Labeling of Lymphocyte Surface Antigens by the Lipophilic, Photoactivatable Reagent Hexanoyldiiodo-*N*-(4-azido-2-nitrophenyl)tyramine†

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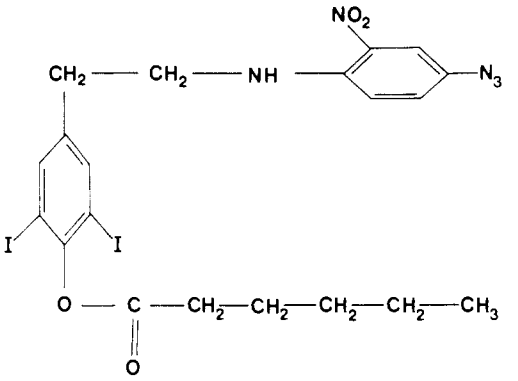
ABSTRACT: Hexanoyldiiodo-*N*-(4-azido-2-nitrophenyl)tyramine has been used after photochemical conversion into the reactive nitrene to label lymphocyte plasma membrane proteins. A wide variety of evidence indicates that the reagent most probably labels only hydrophobic sections of membrane proteins. Most importantly, analysis of the distribution of label in the human major transplantation antigens (HLA-A, -B) showed that labeling was restricted to the membrane-buried portion of the 43 000 molecular weight glycosylated polypeptide; no radioactivity was detected in the papain-cleavable fragment or in β_2-microglobulin, both of which are extrinsic to the lipid bilayer. Immunoprecipitation of human and mouse membrane-associated immunoglobulin demonstrated that the heavy chains (μ and δ) were labeled, whereas, in contrast, the light chains were unlabeled. Secreted human immunoglobulin M (IgM) was not labeled in the presence of detergent and, although some labeling was detected in the presence of added liposomes, it was common to both heavy and light chains and was not restricted to the heavy chain as in the case of membrane-associated Ig. This result argues strongly in support of the view that membrane-associated Ig differs from secreted Ig in possessing a hydrophobic domain which is integrated into the lipid bilayer. Both polypeptide chains of human HLA-DRw antigens reacted with the reagent when present in membrane preparations or as detergent-solubilized antigens. The 33 000 molecular weight component was labeled intensely when compared to the 28 000 molecular weight chain. This result may indicate the presence of protein–protein associations within the bilayer. The Thy-1 rodent differentiation antigen was also labeled strongly.

The currently accepted model for the structure of the plasma membrane is one in which proteins are associated with a fluid lipid bilayer by polar and/or hydrophobic interactions with phospholipid (Singer & Nicolson, 1972). Of particular interest in this model are the transmembrane proteins, since they can in principle act as mediators of information transfer across the phospholipid barrier. Although a variety of effective reagents exist to probe regions of polypeptides exposed at the membrane surfaces, only recently have attempts been made to characterize regions of the polypeptide situated within the bilayer. Ideally such reagents should partition into the lipid bilayer and be convertible into a form which reacts indiscriminately with proteins. Reactive lipophilic reagents which are generated photochemically within the membrane have generally been used (see, for example, Klip & Gitler, 1974; Klip et al., 1976; Bercovici & Gitler, 1978; Goldman et al., 1979; Wells & Findlay, 1979a,b).

In this paper we describe the use of the lipophilic, photoactivatable reagent hexanoyldiiodo-*N*-(4-azido-2-nitrophenyl)tyramine (Chart I) to label lymphocyte surface antigens. This reagent has a partition coefficient between octanol and water of at least 10^4 and is thus extremely lipid soluble (G. M. Hebden, J. C. A. Knott, and N. M. Green, unpublished experiments). The reactive nitrene is generated photochemically. Evidence is presented that only portions of membrane proteins associated with the interior of the lipid bilayer are labeled. The implications of the results for the modes of attachment of several lymphocyte surface antigens, especially membrane-associated Ig,[1] are discussed. Previously, it has proved difficult to detect any differences between the structure of lymphocyte Ig and that of its secreted counterpart, leading to the suggestion that membrane-associated Ig is a peripheral protein which is attached to the cell surface via its interaction with an integral membrane receptor (reviewed by Feinstein et al., 1980). Here we present evidence that membrane-associated immunoglobulin differs from its secreted counterpart in having a nonpolar portion which inserts the heavy chain into the lipid bilayer.

Chart I: Hexanoyldiiodo-*N*-(4-azido-2-nitrophenyl)tyramine[a]



[a] Conversion to the reactive nitrene is achieved by photolysis at 480 nm.

Materials and Methods

All chemicals and buffer components were of A.R. purity or the highest commercial grade obtainable. NAP-tyramine was a kind gift from Dr. N. M. Green and was synthesized by him as described (G. M. Hebden, J. C. A. Knott, and N. M. Green, unpublished experiments). Sodium deoxycholate

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[1] Abbreviations used: Ig, immunoglobulin; NAP, (4-azido-2-nitrophenyl); $NaDodSO_4$, sodium dodecyl sulfate; HLA-A, -B, human major transplantation antigens coded by the A and B loci of the major histocompatibility gene complex; HLA-DRw, human Ia antigens coded by the D locus or an adjacent locus of the major histocompatibility gene complex (Barnstable et al., 1978).

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was obtained from Sigma Chemical Co. and carrier-free $Na^{125}I$ was from The Radiochemical Centre, Amersham. Trypsin (pretreated with diphenylcarbamoyl chloride), lactoperoxidase, and standard proteins for $NaDodSO_4$–polyacrylamide gel electrophoresis were purchased from Sigma Chemical Co. Papain and Pronase were obtained from Worthington Corp. Rabbit anti-(HLA-DRw) was a kind gift from Dr. A. Sullivan, and rabbit anti-(mouse Ig) was from Dr. R. M. E. Parkhouse. Rabbit anti-(rat brain Thy-1) and mouse monoclonal (hybridoma) antibodies against rat Thy-1 antigen were generously provided by Dr. A. F. Williams. Rabbit anti-(human β_2-microglobulin) was raised in this laboratory and rabbit anti-(human Ig) was purchased from Nordic Immunological Laboratories. Human serum IgM was a gift from Dr. K. B. Cooke. *Staphylococcus aureus* bacteria were killed and fixed prior to their use in immunoprecipitations as described (Kessler, 1975). Phosphatidylcholine liposomes were a gift from Dr. G. Kenna.

Synthesis of Hexanoyldiiodo-N-(4-azido-2-nitrophenyl)-tyramine. This was synthesized from NAP-tyramine as described (G. M. Hebden, J. C. A. Knott, and N. M. Green, unpublished experiments) and purified before use by thin-layer chromatography on silica gel–Polygram Sil G (Macherey-Nagel & Co.) using an authentic nonradioactive sample as a marker and benzene–butanone (99:1 by volume) as the solvent. The yield was routinely 10–15%, and the specific activity was approximately 200 Ci/mmol. The reagent was stored in the dark and used within 24 h of synthesis.

Plasma Membrane Preparation. Cell suspensions were prepared from rat thymus, mouse spleen, and mouse thymus as described previously for pig lymphocytes (Perlés et al., 1977). The human B lymphoblastoid cell line BRI 8 and the Burkitt lymphoma line Daudi were cultured in RPMI 1640 medium containing the antibiotics penicillin (100 units/mL) and streptomycin (50 μg/mL) and supplemented with 10% (v/v) fetal calf serum (Flow Laboratories, Irving, Ayrshire, U.K.). Plasma membrane was prepared from each cell suspension as described by Allan & Crumpton (1970) except that a commercial cell disrupting pump (Stansted Fluid Power) was used.

Labeling Procedures. Nitrene labeling was carried out by adding 50 μCi of the lipophilic azide, dissolved in methanol at a concentration of 5 μCi/μL, to plasma membrane (1 mg of protein) suspended in 1 mL of 10 mM Tris-HCl, pH 7.4, at 20 °C with rapid mixing (final methanol concentration 1%, v/v). Mixtures were then irradiated for 20 min at 20 °C, 10 cm from a 150-W Xenon arc lamp with 430-nm cutoff. After photolysis, plasma membrane was recovered by centrifugation (100000*g* for 45 min), and the pellet, which contained more than 98% of the initial radioactivity, was resuspended to 0.9 mL with 10 mM Tris-HCl, pH 8.2.

Lactoperoxidase-catalyzed iodination of plasma membrane vesicles was carried out as described previously (Walsh & Crumpton, 1977), except that 10 mM Tris-HCl, pH 7.4, was used as buffer, and the vesicle concentration was 1 mg of protein/mL. Labeled plasma membrane was resuspended after washing to 0.9 mL with 10 mM Tris-HCl, pH 8.2.

Plasma membrane, labeled by either the lipophilic nitrene or lactoperoxidase-catalyzed iodination, was solubilized by addition of 0.1 mL of 10% (w/v) sodium deoxycholate in 10 mM Tris-HCl, pH 8.2, for 10 min at 20 °C and diluted after centrifugation (100000*g* for 45 min) with an equal volume of 10 mM Tris-HCl, pH 8.2 (Allan & Crumpton, 1971).

Serum (secreted) IgM was radioiodinated with sodium [^{125}I]iodide using the chloramine T method (Greenwood et al., 1963). Nitrene labeling was carried out by adding the lipophilic nitrene (5 μCi) to serum IgM (20 μg in 1 mL of 10 mM Tris-HCl, pH 7.4), with no addition, after preincubation with 1% (w/v) Triton X-100 or with 100 μg of phosphatidylcholine liposomes.

Immunoprecipitation. Immunoprecipitation with rabbit antisera was carried out by using fixed *S. aureus* organisms to precipitate antigen–antibody complexes (Kessler, 1975). Solubilized plasma membrane (1 mg of protein in 2 mL of 10 mM Tris-HCl, pH 8.2, containing 0.5% sodium deoxycholate) was precleared by incubation (30 min at 4 °C) with 50 μL of normal rabbit serum followed by two incubations (1 h at 4 °C each) with 200 μL of a 10% (w/v) suspension of fixed *S. aureus* in 10 mM Tris-HCl, pH 8.2, containing 0.5% sodium deoxycholate. Bacteria were removed by centrifugation (3000*g* for 10 min), and the final supernatant was further centrifuged (100000*g* for 1 h). The resulting supernatant was divided into two to four samples, each of which contained 0.25–0.5 mg of protein and about 5×10^6 cpm. Each sample was diluted to 1 mL with 10 mM Tris-HCl, pH 8.2, containing 0.5% sodium deoxycholate. Specific antiserum or normal rabbit serum (50 μL) was added, and the mixture was incubated for 30 min at 20 °C followed by 4 h at 4 °C with 200 μL of fixed *S. aureus*. Sedimented bacteria were washed twice with 10 mM Tris-HCl, pH 8.2, containing 0.5% sodium deoxycholate, and the washed pellet was resuspended in 100 μL of water. A sample (50 μL) was removed and heated at 100 °C for 2 min with an equal volume of $NaDodSO_4$ sample buffer prior to analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Immunoprecipitation with monoclonal antibodies was carried out by using rabbit anti-(mouse Ig) to precipitate antigen–antibody complexes. Solubilized plasma membrane was precleared as described above. After the bacteria were removed by centrifugation, the supernatant was further centrifuged at 100000*g* for 1 h before being divided into 0.5-mL samples. Each sample was incubated with 20 μg of either purified normal mouse IgG or monoclonal antibody for 1 h at 37 °C, followed by 1 h on ice, before it was left overnight at 4 °C with rabbit anti-(mouse Ig) (100 μL; twofold excess over equivalence). The resulting precipitate was recovered by centrifugation (12000*g* for 15 min) and washed twice with 1 mL of 10 mM Tris-HCl, pH 8.2, containing 0.5% sodium deoxycholate. The washed pellet was dissolved by boiling for 2 min in 100 μL of $NaDodSO_4$ sample buffer and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Electrophoretic Analysis. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out according to Laemmli (1970) in 0.1% $NaDodSO_4$–Tris-glycine buffer on 10 or 15% (w/v) acrylamide slab gels using a 5% (w/v) stacking gel. Samples for electrophoresis were dissolved in 2% (w/v) $NaDodSO_4$, 10% (v/v) glycerol, 80 mM Tris-HCl, pH 8.6, 0.1 M dithiothreitol, and 0.02% bromophenol blue ($NaDodSO_4$ sample buffer) and heated at 100 °C for 2 min before layering onto the gel. Gels were run at 25 mA per slab until the tracker dye had reached the bottom of the gel and then stained with 0.01% Coomassie blue in methanol–water–acetic acid (41:52:7, by volume) and destained by using the same solvent. Autoradiography was performed on the dried gel using Fuji RX film. Alternatively, the dried gel was divided into 1-mm slices and assessed for ^{125}I radioactivity in a γ counter (Packard). Apparent molecular weights of bands were determined by reference to the mobilities of the following standard proteins (subunit molecular weights in parentheses): myosin heavy chain (200 000); phosphorylase *b* (95 000); transferrin (78 000);

bovine serum albumin (68 000); ovalbumin (45 000); glyceraldehyde-3-phosphate dehydrogenase (34 000); Ig light chain (25 000); cytochrome *c* (12 500).

Protease Digestion. ^{125}I-Labeled plasma membrane vesicles (1 mg of protein in 1 mL of 10 mM Tris-HCl, pH 7.4) were digested with 0.1% Pronase for 15 min at 37 °C. The mixture was cooled to 0 °C, and plasma membrane was recovered by centrifugation (100000*g* for 30 min). The pellet was washed twice by centrifugation, and the radioactivity associated with the washed pellet was determined on a sample (5 μL from 1 mL) after precipitation with 10% (w/v) trichloroacetic acid and washing with diethyl ether.

Papain digestion of detergent-solubilized histocompatibility antigens was performed by using a purified glycoprotein fraction prepared from nitrene-labeled BRI 8 plasma membrane (1 mg of labeled plus 10 mg of unlabeled plasma membrane). The glycoprotein fraction was separated as previously described (Bridgen et al., 1976) by fractionation of the deoxycholate-solubilized plasma membrane on a column of AcA 34 followed by affinity chromatography on a 6-mL column of *Lens culinaris* (lentil) lectin–Sepharose (10 mg of lectin per mL of gel sediment); the adsorbed glycoproteins were eluted with 2% (w/v) methyl α-mannopyranoside. When this fraction was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis, only five bands, whose positions corresponded to molecular weights of 43 000, 39 000, 33 000, 28 000, and 12 000, were detected. The 43 000 and 12 000 molecular weight chains were derived from the HLA-A and -B antigens, whereas those of molecular weights 33 000 and 28 000 were derived from the HLA-DRw antigens. Enzymatic activation and digestion conditions were those described by Springer et al. (1974) (papain to protein ratio, 1:100; protein concentration about 100 μg/mL; radioactivity 50 000 cpm), except that 0.5% (w/v) sodium deoxycholate was used in place of Brij 99. Digestion was terminated by addition of sodium iodoacetate (20 mM final concentration), and protein was precipitated by the addition of 4 volumes of ethanol at −20 °C for 48 h. Protein was recovered by centrifugation and solubilized by boiling for 2 min in 100 μL of $NaDodSO_4$ sample buffer. The papain-digested sample was compared to a control sample, which had not been exposed to the enzyme, by $NaDodSO_4$–polyacrylamide gel electrophoretic analysis followed by autoradiography of the stained and dried down gel.

Results

A preparation of plasma membrane, purified from human BRI 8 lymphoblastoid cells, was illuminated at 20 °C for 30 min with the lipophilic nitrene and then recovered by centrifugation. More than 98% of the added radioactivity remained with the plasma membrane pellet. This result indicates that the nitrene has an exceptionally high affinity for the membrane relative to the aqueous environment. Between 10 and 15% of the added radioactivity was incorporated into BRI 8 plasma membrane proteins as assessed by precipitation of labeled plasma membrane by 10% (w/v) trichloroacetic acid followed by extensive washing with ethanol and diethyl ether. Digestion of nitrene-labeled BRI 8 plasma membrane with Pronase failed to solubilize to any significant extent the trichloroacetic acid precipitable radioactivity of the plasma membrane. In contrast, at least 70% of the radioactivity incorporated into BRI 8 plasma membrane protein by lactoperoxidase-catalyzed iodination was removed by Pronase digestion. These results indicate that the majority of the protein-bound radioactivity was associated with portions of the polypeptide chains that are not readily accessible to Pronase (i.e., are not exposed to the external environment).

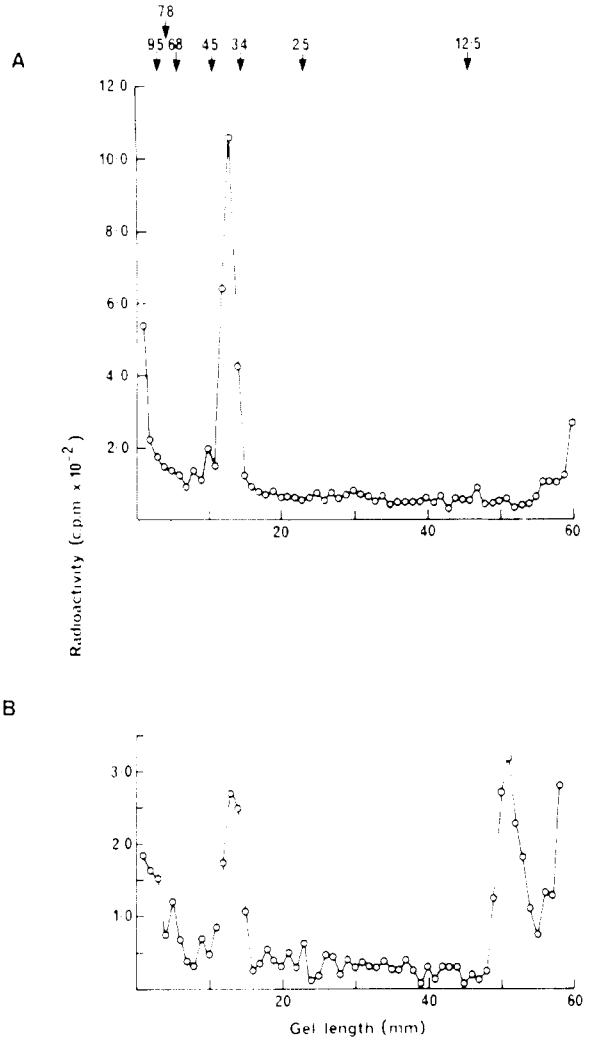

FIGURE 1: Immunoprecipitation of BRI 8 plasma membrane with rabbit anti-(human β_2-microglobulin) serum. Plasma membrane was radioiodinated (^{125}I) by either the lipophilic nitrene (A) or lactoperoxidase-catalyzed iodination (B). Immunoprecipitation and electrophoresis were as described under Materials and Methods. Immunoprecipitates were analyzed on a 15% (w/v) polyacrylamide gel which was stained, dried, and divided into 1-mm sections.

Direct evidence that the lipophilic nitrene preferentially labeled the intrabilayer regions of membrane proteins was obtained from a detailed characterization of the labeling pattern of the major transplantation antigens (HLA-A, -B) of BRI 8 plasma membrane. These antigens comprise two polypeptides, namely, a transmembrane, glycosylated polypeptide of 43 000 molecular weight that is noncovalently associated with β_2-microglobulin (molecular weight 11 600), which is judged to be completely extrinsic to the lipid bilayer (Barnstable et al., 1978). As shown in Figure 1A, immunoprecipitation of nitrene-labeled BRI 8 plasma membrane revealed that the 43 000 molecular weight polypeptide was intensely labeled, whereas no detectable radioactivity was observed in a position corresponding to the mobility of an authentic sample of β_2-microglobulin. In contrast, when HLA-A and -B antigens were immunoprecipitated from lactoperoxidase-labeled BRI 8 plasma membrane, both the 43 000 molecular weight transmembrane polypeptide and β_2-microglobulin were labeled (Figure 1B).

The distribution of radioactivity within the nitrene-labeled 43 000 molecular weight polypeptide was characterized further by using papain cleavage of a detergent-solubilized purified glycoprotein fraction comprising a mixture of HLA-A and -B

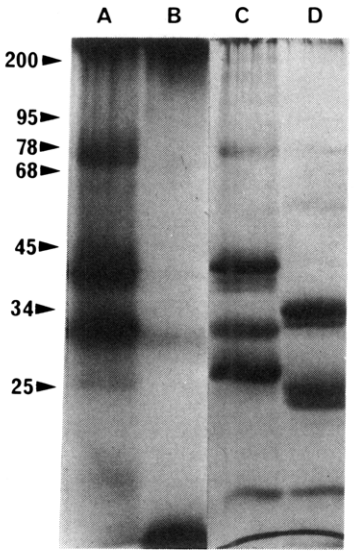

FIGURE 2: Papain cleavage of a sodium deoxycholate solubilized, purified glycoprotein fraction containing HLA-A and -B antigens labeled by the lipophilic nitrene. Tracks A and B represent autoradiographs of uncleaved and papain-cleaved glycoprotein fractions after ethanol precipitation and analysis on a 10% (w/v) polyacrylamide gel. The Coomassie blue stained profiles are depicted in tracks C and D.

and HLA-DRw antigens. The results of papain cleavage are presented in Figure 2. The 43 000 molecular weight polypeptide of the HLA-A and -B antigens (Figure 2C) was completely degraded to a cleavage product of apparent molecular weight of about 34 000 (Figure 2 D), as visualized by Coomassie blue staining of $NaDodSO_4$–polyacrylamide gels (Springer et al., 1974). The autoradiograph of the same $NaDodSO_4$–polyacrylamide gel, however, revealed that the radioactivity initially associated with the 43 000 molecular weight component (Figure 2A) was completely absent from the 34 000 molecular weight fragment (Figure 2B). Instead, a greatly increased proportion of radioactivity was observed associated with the tracker dye front (Figure 2B). These results are in agreement with a similar study using a lipophilic carbene (Goldman et al., 1979).

Labeling of the HLA-DRw antigens of BRI 8 plasma membrane by the lipophilic nitrene was also investigated by immunoprecipitation of labeled BRI 8 plasma membrane. HLA-DRw antigens comprise two polypeptides of apparent molecular weights of about 33 000 and 28 000 as evidenced by immunoprecipitation and $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of lactoperoxidase-labeled plasma membrane (Figure 3B). Immunoprecipitation of nitrene-labeled plasma membrane revealed that the 33 000 molecular weight component was strongly labeled, whereas the radioactivity associated with the 28 000 molecular weight polypeptide was barely discernible (Figure 3A). The ratio of labeling of the two chains was typically about 20:1. The minor labeling of the 28 000 molecular weight polypeptide of HLA-DRw is seen more clearly in the autoradiograph of a partially purified preparation of the nitrene-labeled antigen analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4A). This uneven distribution of label between the two chains of HLA-DRw was also characteristic of the purified protein that had been reacted with the lipophilic nitrene in association with Triton X-100 micelles (Figure 4B) rather than in association with a phospholipid bilayer. Nitrene labeling of rat spleen plasma membrane also gave a disproportionate distribution of radioactivity (about 4:1) among the polypeptides of the rat Ia antigens (McMaster & Williams, 1979). This

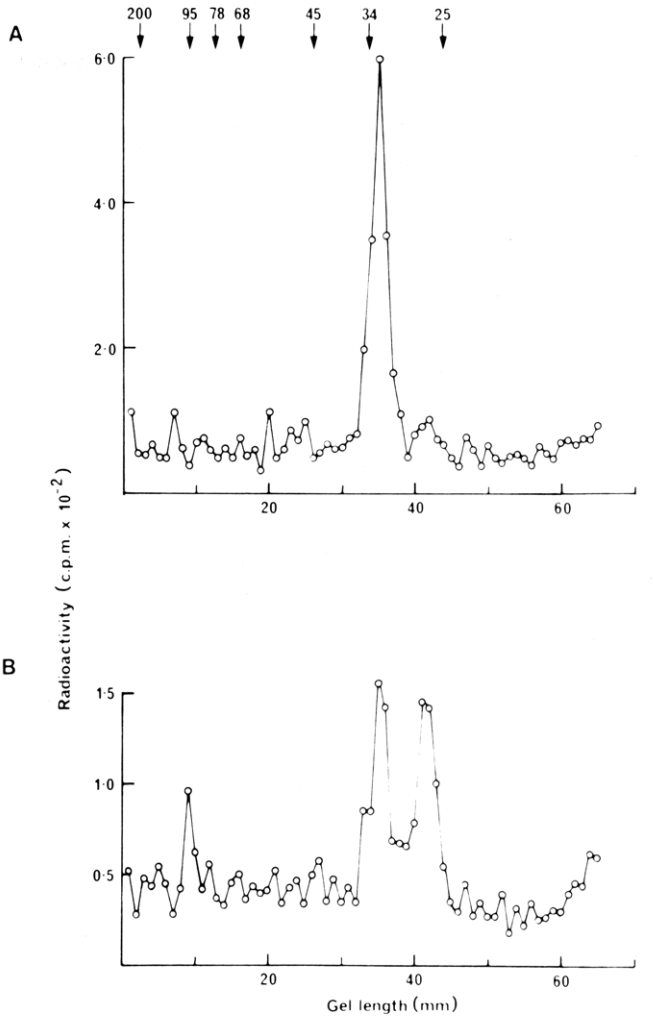

FIGURE 3: Immunoprecipitation of BRI 8 plasma membrane with rabbit anti-(HLA-DRw) serum. Plasma membrane was radioiodinated by either the lipophilic nitrene (A) or lactoperoxidase-catalyzed iodination (B). Immunoprecipitates were analyzed on a 10% (w/v) polyacrylamide gel.

result suggests that the preferential labeling of the 33 000 molecular weight polypeptide relative to that of the 28 000 molecular weight component is probably a property of Ia antigens in general.

The effects of papain digestion on the molecular sizes and radioactivity of the HLA-DRw polypeptides are presented in Figure 2, C and D. Both the 33 000 and 28 000 molecular weight polypeptides were degraded by papain, fragments of molecular weight about 23 000 being formed; it has not been determined whether the fragments represent the degradation products of one or of both chains of HLA-DRw. However, no labeling was associated with the Coomassie blue stained bands as evidenced by the autoradiograph (Figure 2B).

Labeling of the membrane-associated Ig of Daudi lymphoma and BRI 8 lymphoblastoid cells was assessed after reaction of the plasma membrane fractions with the lipophilic nitrene. The profile of the membrane-associated Ig isolated by immunoprecipitation from lactoperoxidase-labeled Daudi plasma membrane and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis is presented in Figure 5A. Three peaks of radioactivity were revealed. One peak corresponded in mobility to that of the μ heavy chain of human IgM, and another migrated in a position corresponding to the generally accepted mobility of immunoglobulin light chain (molecular weight about 25 000). An additional peak was also present

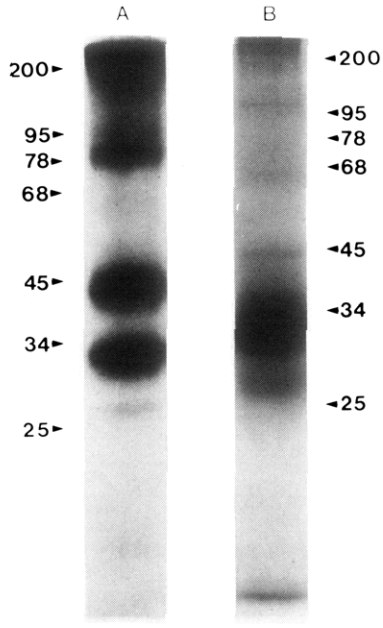

FIGURE 4: Nitrene labeling of HLA-DRw antigens in intact BRI 8 plasma membrane or in a purified preparation solubilized in 0.5% (w/v) Triton X-100. The HLA-DRw antigens of the nitrene-labeled BRI 8 plasma membrane were separated by gel filtration and lentil lectin chromatography as described under Materials and Methods (track A). Alternatively, HLA-DRw antigens were purified before labeling as described previously (Ikeman et al., 1978) and subsequently labeled by the lipophilic nitrene (5 μCi) in the presence of Triton X-100. After ethanol precipitation, the labeled products were analyzed on a 10% (w/v) polyacrylamide gel (track B). The gels represented in tracks A and B were run on separate occasions.

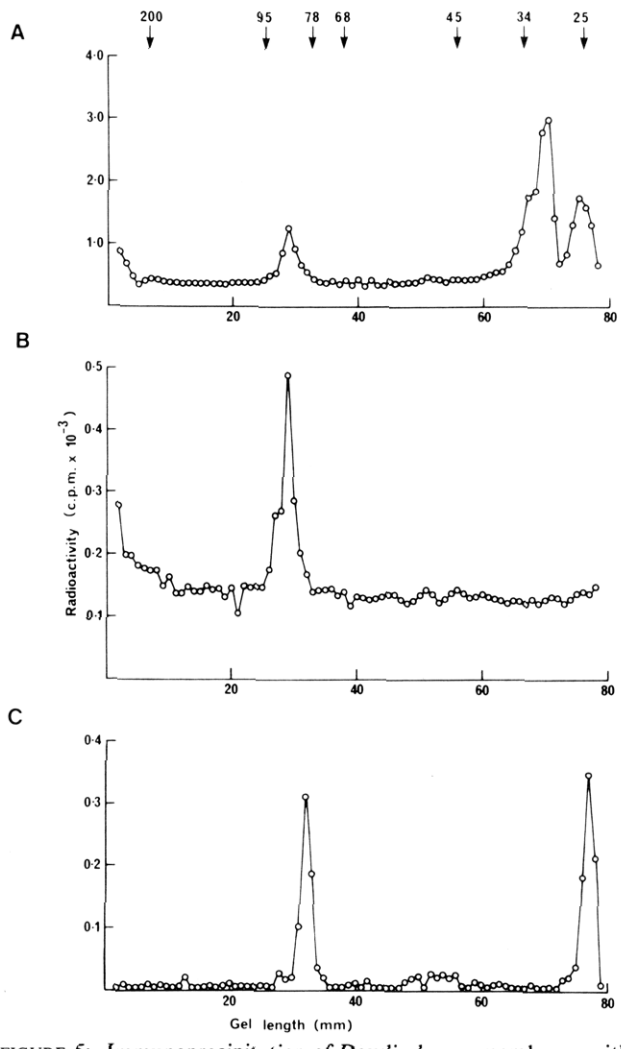

FIGURE 5: Immunoprecipitation of Daudi plasma membrane with rabbit anti-(human Ig) serum. Immunoprecipitates from plasma membrane, labeled by either lactoperoxidase-catalyzed iodination (A) or the lipophilic nitrene (B), were analyzed on a 7.5% (w/v) polyacrylamide gel. A sample of serum IgM, iodinated using the chloramine T method, was electrophoresed on the same gel (C) (2000 cpm added). The bromophenol blue tracker dye was electrophoresed completely off the end of the gel to ensure adequate migration of the μ heavy chains.

which migrated with a mobility corresponding to an apparent molecular weight of about 30 000. An identical labeling pattern was obtained by polyacrylamide gel electrophoretic analysis of Ig precipitated from lactoperoxidase-labeled BRI 8 plasma membrane (data not shown). The presence of a strongly labeled 30 000 molecular weight band in immunoprecipitates of Ig from Daudi plasma membrane has been reported previously (Kennel, 1974; Singer & Williamson, 1979). The analyses of the immunoprecipitates from nitrene-labeled Daudi (Figure 5B) and BRI 8 (data not shown) plasma membranes revealed that the μ chain was labeled, whereas no radioactivity was detected in the region of the gel corresponding to either the 30 000 or 25 000 molecular weight polypeptides. Furthermore, comparison of electrophoretic mobilities demonstrated that the μ chain of Daudi membrane associated IgM, labeled by the lipophilic nitrene or by lactoperoxidase-catalyzed iodination, had a greater apparent molecular weight (equivalent to about 7000) than the μ chains of serum IgM (Figure 5C). Similar labeling patterns of membrane-associated Ig were also obtained by nitrene labeling of mouse spleen plasma membrane. Thus, the nitrene labeled both μ and δ heavy chains, but no labeling of light chain was observed (Figure 6A), whereas lactoperoxidase-catalyzed iodination labeled μ aand δ heavy chains together with light chain (Figure 6B).

Knowledge of the extent of labeling of secreted (serum) Ig by the nitrene in the presence of detergent or liposomes, as well as the distribution of label between the heavy and light chains, is essential in order to evaluate the above results for the membrane-associated Ig. Reaction of the lipophilic nitrene with serum IgM in aqueous solution (10 mM Tris-HCl buffer, pH 7.4) resulted in extensive labeling. The heavy and light chains were, however, labeled to about an equal intensity (Figure 7A) compared with the specific labeling of the heavy chain of membrane-associated IgM (cf. Figure 5B). In the presence of phosphatidylcholine liposomes, nitrene labeling of serum IgM was reduced substantially, although some labeling of both heavy and light chains was still observed (Figure 7C). Labeling of serum IgM was, however, not detectable in the presence of 1% (w/v) Triton X-100 during the exposure time used (Figure 7B), whereas under the same conditions of labeling a purified membrane glycoprotein fraction from BRI 8 cells gave the characteristic labeling patterns (see above) of HLA-A, -B, and -DRw antigens (Figure 7D).

Nitrene labeling of the Thy-1 differentiation surface antigen of rat and mouse thymus was also investigated. This antigen is of interest since it has been extensively characterized. It comprises a single glycosylated polypeptide of about 25 000 molecular weight (Barclay et al., 1976). This polypeptide binds detergent and on this basis can be judged to be an integral membrane protein, although amino acid sequence studies have failed to reveal an obvious hydrophobic region (Campbell et al., 1979). When Thy-1 was immunoprecipitated from nitrene-labeled rat and mouse thymus plasma membrane, an intensely labeled polypeptide of molecular weight about 25 000 was revealed by $NaDodSO_4$–polyacrylamide gel elec-

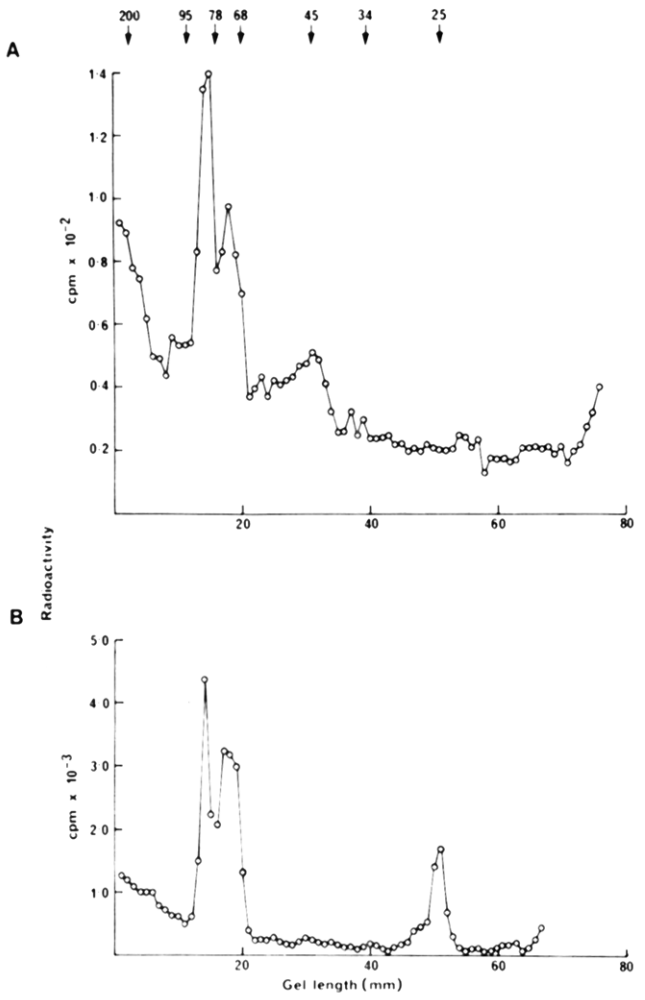

FIGURE 6: Immunoprecipitation of mouse spleen plasma membrane with rabbit anti-(mouse Ig) serum. Immunoprecipitates from plasma membrane labeled by the lipophilic nitrene (A) or lactoperoxidase-catalyzed iodination (B) were analyzed on a 10% (w/v) polyacrylamide gel.

trophoretic analysis (Figure 8, A and B, respectively).

Discussion

An important consideration in the interpretation of the results presented here is whether the lipophilic nitrene labels only those portions of polypeptide chains within the lipid bilayer. Restricted labeling is supported by various arguments and results. Thus, the marked hydrophobicity of the reagent (partition coefficient between octanol–water of at least 10^4) argues strongly in support of an almost exclusive partitioning into the bilayer when added to aqueous suspensions of membranes. Direct evidence in support of this argument is provided by the observation that >98% of the nitrene is associated with the membranes under these conditions. Moreover, the failure of Pronase to release any radioactivity from nitrene-labeled BRI 8 plasma membrane is consistent with labeling being restricted to amino acid residues inside or close to the bilayer. Most importantly, analysis of the distribution of the nitrene label in the human major transplantation (HLA-A and -B) antigens suggests that the transbilayer peptide only is labeled. Detailed structural information is available concerning the mode of association of these membrane proteins with the lymphocyte plasma membrane. Thus, the 43 000 molecular weight polypeptide is known to span the lipid bilayer, whereas β_2-microglobulin is most probably completely extrinsic to the outer surface of the lipid bilayer (Walsh & Crumpton, 1977; Strominger et al., 1976). Furthermore, papain cleavage of both

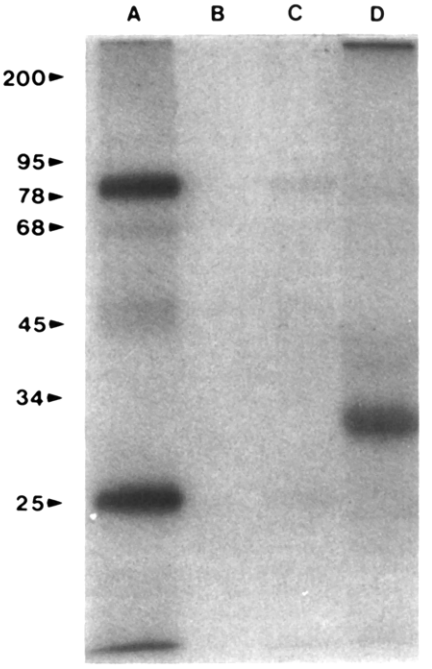

FIGURE 7: Nitrene labeling of human serum IgM in aqueous solution and in the presence of Triton X-100 or phosphatidylcholine liposomes. Serum IgM was labeled with the lipophilic nitrene either with no addition (A) or after preincubation with Triton X-100 (B) or with phosphatidylcholine liposomes (C). Nitrene labeling of the purified BRI 8 glycoprotein fraction, dissolved in Triton X-100, was used as a control (D). After ethanol precipitation, the labeled products were analyzed on a 10% (w/v) polyacrylamide gel followed by autoradiography.

plasma membrane associated and detergent-solubilized HLA-A and -B antigens releases a water-soluble, nondetergent binding fragment of molecular weight 34 000, which is associated with β_2-microglobulin and situated externally to the lipid bilayer (Springer et al., 1974). The present results show that neither β_2-microglobulin nor the water-soluble, 34 000 molecular weight proteolytic fragment was labeled to a significant extent by the lipophilic nitrene. Instead, the label was associated exclusively with a low molecular weight fragment(s) (Figure 2, track B) which may correspond to the intrabilayer peptide. Further analysis of the distribution of label by high-pressure liquid chromatography of *S. aureus* V8 protease-digested, nitrene-labeled HLA-A and -B antigens showed that greater than 90% of the radioactivity remained adsorbed to the column material under conditions when peptides accounting for the majority of the protein were eluted (unpublished observation).

A similar analysis of the labeling of HLA-A and -B antigens has been used by Goldman et al. (1979) to characterize a lipophilic carbene, [^{3}H]adamantylidene. In this study, labeling was claimed to be restricted to the 43 000 molecular weight chain and to be associated with or lie close to the transmembrane segment.

Although various results suggest that labeling by the lipophilic nitrene is restricted to integral membrane proteins, the possibility that it also labels nonintegral membrane proteins has not been ruled out. Indeed, any water-soluble protein is likely to be labeled in the absence of lipid or detergent (see, for example, Figure 7A), since under these conditions the protein is more hydrophobic than the water phase. However, in the presence of a competing hydrophobic phase, such as a detergent or liposomes, water-soluble (nonintegral) proteins should not, in principle, react with the lipophilic nitrene. Of three water-soluble proteins tested to date, namely, serum IgM

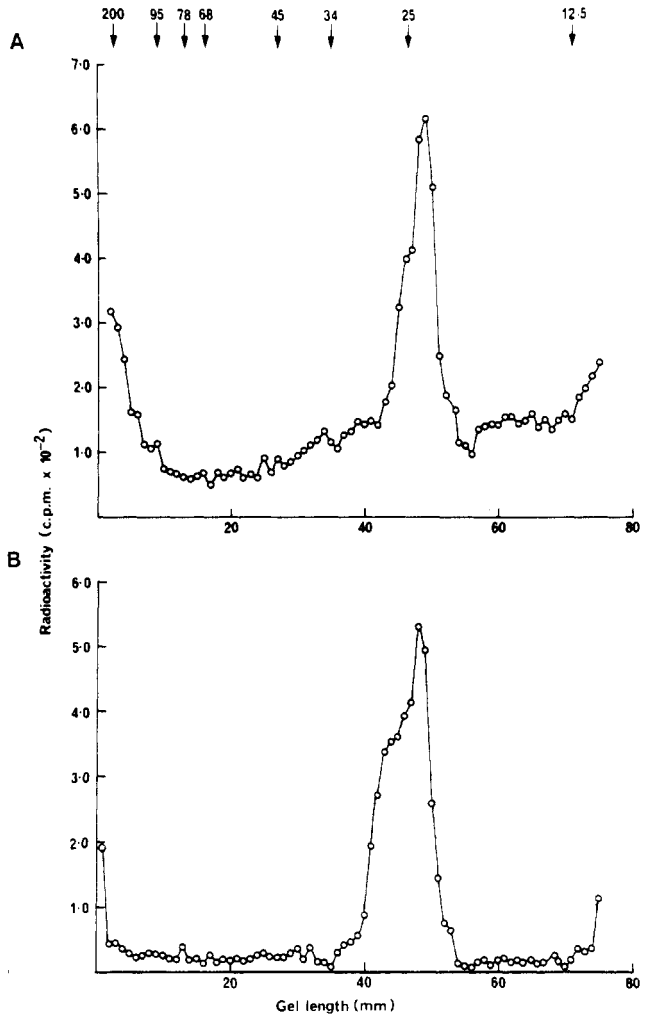

FIGURE 8: Immunoprecipitation of nitrene-labeled mouse and rat thymus plasma membranes with rabbit anti-(rat brain Thy-1) serum (A) and monoclonal mouse anti-(rat brain Thy-1) serum (B), respectively. Immunoprecipitates were analyzed on a 10% (w/v) polyacrylamide gel.

(Figure 7B), transferrin, and *Lens culinaris* (lentil) lectin (C. Bron, M. J. Owen, and M. J. Crumpton unpublished observations), none were labeled by the lipophilic nitrene in the presence of Triton X-100. In contrast, under the same conditions integral membrane proteins as represented by HLA-A, -B, and -DRw antigens were efficiently labeled.

A recent report has suggested that nitrenes are in principle poor reagents for this type of study since their long lifetime and electrophilic character might result in labeling of extrinsic membrane components (Bayley & Knowles, 1978a,b). In order to examine this possibility we labeled BRI 8 plasma membrane in the presence of 15 mM reduced glutathione, which acts as a water-soluble scavenger of reactive intermediates (Bayley & Knowles, 1978a,b). The overall incorporation of label into protein and the labeling of HLA-A, -B, and -DRw antigens in particular were identical in the presence and absence of glutathione (not shown). This specific objection would not, therefore, appear to be valid, at least for this particular nitrene.

From the above considerations it appears that labeling of lymphocyte surface proteins with the lipophilic nitrene is an accurate reflection of their association with the lipid bilayer. In this case the nitrene can be used to explore the mode of association of particular lymphocyte surface proteins with the membrane. A particularly interesting surface protein is the membrane-associated Ig which has often been surmised to be a peripheral protein that is attached to the cell surface via interaction with a Fc receptor. Thus, cell surface IgM fails to satisfy completely some of the criteria for an integral membrane protein, particularly with respect to its rather poor detergent-binding capacity (Melcher & Uhr, 1977). Evidence has also been presented that its C-terminal amino acid is identical with that of μ chains of serum IgM, which suggests that it lacks an extra C-terminal hydrophobic domain (McIlhinney et al., 1978), although other workers have claimed that the μ chains of membrane-associated Ig have a different C-terminal sequence than secreted μ chains (Williams & Grey, 1978). The lipophilic nitrene labeled the μ and δ heavy chains, but not the light chains (see below), of human and mouse membrane associated IgM and IgD. This result suggests that the heavy chains are inserted into the phospholipid bilayer. Attempts to show that the nitrene failed to label secreted IgM were not completely clear-cut. However, although some labeling of serum IgM occurred in aqueous solution, this labeling may be judged to be nonspecific on the basis that heavy and light chains of IgM were labeled equally. Moreover, labeling was reduced substantially in the presence of phosphatidylcholine vesicles and was not detectable in the presence of Triton X-100. The apparent molecular weight of the membrane-associated μ chain of Daudi cells was clearly greater than that of μ chains of secreted IgM, as assessed by polyacrylamide gel electrophoresis in $NaDodSO_4$. Although this difference in molecular weights (about 7000) is enough to account for an extra polypeptide spanning the bilayer, it cannot be ruled out that it reflects differences in glycosylation. In this context, it is noteworthy that no difference in mobility between the μ chains of serum IgM and membrane-associated IgM was observed in BRI 8 cells (not shown).

The light chains of mouse spleen membrane-associated Ig were not labeled by the lipophilic nitrene. This distinction between light and heavy chains also argues that labeling was restricted to the bilayer. Similarly, no nitrene labeling was observed of polypeptides of the generally accepted apparent molecular weight of light chains of human membrane-associated IgM. In both Daudi and BRI 8 cells, however, an extra polypeptide of about 30 000 molecular weight was observed in immunoprecipitates from lactoperoxidase-iodinated plasma membrane. This polypeptide has been observed previously in Daudi and claimed to be an aberrent immunoglobulin light chain (Kennel, 1974) or possibly a heavy chain of HLA-DRw antigens (Singer & Williamson, 1979). Whatever the identity of this polypeptide, it was not labeled by the lipophilic nitrene in both Daudi and BRI 8 cells and is thus most probably situated extrinsically to the bilayer.

Reaction of HLA-DRw antigens with the lipophilic nitrene, either in association with the plasma membrane or after solubilization in Triton X-100, resulted in strong labeling of the 33 000 molecular weight polypeptide but weak labeling only of the 28 000 molecular weight component. This result is unexpected since both chains are apparently transmembrane (Walsh & Crumpton, 1977) and would, therefore, be expected to be labeled equally. This unequal reactivity may be a general property of these antigens, since the pattern was also obtained with rat Ia-like antigens. Two possible explanations would account for this result. First, the rate constants for reaction of nitrenes with different protein groups may vary. If a particularly reactive group was present only in the intrabilayer portion of the 33 000 molecular weight polypeptide, then the observed distribution of label would be obtained. Second, interchain associations within the bilayer may shield the 28 000

molecular weight polypeptide from reaction with the nitrene. The observation that the two polypeptide chains of HLA-DRw are extremely resistant to dissociation suggests extensive polypeptide interaction (Springer et al., 1977), which is consistent with the latter explanation. Furthermore, experiments in which detergent (Triton X-100)-solubilized HLA-DRw antigens had been dissociated by heat treatment prior to reaction with the nitrene have suggested that the 28 000 molecular weight component is not intrinsically unable to react (M. J. Owen, A. Sullivan, and M. J. Crumpton, unpublished observation). The possibility that the lipophilic nitrene is able to probe protein–protein associations within the lipid bilayer is particularly attractive, since if correct it renders feasible a study of changes in such associations during biological reactions. No other method exists at present for investigating this aspect of membrane protein structure.

The potential of various lipophilic, photogenerated reagents for labeling selectively hydrophobic sequences of intrinsic membrane proteins has been explored (Klip & Gitler, 1974; Klip et al., 1976; Bayley & Knowles, 1978a,b; Bercovici & Gitler, 1978; Goldman et al., 1979; Wells & Findlay, 1979a,b). Prior to the present study, two reagents, namely, 5-[^{125}I]-iodonaphthyl 1-azide (Bercovici & Gitler, 1978) and [^{3}H]-adamantylidene (Bayley & Knowles, 1978b; Goldman et al., 1979), appeared especially promising. Thus, as judged by using sarcoplasmic reticulum and human erythrocytes, iodonaphthyl azide preferentially labeled intrinsic proteins. More recently, evidence has been obtained by using several membrane systems that adamantylidene distinguishes effectively between intrinsic and peripheral membrane proteins, labeling only the intramembrane segments of polypeptide chains. The results of analyses of adamantylidene labeling of HLA-A and -B antigens and influenza virus hemagglutinin were especially persuasive. However, the present lipophilic nitrene, which has properties similar to iodonaphthyl azide, gave similar labeling patterns of HLA-A and -B antigens and influenza virus hemagglutinin (Wiley & Skehel, 1978) to the carbene. These results do not, therefore, endorse the previous conclusion that nitrenes are unsatisfactory reagents for preferential labeling of the membrane interior, nor do they provide any striking evidence that carbenes are superior as photochemical labeling reagents. More detailed analyses of labeled proteins, particularly with respect to the exact location of the label relative to the intramembranous segment, are essential before one or the other reagent emerges with clear-cut advantages.

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