Spectroscopic Assays for Measuring Quantities of Erythrocyte Membrane "Halves"

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ABSTRACT The quantities of outer and inner "halves" produced by freeze-fracturing human erythrocyte membranes have been measured by visible and fluorescence spectroscopy. Assays have been developed that are based on the use of two membrane surface markers: hemoglobin (Hb), a native marker for the cytoplasmic side of the membrane, and fluoresceinated concanavalin A (FITC-Con-A), a marker for the extracellular side. Hb absorbance is proportional to the fraction of cytoplasmic "half" membranes, and FITC fluorescence is proportional to the fraction of extracellular "halves." A procedure is described for the preparation of surface-labeled, intact erythrocytes suitable for the formation of homogeneous, planar cell monolayers of square-centimeter dimensions on polylysine-treated glass (PL-glass). Cell monolayers were frozen and fractured, and the fractions of absorbance and fluorescence in each of the two split portions determined. The PL-glass portion of the membrane contained a substantially higher ratio of fluorescence to absorbance than unsplit controls, and its paired portion, a complementary lower ratio, demonstrating that the PL-glass portion was significantly enriched in extracellular "half" membrane. Experiments investigating split membrane recovery show that the double labeled membrane splitting technique is well suited to analysis of the transmembrane distribution of membrane lipids and polypeptides using methods that do not require quantitation by electron microscopy.

The method of freeze-fracture, although used primarily to gain information about the physical structure of model membranes and biomembranes, can also be used to examine the chemical composition of membrane "halves" (18). This latter approach relies on membrane bilayer splitting to produce two fractions: one enriched in extracellular "half" membranes, one enriched in intracellular "halves." Such fractions can be produced by the method of monolayer freeze-fracture in which cells or membrane fragments are attached to a planar cationic glass surface. After freezing, the flattened membranes fracture in preference to those that are unattached, leaving the planar surface rich in outer "half" membrane (15). This monolayer freeze-fracture method has been used to examine the transmembrane concentration of human erythrocyte (RBC) cholesterol (16) and the fracturing properties of certain erythrocyte glycopeptides (12) and purple membrane bacteriorhodopsin (21, 22).

A key component in the analysis of transbilayer concentration is quantitation of the fractions of "half" membranes and intact membranes in the two split portions and in unsplit controls (16, 17, 34). In past studies such quantitation has been provided by transmission electron microscopy (TEM) and photographic methods (15, 16). Although TEM can directly verify membrane splitting, its use to determine amounts of "half" membranes in split fractions is limited. Microscopic methods require duplicate samples, one for TEM and one for chemical analysis, and at best allow examination of only a tiny portion of the total sample. Moreover, cells of irregular shape and unknown surface area present a special problem for quantitative TEM, where measurements of split membrane profiles on micrographs become unrealistically time-consuming. Any multiple sample experiment, such as a study of transbilayer flip-flop kinetics, is also unwieldy.

If the planar cell monolayer approach is to become generally useful, it must be coupled with rapid quantitative assays of "half" membrane concentrations. Ideally those assays should not only be rapid but also capable of evaluating concentrations of "half" membranes in the same sample used to examine the molecule or probe of interest, and applicable to cells of unknown surface area or of irregular morphology. The assays must also be sensitive because planar monolayer freeze-fracture produces submicrogram amounts of membrane components.

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In this report I describe a new technique, double labeled membrane splitting, for evaluating the concentrations of outer and inner "half" membranes in split planar RBC monolayers. The monolayers used in this study were 2.4 cm^2 and contained ~4.8 × 10^6 intact RBC's. The double labeling rationale was as follows. If one labels both surfaces of the plasma membrane before splitting, fracturing the membrane bilayer should physically separate the labels. The quantities of surface labels in the split fractions would thus be proportional to the quantity of associated "half" membranes. Hemoglobin (Hb) was used as a native marker for the fraction of RBC cytoplasmic "half" membrane. Although Hb is a soluble protein, not strictly a surface label, several observations suggested that it would be suitable. First it is exclusively cytoplasmic and thus sidedness of the label is known. Second, TEM observations of split monolayers (15, 16) indicated that the residual pink regions contained almost exclusively intact cells, suggesting that the fraction of Hb would be directly proportional to the fraction of cytoplasmic surface. And third, TEM has shown that where cells are cross or partially fractured, neither large areas of cytoplasmic surfaces nor cytoplasmic fracture faces devoid of residual cytoplasm are found. Because the Soret absorbance peak of Hb has a high molar extinction coefficient (3), the concentration of Hb derived from small planar areas could be determined accurately by conventional UV-visible spectroscopy. A fluorescent lectin, fluorescein isothiocyanate modified concanavalin A (FITC-Con-A), was used as a marker for the fraction of extracellular "half" membrane. Conditions for labeling cells in suspension that would prevent penetration of the label and provide quantitative retention during monolayer formation and fracturing were evaluated so that fluorescence relative intensity would be proportional to the fraction of extracellular "half" membrane. Conditions for optimizing the fluorescence relative intensity for fluorescein-protein conjugates were also examined. The direct determination of the concentrations of intra- and extracellular "half" membranes allowed the analysis of the transmembrane distribution of native molecules or probes (16) to be simplified. A radioiodinated derivative of FITC-Con-A was prepared and its distribution evaluated as an example of the application of split monolayer analysis to studies of the transbilayer distribution of radioisotopic probes.

MATERIALS AND METHODS

A synopsis of the double labeled membrane splitting technique is shown in Fig. 1. A variety of potential cell surface labels were evaluated including several lectins, antibodies, and cyanine dyes. Results of preliminary experiments comparing properties of FITC-Con-A (1, 6, 39, 43, 45) with commercially available derivatives indicated that FITC-Con-A from Vector Laboratories (Vector Laboratories, Inc., Burlingame, Calif.) would be a suitable label. This conjugate was used for all experiments described in this report.

FITC-Con-A Conjugates

The protein concentrations of the FITC-Con-A conjugates were determined using four methods: dry weight; absorbance at 280 nm (2) corrected for fluorescein (39); Lowry protein assay (35); and Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) (7). The fluorescein concentration was determined from its absorbance at 495 nm corrected for fluorescence and for protein conjugation (6, 31, 33, 38, 39). Conjugates were stored in the dark at 5°C. Radioiodinated derivatives of FITC-Con-A were prepared following the Bolton Hunter procedure (9) and details will be presented elsewhere.[1]

[1] Fisher, K. A. 1981. Monolayer freeze-fracture autoradiography: quantitative analysis of the transmembrane distribution of radioiodinated concanavalin A. *J. Cell Biol.* In press.

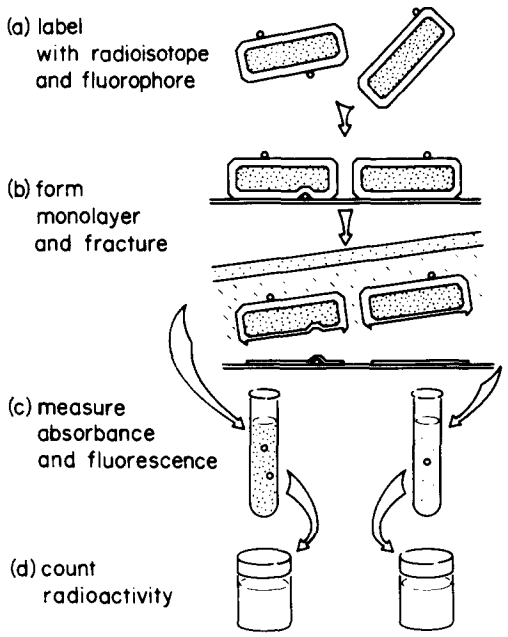

FIGURE 1 Synopsis of the double labeled membrane splitting technique. Well-washed erythrocytes, randomly labeled with a radioisotope and an extracellular fluorescent surface marker (a), are applied to PL-glass and freeze-fractured (b). The split portions are dissolved in SDS-borate buffer and hemoglobin absorbance and fluorophore fluorescence measured (c). The solutions are transferred to a scintillation vial, dried, isotope solubilized with scintillation cocktail, and the radioactivity is counted (d).

Erythrocyte Labeling

Fresh human RBC's, type A positive, were obtained from a local blood bank and used within 14 d of the draw date. RBC's were washed three times in 0.9% NaCl and three times in HEPES-buffered saline, HBS (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 143 mM NaCl, 5 mM $MgCl_2$ at pH 7.4), and separated from other blood cells as previously described (16). Several labeling conditions were examined in an effort to maximize the amount of FITC-Con-A adsorbed to washed RBC's in suspension. The following labeling procedure was found to be satisfactory and was routinely used for the RBC membrane splitting experiments. FITC-Con-A, 1.5 mg (e.g., 75 μl of 20-mg/ml stock solution), was added to 7.5 ml HBS, and washed, pelleted RBC's (100 μl) were suspended separately in 7.5 ml HBS. Diluted label and cells were quickly mixed together and the final 15 ml was vortexed and incubated on ice for 60 min. Labeled cells were centrifuged at ~500 *g* for 2.5 min and supernatants were aspirated. Pellets were resuspended in 15 ml buffer, pelleted, and the final pellet resuspended in buffer (1 vol pellet plus 4 vol HBS) and applied to a 1.5 × 12-cm Bio-Gel A-15m column (100–200 mesh; Bio-Rad Laboratories). Columns were pretreated with 0.01% bovine serum albumen (BSA) (fraction V; Sigma Chemical Co., St. Louis, Mo.) in HBS for 30 min followed by HBS alone for 45 min, both at 1.2 ml/min at 4°C. Cells were eluted with HBS at 0.6 ml/min at 4°C; fractions 13 through 23 were pooled (see Results) and centrifuged, and the pellets resuspended to a final dilution of ~2 vol cells plus 1 vol HBS. Suspended cells were sampled for fluorescence and absorbance measurements. FITC-Con-A labeled cells were kept on ice and usually applied to polylysine-treated cover glass (PL-glass) within 6 h of labeling.

Planar Monolayer Preparation and Fracturing

Cover glasses, 11 × 22 mm, were notched at one corner for orientation, cleaned, dried, treated with 50 μl 5 mM poly-L-lysine HBr, mol wt 2,000–4,000 (Miles Laboratories, Inc., Elkhart, Ind.) for 30 s and washed and nitrogen dried as previously described (20). Usually 50 μl of 2 vol RBC's plus 1 vol HBS were applied to the dry cationic PL-glass, gyrated for 5 s and washed by gentle agitation in 40 ml HBS for 20 s, followed by two more identical 20-s washes in fresh HBS. After the last wash, monolayers were processed in several ways.

For fracturing, monolayers were placed against 12 × 24-mm, 0.6- to 0.75-mm thick, acid-cleaned and nitrogen-dried glass plates cut from 2 × 2-in. ultrathin cover glass (Emde Products, Inc., Los Angeles, Calif.). These sandwiches were frozen in liquid Freon-22 at −150°C and briefly stored in liquid nitrogen.

Monolayers were fractured by prying the glasses apart under liquid nitrogen. Frozen splits as well as control unfractured monolayers, frozen or unfrozen, were dropped into 2.5 ml fluorescence cocktails (see Fluorescence Spectroscopy) in glass scintillation vials, and sonicated for 5 s in an 80-kHz bath-type sonicator (Laboratory Supplies Co., Hicksville, N. Y.).

Light and Electron Microscopy

For quantitative light microscopy, RBC monolayers were fixed in 1% glutaraldehyde (42) in HBS on ice for 1 h, stored in fixative overnight at 5°C, and then rinsed with HBS before photomicrography. Light micrographs were taken with a Zeiss Photomicroscope. Monolayers were photographed at random, 8 to 10 micrographs per monolayer. For calibration a grid and a stage micrometer were photographed under identical optical conditions. A micrograph of the grid was printed with each set of monolayer pictures for use as a template for cell counting.

For electron microscopy unfixed, freeze-fractured monolayers were lyophilized and warmed to 35°C before shadowing at room temperature with platinum-carbon at an angle of 20 degrees (16, 20). Shadowed samples were cut into 2 × 2-mm strips and the replicas floated off the glass onto 1 part HF plus 1 part distilled water. After 3 loop transfers through distilled water, replicas were picked up on flamed grids and examined using a Siemens 101 electron microscope.

UV-Visible Spectroscopy

Samples in fluorescence cocktails were transferred to 4-ml quartz cuvettes, 1-cm pathlength, and spectra scanned from 250 nm to 750 nm using a Cary-14 spectrophotometer (Varian, Palo Alto, Calif.) equipped with scattered transmission accessory. RBC Hb concentration was based on an Hb millimolar extinction coefficient of 125 at 415 nm; i.e., $\epsilon = 125$/millimole monomer of mol wt 16,000 (3), and compared to a concentration of 30 ± 10 pg Hb/cell (4, 5, 30). For routine assay of Hb in SDS-borate, absorbance was measured at 403 nm. RBC's dissolved in SDS-borate (below) were minimally light scattering and for routine measurements did not require the scattered transmission accessory.

Fluorescence Spectroscopy

Various buffered detergent solutions were examined to design a cocktail that would satisfy the requirements imposed by studies of small planar monolayers. The cocktail selected for routine use was 1% (wt/vol) sodium dodecyl sulfate, SDS (Bio-Rad Laboratories), in 10 mM sodium tetraborate adjusted to pH 9.5 with 10 N NaOH. Samples were solubilized in 2.5 ml of this cocktail in 13 × 100-mm glass test tubes or glass scintillation vials and sonicated for 5 s.

Fluorescence measurements were made of samples in 13 × 100-mm glass test tubes using a home-built, right-angle spectrofluorometer. Design details of the fluorometer are available on request. Fluorometer performance was routinely calibrated either with fluorescent protein standards (FITC-Con-A of measured protein concentration) and/or with solid polymethylmethacrylate fluorescence intensity standards (Perkin-Elmer, Moutain View, Calif.). For routine assays sample excitation was fixed at 495 nm and emission at 525 nm. For most fluorescence measurements triplicate SDS-borate blanks with and without equivalent Hb additions were measured at the beginning and end of each group of readings of fluorescent unknowns to determine mean background values. Fluorescent signals were recorded for a minimum of 30 s and a single value derived for each trace. Only those traces that were parallel to the baseline were averaged and the value determined by visual integration. Usually samples were measured in triplicate and means and standard deviations calculated.

Scintillation Counting

After absorbance and fluorescence were measured, solubilized samples were returned to their scintillation vials, dried in a heating block at 75°C under filtered compressed air (45–60 min), cooled to room temperature, and suspended in 10 ml Aquasol (New England Nuclear, Boston, Mass.) with 30 s to 60 s sonication. Radioactivity was measured with an LS-7000 scintillation counter (Beckman Instruments Inc., Fullerton, Calif.) optimized for, and calibrated with, ^{125}I. Background counts from nonradioactive RBC/Aquasol were subtracted from matching ^{125}I-labeled RBC/Aquasol samples.

Transmembrane Concentration Analysis

The fraction of cytoplasmic membrane surface (f_{PS})[2] in a bilayer preparation was determined by hemoglobin absorbance and the fraction of extracellular membrane surface (f_{ES}) by FITC-Con-A fluorescence. Transbilayer distributions were expressed as two unknowns, n_E and n_P (n_E: number of moles of X in extracellular "half" membrane; and n_P: number of moles of X in cytoplasmic "half"), in two equations: (*a*) $N_T = n_E + n_P$ where N_T = number of moles in total, unsplit, or summed split preparations; and (*b*) $N_S = (n_E)(f_{ES}) + (n_P)(f_{PS})$ where N_S = number of moles in split preparations (PL-glass side).

[2] Although the cytoplasmic surface could logically be designated CS, I have used P, for "protoplasmic", to be consistent with accepted freeze-fracture nomenclature (10).

RESULTS

Preliminary tests of a variety of lectins and antibodies revealed that few were suitable for labeling RBC's for monolayer preparation. In general, large fluorescent molecules or complexes that labeled the cell surface brightly (as for fluorescence microscopy) also aggregated cells in suspension, making homogeneous monolayer formation impossible. Although Fab fragments and small lipid-soluble fluorophores did not aggregate cells, their size and unknown fracturing and partitioning properties precluded their use for these initial studies.

Properties of FITC-Con-A Conjugates

FITC-Con-A was selected as the most satisfactory of the labels tested for the RBC cell surface. Although phase contrast microscopy revealed that some aggregation of cells treated with FITC-Con-A on ice occurred during low speed pelleting, the pellets could be resuspended by vigorous vortexing. Absorbance spectra from 250 nm to 750 nm of both commercial and synthesized FITC-Con-A solutions after repetitive gel filtration through Sephadex G-25 revealed a constant 495 nm/280 nm ratio, indicating that they contained undetectable amounts of free FITC. Radioiodinated FITC-Con-A derivatives were similar, by spectroscopic and adsorption criteria, to their parent noniodinated molecules.

Erythrocyte Labeling

Adsorption of FITC-Con-A to RBC's under the nonoptimal labeling conditions of low temperature and physiological pH (14, 25, 29) was evaluated as a function of FITC-Con-A concentration, erythrocyte concentration, cation additions, and incubation time. As determined by the fluorescence to absorbance ratio (F/A), the level of RBC labeling increased with increasing FITC-Con-A concentration and with decreasing hematocrit. Addition of the divalent cations Ca^{++} and Mn^{++} at 1 mM also slightly increased the amount of FITC-Con-A adsorbed to the RBC's. Incubation at 25°C increased the level of cell labeling, but cells tended to lyse at the higher temperature. Although the period of labeling was routinely 30 to 60 min at 0–4°C, longer times, e.g. to 90 min, produced higher F/A ratios.

If FITC-Con-A-labeled RBC's were repeatedly washed after labeling, the F/A ratio continued to decline indicating that FITC-Con-A was being continually removed from the cell surface. Furthermore, regardless of how many times the cells were washed before monolayer formation, the F/A ratio of the monolayers was significantly lower than that of the corresponding cells in suspension, suggesting that surface label continued to be removed during monolayer formation.

A constant F/A ratio before and after monolayer formation was produced by agarose gel filtration of labeled cells (Fig. 2). Given the methods described in this report >90% of the cells were recovered intact in the void volume. Both hemoglobin absorbance at 403 nm and FITC-Con-A fluorescence at 525 nm peaked at fraction 18 (Fig. 2). A small but detectable amount of fluorescence was broadly spread between fractions 35 and 55. The F/A ratio was constant for the void volume

fractions 13 through 23 and began to increase at fraction 26 to a peak at fraction 44.

Planar Monolayer Formation

When column-purified, labeled cells were applied to PL-glass as a thick slurry (1.5 to 2 vol cells plus 1 vol HBS), their F/A ratios remained essentially unchanged from those of cells in suspension (Table I). The reproducibility of monolayer formation from a single dilution and within a single experiment (e.g., #2 when n=6) was good (SD 2.5%).

The attachment of FITC-Con-A–labeled cells to PL-glass was evaluated by quantitative light microscopy. Fig. 3 shows an area representative of those tabulated. Note that flattened cells predominate although there are a few cells attached on edge. The results of counts of cells applied at 3 slightly different dilutions are shown in Table II. Cells recycled twice were slightly more dilute than the initial nonrecycled cells and accordingly fewer cells bound.

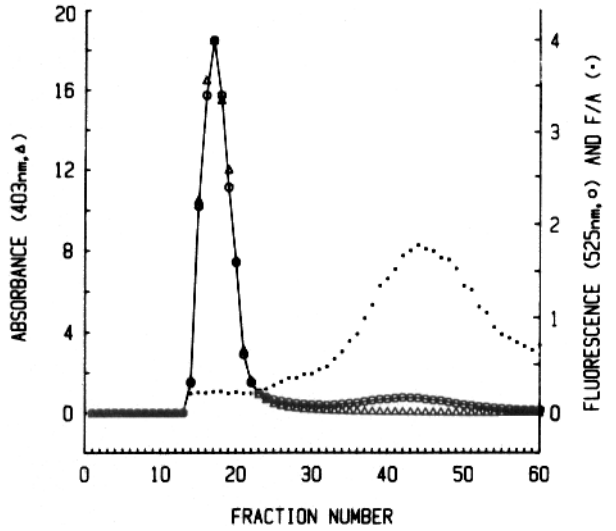

FIGURE 2 Agarose gel filtration of FITC-Con-A labeled erythrocytes. Washed erythrocytes (100 μl) incubated in 15 ml HBS containing 2.25 mg FITC-Con-A, for 60 min, 0–4°C. Cells pelleted, washed once with 15 ml HBS. Two pellets diluted with 4 vol buffer, applied to an agarose column kept at 4°C and previously washed with BSA and HBS. Cells were eluted with HBS at 0.6 ml/min. Fractions (500 μl) were collected and sampled into SDS-borate for absorbance (Δ) and corrected fluorescence relative intensity (O) measurement. The F/A ratio for each sample (*dots*) is also shown. (F/A), corrected fluorescence relative intensity at 525 nm divided by hemoglobin absorbance at 403 nm (equivalent to FITC-Con-A bound per cell).

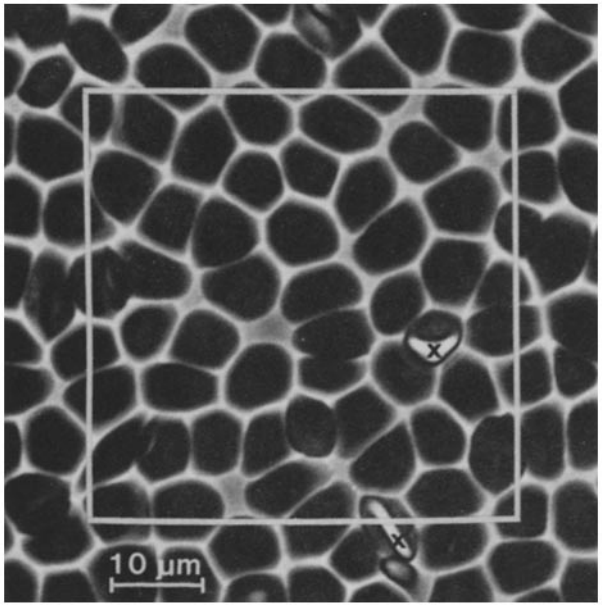

FIGURE 3 Phase contrast light micrograph of FITC-Con-A-treated erythrocytes attached to PL-glass. Washed cells (50 μl, 2 vol cells plus 1 vol HBS) were applied to dry PL-glass (11 mm × 22 mm) for 15 s, given 3 × 20-s washes in HBS and fixed in 1% glutaraldehyde (for LM only), all at 0–4°C. Outline indicates size of unit area randomly tabulated (2,500 μm^2). Cells scored as "on edge" are indicated by X. The micrograph is printed at actual size used for quantitation. ×1,160.

TABLE II

Density of FITC-Con-A-Labeled RBC's Adsorbed to PL-Glass

Sample	Cells/2500 μm^2	Orientation	
		Flattened	On edge
Nonrecycled (n = 72)	53 ± 3	51 ± 3	2 ± 2
Recycled once (n = 72)	50 ± 3	49 ± 3	1 ± 1
Recycled twice (n = 64)	48 ± 3	46 ± 3	2 ± 1
Overall mean = 50 ± 2.5 cells/2500 μm^2 (4.84×10^6 cells/242 mm^2 PL-glass)			

Recycled: number of times RBC's used after initial application; i.e., times that applied, but nonadsorbed, cells were pelleted from the first wash and re-applied. n = number of 50 μm × 50 μm areas counted; mean ± SD.

Electron microscopy of a freeze-fractured PL-glass monolayer of FITC-Con-A labeled RBC's revealed large areas containing split membrane profiles, exclusively E-faces (10), on the PL-glass surface (Fig. 4). The appearance of the E-faces in

TABLE I

Effect of Monolayer Preparation on F/A Ratio

Experiment	Cells in Suspension			Cells Attached to PL-Glass		
	A(403 nm)	F(525 nm)	F/A	A(403 nm)	F(525 nm)	F/A
1 (n = 3)	0.49 ± 0.01	0.19 ± 0.01	0.38 ± 0.02	0.36 ± 0.02	0.15 ± 0.01	0.42 ± 0.00
2 (n = 6)	0.40 ± 0.01	0.17 ± 0.00	0.43 ± 0.01	0.36 ± 0.01	0.17 ± 0.00	0.46 ± 0.01
3 (n = 2)	0.42 ± 0.04	0.12 ± 0.02	0.30 ± 0.00	0.35 ± 0.02	0.11 ± 0.00	0.31 ± 0.01
4 (n = 2)	0.40 ± 0.02	0.17 ± 0.01	0.42 ± 0.01	0.42 ± 0.01	0.19 ± 0.00	0.46 ± 0.00
5 (n = 2)	0.32 ± 0.00	0.10 ± 0.00	0.33 ± 0.01	0.37 ± 0.00	0.14 ± 0.00	0.36 ± 0.00
Mean F/A			0.37 ± 0.06			0.40 ± 0.07

FITC-Con-A labeled RBC's, purified on A-15m; 50 μl of 2:1::cells:HBS, pH 7.4, applied to 11 × 22 mm PL-glasses, given three 20-s washes in ~50 ml HBS on ice, excess buffer removed, dropped into 2.5 ml borate buffer, 10 mM, pH 9.5, containing 1% SDS (wt/vol).
n = number of glasses; mean ± SD.

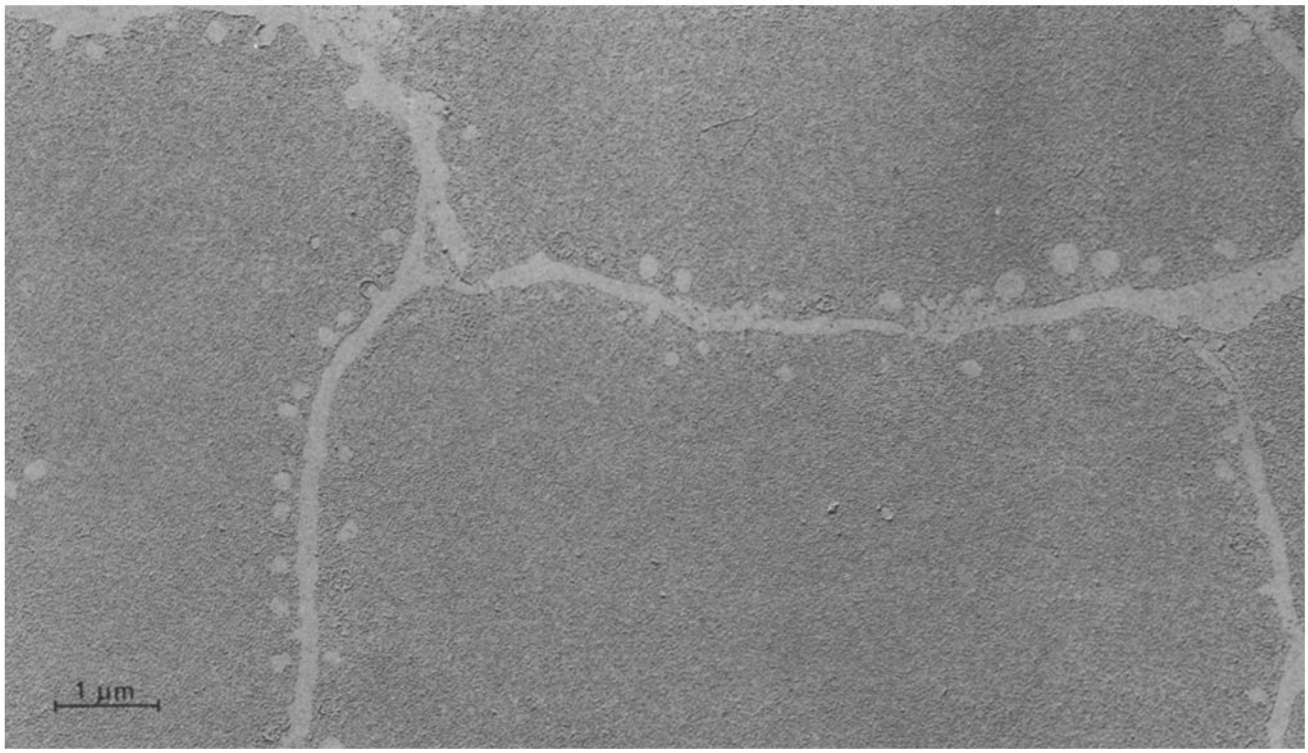

FIGURE 4 Electron micrograph of split erythrocyte E-faces on PL-glass. Cells were labeled with FITC-Con-A and column purified. PL-glass/glass sandwiches (11 mm × 22 mm) were frozen in Freon-22, fractured under liquid nitrogen, rapidly lyophilized, warmed to 35°C, and shadowed at room temperature with platinum-carbon at an angle of 20 degrees. Shadowing direction, bottom to top. ×13,250.

Fig. 4 differs from that of conventional freeze-fracture preparations. The surface is rougher with little definition between smooth areas and particles. This difference is due to preparative procedure (see Fig. 4, legend). An examination of samples that were deep-etched and shadowed at low temperature revealed "half" membranes similar in structure to those previously shown for unlabeled RBC's (12, 15, 16, 17).

Hemoglobin Assays

The absorbance spectra of briefly sonicated cells before and after being dissolved in SDS-borate are shown in Fig. 5. SDS caused a blue shift in the maximum Soret band from 415 nm to 403 nm and a concomitant decrease in absorbance to ~68% of the initial value. The solutions were optically homogeneous and minimally light-scattering.

Absorbance at 415 nm was linearly proportional to Hb concentration over the range derived from intact and split 11 × 22-mm monolayers, as was the absorbance at 403 nm after addition of 1% SDS and at pH 9.5.

FITC-Concanavalin-A Assays

Because the fluorescence intensity of FITC-modified proteins is known to be sensitive to hydrogen ion concentration (13, 28, 31, 39), the effect of pH on the relative intensity of FITC-Con-A fluorescence in 1% SDS was examined (Fig. 6). Fluorescence was about four times brighter at pH 9.5 than at pH 7.5. Borate buffer at pH 9.5 was chosen for routine fluorescent cocktail preparation because its pK is near the peak of fluorescence enhancement. Addition of 1% SDS to solutions of FITC-conjugates also significantly increased fluorescence relative intensity. Fluorescence of FITC-Con-A in SDS borate, pH 9.5, was proportional to its concentration over several

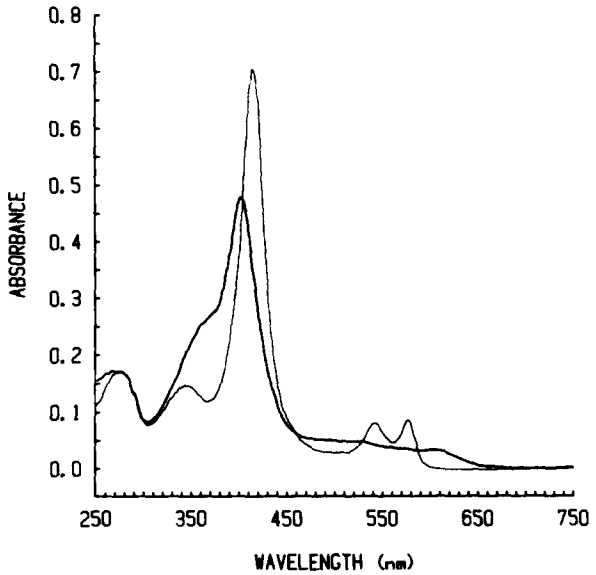

FIGURE 5 Absorbance spectra of erythrocytes: buffered detergent effect. Thin line, spectrum of lysed erythrocytes in 10 mM phosphate buffer, pH 7.4 (Soret peak at 415 nm). Thick line, spectrum of erythrocytes (at the same concentration) in 1% SDS-borate buffer, pH 9.5 (peak at 403 nm). Baselines (not shown) of appropriate buffer blanks lie within 5 milliOD of zero.

orders of magnitude (Fig. 7). The fluorometer could detect concentrations of fluorescein as low as 10 pM; below that level background scattering became limiting.

The effect of Hb concentration (monitored at 403 nm) on FITC-Con-A fluorescence relative intensity is shown in Fig. 8. Fluorescence was significantly enhanced by Hb and enhance-

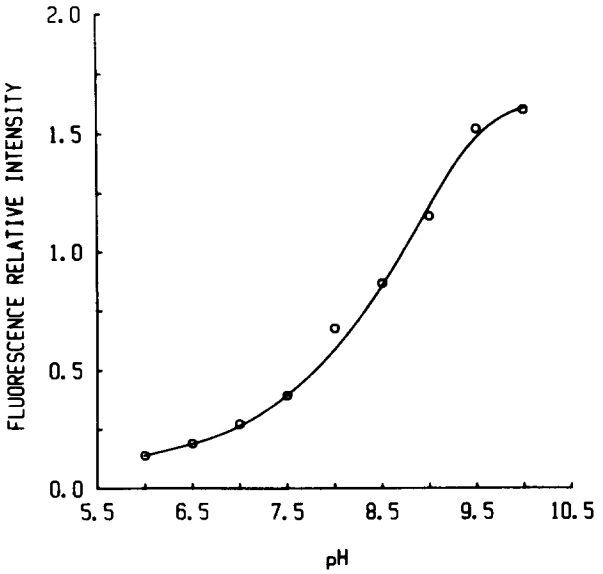

FIGURE 6 FITC-Con-A fluorescence: pH effect. 10 ng FITC-Con-A in 2.5 ml 10 mM buffers (phosphate pH 6 to 8, and borate pH 8 to 10) containing 1% SDS. Fluorescence at 525 nm corrected for background. Each point encompasses the mean ± SD of three or more samples.

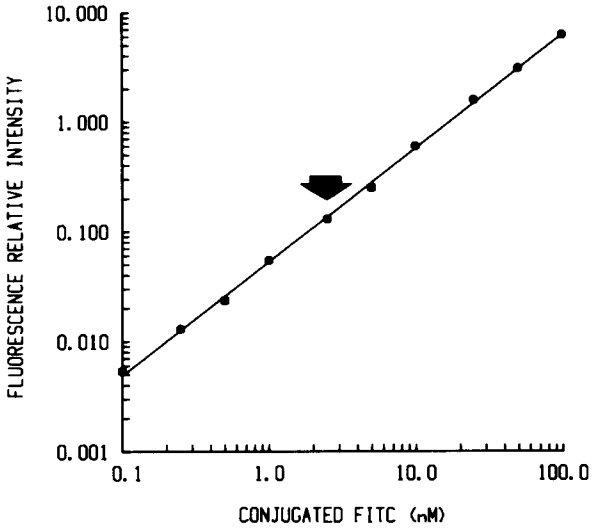

FIGURE 7 FITC-Con-A fluorescence: concentration dependence and fluorometer performance. Conjugated FITC concentration determined by its absorbance at 495 nm. Arrow indicates the representative level of fluorescence of a single 11 mm × 22 mm monolayer of FITC-Con-A-labeled, column-purified erythrocytes.

ment increased with decreasing Hb absorbance between 0.50 and 0.01 OD. Between 0.01 and 0, OD fluorescence enhancement decreased, and above 0.5, OD fluorescence was increasingly quenched. Although free FITC showed no Hb enhancement, the fluorescence of other FITC-protein conjugates (IgG, avidin) was also enhanced by the addition of Hb or of other colored (cytochrome-c, bacteriorhodopsin) and noncolored (BSA, ovalbumin), soluble and membrane associated proteins. The curve shown in Fig. 8 (y intercept: 31.8; slope: −62.6) was routinely used to correct fluorescence relative intensity for Hb enhancement. For each absorbance value fluorescence was calculated as follows: corrected F_{525} = $[(F_{525,obs})(100)]/(100 +$ % enhancement), where $F_{525,obs}$ = observed F_{525} minus background.

The stability of SDS borate cocktails was evaluated. Samples stored in the dark at room temperature showed only slight changes (±1%) in both fluorescence and absorbance during the first 4 d. Samples were routinely stored in the dark and measured within 4 d, usually within 2 h.

RBC Monolayer Splitting

A synopsis of several monolayer splitting experiments is given in Table III. In each experiment absorbance and corrected fluorescence values for unsplit controls and split monolayers were determined. Unsplit monolayer absorbance ranged from 0.30 to 0.42 OD units with a mean value of 0.36. The mean sum of split absorbances, 0.35, was 96% of that of the unsplit controls. Similarly the sum of the corrected fluorescence values, 0.137, was 94% of the intact values (0.146). The F/A ratios for unsplit (0.40) and split samples (0.39) were essentially identical. The bulk of the absorbance (79%) in split preparations partitioned with the glass side, and the complementary fraction (21%) with the PL-glass side. Concomitantly a greater fraction of fluorescence, 39%, was measured in the PL-glass portion consistent with an enrichment of extracellular membrane surface (or "half") relative to cytoplasmic surface. PL-glass alone when added to SDS-borate solutions containing FITC-Con-A had no effect on fluorescence relative intensity.

Absorbance, fluorescence, and radioactivity data for unsplit and split monolayers of RBC's labeled with ^{125}I-FITC-Con-A are given in Table IV. The sums of the split absorbance and fluorescence values for these experiments were within a few percent of the unsplit control values. As was found for nonisotopically labeled preparations (Table III), the percentage of radioactive FITC-Con-A partitioning with the PL-glass surface (44% and 52% in experiments no. 1 and 2 respectively) was greater than the percentage of absorbance (33% and 38%). The

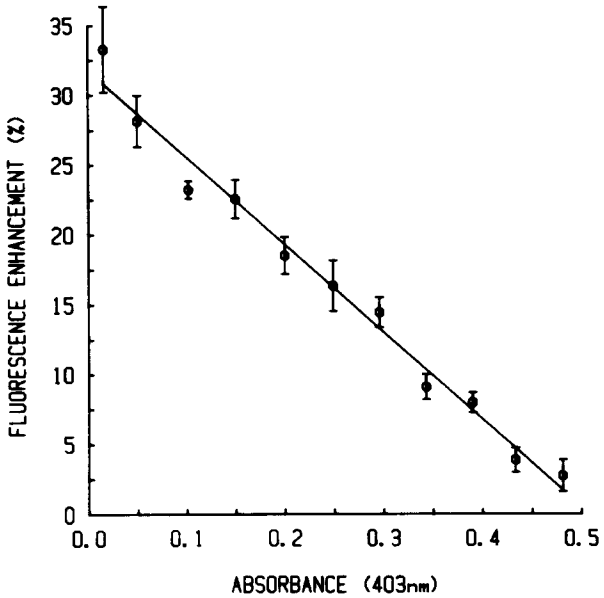

FIGURE 8 FITC-Con-A fluorescence: hemoglobin effect. Dilution series of erythrocytes prepared in SDS-borate containing FITC-Con-A at 0, 10, and 100 ng/ml. Samples (2.5 ml) were sonicated, stored in the dark for 3 h, and A_{403} and F_{525} measured at 20°C. Fluorescence enhancement (%) = $[(F_{cells} - F_{blank})/F_{blank}](100\%)$ where the blank is an identical concentration of FITC-Con-A without Hb. Points and error bars represent the means (n = 6) ± SD of pooled data of triplicate low and high fluorescence samples. Straight line plot derived from a least squares linear regression computation.

TABLE III

Absorbance and Fluorescence Data for Intact and Split Monolayers

Experiment	Unsplit Monolayer		Split Monolayer			
			A(403 nm)		F(525 nm)	
	A(403 nm)	F(525 nm)	PL-Glass	Glass	PL-Glass	Glass
Nonrecycled						
1 (n = 3)	0.360 ± 0.015	0.153 ± 0.006	0.055 ± 0.021	0.296 ± 0.030	0.048 ± 0.014	0.093 ± 0.016
2 (n = 3)	0.363 ± 0.013	0.166 ± 0.004	0.050 ± 0.005	0.330 ± 0.021	0.065 ± 0.010	0.107 ± 0.009
3 (n = 2)	0.348 ± 0.025	0.106 ± 0.005	0.100 ± 0.064	0.190 ± 0.000	0.044 ± 0.011	0.054 ± 0.006
4 (n = 3)	0.420 ± 0.010	0.194 ± 0.005	0.066 ± 0.015	0.326 ± 0.016	0.060 ± 0.013	0.106 ± 0.010
Recycled						
1 (n = 1)	0.395	0.152	0.020	0.350	0.028	0.109
2 (n = 3)	0.295 ± 0.000	0.131 ± 0.002	0.053 ± 0.016	0.235 ± 0.009	0.044 ± 0.009	0.077 ± 0.002
3 (n = 2)	0.330 ± 0.000	0.096 ± 0.000	0.095 ± 0.028	0.238 ± 0.011	0.048 ± 0.005	0.050 ± 0.007
4 (n = 3)	0.403 ± 0.006	0.166 ± 0.004	0.157 ± 0.042	0.247 ± 0.038	0.088 ± 0.016	0.073 ± 0.010
Overall mean	0.364 ± 0.041	0.146 ± 0.033	0.074 ± 0.042 (21%)	0.276 ± 0.057 (79%)	0.053 ± 0.018 (39%)	0.084 ± 0.024 (61%)
Σ F/n		0.146			0.137 (94%)	
Σ A/n	0.364		0.350 (96%)			
F/A or Σ F/Σ A	0.40			0.39		

Cell monolayers frozen in Freon-22; split or unsplit dropped into 2.5 ml SDS-borate. Unsplit monolayer data (left two columns) are controls for split pair data (right four columns); mean ± S.D.

Fluorescence relative intensity values corrected individually for background and enhancement. Nonrecycled: RBCs applied immediately after column purification; Recycled: RBCs not bound during first application, pelleted, re-applied to PL-glass.

n = number of glasses per experiment.

TABLE IV

Transmembrane Distribution of ^{125}I-FITC-Con A

Experiment	Experimental data				
	A(403 nm)	f_{PS}	F(525 nm)	f_{ES}	CPM (N)
1					
Unsplit (n = 4)	0.326 ± 0.010		0.028 ± 0.003		11,044 ± 246
Split PL-glass (n = 2)	0.108 ± 0.039	0.329	0.011 ± 0.000	0.440	4,833 ± 277
Split glass (n = 2)	0.220 ± 0.021	0.671	0.014 ± 0.001	0.560	6,313 ± 89
2					
Unsplit (n = 4)	0.236 ± 0.006		0.028 ± 0.003		10,825 ± 547
Split PL-glass (n = 2)	0.092 ± 0.032	0.383	0.014 ± 0.002	0.518	5,495 ± 795
Split glass (n = 2)	0.148 ± 0.039	0.617	0.013 ± 0.004	0.482	5,090 ± 1,603

Labeling conditions: 1 ml HBS, pH 7.4, contained 100 μl washed RBC pellet, 16 μg [^{125}I]FITC-Con A, 1 mM Ca^{++}, and 1 mM Mg^{++}. Cells were incubated for 90 min, on ice, diluted, centrifuged, column purified on BSA-treated A-15m, and applied to PL-glass.

n = number of glasses per experiment.

Data expressed as mean ± SD.

ratios of PL-glass/glass cpm's in both experiments are similar to the fluorescence ratios as would be expected if both fluorophore and isotope were partitioning in the same fashion after freeze-fracturing. Values for the fractions of cytoplasmic surface (f_{PS}), extracellular surface (f_{ES}), and number of moles of radioisotope (N), expressed as cpm, are included in Table IV.

Table V illustrates the application of equations derived for the transmembrane analysis of radioisotopes using data from Table IV. In a given experiment four values are measured: the number of moles of isotope in total, unsplit preparations, N_T; the number of moles in the split PL-glass portion, N_S; the fraction of cytoplasmic surface, or Hb, in the PL-glass portion, f_{PS}; and the fraction of extracellular surface, or FITC-Con-A, in the PL-glass portion, f_{ES}. Similar equations can be derived for the complementary split glass portion. The calculated percent transmembrane distribution of radioiodinated FITC-Con-A for each of the two experiments is shown at the bottom of Table V.

DISCUSSION

This report describes rapid assays for quantifying the fractions of "half" membranes in split membrane preparations. Hemoglobin is used as a cytoplasmic surface marker and FITC-Con-A as an extracellular surface marker, with absorbance at 403 nm being proportional to the fraction of cytoplasmic "half" and fluorescence at 525 nm proportional to the extracellular "half."

Hemoglobin was selected as a marker of the cytoplasmic "half" for several reasons. In addition to its exclusive cytoplasmic location and high molar extinction coefficient (3) electron microscope studies (15) have shown that Hb partitioning with the PL-glass after splitting is almost exclusively associated with intact cells. Electron microscopy of split FITC-Con-A-labeled RBC monolayers confirmed this observation. Furthermore, where cells were cross-fractured or partially fractured, the residual mass of cytoplasm (Hb) was qualitatively proportional

TABLE V

Transmembrane Distribution of ^{125}I-FITC-Con A

Data for calculations		
	Experiment 1	Experiment 2
Total cpm (sum of splits), N_T	11,146	10,585
PL-glass cpm, N_S	4,833	5,495
Fraction Hb PL-glass, f_{PS}	0.33	0.38
Fraction FITC PL-glass, f_{ES}	0.44	0.52

Equations for Calculations

$$n_E = [N_S - (N_T)(f_{PS})]/(f_{ES} - f_{PS})$$
$$n_P = N_T - n_E$$

Transmembrane distribution

Experiment 1	Experiment 2
n_E = 10,498 cpm (94%)	n_E = 10,519 cpm (99%)
n_P = 648 cpm (6%)	n_P = 66 cpm (1%)

Data taken from Table IV. n_E: number of moles of unknown (expressed here in cpm) in extracellular split membrane portion; n_P, in cytoplasmic portion. N_T: number of moles in unsplit, total membrane portion; N_S, in split PL-glass portion. f_{PS}: fraction of cytoplasmic surface, and f_{ES}: fraction of extracellular surface in PL-glass portion.

to the fraction of cytoplasmic surface.

Hemoglobin absorbance was also used to calculate the number of cells attached to PL-glass and to verify that the bound cells had properties similar to unattached cells. Given the extinction coefficient of Hb, the blue shift and absorbance reduction by SDS-borate, and the concentration of Hb per cell, one can calculate that 0.36 OD is equivalent to 4.8×10^6 cells per 11 × 22-mm glass, close to the value derived directly by light microscopy. Moreover, absorbance per cell was identical for cells in suspension or attached to PL-glass. Hb absorbance also provided a quantitative index of the reproducibility of monolayer formation. The mean absorbance value of unsplit monolayers of 8 preparations using different batches of blood, dilutions, polylysine, cover glasses, etc. (Table III) was 0.364 ± 0.041 (mean ± SD). These numerical data stand in sharp contrast to qualitative observations (44).

The extracellular surface marker for labeling cells for monolayer freeze-fracturing should meet several requirements. It should be nonaggregating so that cells may be labeled before monolayer formation, nonpenetrating both before and after fracturing, attached so as to be retained during cell monolayer formation, randomly distributed over the surface of the cell, and sensitive enough to produce data with a good signal-to-noise ratio. Under certain conditions FITC-Con-A appears to satisfy these criteria.

Con A has the further advantage that its properties (2, 8, 11, 23, 26) and interaction with the human RBC surface (24, 25, 41) have been well studied. Although the labeling conditions used in the present study, especially those of temperature and pH, are known to provide less than optimal labeling (14, 24, 29, 32, 37), the level was still high enough for convincing fluorescence data. By keeping the temperature low and pH physiological, cells could be labeled while minimizing the possibility of aggregation, lysis, endocytosis, or enzymatic modification of the label, conditions that would alter the assumed exclusive extracellular location of the fluorophore.

The present report describes methods designed to label the cell surface so that the label remains random even after monolayer formation. This was not a trivial consideration because the method of transmembrane analysis described here assumes that the extracellular label is randomly and homogeneously distributed over the entire surface of the cell. Gel filtration was effective in preparing labeled intact RBC's whose F/A ratios remained constant before and after cell monolayer formation.

Sensitivity appropriate to the quantitative analysis of 4.8×10^6 RBC's was provided by using fluorescent and radioisotopic derivatives of the lectin. To maximize fluorescence and simultaneously detect low signal levels, two approaches were taken: conditions known to influence fluorescence intensity were examined, and a fluorometer capable of detecting low levels of fluorescence was designed and built. For planar cell monolayer studies, the ideal fluorescent cocktail should satisfy several requirements. It must solubilize all cellular components attached to the cationic PL-glass. It must have good optical properties and be compatible with hemoglobin absorbance measurements. It should optimally enhance fluorescence while retaining quantitative linearity over a wide range of concentrations. It should be convenient to prepare and have good long-term stability. A 1% SDS solution buffered to pH 9.5 with borate appeared to satisfy these requirements. The anionic detergent readily solubilized electrostatically-attached membrane components producing an optically clear, nonscattering sample suitable for spectroscopy (27, 36, 40).

The enhancement by soluble proteins of fluorescence of FITC-protein conjugates dissolved in SDS-borate was unexpected. Fortunately enhancement was linearly proportional to 403 nm absorbance between 0.01 and 0.50 OD units and thus any fluorescence intensity value could be corrected for the presence of Hb. The necessity for such corrections was a nuisance, however, and other fluorophore-Con-A conjugates are being investigated.

For split membrane preparations, the sum of the moles of unknown in the splits should equal the number of moles in the intact control. For proteins, this quantitative criterion could not be met by the copper/glass sandwiches previously used to study cholesterol (16) and RBC polypeptides (12). However, use of the PL-glass/glass sandwich allowed for >95% recovery of both Hb and FITC-Con-A from splits. Spectroscopic data, specifically the F/A ratios, clearly indicated an enrichment of extracellular "half" membrane in the PL-glass fraction and a complementary depletion in the glass fraction. For example, the F/A ratio (Table III) for the PL-glass fraction is 0.716 (i.e., 0.053/0.074) and for the glass fraction 0.304 (0.084/0.276). The PL-glass ratio is much higher and the glass ratio much lower than the intact ratio of 0.390.

The ^{125}I-FITC-Con-A experiments illustrate how the double labeled membrane splitting technique can be used to examine the transmembrane distribution of a radioisotopic probe. However, the data could not be used to determine the sidedness of FITC-Con-A because both isotope and fluorophore were covalently attached to the same molecule. Nevertheless, because the fractions of radioactivity and fluorescence were nearly identical in the split preparations, monolayer freeze-fracture autoradiography could be used to determine sidedness independently. Although results of that autoradiographic study will be reported separately,[1] an observation relevant to this report was that essentially all of the radioactivity, and thus all of the FITC-Con-A, was associated with the E-face. In other words, no portion of the FITC-Con-A molecule partitioned to the cytoplasmic side during freeze-fracturing.

In summary, labeling RBC's with FITC-Con-A followed by gel filtration, monolayer splitting, and spectroscopic assays provided a method for determining quantities of split membranes derived from small planar surfaces. Analysis of the transmembrane concentrations of a native or probe molecule was simplified relative to earlier analyses (16) by quantifying

the fractions of cytoplasmic and extracellular "halves" directly. This analysis does not require knowledge of the mode of cell fracturing; i.e., it is unnecessary to determine the number of crossfractured, partially fractured, and intact cells in each split portion. Nor is it necessary to measure the total surface area of the cell and determine the split fraction of that surface as previously required by TEM methods (16).

This double labeled membrane splitting method is currently being applied to an examination of the transmembrane distribution of tritiated cholesterol and the fracturing properties of RBC polypeptides. Although the RBC has many advantages for plasma membrane studies, and especially for development of new techniques, the principles of monolayer splitting and spectroscopic assays should be applicable to other cells as well.

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