Biochimica et Biophysica Acta, 553 (1979) 54—65

BBA 78355

DIAZOTIZED [^{125}I]DIIODOSULFANILIC ACID AS A LABEL FOR CELL SURFACE MEMBRANES

STUDIES ON ERYTHROCYTES *

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(Received August 29th, 1978)

Key words: Diiodosulfanilic acid; Membrane labeling; α-Naphthol; (Erythrocyte)

Summary

1. Diazotized 2,6-diiodosulfanilic acid (DDISA) appears to have properties suitable to serve as an artificial, non-penetrating label of cell surface membranes. Therefore, the conditions for selective labeling of cell surface membranes as compared to intracellular proteins as well as a method for its chemical determination were explored in the present study.

2. DDISA reacts with α-naphthol at neutral pH to produce a compound (1-hydroxy-4-(2,6-diiodo-4-sulfo-1-phenylazo-(naphthylene)), DSPN) with a characteristic spectrum in the visible range (A_{max} 430 nm). The absorbance of the reaction product, DSPN, is linearly proportional to the concentration of DDISA and can be used as a method for the colorimetric determination of DDISA. Reaction of DDISA with a molar excess of α-naphthol was also used as a method for inactivating unreacted DDISA to terminate labeling prior to cell fractionation.

3. [^{125}I]DDISA reacts avidly with a variety of basic, neutral and acidic proteins as well as with cell membranes to form an acid-stable covalent azo linkage.

4. Effectiveness of labeling of the surface membrane of intact erythrocytes after incubation with [^{125}I]DDISA was assessed by the ratio of ^{125}I incorporated into membrane proteins compared to intracellular proteins. When intact erythrocytes were exposed to [^{125}I]DDISA, the optimal labeling of membranes occurred at 37°C after 20 min of incubation time and at a concentration of 10^{-4} M [^{125}I]DDISA in the incubation media. Under these conditions the ratio

* A preliminary report of this work was published as an abstract (Physiologist (1978) 21, 32).
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of the specific activity (cpm ^{125}I/mg protein) of the membrane fraction to the specific activity of the soluble protein fraction (membrane/supernatant ratio) was greater than 500. When incubations were conducted at 4° C this ratio was less than 50. However, when osmotically lysed erythrocytes were incubated with [^{125}I]DDISA the majority of the label reacted with the soluble protein fraction resulting in a membrane/supernatant ratio of 0.14.

5. The results thus suggest that [^{125}I]DDISA used under the appropriate incubation conditions, including the inactivation and removal of [^{125}I]DDISA by washing with α-naphthol, can serve as a highly selective membrane label with minimal incorporation into intracellular soluble proteins. The general applicability of this method for other cell types remains to be explored.

Introduction

In studies on plasma membranes of a variety of mammalian cells, several basic criteria are used to identify this subcellular structure in the course of isolation and purification [1]. Typical morphologic characteristics and the presence of specific enzymes can be used as markers for identification of the whole cell or specific regions of cell surface membranes. For example, the brush border luminal membrane of small intestine and renal epithelial cells can be identified both by typical morphological features (microvilli) and the presence of typical enzymes [1—3]. However, in many tissues composed of heterogeneous populations of cells, the morphologic or enzymatic markers specific enough for identification purposes are either not present or have not yet been identified [1]. In the past, several attempts were made to develop an artificial chemical marker which, after exposure to intact cells would selectively bind to the plasma membrane but not to intracellular structures and would be readily identifiable in the course of the preparative procedure [4—6].

Radioactively labeled diazotized [^{125}I]diiodosulfanilic acid ([^{125}I]DDISA) or diazotized [^{35}S]sulfanilic acid have been synthesized and used in several studies as non-penetrating reagents for labeling of cell membranes [7—13]. These compounds offer several advantages as cell surface membrane labels: they react rapidly and covalently with proteins under mild conditions [14,15]; the negative charge on the parent molecule, sulfanilic acid, retards entry into cells; radioactive analogs of sulfanilic acid can be easily synthesized at high specific activities [16,17]; and at low concentrations cause minimal cell damage [8,17].

Past studies with diazotized derivatives of sulfanilic acid [7—10] have been directed more to the labeling of individual surface membrane proteins and little emphasis has been placed on the extent of binding to intracellular proteins which is of critical importance if the label is to be used to specifically identify the cell membranes compared to intracellular structures in the course of isolation procedures. Observations reported in previous studies [7—13] suggest that [^{125}I]DDISA could be a suitable marker of plasma membranes. However, the optimal conditions for selective labeling of the cell membrane as compared to intracellular proteins as well as a method for determining the specific activity and concentration of [^{125}I]DDISA have not been investigated in detail.

To define the conditions for the use of [^{125}I]DDISA as an artificial marker

of the cell surface (plasma) membrane, we developed a method for the determination of the concentration and specific activity of the compound and explored the conditions of time, temperature and reagent concentration which are necessary for the optimal labeling of erythrocyte cell membranes compared to intracellular soluble proteins.

Materials and Methods

2,6-Diiodosulfanilic acid (DISA) was synthesized from sulfanilic acid by the method of Boyle [18] and was stored in the dark at 0° C until needed. ^{125}I-labeled 2,6-diiodosulfanilic acid (Fig. 1) was prepared by the exchange reaction between DISA and Na^{125}I in the presence of ICl as described by Helmkamp and Sears [16]. Following the exchange reaction, the solution containing ^{125}I-labeled DISA was cooled to —5° C and 0.8 ml of cold 0.12 M $NaNO_2$ was added which resulted in the precipitation of the ^{125}I-labeled diazonium salt of DISA ([^{125}I]DDISA). After 20 min the solution was centrifuged at 0° C and the supernatant containing unreacted ^{125}I and excess reagents was removed. The precipitated [^{125}I]DDISA was then washed three times by centrifugation with 1 ml of cold H_2O. The yield of the diazo product ranged from 51 to 74% and the extent of incorporation of ^{125}I into [^{125}I]DDISA was from 45 to 71%. [^{125}I]-DDISA was stored at —70° C and used within 1 week of preparation during which time the compound is stable [8].

Since the yield of DDISA in the synthesis as well as the extent of incorporation of ^{125}I into [^{125}I]DDISA was variable a method based on the reaction between DDISA and α-naphthol was used to determine the molar concentration and specific activity of [^{125}I]DDISA. To 50 µl of DDISA dissolved in distilled water (average solubility 1.60 mM at 25° C) was added 1 ml of a 1 mM solution of α-naphthol in distilled water. The absorbance of the resulting orange-colored azo compound (1-hydroxy-4-(2,6-diiodo-4-sulfo-1-phenylazo-(naphthylene)), DSPN) (Fig. 2) was determined at an optimum wavelength (430 nm, Fig. 3) at which the calculated log molar extinction coefficient was 6.50. The specific activity of [^{125}I]DDISA was determined by measuring the radioactivity of the same aliquot as was used to determine concentration (see Results). Specific activities on the order of 10 Ci/mol were used in all experiments and could be routinely obtained by using 80 µmol of DISA and approx. 1 mCi of ^{125}I in the above-mentioned exchange reaction.

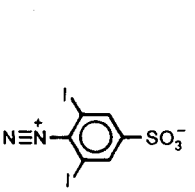

Fig. 1. Diazotized 2,6-diiodosulfanilic acid (DDISA).

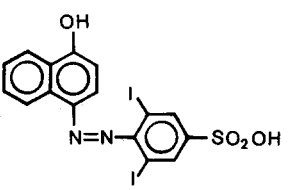

Fig. 2. 1-Hydroxy-4-(2,6-diiodo-4-sulfo-1-phenylazo-(naphthylene)) (DSPN). Product of reaction of α-naphthol with diazotized 2,6-diiodosulfanilic acid.

Erythrocytes were obtained by mixing freshly drawn human blood (25 ml) with 1 ml 0.2 M EDTA and an equal volume of cold modified Kreb's Ringer buffer (final concentrations; 150 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 0.8 mM $CaCl_2$, 10 mM sodium acetate, 10 mM glucose, 2 mM sodium phosphate, pH 7.4). The suspension was centrifuged at 1000 × *g* for 10 min at 4°C and the supernatant and buffy coat were removed by aspiration. The packed cells were resuspended in buffer and washed three additional times using the same procedure.

Labeling of intact cells was initiated by rapidly adding a small volume of [^{125}I]DDISA dissolved in Krebs-Ringer buffer to 20% (v/v) suspensions of washed erythrocytes pre-equilibrated to the specified temperatures. Aliquots (0.5 ml) were removed at specified time intervals as indicated in Results and the reaction was stopped by adding 1 mM α-naphthol in buffer to produce an α-naphthol/[^{125}I]DDISA molar ratio of 1.5/1.0. The samples of reacted cells were washed twice by resuspension in 20 ml of the appropriate concentration of α-naphthol in buffer to remove any residual unreacted label and two additional times with 20 ml of buffer without α-naphthol, and all recentrifugations were at 1000 × *g* for 10 min at 4°C. In preliminary experiments it was found that the concentrations of α-naphthol used in these experiments (maximum 2.1 mM) produced no hemolysis or morphological abnormalities. Following removal of the unreacted label the packed cells were lysed by suspending them in 20 ml of ice-cold 5 mM sodium phosphate buffer, pH 7.4, and centrifuged at 30 000 × *g* for 10 min. At this time aliquots of the supernatant were taken for protein and radioactivity measurement. The pellet of ghosts was resuspended and washed two additional times using the same procedures as above to remove residual hemoglobin.

Radioactivity incorporated into membrane and soluble protein was determined by a method previously described [19]. Briefly, 2 ml 10% trichloroacetic acid was added to 100-μl aliquots of supernatant and membrane samples (50—200 μg protein). The acid precipitate was dissolved in 0.1 ml 0.5 M NaOH and reprecipitated with 2 ml 10% trichloroacetic acid. The precipitate was collected on glass fiber filters (GF/C, Whatman) and washed five times with 5 ml 5% trichloroacetic acid-0.1 mM KH_2PO_4, and the radioactivity retained by the filters was measured in a gamma counter (Searle Analytic Gamma Counter, Model 1285). The amount of [^{125}I]DDISA bound to the two fraction is expressed as cpm ^{125}I/mg protein. Protein in the same aliquot was measured by the method of Lowry et al. [20].

Carrier-free Na^{125}I (17 Ci/mg) was obtained from New England Nuclear (Boston, MA). Sulfanilic acid and α-naphthol were purchased from Fisher Scientific Co. (Fairlawn, NJ). Histone fractions, protamine and casein were purchased from Sigma Co. (St. Louis, MO). Myoglobin and γ-globulin were from Schwartz-Mann, Orangeburg, NY. All other chemicals were of ACS reagent grade.

When appropriate, results were statistically evaluated with the use of Student's *t*-test.

Results

Colorimetric determination of DDISA

As shown in Fig. 3, the absorbance maximum of DDISA in the visible range is shifted from 385 to 430 nm upon reaction with α-naphthol. α-Naphthol alone did not absorb at this wavelength and enough α-naphthol was present to ensure the complete coupling of DDISA. The formation of the azo product, DSPN, with an absorption maximum at 430 nm, is linearly proportional (r = 0.99) to the concentration of DDISA from 0 up to its upper solubility limit (1.6 mM) and obeys Beer's law; absorbance at 430 nm of a 1 mM solution of DSPN was 0.67. The reaction is complete in less than 1 min, even at 4°C, and the color is stable for at least 2 h.

Protein labeling

To determine whether [^{125}I]DDISA reacts with proteins of different physical and chemical properties a series of basic neutral and acidic proteins was incubated with [^{125}I]DDISA. 50 μg of protein dissolved in 200 μl of Krebs-Ringer buffer was incubated with 100 μl of [^{125}I]DDISA (final concentration, 0.47 mM) at 37°C for 20 min. The reaction was stopped by addition of an excess of α-naphthol and the incorporation of the label into the proteins was determined as outlined in Materials and Methods. As shown in Table I, [^{125}I]-DDISA reacted avidly with all proteins tested. The effect of temperature and reagent concentration on the degree of labeling of soluble proteins was examined for bovine serum albumin and soluble proteins from osmotically lysed cells. The incorporation of [^{125}I]DDISA incubated with 50 μg of bovine serum albumin for 20 min was proportional to the molar concentration of [^{125}I]-DDISA; the fraction of added [^{125}I]DDISA which bound to the same amount of protein increased from 20% at 1.0 mM [^{125}I]DDISA to 45% at 1.4 mM [^{125}I]DDISA. There was no difference in the extent of [^{125}I]DDISA binding to bovine serum albumin or soluble proteins of hemolysates at 4 and 37°C.

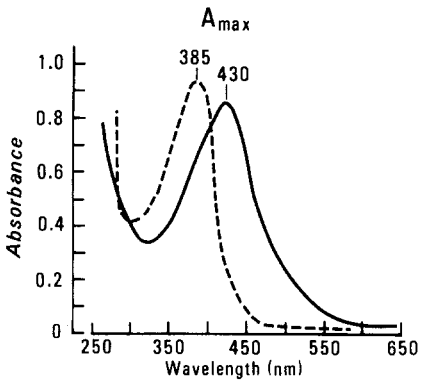

Fig. 3. Comparison of the absorption spectra in the range 250—650 nm of diazotized 2,6-diiodosulfanilic acid (DDISA) (- - - - -) and 1-hydroxy-4-(2,6-diiodo-4-sulfo-1-phenylazo-(naphthylene)) (DSPN, product of reaction of α-naphthol with DDISA) (———). The spectra were recorded from solutions of the compounds dissolved in Krebs-Ringer buffer, pH 7.4. A_{max} for DDISA is 385 nm and A_{max} for DSPN is 430 nm.

TABLE I

PROTEIN LABELING

50 μg of protein were incubated in Krebs-Ringer buffer with a final concentration of 0.47 mM [^{125}I]-DDISA at 37°C for 20 min, pH 7.4.

Protein	Specific activity (^{125}I cpm X 10^{-6}/mg protein)
Histone II_a	5.8 ± 0.03 *
Histone F_3	6.7 ± 0.01
Histone F_{2b}	7.4 ± 0.70
Protamine	20.9 ± 0.96
Myoglobin	14.2 ± 0.88
γ-Globulin	6.4 ± 0.44
Casein	10.1 ± 1.27
Bovine serum albumin	6.7 ± 0.67

* Mean ± SE, $n = 3$.

Effects of time and temperature on the labeling of intact erythrocytes

The extent of binding of [^{125}I]DDISA to membrane and supernatant fractions of intact cells as a function of time and temperature is shown in Fig. 4. At both 4 and 37°C membrane labeling was rapid, reaching a plateau in 20—25 min. At both temperatures the reaction was nearly 50% complete by 2 min. At each sampled time period the amount of membrane-bound [^{125}I]DDISA per

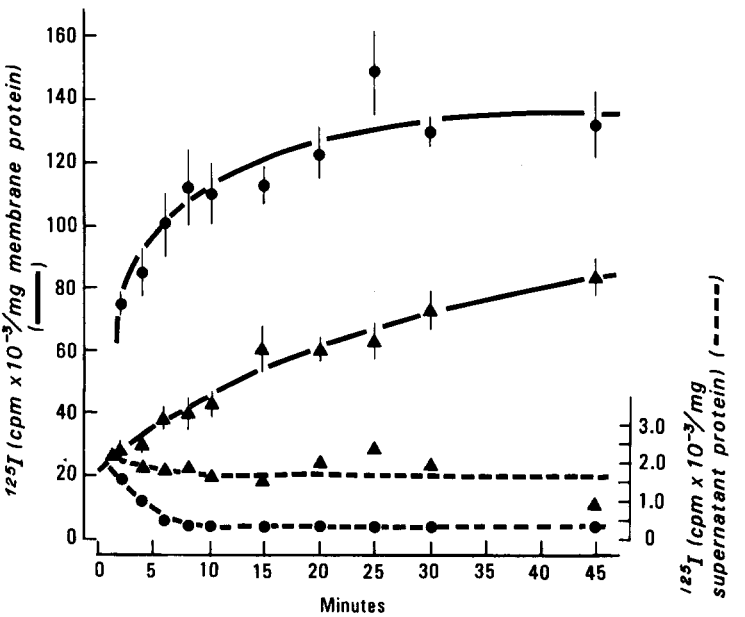

Fig. 4. Time course of the incorporation of diazotized [^{125}I]diiodosulfanilic acid ([^{125}I]DDISA) into membrane and supernatant fractions of intact erythrocytes incubated at 4 and 37°C (for details, see Materials and Methods). Final concentration of [^{125}I]DDISA was 0.093 mM. Membrane fractions: 4°C (▲——▲), 37°C (●——●); supernatant fractions: 4°C (▲- - - -▲), 37°C (●- - - -●). Each point represents the mean ± S.E. of five experiments analyzed in duplicate. Each value at 37°C is significantly different from the corresponding value at 4°C, $P < 0.05$ or better, t-test.

mg protein was significantly greater ($P < 0.05$) at 37°C than at 4°C. Following an initial decline at 37°C the amount of label bound to soluble proteins remained constant. At 4°C a transient increase in incorporation into soluble proteins occurred from 15 to 30 min followed by a decline at 45 min. At all time periods the amount of label bound to soluble proteins was significantly less ($P < 0.05$) at 37°C as compared to 4°C.

The electrophoretic profile of [^{125}I]DDISA-labeled erythrocyte membrane proteins was analogous to those reported from other laboratories [12,13].

The degree to which [^{125}I]DDISA labels the erythrocyte membrane and penetrates into the cell interior expressed as the ratio of the specific activity of the membrane fraction to the specific activity of the supernatant fraction is shown in Fig. 5. At 4°C this ratio was less than 100 and remained relatively constant with time. At 37°C the ratio continuously increased and was more than 10-fold greater after 20 min of incubation when compared to the corresponding value at 4°C.

Effects of concentration of [^{125}I]DDISA on erythrocyte labeling

The amount of [^{125}I]DDISA incorporated into membrane proteins increased proportionally to the concentration of [^{125}I]DDISA in the incubation medium (Fig. 6). No saturation of membranes with [^{125}I]DDISA was seen over the range of concentrations of [^{125}I]DDISA used. In the supernatant fraction from the same cells incorporation of [^{125}I]DDISA, which was miniscule, increased up to a concentration of 0.8 mM [^{125}I]DDISA and then remained relatively

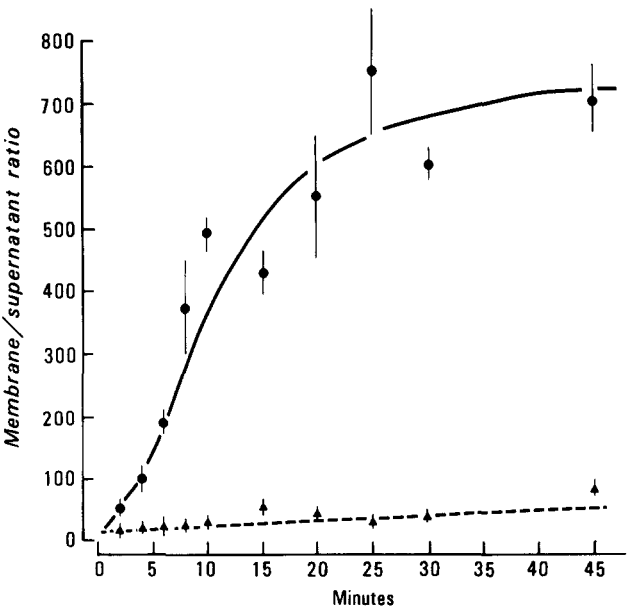

Fig. 5. Effects of time of incubation on the ratio of the specific activity (^{125}I cpm/mg protein) of the membrane fraction to the specific activity of the supernatant fraction (membrane/supernatant ratio) of intact erythrocytes incubated with [^{125}I]DDISA at 4°C (▲- - - - -▲) and 37°C (●————●). Final concentration of [^{125}I]DDISA was 0.093 mM. Each point represents the mean ± S.E. of five experiments analyzed in duplicate (for details, see Methods).

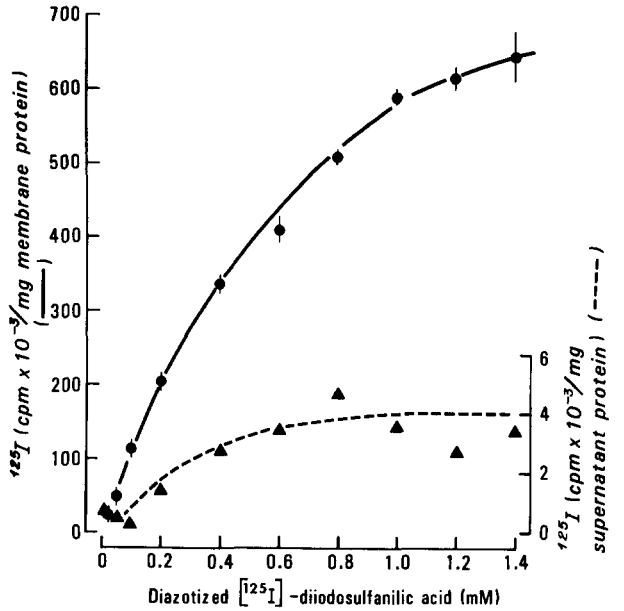

Fig. 6. Effects of various concentrations of [^{125}I]DDISA on the incorporation of the label into membrane (●——●) and supernatant (▲- - - - -▲) fractions of intact erythrocytes incubated at 37°C for 20 min. Each point represents the mean ± S.E. of five experiments analyzed in duplicate.

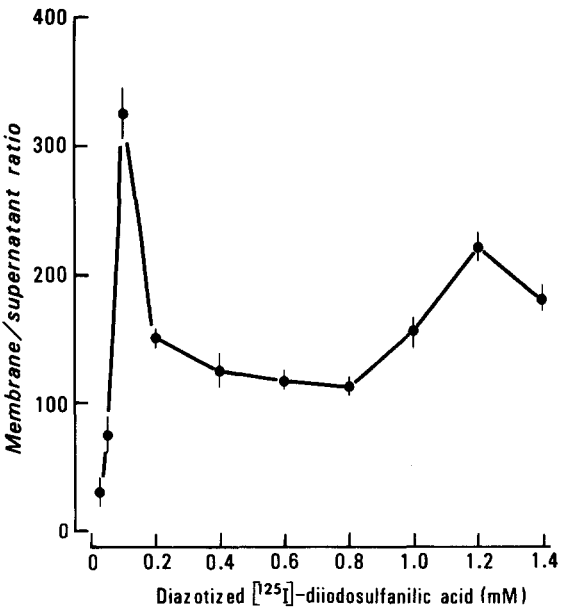

Fig. 7. Effects of various concentrations of [^{125}I]DDISA on the ratio of the specific activity (^{125}I cpm/mg protein) of the membrane to the specific activity of the supernatant fractions of intact erythrocytes incubated at 37°C for 20 min. Each point represents the mean ± S.E. of five experiments analyzed in duplicate.

TABLE II

LABELING OF WHOLE CELLS VERSUS LYSED CELL SUSPENSIONS WITH [^{125}I]DDISA

Equal volumes of packed cells were diluted 1 : 1 with either Krebs-Ringer buffer to prepare whole cell suspension or 5 mM sodium phosphate buffer to prepare lysed cell suspensions. Two preparations were incubated at 37°C for 20 min, pH 7.4, with a final concentration of 0.058 mM [^{125}I]DDISA. Membranes and supernatant were separated and prepared as described in Materials and Methods.

	Specific activity (^{125}I cpm × 10^{-3}/mg protein)		Membrane/ supernatant
	Membrane	Supernatant	
Whole cells	66.6 ± 3.26 *	0.21 ± 0.02	343 ± 47
Lysed suspension	1.19 ± 0.06	9.73 ± 0.47	0.14 ± 0.014

* Mean ± S.E., n = 5.

constant (Fig. 6). Even at the highest concentration of [^{125}I]DDISA (1.4 mM) no hemolysis was noted.

The membrane to supernatant ratios achieved with different concentrations of [^{125}I]DDISA are shown in Fig. 7. A maximum membrane/supernatant ratio of 325 was achieved at a concentration of 0.1 mM [^{125}I]DDISA. At higher concentration of [^{125}I]DDISA the ratio was reduced primarily due to increased labeling of soluble proteins (Fig. 6).

Labeling of whole cells compared to osmotically lysed cell suspensions

Additional experiments were conducted to compare the degree of incorporation of [^{125}I]DDISA into supernatant and membrane fractions under conditions when whole cells and osmotically lysed cell preparations are exposed to the labeling compound. Equal volumes of packed cells were diluted 1 : 1 with either cold Krebs-Ringer-buffer to prepare whole cell suspension or 5 mM sodium phosphate buffer to prepare osmotically lysed cell suspensions and then equilibrated to 37°C. Experiments were started by adding equal concentrations of the label (0.058 mM, final concentration) to the two preparations. The reaction was stopped at 20 min with addition of α-naphthol. Supernatant and membrane fractions were obtained and protein and radioactivity measured as described in Materials and Methods. When intact cells were exposed to [^{125}I]-DDISA the majority of [^{125}I]DDISA labeled the membrane while only traces entered the cells, resulting in a membrane/supernatant ratio of 343 (Table II). However, in the osmotically lysed cells exposed to [^{125}I]DDISA the supernatant fraction contained more than eight times the amount of [^{125}I]DDISA per mg protein than in the membrane fraction resulting in a membrane/supernatant ratio of 0.14 which was 3000-fold less than in labeled intact cells.

Discussion

The use of enzymatic and morphological markers for identification of plasma membranes presents many difficulties. For instance, enzyme localization may vary between the same tissue in different species [21] or the distribution of enzyme activity may change during fractionation [4]. The greatest

difficulty with morphological markers is the lack of a satisfactory method of quantification [1]. In tissues in which the enzymatic and morphological characteristics of the plasma membrane have not yet been identified or in which they are not very distinct, the use of artificial markers which selectively bind to the plasma membrane presents an alternative approach which could be used to isolate this structure from other cell components.

A major determinant of the usefulness of a compound in the vectorial labeling of surface membranes is under what conditions and to what degree the reagent penetrates into the cell interior [6]. In addition, the labeling compound should bind quickly and irreversibly with the membrane under physiological conditions and that portion of the compound which does not react with the membrane should be completely removed prior to homogenization and fractionation to avoid spurious labeling of intracellular components. In the present study, using erythrocytes as a model system, we assessed the effects of time, temperature and reagent concentration on the efficiency of labeling and on the penetration characteristics of [^{125}I]DDISA by measuring the specific activity (cpm ^{125}I/mg protein) of membrane and soluble protein fractions and by calculating the membrane/soluble protein ratio.

Since the efficiency of the exchange reaction between Na^{125}I and DISA varied from preparation to preparation it was necessary to develop a method of determining the specific activity of [^{125}I]DDISA, which would determine exactly the experimental conditions for labeling. The product of the reaction between DDISA and α-naphthol, DSPN, has a characteristic spectrum in the visible range which is clearly distinguishable from that of the parent compound, DDISA. The reaction is linear with the concentration of DDISA and can be used as a quick and convenient method for the colorimetric determination of DDISA. Moreover, the rapid reaction of DDISA with α-naphthol or other phenolic coupling agents can be employed as a method for removing excess unreacted [^{125}I]DDISA and thus prevent labeling of structures other than the surface membrane by residual [^{125}I]DDISA during subsequent fractionation and preparative procedures.

Temperature and reagent concentration have marked effects on the distribution of [^{125}I]DDISA across the cell membrane. Not only was the amount of label bound to membrane proteins greater at 37°C but the labeling of the soluble proteins at 37°C was decreased more than 2-fold compared to 4°C. Since there was little or no effect of temperature on the binding of [^{125}I]-DDISA to supernatant from lysed cells or bovine serum albumin, as was also found in a recent study [13], the marked effect of temperature is likely due to an increased permeability of the erythrocyte membrane to [^{125}I]DDISA at low temperatures caused perhaps by decreased fluidity and deformability of the membrane at lower temperatures or other changes in membrane conformation [22—24]. The observation that the membrane/supernatant ratio is one order of magnitude greater at 37°C compared to 4°C is significant in view of the possibility of using [^{125}I]DDISA in in vivo situations.

Additional evidence to support the interpretation that it is the low permeability of the membrane to [^{125}I]DDISA and not its differential reaction with soluble and membrane proteins which determines the distribution of the reagent is the finding that the membrane/supernatant ratio was 3000 times

greater in intact cells exposed to [^{125}I]DDISA as compared to osmotically lysed cell suspensions in which the reagent has direct access to both the membrane and soluble proteins.

As expected increasing the concentration of [^{125}I]DDISA in the labeling medium increased the incorporation of [^{125}I]DDISA into the membrane proteins. However, concomitant with this increase, the penetration of the reagent also increased. Thus, the highest and for labeling purposes optimal membrane/supernatant ratio was achieved at a relatively low concentration of 0.1 mM [^{125}I]DDISA.

The present study thus demonstrates that [^{125}I]DDISA used under the appropriate conditions can serve as a selective marker of the plasma membrane with minimal labeling of intracellular proteins. [^{125}I]DDISA reacts avidly with a number of basic, neutral and acidic proteins as well as cell membranes forming an acid-stable covalent linkage. The length of incubation time, temperature and reagent concentration are critically important in determining the degree of penetration of [^{125}I]DDISA across the cell membrane and in achieving the most favorable surface membrane labeling. By using [^{125}I]DDISA of high specific activity, low molar concentrations and relatively short incubation times the most favorable membrane/soluble protein ratios can be obtained. The reaction with α-naphthol can be used as a simple and effective method for removing unreacted [^{125}I]DDISA prior to fractionation without apparent damage to the cells. Moreover, it provides a convenient method for the chemical assay of DDISA. Applicability of this method to other cell systems is suggested by our recent observations in which specific labeling of the luminal plasma membranes of renal papillary collecting ducts with [^{125}I]DDISA was obtained. The general applicability of this method for labeling other cell types in vitro or in vivo remains to be explored.

Acknowledgments

This study was supported by the U.S.P.H.S. research grant AM-16105 from the National Institute of Arthritis Metabolism and Digestive Diseases, and by the Mayo Foundation. This study was done by R.M.E. in partial fulfillment of the requirements for the Ph.D. degree, Mayo Graduate School of Medicine. S.A.K. is recipient of a postdoctoral research fellowship of the Minnesota Heart Association. T.P.D. is an Established Investigator of the American Heart Association. We thank Mrs. Ardith Walker for her excellent secretarial assistance.

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