methionine and S-adenosyl-L-[methyl-3H]methionine were purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). Polyacrylamide gel electrophoresis was performed from Research Products International. Cycloleucine, γ-globulin, S-adenosylhomocysteine and S-adenosylmethionine were purchased from Sigma. Reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad. All other chemicals were obtained from commercial sources and were of the highest purity available. Normal human blood was collected in heparin from healthy volunteers, stored in acid-citrate-dextrose at 0-4°C, and used within 4-5 h. Sickle cell blood was obtained from ambulatory outpatients with (homozygous-S) sickle cell anemia through the Comprehensive Sickle Cell Center, USC-Los Angeles County Hospital. All of these patients had leg ulcers. The blood samples were made up in 50 ml of 1 volume of acid-citrate-dextrose and kept on ice. The red cells were obtained by centrifugation at 3000 × g for 5 min, washed with Buffer A (buffered saline glucose: NaCl, 8.1 g; Na2HPO4·7H2O, 2.302 g; NaH2PO4·H2O, 0.194 g; glucose, 2.0 g made up to 1 liter with distilled water, the pH and osmolarity adjusted to 7.4, and 290-295 mosm/kg) to remove buffy coat.

**Separation of Irreversibly Sickled Cells**—The sickle erythrocytes were made up to 50% hemocrit with buffered saline glucose (Buffer A) and fractionated on a Stractan density gradient at 5°C according to the procedure of Clark and Suhor (20). The reticulocyte rich top fraction was discarded. The pooled intermediate fractions and most dense fractions were taken as reversibly sickled cells and irreversibly sickled cells, respectively. ISC were counted on oxygenated wet whole blood in a Coulter model S counter. The fractions were incubated with L-[methyl-3H]methionine to a final concentration of 30-40% less 14C incorporation, the [3H]carboxylmethyl esters contained on the ghost protein (membrane) of ISC cells, respectively. ISC were counted on oxygenated wet whole blood in a Coulter model S counter. The tritium counts present in an aliquot of this fraction were determined by scintillation counting. The specific activity of fraction II was reported as 14C cpm/nmol of S-adenosylmethionine.

**Assay of Protein Methylation**—The activity of protein methylase II was assayed according to the procedure of Kim and Peck (25).

**Carboxymethylation of Intact Cells**—The erythrocytes were washed three times with 20 volumes of Buffer A by centrifugation at 3000 × g for 15 min. The procedure for methylation of erythrocytes was essentially similar to that employed by Kim et al. (19). The basic incubation mixture contained 0.25 ml of erythrocytes, 12.5 μmol of sodium phosphate, pH 7.2, 1.25 μmol of MgCl2, 0.5 μmol of ATP, 3.2 pmol of L-[methyl-3H]methionine (56 μCi) in a total volume of 0.1 ml. The reaction mixture was scaled up 2-10-fold as required by subsequent assay. The mixture was incubated in a shaking water bath at 37°C for 20 min and the pellet was washed three times with phosphate buffer, pH 6.8, then once with ethanol as described (19). To determine the alkali-labile methyl group incorporation, the [3H]carboxymethylated proteins contained on the ghost membranes were hydrolyzed under mild alkaline hydrolytic conditions as described (10). Radioactivity was counted in a liquid scintillation spectrophotometer.

**SDS-Polyacrylamide Gel Electrophoresis of Membrane Proteins**—The methylation of human erythrocytes was carried out by the procedure described above with the reaction mixture scaled up 10 times. The methylated membranes were washed 3-4 times with 10 volumes of 5 mm sodium phosphate, pH 6.8. The washed ghost pellet was divided into 100 μg of protein aliquots, made up to 50 mM dithiothreitol, 0.1% SDS as described (19), and incubated for 10 min at 37°C. Electrophoresis of these samples (100 μg of protein/gel) was then carried out on SDS/5.6% polyacrylamide or SDS/7.5% polyacrylamide (1% SDS) according to the procedure of Fairbanks et al. (21) and modifications of Steck and Yu (22). For 7.5% gel preparation, the ratio of acrylamide to bisacrylamide was the same as described for the 5.6% gels. After electrophoresis, the gels were stained with Coomassie blue or periodic acid Schiff-stained gels were performed at 555 nm and 565 nm, respectively, utilizing a Perkin-Elmer spectrophotometer, model 552, equipped with gel scanner.

**Estimation of H Incorporated into AdoMet**—The concentration of S-adenosylmethionine and tritium counts incorporated into endogenous protein was determined by the 30-min incubation at 37°C of the cells with L-[methyl-3H]methionine were determined in lysates obtained after the termination of the carboxymethylation reaction. The concentration of AdoMet was quantified utilizing the isotope dilution technique employed by Kim et al. (10). To aliquots of the lysate was added 0.25 μCi (8.62 nmol) of [methyl-3H]AdoMet as an internal recovery standard. The lysates were then deproteinized by adding an equal volume of 1.5 N perchloric acid. The samples were centrifuged at 10,000 × g for 15 min at 5°C. Saturated KHCO3 was added to the supernatants obtained to adjust the pH to 6.0. The supernatant was then adsorbed onto a column (0.3 × 3 cm) of Dowex 50 (Na+) which had previously been equilibrated with 0.1 M NaCl. The column was washed with 0.1 M NaCl until no more UV-absorbing materials or radioactivity were detectable in the wash effluent. S-Adenosylmethionine was then eluted with 6 M HCl; 1 ml fractions were collected. The AdoMet concentration was estimated in fraction two by determining the absorbance at 256 nm and using a molar absorbance for AdoMet of 14,700 (24). The tritium incorporation in an aliquot of this fraction was determined by scintillation counting. The specific activity of fraction II was reported as 14C cpm/nmol of S-adenosylmethionine.

**Results**

**Methylation of Membrane Proteins of Normal and Sickle Erythrocytes—Examination of normal, reversible sickled, and irreversibly sickled erythrocytes with L-[methyl-3H]methionine and ATP at 37°C resulted in the incorporation of [3H]methyl groups into the membrane (Table I). As shown, the incorporation of [3H]methyl groups into ISC (14.9 ± 2.0 pmol/mg of ghost protein) is greater compared to RSC (9.6 ± 1.8 pmol/mg of ghost protein) and AA (5.70 ± 1.0 pmol/mg of ghost protein) erythrocytes. It is pertinent to mention that unfractionated sickle cells incorporated 30-40% less [3H]methyl groups into the membrane compared to fresh unfractinated normal cells (data not shown). The incorporation of [3H]methyl groups into the membrane was inhibited 70% in AA and RSC cells as compared to 32% inhibition in ISC cells by cycloleucine, a known inhibitor of S-adenosylmethionine synthetase (27). Similar inhibition of cycloleucine in the incorporation of [3H]methyl groups in membrane protein of AA cells has been reported by Kim et al. (10) These findings indicate that in sickle cells, as demonstrated for normal erythrocytes, S-adenosylmethionine serves as the methyl donor. It is known that the methyl group of S-adenosylmethionine is incorporated into various amino acid residues in protein. This reaction is catalyzed by specific protein methyltransferases (5) yielding stable methyl substitutions of lysyl, arginyl, and histidyl residues in protein and alkali-labile protein methyl esters of aspartic and glutamic acid residues. Therefore, studies were carried out to determine the extent of alkali-labile and alkali-stable methyl incorporation into membrane proteins of AA, RSC, and ISC cells by carrying out hydrolysis of [3H]methylated membrane proteins under mild alkali conditions. As shown in Table I, 92 ± 2% of [3H]methyl group
Erythrocytes prepared and fractionated as described under "Experimental Procedures" were incubated with L-[methyl-3H]methionine for 90 min at 37 °C, with and without 25 mM cycloleucine included in the reaction mixture. An aliquot (30 μl) of sample was subjected to mild alkaline hydrolysis at pH 7.4, 100 °C for 5 min. The radioactivity profile of [3H]methyl incorporation in these substrates was identified as glycophorin A (PAS I and PAS II) and Coomassie blue staining band in the 4.5 region (4). We therefore analyzed the [3H]methylated membrane polypeptides contained in the membrane preparations, using SDS-polyacrylamide gel electrophoresis, in order to determine if irreversibly sickled cells and reversibly sickled erythrocytes incorporated more [3H]methyl groups into these substrates. Since it has been shown (4) that glycophorin A co-migrates with band 3 in the dimeric form (PAS I), and band 4.5 co-migrates with the monomeric form (PAS II) of glycophorin A on 5% polyacrylamide gel, analysis on 7.5% polyacrylamide gel (4, 28, 29) were carried out to resolve and distinguish between the methylation of glycophorins and other membrane protein components. As shown in Fig. 1, electrophoresis of [3H]methylated AA membrane proteins on 7.5% polyacrylamide gel resulted in an apparent separation of PAS I and band 3, as well as clear resolution of PAS II and the band 4.5 region.

The radioactivity profile of [3H]methyl incorporation in AA (Fig. 1C), RSC (Fig. 1D) and ISC (Fig. 1E) erythrocytes demonstrates distinct differences in the distribution of [3H]label into membrane polypeptide constituents of normal versus sickle cells. As shown in Fig. 1E, ISC incorporate more [3H]methyl groups into lower molecular weight membrane proteins, tentatively identified as bands 5, 6, and 7 as compared to RSC and AA cells. These low molecular weight components have not been previously identified as substrates for protein methylase II (4, 10).

Quantitation of [3H]Methyl Incorporation into Specific Polypeptides of Normal and Sickle Cells—Since sickle cells incorporated more [3H]methyl groups into the membrane and also showed altered distribution profile of tritium label in normal erythrocytes, D, reversibly sickled cells. E, irreversibly sickled cells.

Table I

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>Total [3H]Methyl incorporation</th>
<th>After mild alkaline hydrolysis</th>
<th>Alkal-labile</th>
<th>Hydrolyzed by alkali</th>
<th>Inhibited by cycloleucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA (5)</td>
<td>5.7 ± 0.99</td>
<td>0.44 ± 0.04</td>
<td>5.3 ± 1.0</td>
<td>92.4 ± 2.3</td>
<td>71.0 ± 2.1</td>
</tr>
<tr>
<td>RSC (5)</td>
<td>9.6 ± 1.8</td>
<td>1.1 ± 0.05</td>
<td>8.5 ± 1.7</td>
<td>88.4 ± 3.0</td>
<td>71.8 ± 3.9</td>
</tr>
<tr>
<td>ISC (6)</td>
<td>14.9 ± 2.0</td>
<td>4.6 ± 0.8</td>
<td>10.3 ± 1.0</td>
<td>70.0 ± 4.5</td>
<td>32.8 ± 3.5</td>
</tr>
</tbody>
</table>

The data represents mean ± S.E. Measurements expressed as percentage represent the fraction of the total [3H]methyl group incorporation. Numbers in parentheses indicate the number of experiments carried out.

Fig. 1. Polyacrylamide gel electrophoresis of [3H]methylated membranes from normal and sickle erythrocytes. Methylated membrane preparations (100 μg of protein aliquots/gel tube) were electrophoresed on 7.5% polyacrylamide in the presence of 1% SDS as described under "Experimental Procedures." Densitometric scans of normal methylated membrane preparations are presented (A and B). A, PAS-stained gel scanned at 565 nm. B, Coomassie blue-stained gel scanned at 555 nm. Radioactivity profiles were determined from gels slices (1.6 mm) obtained after electrophoresis of [3H]methylated membranes prepared from intact cells incubated with L-[methyl-3H]methionine for 60 min at 37 °C, in the absence of cycloleucine (---), and in the presence of 25 mM cycloleucine (· · · · ·). C, incorporated into AA erythrocyte membranes were alkalilabile compared to 88 ± 3% and 70 ± 4% for RSC and ISC, respectively. In contrast, the results show (Table I) that ISC incorporated more total (3-fold) radiomethyl groups into the membrane compared to normal cells.

The total amount of alkalilabile methyl esterification of substrates available in ISC were approximately 2-fold greater compared to AA cells, while RSC exhibited intermediate levels of alkalilabile [3H]methyl incorporation. However, the stable methyl incorporation was 10 times higher in ISC compared to AA cells, while in RSC stable methyl group incorporation was 2-fold higher relative to AA erythrocytes (Table I).

Characterization of Methylated Polypeptides in Normal and Sickle Erythrocyte Membranes—Previous investigations by Kim et al. (10) and Galletti et al. (4) demonstrated that certain human erythrocyte membrane proteins serve as substrates for the protein methylase activity found in the cytosol. These substrates were identified as glycophorin A (PAS I and PAS II) and Coomassie blue staining band in the 4.5 region (4). We therefore analyzed the [3H]methylated membrane polypeptides contained in the membrane preparations, using SDS-polyacrylamide gel electrophoresis, in order to determine if irreversibly sickled cells and reversibly sickled erythrocytes incorporated more [3H]methyl groups into these substrates. Since it has been shown (4) that glycophorin A co-migrates with band 3 in the dimeric form (PAS I), and band 4.5 co-migrates with the monomeric form (PAS II) of glycophorin A on 5% polyacrylamide gel, analysis on 7.5% polyacrylamide gel (4, 28, 29) were carried out to resolve and distinguish between the methylation of glycophorins and other membrane protein components. As shown in Fig. 1, electrophoresis of [3H]methylated AA membrane proteins on 7.5% polyacrylamide gel resulted in an apparent separation of PAS I and band 3, as well as clear resolution of PAS II and the band 4.5 region.

The radioactivity profile of [3H]methyl incorporation in AA (Fig. 1C), RSC (Fig. 1D) and ISC (Fig. 1E) erythrocytes demonstrates distinct differences in the distribution of [3H]label into membrane polypeptide constituents of normal versus sickle cells. As shown in Fig. 1E, ISC incorporate more [3H]methyl groups into lower molecular weight membrane proteins, tentatively identified as bands 5, 6, and 7 as compared to RSC and AA cells. These low molecular weight components have not been previously identified as substrates for protein methylase II (4, 10).
methylene membranes were subjected to electrophoresis on 7.5% polyacrylamide (with SDS) as described in Fig. 1. The total radioactivity contained in gel slices which co-migrated with periodic acid-Schiff-staining bands (PAS I and PAS II) and Coomasie blue staining region 4.5 was quantified. The data expressed as percentage represents the fraction, present in PAS I, PAS II, and band 4.5 of the total [3H]methyl radioactivity contained in the entire protein staining region of the gels. Cycloleucine-sensitive methylation represents the difference between the total [3H]methyl group incorporated into PAS I, PAS II, and band 4.5 in the absence of cycloleucine and the total radioactivity incorporated into these bands in the presence of 2.5 mM cycloleucine. This data is representative of three separate experiments.

### Table II

<table>
<thead>
<tr>
<th>Erythrocytes</th>
<th>PAS I Band 4.5</th>
<th>PAS II Band 4.5</th>
<th>Cycleiolne-sensitive [3H]methyl incorporation into PAS I, PAS II, and band 4.5</th>
<th>Percentage of total in PAS I, PAS II, and band 4.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>10.6 ± 0.5</td>
<td>29.9 ± 2.3</td>
<td>10.8 ± 5.7</td>
<td>51.3 ± 3.0</td>
</tr>
<tr>
<td>RSC</td>
<td>7.8 ± 0.3</td>
<td>16.4 ± 7.3</td>
<td>12.0 ± 5.0</td>
<td>50.1 ± 3.8</td>
</tr>
<tr>
<td>ISC</td>
<td>6.3 ± 3.0</td>
<td>17.2 ± 10.8</td>
<td>14.0 ± 9.0</td>
<td>49.1 ± 3.5</td>
</tr>
</tbody>
</table>


### Protein Methylase II Activity and S-Adenosyl-L-methionine Concentration in Normal and Sickle Cells

Since the data showed that irreversibly sickled cells incorporated more total [3H]methyl groups into the membrane, we assessed the activity of protein methylase II present in these cells in order to determine if the increased methylation was due to differences in this enzyme activity. As shown in Table III, the protein methylase activity using y-globulin as a substrate, in control and sickle cells was not significantly different. Moreover, the [3H]methyl groups incorporated into newly synthesized S-adenosylmethionine did not show significant differences in the specific activity of [3H]AdoMet in normal or sickle erythrocytes. The comparatively similar incorporation of [3H]methyl groups into endogenous S-adenosylmethionine by normal and sickle cells suggest that the pools of S-adenosylmethionine available for methylation were not significantly different. Moreover, these results indicate that there is little difference in the S-adenosylmethionine synthetase activity.

### Effect of Age or ATP-depletion of Erythrocytes on Methylation of Membrane Proteins

We next investigated whether the observed increased methylation of ISC membrane protein was due to rapid aging of these cells, as compared to normal cells, which consequently resulted in altered topology of membrane protein substrates available for methylation. To study these aspects in vitro (39), normal and sickle cells were stored in Buffer A at 0–4°C for various lengths of time.

### Table III

| Protein methylase II activity and specific activity of endogenous S-adenosylmethionine |
|------------------------------------------|-----------------------------------------------|
| Exogenous activity | Ratio of exogenous to endogenous activity | Specific activity |
| pmol/min/10⁶ cells | | H cpm/nmol |
| AA | 0.136 ± 0.01 | 6.9 ± 1.3 (6.0–11.5) | 363 ± 10 |
| RSC | 0.194 ± 0.01 | 10.2 ± 1.9 (8–15) | 303 ± 47 |
| ISC | 0.110 ± 0.01 | 8.3 ± 1.0 (2.5–5) | 348 ± 27 |
time (0–5 days) prior to initiating the methylation reaction. As shown in Table IV, control and sickle cells exhibited increased amounts of [3H]methyl group incorporation when these cells were stored for 18 h and 5 days. Analysis of the alkali-labile and -stable methyl incorporation revealed that as the normal cells were stored, with glucose as energy source, the amount of stable methyl incorporation increased more compared to alkali-labile methyl incorporation. As shown in Table IV, AA cells stored for 18 h resulted in a lowered ratio (3.4) of alkali-labile to alkali-stable methyl incorporation as compared to fresh cells (12:1). However, in ISC no significant changes in the ratio of alkali-labile to alkali-stable methyl incorporation was observed when these cells were stored for 18 h or 5 days.

It is possible that partial ATP-depletion of AA cells during storage may contribute to the observed increased [3H]methyl incorporation into these membranes. Studies were therefore carried out with control cells in an attempt to determine whether ATP-depletion caused an increase in methyl incorporation. When fresh AA cells were subjected to rapid ATP-depletion by incubation with ionophore A23187 according to the procedure of Taylor et al. (31), there was no increase in [3H]methyl incorporation into membrane proteins compared to control samples. However, a 33% decline in the alkali-labile methyl incorporation was observed (data not shown). In a separate set of experiments, fresh normal blood was fractionated into two red cell subpopulations after centrifugation on a density gradient similar to that used to fractionate sickle erythrocytes, prior to methylation by the procedure described under “Experimental Procedures.” These results showed that the most dense fraction of normal erythrocytes, which correspond to the increased age of these cells (32), incorporated approximately 3-fold more [3H]methyl groups into the membrane (Table V) compared to the least dense, younger, red cell subpopulation. As shown in Table V, ISC-rich fraction compared to the most dense normal AA cell fraction incorporated 1.3-fold more [3H]methyl group into the membrane.

TABLE IV
Effect of storage on [3H]methyl incorporation into erythrocyte membranes

Fresh erythrocytes washed and fractionated as described under “Experimental Procedures” were subjected to methylation immediately after preparation (fresh), and after storage at 0–4 °C in Buffer A at 50% hematocrit for the time periods listed in the table. Mild alkaline hydrolysis of aliquots of the membrane preparation was carried out as described previously (“Experimental Procedures”). The data represents the mean ± S.E. for the number of experiments indicated in parentheses. The data expressed as percentage represents the fraction of the total [3H]methyl incorporation. Ratio represents: pmol of [3H]methyl/mg of protein hydrolyzed to pmol/mg of alkali-stable protein.

<table>
<thead>
<tr>
<th>Erythrocyte samples</th>
<th>Storage time</th>
<th>[3H]Methyl incorporation</th>
<th>Ratio alkali-labile to alkali-stable</th>
<th>pmol/mg ghost protein</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>Alkal Stable</td>
<td>[3H] Methyl groups</td>
<td>%</td>
</tr>
<tr>
<td>AA (4) 5 days</td>
<td>13.77 ± 2.57</td>
<td>3.98 ± 1.15</td>
<td>71.1</td>
<td>2.45</td>
<td></td>
</tr>
<tr>
<td>(5) 18 h</td>
<td>9.52 ± 1.23</td>
<td>2.16 ± 0.13</td>
<td>77.4</td>
<td>3.40</td>
<td></td>
</tr>
<tr>
<td>(5) Fresh (3-4)</td>
<td>5.70 ± 0.99</td>
<td>0.43 ± 0.04</td>
<td>92.4</td>
<td>12.1</td>
<td></td>
</tr>
<tr>
<td>RSC (3) 5 days</td>
<td>19.2 ± 3.82</td>
<td>7.17 ± 1.88</td>
<td>62.7</td>
<td>1.67</td>
<td></td>
</tr>
<tr>
<td>(4) 18 h</td>
<td>13.3 ± 1.85</td>
<td>4.57 ± 1.10</td>
<td>65.0</td>
<td>1.91</td>
<td></td>
</tr>
<tr>
<td>(5) Fresh (3-4)</td>
<td>9.63 ± 1.80</td>
<td>1.12 ± 0.12</td>
<td>88.4</td>
<td>7.59</td>
<td></td>
</tr>
<tr>
<td>ISC (4) 5 days</td>
<td>24.6 ± 2.48</td>
<td>7.94 ± 0.22</td>
<td>67.7</td>
<td>2.09</td>
<td></td>
</tr>
<tr>
<td>(4) 18 h</td>
<td>24.8 ± 2.44</td>
<td>10.19 ± 1.29</td>
<td>65.0</td>
<td>1.43</td>
<td></td>
</tr>
<tr>
<td>(6) Fresh (3-4)</td>
<td>14.5 ± 2.10</td>
<td>4.64 ± 0.8</td>
<td>70.0</td>
<td>2.22</td>
<td></td>
</tr>
</tbody>
</table>

Comparison of [3H]methyl group incorporated into fractionated normal and sickle erythrocyte membranes

Fresh washed normal and sickle erythrocytes were fractionated on similar discontinuous Stractan gradients as described under “Experimental Procedures.” The single major broad band obtained with normal cells was divided into two fractions. For sickle cells, four major bands were obtained. The two intermediate bands (density range 1.1-1.134 g/ml) were pooled and treated as RSC in this study, while the bottom (most dense) 20% of the sickle cells were taken as ISC-rich fractions. Cells washed free of Stractan were incubated with [3H] methionine, ghosts prepared, and membrane incorporated radioactivity quantified as described under “Experimental Procedures.” Results express the mean ± S.E. of three separate experiments; total [3H] methyl incorporated into RSC and ISC fractions represent five and six experiments, respectively.

![Table V](image)

Comparison of the methylation of erythrocyte membrane proteins with S-adenosyl-L-methionine donor revealed that fresh irreversibly sickled cells incorporated approximately three times more [3H]methyl groups into the membrane compared to fresh normal cells. Methylation of membrane proteins in normal human erythrocytes has been characterized by Kim et al. (10) to be catalyzed by endogenous protein methylase II. Several amino acid residues of proteins have been shown to serve as methyl acceptors. For example, N-methylation of lysyl, arginyl, and histidyl residues; O-methylation of free COOH-terminal groups and the free carboxyl groups of aspartyl and glutamyl residues, are known to be catalyzed by specific protein methylases utilizing S-adenosyl-L-methionine as the methyl donor (5). In this investigation, the extent of methyl esterification of free carboxyl groups on protein in [3H]methylated membranes was quantified by carrying out hydrolysis, under mild alkaline conditions which result in hydrolysis of esters, while N-methylated amino acid residues are stable under these conditions (10). Studies show that normal cells incorporated a greater percentage (60%) of alkali-labile [3H]methyl groups into membrane compared to RSC and ISC, indicating that relatively more specific carboxymethylation of membrane protein occurs in normal cells. However, the total alkali-labile and alkali-stable [3H]methyl groups incorporated by ISC were consistently greater compared to RSC and normal AA cells. Since previous investigations (30, 32) have shown that membrane protein constituents are not increased in ISC, these findings indicate a qualitative difference in the nature of protein substrates available for methylation in ISC compared to normal cells.

Analysis of the [3H]methylated membrane proteins by SDS-gel electrophoresis revealed that ISC incorporated more [3H]methyl groups into membrane proteins, PAS I, PAS II, and band 4.5, which have been identified as substrates for protein methylase II in normal human erythrocytes (4). Our results show a distinct difference in the radioactivity distri-
Carboxy whole view of ISC compared to RSC and normal cells. Moreover, these data revealed that ISC incorporate substantially more [3H]methyl groups into smaller molecular weight membrane proteins in contrast to RSC and normal cells. Studies also demonstrate that [3H]methyl groups incorporated into ISC protein substrates were sensitive to cyclooxygenase inhibition. The observed increased methylation of polypeptides in sickle erythrocytes cannot be accounted for by quantitative differences in the polypeptide composition of ISC, since protein methylase II substrate, glycophorin A, has been previously shown by Riggs and Ingram (30) to be reduced by approximately 22%, while band 4.5 is not significantly altered in ISC compared to AA cells. The ISC incorporated about 2-fold more cyclooxygenase-sensitive [3H]methyl groups into glycophorin A compared to RSC and AA, although the fraction of the total methyl groups incorporated into glycophorin A was similar for normal and sickle cells. Moreover, the total quantity of [3H]methyl groups incorporated into band 4.5 was similar for AA and ISC and greatly diminished (60%) in RSC, a finding which further suggests the morphological heterogeneity of RSC, ISC, and normal cells.

The increased methylation of membrane polypeptides observed in ISC could be due to altered protein methylase II activity or levels of endogenous S-adenosylmethionine. However, we did not observe significant differences in either the protein methylase II activity or changes in the specific activity of endogenous S-adenosylmethionine measured (after the 60-min incubation with [3H]methionine) in sickle erythrocytes compared to normal cells. Since various investigations (33-37) have correlated erythrocyte shape transformations with changes in the intracellular ATP content, and, since it has been suggested that ISC arise from RSC as a result of qualitative changes in the ISC was due to ATP-depletion, and are independent of relative ATP depletion exhibited by normal aged or senescent cells. The present findings may reflect distinct differences in the orientation of the protein constituents of the ISC membrane, and would suggest that the increased availability of methyl acceptor sites may arise from altered protein-protein associations involving the ISC cytoskeleton.

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REFERENCES


2 G. A. Green and V. K. Kalra, unpublished data.
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