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A RADIOACTIVE LABEL FOR THE ERYTHROCYTE MEMBRANE

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SUMMARY

A new radioactive reagent, diazotized [^{131}I]diiodosulfanilic acid, which binds specifically to protein groups of the erythrocyte membrane, is described. The labeling reaction and some of the effects of varying amounts of the bound isotope on red cell structure and function have been described. Among the advantages of this membrane label over others available are the following:(1) The diazonium salt of the diiodo-compound is less soluble than that of sulfanilic acid and can therefore be precipitated from the diazotization mixture, washed, and rendered free of reactants and impurities. (2) Effective membrane labeling can be achieved with relatively little damage to the cell. (3) It can be prepared as a γ-emitting radioisotope at very high specific activity. (4) Preliminary data suggest that it may be utilized to study the fate of the red cell membrane *in vivo*.

In recent years certain agents that combine with outer components of the cell membrane have been developed and used to study membrane anatomy and function[1–3] The properties essential for such a reagent, as defined by MADDY[1], include the following: (1) It should contain a reactive group that binds covalently with membrane groups under mild conditions. (2) Its polarity and size should be sufficient to prevent its penetrating the lipoid membrane. (3) It should be detectable in small amounts. MADDY's compound, a stilbene isothiocyanate, fulfills these criteria. However, it cannot be measured on the intact erythrocyte because of quenching by heme in the fluorimetric determination. Diazonium compounds bind to proteins at reactive sites of tyrosine, histidine, lysine, and perhaps other amino acids[4]. Binding of the diazonium salt of [^{35}S]sulfanilic acid to red cells was utilized by BERG[3] to study membrane structural components and by HOYER AND TRABOLD[5] for immunological investigations. This report describes the use of a new reagent, diazotized [^{131}I]diiodosulfanilic acid as a label for the human erythrocyte membrane.

Diazotized [^{131}I]diiodosulfanilic acid was synthesized as previously described[6]. The water-washed diazonium salt was stable in the frozen state for at least 6 weeks. Its concentration in solution was determined by comparing its radioactivity to that

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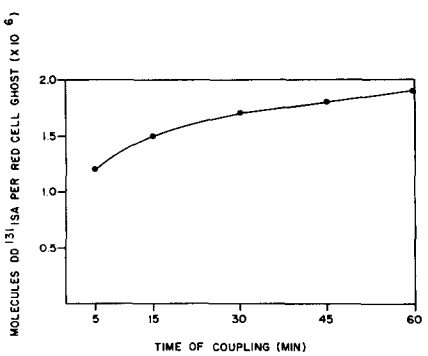
of an aliquot of the $Na^{131}I$ used in its preparation. Incorporation of 95% of the isotope into diiodosulfanilic acid was assumed for this calculation since the exchange reaction was shown to be over 95% complete[6]. For each experiment the reagent was dissolved in water at 4° (average solubility 1.27 μmoles/ml) and made isotonic by addition of phosphate buffer. For coupling to erythrocytes a small volume was added rapidly to a 20% suspension of washed cells in phosphate-buffered saline (0.05 M phosphate, 0.1 M saline, pH 7.4) at 4°. After 20 min the suspension was centrifuged. The red cells were washed at least 6 times with cold phosphate-buffered saline containing 0.5–1.0% bovine or human albumin. Red cell ghosts were prepared as previously described[7,8]. Red cells and ghosts were counted in an electronic particle counter (Coulter Counter, Model B). Radioactivity was measured in a well-type γ counter (Model 4233, Nuclear-Chicago Corp.). Radioactivity was converted to molecules of diazotized [^{131}I]diiodosulfanilic acid per cell from the activity of the isotope standard. The lipids were extracted from labeled red cells and subjected to paper chromatography as described by REED *et al.*[9]. A solution of the extracted lipids in methanol and chloroform (1:2, v/v) was further extracted twice with 0.1 M KCl.

Binding of diazotized [^{131}I]diiodosulfanilic acid by the cell membranes was rapid (Fig. 1). The influence of concentration of reagent on the degree of labeling is shown in Fig. 2.

At temperatures above 4° (24° or 37°), less diazotized [^{131}I]diiodosulfanilic acid was bound to the membrane. Labeling was diminished at pH 7.0 and 7.8, as compared to pH 7.4. Labeling of human cells was unaffected by blood group. Dog and rabbit erythrocytes bound the isotope similarly to human cells.

When the cells were washed at least 6 times with protein-containing buffer, over 85% of the label was usually found with the isolated membranes. The requirements for thorough washing of the labeled cells and the use of albumin in the washing buffer are illustrated in Table I. There appears to be some loosely bound diazotized



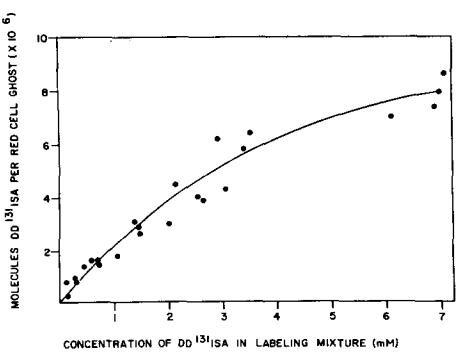


Fig. 1. Time-course of membrane binding of diazotized [^{131}I]diiodosulfanilic acid (DD^{131}ISA). The labeling mixture contained a 20% suspension of human red cells in phosphate-buffered saline pH 7.4, and 0.89 mM diazotized [^{131}I]diiodosulfanilic acid at 4°. The time of coupling was measured from the time of addition of isotope to the beginning of centrifugation of the cell suspension.

Fig. 2. Relationship of concentration of diazotized [^{131}I]diiodosulfanilic acid (DD^{131}ISA) to binding by cell membrane. The labeling mixture contained a 20% suspension of human red cells in phosphate-buffered saline, pH 7.4, at 4°. The points shown represent the results of several experiments; the line was fitted by eye. The fraction of isotope bound was usually less than 10% of the amount added and varied inversely with its concentration.

TABLE 1

REMOVAL OF LOOSELY BOUND DIAZOTIZED [^{131}I]DIIODOSULFANILIC ACID FROM LABELED ERYTHROCYTES

A 20% suspension of human red cells in phosphate-buffered saline was labeled with 0.02 mM diazotized [^{131}I]diiodosulfanilic acid. Aliquots were washed repeatedly with 6–8 vol. of phosphate-buffered saline with or without added 0.5% bovine albumin (Fraction V).

Red cells washed with	*Number of washes*	*Diazotized [^{131}I]diiodosulfanilic acid*		
		Molecules per red cell ($\times 10^{-6}$)	*Molecules per ghost* ($\times 10^{-6}$)	*% firmly bound to membrane*
Buffer	6	0.693	0.580	83.7
	12	0.568	0.550	96.8
Buffer + albumin	6	0.605	0.567	93.7
	12	0.570	0.542	95.1

[^{131}]diiodosulfanilic acid that requires vigorous washing to remove. HOYER AND TRABOLD[5] reported similar findings with diazotized sulfanilic acid.

That the membrane-bound isotope was associated primarily with stromal protein was demonstrated by the fact that the washed lipid extract contained less than 6% of the radioactivity of the red cells. All components of the lipid extract migrated normally on paper chromatography. Thus even the small amount of radioactivity associated with the lipid fraction may have been a contaminant and not covalently bound to reactive lipid sites such as the free amino groups of phosphatidylserine or phosphatidylethanolamine, since such a large polar residue bound to those lipids would have been expected to alter their chromatographic migration. In addition those two lipids stained with ninhydrin indicating that their free amino groups were preserved.

Concentrations of diazotized [^{131}I]diiodosulfanilic acid even as high as 7 mM usually produced no hemolysis. However, with more than $1.5 \cdot 10^6$ molecules of bound

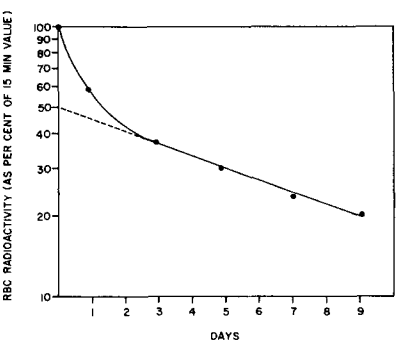


Fig. 3. *In vivo* survival of rabbit red cells labeled with diazotized [^{131}I]diiodosulfanilic acid. Washed cells were labeled with 0.022 mM diazotized [^{131}I]diiodosulfanilic acid resulting in $0.16 \cdot 10^6$ molecules per cell membrane. The cells were washed 6 times with albumin-containing buffer, suspended in buffer, and an aliquot reinjected into the animal. A blood sample drawn at 15 min provided the 100% point for red blood cell (RBC) radioactivity which was expressed as red cell counts/ml of whole blood.

isotope per cell, over 10% of the cells were crenated. With less than $1 \cdot 10^6$ molecules per cell, morphological abnormalities were not observed by phase microscopy. The osmotic fragility of cell suspensions with up to $2.5 \cdot 10^6$ molecules per cell was normal. Erythrocytes carrying up to $0.2 \cdot 10^6$ molecules of diazotized [^{131}I]diiodosulfanilic acid per cell could be incubated with shaking for 48 h at 37° in their own plasma with no more hemolysis than similarly treated unlabeled cells (less than 2%). When there were over $0.3 \cdot 10^6$ groups per cell, hemolysis after 48 h incubation slightly exceeded that of control cells.

A preliminary experiment was carried out to determine the behavior of labeled cells *in vivo*. Autologous rabbit cells labeled with $0.16 \cdot 10^6$ molecules of diazotized [^{131}I]diiodosulfanilic acid per cell membrane were reinjected into the animal and the red cell radioactivity followed. The results are shown in Fig. 3. After a rapid early loss of 60% of the radioactivity in 3 days, there was a slower decline with a half-life of about 7 days. These data do not, of course, distinguish among loss of label, loss of membrane protein, and destruction of whole cells, but the persistance of significant activity for the time shown indicates that the isotope may provide a means of studying the fate of the red cell membrane *in vivo*.

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