Microdetermination of Adrenocortical Steroids by Double Isotope Method.

V. Interaction between Corticosteroids and Human Erythrocytes

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Interaction between corticosteroids and human erythrocytes, and the mode of incorporation of corticosteroids into erythrocytes were examined by electrophoresis, gel filtration, and other chromatographic methods, in combination with the double isotope derivative dilution method.

Corticosteroids were incorporated into erythrocytes in physiological saline and subfractions of erythrocytes were separated and analyzed by the above methods. For example, cortisol was found to be bound or adsorbed on the erythrocyte surface layer to 38% (55.8% bound and 44.2% adsorbed), incorporated into the membrane to 1.7% (50% in free form, 40% bound to lipid, and 10% bound to protein), and 25.3% incorporated into the internal fluid of the cell (60% in free form and 40% bound to hemoglobin and other internal fluid components). These results indicated that a part of corticosteroids is adsorbed in the free form on the erythrocyte surface and also bound to sialoglycoprotein, and also incorporated into the membrane by binding with membrane components, lipid and protein. Further, the steroids are transported into the internal fluid by penetration through the membrane, a part of which are bound to hemoglobin and other internal fluid components and others are present in the free form.

Thus, the passage of corticosteroids through the erythrocyte membrane is a passive and nonspecific transport, which, would be based on the diffusion restricted by a lipid barrier and diffusion facilitated by lipid and protein in the membrane as a carrier according to the classification of Park.

Keywords—human erythrocytes; corticosteroids; erythrocyte subfractions; incorporation and penetration of membrane; microdetermination; double isotope method; thiosemicarbazide sulfur-35; derivative dilution analysis; tritium and sulfur-35 radioactivity

Incorporation of corticosteroids into erythrocytes has been reported by Agarwal and Carstensen,3) Peterson et al.4) and by Sandberg et al.5) but there is no report on the details of the passage of corticosteroids through the erythrocyte membrane. As reported in our preceding paper,6) corticosteroids are distributed in intact erythrocytes in physiological state, in vitro incorporation experiments have shown that corticosteroids with smaller polarity tend to be more easily incorporated into erythrocytes, and the saturation volume of the incorporation by erythrocytes is larger than the binding capacity of corticosteroids and transcortin. Corticosteroids are incorporated into various blood cells, such as erythrocytes, granulocytes, lymphocytes, and platelets,6) but the total amount of incorporation is far larger in erythrocytes.

In the present series of work, interaction of steroids and erythrocytes, and the mode of steroid incorporation were examined by use of erythrocyte membrane as the experimental

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material because its membrane structure and constituting composition have been studied in detail, and has been used as an experimental model for the penetration of biomembrane. In this experiment, erythrocytes were allowed to incorporate corticosteroids, fractionated into various subfractions (cell-surface, cell-membrane, cell-internal fractions), and quantitative analysis were made on the sialoglycoprotein localized on the cell surface, with the cell-surface fraction, the main components of the membrane, lipid and protein, with the cell-membrane fraction, and intracellular liquid component, mainly of hemoglobin, with the cell-internal fraction, by the various methods such as gel filtration and disc electrophoresis, and also the double isotope derivative dilution method reported previously,

in which the carbonyl group in C-3 position alone is derived to \(^{35}\)S-thiosemicarbazone. Based on these experimental results, some discussions will be made on the mode of penetration of corticosteroids through the human erythrocyte membrane.

Materials and Method

Reagents—The following labeled steroids were purified by thin-layer chromatography (TLC) with CHCl\(_3\)–EtOH (9:1, v/v) as the solvent system: Cortisol[1,2-\(^{3}H\)], specific activity, 20 Ci/mmol (Radiochemical Centre, Amersham, England); corticosterone[1,2-\(^{3}H\)], specific activity, 10 Ci/mmol (Radiochemical Centre); cortisone[1,2-\(^{3}H\)], specific activity, 53 Ci/mmol (Radiochemical Centre); aldosterone[1,2-\(^{3}H\)], specific activity, 17 Ci/mmol (Radiochemical Centre); 11-deoxycorticosterone[1,2-\(^{3}H\)], specific activity, 30 Ci/mmol (New England Nuclear, Boston, U.S.A.); 11-dehydrocorticosterone[1,2-\(^{3}H\)], specific activity, 35 Ci/mmol (Radiochemical Centre); dexamethasone[1,2-\(^{3}H\)], specific activity, 40 Ci/mmol (New England Nuclear); progesterone[1,2-\(^{3}H\)], specific activity, 33.5 Ci/mmol (Radiochemical Centre). \(^{35}\)S-Thiosemicarbazide was synthesized by the method described previously.

Neuraminidase from Clostridium perfringens was purchased from Sigma Chemical Company and trypsin from bovine pancreas was obtained from Miles-Seravac, Maidenhead, England. Sephadex LH-20 and G-75 (medium) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The hydrophobic liquid scintillator was prepared by dissolving 4 g of PPO and 0.1 g of dimethyl-POPOP in 11 of toluene. The hydrophilic liquid scintillator was prepared by dissolving 4 g of PPO, 0.4 g of dimethyl-POPOP, and 100 g of naphthalene in 11 of dioxane–toluene–methyl cellosolve (15:3:2, v/v) mixture.

Analytical Procedures

Examination of Factors affecting Incorporation of Corticosteroids into Erythrocyte Subfractions—Temperature: To 1 ml of a suspension of human erythrocytes (5 \(\times\) 10\(^8\) cells) in isotonic physiological saline solution, 100 \(\mu\)g of each of the corticosteroids was added and the mixture was incubated at 4\(^\circ\) or 37\(^\circ\) for 30 min. The erythrocyte mixture was fractionated into various subfractions and the amount of corticosteroids in each fraction was determined.

Ouabain and Sodium Fluoride: Intact erythrocytes (5 \(\times\) 10\(^8\) cells) were suspended in 1 ml of physiological saline containing 1 \(\times\) 10\(^{-4}\) or 1 \(\times\) 10\(^{-4}\) g/ml of ouabain and 1 \(\times\) 10\(^{-4}\) g/ml of NaF, the mixture was pre-incubated at 37\(^\circ\) for 60 min, and centrifuged. To 1 ml of erythrocytes suspended in physiological saline so obtained, 100 \(\mu\)g of each of the corticosteroids was added, the mixture was incubated at 37\(^\circ\) for 90 min, and the mixture was fractionated into various erythrocyte subfractions. Corticosteroid in each fraction was determined.

Protein Binding: A mixture of 100 \(\mu\)g of each of the corticosteroids and 1 ml of human plasma was incubated at 37\(^\circ\) for 90 min, the mixture was submitted to gel chromatography as described in the previous paper,

the protein-bound corticosteroid fraction so obtained was added to intact erythrocytes (5 \(\times\) 10\(^8\) cells), and the mixture was incubated at 37\(^\circ\) for 90 min. Corticosteroid in each fraction was then determined.

Fractionation of Erythrocytes into Subfractions—After incubation of erythrocytes with one of corticosteroids, erythrocytes were fractionated by the previously described method into cell-surface, cell-membrane, and cell-internal fractions.

Enzyme Treatment of Cell-surface Fraction—To 1 ml each of the cell-surface fraction, 3 ml of 0.05 \(m\) acetate buffer solution (pH 4.5), 0.5 mg of neuraminidase, and 3 ml of 0.01 \(m\) phosphate buffer solution (pH 7.4), 1.5 mg of trypsin were added and the mixture was incubated at 37\(^\circ\) for 24 hr.

Sulfolization of Erythrocyte Membrane—The cell membrane was solubilized by the addition of 5 ml of 2-chloroethanol to the cell membrane fraction.

(pH 7.4) containing 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol was added to the cell membrane fraction and the mixture was warmed at 30° for 2 hr.10

**Extraction of Lipids and Corticosteroid in Erythrocyte Membrane**—Lipids and corticosteroid were extracted from the cell-membrane fraction according to the method of Folch, et al.,11) by use of CHCl₃–MeOH, or by the method of Hanahan, et al.,12) with 95% EtOH.

**Gel Filtration**—One ml each of cell surface, SDS-solubilized cell-membrane, and cell-internal fractions was loaded on a column (1.5 x 50 cm) of Sephadex G-75 (medium) swollen with physiological saline and the column was eluted with the same physiological saline. For the cell-membrane fraction solubilized with 2-chloroethanol, its 1 ml was loaded on a column (1.5 x 50 cm) of Sephadex LH-20 swollen with 2-chloroethanol–H₂O (9:1, v/v) and the column was eluted with the same solvent. If and when necessary, measurements were made with each eluate for protein by the method of Lowry, et al.,13) N-acetylneuramic acid by the thiobarbituric acid method,14) saccharides by the anthrone–H₂SO₄ method,15) hemoglobin by absorbance at 540 nm, lipid by the phosphomolybdic acid method, and the radioactivity.

**Determination of Corticosteroids**—Determination was made by the double isotope derivative dilution method16) for corticosteroids in each of subfractions.

**Results**

**Amount of Corticosteroids and Related Steroids Incorporated into Erythrocyte Subfractions**

Amount of corticosteroids and related steroids incorporated into intact human erythrocytes in physiological saline suspension was determined. Relative ratio of the amount of steroids in each subfraction, to the total amount of steroids added as 100, is shown in Fig. 1.

There was no great difference in the amount of steroids bound or adsorbed on the surface of erythrocytes according to their polarity. This fact suggests that polarity of steroids does not affect the binding to sialoglycoprotein or adsorption on the erythrocyte surface, although incorporation of steroids into erythrocyte membrane and intracellular fluid tend to be greater, the smaller the polarity of steroids. On the other hand, testosterone showed little tendency to be incorporated, in spite of its small polarity, while estradiol was incorporated into the membrane in a large amount, although its polarity was similar to that of corticosteroids which were incorporated more into the intracellular fluid.

In order to examine the mode of penetration of corticosteroids through the erythrocyte membrane, effect of temperature dependence, ouabain (ATPase inhibitor), and sodium fluoride (glycolytic enzyme inhibitor), and protein-bound corticosteroid on the incorporation was examined. As shown in Table I, incorporation of corticosteroids into erythrocytes did not depend on temperature, and the incorporation was not suppressed in erythrocytes treated with ouabain or sodium fluoride, suggesting that this incorporation is not an active transport in which an enzyme system like ATPase takes part but is a passive transport. Protein-bound corticosteroids were found not to penetrate through the erythrocyte membrane.

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Table I. Effect of Temperature, Plasma Protein, Ouabain and Sodium Fluoride on Incorporation of Corticosteroids into Human Erythrocyte Subfraction

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Condition&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Distribution of corticosteroids&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Cell surface</th>
<th>Cell internal</th>
<th>Cell membrane</th>
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<tbody>
<tr>
<td>Cortisol</td>
<td>a</td>
<td>37.53</td>
<td>25.15</td>
<td>1.73</td>
<td></td>
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<tr>
<td></td>
<td>b</td>
<td>37.02</td>
<td>24.78</td>
<td>1.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>36.92</td>
<td>26.51</td>
<td>1.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>37.05</td>
<td>25.75</td>
<td>1.87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>e</td>
<td>37.49</td>
<td>24.10</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>f</td>
<td>2.31</td>
<td>1.39</td>
<td>0.38</td>
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<tr>
<td>Corticosterone</td>
<td>a</td>
<td>43.63</td>
<td>30.95</td>
<td>4.69</td>
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<tr>
<td></td>
<td>b</td>
<td>43.73</td>
<td>31.25</td>
<td>5.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>43.51</td>
<td>31.58</td>
<td>4.38</td>
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<tr>
<td></td>
<td>d</td>
<td>42.98</td>
<td>29.73</td>
<td>4.21</td>
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</tr>
<tr>
<td></td>
<td>e</td>
<td>43.85</td>
<td>29.50</td>
<td>4.02</td>
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<tr>
<td></td>
<td>f</td>
<td>1.89</td>
<td>0.78</td>
<td>0.35</td>
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<tr>
<td>11-Deoxycorticosterone</td>
<td>a</td>
<td>42.85</td>
<td>45.00</td>
<td>6.27</td>
<td></td>
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<tr>
<td></td>
<td>b</td>
<td>42.90</td>
<td>45.91</td>
<td>7.01</td>
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<tr>
<td></td>
<td>c</td>
<td>41.73</td>
<td>43.88</td>
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<tr>
<td></td>
<td>d</td>
<td>42.30</td>
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<td>f</td>
<td>3.45</td>
<td>2.15</td>
<td>0.41</td>
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</table>

<sup>a</sup> Incubation in isotonic physiological saline.
<sup>b</sup> Condition: a) incubation at 37°C, b) incubation at 4°C, c) treated erythrocytes with ouabain 1 x 10⁻⁴ g/ml,
<sup>c</sup> d) treated erythrocytes with ouabain 1 x 10⁻⁴ g/ml, e) treated erythrocytes with NaF 1 x 10⁻⁴ g/ml,
f) plasma protein-bound corticosteroid.
<sup>c</sup> Amount of incorporated steroid (μg) into erythrocyte subfraction when added 100 μg of steroids.

Interaction between Corticosteroids and Constitutive Components of Erythrocyte Surface

Examination was made on the possible interaction between cortisol, the main corticosteroid in human blood, and constitutive components on the outermost layer of erythrocyte membrane (mainly sialoglycoprotein) on the erythrocyte surface. A suspension of intact human erythrocytes was incubated (37°C, 90 min) with cortisol and ³H-cortisol in physiological

![Fig. 2. Gel Filtration of Erythrocyte Cell-Surface Fraction<sup>a</sup> on Sephadex G-75](image)

Bed dimension, 1.5 x 50 cm; eluate, physiological saline; flow rate, 0.2 ml/min.

--- radioactivity of ³H-cortisol, --- protein (Lowry's method, 750 nm), —— N-acetylneuramic acid (thiobarbituric assay, 549 nm), --- saccharide (anthrone-H₂SO₄ assay, 620 nm).

<sup>a</sup> To 50 ml of suspension of 10 ml of intact human erythrocytes in physiological saline, 200 μg of cortisol and 1 x 10⁶ dpm of ³H-cortisol were added, the mixture was incubated at 37°C for 90 min, and erythrocytes were separated by centrifugation. The erythrocytes were washed five times with 10 ml of physiological saline and a fraction containing sialoglycoprotein was separated.

![Fig. 3. Acrylamide Gel Disc Electrophoresis of Erythrocyte Cell-surface Fraction](image)

a : human plasma stained for protein.
b : cell surface fraction stained for saccaride.
c : cell surface fraction stained for protein.
saline. The erythrocytes were washed with saline to separate sialoglycoprotein localized on the membrane surface from the membrane structural protein, and a fraction containing sialoglycoprotein, easily soluble in salt solution, was separated. This cell-surface fraction was submitted to gel filtration on Sephadex G-75. As shown in Fig. 2, a part of cortisol was present in the high-molecular fraction containing N-acetylneuramic acid, polysaccharides, and proteins. In this fraction, the peaks of N-acetylneuramic acid, polysaccharides, proteins, and tritium radioactivity agreed, so that cortisol was considered to be bound to sialoglycoprotein. Therefore, this high-molecular fraction was submitted to acrylamide gel disc electrophoresis and, as shown in Fig. 3, 10 zones by protein staining and 3 zones by saccharide staining were detected, and tritium radioactivity was found in 5 of these zones. This result indicated that cortisol is bound to glycoprotein and protein, though the amount is small considering the total amount present in the cell-surface fraction. Therefore, cortisol is not only adsorbed on the erythrocyte surface but a part of it is bound to glycoprotein localized on the outermost layer of erythrocyte membrane.

It has been revealed that N-acetylneuramic acid is present in the terminal end of the polysaccharide chain in the outermost layer of the erythrocyte membrane as its constitutive components and is present in the cell-surface fraction by the thiobarbituric acid method. Therefore, cell-surface fraction was treated with neuraminidase to liberate the N-acetylneuramic acid, and interaction between cortisol and N-acetylneuramic acid residue was examined. Trypsin is known to act on the sialoglycopeptide chain in the outermost layer of the erythrocyte membrane and liberates the peptide. Therefore, cell-surface fraction was

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![Diagram](image-url)

**Fig. 4. Distribution of Free and Sialoglycoprotein-bound Cortisol on Paper Chromatogram of Low-molecular Weight, Cell-surface Fraction**

- N-acetylneuramic acid (thiobarbituric acid assay, 549 nm).
- Protein (Lowry's method, 760 nm).
- Free cortisol.
- Bound cortisol.

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treated with trypsin to liberate the glycopeptide and possible binding of cortisol with the peptide chain was examined.

The cell-surface fraction was treated with two kinds of enzyme as described above and the lysate was submitted to gel filtration on Sephadex G-75. Majority of cortisol was found to be present in the low-molecular fraction which also contained N-acetyleneuramic acid, peptides, and saccharides. Therefore, this low-molecular fraction was submitted to paper chromatography, with butanol–acetic acid–water (3:1:1, v/v/v) as a solvent system suited for the separation of peptides. As shown in Fig. 4, the tritium radioactivity increased in the spots at Rf 0.0–0.3, where N-acetyleneuramic acid liberated by neuraminidase would present, indicating that cortisol is bound to N-acetyleneuramic acid in the terminal end of the polysaccharide chain. Tritium radioactivity also increased in the spots at Rf 0.3–0.5, where the peptides liberated by trypsin would present, indicating that cortisol is also bound to the peptide chain. Further, the spots at Rf 0.7–0.9 would contain free and bound cortisol but this could not be separated by this solvent system. Therefore, the solvent system suitable for the separation of these two, toluene–ethyl acetate–methanol–water (9:1:5:5, v/v/v/v), was used for the development, and about 46% of cortisol was found to be present in the free form in the cell-surface fraction.

These experimental results showed that, while bound cortisol increased by 16% accompanying liberation of N-acetyleneuramic acid by neuraminidase, bound cortisol increased by about 18% accompanying glycopeptides liberated by trypsin. This fact indicates that majority (ca. 54%) of cortisol bound on the erythrocyte surface is bound to N-acetyleneuramic acid in the terminal end of the polysaccharide chain and that ca. 46% of cortisol is adsorbed on the erythrocyte surface in a free form.

Interaction between Corticosteroids and Constitutive Components of Erythrocyte Membrane

The main constitutive components of an erythrocyte membrane are phospholipids, neutral lipids, and proteins. Possible interaction between corticosteroids and lipids or proteins during transport through the erythrocyte membrane was examined.

The cell-membrane fraction solubilized with 2-chloroethanol was submitted to gel filtration on Sephadex LH-20. As shown in Fig. 5, about 10% of cortisol was bound to the membrane–constituting proteins and the remaining 90% was thought to be present by some interaction with lipids. Therefore, interaction between corticosteroids and lipids was examined by use of 11-deoxycorticosterone and cortisol. Since it was observed in our previous paper,6) that the former steroid was incorporated most readily into erythrocytes due to the lowest polarity among the corticosteroids investigated, while the latter was incorporated to the smallest extent due to the highest polarity.

The lipid fraction extracted from the erythrocyte membrane was submitted to TLC on a silica gel plate by use of the solvent systems of chloroform, chloroform–methanol (19:1, v/v), and chloroform–methanol–conc. ammonia aq. (170:30:10, v/v/v). As shown in Fig. 6, the Rf values of free cortisol and 11-deoxycorticosterone were 0 and 0.05, respectively, when chloroform was used as a solvent, and spots with tritium radioactivity and positive to blue tetrazolium reaction were found in these regions. A lipid spot and tritium radioactivity were detected at Rf 0.25 and 0.40, respectively. The same results were obtained with other solvent systems.
We calculated the polarity values of corticosteroids according to the empirical method proposed by Fujita\textsuperscript{21} and these values were plotted against the radioactivity of incorporated steroids into the lipid layer constituting the erythrocyte membrane. There was a nearly linear relationship between the polarity value and the incorporated radioactivity as shown in Fig. 7. Corticosteroids with smaller polarity such as progesterone and 11-deoxycorticosterone are incorporated more into the lipid layer constituting the erythrocyte membrane as lipid and protein bound forms as well as free form than in those with larger polarity such as cortisol and cortisone.

Interaction between corticosteroids and erythrocyte-constituting proteins was examined by gel filtration and disc electrophoresis. As shown in Fig. 8, gel filtration on Sephadex G-75 indicated that proteins, polysaccharides, and tritium radioactivity were present in the high-molecular fraction which was eluted immediately after void volume, suggesting that a part of corticosteroids would be bound to glycoproteins.

Acrylamide gel disc electrophoresis of the solubilized cell membrane indicated the presence of tritium radioactivity in the part in which proteins and polysaccharides must be present, and this fact proved that about 10\% of corticosteroids was present bound to the proteins constituting the erythrocyte membrane.

**Interaction between Corticosteroids and Internal Fluid Components of Erythrocytes**

Interaction between corticosteroids penetrating through the erythrocyte membrane and components of internal fluid of erythrocytes (hemoglobin and soluble enzyme proteins) was examined by gel filtration and disc electrophoresis. As shown in Fig. 9, gel filtration of the cell-internal fraction on Sephadex G-75 indicated the presence of tritium radioactivity in the fraction which was eluted immediately after void volume, containing hemoglobin, which suggested that about 30% of cortisol was bound to hemoglobin, about 10% with other soluble proteins, and the remaining about 60% was present as free cortisol. Electrophoresis of the
cell-internal fraction showed the presence of 8 bands by protein staining, and 4 of these bands were found to have tritium radioactivity. Electrophoresis of human hemoglobin sample under the same condition showed the presence of tritium radioactivity in the locus corresponding to HbA (α2, β, γ), proving that cortisol is bound to hemoglobin. The same results were obtained with corticosterone, cortisone, 11-deoxycorticosterone, and progesterone.

Discussion

Mechanism for the transport of a substance through a membrane is complicated and various forms of classification have been suggested. Park(22) classified membrane transport into 9 categories of simple diffusion, solvent drag, diffusion restricted by membrane charge, diffusion restricted by a lipid barrier, facilitated diffusion, exchange diffusion, active transport, pinocytosis, and phagocytosis.

Our experimental results showed that penetration of corticosteroids through the erythrocyte membrane dose not depend on temperature and is not inhibited by ATPase inhibitors or glycolytic enzyme inhibitors, suggesting that the penetration of corticosteroids is a passive transport in which the energy system does not take part. Therefore, the correlation of the membrane structure with the mechanism of the transport of corticosteroids through the erythrocyte membrane was examined. It was thereby found that cortisol, which is the main component in human blood, was bound approximately 38% (54% bound, 46% adsorbed) with the erythrocyte surface layer, 1.7% was bound with the membrane constituting components (90% interaction with lipids, 55% of which was in free form, 45% bound with lipid, and 10% bound with proteins), and about 25% incorporated into the internal fluid of the cell (40% bound with hemoglobin and other internal components and 60% in free form).

These results indicate that some of corticosteroids are present as a free form adsorbed on the surface of erythrocyte membrane and some are bound to sialoglycoprotein, the constitutive component in the outermost layer of the membrane. This bound steroid is dissociated as a free form by solvent extraction. It was also found that the steroids with lower polarity tend to interact with lipids to a larger extent and are incorporated more into the lipid layer of the erythrocyte membrane. Moreover the steroids were present in the membrane both in the bound (with lipid and with protein) and unbound forms. After passing through the membrane, the steroids were either in the hemoglobin-bound or in the unbound form.

In our previous paper,(6) we reported that the passage of corticosteroids through the membrane of whole erythrocyte cells may be due to a passive and nonspecific transport and that the steroids with lower polarity passed through the membrane more readily than those with higher polarity. These findings were consistent with the reports of Danielli and Davison(23) and of Eyring and Parlin(24) in that substances with higher lipid solubility pass through a cell membrane to a larger extent than those with lower lipid solubility. It is not at present possible to decide whether the passage of corticosteroids through the erythrocyte membrane is due to the simple diffusion, the diffusion restricted by a lipid barrier, or the carrier-facilitated diffusion.