The exterior surface of the chicken erythrocyte* 

ROBERT C. JACKSON†

From the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138

(Received for publication, June 24, 1974)

SUMMARY

The exterior surface of mature chicken erythrocytes has been labeled with a cationic membrane impermeable reagent, p-nitrophenyl-N,N,N-trimethyl-125Iiodotyrosinate. This reagent forms stable covalent bonds with the amino groups of exposed protein and lipid components. Two major protein components with subunit molecular weights of 100,000 and 55,000 are found on the outer surface of mature chicken erythrocytes. Both of the labeled components coincide with periodic acid-Schiff base-stained areas on sodium dodecyl sulfate polyacrylamide gel electrophoresis indicating that they may be glycoproteins.

The exterior surface of a cell mediates the interaction of the cell with its external environment. Uptake of nutrients, maintenance of ionic balance, and cellular response to hormones and neighboring cells are all processes which necessarily involve cellular components exposed to the external environment. In recent years an arsenal of chemical and enzymatic probes has been used to investigate this surface. Each of these probes has its advantages and disadvantages. The chemical probes, by virtue of their smaller size, are able to label membrane components which are sterically unavailable to enzymatic probes. On the other hand, enzymatic probes (1-5) are generally easier to use and are completely impermeant. The chemical probes can be divided into four classes: anionic (6, 5); neutral (8); zwitterionic (9); and cationic (10, 11). This communication reports the synthesis of a cationic reagent, [125I]NTIT, of high specific activity and its use in the investigation of the external surface of the mature chicken erythrocyte.

EXPERIMENTAL PROCEDURES

Materials

White leghorn roosters (Gallus domesticus) were obtained from Spafas Farms, Norwich, Conn. Human blood drawn into acid-citrate-dextrose was provided by the Blood Research Institute, Boston, Mass. Bovine lactoperoxidase, phenylmethanesulfonyl fluoride, heparin, bovine serum albumin, Escherichia coli 8-galactosidase, cytochrome c, and sodium dodecyl sulfate were from Sigma Chemical Co. Bovine pancreatic deoxyribonuclease I, grade 1PPF, was obtained from Worthington Biochemical. N,N-dicyclohexylcarbodiimide was purchased from Mann Chemical Co. Chicken erythrocyte ghosts were prepared by DNase treatment of the chicken erythrocyte ghosts. The ghosts were prepared by hypotonic lysis as described by Dodge et al. (13). Chicken erythrocyte ghosts were prepared by hypotonic lysis in 20 to 30 volumes of 10 mM NaH₂PO₄, pH 7.5-150 mM NaCl. After centrifugation (1000 X g, 5 min), the supernatant and buffy coat were removed by aspiration. The red blood cells were resuspended in 10 mM NaH₂PO₄, pH 7.5-150 mM NaCl and the washing and aspiration procedures were repeated twice. Human red blood cells drawn into acid-citrate-dextrose were prepared by the same procedure. Human red blood cell ghosts were prepared by digestion with heparin (12) as described by Dodge et al. (13). Chicken erythrocyte ghosts were prepared by hypotonic lysis in 20 to 30 volumes of 10 mM NaH₂PO₄, pH 7.5-150 mM MgCl₂ followed by repeated washings (at least three) in this buffer. All procedures were conducted at 4°. Chicken erythrocyte membranes were prepared by DNase treatment of the chicken erythrocyte ghosts. The ghosts were resuspended at room temperature in 0.1 mM MgCl₂, 1 mM sodium tetrathionate, and 0.1 mM phenylmethanesulfonyl fluoride to a final concentration of about 10 μg of protein per ml, and vortexed vigorously. This treatment lyses the nuclei and greatly increases the viscosity of the suspension. DNase was added to a concentration of 10 μg per ml. Finally, 4 volumes of 0.1 mM MgCl₂, 10 mM NaH₂PO₄ (pH 7.5), 1 mM sodium tetrathionate, and 0.1 mM phenylmethanesulfonyl fluoride were added. Digestion was allowed to proceed for 15 min at room temperature. The membranes were pelleted at 12,000 X g for 20 min in a Sorval RC2-B refrigerated centrifuge, and were resuspended in an appropriate buffer.

Labeling Procedure—The material to be labeled was washed once in 0.1 M sodium chloride-0.1 M sodium borate, pH 8.5, resuspended in this buffer, and then added to a test tube containing the desired amount of [125I]NTIT. The test tube was immediately vortexed. Reaction was allowed to proceed at room temperature for 1 hour. The labeled cells or membranes were washed free of unreacted reagent by at least 3 cycles of centrifugation and resuspension in the same phosphate buffer which had been used in their preparation.

Synthesis of p-Nitrophenyl-N,N,N-trimethyl-125Iiodotyrosinate

Printed in U.S.A.
Synthesis of p-Nitrophenyl-N,N,N-trimethyl-[125I]iodotyrosinate

As described under "Methods" N,N,N-trimethyltyrosine was iodinated with lactoperoxidase and H2O2. Since the concentration of N,N,N-trimethyltyrosine was always at least 10-fold greater than that of 1-125I-N,N,N-trimethyltyrosine was the only iodinated product. Analysis of the products and starting materials by thin layer chromatography produced the following results.

N,N,N-Trimethyltyrosine was mixed with an Rf of 0.58 and stained with the 1-nitroso-2-methyl and Dragendorff's (quaternary amine) stains, but failed to react with the ninhydrin reagent. Tyrosine migrated with an Rf of 0.45 and stained with the ninhydrin and 1-nitroso-2-methylthiol reagents, but not with Dragendorff's reagent. N,N,N-Trimethyl-[125I]iodotyrosine mixed with an Rf of 0.75 while free [125I]iodide mixed with an Rf of 0.46.

The percentage of 125I incorporated into N,N,N-trimethyl-[125I]iodoiodotyrosine always exceeded 90% under the conditions described. N,N,N-Trimethyltyrosine can also be iodinated with I- and chloramine-T (20); however, lactoperoxidase iodinated material was used for the experiments reported here.

N,N,N-Trimethyl-[125I]iodoiodotyrosine and carrier N,N,N-trimethyltyrosine were activated to their respective p-nitrophenyl esters with N,N-dicyclohexylcarbodiimide as described under "Methods." The rate of hydrolysis of p-nitrophenyl-N,N,N-trimethyltyrosine was determined spectrophotometrically by following the release of p-nitrophenol at 400 nm, at room temperature (20°C). The half-time for hydrolysis in 0.1 M borate buffer at pH 8.5 is 100 min. At pH 9.5 (0.1 M bicarbonate buffer) the half-time for hydrolysis is 17.5 min.

Labeling of Human Red Blood Cell—Although the exterior surface of the human erythrocyte has been thoroughly investigated with anionic (9, 13), zwitterionic (9), and enzymatic (1-4) probes, no studies of this surface have been made with cationic probes. It was of interest, therefore, to label human red blood cells with [125I]NTIT, thereby demonstrating the impermeability of the reagent to this particularly well-studied membrane. Fig. 1 shows a 7.5% sodium dodecyl sulfate gel of [125I]NTIT-labeled human erythrocytes. The sodium dodecyl sulfate gel was stained with periodic acid and Schiff reagent and scanned at 560 nm. Next the gel was overstained with Coomassie blue and scanned at 600 nm. Finally the gel was manually sliced into 1-mm slices and counted. All of the well known major exterior components of the red cell are labeled with [125I]NTIT. The peak of label with an apparent mass of 28,000 daltons corresponds to PAS 3 of Steck and Jawson (21). The peaks of label with apparent molecular weights of 52,000 and 60,000 correspond to PAS 2 and PAS 4, respectively, and the peak of label with an apparent molecular weight of 100,000 represents both PAS 1 and Band 3 which are inadequately separated on 7.5% gels. In gels containing a higher percentage of acrylamide Band 3 and PAS 1 can be resolved (22). Under these conditions both Band 3 and PAS 1 are labeled (data not shown). When erythrocyte ghosts are treated with [125I]NTIT under the conditions described under "Methods" all of the proteins are labeled. Fig. 2 presents data from a labeled ghost preparation. The ability of [125I]NTIT to label the known exterior components of the erythrocyte without labeling the known interior components, e.g. spectrin (23, 24) demonstrates the impermeability of [125I]NTIT. [125I]NTIT also labels the lipids of the red cell; however, during the prolonged staining and destaining procedures employed most of the lipids leach out of the gel. In summary, despite its cationic nature, [125I]NTIT fails to label any new polypeptides on the exterior surface of the human red blood cell.

Labeling of Chicken Erythrocyte—Labeling of the exterior surface of the chicken erythrocyte proved to be more difficult than labeling the exterior surface of the human erythrocyte. The major problem encountered in dealing with the chicken erythrocyte involved cellular proteases. Fig. 3 compares scans of sodium dodecyl sulfate gels of chicken erythrocyte membranes prepared without (Trace A) and with (Trace B) the protease...
FIG. 1. [¹²⁵I]NTIT-labeled human red blood cells. A 50% suspension (0.12 ml) of human red blood cells was labeled with 1 μCi of [¹²⁵I]NTIT (50 mCi per mmol) as described under "Methods." Erythrocyte ghosts were prepared by hypotonic lysis, dissolved in 1% sodium dodecyl sulfate, and subjected to electrophoresis on 7.5% sodium dodecyl sulfate gels. Gels were oxidized with 1% periodic acid, stained with Schiff reagent, and scanned at 560 nm on a Zeiss spectrophotometer. Gels were overstained with Coomassie blue and scanned at 500 nm. Finally, gels were sectioned into 1-mm slices and counted. Each slice was counted for 10 min; 1.4% of the cells lysed during the labeling procedure. -- , absorbance at 500 nm (Coomassie); --- , absorbance at 500 nm (periodic acid-Schiff base); -- -- , counts per min.

FIG. 2. [¹²⁵I]NTIT-labeled human red blood cell ghosts. The procedure used was exactly the same as in Fig. 1 except that erythrocyte ghosts from 60 μl of packed cells in 120 μl of buffer were labeled. -- , absorbance at 500 nm (Coomassie); --- , absorbance at 500 nm (periodic acid-Schiff base); -- -- , counts per min.

FIG. 3. Effect of protease inhibitors on chicken erythrocyte membrane sodium dodecyl sulfate gel pattern. Trace A, chicken erythrocyte membranes prepared in the absence of protease inhibitors; Trace B, chicken erythrocyte membranes prepared in the presence of 0.1 mm phenylmethylsulfonyl fluoride and 1 mm sodium tetrathionate as described under "Methods."
inhibitors phenylmethanesulfonyl fluoride and sodium tetra-
thionate, a sulphydryl reagent (25-27), which has been shown to
prevent effectively proteolysis of mitochondrial proteins. The
canadian erythrocyte membranes prepared without the protease
inhibitors have been altered by proteolysis. The major differ-
ences between the two traces are denoted by arrows. The most
characteristic differences are the appearance in the proteolyzed
gel of bands with apparent molecular weights of 175,000, 150,000,
and 140,000 and the disappearance of a band with an apparent
molecular weight of 60,000. It should be emphasized that the
proteolized membranes (Fig. 3, Trace A) remained in the lysate
for only a very brief period (15 min) and were maintained at 4º
during this period. Fig. 3 demonstrates that even under these
optimal conditions some proteolysis is inevitable unless protease
inhibitors are included. In washing the erythrocytes the buffy
coat was meticulously removed by aspiration. Between 25% and
50% of the red cell pellet was sacrificed during the multiple
aspiration steps in order to assure that white cell contami-
nation was minimal. For this reason it is believed that the pro-
tease(s) responsible for this difficulty are of red cell origin. It is
not clear whether the protease(s) are nuclear or cytoplasmic in
origin.

Although the chicken erythrocyte membrane preparation
described here contains nuclear as well as plasma membranes,
the plasma membrane proteins dominate the sodium dodecyl
sulfate gel pattern both in variety and in staining intensity.
Isolated nuclear membrane gives a simple sodium dodecyl
sulfate gel pattern. Consequently, a direct comparison of the
sodium dodecyl sulfate gel patterns of human and chicken
erythrocyte membranes is worthwhile. The two membranes are
strikingly similar (Fig. 4). The major difference between the
two patterns is the large amount of histones (apparent molecu-
lar weights of 28,000, 15,000, and 10,000) in the chicken eryth-
rocyte preparation. The amount of histone observed is variable,
depending upon the effectiveness of the DNase treatment.
Sodium dodecyl sulfate gels of both human and chicken eryth-
rocyte membranes are dominated by a doublet with an apparent
molecular weight of 200,000 to 250,000, and a broad band with an
apparent mass of 100,000 daltons. When less chicken eryth-
rocyte membrane protein is loaded on the gel the band at 100,000
splits into a closely spaced doublet with apparent molecular
weights of 100,000 and 105,000.

Table 1 compares the periodic acid-Schiff base-staining com-
ponents of the human and chicken red blood cell sodium dodecyl
sulfate gels. The periodic acid-Schiff base-stained bands on the
chicken erythrocyte gels are extremely faint, whereas, the
periodic acid-Schiff base-bands of the human erythrocyte mem-
branes are easily detected. One of the most striking differences
between the two cells is the high molecular weight (190,000)
PAS 1 band of the chicken erythrocyte membranes. Human
PAS 1 stains much more intensely and runs with an apparent
molecular weight of 86,000 on these 7.5% acrylamide gels.

When intact chicken red blood cells are labeled with [35S]-
NNNTT, two major peaks of radioactivity are detected (Fig. 5).
The labeled components run with apparent molecular weights

---

**Table 1**

Comparison of human and chicken erythrocyte periodic acid-Schiff
base-stained components

<table>
<thead>
<tr>
<th>Chicken PAS designation</th>
<th>Apparent mol wt $\times 10^5$</th>
<th>Human PAS designation</th>
<th>Apparent mol wt $\times 10^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS 1</td>
<td>190</td>
<td>PAS 1</td>
<td>85</td>
</tr>
<tr>
<td>PAS 2</td>
<td>99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAS 3a</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAS 3b</td>
<td>60</td>
<td>PAS 4</td>
<td>54</td>
</tr>
<tr>
<td>PAS 3c</td>
<td>53</td>
<td>PAS 2</td>
<td>48</td>
</tr>
<tr>
<td>PAS 4</td>
<td>36</td>
<td>PAS 3</td>
<td>25</td>
</tr>
<tr>
<td>PAS 5</td>
<td>10.5</td>
<td>Glycolipid</td>
<td>10.5</td>
</tr>
</tbody>
</table>

*a* Adapted from Steck and Dawson (21) and Fairbanks et al.
(28). Sodium dodecyl sulfate gels were stained with periodic
acid-Schiff base stain according to the procedure of Glosmann
and Neville (19). Molecular weight standards used in assigning
subunit molecular weights were myosin (220,000), $\beta$-galactosidase
(130,000), bovine serum albumin (68,000), ovalbumin (43,000),
DNase I (31,000), and cytochrome c (11,700).

---

*S. G. Clarke, personal communication.*
of 96,000 and 55,000 and correspond to l'AS 2 and l'AS 3b (see Table 1). Minor, although reproducible peaks of radioactivity run with apparent molecular weights of 200,000, 36,000, and 10,000. The 10,000 molecular weight material may represent labeled lipid which has not completely leached out of the gel despite the prolonged staining and destaining procedures, or it may represent a genuine glycoprotein. Six per cent of the cells lysed during the labeling procedure resulting in a small peak of labeled hemoglobin (16,000 daltons). Fig. 6 demonstrates that [l25I]NTIT labels no new bands in the sodium dodecyl sulfate gel profile of the human erythrocyte. The most reasonable interpretation of this result is that if [l25I]NTIT does label any new cation-specific components, they are present to such a small extent that they cannot be observed at the present level of resolution. The level of resolution possible with [l25I]NTIT can be significantly improved by increasing the

**FIG. 5.** [l25I]NTIT-labelled chicken red blood cells. One milliliter of a 5% suspension of cells was labeled with 40 μCi of [l25I]NTIT (11.6 mCi per mmol). Membranes were prepared as described under "Methods." Membranes were solubilized in 1% sodium dodecyl sulfate and subjected to electrophoresis on 7.5% sodium dodecyl sulfate gels. The gels were stained successively with periodate-Schiff reagent and Coomassie blue as described in the legend to Fig. 1. Six per cent of the cells lysed during the labeling procedure. ---, absorbance at 500 nm (Coomassie); - - -, absorbance at 560 nm (periodic acid-Schiff base); - - -, counts per min.

**FIG. 6.** [l25I]NTIT-labeled chicken erythrocyte membranes. Chicken erythrocyte membranes from 0.1 ml of packed cells were solubilized in 0.8 ml of 1% sodium dodecyl sulfate buffered with 0.1 M borate (pH 8.5), and labeled with 40 μCi of [l25I]NTIT (11.6 mCi per mmol). The membranes were subjected to electrophoresis and stained as described in the legend to Fig. 1. ---, absorbance at 500 nm (Coomassie); - - -, absorbance at 560 nm (periodic acid-Schiff base); - - -, counts per min.

**DISCUSSION**

Although other cationic labeling reagents have been synthesized (10, 11), no sodium dodecyl sulfate gel profiles of cationically labeled human red blood cells have been published. Despite its cationic nature, [l25I]NTIT labels no new bands in the sodium dodecyl sulfate gel profile of the human erythrocyte. The most reasonable interpretation of this result is that if [l25I]NTIT does label any new cation-specific components, they either co-migrate with other known exterior components or they are present to such a small extent that they cannot be observed at the present level of resolution. The level of resolution possible with [l25I]NTIT can be significantly improved by increasing the
specific activity of the reagent. Co-electrophoresis of various external components is a more difficult problem requiring an alternate means of protein separation or competition studies using membrane impermeable reagents with different specificities.

Chicken Red Blood Cell—Recently there has been considerable interest in the membranes of avian erythrocytes. Procedures have been described for the preparation of plasma and nuclear membranes from this cell (29-31). A number of reports have dealt with the hormone sensitivity and adenylate cyclase of avian plasma membranes (32-35). The chemical composition of the plasma and nuclear membranes has been determined (30) and a developmental study has been published (29). All of these studies have tacitly assumed that proteolysis is not a significant problem in this system. As demonstrated in Fig. 4 this is an assumption which cannot be made. In fact, the Coomassie-stained sodium dodecyl sulfate gel profiles presented in this paper differ considerably from the profiles of chicken erythrocyte plasma and nuclear membrane of Ulanchet (29), who used no protease inhibitors in his membrane preparation. He observes three major bands of stain between his high molecular weight doublet and the closely spaced doublet which runs with an apparent molecular weight of 100,000 on the gel system described here. These three bands correspond closely to the protease produced bands of Fig. 4.

When chicken erythrocytes are labeled with [125I]NTIT radioactivity is primarily incorporated into two components which move with apparent molecular weights of 96,000 and 55,000. Both bands correspond to periodic acid-Schiff base-stained areas of the sodium dodecyl sulfate gel. Other minor components appear to be reproducibly labeled with [125I]NTIT, however these may or may not be significant.

Acknowledgments—I would like to thank Professor Guido Guidotti for his generous support and advice. I would also like to thank Dr. Lloyd Waxman and Steven Clarke for their helpful discussions.

REFERENCES

34. Davoren, P. R., and Sutherland, E. W. (1963) J. Biol. Chem. 238, 3000-3015
The exterior surface of the chicken erythrocyte.
R C Jackson


Access the most updated version of this article at http://www.jbc.org/content/250/2/617

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/250/2/617.full.html#ref-list-1