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5-[^{125}I]Iodonaphthyl Azide, a Reagent to Determine the Penetration of Proteins into the Lipid Bilayer of Biological Membranes†

T. Bercovici and C. Gitler*

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ABSTRACT: 5-[^{125}I]Iodonaphthyl 1-azide is shown to be a useful reagent for the determination of the extent of penetration of proteins into the lipid bilayer of biological membranes. The label can readily be made highly radioactive and stored for reasonable times or repurified and then used. In the dark it has a high partition coefficient into membrane lipids. It has a high extinction coefficient for the light-mediated conversion of the azide into the reactive nitrene. It can be activated by short periods of light at wavelengths which membrane proteins and lipids do not absorb so that their radiation damage is minimal. The light-generated nitrene inserts covalently with very high efficiencies into the membrane components. With different membrane preparations, 20 to 55% of the added label inserts into the membrane proteins and lipids. It appears to insert from within the lipid bilayer mainly into intrinsic membrane proteins; very little, if any insertion occurs into extrinsic proteins. In rabbit skeletal muscle sarcoplasmic reticulum, the main insertion of the nitrene occurs into the Ca^{2+}-sensitive ATPase. The ATPase activity is not affected by the labeling procedure. Mild tryptic cleavage of the 100 000 molecular weight Ca^{2+}-ATPase results in the formation of two fragments of molecular weight 52 000 and 46 000. The two fragments are equally labeled, suggesting that the protein is in contact with the bilayer by at least two segments of its polypeptide chain. In intact erythrocytes and hemoglobin-free erythrocyte membranes, little, if any, label is inserted into bands 1, 2, or 5 (spectrin and erythrocyte actin, respectively). Label occurs in the region of bands 3, periodic acid-Schiff 1, 2, and 3 and in band 7.

Previous communications have described a new method for labeling those portions of membrane proteins that are in contact with the lipid bilayer (Klip & Gitler, 1974, 1976; Klip et al., 1976). The method was based on the following properties of the labeling agents as studied with rabbit skeletal muscle sarcoplasmic reticulum (SR).[1] Firstly, when the apolar radioactive azides [^{3}H]-1-azidonaphthalene and 4-[^{125}I]-iodobenzene 1-azide were added to the membrane suspension in the dark, they partitioned rapidly and nearly quantitatively into the liquid hydrocarbon regions of the membrane lipids. Furthermore, the azides effectively quenched the fluorescence of perylene present in liposomes and in the SR membranes. Since the quenching was due to molecular encounter, a significant portion of the azides must dissolve in sites equivalent to those of the perylene (Klip & Gitler, 1974). Secondly, exposure to light after the partition step converted the aromatic azides into reactive nitrenes which were capable of covalent incorporation into both the membrane proteins and the membrane lipids.

That the labeling occurred within the bilayer was suggested by the majority of the evidence. Thus, the nitrene products were incorporated to an extent greater than 88% into the fatty-acyl chains of the SR membrane phospholipids. Furthermore, Pronase digestion of the SR resulted in a significant release of peptides from the membrane proteins without appreciable release of radioactivity (Klip & Gitler, 1974; Klip, 1974). Even though the incorporation of the label was low, only the "integral" and not the "peripheral" proteins appeared to be labeled.

The aromatic azides used in these studies had several limitations. Relatively high concentrations of the labels had to be used because of their low specific radioactivities. Furthermore, the iodo derivative had an absorption maximum (258 nm) which overlaps with that of the membrane components. It therefore had to be excited in the edge of its absorption band (>300 nm) where the efficiency of nitrene formation is low. Initial studies with [^{3}H]-1-azidonaphthalene with a specific radioactivity of 3 Ci/mmol showed that radiation damage of the compound was extensive during storage. Evidence also was obtained that on irradiation it formed artefactual polymeric products.

The above limitations have been overcome by the use of the label 5-[^{125}I]iodonaphthyl 1-azide (INA). The radioisotope can be incorporated in the final step of the synthesis and the azide can be stored for reasonable periods of time without degradation. Its absorption maximum is at 310 nm with an extinction coefficient of 21 400 M^{-1} cm^{-1}. No polymeric products have been detected upon irradiation and it is covalently inserted into the membrane components to the extent of 20 to more than 50%. It does not appear to label "peripheral" proteins significantly and may therefore be a useful reagent

† From the Department of Membrane Research, Weizmann Institute of Science, Rehovot, Israel. *Received July 18, 1977.* Supported in part by a grant from the Israel National Academy of Sciences.

[1] Abbreviations used: INA, 5-iodonaphthyl 1-azide; [^{125}I]INA, 5-[^{125}I]iodonaphthyl 1-azide; PAS, periodic acid-Schiff; $NaDodSO_4$ gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SR, rabbit skeletal muscle sarcoplasmic reticulum.

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to define these proteins and their polypeptide regions in contact with the membrane lipids.

Experimental Section

Materials. Bovine serum albumin was A grade from California Biochemical Corp.; sodium azide and isoamyl nitrite were from British Drug Houses. Acetylated trypsin was from Sigma Chemical Corp.

Synthesis of 5-Iodonaphthyl 1-Azide. All operations where an azide was formed were performed in a room with light passed through a red filter. Three different routes were used to synthesize INA. The first method was used for comparison of the final product with the other two methods which could be scaled down to micromethods and used to synthesize the highly radioactive label.

(1) From 5-Iodonaphthyl-1-amine (Scholl et al., 1921). To the amine dissolved in 50% sulfuric acid and cooled to 4 °C was added 1 equiv of sodium nitrite. After 30 min at 4 °C, 1.7 equiv of sodium azide water solution was added in small portions. Immediate evolution of nitrogen was observed. The solution was kept at 4 °C for 2 h and then at room temperature overnight. The precipitated azide was filtered, washed extensively, and dried. It was then dissolved in *n*-hexane and passed through a column of silica gel (Merk). The material eluted with an R_f of 0.57 with hexane. By evaporation in vacuo, pale yellow needle crystals were obtained, mp 88 °C.

(2) From 1,5-Diaminonaphthalene (Eastman Kodak). One equivalent of the diamine was diazotized with 2 equiv of sodium nitrite in 50% aqueous sulfuric acid at 4 °C. After solubilization (30 min), 1 equiv of sodium azide in water was added in small portions. The reaction mixture was kept at 4 °C for 24 h. One equivalent of sodium iodide was then added and the solution kept at 4 °C for 4 h and at room temperature for 20 h. The precipitate was obtained by filtration or by centrifugation depending on the quantities involved. After washing with water and drying, it was dissolved in *n*-hexane and the solution was passed through a silica gel column as detailed above. Pale yellow crystals were obtained, mp 88 °C. This procedure could be scaled down to obtain high specific radioactivity INA.

(3) From 5-Aminonaphthyl Azide. 5-Aminonaphthyl 1-azide was obtained by reduction of 5-nitronaphthyl 1-azide (prepared as above from 5-nitronaphthyl-1-amine, Eastman Kodak; Foster & Fierz, 1907), with sodium dithionite in ethanol as described for halogenonitronaphthalenes (Hodgson & Ward, 1947), mp 101–103 °C. The micromethod used routinely to prepare high specific radioactivity [^{125}I]INA is detailed.

One milligram of 5-aminonaphthyl 1-azide was dispersed in 200 μL of a solution of 11 μL of concentrated H_2SO_4 in 1 mL of glacial acetic acid. To this mixture was added 20 μL of a solution of 70 μL of isoamyl nitrite in 1 mL of glacial acetic acid. After 10 min the solution became clear, then 1 mL of diethyl ether was added. The precipitated diazonium salt was obtained by centrifugation for 5 min in a clinical centrifuge and washed twice more with 0.5 mL of diethyl ether. The sediment was dissolved in 100 μL of 10% aqueous sulfuric acid. Any undissolved material was removed and the solution was then ready for the step in which the ^{125}I-labeled iodide was added. This stock solution may be stored for at least 3 weeks at −20 °C without deterioration. When the [^{125}I]INA was required, 10 μL of this solution was combined at 4 °C with 10 μL of a NaI solution (9 mg/mL water) containing 1 to 5 mCi of carrier-free [^{125}I]NaI. The reaction mixture was allowed to stand at 4 °C for 2 h and at room temperature for another 2 h. It was then extracted three times with 100 μL of *n*-hexane. The combined extracts were applied to a silica gel column 0.2 × 1.0 cm and eluted with *n*-hexane (R_f 0.57). The hexane was evaporated under vacuum with an adequate trap to collect the radioactive products which may coevaporate. The dry INA was then dissolved in ethanol. It was stored at −20 °C. It could be used without marked deterioration during the following 4 days. After longer periods of storage, it was first repurified by passage through the silica gel column, repeating the last step of the synthesis.

Mixed crystals of the INA prepared by the three methods gave the same melting point, 88 °C. A single radioactive spot with an R_f 0.57 was obtained in thin-layer chromatography using silica gel (Kiesselgel mit fluoreszentindicator, Riedel-Dehaem A.G.) with *n*-hexane as solvent. On exposure to light the spot darkened immediately.

The IR spectra show a peak at 4.76 μm characteristic of azides. Mass spectra of the INA at room temperature of 70 eV gave the following peaks of molecular weight and intensities, respectively: 295, 8.9; 267, 35.5; 217, 13.3; 140, 100; 104, 44.5, consistent with the expected structure.

Membrane Preparations. Rabbit skeletal muscle sarcoplasmic reticulum was obtained from albino rabbits by the procedure of MacLennan (1970). Human erythrocytes were obtained from normal donors with heparin as anticoagulant. They were washed with phosphate-buffered saline, care being taken to remove the buffy coat. Hemoglobin-free erythrocyte membranes were prepared by the method of Dodge et al. (1963) at pH 7.6.

INA Labeling Procedure. The membranes to be labeled were suspended at a concentration of 1 to 2 mg of membrane protein/mL of the desired buffer in a round glass cuvette. Usually 2 mL of solution was irradiated at a time. The cuvette was placed in a glass holder through which water could be circulated to keep the membrane suspension at 37 °C. The membrane suspension was stirred with a magnetic stirrer during the period of irradiation. Light from a Wild microscope UV source containing a 200-W mercury arc lamp (Osram HBO) was focused to 1 cm^2 in the center of the cuvette. Corning 7-60 and 0-54 filters were used to remove light below 305 and above 400 nm. The INA in alcohol was added to the membrane suspension (1% final alcohol concentration, 1 to 10 × 10^6 count/min per mL of membrane suspension) under red filtered light, the suspension was stirred for 5 min and then it was exposed to the UV light for the desired time.

Removal of Noncovalently Attached [^{125}I]INA Products. After irradiation, the membranes were sedimented by centrifugation, the supernatant fluid was discarded, and the membranes were resuspended in buffer and treated in any of the three following procedures: (a) bovine serum albumin (Sigma grade) was added to a final concentration of 12.5 mg/mL; the membrane suspension was kept at 4 °C for 15 min and centrifuged, the supernatant was discarded and the membranes were resuspended in buffer containing albumin two more times. The remaining albumin was removed by washing with buffer twice. (b) Nine volumes of acetone at −20 °C was added to the membrane suspension; after 15 min at −20 °C, the precipitated material was sedimented for 10 min at 2000*g*. The sediment was washed two more times with an acetone/water solution (9:1) at −20 °C and once with pure acetone, dried, and then resuspended in a $NaDodSO_4$–mercaptoethanol solution for electrophoresis. (c) The membrane suspension was dialyzed against a bovine serum albumin–buffer solution (1.25 mg of bovine serum albumin/mL) overnight. Several changes in the external solution were effected.

$NaDodSO_4$ Gel Electrophoresis. The procedures of Fair-

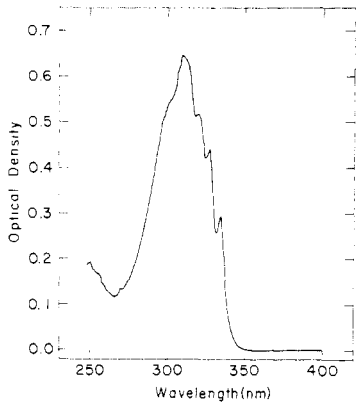

FIGURE 1: Absorption spectrum of 5-iodonaphthyl 1-azide in *n*-hexane (3×10^{-5} M).

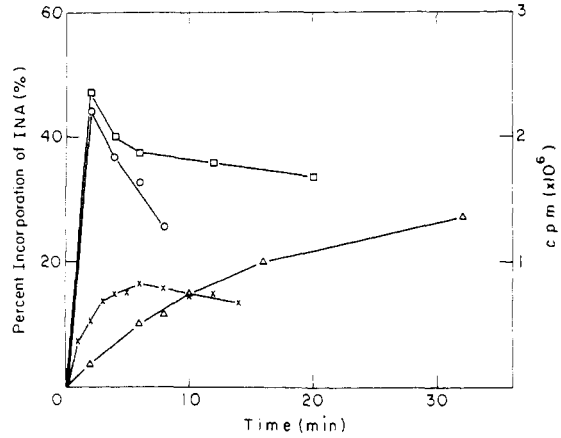

FIGURE 2: Covalent incorporation of [5-^{125}I]iodonaphthyl-1-nitrene as a function of time of exposure to light (314 nm). Each curve represents the incorporation of INA (ca. 10^{-6} M) in 2 mL of phosphate-buffered saline containing: (—○—) 8 mg bovine serum albumin; (—□—) SR membranes, 8 mg of protein; (—X—) hemoglobin-free erythrocyte membranes, 3.5 mg of protein; (—△—) 10% erythrocyte suspension. At each point 100 μL of the suspension was added to 0.9 mL of acetone at −20 °C. The precipitate was washed twice with acetone–10% water and once with acetone and dried and ^{125}I content determined. For details, see Experimental Section.

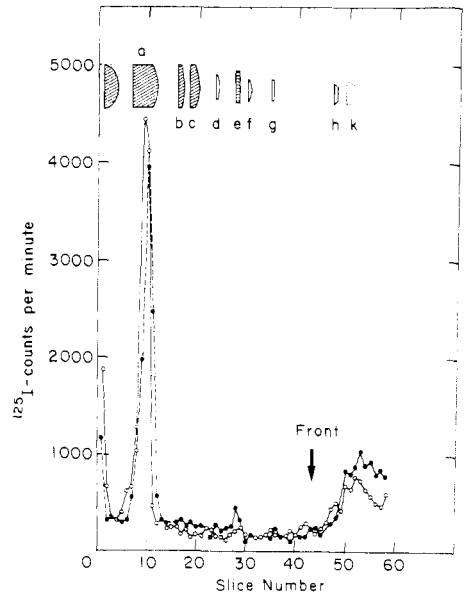

FIGURE 3: Distribution of the INA-insertion products in the SR membrane components as a function of the method used to remove the noncovalently attached INA-irradiation products. SR membranes (8 mg of protein in 2 mL) were labeled with [^{125}I]INA by exposing to light for 2 min. A 1-mL fraction was treated with bovine serum albumin (—●—) and the other with acetone (—○—) by the procedures detailed in the Experimental Section. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis tube gels were then run, fixed, and stained (Laemmli & Favre, 1973), sliced into 1.5-mm slices, and counted. The Coomassie pattern shows the position of the Ca^{2+}-ATPase (a), high affinity Ca^{2+}-binding protein (b), calcequestrin (c), and acidic proteins (d–g). The proteolipid (h) and lipids (k) appear after the tracking dye. Some unidentified material is present on the top of the gel.

banks et al. (1971) and of Laemmli & Favre (1973) were used for erythrocyte membranes and SR, respectively.

Results

Characteristics of the INA. Essentially equal results in the labelling characteristics could be obtained, as expected, with highly radioactive [^{125}I]INA synthesized by the two micromethods detailed above. The last procedure was chosen due to the shorter time required.

The absorption spectrum of INA is shown in Figure 1. The maximum occurs at 310 nm so that the 314-nm line of a mercury lamp can effectively be used for irradiation. The high molar extinction (ϵ 21 400 M^{-1} cm^{-1}) allows the generation of nitrenes with minimal times of exposure to relatively low light fluxes. INA has a very low solubility in water and partitions even at low concentrations into the membranes to an extent greater than 98%.

Time Course in the Incorporation of INA into Bovine Serum Albumin and the Membrane Preparations. The time of the light exposure to obtain maximum conversion of INA into the nitrene depends on the turbidity and light absorption of the system under study. As shown in Figure 2 in the clear bovine serum albumin solutions, the conversion was complete in some 2 min, while, with intact erythrocytes, periods up to 30 min were required. No hemolysis was observed during irradiation. In the relatively transparent solutions, the degree of covalent incorporation of the INA decreased after an initial maximum was obtained. Presumably the insertion products are themselves photolabile.

The results shown in Figure 2 were obtained by the acetone extraction procedure detailed in the Experimental Section. As shown below, some lipids are extracted by this procedure. Therefore, the values reported in Figure 2 are slightly lower than the actual total incorporated into the membrane elements. It can be observed that surprisingly high efficiencies of labeling were obtained.

Removal of the Nitrene Products Not Covalently Attached. It is unlikely that all the nitrene products not covalently attached to the membrane elements will be readily soluble in water. Therefore, the procedure designed to remove them from the membranes (see Experimental Section) included bovine serum albumin or acetone to effect their quantitative removal. In a typical experiment with SR labeled with INA, 27.2% of the added counts were found in the supernatant after irradiation, another 14% were removed in two subsequent bovine serum albumin washes. Additional washes removed less than 1% of the radioactivity. Comparisons of the electrophoretic patterns obtained after such washings and after acetone precipitation are shown in Figure 3. The patterns are essentially similar. They are only different in the fraction of the label associated with the lipids. This difference is probably due to the fact that the acetone removes some of the labeled phospholipids. The total amounts incorporated into the membranes were 55 and 49% of the total added, after bovine serum albu-

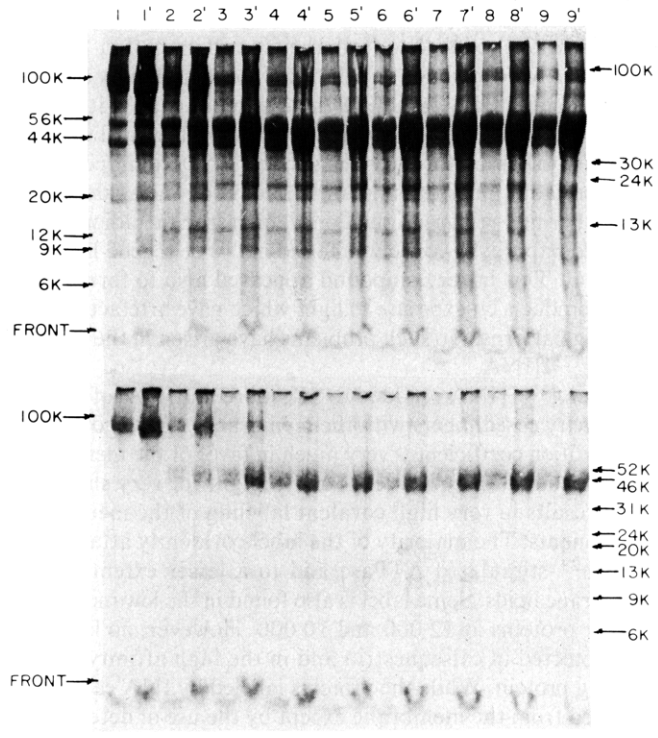

FIGURE 4: Distribution of polypeptides and of radioactivity of [^{125}I]-INA-labeled sarcoplasmic reticulum membranes before and in the course of acetyltrypsin digestion. The upper pattern is that of the Coomassie-stained NaDodSO$_4$ gel electrophoresis slab gel. The lower pattern shows the derived radioautogram. Samples (10 and 20 μg of protein) were taken before (1, 1') and at various times after the addition of acetyltrypsin (1 μg/mg of SR protein): 10 s (2, 2'), 30 s (3, 3'), and 1(4, 4'), 2(5, 5'), 4(6, 6'), 8(7, 7'), 16(6, 8'), and 32 min (9, 9'). The reaction was stopped by the addition of NaDodSO$_4$–mercaptoethanol. Incubation was at 37 °C. Noncovalently attached INA products were removed by the albumin wash procedure detailed in the Experimental Section.

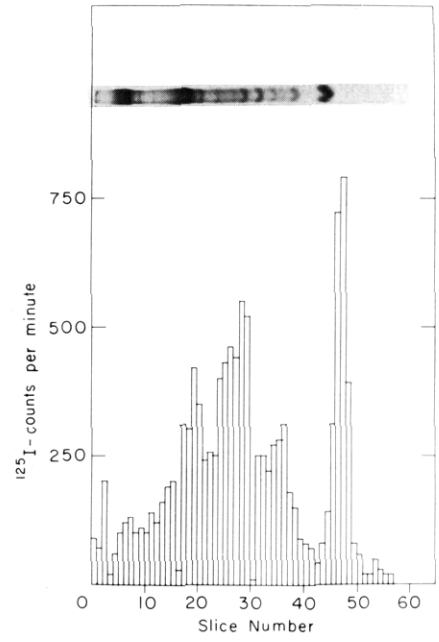

FIGURE 5: Distribution of [^{125}I]INA insertion products in the components of the erythrocyte membrane. Hemoglobin-free erythrocyte membranes were labeled with [^{125}I]INA as described in the Experimental Section. Without removal of the noncovalently attached irradiation products, a sample containing 10 μg of protein was applied to a slab gel. After fixation, staining, drying, the gel was sliced into 2-mm slices and the ^{125}I content determined. The Coomassie stained pattern is shown in the upper part of the figure.

min and acetone washing respectively. Thin-layer chromatography of the lipids extracted from the SR membranes (not shown) indicated that after either of these washing procedures, no free nitrene products were present.

During the bovine serum albumin washing of erythrocyte membranes, evidence was found that part of the spectrin and actin bands dissociated from the membranes and was removed. For this reason, these membranes were either dialyzed against a bovine serum albumin solution or applied directly to the electrophoresis gels since, during fixation, staining, and destaining of the gels, all the noncovalently attached radioactivity was removed.

Activity of the Ca^{2+}-Stimulated ATPase and INA Labeling. The INA labeling was usually performed at concentrations of the label less than 1% of the Ca^{2+}–ATPase concentration. In order to determine whether inhibition of the enzyme occurred during labeling, experiments were performed using INA concentrations up to 1.25 times the enzyme concentration (assuming molecular weight 100 000 and 70% of the total SR protein to be enzyme). After irradiation under these conditions, approximately 0.5 molecule of INA was incorporated per molecule of enzyme, while the activity was reduced by less than 10%. This reduction was in part due to the many maniuplations involved rather than to the labeling itself.

Products of Acetyltrypsin Digestion of INA-Labeled SR. Following irradiation and removal of noncovalently attached products by bovine serum albumin washing, the distribution of the label was determined by performing radioautograms of SR membranes analyzed by NaDodSO$_4$ gel electrophoresis slab gels. The results (Figure 4, 1 and 1') are in agreement with those shown in Figure 3 that the main labeled components are the Ca^{2+}–ATPase and the lipids. The 56 000 calcium binding protein and the 44 000 calcequestrin are not labeled at all. Minor insertion of the nitrene also occurs in two of the acidic proteins (molecular weights 20 000 and 10 000) and to a smaller molecular weight component that might correspond to the proteolipid.

Prior to attempts to purify the Ca^{2+}–ATPase to determine those polypeptides which are labeled, it appeared of interest to establish the patterns of acetyltrypsin digestion in the intact membrane. Acetyltrypsin was used because it does not bind to membranes (Banik & Davison, 1974). It can be observed in Figure 4 that as soon as 10 s after the addition of the acetyltrypsin, degradation of the Ca^{2+}–ATPase ensues with the appearance of two labeled polypeptides of molecular weights 52 000 and 46 000; as time of digestion proceeds, this general tendency is amplified and at longer times the process appears to be complete.

Preliminary evidence is also derived from the results presented in Figure 4 that acetyltrypsin further digests the 52 000 and 46 000 fragments and releases labeled peptides of molecular weights 31 000, 24 000, 20 000, 13 000, and 6000–10 000. Furthermore, data not shown indicate that exhaustive digestion with higher levels of acetyltrypsin leaves only two labeled bands in the region of 20 000 and of 6000–10 000.

Patterns of INA Labelling of Erythrocyte Membranes. No differences in label distribution could be detected in the NaDodSO$_4$ electrophoretic patterns of labeled erythrocytes when compared with labeled hemoglobin-free erythrocyte membranes. Both showed very little labeling in the position of the spectrin, while a broad region of labeled polypeptides was found in the position of the PAS-positive bands (Figure 5). It is likely that, in addition to the main polypeptides described

TABLE I: Distribution of [^{125}I]INA-Insertion Products in Erythrocyte Membranes Subjected to Different "Protein Perturbants" and Detergents.[a]

Treatment	Fraction	[^{125}I]INA (cpm × 10^{-3})	[^{125}I]INA % of total recovd	Protein mg	Protein % of total recovd
0.1 N NaOH	Precipitate	30.9	92.2	2.04	59.9
	Supernatant	2.6	7.8	1.36	40.0
5 mM pCMB	Precipitate	114.0	95.8	1.83	53.8
	Supernatant	4.9	4.2	1.58	46.2
1.0 mM EDTA	Precipitate	178.0	96.9	2.14	63.1
	Supernatant	5.7	3.1	1.26	36.9
0.5% Triton X-100, 56 mM sodium borate, pH 8.0	Precipitate	10.5	16.8	1.09	31.8
	Supernatant	52.3	83.2	2.32	68.3

[a] Packed ghosts (3.5 mg of protein/mL) were subjected to the treatments described following essentially the procedures of Steck & Yu (1973) and of Yu et al. (1973). The EDTA treatment involved exposure of packed ghosts to 1.0 mM EDTA (final concentration) in 10 mM phosphate buffer, pH 8.0, for 20 min at 37 °C followed by centrifugation at 18 000*g* for 30 min.

in the erythrocyte membrane, many other minor bands are present. Therefore, it is difficult to ascribe labeling to single bands. In order to establish whether so-called extrinsic and intrinsic proteins were labeled, the membranes were subjected to general "protein perturbants" following essentially the procedures described by Steck & Yu (1973) and Yu et al. (1973). It can be observed (Table I) that release of proteins by 0.1 N NaOH, 5 mM *p*-chloromercuribenzoate, or 1.0 mM EDTA was accompanied by minor release of radioactivity. $NaDodSO_4$ electrophoresis of these released proteins (not shown) indicated minor incorporation of [^{125}I]INA products into bands 1, 2, and no label in bands 4.1, 4.2, 5, and 6 (using the nomenclature of Fairbanks et al. (1971)). The majority of the label remained in the sedimented lipid rich material. On the other hand, solubilization with Triton X-100, a procedure which releases mainly the intrinsic proteins including bands 3, PAS-1, 2, and 3, and band 7, is accompanied by the release of the majority of the radioactivity, while little remains in the sedimented material even though it contains an appreciable amount of protein. The Triton extraction was performed once so that the solubilization was not quantitative. The pattern of release was similar when unlabeled membranes were subjected to the same protein perturbants.

Discussion

A new label, patterned after those previously described (Klip & Gitler, 1974), has been synthesized by two different micromethods. In both procedures Na^{125}I can be used in the final step to obtain highly radioactive [^{125}I]INA. One procedure is based on the fact that, in 1,5-didiazoniumnaphthalene, one of the diazonium groups probably reacts preferentially with azide because it is activated by the very high electron-withdrawing capacity of the second diazonium group (Zollinger, 1961). This allows the formation of the monosubstituted azidodiazoniumnaphthalene which then can be treated with Na^{125}I to obtain the [^{125}I]INA. A second method which is simpler is made possible by the finding that 5-nitronaphthyl 1-azide can be reduced to form the 5-aminonaphthyl 1-azide derivative in good yields and that this compound may then be readily iodinated by reacting the diazonium salt with NaI. These procedures allow the ready synthesis of the label without necessity to store it for extended periods. This is of importance because radiation appears readily to destroy the azide derivatives. For this reason, as mentioned, high specific radioactivity tritiated naphthyl 1-azide was found not to be a good labeling material. This latter compound appeared also to form polymeric products on exposure to light which gave artefacts in the labeling patterns. No such problems have arisen in the studies using [^{125}I]INA.

When [^{125}I]INA is added to SR, more than 98% of the radioactivity cosediments with the membranes. This shows that the partition coefficient is very much in favor of the membrane components. Subsequent exposure to light for very short periods results in very high covalent labeling of the membrane components. The majority of the label covalently attaches to the Ca^{2+}-stimulated ATPase and to a lesser extent to the membrane lipids. Some label is also found in the low molecular weight proteins at 22 000 and 10 000. However, no labeling was detected in calsequestrin and in the high affinity Ca^{2+}-binding protein. While the proteins labeled by INA cannot be released from the membrane except by the use of detergents, those not labeled are readily released and may be purified as water soluble molecules (MacLennan & Wong, 1971; Ostwald & MacLennan, 1974).

Tryptic cleavage of the INA labeled Ca^{2+}-ATPase results in two fragments of approximate molecular weights 52 000 and 46 000. Both fragments are nearly equally labeled. Stewart et al. (1976) have established the ATP phosphorylation site on the larger fragment formed after short trypsin digestion; the smaller fragment they consider to be the more apolar segment. The fact that the Ca^{2+}-ATPase is not inactivated during INA labeling at high concentrations suggests that it does not label the 52 000 fragment in the active ATP binding site. If, indeed, the INA labels from the bilayer, the findings that the two initial tryptic cleavage products are nearly equally labeled suggests that the polypeptide of the Ca^{2+}-ATPase comes in contact or spans the bilayer *in at least two sites*. The fact that these tryptic cleavage products retain ATPase activity and are not released by washing supports this view. Evidence for membrane proteins with similar properties may be derived from studies on bacteriorhodopsin (Henderson & Unwin, 1975) and for band 3 of the erythrocyte by Jenkins & Tanner (1975).

These initial studies also indicate that more prolonged trypsin cleavage of the ATPase results in the liberation of radioactive peptides of molecular weights 31 000, 24 000, 20 000, 13 000, and 6000. Exhaustive tryptic cleavage (not shown) results in two radioactive bands in the region of 20 000 and approximately 6000–10 000. These studies will be extended in purified preparations of the Ca^{2+}-ATPase. It may be mentioned that a similar study where INA was used to label a highly purified preparation of the [Na^+-K^+]-stimulated ATPase shows that label is mainly inserted into the 100 000 polypeptide. Minor insertion occurs into the glycoprotein. Tryptic cleavage indicates that only one-half of the 100 000 polypeptide is labeled and exhaustive digestion with trypsin or thermolysin converts quantitatively the 100 000 polypeptide into a fragment(s) of 13 000 molecular weights (Karlish et al., 1977). Thus, this approach might allow for the identification of the polypeptide segments in contact with the bilayer.

[^{125}I]INA labelling of the intact erythrocyte or of hemoglobin-free erythrocyte membranes gives essentially equal

$NaDodSO_4$ electrophoretic patterns of label distribution. In this more complex membrane, it is more difficult to derive precise information on which bands are labeled from the $NaDodSO_4$ electrophoretic patterns since many minor bands might also be labeled.

In any case, a consistent finding was a low incorporation into the spectrin region, while the majority of the incorporation was found in the band 3 and PAS 1-3 regions and into the lipids. Before purifying single proteins to ascertain their mode of labeling, experiments were performed to define general classes of membrane proteins labelled (Steck & Yu, 1973; Yu et al., 1973). The results indicated that proteins liberated by treatments such as high pH, EDTA, or PCMB are only weakly labeled. Analysis of these proteins indicated minor labeling of released spectrin and no radioactivity in the released band 5 (erythrocyte actin) region. It seems that the majority of the INA insertion occurs into the "intrinsic" proteins and of those into the PAS positive bands. Preliminary results with purified glycophorin indicate that it is significantly labeled and that the label is not released by tryptic digestion (Kahane & Gitler, unpublished results). These findings suggest that spectrin and actin are located in the erythrocyte membrane such that there exists little insertion of their polypeptide chains into the lipid bilayer. The mode of attachment to the membrane of spectrin and actin might be through other intrinsic membrane proteins (Yu & Branton, 1976; Scott et al., 1974, 1977; Kirpatrick, 1976). Apolar interaction of spectrin with phospholipids of the type described by Juliano et al. (1974) and by Sweet & Zull (1970) are not in general accord with the present findings.

One problem in the use of INA is that it labels a soluble protein such as bovine serum albumin. Thus, presumably it could also label membrane proteins containing similar apolar pockets. However, our previous studies (Klip & Gitler, 1974) suggest that the bulk of the label is rapidly dissolved in the liquid hydrocarbon regions of the membrane. Thus, label would not be available to bind from the aqueous phase to such apolar crevices, especially since the label is used at very low concentrations. The finding that the Ca^{2+}-ATPase is not inhibited by high concentrations of INA supports this conclusion. However, these results may not be generalized since the $[Na^+-K^+]$ATPase is inhibited by the INA-labeling procedure. However, this inhibition is not prevented by ATP nor is the ATP binding altered after labelling (Karlish et al., 1977).

In general, little if any label is released by proteolysis (see Klip & Gitler, 1974; Sigrist-Nelson et al., 1977; Karlish et al., 1977; Klip et al., 1976). Furthermore, all our studies have indicated that only intrinsic proteins are labeled. One general finding in the labeling studies has been that as the concentration of INA is decreased, the fraction of the label attached to proteins increases while that inserted into the lipids decreases. The reasons for these results are not immediately apparent. They could be due to the greater reactivity of the nitrene formed with the side chains of the protein. Nitrenes are known to insert best into tertiary, then into secondary, then into primary carbons (Knowles, 1972). Alternatively, binding of the aromatic azide to aromatic side chains of the proteins might occur due to the low dielectric constant of the environment. Clearly, more data are required before definite conclusions may be reached.

The present findings suggest that the use of INA together with other similar labelling techniques (Chakrabarti & Khorana, 1975; Greenberg et al., 1976), in combination with labels reactive from the aqueous environments, might allow the identification of those polypeptide chains responsible for the interaction of the membrane proteins with the bilayer lipids.

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