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MEMBRANE ASYMMETRY

A SURVEY AND CRITICAL APPRAISAL OF THE METHODOLOGY

I. METHODS FOR ASSESSING THE ASYMMETRIC ORIENTATION AND DISTRIBUTION OF PROTEINS

ABOL-HASSAN ETEMADI

Laboratoire de Biochimie et de Biologie Moléculaire des Lipides Bactériens, Faculté des Sciences de l'Université Paris VI, 12, rue Cuvier, 75005 Paris (France)

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Abbreviations: SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

I. Summary

This and the companion article are aimed at surveying the methods used for the study of membrane asymmetry. The techniques employed for the assessment of the asymmetric distribution and orientation of membrane proteins are reviewed in this article, whereas those pertaining to the unequal distribution of lipids are detailed in the companion paper. The use of immunological techniques and lectins, functions of proteins and their perturbations, chemical reagents, enzymatic isotopic labeling and enzymatic cleavage of membrane proteins and physical techniques are discussed and illustrated using recent examples of their application. Whenever appropriate, problems involving crypticity and non-availability or non-reactivity of functional sites, relevant chemical functions or protein fragments to appropriate ligands, reagents or modifying enzymes are envisaged and possible modification of the exposure of proteins during preparation of ghosts and other drawbacks are discussed, the use of different techniques and control experiments in conjunction is recommended for a more realistic assessment of the distribution and orientation of proteins.

II. Introduction

We owe to Pasteur the recognition of the introduction of molecular dissymetry by reactions taking place in living cells and also the specific use of one of the two enantiomers by living matter when both are available in the environment. The notion of complementarity, reflected in the classical image of 'lock and key', for enzyme-substrate interactions was introduced by Fischer (1894) and then extended by Ehrlich (1906) to immune (antigen-antibody, hapten-antibody) interactions. The complementarity of partners is now recognized as the basis for many biologically important interactions encompassing many of those involving biological membranes. However, though the recognition of molecular asymmetry and its consequences have been long established (see Refs. 1 and 2 for more details and references) to the point where Cornforth [3] could write: "The organic chemist tends to look on asymmetry as something a little extraneous to his scheme of things, as when the successful synthesis of a racemic natural product is incomplete without an often tedious or capricious optical resolution. Perhaps this is why there has so often been speculation on the origin of optical activity as an attribute of life - it seemed to need a special explanation. But there are no racemic molecules; and if the replication characteristic of life is seen in modern terms as an event at the molecular level, it is much more difficult and complicated to imagine either the origin or the continuance of a 'racemic' life than of the lopsided variety that we enjoy", recognition of membrane asymmetry, despite the fact that its acceptance presents no intuitive, particular difficulty – and Cornforth's consideration could well be paraphrazed – is, in its modern sense, more recent. The reason for this seems to reside in the fact that in general for a membrane to be functional, its particular 'acceptor' should be able to interact with a specific 'ligand', which in most relevant cases (hormones, neurotransmitters, antigens, antibodies to surface antigens, etc.) is asymmetrically distributed [2] so that the mere interaction is not proof of its asymmetric orientation and, as mentioned before [2], even a 'functional symmetry' cannot be taken as evidence for 'orientational' symmetry. Thus, the orientational asymmetry of the membrane protein molecules had to be demonstrated since, conversely, it is not possible to accept, a priori, that a device by itself symmetric but shaped by an asymmetric environment (which is, in fact, the situation with biological membranes)

would not act asymmetrically. In this regard, the question as to oligomeric membrane protein components may be raised. Asymmetry of orientation is, however, explicitly or implicitly admitted for a variety of membrane proteins [2,4–6]. The consensus is that for lipids the asymmetry is quantitative and not qualitative, i.e., the number of molecules of a given lipid in the two leaflets of a membrane is different so that the term 'asymmetry' appears somewhat inappropriate and, more pertinently, one could replace it with 'unequal'. This asymmetry or unequal distribution, notwithstanding its degree, can easily be accepted since the reverse, symmetric or equal distribution, would be difficult to accept, particularly because of the asymmetry of the environment on both sides of a membrane with its many consequences [2,5] and because of the asymmetry of membrane proteins [2]. In fact, the unequal distribution of lipids has been observed in many cases such as erythrocytes, platelets and other plasma membranes, intracellular membranes, and bacterial and viral membranes, though, as we will see in the companion paper [2a], contradictory results have also been reported in most cases.

The aim of the present review is to survey the methodology used in assessing membrane sidedness. Emphasis will be placed on salient facts, on the caution that should be exercized in applying the methods and some problems that are likely to be encountered. Care has been taken to include recent pertinent applications so that the reader will not be faced with an indigestible catalog of techniques and will be able to appreciate the advantages gained by workers who have used these techniques. The present article will describe methods for assessing the orientation and distribution of proteins in membranes; the accompanying paper will deal with those related to the assessment of the asymmetric (or unequal) distribution of lipids. Numerous methods are available for the examination of the localization of proteins in membranes, these are: immunological techniques and the use of lectins, the functions of proteins and their perturbations, chemical reagents, enzymatic isotopic labeling of membrane proteins, enzymatic cleavage of these proteins and certain physical techniques. Obviously, some of these methods are interconnected or have been used jointly, but it seems useful to delineate the topics.

III. Immunological techniques and the use of lectins

These techniques take advantage of the properties of antibodies and lectins, acting specifically with antigens or molecules bearing sugar units of a specific nature and configuration.

IIIA. Immunological techniques

Immunological techniques have been used in various ways for assessing the exposure and orientation of membrane proteins. Among these methods are crossed immunoelectrophoresis, immunofluorescence microscopy, immunoelectron microscopy, immunoenzymatic studies and immunoautoradiography.

Some immunological techniques aim at studying the in situ exposure of membrane antigens. The antibody raised against a membrane protein will only interact from that side of the membrane from which it is allowed to approach the antigen. Antibodies, being high molecular weight proteins (approx. 200 000), cannot penetrate the membrane. Different artifices were used; if a membrane protein is weakly antigenic, even when emulsified with Freund's adjuvant, it can be injected as a polymerized protein [22]. If the amount of protein available is small, it can be cross-linked to a non-immune γ-globulin fraction [23].

reacting it with the cells, protoplasts, spheroplasts or lysed leaky cells. Relevant antibodies are complexed, removed by spinning the preparation and will then be missing in immunoplates showing immunoprecipitate lines. Enzymes can be detected in immunoprecipitates by zymogram preparation [8]. In the first experiment [7] which used this technique, antiserum was depleted either with cells or with lysed, washed (leaky) cells and it was found that probably only one of the 20 detergent-soluble proteins of *A. laidlawii*, detected by 20 precipitation lines, was exposed to the outside and only three to the inner face. The analytical value of this technique can be judged by the fact that, upon SDS-polyacrylamide gel electrophoresis, the Tween 20 extract of the membrane of the same organism gave only five bands [9]. A drawback of this method is that if a protein exposed to one side has no antigenic determinant on that side, it will not be detected by antibodies on that side. This is, however, applicable to many other techniques such as the chemical or enzymatic modification of cell surface molecules. Consequently, convergent results from different procedures should be obtained before a definitive assessment is made.

Analysis of *Micrococcus lysodeikticus* membrane using this technique [10] led to the detection of 17 antigens in Triton X-100-solubilized membranes. In all, eight antigens appeared to be at the surface. Two major surface immunogens exposed to the outside were detected. One of these was a succinylated mannan existing as lipomannan in the membrane (for references see Ref. 8). Five of the major membrane antigens unaffected by absorption of antiserum antibodies on intact protoplasts were found to be enzymes by using a zymogram-staining technique. The internal localization of one of these enzymes, the ATPase [8,10], was confirmed on iodination and by the use of ferritin-anti-ATPase labeling [8]. We will return to the latter techniques later in this review.

Other applications of crossed immunoelectrophoresis were used on *Neisseria gonorrhoeae* [11] and *Escherichia coli* [12-14]. In the case of *E. coli*, the outer and inner membranes were examined. 46 antigens were identified in the inner membrane, 12 of which were identified as enzymes by using the zymogram-staining technique and/or by the use of specific antiserum; 25 antigens were detected in the outer membrane, among which were the lipopolysaccharides and the lipoprotein. However, the matrix protein was not detected in the crossed immunoelectrophoretic profile (for the terminology of the outer membrane components of Gram-negative bacteria see Ref. 15).

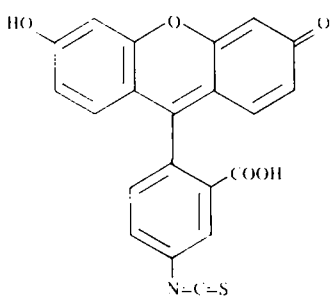
In the case of *E. coli* ML 308-225, analysis was conducted using intact and physically disrupted vesicles. Many enzymes (NADH dehydrogenase, lactate dehydrogenase, dihydro-orotate dehydrogenase, 6-phosphogluconate dehydrogenase, polynucleotide phosphorylase and β-glucosidase) had miminal exposure. An interesting conclusion from these studies was that 95% of the vesicles have the same orientation of molecules as that in the intact cells. The distribution of antigens was asymmetric, indicating that contrary to some other reports [16], the dislocation of components from the inner to the outer surface did not occur to an extent greater than 10% during the vesicle preparation procedure described by Kaback [17-20] and Short et al. [21].

IIIA-1. Crossed immunoelectrophoresis

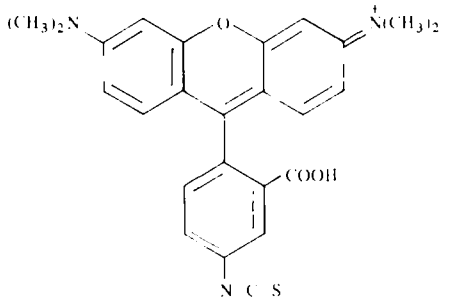
This technique was first applied by Johansson and Hjertén [7] to *Acholeplasma laidlawii* membrane. The principle of this technique is to prepare antiserum to membrane proteins. Detergent-(Tween 20 [7] or Triton X-100 [8])-solubilized membrane proteins are then used as antigens and analyzed by two-dimensional immunoelectrophoresis. The total antiserum is then depleted of antibodies to the exposed antigenic determinants by

IIIA-2. Immunofluorescence microscopy

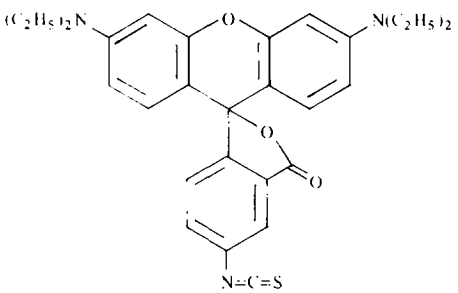
In this method, the antibody is covalently coupled to a fluorochrome such as fluorescein isothiocyanate (green fluorescence) or tetramethylrhodamine isothiocyanate (red fluorescence). Other rhodamine derivatives which have been used are rhodamine B isothiocyanate and lissamine rhodamine B sulfonyl chloride [24,25]. Isothiocyanate derivatives react covalently with the free amino groups of proteins, either the ϵ-amino groups of lysine residues or those of N-terminal residues, and form thiocarbamide (thiourea) derivatives (see below). Lissamine rhodamine B sulfonyl chloride reacts with free amino groups to form sulfonamide derivatives; sulfonyl chlorides react also with phenolic hydroxyls.



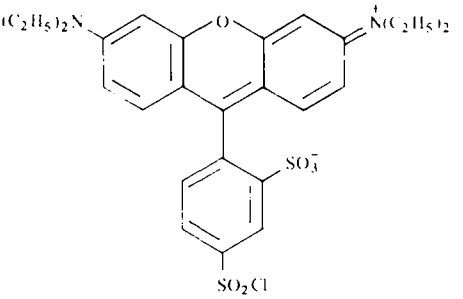
fluorescein isothiocyanate



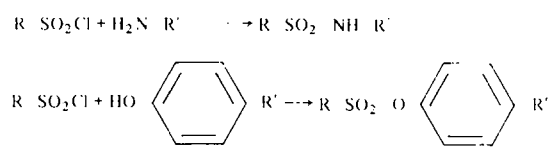
tetramethylrhodamine isothiocyanate



rhodamine B isothiocyanate



lissamine rhodamine B sulfonyl chloride



The fluorescent antibody is then reacted with the surface antigen and the preparation examined by light microscopy.

This technique has often been used for the study of the aggregation of surface proteins (patching, capping) which demonstrates their lateral mobility [26–30] and, in some cases, surface molecules were directly labeled for this purpose [31]. A variant of the immunofluorescence technique is the use of double immunofluorescence, which has found useful applications in the study of the asymmetric distribution of membrane proteins. Examples of the use of this method will be given after we have examined the indirect method of labeling surface molecules.

III.A-3. Immunoelectron microscopy

Immunoelectron microscopy uses markers which, when conjugated to antibodies raised against surface antigens, are detectable with the electron microscope. Markers may be recognized by their high electron density as in the case of ferritin [32–37] or by their shape as in mollusc hemocyanins [38,39], viruses [40–42] and spherical acrylic latex [42,43]. The antibody may be directly conjugated to or indirectly [44] labeled with the various markers. The direct method (Fig. 1A), apart from its simplicity, has the particular property that the number of antibody molecules determines the number of markers bound to the surface. This method allows the detection of more than one antigen if different markers are conjugated to specific antibodies raised against different surface molecules. However, since the yield in chemically synthesized active conjugate may be low when only small amounts of antibody or low titer antisera are available, the indirect methods may be used. Different means of indirect labeling exist (see Fig. 1B–F); the simplest is shown in Fig. 1B. Indirect methods used for immunoelectron microscopy can be adapted to immunofluorescence or immunoenzymatic studies (see below for examples). A recent application of the indirect method was in the determination of the membrane location of a 15 000 dalton protein of the outer membrane of *Salmonella minnesota*, which was purified to homogeneity [45]. The localization of this protein, which is bound by ionic forces to phosphate or pyrophosphate of the lipopolysaccharides, was determined by using the antibodies raised in a rabbit. Its even distribution at the cell surface was assessed by using ferritin-conjugated antibodies (direct method) or ferritin-conjugated goat antibodies to rabbit IgG that react with the cell surface after non-marked antibodies (raised in rabbit) are fixed. Another example of the use of indirect immunoelectron microscopy is the demonstration of the presence of an antigen on the cell surface of chick embryonic erythrocytes. Goat anti-rabbit IgG were conjugated to hemocyanin and the specific antibodies were prepared in rabbit. The expression of this antigen diminished with age so that it was no longer detectable at 7 months [46].

A recent and interesting application of the indirect method concerns studies of the degree of coverage of the inner surface of erythrocytes and reticulocytes by spectrin. The lateral mobility of integral proteins of the erythrocyte membrane is known to be restricted [47–49]. In erythrocyte ghosts, particles can be aggregated under particular experimental conditions such as pH 5.5 [50]. However, membrane receptors for concanavalin A, and blood group A antigens when assayed with the respective ligands, can be clustered to a limited extent if neonatal erythrocytes are used [51]. Experiments using ferritin-labeled concanavalin A show that clustered regions can be formed in membrane invaginations and endocytotic vesicles. It was suggested that in neonatal erythrocytes discrete domains exist or were induced, within which lateral mobility can take place. Recent experiments [51] using the indirect method and intracellular immunoferritin staining led to interesting results. Antispectrin antibodies were prepared in rabbit and after reacting these antibodies in situ on ultra-thin frozen sections [52,53], ferritin-conjugated goat anti-rabbit Ig were allowed to label regions covered with spectrin. It was shown that membrane invaginations and endocytotic vesicles of concanavalin A-treated neonatal human erythrocytes and reticulocytes were differentiated from unperturbed regions by the absence of spectrin labeling [51]. This observation is in keeping with the generally accepted concept that the spectrin complex forms a 'scaffolding' under the erythrocyte membrane [48,54,55]. The use of ferritin-labeled concanavalin A (see below) and transmission electron microscopy of thin sections showed that endocytosis was induced in rabbit reticulocytes and that this induction decreased with reticulocyte maturation [56]. It

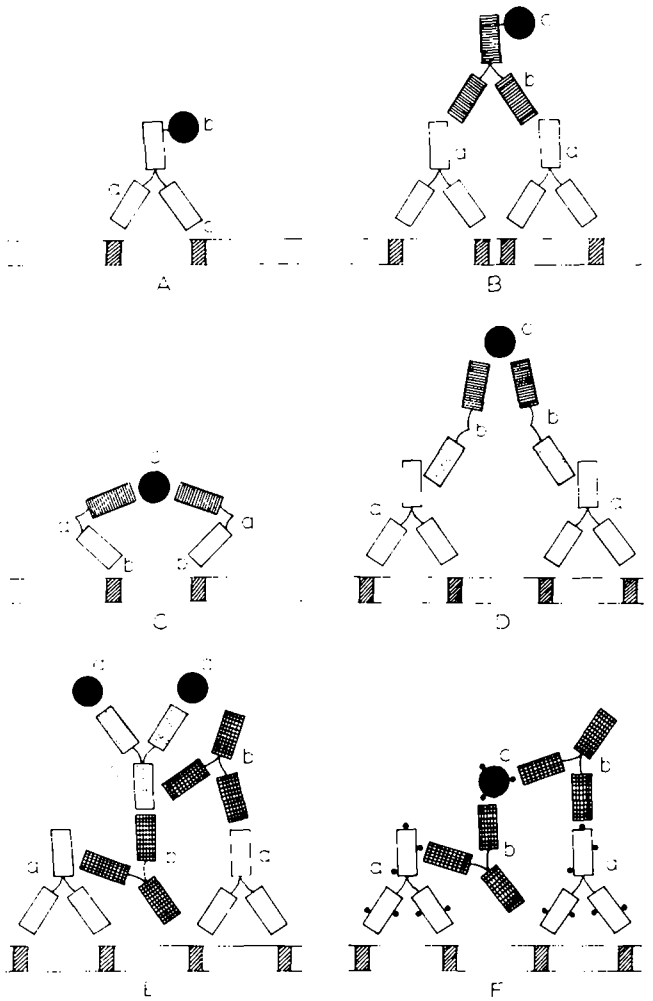


Fig. 1. (A) Direct method. The antibody (or the lectin) (a) is itself labeled (b) and reacts with the surface antigen (or receptor) (c). (B) Indirect method. The antibody (a) to the membrane antigen is itself not labeled but the antibody (b) to it is conjugated with a marker (c). For example, goat or sheep antibodies are raised against the immunoglobulins of the animal which will serve for preparation of antibodies to the surface molecules and the former antibodies are labeled. The staphylococcal protein A can be used for conjugation. (C) Indirect method (hybrid antibody method). Each hybrid antibody molecule (a) (see Ref. 73) reacts with a surface protein (b) and with the marker molecule (c). Note that the hybrid antibody is linked monovalently to the surface molecule and that the complex is henceforth relatively fragile. Note also that no chemical coupling is carried out. (D) Indirect method (hybrid antibody bridge method). An antibody (a) is directed to the surface antigen, then a hybrid antibody (b) reacts with this antibody and with the marker (c). Here again no chemical coupling is carried out. (E) Indirect method (mixed antibody bridge method). An antibody (a) to the cell surface molecules is allowed to react, followed by an antibody (b) directed to the first one, then an antibody (c) to a marker (e.g., ferritin, viruses, enzymes); c being prepared from the same animal species as a then reacts with b and with the marker. The binding of antimarker antibody (c) depends upon the sites on the bridging antibody (b) left after its reaction with antimembrane antibody (a). Thus, a quantitative relationship between membrane-bound antibody and the marker molecules might not exist. The staphylococcal protein A can replace the b antibody. (F) Indirect method (hapten bridge or hapten sandwich method). The antibody (a) to the cell surface molecule is allowed to react, this antibody being previously conjugated to a hapten (•). Then an anti-hapten antibody (b) is reacted. The marker (c) (ferritin, hemocyanin or a small virus) is also conjugated with the same hapten; it is possible to use a fluorescent anti-hapten antibody (see Ref. 85).

was suggested that the progressive decrease in concanavalin A receptor mobility and in endocytosis with the reticulocyte maturation might be related to the 'elimination of gaps or imperfections in the spectrin scaffolding' [56]. It is worthwhile mentioning in this respect that the involvement of spectrin in the lateral mobility of human erythrocyte transmembrane proteins has been challenged [57] on the basis of the observation that low concentrations of Triton X-100 aggregate ghost intramembrane particles under conditions in which spectrin is neither extracted nor precipitated. This indicates that the precipitation of this protein is not a prerequisite for particle aggregation. On the other hand, particles of ghosts from which extrinsic proteins (including spectrin) were thoroughly removed (alkali treatment) could still undergo aggregation by such procedures as the modification of ionic strength or pH, or by the addition of Ca^{2+}. In recombination experiments with vesicles of different lipids, the mobility of detergent-extracted erythrocyte proteins in the above-mentioned methods depended on the nature of the lipids used (for more discussion and, in particular, concerning the suggested role of spectrin in stabilizing membrane lipids, see Ref. 57 and references therein).

A double-immunofluorescence method, using the indirect method of labeling of surface molecules, was recently employed for the study of the location of α-actinin in murine lymphocytes and its correlation with certain surface molecules. α-Actinin, a component of the striated muscle Z-discs [58–60], has been detected in non-muscle cells at the attachment sites of microfilaments to membranes [61–64]. It has also recently been detected in murine lymphocytes associated with the membrane and its location and correlation with surface components were studied by using immunofluorescence and double-fluorescence techniques [65]. Murine lymphocyte α-actinin was found to be inaccessible to lactoperoxidase-catalyzed surface iodination (see below for details on this technique); however, it was associated with isolated membranes and could be immunoprecipitated from the membrane detergent lysates by anti-α-actinin antibodies directed against skeletal muscle α-actinin. The absence of exposure of the α-actinin antigenic determinants at the external surface was further substantiated by preparing an anti-membrane antiserum and reacting it with fixed murine lymphocytes. Anti-α-actinin antibodies of this serum were not fixed to the cell surface. The association of α-actinin with the internal surface of the membrane and its correlation with surface receptors was confirmed by using a combination of direct and indirect methods. Murine spleen lymphocytes were treated with rhodamine-conjugated goat antiserum against murine immunoglobulins which promotes patching and capping. At intervals, samples of the preparation were fixed first by paraformaldehyde, then with absolute alcohol. The sample was then incubated with anti-α-actinin antibodies prepared in rabbit followed by incubation with fluorescein-conjugated goat antiserum against rabbit IgG. It was observed that α-actinin co-capped with surface immunoglobulins of lymphocyte cells [65]. In a parallel experiment, T-cells were separated from peripheral lymph nodes and, in some assays, enriched by filtration through a nylon-wool column [66]. These cells were then incubated with anti-Thy-1,2 alloantiserum and then with fluorescein-conjugated goat antiserum against murine immunoglobulins. In this case, α-actinin was revealed by treatment with rabbit anti-α-actinin antibodies followed by rhodamine-conjugated goat antiserum against rabbit Ig [65]. Here again, α-actinin co-capped with Thy-1 antigent. These experiments show that α-actinin remained stably associated with surface immunoglobulins and Thy-1 antigen for up to 30 min. Other experiments had previously revealed that α-actinin co-capped with other lymphocyte surface antigens [67].

An interesting variant of the indirect method is to use a staphylococcal protein called

protein A. This protein, a component of the cell wall of different strains of *Staphylococcus aureus*, binds to the FC fragment of immunoglobulins and presumably plays a role in the modulation of opsonization of bacteria. This peculiar and useful protein can be marked, for example, with ferritin, and used as a substitute for antibodies (in the previous examples, goat antibodies) to the Ig of the animal (rabbit in the previous examples) in which antibodies to surface molecules are prepared [68] *.

Another indirect method for linking a marker to a surface molecule is the use of hybrid antibodies (see Fig. 1C) [73] which generally involves the Fab′ fragment of two antibodies (for details on the fine structure of antibodies see, for example, Refs. 74–76). Here again, different types of marker can be linked to the surface molecules, e.g., ferritin [77], viruses [41] and enzymes [78].

The hybrid antibody bridge method (Fig. 1D) is another indirect method which was used, for example, to assess the presence of IgE on basophil lymphocytes. Human basophil lymphocytes could be marked by hybrid antibody molecules directed against anti-IgE and ferritin after treatment of cells with anti-IgE antibodies [79]. In this method, since no covalent binding of the markers is involved, discrepancies with direct methods during quantitative studies of surface molecules may appear [41].

Yet another indirect method is the mixed antibody method (Fig. 1E). Again, different markers can be used, e.g., ferritin, viruses or enzymes (see, for example, Ref. 80).

Still another indirect method is the hapten bridge or hapten sandwich method (Fig. 1F) [81–85]. The latter method can be adapted for simultaneous labeling of multiple cell surface antigens for fluorescence and electron microscopic studies or in circumstances where amplification is required for observing alloantigens [81–85].

IIIA-4. Immunoenzymatic detection

In this method, antigens are detected by using the conjugate of an antibody with an enzyme. The antigen corresponding to the antibody can then be located by detection of the complex, taking advantage of the catalytic property of the enzyme [86–90]. The most widely used enzymes are peroxidases (particularly horseradish peroxidase) and alkaline phosphatase. With peroxidases, for example, using diaminobenzidine (3,3′,4,4′-tetraaminobiphenyl) and H_2O_2 as substrates, an amorphous polymer is formed, which can be stained by post-fixation with OsO_4. The reaction is the same as that for the cytochemical detection of peroxisomes where the peroxidase activity of the microbody catalase is detected [91–93]. This method has the advantage that the same preparation can be examined by either light or electron microscopy. It appeared suitable for ultrastructural studies of antigen distribution after making permeable surface membranes. Horseradish peroxidase (molecular weight 40 000), for example, is much smaller than electron-dense markers such as ferritin (molecular weight 445 000) [33,37,78,87] and therefore, penetration of antibody-enzyme conjugate into the cell is facilitated [78,87]. Besides the

* Protein A found other applications. Covalently linked to agarose, it was used to purify rabbit, human and murine IgG [69]. Protein A-bearing staphylococci can be used to adsorb IgG and to separate immune complexes in solution [70,71]. Recently, it was observed that the binding of antigen to immunoglobulin-bearing staphylococci is unaltered with respect to affinity and capacity in comparison with the binding of antigen to soluble immunoglobulin; thus, protein A-bearing formalin-treated, heat-inactivated *Staphylococcus aureus* binds rabbit ^{125}I-labeled IgG with a high affinity (K_d = 10^{-10} M). The affinity and capacity of the IgG for the antigen are preserved and it can be used as a primary solid-phase immunoadsorbant [72].

direct marking of antibodies with enzymes, indirect labeling by the hybrid antibody method and mixed antibody bridge method [41,86] is possible.

In a recent application of an enzyme immunoassay, Avrameas et al. [90] studied quantitatively the immunoglobulin antigenic determinants on the murine B and T lymphocytes. Fab fragments of anti-κ, anti-γ and anti-μ immunoglobulins were conjugated to *E. coli* β-galactosidase. Measurement of the enzyme activity was made with a fluorescent substrate (4-methylumbelliferyl-β-D-galactopyranoside). From 0 to 79 000 membrane Ig molecules per lymphocyte could be measured, spleen lymphocytes being richer in surface immunoglobulins than lymph node lymphocytes. On average, B cells carried 2–2.5-fold more κ and γ antigenic determinants, but interestingly, T cells were also provided with these determinants. Moreover, when these T cells were stripped from antigenic determinants they were found to be able to synthesize immunoglobulins. Another recent use of the immunoenzymatic reaction combines this technique with the enzyme-catalyzed chemical labeling of surface molecules. Here, an enzyme that catalyzes the chemical labeling of surface molecules is linked to antibodies to surface antigens. I will discuss below an example in which immunolactoperoxidase was used for this purpose [94].

IIIA-5. Immunoautoradiography

This method uses isotopic labeling (^{3}H, ^{14}C or ^{125}I) of antibodies. The labeled antibodies are allowed to react with the membrane preparation and the location of the membrane antigen is examined using autoradiography. This technique has found some applications [95–97] but isotopic labeling, especially with ^{125}I, is more generally used directly on the membrane [96], often in order to label the exposed molecules for further analysis (see below). In some cases, the labeled antibodies are allowed to react with the surface antigen, then the preparation is spun and the radioactivity counted [23].

IIIB. The use of lectins

Lectins are polyvalent proteins that react specifically with surface glycoproteins or glycolipids [98–100] *. The direct and indirect techniques mentioned above for marking surface molecules with antibodies can also utilize lectins. For example, in an application of the direct method, Nicolson and Singer [101] directly conjugated ferritin to concanavalin A, and by using a technique for preparing flattened, permeable membrane specimens, they could examine both sides of the plasma membrane of erythrocytes as well as of a variety of other mammalian cells. Cells including lymphocyte, lymphoma, myeloma, normal and spontaneously or virally transformed fibroblast cells were examined using this technique and in each case the ferritin conjugate could be localized only on the outer surface [101]. Thus, the sidedness of mammalian cell surface saccharides could be assessed.

* First isolated from plants, lectins were also found in other sources (bacteria, social amoebae, snail, electric eel, hepatocytes, etc.). Chemically, they constitute a heterogeneous family of compounds with different molecular weights (120 000, with four subunits for the bivalent agglutinin of soybean which is a glycoprotein; 36 000, with two subunits for the tetravalent lectin of wheat germ). They have in common the property of binding specifically to surface glycoproteins or glycolipids of different cell types and agglutinating them. The specificity of lectins stems from their specific interaction with sugar units. Thus, the jackbean hemagglutinin, concanavalin A, is specific for α-D-mannopyranosyl-like residues. The *Ricinus communis* lectin, ricin, reacts specifically with α-D-galactopyranosyl-like residues and the agglutinin of wheat germ is specific for *N*-acetylglucosamine [98–100].

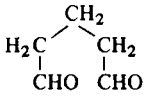
As for antibodies, different markers such as ferritin, enzymes and iron-dextran can be covalently linked to lectins. It should be mentioned, however, that good use was made of the fact that some marker molecules are themselves glycoproteins and, therefore, are able to bind lectins for indirect labeling of surface glycoproteins. Thus, if a sufficient excess of a lectin such as concanavalin A is reacted with a membrane surface receptor so that the valences of its molecules remain partially unreacted, these free valences can link the glycoprotein markers. Horseradish peroxidase [102,103], hemocyanin [104,105] and iron-dextran [106] have been non-covalently linked using this method.

Experiments in which tagged lectins are used are numerous. Fluorescence-labeled lectins were employed to measure the mobility of ligand-receptor complexes by photobleaching [107–110]. Taking advantage of the presence of lectin receptors on lymphocyte cell membranes, immobilized lectins were used to fractionate calf thymocyte plasma membrane vesicles: it could thus be observed that specialized membrane enzymes were enriched in retained (mitogen receptor-rich) vesicles whereas other enzymes were more or less evenly distributed in retained and non-retained vesicles, revealing the 'mosaicism' of the cytoplasmic membrane [111,112]. In other experiments, the heterogeneity of Thy-1 lymphocytes could be demonstrated [113]. Other observations indicate the presence of lectins on the cell surface of hepatocytes and their implication in cell adhesion [114–117]. Social amoebae are known to aggregate upon starvation as a result of a chemotactic signal [118–120]. Migrating cells acquire the ability to adhere to each other [118–121]. Proteins have been implicated in competence acquisition, since trypsinization inhibits aggregate formation [122]. A recent analysis has demonstrated the quantitative and qualitative changes that take place during differentiation of *Dictyostelium discoideum* [123]. Some studies indicate the appearance of surface sites for intercellular adhesion during the differentiation period [124]. If antibodies are raised against whole cell surface lysate of differentiated *D. discoideum*, Fab fragments of these antibodies inhibit cell cohesion [124]. However, Fab fragments of antibodies against *Polyspondilium pallidum* prepared under the same conditions do not inhibit adhesion of *D. discoideum* [125]. The cohesion of differentiated cells is not sensitive to EDTA, but that of vegetative cells is sensitive to the action of this chelating agent [126,127]. Modification of lectin-binding glycoproteins of the social amoebae, *D. discoideum*, during successive developmental stages was studied by using radioactively iodinated lectins applied to SDS-polyacrylamide gels of solubilized cell membranes [128]. In addition, in *D. discoideum*, two lectin-like compounds (discoidins I and II [129,130]) and in *P. pallidum*, three isolectins called pallidins were identified [131–133] which are believed to be involved in cell adhesion. Interestingly, 'affinity matrices', i.e., simply Sepharose 4B or 6B polymers containing D-galactose, were used during the purification [131–133]. Sepharose 4B-isolated lectins from *Dictyostelium purpureum* constitute up to 5% of the total soluble proteins of cohesive cells [134]. The surface exposure of discoidins [135] was assessed using immunofluorescence and immunoferritin-labeling techniques. In these studies, the indirect method of labeling was used, surface discoidin was reacted with antibodies prepared in rabbit, and then fluorescein or ferritin-labeled goat anti-rabbit immunoglobulins were used [135]. By means of this technique, the diffuse distribution and the lateral mobility of discoidins in the cell surface could be assessed [135]. Similar observations were made on the distribution and mobility of pallidins in the cell surface of *P. pallidum* [136]. Qualitative and quantitative changes take place during differentiation. Vegetative cells of the social amoebae bind lectins with no apparent species specificity. After differentiation, a 20-fold greater affinity of cells for discoidin and pallidin is observed [137]. Interestingly, a lipid component

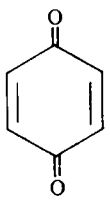
appeared to be necessary for the carbohydrate binding site of discoidin to be functional [138]. Thus, purified discoidin lacked the agglutination activity it exerts, as a lectin, upon erythrocytes. The addition of aqueous dispersions of a $CHCl_3/CH_3OH$ extract of *D. discoideum* particulate fraction to the purified discoidin restores this agglutination activity. Analysis of extracted lipids suggested that an unsaturated fatty acid may be involved and of many lipids assayed, *cis*-vaccenic acid ($18:1\Delta^{11}$) and oleic acid ($18:1\Delta^{9}$) at high concentrations, exhibited significant reconstitution activity [138]. Lectin binding sites were found on the surface of the cells [139,140], but their correlation with the receptor for discoidin and pallidin is not clear [141]. Studies on cell adhesion of embryonic retinal and tectal cells give equally interesting examples of the involvement of lectin receptors in cell adhesion [142]. Concanavalin A was observed to prevent cell adhesion. Fluorescence microscopy allows the visualization of the redistribution of concanavalin A-receptor complexes. The filopodia flattened regions of growth cones and edges of axons were found to be free of receptors. These regions are thought to be sites of adhesive contact between cells and between cells and artificial substrata (see Refs. 60,143–145 and references therein). In other studies, the use of ferritin-conjugated *R. communis* I agglutinin indicated that lectins mediate agglutination of murine S49 lymphoma cells which grow singly in suspension and do not form homotypic adhesion [146].

IIIC. Preparation of conjugates and hapten derivatives

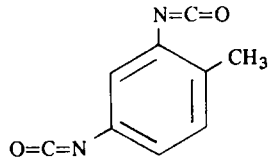
The preparation of conjugates for immunofluorescence, immunoelectron microscopy or immunoenzymatic studies, as well as the preparation of lectin conjugates and more generally the cross-linking of proteins, make use of a variety of bifunctional coupling reagents. Glutaraldehyde [86,87,147], benzoquinone [88,148], toluene-2,4-diisocyanate, *m*-xylylenediisocyanate [33,40] and bis(4-fluoro-3-nitrophenyl)sulfone [68] are a few of these reagents. Aldehydes react with the free amino groups of proteins to form Schiff bases. This is the principle of the use of glutaraldehyde for conjugation of proteins. However, the situation is much more complicated (for a discussion see Ref. 147). The use of benzoquinone has recently been introduced in conjugation studies [88,148].



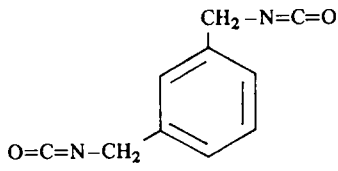
glutaraldehyde



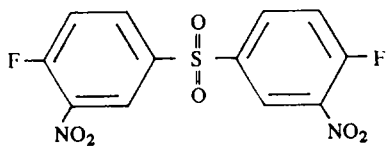
benzoquinone



toluene-2,4-diisocyanate



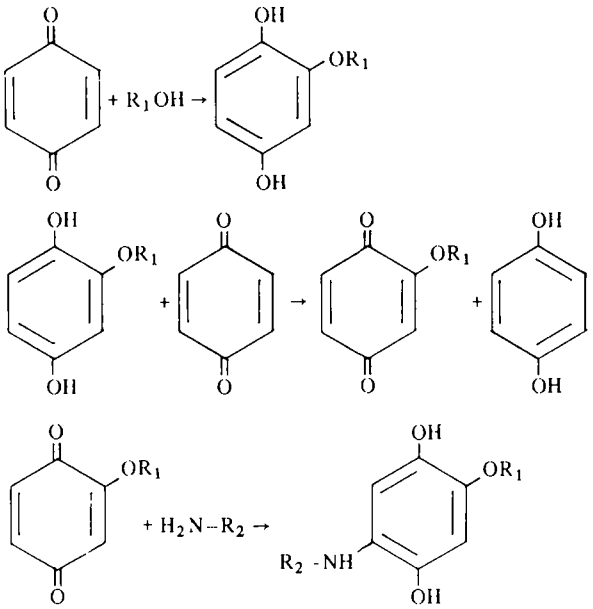
m-xylylenediisocyanate



bis(4-fluoro-3-nitrophenyl)sulfone

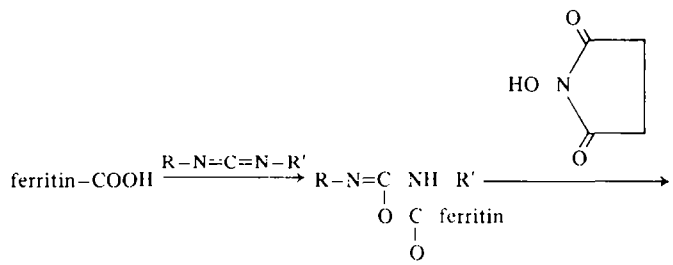
The suggested mechanism for the covalent attachment of proteins to polysaccharide

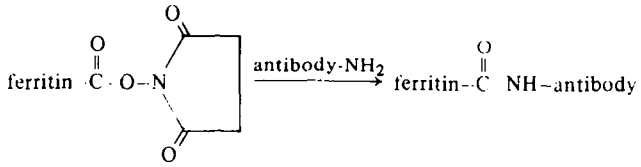
carriers (agarose; cross-linked dextran) involves an activation step and a coupling step [148]. Benzoquinone is then used for coupling proteins to horseradish peroxidase [88].



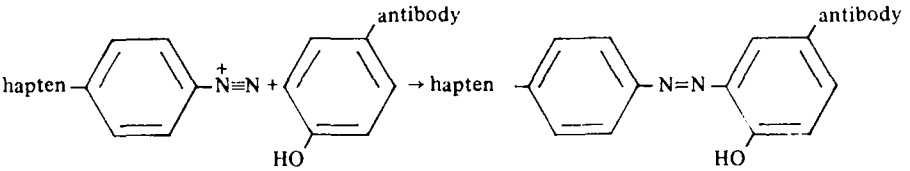
Isocyanates are able to react with the free amino group of molecules and therefore diisocyanates conjugate two suitable molecules [33,36]. Bis(4-fluoro-3-nitrophenyl)-sulfone, which also links two amino groups, has been used during the conjugation of staphylococcal protein A to ferritin [68].

Methods in which the conjugate formation is carried out in a single step lead inevitably to the formation of aggregates, alteration of antibodies and inter- and intramolecular reactions within each coupled compound. These are some of the reasons which led workers to carry out the two-step coupling technique. For example, ferritin is first reacted with glutaraldehyde in such a way that one of its reactive functions remains free; then in a second step, the antibodies react with this 'activated' ferritin [149,150]. Horseradish peroxidase has been conjugated to antibodies using this technique [151,152]. The two-step method has also been used for conjugation with diisocyanates [33,36,153]. An interesting technique for the conjugation of ferritin to antibodies is called the three-step method [85] and has been developed by Kishida et al. [149]. In this method, all the free amino groups of ferritin are first succinylated by treatment with succinyl anhydride, then the molecule is succinylated on its carboxyl groups by using *N*-hydroxysuccinimide and a water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride). The active ester then reacts with the antibody [149].



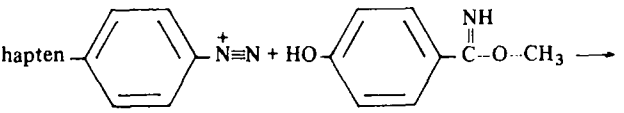


Haptens which have been used in the hapten bridge method are *p*-azophenyl-β-D-lactoside, *o*-azophenyl-β-D-galactoside and *p*-azophenyl arsonate [82,95], fixed by using the diazonium method, or ε-*N*-2,4-dinitrophenyllysine [82] formed by the reaction of dinitrofluorobenzene or dinitrobenzenesulfonate. The diazotation reaction is shown below. The diazonium procedure also labels amine and histidine groups of proteins. A variant of the technique allowing the labeling of only the amino groups is to convert the diazo derivative of the hapten into an imodoester, for example, by reaction with methyl-*p*-hydroxybenzimidate, or methyl-3,5-dihydroxybenzimidate. The azo-coupled methyl-*p*-

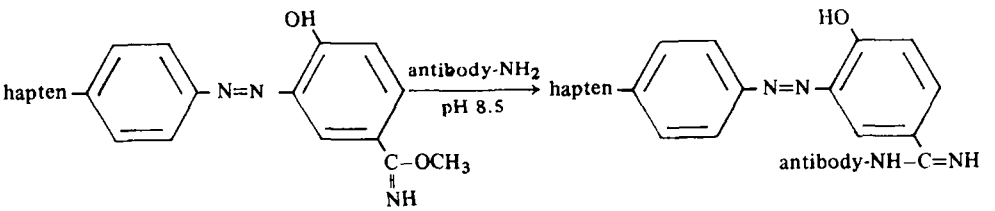


diazophenyl hapten

hydroxybenzimidate or methyl-3,5-dihydroxybenzimidate imidoester obtained is then allowed to react with the antibody [84,154]. Thus, histidine and tyrosine residues of proteins are not involved (see also below).



diazophenyl hapten methyl-*p*-hydroxybenzimidate

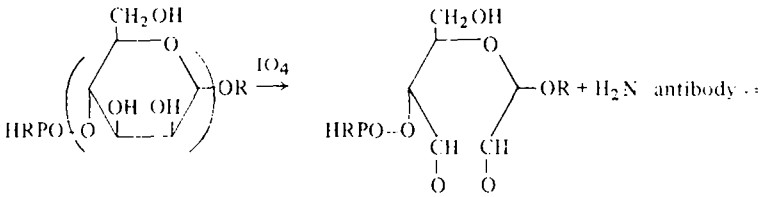


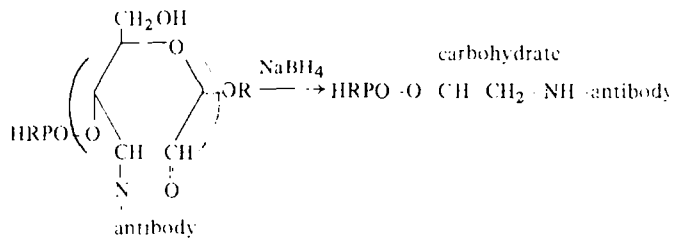
hapten methyl-*p*-hydroxybenzimidate (amidinating reagent) hapten-antibody conjugate

The hapten-antibody conjugate is then available as a 'first-layer reagent' to label cell surface antigens. The conjugate is able to react to a high degree with anti-hapten antibodies which react in turn with markers tagged with the same hapten (Fig. 1F). Recently, an improved procedure leading to increased solubility of the conjugate was described [155].

A method used during the conjugation of horseradish peroxidase is the periodate oxi-

dation of the enzyme [89,152]. This is possible as this peroxidase is a glycoprotein bearing sugar units with vicinal OH groups, which are converted upon oxidation into aldehydes. Oxidation is carried out on the fluorodinitrobenzene-blocked peroxidase [89], then the horseradish peroxidase aldehyde forms a Schiff base with the antibody to the surface antigen. The conjugate is stabilized by $NaBH_4$ treatment. After purification, two enzyme molecules were found to be linked to each antibody molecule [89,152]. The reactions are represented schematically as follows [85]:





Immunolatex studies have used cross-linked latex spheres ranging in diameter from 300 to 3400 Å. The small-diameter spheres (less then 400 Å) can be used for transmission electron microscopy and the large-diameter spheres for scanning electron microscopy [85]. Acrylic spheres are copolymers of methacrylate derivatives containing hydroxyl and carboxyl groups. These functions in the polymer allow their versatile use for coupling; for example, they can first be labeled by a fluorescent or a radioactive marker using CNBr:

$$HO\text{-latex-}COOH \xrightarrow[CNBr]{X} X\text{-latex-}COOH \qquad (X = \text{fluorescent or radioactive marker})$$

then conjugated to an antibody according to the following scheme using a diamine:

$$X\text{-latex-}COOH \xrightarrow[\text{carbodiimide}]{H_2N-(CH_2)_7-NH_2} X\text{-latex-}CONH\text{-}(CH_2)_7\text{-}NH_2 \xrightarrow[\text{glutaraldehyde}]{1} \xrightarrow[\text{antibody}]{2} X\text{-latex-CO-NH-}(CH_2)_7\text{-NH}\sim\text{antibody}$$

or an ω-amino acid [42].

$$X\text{-latex-}COOH \xrightarrow[\text{carbodiimide}]{H_2N\text{-}(CH_2)_7-COOH} X\text{-latex-CO-NH-}(CH_2)_7\text{-}COOH \xrightarrow[\text{carbodiimide}]{\text{antibody}}$$

$$X\text{-latex-CO-NH-}(CH_2)_7\text{-CO-NH-antibody}$$

Obviously, besides antibodies, lectins and other proteins can be tagged by similar reactions [156]. It is worthwhile mentioning that antigen tagging can be performed by simple adherence of polymethyl methacrylic plastic particles coated with antibody. The coating was effected by simple incubation of particles (120 min at 24°C) with the antibody [43].

IV. Functions of proteins and their perturbations

If a membrane protein has a particular function as an enzyme, transporter, receptor, etc., the assessment of its asymmetric orientation may be aided by taking advantage of this function and/or modifications which it will undergo asymmetrically. However, as mentioned in Introduction, this cannot always be taken as an absolute proof.

The latency or inactivity of enzymes may be interpreted as indicating an unsuitable orientation of their active site or their asymmetric distribution. This approach has been used in studies on the organization and orientation of components of mitochondrial inner membrane complexes and of chloroplast photosystems. Thus, mitochondrial NADH dehydrogenase (an FMN enzyme [157]) and succinate dehydrogenase (an FDN enzyme [158]) have their catalytic site exposed to the matrix side, since their respective substrates react only from this side. Cytochrome *c* is a peripheral protein of the inner mitochondrial membrane and it is well known that it is easily released from mitochondria [159,160]. It should then have an intermembrane exposure (for more details see, for example, Ref. 161) and, accordingly, be accessible to ferricyanide, an artificial electron acceptor with a high redox potential ($E = 430$ mV) which reacts with electron donors such as cytochrome *c* and flavin-linked dehydrogenases [162]. The use of ferricyanide confirms that glycerophosphate dehydrogenase, the substrate of which (glycerol phosphate) is impermeant, is localized on the outer leaflet of the inner mitochondrial membrane and that succinate dehydrogenase is localized on the inner leaflet facing the matrix compartment. Mitochondria contain, besides the above-mentioned NADH dehydrogenase of the inner leaflet, an NADH (NADPH) dehydrogenase exposed to the intermembrane compartment. The use of ferricyanide allows the recognition of these enzymes. Although both enzymes can reduce ferricyanide in the presence of their substrates, the reduction of external ferricyanide through the internal dehydrogenase implies the oxido-reduction chain: internal dehydrogenase-ubiquinone-cytochrome *b*-cytochrome c_1-cytochrome *c*-ferricyanide, and this pathway is inhibited by antimycin. However, if inside-out vesicles of mitochondria are formed [161–167], then external ferricyanide can be reduced by internal NADH dehydrogenase in the presence of substrate and this reduction is not inhibited by antimycin [162].

Antibodies may be used to inhibit or modify the function of membrane components. Thus, antibodies raised against cytochrome *c* and the inner mitochondrial membrane ATPase, F_1 (see also below), react in such a way that the first can be located on the external leaflet of the inner mitochondrial membrane exposed to the intermembrane compartment and the second, on the internal leaflet, exposed to the matrix side. However, antibodies against cytochrome oxidase reach this complex from both sides of the membrane [163].

When appropriate particles (N-particles) of bovine heart mitochondrial membrane which are deficient in F_1 factor were fortified with yeast F_1, phosphorylation was inhibited by antibodies against bovine F_1. However, although antibodies against yeast F_1 inhibited the phosphorylation in yeast particles containing yeast F_1, they were not operative in the hybrid particles. The action of antibodies against yeast F_1 inhibited its ATPase activity. The yeast F_1, the ATPase activity of which was inhibited by antibodies, still stimulated the function of the residual F_1 of bovine heart mitochondria which by itself was insufficient to carry out the phosphorylation. Thus, it appeared that yeast ATPase has two roles, a structural role which is not inhibited in the hybrid particles and a catalytic role which is susceptible to antibodies [165].

NADP, the final natural electron acceptor of chloroplast photosystem I (PSI) as well as artificial acceptors like ferricyanide and quinone compounds, can be reduced by thylakoid membranes [168–172]. The reduction of NADP is hampered by antibodies raised against ferredoxin-NADP oxidoreductase [173], indicating that the active site of the enzyme is exposed to the chloroplast stroma. However, this antibody does not precipitate chloroplasts, but if the chloroplast coupling factor (CF_1) is eliminated by EDTA treatment then the agglutination occurs [173]. This constitutes a particular example of crypticity, since the antibodies reach the enzyme molecule and inhibit its function. However, they cannot cross-link membrane preparations as if the enzyme were localized in a cleft, in the neighborhood of the coupling factor [168]. The exposure of the enzyme was further confirmed in an interesting way. Its substrate (ferredoxin) binds and forms a stoichiometric complex with the reductase. Antibodies to ferredoxin were found to precipitate chloroplasts, indicating the exposure of ferredoxin as well as that of ferredoxin-NADP reductase [174]. The exposure of NADPH-producing enzyme as well as that of CF_1 in chloroplasts is such that both NADPH and ATP are liberated in the stroma compartment [175–177] where they are attacked by enzymes of the Calvin cycle.

It is worth recalling that in bacteria, the exposure of the coupling factor BF_1 is cytoplasmic [172,178,179]. Therefore, protons ejected externally from the cell during respiration, on returning to the cell, allow the synthesis of ATP by BF_1 following the premises of the chemiosmotic hypothesis of Mitchell [180,181] (see also Boyer et al. [182]). ATP is propelled into the cytoplasm where it is used. In mitochondria, the situation differs; the coupling factor F_1 is oriented towards the matrix side [161,172,182], equivalent to the interior of bacteria, but remote from the cytoplasmic compartment where protons are liberated. The backward movement of protons to the matrix allows the synthesis of ATP by F_1. ATP is thus formed on the matrix side, then a transporter exchanges ATP molecules with ADP from the cytoplasm [183–190]. Here, we have an example of a transporter which has an asymmetric exposure [183,184] in the membrane. As reported by Vignais [183], a notable aspect of the function of this transporter is that the ATP synthesized is not mixed with the pool of the matrix ATP, thus indicating that the transporter may be located near the F_1 factor. The adenine nucleotide transporter has been recently purified [188,189]. The fact that its external site has a higher affinity for ADP and its internal site a higher affinity for ATP already indicates that at least this transporter behaves asymmetrically [183]. The use of inhibitors confirms the asymmetry. Non-permeant inhibitors such as atractyloside, carboxyatractyloside and acyl-CoAs act on external sites and bongkrekic acid, a permanent inhibitor, on internal sites. Atractyloside and carboxyatractyloside do not inhibit transport in inside-out vesicles. Inhibition is observed, however, if these vesicles are charged with atractyloside, i.e., inhibitor is presented from the outside of the normal internal mitochondrial membrane [183]. Spin-labeling experiments confirm the asymmetric nature of the adenine nucleotide transport system (see below).

Another example of the presumably asymmetric orientation of transport proteins is shown in glutamate transport in the internal mitochondrial membrane. This transport protein is inhibited by liposoluble neutral SH group reagents such as fuscin, avenaciolide and *N*-ethylmaleimide, but not by ionic reagents such as mersalyl that do not penetrate the membrane [183,191,192]. However, the exact location of SH groups (hydrophilic part, matrix-exposed moiety) remains to be elucidated [191,192].

ATPases of different origin involved in energy transduction, such as those from mitochondrial membrane [193–196], chloroplasts [177,197,198], bacteria [199–201] or

algae [202] show structural similarities. All consist of highly asymmetric devices formed of a hydrophobic (F_0) part embedded in the membrane and a hydrophilic part (F_1) exposed to the inside of bacteria and mitochondria but exposed to the outside of thylakoid membranes (see above). Both parts are involved in energy transduction. Dicyclohexylcarbodiimide blocks proton translocation, ATP synthesis and ATP hydrolysis [203,204]. Each part (F_1 and F_0) is formed of a number of subunits termed $\alpha, \beta, \gamma, \delta$ and ϵ for F_1 (at present the stoichiometry of the subunits is disputed, but, since the chloroplast CF_1 and the thermophilic bacterial BF_1 can now be crystallized, X-ray studies are likely to solve this problem [177]) and a, b and c for F_0. Inactivation and binding studies using dicyclohexylcarbodiimide have been carried out [195,196,201]. In the case of *E. coli*, the inactivation of isolated ATPase (BF_1) with dicyclohexylcarbodiimide has been correlated with the modification of the β subunit [200]. However, it has been reported that labeled DCCD links to the c subunit of F_0 of purified ATP synthetase of *E. coli* [201]. Apparently, isolation of BF_1 uncovers a carboxyl group [200] to which dicyclohexylcarbodiimide is bound. An 8000 dalton protein has been isolated from chloroplasts which binds dicyclohexylcarbodiimide and which may act as the chloroplast ionophore [177]. However, recent studies on dicyclohexylcarbodiimide binding of chloroplast CF_1 confirm that, here again, the β subunit is involved [205]. This treatment inactivated the ATPase activity. Upon reconstitution, the modified CF_1 linked to and restored the proton uptake by CF_1-depleted spinach chloroplasts; but upon illumination, light-dependent ATP synthesis and adenine nucleotide exchange remained inhibited.

Orientational studies were performed on formate dehydrogenase and fumarate reductase which are involved in electron transport-coupled phosphorylation in *Vibrio succinogenes*. Using bacterial cells or French-press particles (70% inside-out) obtained after lysozyme and EDTA treatment, sites for substrates and dyes (used as artificial markers for oxido-reduction reactions) for both enzymes were found to be asymmetrically displayed [206]. The catalytic sites of the formate oxidase were exposed to the cell exterior and those for fumarate reductase to the interior; thus, the first enzyme acted on non-permeant dyes when cells were used and the K_m value for formate remained unaffected by cell lysis. Upon lysis of French-press particles, however, a 3-fold increase in formate oxidation with both permeant and non-permeant acceptors occurred. Lysis of these particles exposed the external surface. In contrast to the case of formate oxidase, when succinate dehydrogenase function was assayed it was observed that if cells were used, sites for non-permeant dyes were inaccessible. They were accessible, however, when French-press particles were used. Results of other observations using 4-chloromercuriphenylsulfonate or 4-diazophenylsulfonate as inhibitors generally confirmed the suggested orientation for these enzymes [206].

In many cases, asymmetry of membrane proteins was assessed by the demonstration of their asymmetric functioning. Thus, erythrocyte ($Na^+ + K^+$)-dependent ATPase acts as an Na^+ and K^+ pump, hydrolyzes ATP and is transiently phosphorylated [207]. The formation of this intermediate is stimulated by Na^+ and its hydrolysis by K^+ [208]. The general picture of ($Na^+ + K^+$)-ATPase is of a quaternary structure of the $\alpha_2\beta_2$ type, consisting of two catalytic subunits of 100000 daltons each and two glycoprotein subunits of 50000 daltons each [209–220]. The ability to be phosphorylated was used to assess the purification of the ATPase [221–223]. Using resealed inside-out vesicles prepared according to the method of Steck et al. [224,225], it was shown that phosphorylation of the enzyme is stimulated by addition of Na^+ to the surrounding medium (i.e., the cytoplasmic side) and not to the intravesicular compartment (i.e., the external surface). Dephosphorylation

of the enzyme is enhanced by K^+ in the intravesicular compartment. Phosphorylation of the enzyme is also inhibited by K^+ by its interaction with the dephosphoenzyme [226, 227].

Interesting observations were made by using inside-out and right-side-out erythrocyte membrane vesicles and ouabain, a cardiac glycoside. The ATPase of inside-out vesicles was relatively insensitive to ouabain inhibition whereas the enzyme of ghosts and right-side-out vesicles was sensitive. The specific [^{3}H]ouabain binding of inside-out vesicles was negligible, whereas right-side-out vesicles bound this cardiac glycoside. These observations indicated that ouabain sites are located on the outer membrane surface [228]. Other workers, using inside-out vesicles of murine plasmocytoma membrane, observed that the sensitivity of ATPase to ouabain depended on the absence or presence of some proteins located at the extravesicular (i.e., cytoplasmic) surface of the membrane. Removal of proteins by EDTA treatment resulted in an increased sensitivity of ATPase to ouabain. Addition of the supernatant of EDTA-treated vesicles in the presence of Ca^{2+} restored the original level of ouabain sensitivity. Among the three proteins found in EDTA extracts, one has the same molecular weight and isoelectric point as tropomyosin [229].

The cardiac glycoside receptor of erythrocyte ATPase was studied by using a labeled ethyldiazomalonyl derivative of cymarin. The photoaffinity labeling (see below) of the receptor was carried out using this derivative. It was observed that the large subunit of the enzyme was labeled [230].

Antibodies raised against either the catalytic or the glycoprotein subunits inhibit the $(Na^+ + K^+)$-ATPase [231]. Recently, it was observed that antibodies to purified $(Na^+ + K^+)$-ATPase from *Squalus acanthia* as well as antibodies against its catalytic subunit inhibited the ouabain binding by as much as 50%. Antibodies against the glycoprotein subunit did not influence ouabain binding. Thus, the topology of the ouabain site is such that it is either covered by antibody to the catalytic subunit or undergoes conformational changes preventing access to or affinity for ouabain [232]. The fact that only 50% of ouabain binding is inhibited indicates that two types of ouabain binding site may exist, as has also been suggested [233]. In another study using *Electrophorus electricus* ATPase, antibodies against the glycoprotein but not those against the catalytic subunits inhibited the ouabain binding. The reason for this discrepancy is not clear but, remarkably, only 50% of the binding sites were affected [234]. It is noteworthy in this respect, that the $(Na^+ + K^+)$-dependent ATPase from ovine kidney medulla obtained as vesicular membrane preparations showed a slow rate of ouabain binding in the presence of isotonic sucrose. Hypoosmotic shock (or phospholipase A treatment) enhanced the rate of ouabain binding by at least 3-fold. At equilibrium, however, the concentration of the ouabain-enzyme complex was twice as high as that with untreated vesicles. The presence of two types of ouabain binding site with an approximate stoichiometry of 1 : 1 is envisaged. A further clue to the heterogeneity of the ouabain binding sites was obtained when it was observed that a minor fraction of the sites were occupied by ouabain only after a long period of incubation with Mg^{2+} [235].

The red blood cell membrane contains an independent, Ca^{2+}-dependent ATPase [211, 236], the low level of Ca^{2+} in the intracellular compartment being maintained by a calcium pump. Metabolic depletion of ATP and subsequent accumulation of Ca^{2+} in erythrocytes or ghosts lead to a rigid membrane which has lost its deformability [237]. Leaky ghosts exhibit a Ca^{2+}-stimulated ATPase activity [236,238,239]. The divalent cation-stimulated site of the ghost ATPase must be located on the inner side of the membrane, since the activity is observed in leaky ghosts [236,238,239] but not in resealed ghosts if

ATP and Ca^{2+} are added only to the external compartment [238]. The Ca^{2+}-dependent ATPase of red blood cells is also transiently phosphorylated during its function and [γ-^{32}P]ATP labels a polypeptide of 150 000 daltons which has been previously isolated [240]. Similarly, sarcoplasmic reticulum ATPase with a molecular weight of 120 000 has shown a phosphorylated intermediate [241].

Acetylcholinesterase of erythrocyte membranes was identified by means of covalent labeling. Diisopropyl fluorophosphate acts as a pseudo-substrate for this enzyme and reacts with its exposed sites. The membrane was first reacted with non-labeled diisopropyl fluorophosphate in the presence of butyrylcholine, an enzyme inhibitor which protects the active site. This treatment saturates diisopropyl fluorophosphate-reactive sites not associated with acetylcholinesterase. Subsequently, diisopropyl fluorophosphate is removed and the membrane reacted with [^{3}H]diisopropyl fluorophosphate. Analysis showed that a single peak became radioactive and in the presence of 2-mercaptoethanol the material shows a molecular weight of 90 000. In the absence of the reducing agent, the enzyme has a molecular weight of 180 000. Thus, it may exist as a dimer in the membrane [242]. On the other hand, mild reduction using mercaptans followed by alkylation of human erythrocyte stroma was reported to result in a loss of acetylcholinesterase activity [243]. When mercaptans and iodoacetamide were tested separately, no significant effect on the enzyme activity could be observed.

Surface sites of membranes, when reacted with their specific ligands, often undergo conformational changes which can be observed by the use of different physical techniques and by modification of their reactivity to different reagents. *N*-Ethylmaleimide treatment of *E. coli* cells strongly inhibits β-galactoside transport and in the presence of β-D-galactopyranosyl-1-thio-β-D-galactoside (thiodigalactoside), an analogue of the substrate, inhibition by *N*-ethylmaleimide is reduced [244,245]. This fact was taken advantage of in the labeling of inducible lactose transport protein (M protein, which is encoded by gene y of the lactose operon) by Fox and Kennedy [244]. This protein constitutes 4% of the total membrane proteins in induced cells. Non-induced cells and cells induced in the presence of isopropyl-β-thiogalactoside were first treated with unlabeled *N*-ethylmaleimide in the presence of thiodigalactoside. Then the inhibitor was removed by treatment with 2-mercaptoethanol and the induced cells labeled with *N*-[^{14}C]ethylmaleimide and the non-induced cells with *N*-[$^3H_3$]ethylmaleimide. The M protein was identified by its relatively high $^{14}C : {}^3H$ ratio in the mixed extracts of mixed membranes. It is worthwhile mentioning an interesting and more simple method of purification of the M protein used later in the same laboratory [246] which is as follows. A sample of *E. coli* was divided into two parts, one was cultivated in the presence of [^{14}C]leucine and the other in the presence of [^{3}H]leucine and the permease inducer, isopropyl-β-thiogalactoside. Samples of both cultures were mixed and extracted using an SDS-containing buffer and the M protein was detected by its high $^3H : {}^{14}C$ ratio. It appeared in SDS-polyacrylamide gels in the position expected for M protein, the subunit of which has a molecular weight of 30 000 [245,246]. The principle of this work was used during the purification of the inducible L-malate transport protein of *Bacillus subtilis* membrane [247].

Among the group translocation systems implied in the transport of molecules through membranes [17,18,248–251], the most actively studied is the phospho*enol*pyruvate-phosphotransferase system of bacteria discovered by Kunding et al. [248]. This system phosphorylates sugars during transport by using phospho*enol*pyruvate. It is comprised of different proteins (enzyme I, HPr, enzyme II, factor III). Haguenauer-Tsapsis and Kepes [250] observed that if *N*-ethylmaleimide (or fluorodinitrobenzene) is added to a suspen-

sion of bacteria during the incorporation of α-methylglucose phosphate, incorporation and phosphorylation are stopped. The rate of this inactivation is proportional to the rate of incorporation of the phosphorylated sugar. Thus, the above-mentioned reagents act on a thiol group 'unmasked' during the functioning of the system. Pretreatment of bacteria with these reagents does not have this inhibitory effect and if the sugar to be phosphorylated is absent, the essential thiol groups do not react. Studies with *E. coli* mutants able to incorporate β-glucosides after their phosphorylation (wild strains cannot) show that transport of *p*-nitrophenyl-β-D-glucose by the phospho*enol*pyruvate-phosphotransferase system is similarly inactivated by thiol-reactive agents. Here again, it is the thiol group of a specific enzyme (enzyme II) that reacts, and furthermore, inhibition is possible only during the functioning of the transport system [251].

N-Ethylmaleimide inhibits the photophosphorylation in thylakoid membranes only after illumination in the presence of this sulfhydryl reagent. 0.5 0.7 mol of the reagent is bound per mol of CF_1, the γ subunit of which is the site of the reaction. ADP and ATP hinder the inhibition by *N*-ethylmaleimide whereas P_i, which alone has no effect, enhances that of ADP and ATP [177]. Further proof of a light-dependent conformational change in CF_1 is furnished by the fact that thylakoid membranes pretreated with an amino group-specific reagent, methylacetimidate (see below), in the dark, still react in the light with trinitrobenzenesulfonate, another such reagent. 4 mol of the latter reagent react with 1 mol of CF_1 and α, β and γ subunits have been found to be involved in the reaction [177].

An intriguing observation is that when erythrocyte membranes were labeled with a low concentration of the general reagent, iodoacetamide, only band 8, i.e., the glyceraldehyde-3-phosphate dehydrogenase, reacted due to its 'super-reactivity' [252]. However, after hemolysis in the presence of ATP, labeled band 8 is absent, indicating that enzyme-membrane association is sensitive to metabolic products and effectors. It was suggested that the enzyme is released by the direct action of ATP on the membrane [253] *.

Glucagon has been shown to enhance the interaction of a presumptive component of membrane adenylate cyclase with iodoacetamide: this reaction inactivates the enzyme [259]. Taking advantage of this fact, a double-labeling procedure was used to separate the membrane component of the enzyme. First, in the absence of glucagon, iodo[^{3}H]-acetamide is allowed to react with the membrane. Then in the presence of glucagon, iodo-[^{14}C]acetamide is reacted. An increased $^{14}C : {}^3H$ ratio due to the interaction of glucagon and the receptor exposed to the outside of the membrane was used to detect a 240 000 dalton material which was subsequently isolated [260].

Ion channels in excitable membranes have an asymmetric orientation. Thus, an inhibi-

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* It should be noted that in the original numbering of erythrocyte membrane proteins by Fairbanks et al. [254], e.g., as illustrated in a figure given by Marchesi et al. [255], glyceraldehyde-3-phosphate dehydrogenase is numbered as band 6, however, Shin et al. [253] numbered the same enzyme as band 8 (see also the figure given by Carraway [256]). Polypeptides are numbered according to their migration on SDS-polyacrylamide gels, the molecules migrating to a greater degree bearing the higher numbers; consequently, the numbering will depend on whether or not all bands are taken into account. It is worth recalling that 200 polypeptides have been reported to be detected using two-dimensional gel electrophoresis of erythrocytes [257]. The numbering [254,256,258] takes into account the prominent spots. In some cases, a given band may appear double; double bands are denoted by a, b, etc.; thus, we have bands 4a and 4b that are alternatively termed 4.1 and 4.2. In addition, four sialoglycoproteins, PAS1 PAS4, are labeled on SDS-polyacrylamide gels by the periodic acid-Schiff (PAS) reaction.

tor of the Na channel, tetrodotoxin, links to the external opening of this channel with a high affinity [261,262]. Saxitoxin behaves like tetrodotoxin. These toxins have, respectively, one and two guanidinium groups which seems to be responsible for the linkage *. Toxins which have diffused into the axon, i.e., into the opposite side of the channel, have no effect.

Bacterial chemotactic receptors [264–267] are also asymmetrically located, as can be judged from their behavior. Thus, glucose and α-methylglucoside, both substrates of the phospho*enol*pyruvate-phosphotransferase system, are chemoattractants. The same holds true for galactose and β-methylgalactoside involving an active transport. However, lactose, the transport of which implies an independent system [244–246,268–272], has no chemotactic effect, although it is converted in the cell to glucose and galactose both of which elicit positive chemotactic responses in bacteria when acting from the environmental side.

Interferon must act from the outside of the cell in order to induce the antiviral state. Cells induced to produce interferon by the double-stranded RNA (poly rI : rC) [273–275] were not protected from viral infection when antiserum to interferon was added to the medium. The synthesis and release of interferon are not inhibited under these conditions. Similarly, ouabain, which inhibits the membrane-bound ATPase, blocks the antiviral action of interferon without affecting either its synthesis or release [276]. These observations confirm others, indicating that interferon acting as a genuine hormone reacts with the externally exposed receptors (gangliosides) of the cell membrane [277–280] and, furthermore, show the remarkable situation that interferon produced in the cell is inactive if it is not first released to react from the outside, presumably with membrane receptors of other cells, since the cell which was triggered to produce interferon would have had the time to be infected by the virus, to be damaged and even to die.

V. Chemical reagents

Chemical reagents tagging membrane proteins by means of chromophores, fluorescent

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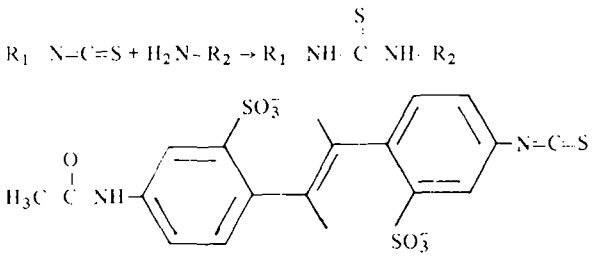
* Amiloride which blocks the Na^+ channel, for example, at the apical surface of the proximal tubules of frog kidney [263], also has a guanidinium group.

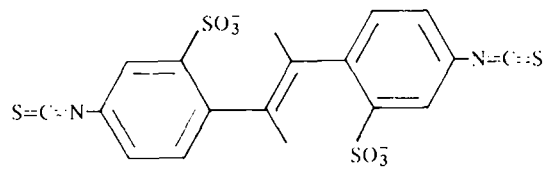
** I will not give details on the (SDS) interaction with proteins and the practical use of the method [281–284], but rather mention some of the problems encountered or variants of the technique used. Many difficulties have been encountered with glycoproteins [285–287]. Their possibly incomplete dissociation by SDS has been reported [288,289]. Sialidase treatment of sialoglycoproteins was observed by different authors to decrease significantly their electrophoretic mobility so that their apparent molecular weights seemed to be increased [290–294]. Sialoglycoproteins can possibly be oxidized using periodate, then labeled by $NaB[^3H]H_4$ before analysis [295]. The necessity of removal of SDS for a quantitative estimate of glycoproteins [296] and the possibility of nondetectability of glycoproteins with low carbohydrate content and which are deficient in sialic acid [297] have been reported. Problems were also encountered with the staining of highly glycosylated proteins [298]; likewise, modification of proteins by reagents that introduce high negative charges into the molecule perturb the dye binding [256]. Integral membrane proteins can bind more SDS (approx. 3 g/g protein) than soluble proteins (1.4 g/g protein) [299,300]. The electrophoretic mobility and the number of proteins of the outer membrane of Gram-negative bacteria were reported to be 'heat modifiable', i.e., determined by the solubilization temperature [301–303]. Slab gel electrophoresis [304], the use of a discontinuous buffer system [305] and two-dimensional gel electrophoresis [306–308] improve the resolution of bands. After electrophoresis of radioactive membrane proteins, SDS-polyacrylamide gels may be sliced longitudinally for autoradiography [309]. Radioactive proteins may be revealed by means of a linear transport system

probes or radioactive isotopes were used to study their distribution and orientation, often in conjunction with SDS-polyacrylamide gel electrophoresis **.

Reagents that do not penetrate membranes can be used for probing accessible, reactive molecules. Such reagents are numerous. Many react with free amine functions and are common to proteins and relevant lipids. Various isothiocyanate derivates have been used; the first, a fluorescent compound introduced by Maddy [311], is 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate (SITS). Another derivative, 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) which, being bifunctional, acts as a cross-linking reagent has been introduced by Cabantchik and Rothstein [312,313]. These derivatives react under mild conditions with other groups such as sulfhydryl, tyrosyl, histidyl and sometimes guanidyl



4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonate



4,4′-diisothiocyanostilbene-2,2′-disulfonate

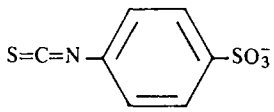
groups of proteins. Isothiocyanate derivatives react with amino groups to yield thiourea derivatives.

Sulfhydryl and tyrosyl derivatives of SITS undergo rapid hydrolysis at pH 11. Since SITS was not removed at this pH, Maddy [311] concluded that it reacted with amino, histidyl or guanidyl groups. SITS and DIDS have found numerous applications [255, 311–316], especially with erythrocyte membranes. They are non-penetrating reagents. [^{3}H]DIDS, (i.e., 4,4′-diisothiocyano-2,2′-[$^3H_2$]stilbenedisulfonate) labels specifically about 30 000 sites on the erythrocyte surface [314], predominantly the band 3 protein. The only other protein that binds this reagent is glycophorin, but this linkage accounts for less than 5% of the total bound [^{3}H]DIDS. The site of binding is suggested to be the substrate binding center of the anion transport system. If leaky erythrocyte ghosts are used, extensive labeling of other membrane proteins takes place [314]. Interestingly, even high concentrations of DIDS do not label more than 4% of lipids in intact erythrocytes, in contrast to the extensive labeling of lipids in leaky ghosts. This is in agreement with the known asymmetric distribution of aminophospholipids in erythrocyte membranes and with earlier experiments with SITS reported by Maddy [311]. SITS and DIDS have been used by Juliano and Behar-Bannelier [316] for labeling of mammalian cells (CHO and

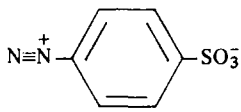
scanning the gel; scanning spectroscopy and scanning fluorimetry are also possible. If thin-layer plates are used, labeled compounds can be analysed using scanning spectroscopy or autoradiography [310].

N_2A cells), and it has been confirmed that these reagents do not penetrate membranes except those of dead or dying cells.

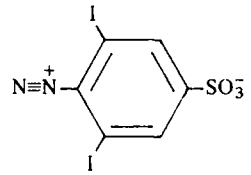
Among the isothiocyanate derivatives which have been used is isothiocyanosulfanilate, which was shown to bind to and inhibit the erythrocyte anion transporter (band 3 protein [317]).



isothiocyanosulfanilate



diazosulfanilate



2,6-diiodiazosulfanilate

Another non-permeant reagent of amino groups, as well as tyrosine and histidine introduced by Berg [318], is diazo[^{35}S]sulfanilic acid. This reagent reacts with ϵ-amino groups of lysine residues as well as with the tyrosine and histidine groups [319] of proteins. Recently, resonance Raman spectroscopy has been used in order to identify the enzyme coupling sites with aromatic diazonium salts [320]. Though Raman spectroscopy has been used in membrane studies, an application to the particular field mentioned above has not yet been reported. Diazosulfanilate has been found to cause damage to membrane functions [321], however, with low concentrations the damage appears minimal [322, 323]. The use of this reagent confirms that a limited number of erythrocyte membrane proteins (only band 3 protein and glycophorin) are accessible [318] and, in particular, spectrin and hemoglobin are not labeled, thus confirming the non-permeant nature of the reagent. The lack of labeling of the major portion of erythrocyte membrane proteins with this reagent is not correlated with a hypothetical lack of reactivity of these components, since penetrating agents such as *N*-ethylmaleimide [252] and acetic anhydride [324] labeled all the membrane proteins. To determine the distribution of the components of ubiquinone-cytochrome *c* reductase (complex III) between the cytoplasmic and the matrix faces of mitochondrial inner membrane, mitochondria or sonicated submitochondrial particles were treated with diazo[^{35}S]sulfanilate [325]. After solubilization, cytochrome *b*, cytochrome c_1 and protein VI were heavily labeled in mitochondria, indicating that they are exposed on the cytoplasmic face of the membrane. Proteins I, II, V and VII showed limited labeling and proteins VIII and IX none. With inverted particles, protein II and cytochrome *b* were extensively labeled and proteins III, VIII and IX did not react. Thus, it is proposed that protein II spans the membrane. However, complex III contains 2 mol of cytochrome *b* and it has not yet been determined whether 1 mol is present on each face or 1 or 2 mol span the membrane.

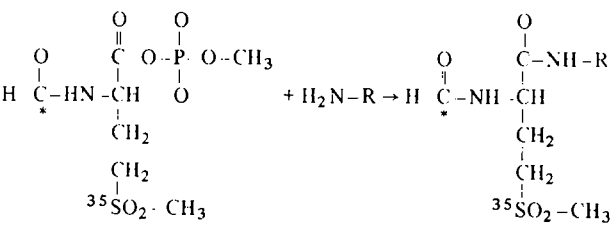
Diazo[^{35}S]sulfanilate has been similarly used for assessing the transmembrane distribution of components of bovine heart mitochondrial cytochrome *c* oxidase (complex IV) [326]. The non-permeability of this reagent has been shown previously [327] by demonstrating that radioactive coupling factor 1 (ATPase) could be isolated after treatment of submitochondrial particles and that labeling of mitochondria resulted in labeling of cytochrome *c*. The coupling factor, isolated from mitochondria, as well as the cytochrome *c* isolated from submitochondrial particles, showed low radioactivity. In order to assess the distribution of the components of cytochrome oxidase after reaction of the reagent with mitochondrial or submitochondrial particles, membranes were lysed with cholate and cytochrome *c* oxidase was isolated by immunoprecipitation. The six subunits

of the oxidase were analyzed using SDS-polyacrylamide gel electrophoresis and scanned for radioactivity. Labeling of intact mitochondria led to the formation of radioactive subunits II, V and VI and that of submitochondrial particles to radioactive subunit III. Thus, cytochrome oxidase is transmembranous and asymmetrically oriented. Subunits I and IV were not labeled; even in a purified complex they did not react due to their inaccessibility. Cytochrome *c* oxidase from *Saccharomyces cerevisiae* is known to contain an additional component VII (see below) and bovine heart cytochrome *c* oxidase has recently been reported to be comprised of nine subunits [328].

Diazosulfanilate has been used to study the exposure of Photosystem I (PS I) and Photosystem II (PS II) in chloroplast thylakoid membranes [329]. These systems were isolated by deoxycholate treatment of thylakoid membranes and antibodies directed against PS I prepared. Among these antibodies, some were able to react with PS I in intact membranes, others only if the membrane was disrupted giving access to the intrathylakoid compartment. This has been taken as an indication of the asymmetric orientation of PS I on the one hand and of its accessibility from both sides on the other [330]. When, in other experiments, PS I- and PS II-enriched particles were prepared from corn leaf chloroplasts and the corresponding antibodies prepared in rabbits, antibodies to PS II were found to be less reactive. Thus, PS II behaves as if it were less accessible [331]. This has been confirmed by the action of diazosulfanilate. After reaction of this isotopically labeled reagent, the separation of PS I and PS II from the treated chloroplasts showed that radioactivity was associated mainly with PS I [168].

In the above-mentioned discussion it is assumed that the reagent does not penetrate the membrane, however, diazosulfanilate has been reported to penetrate the membrane in the case of *A. laidlawii* [332]. Also, recently, the synthesis of diazotized [2,6-^{125}I]-diiodosulfanilic acid was reported which can be used as a non-penetrating reagent with membrane proteins [333]. It should be noted that here the labeling uses an isotope of iodine, not sulfur.

Formyl[^{35}S]methionyl(sulfone)methyl phosphate, the use of which was introduced by Bretscher [334], reacts with amino groups and labels proteins. This reagent should be



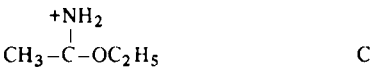
formylmethionyl(sulfone)methyl phosphate

used at pH 9.6, at lower pH values it is hydrolyzed to formylmethionyl sulfone. This reagent is a non-penetrating compound. When used with intact erythrocytes, it labels only two transmembrane proteins, namely band 3 protein and glycophorin. If it is allowed to reach the cytoplasmic side (leaky ghosts) then all membrane proteins are labeled [334]. Labeling with formylmethionyl(sulfone)methyl phosphate was used for determination of the transmembrane nature of proteins, the basic procedure being to treat erythrocyte cells or leaky membranes with the reagent, to separate proteins and to examine their proteolysis fingerprint. Those proteins exposed to the outside of the erythrocyte will show a number of labeled peptides different from the fingerprint of the proteolysis products of leaky membranes which will show additional labeled peptides. By using this tech-

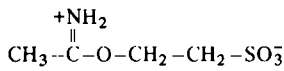
nique, it was observed that band 3 protein and glycophorin span the membrane. Although the conclusions drawn from these studies are generally accepted, Carraway [256] remarked that the mere change in the number of labeled peptides cannot be taken as evidence that, necessarily, the cytoplasmic side of the protein reacted. If, during ghost preparation, a conformational change occurs so that new reactive groups appear on the exposed surface or if a composite aqueous channel is opened during this preparation, new labeled peptides on the fingerprint of the proteins will now be observed.

Experiments with formylmethionyl(sulfone)methyl phosphate have confirmed the hidden nature of the aminophospholipids of erythrocytes [334] observed implicitly by Maddy [311] using the fluorescent reagent, SITS.

Imidoesters constitute another type of amino group reagent; amidines and alcohols are formed as products of the reaction. Two synthetic homologues of imidoesters were used by Whiteley and Berg [321], one being a permeant reagent and the other non-permeant, the latter reacting only as a surface marker. The use of this technique confirmed that only two proteins of the erythrocyte membrane, band 3 protein and glycophorin, are accessible from the external surface.



ethylacetimidate (permeant)

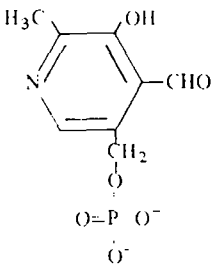


isethionylacetimidate (non-permeant)

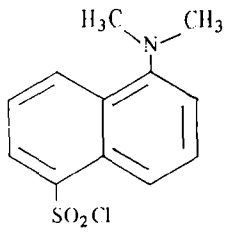
The permeant reagent is ethylacetimidate and the non-permeant reagent isethionylacetimidate. Ethylacetimidate is relatively non-polar and can be solubilized in and pass through lipids. Isethionylacetimidate is polar and reacts only with surface molecules from the outside of the membrane. These reagents allow double-labeling experiments to be performed by labeling one molecule with ^{3}H and the other with ^{14}C. For example, if the exposed amine functions are first saturated with the ^{3}H-labeled non-penetrating reagent, and then reaction is followed by that of the ^{14}C-labeled penetrating reagent, one can discriminate between membrane molecules with only an external exposure, those with only an internal exposure and transmembrane molecules exposed on both sides of the membrane. If a doubly labeled membrane preparation is treated with pronase, transmembrane molecules can be further distinguished from those with a single exposure. The use of the non-permeant reagent alone is also possible. In this case, after the reaction of exposed molecules, leaky membranes are prepared and the reagent is allowed to react further. An application of this method to erythrocytes led to the conclusion that 10-times more amino groups of proteins are exposed to the internal leaflet of the membrane than to the outside and that almost all the reactive amino groups of lipids, in accordance with other studies, are localized internally. Using imidoesters, band 3 protein, glycophorin, component 4a and a polypeptide of molecular weight 170 000 were found to be exposed on the external surface.

Pyridoxal phosphate is another non-permeant reagent. Reduction of the Schiff base, formed by its reaction with amino groups, with $NaB[^{3}H]H_4$ labels isotopically protein molecules. This method was first applied by Rifkin et al. [335] to influenza virus that retained its infectivity and hemagglutinating activity after this treatment. Other uses of this reagent are reported elsewhere [316,336]. The pyridoxal phosphate-$NaB[^{3}H]H_4$ system was found to penetrate the microsomal membrane as demonstrated by labeling of proteins present in the vesicular cavity [337]. Recently, this technique was used in a study on chick brain synaptosomal plasma membrane. Careful adjustments of the reac-

tion conditions were carried out to label exclusively proteins exposed to the external surface. It was found that 14 major external polypeptides were labeled, three chains of 42 000, 29 000 and 26 000 daltons sharing most of the label [338].

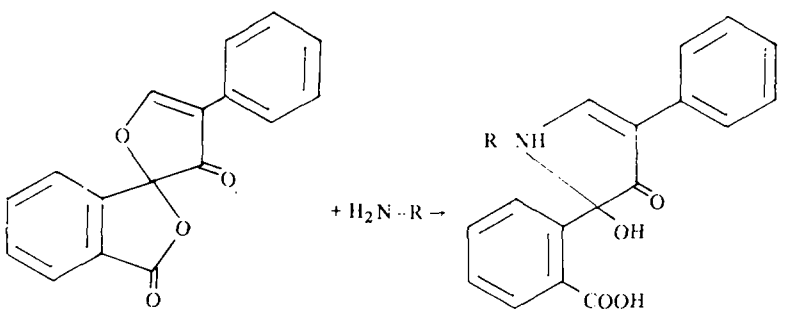


pyridoxal phosphate



1-dimethylaminonaphthalene-5-sulfonyl chloride

As mentioned above, the labeling of surface molecules with fluorescent reagents was first carried out by Maddy [311]. Another fluorescent reagent which is used is 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride). This is a water-insoluble material [339] which is hydrolyzed in water to the corresponding freely soluble sulfonic acid. Addition of this liposoluble reagent in organic solvents has the serious disadvantage that membranes may be damaged to the point of lysis [258]. For this reason, Schmidt-Ullrich et al. [258] used a dispersion of dansyl chloride in a lecithin-cholesterol aggregate. Results obtained with erythrocytes are interesting, since the permeant reagent behaves as an impermeant protein label. In human erythrocytes, a peptide of 90 000 daltons and a (presumed) glycoprotein of 60 000 daltons reacted most vigorously and in sheep erythrocytes three proteins of 155 000, 95 000 and 40 000 daltons were labeled, with more than 90% of the phosphatidylethanolamines not reacting. In erythrocyte ghosts, however, all membrane proteins were labeled. The different labeling of proteins in intact cells and in ghosts cannot be attributed unambiguously to the leakiness of the ghosts. It was suggested that it might be due to the reactivity of proteins changing during ghost isolation or because of the lack of the hemoglobulin 'sink' in the case of ghosts. The results obtained with dansyl chloride were confirmed with the use of fluorescamine, which reacts with amino groups [340]. The observation by Kinoshita et al. [341] of the enhanced fluorescence of dansyl amino acids obtained by using cycloheptaamylose and the interpretation of this observation in terms of the incorporation of dansyl derivatives into the hydrophobic cavity of the cyclic sugar [342] led to the assay of fluorescamine in the same way. It was observed that this reagent could also be complexed [343]. The complex tagged only the exposed surface molecules, thus only band 3 and 5 proteins of



fluorescamine

sheep erythrocytes were labeled and band 1 and 2 proteins were only faintly marked. When leaky ghosts were used all major erythrocyte membrane proteins were labeled [343]. In addition, it was proved by assaying the globin part of the hemoglobin (the heme would quench the fluorescence) that the complex does not penetrate intact erythrocytes, since no fluorescent globin could be isolated. In the case of human erythrocytes, only band 3 protein was labeled. Fluorescamine has been also used for the study of c proteins (see below) in sarcoplasmic reticulum [344]. The permeant nature of fluorescamine, as assayed with sarcoplasmic reticulum [345], and that of the inner membrane of bovine heart mitochondria [346] were found to be temperature and concentration dependent. However, the use of fluorescamine complexed with cycloheptaamylose [342] prevented the concentration-dependent penetration of the reagent [346]. The use of fluorescamine has the advantage that the reagent itself and its hydrolysis products, in contrast to its reaction products with amines, are not fluorescent. The reaction products exhibit excitation and emission maxima at 390 and 480 nm, respectively, and the products obtained with the complex indeed show the same properties [342].

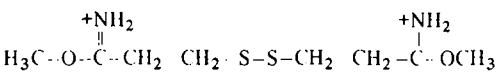
Another chemical modification concerns the surface sialyl residues. Periodate oxidation followed by treatment with $NaB[^3H]H_4$ converts sialyl residues to 5-acetamido-3,5-dideoxy-L-arabino-2-heptulonic acid (also termed 3-deoxy-5-acetamidoheptulonic acid). Erythrocytes, isolated membranes or sialoglycoproteins have been found to be labeled by this procedure. The label appeared in residues which were formerly those of bound sialic acids. No other membrane protein was labeled [347]. Recently, careful use of periodate oxidation induced specific oxidative cleavage of surface sialic acids between carbons 7 and 8 or carbons 8 and 9 [290]. After reduction, ^3H-labeled 5-acetamido-3,5-dideoxy-L-arabino-2-heptulonic acid or 5-acetamido-3,5-dideoxy-L-arabino-2-octulonic acid is obtained, the first component being the major reaction product [348–350]. At low temperature (0°C), a low concentration of periodate and short reaction time, only the exposed sialyl residues are labeled [290]. When inside-out erythrocyte membranes prepared by the vesiculation technique [224,225] are used, surface labeling does not occur. Similarly, fetuin (a sialoglycoprotein) entrapped in resealed, right-side-out ghosts is not labeled. This surface labeling technique, predicted [290] to be very useful in the study of transformed cells (which are extensively sialylated [351]), has been used for the study of normal and transformed T cells [294,352]. Both normal and lymphoblastoid human T cells have been labeled by using this technique and by lactoperoxidase-catalyzed radioactive iodination and galactose oxidase-$NaB[^3H]H_4$ labeling. Both cell types exhibit a sialoglycoprotein of 95 000 daltons. After neuraminidase treatment, the desialylated compound shows a molecular weight of 120 000. As mentioned above, the increase in the apparent molecular weight of sialoglycoproteins has been observed by different authors [290–294].

A variant of the periodate-oxidation technique which has been recently introduced is to use dansyl hydrazine instead of $NaB[^3H]H_4$ to label the generated aldehyde groups. Sialic acid of glycoprotein origin is found to be the sole source of the carbonyl group that reacts with this fluorescent reagent. It is concluded that the fluorescent label is introduced specifically into the sialic acid residues of glycoconjugates either in solution or in the cellular membrane [353] *.

Cross-linking reagents give useful information about the asymmetric orientation of proteins. By using dimethyl-3,3′-dithiobispropionimidate, a penetrating cross-linking

* Recently, by using a mitogenic concentration of periodate and human peripheral blood lymphocytes, oxidation of fucose and probably galactose residues was observed [745].

reagent. Wang and Richards [354] observed that when using intact red cells, the hemoglobin cross-linked, both to itself and to membrane proteins on the cytoplasmic side. Therefore, most of the major erythrocyte membrane proteins (with the possible exception of band 5 (actin)) must be accessible to hemoglobin with which they react in intact cells.



dimethyl-3,3'-dithiobispropionimidate

On the other hand, this experiment shows that although the amount of spectrin present at the inner surface should be sufficient to cover it, proteins exposed to the cytoplasmic surface of membranes have access to the cytoplasmic compartment, since they can be cross-linked to hemoglobin. The 'scaffolding' of the inner surface of erythrocytes [51,56] is therefore not hermetic on the molecular scale. The above-mentioned reagent was designed for its cleavability by disulfide reducing compounds, so that after reduction, it is possible to confirm which components cross-link. Good use has been made of a more common reagent, dimethylsuberimidate, for revealing the presence of M (M, matrix) proteins in avian and murine RNA viruses [355]. In this case, host cells were grown in the presence of [^{14}C]ethanolamine and viruses containing ^{14}C-labeled phosphatidylethanolamines, budding from the cells surface, were treated with the bifunctional reagent. Besides transmembrane proteins, protein P 19 in the case of avian leukemia and sarcoma viruses and protein P 15 in the case of murine leukemia viruses were cross-linked to phosphatidylethanolamine. It is concluded that these proteins are equivalent to a matrix protein known to exist for some other viruses. The use of bifunctional reagents led to other observations with erythrocytes in relation to the asymmetric exposure and functional relationships of membrane proteins. A penetrating bifunctional imidoester, dimethylmalonimidate, with a distance of 4.9 Å between its two functional groups, cannot cross-link spectrin molecules to each other. However, if lectins such as ricin, which binds to band 3 protein, or phytohemagglutinin of *Phaseolus vulgaris*, which links to glycophorin, are added to the ghost suspension, cross-linking of spectrin molecules to a high molecular weight complex does occur. In the absence of lectins, dimethylsuberimidate, with a distance of 11 Å between its functional groups, should be added to promote the cross-reaction of the spectrin molecules [356,357]. These experiments show that spectrin is linked directly or indirectly to transmembranous compounds. The use of dimethyldithiobispropionimidate shows that spectrin can be associated to a compound with the same molecular weight as that of the band 3 protein [358]. Other workers have also reported the cross-linking of band 3 protein with either chain of spectrin [359]. However, the possibility of a direct and specific interaction has been questioned [360,361]. The correlation between integral proteins and spectrin has been proved by the action of anti-spectrin IgG introduced into ghosts. This treatment led to the aggregation of surface sialoglycoproteins. Fab fragments of the antibody do not have this effect [357,362], however, as reported above, the role of spectrin in the lateral mobility of intramembrane particles in erythrocytes has been challenged * [57].

Observations on the asymmetric interaction of membrane integral proteins with cellu-

* Besides the interaction of spectrin with integral proteins, it is noteworthy that other studies have demonstrated interaction of this protein with lipids. This interaction is believed to contribute to the asymmetry of lipid distribution in erythrocytes [363,364].

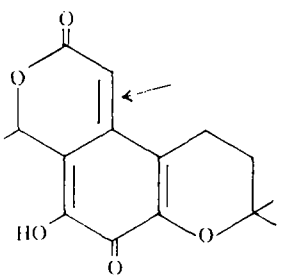
lar cytoskeletal elements in different cases [357,365] and particularly in lymphocytes led Edelman [365] to suggest the presence of a 'surface modulating assembly' which mediates the 'anchorage modulation' of surface receptors by cellular microfilaments and microtubules. The phenomenon of co-capping of surface proteins and cytoskeletal molecules [366,367], as well as the possibility of separating complexes showing protein-protein interactions between externally exposed molecules and cytoskeletal elements [368,369], confirms the asymmetric functional relationship of membrane integral molecules and cytoplasmic components. Thus, actin has been shown to co-cap with concanavalin A receptors in neoplastic and embryonic fibroblast cells [366]. Actin and tubulin have been reported to co-cap with surface immunoglobulin in murine spleen B lymphocytes [65,367,370,371]. In a mastocytoma cell line, P 815 cells, which shed (exfoliate) material, it was shown that actin and the histocompatibility antigen H-2 are complexed. In these experiments, the histocompatibility antigen from exfoliates could be complexed with myosin due to its interaction, either directly or indirectly, with actin. This technique is called the 'myosin affinity technique' [368]. Using this procedure, it was also possible to show that cross-linked surface Ig in lymphocytes and P3 myeloma cells is attached to actin in Nonidet P-40 extracts. Surface Ig was marked with ^{125}I-labeled rabbit anti-mouse immunoglobulin antibodies, either as $F(ab')_2$ or Fab fragments, or with fluorescein isothiocyanate conjugate of rabbit anti-mouse immunoglobulin; binding to myosin under non-cross-linking conditions is very poor. Application of capping conditions, i.e., using ^{125}I-labeled $F(ab')_2$ or fluorescein-treated antibody as labeling reagents prior to detergent extraction, had a strong effect on the binding of Ig to myosin [369]. These procedures can be adapted to complement cross-linking experiments.

As mentioned previously, a pertinent choice of reagent can lead to useful observations even with trivial compounds. Thus, the use of 1,5-difluoro-2,4-dinitrobenzene with erythrocytes led to the linkage of the β-chain of hemoglobin via Cys-93 and some amino group of the membrane. Notably, α-chains were not bound and could be separated. This gives some indication of the relationship between the internal surface of the erythrocyte membrane and the cytoplasmic compartment [372,373].

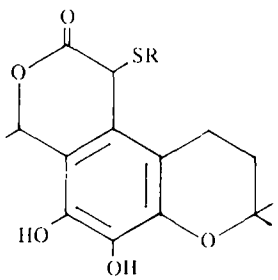
I have mentioned the use of liposoluble sulfhydryl reagents such as fuscin, avenaciolide, *N*-ethylmaleimide and that of a non-permeant reagent, mersalyl [183,191,192]. Other sulfhydryl reagents have been used for the localization of sulfhydryl groups in membrane proteins. Thus, human erythrocytes were treated with mercurials such as *p*-chloromercuribenzoate (PCMB), *p*-chloromercuribenzenesulfonate (PCMBS), 3-chloromercuri-2-methoxypropyl urea (chlormerodrin) and 1-bromomercuri-2-hydroxypropane (BMHP) [374]. PCMB, PCMBS and chlormerodrin react with at least three classes of sulfhydryl group, two groups of which are associated with the Na^+ and K^+ permeability barrier, resulting in the loss of K^+, the accumulation of Na^+ and hemolysis. The sulfhydryl groups involved in ion permeability are accessible to a non-permeant reagent such as glutathione, since it restores the Na^+, K^+ barrier, although removing only a small number of mercurial molecules from the membrane. Thus, most SH groups altered by mercurials are not accessible to glutathione or to 2-mercaptoethylguanidine. Sulfhydryl groups which are inaccessible to glutathione have little effect to the Na^+, K^+ barrier. PCMBS at a high concentration reacts with additional SH groups leading to osmotic hemolysis. BMHP reacts with at least two classes of sulfhydryl group, one of which when altered results in hemolysis in an isotonic solution of lactose or choline chloride, therefore being associated with the permeability. Binding of BMHP is immediate and complete and the sulfhydryl groups modified are very accessible to glutathione which removes almost all the mer-

curial. However, modified sulfhydryl groups appear to be of diverse types, some being also susceptible to *N*-ethylmaleimide. These sulfhydryl groups are not involved in permeability. When they are blocked by *N*-ethylmaleimide, BMHP may react with sulfhydryl groups involved in permeability. This permeability is distinct from that of Na^+ and K^+ [374].

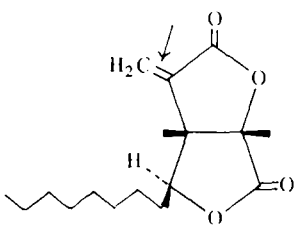
In another study, macromolecular mercurial and SH-regenerating agents were used to study the SH groups of lactose permease in conjunction with other more trivial reagents. *N*-(3-Mercuri-2-methoxypropyl)-poly(DL-alanyl)amide was the macromolecular mercurial used and poly(DL-alanyl)cysteine the non-permeant regenerating compound. The permease inactivated by *N*-(3-mercuri-2-methoxypropyl)-poly(DL-alanyl)amide or PCMBS can be fully reactivated by mercaptoethanol, but the *N*-(3-mercuri-2-methoxypropyl)-poly(DL-alanyl)amide-inactivated permease could only be fully reactivated by poly(DL-alanyl)cysteine whereas the PCMBS-inactivated transporter was partially reactivated. The fact that the sulfhydryl group blocked belongs to permease was demonstrated by the observation that inactivation by *N*-(3-mercuri-2-methoxypropyl)-poly(DL-alanyl)amide is inhibited in the presence of melibiose. It was suggested that the permease contains SH groups in two interconvertible states, only one of which is accessible to macromolecular reagents, whereas both are accessible to PCMB and 2-mercaptoethanol [375].



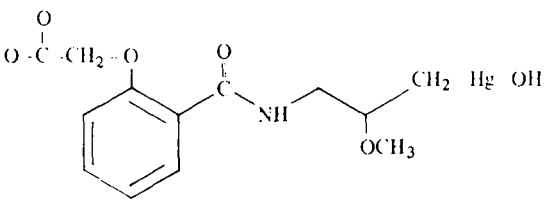
fuscin



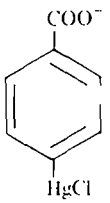
reaction product of fuscin



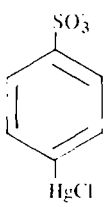
avenaciolide



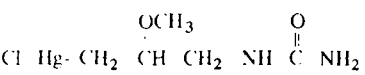
mersalyl



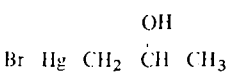
p-chloromercuribenzoate



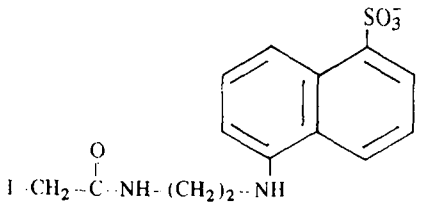
p-chloromercuri-benzenesulfonate



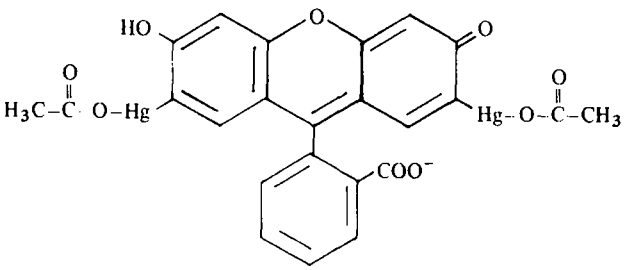
3-chloromercuri-2-methoxypropyl urea



1-bromomercuri-2-hydroxypropane



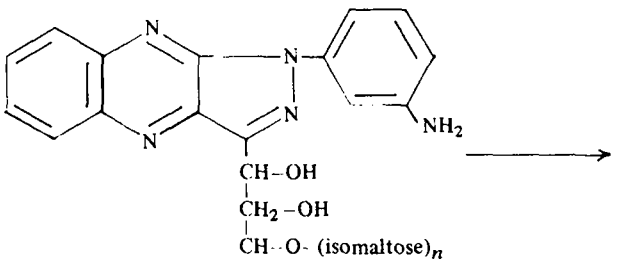
N-iodoacetylaminoethyl-5-naphthylamine-1-sulfonate

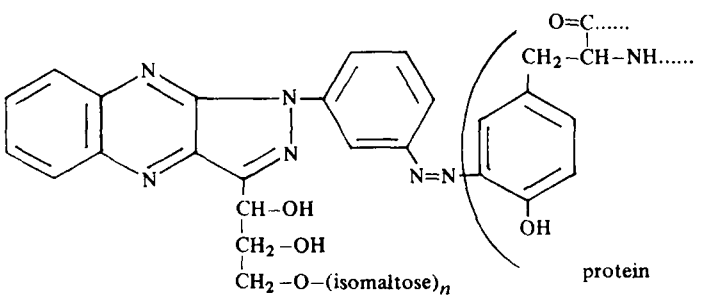


fluorescein mercuric acetate

Some SH blocking reagents mentioned in the text. Arrows (on fuscin and avenaciolide formulae) indicate the sites of reactions.

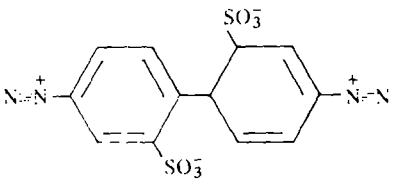
Carbohydrate chains of specified length have been used as carriers for flavazoles which can be diazotized to yield proteins. The sugar moiety may bear NH_2 or SH reagents or photoaffinity labels [376,377].





Contiguous proteins were covalently linked by short polysaccharide bridges. For this purpose, coupling reagents of the dextran (40)-2-(4-aminophenyl)sulfonyl ethyl ether type were used which are capable of multiple azo coupling. Experiments on sheep erythrocytes using this reagent led to the conclusion that at least six proteins are accessible to the external membrane surface [378].

Cross-linking with polylysine using the bifunctional reagent, tetra-azotized 4,4'-diamino-2,2'-biphenyldisulfonic acid, indicated that in mitochondria, cytochrome *a* is



tetra-azotized 4,4'-diamino-2,2'-biphenyldisulfonic acid

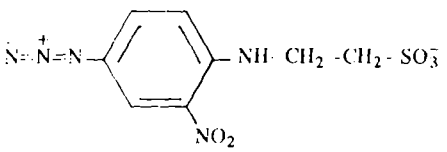
located on the same (external) side as cytochrome *c* and suggested that cytochrome a_3 is located on the same (matrix) side as the coupling factor 1 (ATPase) [327].

Another group of interesting reactants is the azides. These react only when activated by light. First, nitrenes are formed by a photochemical reaction [147,361,379–381], then nitrenes react in a non-selective manner giving rise to different products [147]. An illus-

$$R\text{-}N_3 \xrightarrow{h\nu} R\text{-}\ddot{N} + N_2$$

azide nitrene

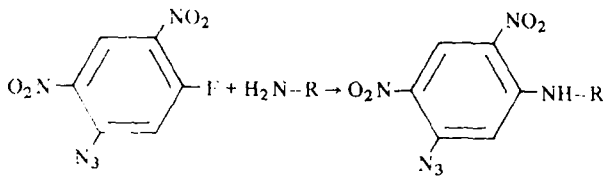
tration of the use of these reagents is provided by *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate [382] of which the permeability in membranes is temperature dependent. At 37°C it penetrates the membrane but at 0°C it is non-permeant. Under conditions in



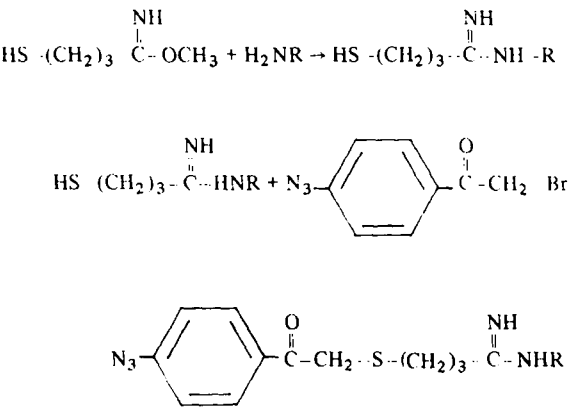
N-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate

which it does not penetrate, it binds, in the dark, to the anion-transporting band 3 protein of erythrocyte membranes. This non-covalent linkage is due to the fact that the reactant is an anion. It is admitted that negatively charged aromatic reagents label significantly the band 3 protein of erythrocyte membranes [312,313,384]. It can then be used as a photoaffinity-labeling marker. Fixed by a non-covalent linkage to band 3 protein, it reacts covalently with it once photochemically activated to a nitrene. In addition, experiments using *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate led to the labeling of glycophorin and of a series of proteins of 120 000 - 200 000 daltons. One of these proteins may be the compound revealed by the use of imidoesters [321].

Another use of photoaffinity labeling in relation to the sideness of membrane components is the reaction of 2,4-dinitro-5-fluorophenylazide with cytochrome *c* to obtain 2,4-dinitrophenylazide derivatives. One or two 2,4-dinitrophenylazide moieties are thus fixed



to this protein in the dark. It was found that reacted cytochrome *c* can still bind non-covalently to the cytochrome *c*-depleted mitochondrial membranes. After photolysis and analysis, cytochrome *c* was found to be covalently, though poorly (less than 5%), linked to cytochrome oxidase [385]. Using a different aryl azide derivative of cytochrome *c*, a quantitative yield of covalently linked cytochrome *c*-cytochrome oxidase was obtained [386]. For the preparation of the aryl azide, first methyl-4-mercaptobutyramidate was reacted with cytochrome *c*, then the newly available SH group was reacted with the photosensitive label, *p*-azidophenacyl bromide [147,361,379,386]. Using this new arylazide, the molar ratio of cytochrome *c* to cytochrome oxidase in the complex was found to be 1 : 1. This complex is able to mediate the electron transfer between



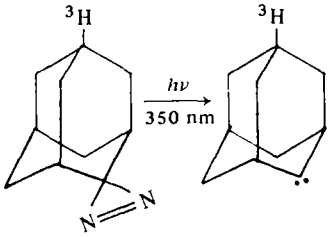
the artificial substrate *N,N,N',N'*-tetramethyl-*p*-phenylenediamine ascorbate and the oxidase [386]. When the same aryl azide derivative from *S. cerevisiae* cytochrome *c* was prepared and linked to mitochondrial cytochrome oxidase, analysis showed the attachment of the cytochrome to a subunit of the oxidase with an apparent molecular weight of 15 000. This is subunit III, which, among the subunits of the complex, probably bears the functional site, since the linked cytochrome *c* can be oxidized [387,388]. In another study, 3-nitrophenylazidocytochrome *c* was prepared by reacting 4-fluoro-3-nitrophenylazide with cytochrome *c*; Lys-13, Lys-22 or both were suggested to react with the reagent. The tagged cytochrome *c* was used for photolabeling isolated cytochrome oxidase. The results obtained are interpreted in terms of specific covalent interactions of the Lys-13 derivative of cytochrome *c* with the 22 000 dalton subunit II of cytochrome oxidase [389]. This result is at variance with that obtained when membrane-linked cytochrome oxidase was used [385,386]. It was noted that the complex obtained with the isolated cytochrome oxidase was not able to transfer electrons from ascorbate to molecular oxygen and that the formation of an inactive complex might occur at a site uncovered during the isolation. This site may be different from a 'native site' when cytochrome *c* binds to the cytochrome *c*-depleted mitochondria. This situation bears a striking similarity to that of the site of interaction of dicyclohexylcarbodiimide with subunits of *E. coli*

and chloroplast ATPases, as discussed above [200,201,206]. Here we face the general, subtle and important problem of the possible variability of the reactivity of molecules when they are removed from their natural membrane environment. However, this is not always the case; for instance, isolated, water-soluble band 2.1 and 4.1 components of erythrocyte membranes were found to exhibit strong binding to spectrin. However, in this case, both enhanced spectrin binding to spectrin-depleted inside-out vesicles which were stripped from both 2.1 and 4.1 proteins. The sites of interaction of spectrin with the membrane-bound and the free proteins are presumably identical [390].

A particular use of photoactivation was made in order to ascertain the presence of a cytoplasmic segment in the intestinal brush border aminopeptidase. The brush border membrane was separated as right-side-out vesicles [391] (see below for more details and references) and the photosensitive reagent, 4-fluoro-3-nitrophenylazide, coupled to the Fab fragment of a human IgG myeloma protein, was trapped in the dark in sealed brush border vesicles. After thorough washing, 'labeling from inside' was carried out using illumination. It was, in fact, observed that both the detergent-solubilized enzyme and the 'hydrophobic' peptide, including the eventual cytoplasmic segment, were labeled and this was ascertained by using peroxidase-labeled antibody raised against the human IgG myeloma proteins. These results are remarkable, since only 3% of the weight of the enzyme studied, as well as that of some other brush border hydrolases such as renal aminopeptidase and intestinal maltase, is removed by proteolysis from the inside. This indicates that the segment exposed to the cytoplasmic side is very short [392].

Photogenerated nitrenes and carbenes were recently used for the specific labeling of membrane-embedded segments of intrinsic proteins. An aryl azide (5-[^{125}I]iodonaphthyl-1-azide), having the remarkable property of exhibiting a high partition coefficient into the lipid core of membranes, reacted only with integral proteins; very little if any insertion occurred into peripheral proteins as reported by Bercovici and Gilter [393]. In rabbit skeletal muscle sarcoplasmic reticulum, where the main insertion of the nitrene was into the Ca^{2+}-sensitive ATPase, the activity of the enzyme was not affected by labeling. In intact erythrocytes and hemoglobin-free erythrocyte membranes, little if any label was inserted into the peripheral protein bands 1, 2 or 5 (spectrin and actin [254,255]). Labeling occurred in the region of band 3, periodic acid Schiff 1, 2 and 3 bands and in band 7, which are all integral proteins. In another application, using 5-iodonaphthyl-1-azide, the labeling of the polypeptide of the acetylcholine receptor-rich membrane fragments from *Torpedo californica* electroplax was examined. Only two membrane polypeptide chains of 90000 and 40000 daltons were extensively labeled, with only minor incorporation into other peptides (55000–60000 dalton region). Specific ligands such as α-bungarotoxin and carbamylcholine did not affect the labeling. Also, an aqueous nitrene scavenger, glutathione, did not affect the labeling. When the acetylcholine receptor was purified by affinity chromatography, it was confirmed that only the 40000 dalton peptide belongs to this receptor. This peptide is the only one of the four 'subunits' of the receptor which was labeled. Trypsinization of the receptor indicated that the label was in fact associated with the membrane-embedded fragment of 13000 daltons; the 27000 dalton segment was not labeled. When purified acetylcholine receptor was solubilized in Triton X-100, all four subunits could be labeled [394]. Carbenes have also been used instead of aryl nitrenes for the labeling of intrinsic proteins. Carbenes have been reported to be more reactive than nitrenes [395–398]. Carbene is generated photochemically from ^{3}H-labeled adamantane diazirine within the biological membrane. Applied to erythrocytes, 70–85% of the label of glycophorin is found in its hydrophobic segment embedded in the mem-

brane (residues 73–95). Here again, the labeling is not changed in the presence of gluta-



1-spiro[^{3}H]adamantane-4,4′-diazirine

thione, a water-soluble scavenger of reactive intermediates. The same reagent has been used to study the buried part of the major human histocompatibility antigens, HLA-A_2 and HLA-B_7. Papain has been found to act on detergent-solubilized HLA antigens in two steps. First, most of the hydrophilic C-terminal sequence is eliminated, then the hydrophobic sequence and a small number of the adjacent amino acids are cleaved from the N-terminus [399]. About 85% of the label of HLA-A_2 and HLA-B_7 antigens in lymphoblastoid cells has been found in this hydrophobic fragment [398]. When applied to influenza virus, 75% of the radioactivity of the hemagglutinin (HA_2) is found in the fragment retained in the membrane after bromalain treatment of the virus. This treatment is known to cleave a 20000 dalton hydrophilic fragment leaving a 5000 dalton fragment, which comprises the embedded segment [400,401]. Furthermore, HA_1 which is exposed to the outside but linked by a disulfide bond to HA_2 [402] is not labeled. However, it was observed that 1/5 of the protein-bound label is in the matrix protein which indicates that this protein may be an integral protein. Nucleocapsid protein was not labeled. Nevertheless, neuraminidase, an intrinsic influenza virus membrane protein, was negligibly labeled for reasons unknown [398].

Let us mention in concluding this section that affinity and photoaffinity markers are used for the labeling of membrane protein binding sites. In many cases, this type of labeling not only facilitates the recognition and isolation of proteins bearing the specific sites, but also is able to provide information about the immediate environment of these sites. Preliminary studies with the peptide hormone gastrin receptors were carried out by using aryl azide or aryl ketone derivatives of a model ligand (in fact the COOH-terminus pentapeptide of gastrin [403]). Photoaffinity labeling, by using bovine insulin derivatives which were in addition ^{125}I-labeled, showed that in the liver plasma membrane of rat, mouse and pig, two proteins of 130000 and 90000 daltons could be labeled [404]. The use of 2-azido-4-nitrophenol, an analogue of the putative uncoupler of oxidative phosphorylation, 2,4-dinitrophenol, allowed the labeling of a mitochondrial inner membrane protein of 20000–30000 daltons [405]. Numerous studies have been made with acetylcholine receptors [406–410]; in particular, it has been shown that this receptor has a reducible disulfide bond. The reduction of this disulfide bond exerts profound effects on the response of the cell to cholinergic agents. The distance between this disulfide bond and the presumably negatively charged site of the receptor reacting with the quaternary ammonium group of acetylcholine was examined by using affinity labeling with α-bromoacetylcholine (a receptor activator) and 4-(*N*-maleimido)-α-benzyltrimethylammonium (a receptor inhibitor), both displaying a quaternary ammonium group and an SH group-reactive residue. The distance between the charge and the SH group-reactive part of the former and the latter molecules is, respectively, 6.6 and 9 Å [407]. Both are about 1000-

times more active than acetylcholine and *N*-ethylmaleimide, the action of which they mimic, respectively. It was concluded that in the resting, inactive state of the receptor, one of the SH groups formed by reduction (and probably the parent disulfide group) is at approx. 1 nm from the negative 'subsite' and that in the active state a shorter distance prevails [407]. The use of quaternary ammonium aryl azides (photoaffinity labels) and that of quaternary ammonium diazonium salts (affinity labels), indicated the presence of serine, tyrosine, histidine and lysine in the vicinity of the binding site of the acetylcholine receptor (for a review, see Ref. 410). This receptor is comprised of a 40 000 dalton protein which carries the acetylcholine site [409,411,412]. The role of another protein of 43 000 daltons and those of minor polypeptides of 50 000 and 65 000 daltons accompanying acetylcholine receptor preparations have been questioned [411,412]. Recent studies using affinity labeling of the receptors (α-bromoacetylcholine, 4-(*N*-maleimido)-α-benzyltrimethylammonium) indicate that these compounds block half of the binding sites for α-bungarotoxin. This fact and the results of complementary studies were interpreted as meaning that two functional sites may exist in the acetylcholine receptor [413]. Lectin receptors can be revealed by affinity labeling, as evidenced by the labeling of peanut agglutinin receptors on neuraminidase-treated human erythrocytes [414]. Recently, an analogue of cyclic AMP (8-N_3-cyclic [^{32}P]AMP) was shown to label the chemotactic receptor of the slime mould, *D. discoideum*, of 40 000 daltons as well as a 42 000 dalton protein (actin) associated with the cytoplasmic side of the membrane. The labeling of the 42 000 dalton protein occurred after the conversion of the label into 8-N_3[^{32}P]AMP by the membrane phosphodiesterase. In addition, different developmentally regulated cytoplasmic proteins were labeled [415].

VI. Enzymatic isotopic labeling of membrane proteins

Two examples of this type of reaction are the iodination of proteins with the help of lactoperoxidase and the oxidation of the non-reducing end of galactose (or galactosamine) groups with galactose oxidase, followed by reduction of the aldehyde thus formed with Na[^{3}H]H_4. Other enzymatic labeling reactions have also been carried out.

Before describing observations made with enzymatic iodination, it is worth recalling that the chemical iodination of proteins including antibodies, lectins, protein hormones or other ligands is possible. This is performed by using ICl [416,417] or by employing chloramine T (*N*-chloro-*p*-toluenesulfonamide) [418,419], which acts as an oxidizing agent and efficiently substitutes ^{125}I into tyrosine residues of proteins in the presence of Na^{125}I. The ^{125}I-labeled 3-(3,5-diiodo-4-hydroxyphenyl)propionic acid *N*-hydroxysuccinimide ester [420], which acts as an acylating agent with amino groups of proteins to form an amide bond, is also used for the iodination of proteins.

Different peroxidases are able to catalyze the formation of carbone-halogen bonds by a nucleophilic acceptor in the presence of H_2O_2 and I^- [421–427]. Thus, the iodination of tyrosine (and histidine) residues with Na^{125}I takes place. The reaction is formally written as follows:

$$^{125}I^- + H_2O_2 + \text{acceptor} \quad H^+ \rightarrow \text{acceptor} - {}^{125}I + H_2O + OH^-$$

Since lactoperoxidase (molecular weight 77 500–78 500 [425,428]) is impermeant, it may be used directly for labeling the exposed membrane proteins. Lactoperoxidase is active at physiological pH values and low I^- concentrations and an enzyme-bound intermediate is suggested to transfer iodine directly to the protein [426]. In order to produce

H_2O_2 in a smooth and controlled manner, glucose and glucose oxidase are used. Under these conditions, only monoiodotyrosine is believed to be formed and therefore the peroxidation of lipids and proteins is avoided [425] *. Murine fibroblasts treated using this technique were shown to be able to grow, to divide and to perform phagocytosis. Different authors have used repeated addition of lactoperoxidase and H_2O_2 in order to avoid iodination and, hence, deactivation of the lactoperoxidase itself [429]. Others have employed 'solid state' lactoperoxidase, which is lactoperoxidase covalently bound to Sepharose 4B [430,431]. This 'insoluble enzyme' was found to be active in the presence of low protein concentrations, whereas with the soluble enzyme, relatively high concentrations of protein are required for iodination to occur. In addition, the complexed form of the enzyme is active in the presence of urea and SDS and at 0°C; thus, its use was judged to be 'versatile' [431]. Some 'self-iodination' was observed, which concerned mostly the supporting beads. The mechanism of this iodination which requires the enzyme is not clear. This iodination and the eventual iodination of the linked enzyme do not deactivate the catalytic activity so that the insoluble enzyme can be re-used [431].

In some cases, as in the study of exposed proteins of the external leaflet of the external nuclear envelope, where there is the possibility of reactants penetrating the nuclear pore complex, the immobilized enzyme is used [432]. One must be cautious in interpreting the results when use is made of the iodination technique, since the isotope may penetrate the membrane, presumably due to I_2 formation [256] or generation of I^+ [255]. Membrane lipids may be iodinated (see the companion paper [2a] for details) and intracellular membrane proteins may show radioactivity which is not related with the external proteins [433,434]. Different experimental conditions have been developed to avoid internal protein labeling. As an example, using erythrocytes, conditions have been found such that the labeling of hemoglobin, which constitutes about 90% of the cytoplasmic proteins, is not significant. This indicates that penetration does not occur [425, 434]. It is pertinent to consider in this respect that attributing the subfractions of cell homogenates to cell surface fragments after enzymatic radioactive iodination has been criticized, since for the reasons just discussed, there seems to be no guarantee that intracellular membranes are not labeled (for a discussion see Refs. 435 and 436) and an alternative technique has been suggested for the detection of the cytoplasmic membrane in homogenates. This technique covalently couples β-galactosidase, an *E. coli* enzyme, to the cell surface using bisdiazobenzidine; the activity of this enzyme allows the recognition of cell surface contaminants in other cell membrane fractions. This method has been applied to different cell types such as lymphoblasts, adenocarcinoma cells and spleen cells [437]. Recently, radioactive iodination of human platelets was carried out [438]. Electrostatic binding of cells to positively charged polylysine beads, due to the negatively charged cell surface molecules [439,440], led to the firm attachment of large areas of surface membrane which remain linked to the beads after washing and sonic disruption. It was found that a 9-fold greater ^{125}I specific activity was associated with the bead-associated membranes, which were enriched in putative marker enzymes (bis(*p*-nitrophenyl)phosphate phosphodiesterase and $(Na^+ + K^+)$-ATPase) for cytoplasmic membranes. In the companion paper, more details and references concerning the employment of positively charged particles will be given.

The use of the lactoperoxidase-$Na^{125}I$ technique demonstrates that erythrocyte extrin-

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* It has been stated by one author that later histidine labeling was also observed. This is quite plausible and will depend on the material examined and on the conditions used.

sic proteins comprising 40% of the total proteins of the membrane are exposed only to the cytoplasmic compartment [425]. It permits the tagging of band 3 protein and glycophorin [441] and, using leaky ghosts, the C-terminal fragment of glycophorin can be labeled, showing that this protein spans the membrane [442]. When sealed ghosts are used, however, band 1 (spectrin), 3 and 4 proteins are labeled and glycophorin shows little or no labeling [443–445]. Nonetheless, some authors have observed a tenuous labeling of glycophorin from the inside in resealed ghosts [446]. Furthermore, in this experiment, in order to avoid problems caused by the release of entrapped lactoperoxidase from resealed ghosts, and thus inadvertent labeling of molecules by reactions from the outside, care was taken to add catalase to the medium to destroy external H_2O_2. H_2O_2 is formed inside the sealed ghosts by the above-mentioned glucose-glucose oxidase system [425]. However, using inside-out ghosts, it has not been possible to label glycophorin using the lactoperoxidase technique [447] and in some experiments, this protein was not significantly degraded proteolytically in inside-out ghosts [448]. The discrepancy between the results of different authors concerning the internal exposure of glycophorin when using the iodination technique may be explained by the fact that the presumptive cytoplasmic segment of this protein has only one tyrosine residue located immediately adjacent to the hydrophobic segment. In addition, it is suggested that iodination might have been effected 'by generating, in excess, ^{125}I radicals' which are able to iodinate tyrosine in spite of its inaccessibility [255]. In a recent study on murine erythrocytes, during which the optimal conditions for lactoperoxidase oxidation (i.e., the use of H_2O_2 or its generation by the glucose oxidase-glucose system for restriction of labeling of the hemoglobin to less than 5%, etc.) were determined, the major labeled protein was found to be a 92 000 dalton protein which is analogous to the anion transport protein, band 3 [449].

To ascertain whether a protein spans the membrane, an interesting trick was used by Hunt and Brown [450] in applying the lactoperoxidase technique to cell surface labeling. After the reaction has occurred, reversed vesicles are prepared by using the polystyrene latex bead procedure [451,452] and then treated with a proteolytic enzyme. If an ^{125}I-labeled protein spans the membrane, it is shortened and this can be monitored by analysis. By using this method of preparation of phagocytic vesicles, it was shown that the murine L-cell membrane contains a high molecular weight (approx. 250 000) transmembrane protein which is also present in normal cells but not in virus-transformed fibroblasts. Trypsinization of the vesicles left a membrane-linked 65 000 dalton radioactive material which did not exist before and the high molecular weight material (fibronectin) disappeared.

The asymmetric distribution and orientation of plasma membrane-associated proteins of murine L929 cells have been studied using the lactoperoxidase-$^{131}I^-$-labeling procedure. Proteins prepared from ^{131}I-labeled latex-filled phagolysosomes (prepared from unlabeled cultured cells), phagolysosomes derived from ^{131}I-labeled cells and phagolysosomes derived from [^{35}S]methionine-labeled cells were analyzed using two-dimensional gel electrophoresis. As a result, differences in labeling were observed. At least two prominent [^{35}S]methionine-labeled 60 000 and 100 000 dalton proteins were found to be available to both sides of the membrane. Furthermore, partial hydrolysis of the 100 000 dalton protein confirmed that different fragments were iodinated when either intact cells or phagolysosomes were labeled [453].

Lactoperoxidase labeling of platelets showed that three major glycoproteins and four additional compounds were labeled [454]. The major glycoproteins are cleavable by trypsin. One of these glycoproteins can be precipitated with concanavalin A and may be the

lectin receptor of platelets [455]. By using this technique, the eventual identity of four surface proteins of platelets and circulating lymphocytes has been reported [456].

Labeling a rat liver rough microsomal fraction, by using the lactoperoxidase and glucose oxidase techniques and subsequent control experiments, led to the conclusion that all membrane proteins are labeled in this case [337]. No major membrane proteins were observed to be exclusively labeled in the presence of a low detergent concentration (0.049%) which increased the labeling or membrane proteins and allowed the entrance of the iodination system into the vesicles, as judged by the labeling of the protein content of the vesicles. This treatment did not disrupt the membrane. However, disruption of vesicles by using a high concentration of detergent did not result in the labeling of new proteins [337]. Other applications of the lactoperoxidase-$^{125}I^-$-labeling technique have been focussed on the outer [457] and the inner [458] mitochondrial membranes. The outer mitochondrial membrane is permeable to cytochrome *c* and proteins of molecular weights of about 10000 (Ref. 459; see, however, Ref. 460) but it should be impermeable to lactoperoxidase. Therefore, it is expected that the labeled proteins are exposed to the outer surface of the outer membrane. It was found that besides some high molecular weight proteins, a protein of molecular weight approx. 14000 was prominently labeled [457]. Adenylate kinase, a soluble enzyme located between the outer and the inner mitochondrial membranes, was assayed for its catalytic activity before and after iodination and the conclusion drawn from the results was that the membrane remains intact during iodination. Interestingly, when the outer membrane was fragmented by digitonin treatment, a portion of its proteins was found to remain selectively with the inner membrane particles. It was concluded that the outer membrane may be heterogeneous with respect to protein components in the plane of the membrane, since the specific, high molecular weight, labeled molecules mentioned above remain with the inner membrane. A possible explanation is that these proteins might contribute to the contact points between the outer and the inner membrane. The presence of such contact points has been reported by Hackenbrock [461,462]. The application of the lactoperoxidase-$^{125}I^-$-labeling technique to inner mitochondrial membrane-matrix preparations led to the observations that few if any proteins were labeled, both sonicated and digitonin-treated inner membrane vesicles could be iodinated and every major band was found to be labeled. It was envisaged that both preparations contain both right-side-out and inside-out vesicles, since both preparations could be fractionated by Ludox gradient centrifugation. The upper bands were slightly labeled and the lower bands were extensively iodinated. It was suggested that in both cases, the upper band corresponds to right-side-out and the lower to inside-out vesicles. When the iodination system was present during sonication, both upper and lower fractions were extensively iodinated [458].

The case of muscle microsomes deserves some development. After reduction of sarcoplasmic reticulum vesicles with 2-mercaptoethanol, the Ca^{2+}-ATPase appears as a dissociated oligomer and, in addition, SDS-polyacrylamide gel electrophoresis shows the presence of minor bands of 45000–55000 daltons (C_1 and C_2 [463]) that have been termed C proteins or named calsequestrin and calcium-binding protein, respectively [464]. On the basis of the inaccessibility of C proteins to digestion with proteolytic enzymes (trypsin) and their non-reactivity with anti-C antibodies, the internal exposure of these proteins was postulated [465,466]. However, other evidence argued in favor of their external exposure. Thus, they were rapidly released by EGTA treatment at pH 8 [463] and easily reacted with fluorescamine [153]. In addition, when sarcoplasmic reticulum was labeled by using the lactoperoxidase-glucose oxidase-glucose system, the specific activity of calse-

questrin was found to be many times greater than that of the Ca^{2+}-ATPase which suggests its exposure on the external surface [153]. C proteins have been considered to be located on the external surface but in such a way that their interactions with antibodies, hydrolytic enzymes and also toluene-2,4-diisocyanate-albumin are prevented due to steric hindrance [153].

Lactoperoxidase labeling was used to study surface modification of the social amoebae, *D. discoideum*. It was found that in the vegetative state, two proteins of apparent molecular weights of 55 000 and 135 000 were labeled. A new glycoprotein was labeled with the development of competent cells [467].

By using the lactoperoxidase-catalyzed iodination technique, immunoglobulin M [468-472] has been localized on the surface of lymphocytes. Murine lymphocytes bear also IgD [471,472]. Iodinated murine IgM and IgD were found to be shed with biphasic kinetics, the rapid phase of IgD shedding being somewhat slower (half-time 12 h) than that of membrane IgM (half-time 7-8 h) [473]. Among other observations, it was noted that shedding of IgD is sensitive to colchicine but not to cytochalasin. Conversely, the shedding of IgM is sensitive to cytochalasin but not to colchicine. Thus, the shedding mechanisms of the two Ig isotypes are different and are regulated by microfilaments or microtubules [473]. Surface changes in different circumstances are reflected in the iodination pattern. Thus, limited trypsinisation led to increased iodination by the lactoperoxidase technique [474]. Similarly, increased labeling was observed in paroxysmal nocturnal hemoglobinuria erythrocytes, and glutathione-treated red blood cells showed decreased labeling. This observation led to the conclusion that glutathione-treated erythrocytes do not constitute a good model for the study of the defective red blood cells in this disease [475]. An important difference in the surface glycoprotein of fibroblasts was observed between normal and virus-transformed cells. Radioactive iodination catalyzed by lactoperoxidase led to the labeling of a surface protein of apparent molecular weight 250 000 on NIL and 3T3 fibroblast cells; virus-transformed cells do not contain this protein [476, 477]. This protein is a glycoprotein of which the amount at the cell surface is dependent upon the growth state [478]. Transformation of chick fibroblasts, by Rous sarcoma virus, greatly decreased the content of a surface glycoprotein, as demonstrated by the lactoperoxidase labeling or immunofluorescence techniques [479].

Recently, immunolactoperoxidase was used in connection with ligand-induced patching and capping. The enzyme catalyzes the iodination of those surface proteins which, after aggregation, possess exposed tyrosine groups in the immediate vicinity of the patch or cap of a particular antigen. As an illustration, the patching and capping of the histocompatibility (H-2) antigen of murine lymphocytes has been studied using this technique [94]. Cytochemical analysis indicated that the antibody alone promoted the same patching and capping of H-2 antigen as did the lactoperoxidase-conjugated antibody. When ^{125}I-labeled proteins were analyzed by using SDS-polyacrylamide gel electrophoresis, it was observed that a large (200 000 dalton) component was labeled. This observation indicated selective redistribution of membrane proteins, since a number of the prominent membrane proteins remained unlabeled.

Another example of the enzymatic labeling of membrane proteins is furnished by the labeling of glycoproteins (and glycolipids) having a galactose or galactosamine residue at their non-reducing hydroxymethylene end. By using this technique, the hydroxyl at the C-6 position is oxidized to an aldehyde with the help of galactose oxidase isolated, for example, from *Dactylium dendroides*. Oxidation does not proceed further to uronic derivatives [480] as has been reported for the enzyme from *Polyporus circinatus* [481].

Galactose oxidase does not penetrate the membrane, since it has a molecular weight of 78 000 and only galactose and galactosamine residues are specifically oxidized [480]. The aldehydes formed can then be reduced and the glycoprotein labeled by $NaB[^3H]H_4$. This reduction may be carried out either during the enzyme reaction [482] or in another step [483]. In some cases, cells have been treated with neuraminidase prior to treatment with galactose oxidase [480,483]. This treatment eliminates surface sialic acids and exposes subterminal galactose or galactosamine residues. It should be noted that with the use of this technique, at least four different molecular species are labeled when human erythrocytes, or ghosts prepared therefrom, are studied. However, as expected, erythrocyte inside-out vesicles are not labeled [482,483], which is in line with other observations indicating that erythrocyte carbohydrate-bearing molecules are externally exposed. A component of molecular weight 150 000 was labeled in isolated membranes but not when whole cells were examined [483]; this may be due to cross-linking of an aldehyde produced during the oxidation reaction with amino groups. Similar observations were made with rabbit erythrocytes and rat reticulocytes; high molecular weight components were observed with membranes but not with cells. A mobility of proteins which is greater in membranes than in intact cells was suggested to facilitate this interaction. Alternative explanations may be envisaged. The possibility that this protein is translocated from the inner surface of erythrocytes during the preparation of ghosts is not supported by the observation that the inner leaflet is devoid of both glycoproteins and glycolipids [482]. The possibility that a glycoprotein of the outer surface becomes unmasked may be considered. The presence of such a glycoprotein is not observed in human erythrocyte membranes by using conventional electrophoretic techniques [155]. However, it has been reported that galactose oxidase-$NaB[^3H]H_4$ labels glycoprotein species 'not clearly demonstrated by conventional stains' [482]. The presence of a material appearing between the SDS-polyacrylamide gel origin and band 3 protein has been mentioned [482], but it is attributed to 'traces of glycoproteins which have become covalently cross-linked Schiff bases formed from galactoaldehydes and reduced by $NaB[^3H]H_4$'. On the other hand, some proteins were reported to be labeled even when galactose oxidase was omitted. This occurred with a 57 000 dalton component for several cell lines (NIL, BHK, 3T3) [480,483–485]. Different possibilities have been envisaged [480]. Labeling was correlated with the eventual reaction of an acyl phosphate, the presence of which has been reported in the membrane ATPase of endoplasmic reticulum [484]. Alternatively, the presence of Schiff bases formed, for example, by lysine-carbohydrate condensation as in aged collagen, has been suspected [485]. Horse erythrocyte glycoprotein was not labeled using either the lactoperoxidase or the galactose oxidase-$NaB[^3H]H_4$ technique. It was not cleaved either by trypsin or by pronase. It can be labeled, however, by using the galactose oxidase technique after treatment with neuraminidase [486]. The application of this method allowed the observation of the crypticity phenomenon of glycoproteins (and glycolipids). Thus, when synaptosomes isolated from rat brain cortex and the external membrane of these synaptosomes were examined, it was observed that in synaptosomes, 80% of the radioactivity was linked to two SDS bands, one of 72 000 daltons and the other of between 7800 and 3200 daltons. Little activity was bound to the bands at 160 000, 96 000 53 000, 39 000, 34 000, 23 000 and 16 000 daltons. However, the radioactivity incorporated into the isolated synaptosome membrane was 5–6-times higher and 80% of this activity was present in polypeptides of three domains: 160 000–96 000, 70 000–40 000 and 7800–3200 daltons. Thus, in isolated synaptosome membranes, more protein is accessible than in synaptosomes, although glycoproteins are exposed to the medium in

both cases [487]. Similar crypticity was observed for glycolipids (see the companion paper).

By using the galactose oxidase-NaB[^{3}H]H_4 method, two glycoproteins were revealed in hamster (NIL) and baby hamster kidney (BHK) cells. One, termed glycoprotein a, has a molecular weight of 200 000 and the other a molecular weight of 130 000 [480]. Galactoprotein a has been reported as being the receptor for ricin and concanavalin A [488]. However, recent studies indicate that after removal of galactoprotein a from BHK cells, as many as 15 glycoproteins can bind ricin [489]. Galactoprotein a, also called fibronectin [489–492], has been the subject of numerous studies, since it is believed to play an important role in cell adhesion [488–492]. This glycoprotein appears in the cell surface early in the G_1 phase of the cell cycle [489–493]. Non-proliferating cells in dense culture are richer in fibronectin than rapidly growing or virus-transformed cells [494] and metastatic cells are deficient in this protein [495]. Studies with heterocaryons, cells which are formed by fusion of two (or more) cells [496–500], however, show that the expression of this protein on the surface is determined by the genotype of the transformed parental cell [501]. A glycoprotein similar, but not identical, to fibronectin is found in the serum [502].

A surface-labeling procedure, using acyl hydrazides acting on membranes after chemical (periodate) or enzymatic (galactose oxidase) oxidation, introducing aldehyde groups into appropriate carbohydrate residues was recently applied to bovine erythrocyte membrane [503]. Oxidation with periodate was rapid and reached completion after 5 min; that catalyzed by galactose oxidase took 240 min.

$$R\text{-}CHO + H_2N\text{-}NH\text{-}\overset{\overset{O}{\|}}{C}\text{-}R_1 \rightarrow R\text{-}CH\text{=}NH\text{-}NH\text{-}\overset{\overset{O}{\|}}{C}\text{-}R_1$$

Reaction of oxidized groups with acetylhydrazide reached completion in 1.5 and 6 h when chemical or enzymatic oxidation, respectively, was carried out. If the latter reactions were in turn catalyzed (by aniline *), then the [^{3}H]acetylhydrazide reaction was completed within 30 and 40 min, respectively. Of practical interest is the fact that when oxidation was carried out using galactose oxidase, the hydrazone formed was stable at 30°C for 24 h. In contrast, when chemically oxidized membranes were used, 50% of the membrane-bound label was lost after 6 h at 30°C, revealing, as expected, the heterogeneity of the oxidized sites appearing after periodate oxidation, whereas the galactose oxidase-catalyzed reaction is specific.

A combination of enzymatic labeling using galactose oxidase-KB[^{3}H]H_4 and lactoperoxidase-$^{125}I^-$, pronase treatment and membrane protein cysteine labeling using *N*-(iodoacetylaminoethyl)-5-naphthylamine-1-sulfonic acid was used to study the sidedness of chromaffin granule membranes. Analysis of these membranes allowed the resolution of at least 60 bands of which 40 were relatively intense and reproducible. Reactions were carried out either with the intact or the broken membranes. 11 membrane proteins were unreactive unless membrane lysis was performed. The carbohydrate moiety of glycoproteins was found to be exposed only on the matrix side of the membrane and at least two proteins were found to span the membrane [505].

* Aniline is known to enhance the rate of hydrazone formation between aldehydes and semicarbazide. The formation of a Schiff base with aniline followed by the displacement of the aniline moiety by semicarbazide has been suggested [504] to be involved in the mechanism of catalysis.

Recently, the use of the Ca^{2+}-dependent transglutaminase was introduced in cell surface modification and labeling. This enzyme catalyzes the cross-reaction of the ϵ-amino groups of lysine residues and the amide groups of protein-bound glutamine residues to form ϵ-(γ-glutamyl)lysyl links in proteins [506,507]. Different amines can replace the lysine residues of proteins. This property of the enzyme has been used in the marking of proteins with differently labeled amines such as the [^{14}C]ethyl ester of glycine, [^{3}H]-tyramine, dansyl cadaverine, [^{14}C]histamine, [^{14}C]noradrenalin and [^{3}H]putrescine [508]. The reaction catalyzed is:

$$\text{protein}-\overset{\overset{O}{\|}}{C}-NH_2 + H_2N-R \rightarrow \text{protein}-\overset{\overset{O}{\|}}{C}-NH-R + NH_3$$

This method is interesting by virtue of the diversity of labels it can introduce into a protein and also by the fact that in contrast to most of the chemical methods involving non-selective amine functions of proteins and lipids, it does not involve lipids. The molecular weight of a transglutaminase isolated from rabbit liver has been estimated to be 80 000 [509]. Thus, only exposed surface molecules will react. Using a model receptor and guinea-pig liver transglutaminase, a transfer reaction between the carboxamide group of the glutamine residues of each chain and both primary amino groups of a diamine or polyamine was observed. Similarly, using a genuine protein in which amino groups were blocked, cross-linking of chains by using diamines such as putrescine or polyamines such as spermine could be performed [510]. Transglutaminase has been employed in cross-linking studies using murine erythrocytes and rabbit skeletal muscle sarcoplasmic reticulum. This method has also been used for labeling the surface proteins of PM_2 virus [506]. Caution should be exercized in using this technique, since the source of the enzyme seems to be important in obtaining successful results [507].

Glycosyltransferases may be used for cell surface labeling. Thus endogenous sialyltransferase has been used for this purpose [256] and, interestingly, by means of *N*-acetylgalactosaminyl transferase action, it was possible to convert an O group erythrocyte to an A group cell [511].

Protein kinases have been used to label serine or threonine residues with [γ-^{32}P]ATP; accordingly, 15 polypeptides of the HeLa cell surface were labeled with an exogenous enzyme [512]. This technique has also been applied to retinal rods. Intact fresh bovine retinal discs were phosphorylated with labeled ATP in the presence of highly purified rhodopsin kinase. After regeneration, the visual pigment was extracted and the ^{32}P-labeled pigment purified. Freeze-thawing led to inverted discs as judged by entrapment of [^{3}H]-inulin, a non-permeant polysaccharide. Inverted discs were now able to bind concanavalin A. This observation shows that glycoprotein has became exposed; this has been also endorsed by the incorporation of [^{3}H]galactose from the precursor, UDP-[^{3}H]galactose, in the presence of galactose transferase. These studies confirm that the carbohydrate moiety of rhodopsin is intradiscal, its phorphorylation site extradiscal and that the protein spans the membrane [513]. The carbohydrate moiety is known to be fixed at two points, Asn-2 and Asn-15′, of the N-terminus of rhodopsin [514].

VII. Enzymatic cleavage of membrane proteins

Another tool is the modification by enzymatic cleavage during studies of the distribution or the orientation of proteins. Sialolysis by sialidase (neuraminidase, *N*-acetylneura-

minidate glycosyl transferase) has been shown to remove all sialic acid units, either from sialoglycoproteins or sialoglycolipids from erythrocytes [515], thus indicating the asymmetric orientation of these molecules. It has been reported that *Vibrio cholerae* sialidase, a Ca^{2+}-requiring enzyme, cleaves protein-bound sialic acid units but few if any lipid-bound moieties of tumorigenic membranes [516]. It is worthwhile mentioning that, during in vivo aging, erythrocytes undergo a 'natural' desialylation. It has been suggested that the newly exposed galactose or galactosamine residues in desialylated glycoproteins may serve as recognition signals triggering the elimination of senescent erythrocytes from the circulation [517]. Another physiopathologically remarkable observation in connection with desialylation, followed by galactose oxidase oxidation, was that when both these enzyme reactions were operated in vitro on peritoneal exudate lymphocytes (PEL) from Listeria specific donors comprising Listeria specific T lymphocytes [518], protection was decreased in liver and more strongly so in the spleen of recipients of these cells. When peritoneal exudate lymphocytes were treated with either of the above-mentioned enzymes alone, only a minor decrease in passive protection was observed. When PEL, after both enzyme reactions, were subjected to chemical reduction by $NaBH_4$, they behaved like cells submitted only to desialylation. The marked loss of protection in the spleen was paralleled by a decrease in the appearance of inflammatory T cells in this tissue, indicating that loss of protection was due to perturbation in the migratory pattern of cells related to the chemical in vitro manipulation of their surface [518].

An important group of cleavage enzymes acting on proteins is obviously the proteolytic enzymes. Plasma membrane proteins appear, in some cases, to be more resistant to proteolytic enzymes in intact cells than in membrane vesicles. Trypsinization of erythrocytes releases only small amounts of peptides derived from surface glycoproteins; though lymphoid cells and Ehrlich ascites cells are rapidly lysed by these enzymes [519].

Trypsinization of erythrocytes releases sialoglycopeptide fragments of molecular weight 37 000, stemming from glycophorin and bearing M or N specificity. 78% of the released fragment is carbohydrate [520,521]. The N-terminus of the glycophorin molecule exposed to the outside of the membranes bears 16 oligosaccharide chains which make this exposed fragment hydrophilic. The C-terminus of the molecule presents, exposed to the inside of the cell, anionic residues which can be eliminated by proteolytic treatment [522,523]. The hydrophobic segment of glycophorin A, embedded in the membrane, comprises residues 73 -95 [524,525]. As reported by Fairbanks et al. [254], upon proteolytic attack of 'leaky' erythrocyte ghosts, all the membrane proteins with one possible exception (peptide 6 which is intrinsically resistant) are cleaved. However, if intact cells or resealed ghosts are attacked by proteolytic enzymes, only two major components are partially hydrolyzed. While trypsin degrades only glycophorin, chymotrypsin and pronase are able to attack also band 3 protein [526]. Erythrocyte band 3 protein with a molecular weight of 95 000 is a glycoprotein, possessing a complex relationship with the membrane [527--532]. When proteolytically attacked from the outside, a fragment of 35 000 daltons from the C-terminus is cleaved. In addition, when a proteolytic attack is carried out on the resealed ghosts, a further and distinct 40 000 dalton fragment is cleaved from the inside. A fragment left in the membrane and having a molecular weight of about 17 000 is still able to transport anions [529,530]. It has been suggested that band 3 protein traverses the bilayers more than once [297], but that only the 17 000 fragment spans the membrane [530 -532]. Externally applied chymotrypsin and pronase (at a low concentration) or internally applied trypsin cleave the peptide chain at some well defined loci and do not inhibit transport. Other proteolytic enzymes such as papain

and pronase (at a high concentration) produce strong inhibition accompanied by degradation of the band 3 protein. A remarkable hypothesis was recently put forward concerning the functional complementarity of cleavage fragments. The 35 000 dalton fragment cleaved externally by the chymotrypsin reaction remains linked to the membrane as does the 60 000 dalton partner. Both these fragments can be cross-linked to each other by the bifunctional reagent, H_2DIDS, and thus a 95 000 dalton cross-linked protein is reconstituted. Cross-linking is suggested to take place in a region involved in the control of anion transport. Papain digestion gives also a 60 000 dalton fragment, but compared to chymotrypsin, six more amino acid residues are eliminated; the 30 000 dalton fragment, however, is partially digested. It was suggested that both the 35 000 and 60 000 dalton fragments are involved in anion transport and the transport inhibition due to papain digestion was thought to be correlated with the removal of a 5000–10 000 dalton fragment from the 35 000 dalton segment rather than, or in addition to, the removal of six amino acid residues from the 60 000 dalton fragment [532]. In other experiments, the effect of an acid proteinase, pepsin, on human erythrocyte band 3 protein was studied by subjecting lactoperoxidase-radioactively iodinated cells to enzyme lysis in the presence of 1 M acetic acid. In particular, three related overlapping products with a common C-terminus were observed and two other fragments from the C-terminal portion of the protein were identified. No well defined, large radioactive fragment could be solubilized from the membrane. The major site of pepsin cleavage leading to the overlapping fragments was estimated to be at 8000–13 000 daltons on the N-terminal side of the major site of extracellular cleavage of neutral proteases [533].

Microsomal cytochrome b_5 of rabbit liver is an endoprotein of 16 700 daltons and is composed of 151 amino acid residues. The major part of the N-terminus of the molecule is exposed to the cytoplasm. Tryptic hydrolysis cleaves this globular part of 11 000 daltons leaving the C-terminus of the molecule enriched in hydrophobic residues attached to the membrane [534]. Similarly, tryptic digestion cleaves a fragment of 33 000 daltons from NADH-cytochrome *c* reductase of 43 000 daltons. Here again, the membrane-linked fragment is enriched in hydrophobic residues [535].

Experiments have been carried out to determine the asymmetric orientation of some intestinal and renal brush border membrane hydrolases (for a review, see Ref. 392) by means of a combination of techniques including the use of hydrolytic enzymes. One of these hydrolases is an endopeptidase which is a specific activator of trypsinogen. Another is an aminopeptidase (aminopeptidase N), which represents about 8% of the proteins of the intestinal brush border membrane; the porcine enzyme contains as much as 23% of carbohydrate. Yet another aminopeptidase (aminopeptidase A) exists in the brush border membrane. Other hydrolases which have been investigated are maltase, sucrase-isomaltase, lactase and alkaline phosphatase [392,536]. These sugar hydrolases have been obtained free from each other and are reported to be independent in the membrane, each maintaining a high degree of translational freedom [536]. EDTA spontaneously separates brush border membranes from enterocytes to give right-side-out, 0.15 μm diameter vesicles. Papain treatment 'solubilizes' all the intestinal hydrolases, though at different rates, indicating that the vesicles are indeed right-side-out. The detergent (emulphogen)-solubilized form of these enzymes displays amphipathic properties and in contrast to the 'soluble form', aggregates upon removal of the detergent [392]. The general structural anatomy of the intestinal hydrolases is that of a hydrophilic domain protruding from the vesicles, a hydrophobic part embedded in the lipid core and a short segment protruding from the inner surface into the cytoplasm. The surface exposure of an important part of the intes-

tinal aminopeptidase N shown by papain cleavage was confirmed by the use of a particularly remarkable technique applied for the first time. The number of antigenic sites of proteins was first established to increase with their molecular weights and consequently with their exposed surface, if they are assumed to be roughly globular [392,537]. Antibodies were raised against the detergent-solubilized enzyme and the number of accessible antigenic determinants in the free and bound (vesicle) forms determined and compared. 12 antigenic determinants were found for the free enzyme and about eight determinants were found in the bound form; only four were masked [538]. It could be expected that if antibodies raised against the free (detergent) form were first depleted by the bound (vesicle) form, antibodies related to masked determinants would remain in solution. However, only antibodies to two determinants remained after exhaustion by vesicles and could be titrated by the detergent-soluble form. Most probably, the exhaustion of antibodies was the cause of the discrepancy if it is assumed that the protruding head groups and the membrane are separated by a significant distance, a situation which confers on the sites limited accessibility; this is in fact what is suggested. Comparable studies with kidney aminopeptidase N have led to similar results, indicating the external exposure of an important portion of the enzyme [539]. Other studies have confirmed the exposed nature of the catalytic site of intestinal aminopeptidase and the independence of its activity of the physical state of the lipid core [540,541]. The hydrophobic domain of hydrolases is rich in apolar residues as has been observed with intestinal and renal aminopeptidases as well as with intestinal isomaltase and maltase; this is probably also true for alkine phosphatase. It should be noted that the NH_2-terminal moiety of some of the hydrolases is oriented towards the cytoplasmic side of the membrane, as has been observed with porcine aminopeptidases N [542] which is a dimer [543], and with the rabbit enzyme which is a monomer [392]. We have seen above that the length of the fragment protruding in the cytoplasm is small.

Certain viruses are endowed with a lipoproteic 'envelope' bearing at least one transmembrane glycoprotein, the carbohydrate moiety of which is exposed to the medium. If tryptic hydrolysis is carried out, a fragment enriched with hydrophobic residues is left in the membrane. Proteolysis has shown that in vesicular stomatitis virus, from 550 amino acid residues of its glycoprotein, only 20 -30 remain attached to the membrane, thus a large part of the chain is cleaved [4,5]. Bromelain treatment of influenza virus cleaves from the hemagglutinin (25 000 daltons) a 20 000 dalton water-soluble fragment leaving a membrane-bound fragment of 5000 daltons [400,401].

A somewhat controversial case of the localization of proteins is that of M-protein in influenza virus. Antiserum to this protein has been reported not to interfere with virion surface properties [544–546]. However, a substantial part of the M-protein was found to be attacked by proteolysis [547] and a 13 000 dalton polypeptide, left after caseinase C treatment of virion, was suggested to constitute the fragment left in the membrane after lysis of M-protein [548]. Recently, antiserum to pure M-protein (of influenza PR8 virion) was found to neutralize the infectivity and to inhibit the hemagglutinating activity of these virions. Antibodies to M-protein were readily adsorbed on either intact or spikeless particles obtained by partial proteolysis of surface molecules [548]. An additional argument favoring the surface exposure of M-protein is that Singapore virus [548] is sensitive to the drug, amantadine, which can prevent the expression of the virus genome [549]. It was observed that the amantadine sensitivity of influenza virus is determined by the M-protein [550].

Trypsinization destroys murine or human cells bearing, respectively, H-2 and HLA

determinants of histocompatibility antigens but limited proteolysis by papain can cleave these determinants [551,552]. Proteolytically cleaved fragments of 57 000 daltons from murine cells containing 10–15% carbohydrate and bearing more than one H-2 determinant (class I fragments) and fragments of 35 000 daltons carrying only one specificity (class II fragments) react in vitro with alloantibodies and in vivo experiments show that these alloantigen fragments induce allo-graft immunity [553], though with considerably less efficiency [554]. Autolysis can release stable H-2 fragments [555]. The structure of histocompatibility antigens is being actively studied (see for example, Refs. 551–563). Human histocompatibility antigens HLA-A and HLA-B are complexes of heavy 44 000 dalton transmembrane glycoproteins and a small β_2-microglobulin of 12 000 daltons (for the particular case of β_2-microglobulin, see Ref. 564). The heavy chain traverses the cell membrane. The N-terminal sequence exposed to the cell surface presents 280 residues, followed by a membrane-embedded 26-residue fragment rich in hydrophobic residues and then by the C-terminal hydrophilic sequence with 32 residues on the cytoplasmic side. HLA-determinant-bearing proteins, resembling the class II fragments mentioned above, have been obtained by simple sonication or detergent (sodium laurylsarcosinate) treatment [556]; intracellular (lysosomal) proteases may be responsible for cleaving these fragments.

The membrane orientation and location of multiple and distinct allogenic determinants of mouse IgD have been studied by "enzymatic and immunochemical dissection" of these molecules [565]. After limited trypsinization of IgD of a (and cross-reacting c) and b allotypes, IgD was no longer detectable using an immunofluorescence technique involving light-chain determinants, namely an allotype specific hetero-antiserum against IgDa and a hybridoma antibody against IgDb allotype. The use of different techniques such as immunoprecipitation, molecular weight analysis (by SDS-polyacrylamide gel electrophoresis) after lactoperoxidase-catalyzed cell radioactive iodination indicated that the respective cleavage fragments of trypsin digestion are liberated in the medium and correspond to the Fab fragment of IgD. Other immunological reactions indicated that the FC fragment of IgD is retained in the cell membrane after trypsin digestion and that it contains, like the Fab fragment, distinct allotypic determinants. It has been reported that both Fab and FC fragments bear carbohydrate moieties as judged by their reactivity with lentil lectin [565].

Other cases of the asymmetric proteolysis of membrane proteins have been reported. Studies with lobster and different squid species showed that extra-axonal application of proteases does not affect axonal function. Thus, lobster giant axons remain excitable and able to conduct impulses after external application of trypsin [566]. Application of proteases from the inside of giant axon, by injection of trypsin, perturbs the nerve function causing decreased excitability (within 2 min) and functional collapse (within 5 min). Similar observations were made by means of perfusion of squid giant axon with many different proteolytic enzymes, confirming in each case the perturbing action of intraaxonal perfusion [567–569].

Proteolytic cleavage of surface components by trypsinization prevents cell aggregation during the recovery period, as observed in experiments with retinal and tectal cells. Ganglion cells of neural retina [570–574] project to optic tectum in such a way that the dorsal-ventral and medial-dorsal axes are inverted. This was suggested to be correlated with surface ligands on ganglion cell axon and tectal cells, so that the axon from the extreme dorsal region of the retina would form synapses with a cell at the extreme ventral region of the tectum; the converse holding true for an axon derived from the extreme ventral

region of the retina [575,576]. This preferential adhesion of trypsin-digested cells is observed only after recovery from trypsinization and is prevented by trypsin and chymotrypsin due to the cleavage of surface proteins implicated in adhesion. If cycloheximide or puromycin are added during the recovery, preferential adhesion does not occur and the treatment of the tectal half with β-*N*-acetyl-D-hexosaminidase also prevents this preferential adhesion. Conversely, treatment of retinal cells with β-*N*-acetyl-D-hexosaminidase or treatment of tectum with proteases prevents the preferential adhesion of dorsal retinal cells to the ventral half of tectum [577]. These observations indicate that one of the partners of adhesion is a protein and the other may be a ganglioside with a terminal *N*-acetylgalactoside, i.e., ganglioside GM_2.

Before leaving this absorbing and multifacetted domain of the proteolysis of membrane proteins, I wish to mention briefly some particular aspects of this cleavage. The first is that the repercussions of proteolysis may be many-fold and sometimes unexpected; thus, as mentioned above, trypsinization is routinely used for separation of cells from tissues [578] and can also detach cells from glass and plastic [579]. It is generally assumed that trypsin treatment eliminates protein fragments involved in cell adhesion and, in fact, with one reported exception [580], glycopeptides were found to be released [478,581]. However, it was recently suggested that trypsin may act in affecting phospholipid status [582–585]. The adhesion behavior of embryonic chick neural retinal cells exposed to phospholipase A_2 and that of cells treated with trypsin appeared similar after pretreatment of cells in both cases with the chelating agent, EDTA. Cells exposed to EDTA alone adhere in the cold but their subsequent exposure to trypsin, phospholipase A_2 or lysolecithin induces non-adherence at low temperature. The components of a diacyl-monoacyl phospholipid turnover system have been reported to be present in the cell membrane in this case [585], as in the case of lymphocytes [585,586]. It was observed that with chick embryonic retinal cells, trypsinization affects the phospholipid turnover machinery; trypsinization of isolated cell membrane has the same effect. In both instances, lysophospholipids accumulate in the membrane. Investigations of the effect of this proteolysis on enzymes involved in phospholipid metabolism revealed that in isolated cell membrane, acyltransferase activity is decreased, CoA ligase activity somewhat stimulated and phospholipase A_2 activity unaffected. As a consequence, lysophospholipids accumulate in the membrane. We have seen above that protein synthesis is necessary for cell adhesion to take place after trypsinization, since it is inhibited by antimetabolite treatment as has been observed by Moscona and Moscona [587]. The protein synthesized could be directly involved in cell adhesion, but it has been suggested that trypsin may affect the reacylating system and that lysophospholipid accumulate and prevent cell adhesion [585], as has been observed with untrypsinized cells exposed to lysophospholipids [582]. It was concluded that 'these results do not preclude the possibility that there is a simultaneous damage of a ligand molecule but there is no need to propose the existence of such a species'. Let us now consider another recent example which will reveal another consequence of proteolysis. Intact, normal rat kidney fibroblasts or purified normal rat kidney plasma membranes were treated with either trypsin or papain. It was observed that a marked increase in the adenylate cyclase activity of confluent cells took place, whereas similar treatment of sparse cells gave only a marginal increase in cyclic AMP formation. Among the exposed cell molecules which were shortened by trypsin treatment of confluent cells was a 46 000 dalton protein. Trypsin treatment of sparse cells which was not accompanied by the modification of this protein did not activate the cyclase. Furthermore, treatment of plasma membrane of normal rat kidney cells resulted in the loss

of numerous bands including that of the 46 000 dalton protein. The proteolytic action of papain, compared with that of trypsin, appeared to be quite specific, causing a discernable loss in only the 46 000 dalton protein. It has been suggested that perhaps the activation of adenylate cyclase is correlated with the proteolytic modification of this protein [588].

The second particular aspect of proteolysis is that of shielding and of non-reactivity. This shielding is commonly encountered with reagents, particularly macromolecular reagents or ligands, and especially enzymes. I have mentioned that it is observed more easily with intact cells than with isolated membranes. Sugar-containing molecules can sometimes shield surface proteins from the action of proteolytic enzymes. The shielding effect of the sugar units of lipopolysaccharides in bacterial outer membrane has been reported [589]. Rough mutants of *Proteus mirabilis* in which the carbohydrate chains of lipopolysaccharides are partially missing [590] present about the same protein-to-phospholipid ratio in the outer membrane as the smooth wild-type strain. However, the susceptibility of the outer membrane polypeptides to proteolytic degradation increases with the shortening of the polysaccharide chains. In this study, the protein content of deep rough mutants appeared unchanged. In the case of *Salmonella typhimurium* [301] and *E. coli* [591] rough mutants, the phospholipid content does not change but the protein content is lower.

A case of non-reactivity to a particular proteolytic enzyme is illustrated by that of microsomal lysophosphatidic acid acyl transferase. The fact that chymotrypsin inactivates this enzyme activity in detergent-disrupted rat liver microsomes but not in intact microsomes falsely indicates a luminal location of the enzyme. In fact, 19% of the enzyme activity was reduced in intact microsomes treated with chymotrypsin, while 90% was lost in the presence of deoxycholate. However, it was realized that when pronase was used, about 87% of lysophosphatidic acid acyl transferase was lost under conditions in which greater than 94% of the mannose-6-phosphatase activity was retained, the latter enzyme being a putative luminal enzyme. Other proteases assayed degraded about 40–90% of lysophosphatidic acid acyl transferase activity in intact microsomes. The enzyme, being thought to be luminal, was found to be not even a transmembrane protein. Microsomes were disrupted by microcavitation and 60% of the luminal enzyme, mannose-6-phosphatase, was inactivated by chymotrypsin treatment, while the activity of lysophosphatidic acid acyl transferase remained unaffected. These results are interpreted to mean that lysophosphatidic acid acyl transferase is exposed to the cytoplasmic surface of the endoplasmic reticulum and that detergent treatment may be unmasking a site of chymotrypsin cleavage which is embedded within the membrane [592].

Let us now examine a third aspect of proteolysis, that of the 'naturally occurring proteolysis', by using only one example. Natural proteolysis modifies the property of the cell surface of sea urchin eggs in a fascinating way [593]. During fertilization, the sperm was reported to adhere non-specifically to the 'jelly coat' of the egg. Eggs (of *Arbacia punctulata*) contain on their cell surface (below the jelly coat) a glycoprotein that is able to inhibit the fertilization of these species but not of others, implying that it may act as a receptor. This activity is destroyed by trypsinization [594]. Significantly, it is suggested that a soluble fraction is cleaved, probably by a protease present in the cortical granules of the egg associated with its membrane. After a sperm fertilizes the egg, cortical granules empty their contents into the previtellin space [595]. Proteases of these granules 'autosolubilize' the factor that can bind the sperm and inhibit further fertilization. The egg surface is stripped of sperm receptors and thus polyspermy avoided. A protein, called

'bindin', of 30 500 daltons [596] has been isolated from sea urchin sperm acrosomal vesicles. It is believed to link to egg receptors. When eggs were labeled with ^{125}I [597] and the labeled eggs activated by a calcium ionophore (A23187) (in the presence of a low concentration of soybean trypsin inhibitor in order to control proteolysis), exocytosis of the content of the cortical granules released a labeled surface material that could bind to bindin [597].

VIII. Physical techniques

Different physical techniques have been used in studying membrane architecture and, incidentally, have given information on the asymmetric distribution and orientation of proteins. Other applications had more refined inquiries as objectives. Significantly, even the 'ancestral' models for the membrane which bear a good deal of accuracy, considered the asymmetry of the localization of proteins [598]. However, only distinctive adsorption on both sides of the lipid bilayer was envisaged, since the penetration of proteins by hydrophobic interactions with lipids which is now accepted [522,523,599–602] was then unknown.

X-ray diffraction and electron microscopic studies of myelin show alternating electron density and staining properties. Myelin lamella stem from Schwann cells. Two cytoplasmic membrane layers of these cells are believed to give rise to one myelin lamella, the dense bands of myelin corresponding to the apposition of cytoplasmic leaflets and the joining outer surface producing the thinner 'interperiod' bands [603–605] (for more recent data, see also, Refs. 606–608). The difference between the bands was attributed to the different proteins (or glycoproteins) of the two sides – hence asymmetry – of Schwann cell membrane [603] and this was supported by the observation that extraction of 98% (!) of lipids from glutaraldehyde-fixed myelin apparently did not alter its electron-microscopic appearance [609]. X-ray studies with *M. laidlawii* confirmed the bilayer arrangement of lipids but differences between the two leaflets could not be defined [610, 611].

Recent X-ray studies using a linear position-sensitive proportional counter allowing the recording of measurable spectra in 10–100 s have led to the conclusion that in retinal rod outer segment discs, the photoreceptor rhodopsin which constitutes 85% or more of the protein [612] spans the membrane and that upon bleaching a small increase in electron density appears at the cytoplasmic side of the membrane [613]. Neutron diffraction measurements on isolated retinal rod outer segment discs confirmed that rhodopsin is embedded in the hydrophobic core of the discs and that a very slight outward shift of protein at the cytoplasmic side of the membrane is associated with external bleaching [614].

Freeze-fracture studies have led to the demonstration of rhodopsin particles on the cytoplasmic fracture face [615] and, as reported above [513], the asymmetry of the orientation of rhodopsin is now well documented. In other cases, such as that of the ATPase in sarcoplasmic reticulum, an asymmetric distribution has been suggested on the basis of X-ray diffraction studies locating the enzyme on the outer half of the membrane [616].

The freeze-etching technique has found applications in the study of membrane asymmetry. It is now admitted that freeze-fracture can cleave the two halves of a bilayer membrane [617–621] (for an interesting and challenging recent review, see Ref. 621). Particles appear on the convex protoplasmic fracture face (face A) or on the concave exoplasmic fracture face (face B). Freeze-etching (sublimation of ice at –100°C) exposes the

protoplasmic or the exoplasmic surface. Particles are most often due to proteins, as shown by their absence on the fracture faces of pure lipid aggregates (see, however, the companion paper) and by their appearance when proteins are added to lipid dispersions [622–627]. In numerous cases such as those of erythrocytes [628,629], sarcoplasmic reticulum [630], rod outer segment [631] and lipid monolayer and bilayers penetrated by hydrophobic proteins [632,633], particles have been attributed to proteins. In general, when particles are present in membranes, their distribution between the two fracture faces is unequal [622,624], the protoplasmic face being richer. However, in the case of platelets, a higher density of particles exists in the outer membrane leaflet [634,635]. Hence, in normal human platelets, by using a computer-linked picture analyser for particle counting, densities of 925 ± 52 and 427 ± 29 particles per μm^2 have been estimated for the outer and the inner leaflets, respectively [635]. Notably, perturbations in the number, size or relative distribution of particles have been observed during some diseases (Glanzmann's thrombasthenia and Bernard-Soulier syndrome) [635].

The unequal distribution of particles on sarcoplasmic reticulum has been attributed to a stronger interaction of the ATPase, which constitutes 70% of the total protein in this organelle, with the cytoplasmic side of the membrane. The ATPase (with a molecular weight of 106 000–120 000 [299,636] is accompanied by two other minor components in this membrane. Brief exposure of the membrane to trypsin degrades all the ATPase molecules into 57 000 and 46 000 dalton fragments [636]; limited hydrolysis is also reported to cleave the molecule into similar halves [299]. The enzyme activity is, however, not lost at this step of degradation. Further exposure degrades the molecule into smaller fragments and leads to the loss of activity. Normally, the etched surface presents 35-Å granules and the cytoplasmic fracture face 90-Å particles. The convex fracture face is almost totally devoid of particles. However, trypsin digestion diminishes the density of particles on the concave fracture face and particles appear now on the convex fracture face [636].

Studies on sarcoplasmic reticulum, submitochondrial particles and plasma membrane were conducted by using a protease of bacterial origin (i.e., nagarase) endowed with a large specificity. All peptide chains were shown to be attacked by this enzyme [637]. In each case, however, 30–50% of the original protein mass was retained in the membrane. The residual, membrane-linked fragments have a molecular weight in the range of 10 000. Incidentally, the amino acid composition of these peptides did not show any specialized feature. Freeze-fracture study of protease-treated sarcoplasmic reticulum and plasma membranes showed that particles were present but that their density and unequal distribution decreased. This result is similar to others [636,638] and although in mitochondrial particles, freeze-fracture features were not different in nagarase-digested and original membranes, it appears that the presence of particles and their unequal distribution in most cases reflect the presence of proteins and their asymmetric interactions, so that proteolysis perturbs the distribution as well as the density of particles. ^{31}P-NMR studies with bovine, rat [639] and rabbit [640] microsomal fractions, ^{1}H-NMR [641] and ^{31}P-NMR [642] studies with sarcoplasmic reticulum, and ^{2}H-NMR [643] and ^{31}P-NMR [642] studies with the inner mitochondrial membrane indicate the possible presence of inverted lipid phases in these biological membranes. Thus, one can legitimately wonder about the implication of these observations in the evaluation of the origin of particles in these membranes (for more details and references see the companion paper).

In many cases, the freeze-etching technique brought forth evidence concerning the relationship between particles and protein components and the asymmetric distribution

of proteins. Studies with erythrocytes, using ferritin-conjugated concanavalin A, a lectin that links to band 3 protein, showed the presence of ferritin particles on the etched surface. This distribution corresponded to that of the particles on the fractured protoplasmic face [628,629]. In these studies, parts of particles were attributed to glycophorin, the major sialoglycoprotein of erythrocytes that interacts presumably with another ferritin-conjugated lectin, conjugated phytohemagglutinin. However, whereas the contribution of band 3 protein to intramembrane particles could be substantiated by reconstitution studies [625-627] it could not be verified with glycophorin [644,645]. On the other hand, freeze-fracture experiments with homozygous EN (a^-) erythrocytes which lack glycophorin showed that fracture faces display the same number of particles with the same morphology as that in normal erythrocytes. As mentioned above, the generality of the attribution of particles to proteins has been questioned [621] and, in particular, cases have been reported in which particles appear on the fracture face remote from the cytoplasmic face and have led to the challenging interpretation that these particles may be due to lipids [621]. We will envisage again these problems in the companion paper.

Observations have been made using electron spin resonance (ESR) spectroscopy which confirm the asymmetric orientation of the mitochondrial adenine nucleotide transport system. Spin-labeled acyl atractylosides inhibit ADP transport in rat heart mitochondria. In experiments on liposome, the comparison of spectra of spin-labeled long-chain acyl atractylosides, the chain of which incorporates into the lipid core, with those of spin-labeled fatty acids shows that when the label is near the head group, the chain appears more immobile for acyl atractylosides than for fatty acids, probably because of the interaction of the chain with the diterpene residue in the former. When spin-labeled acyl atractylosides are added to mitochondria, their chains appear more immobile than in liposomes, especially when the spin label is near the head group. Addition of atractyloside or other specific ligands displaces the label from the transporter and if very short chain acyl atractylosides which do not interact with the lipid core are used, addition of a ligand liberates the label into the aqueous phase. If inverted vesicles are used, long-chain acyl atractylosides are fixed to the membrane but do not react with the transporter. These vesicles transport ADP, however, confirming on the one hand the transbilayer nature of the transporter and on the other its asymmetry [183,184]. Similar studies were carried out with acetylcholine receptors of *Electrophorus* electroplax. The spin-labeled long-chain analogue of acetylcholine, 8-doxylpalmitoylcholine, does not depolarise the membrane but blocks the response to an agonist, carbamylcholine. The probe is completely immobilized in the membrane ($2T_{\parallel} = 65 \pm 1$ G). Addition of carbamylcholine, decamethonium (cholinergic agonists) or (+)-tubocurarin (an antagonist) increases the mobility of the ligand ($2T_{\parallel} = 55 \pm 2$ G) [409,647,648]. Another application of the ESR technique to elucidating the localization and number of SH groups of rhodopsin in the membrane of retinal rod discs deserves discussion. A series of spin-labeled maleimide derivatives, which all react with the SH groups but differ in the length of the 'arms' on which the spin label is fixed, was used. The distance between the double bond of the maleimide and the free radical of the probe varies from 6.8 to 15.3 Å. The spin label is, consequently, depending on the length of the arm, at different distances from the aqueous medium and as a result the spectra vary [649]. The conclusion drawn from these studies was that two SH groups react with the reagents, both embedded in the hydrophobic core, but probably not in a completely identical environment. The distance of these SH groups from the aqueous phase was estimated not to exceed 12 Å; the distance between both SH groups was evaluated to be of the order of 37 Å. The fact that the probe is located at a point remote from

the surface has been supported by the observation that papain treatment, though eliminating 36% of the amino acid residues, does not modify the spectral features [649].

Another use of ESR spectroscopy is to investigate the relative location of SH groups of membrane proteins, as has been applied to human blood platelets [650]. Spin-labeled SH-group reporters such as 4-iodoacetamido-2,2,6,6-tetramethylpiperidine-*N*-oxyl and 3-maleimido-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl were allowed to react with platelets. The spectra revealed the presence of two different classes (mobile and immobile) of sulfhydryl groups. Then, by using a broadening reagent ($K_3Fe(CN)_6$) at concentrations higher than 10^{-3} M, and observing the plot of the decrease in height of the mobile SH peak vs. the concentration of ferricyanide, it was envisaged that three classes of these sulfhydryl groups may exist. 4-Maleimido-2,2,6,6-tetramethylpiperidine-*N*-oxyl has been employed in the study of the topology of SH groups in erythrocyte proteins [651]. Right-side-out erythrocyte ghosts were prepared in the absence of ascorbic acid (a 'scavenger' of the spin label) and labeled or right-side-out ghosts were prepared in the presence of ascorbic acid, washed, then labeled. The spectra obtained were similar in both experiments but differed in the integrated signal intensity, since in the second case proteins exposed internally could not be labeled. This and complementary controls indicated that 80% of the spin label intensity arose from sites at the inner membrane surface. When the spectrin-actin complex was isolated, 70% of the label was found to be attached to this complex [651].

Fluorescence microscopy has found numerous applications in the study of the mobility and transmembrane distribution of surface molecules [27–31,61–66,653–658]. A pertinent fact is the point made recently that photobleaching leads to cross-linking of surface molecules as observed with fluorescein-conjugated concanavalin A linked to erythrocytes [658]. In other studies, it has been reported that not only the cross-linking of surface molecules occurs but also the death of fluorescein-conjugated concanavalin A-linked cells [659], as a consequence of photobleaching. In different investigations, analogues of the ligands of surface-exposed proteins have been used to study conformational changes of these molecules [19,177,408,409]. For example, 2-(*N*-dansyl)aminoethyl-β-D-thiogalactoside and 2-(*N*-dansyl)aminohexyl-β-D-thiogalactoside, which react with the β-galactoside transport system in *E. coli* vesicles but are not transported, have been used for the study of conformational change of the transporter [19]. Dansylcholine was used for the study of the exposed sites of the acetylcholine receptor. Energy-transfer studies (excitation at 287 nm, measurement of fluorescence intensity at 540 nm) in the absence and presence of different ligands have shown that this probe reacts not only with the sites of agonists but also with 'secondary sites' that may be the same as the receptor sites of local anesthetics such as prilocaine and lidocaine (for more details see Ref. 409 and references therein).

Fluorescamine was bound to the chloroplast-coupling factor and the tagged CF_1 was then used for the reconstitution of CF_1-depleted thylakoid membranes. Illumination of these thylakoid membranes led to quenching and blue-shift of the fluorescence, thus confirming the conformational change discussed above and detected by changes in the chemical reactivity of amino groups [177]. Using energy-transfer fluorescence spectroscopy, the location of SH groups in the membrane of *E. electricus* ATPase could be assessed. For this purpose, fluorescein mercuric acetate and anthranoylouabain have been used [660]. Fluorescein mercuric acetate has been shown to react specifically and stoichiometrically with SH groups in proteins and to form mercaptides with a lower fluorescence that the free fluorescein mercuric acetate [661]. Sulfhydryl groups of proteins can therefore be titrated. Using the quenching and red-shift (4–10 nm) of fluorescein mercuric acetate to

titrate the SH groups, it was found that 11–12 mol of the label were linked per mol of the native enzyme. As stated above, the enzyme is a complex of probably two glycoproteins and two catalytic sites [209- 220]. Labeling data were interpreted to mean that each catalytic subunit comprised four to five and each glycoprotein one to two SH groups, respectively. Fluorescein mercuric acetate is an excellent acceptor for energy transfer from anthranoylouabain with a sensitivity between 20 and 80 Å [662]. Anthranoylouabain binds specifically to $(Na^+ + K^+)$-ATPase at the surface-exposed ouabain sites. Measurement of energy transfer between the bound anthranoylouabain and fluorescein mercuric acetate showed that the fluorescence of the ouabain derivative was quenched only by 5–6% when 1 mol of fluorescein mercuric acetate/mol of enzyme reacted. A maximum of 60% quenching was observed at saturation (approx. 10 mol of fluorescein mercuric acetate/mol of enzyme). These results suggest that most or all of the SH sites reacting with fluorescein mercuric acetate are far (approx. 70 Å) from the ouabain site and probably located at the intracellular surface [660]. It was estimated that even if all the measured anthranoylouabain-to-fluorescein mercuric acetate energy transfer has a single site, the evaluated distance must be approx. 40 Å, i.e., of the order of the thickness of the membrane bilayer. These results indicated the lop-sided nature of the distribution of SH groups and gave a clue as to the transmembrane dimension of the enzyme estimated to be of the order of 70 Å.

IX. Conclusions and discussion

I have briefly reviewed techniques used for the establishment of the asymmetric distribution and orientation of proteins in biological membranes. Immunological techniques are used in different ways. I mentioned the drawback of crossed immunoelectrophoresis; if an exposed protein does not have antigenic determinants on the side of the membrane from which the antibody approaches, it will not be detected. This is valid for other immunological techniques such as immunofluorescence, immunoelectron microscopy, immunoenzymatic detection and immunoautoradiography. This remark holds true for many other techniques. Thus, discrepancies have been observed in the study of the iodination of glycophorin [255,441–448] from the cytoplasmic side of the membrane. The explanation is that amino acids of the C-terminus of this protein exposed to the cytoplasmic side present a single tyrosine residue adjacent to the hydrophobic segment embedded in the hydrophobic core [255]. This non-reactivity is a general drawback and is obviously not restricted to immunological or chemical interactions. I have examined the case of lysophosphatidic acid acyl transferase in which a false exposure of the enzyme was suggested on the basis of its non-reactivity to a particular proteolytic enzyme on intact microsomes. Significantly, the mistake was corrected simply by using other proteases [592]. Another instructive example in which a false location for a protein was envisaged on the basis of its non-reactivity to a proteolytic enzyme and to antibodies, is that of muscle microsomal calsequestrin [465,466]. It was realized that this protein is surface-exposed; it can be released by EGTA treatment and react with the fluorescamine-cycloheptaamylose complex [153,344]. A similar but not identical case is that of crypticity, i.e., a molecule is exposed to a given side of the membrane but is hidden from the action of the 'reactant', antibody, hormone, other ligands or appropriate reagents. A particular case is the action of antibody to ferredoxin-nicotinamide adenine dinucleotide phosphate-oxidoreductase which prevents the function of the enzyme in thylakoid membranes but cannot cross-link and precipitate the membrane, the enzyme being presumably localized in a

cleft, in the neighborhood of the chloroplast coupling factor (CF_1) [168]. The use of carbohydrate reagents legitimately presupposes the generally accepted orientation of glycoproteins and glycolipids with the carbohydrate moiety remote from the cytoplasmic side of the membrane. However, one should be aware of possible exceptions [663]. Some studies of the surface exposure and lateral mobility of proteins have used immunological techniques or lectins (see Section III; see also, Refs. 664–672). If so desired, ways exist for probing both sides of membranes for the detection of antigens as well as lectin receptors [51,52,65,86,87,101]. When assaying the transmembranous nature of a protein, comparison of its reactivity to a reagent from one side with that from both sides of the membrane is made. However, the reactivity of a particular protein in native membrane and even in right-side-out ghosts may be different and, on the other hand, if leaky ghosts are prepared the differential reactivity of a protein in these ghosts and in resealed ghosts cannot be attributed necessarily to the part of the molecule exposed by the leakiness, since as previously reported [256], conformational changes in the protein or the opening of aqueous channels may result in the same observations. Comparison of molecules labeled by the galactose oxidase-$NaB[^3H]H_4$ method applied to brain synaptosomes and to isolated synaptosome membranes has shown, quantitatively as well as qualitatively, very different labeling profiles [487]. Another pertinent example is that illustrating the differential reactivity of molecules in intact cells and right-side-out membranes derived therefrom. A lysosomal enzyme, α-L-iduronidase, when treated with an arginine-modifying reagent (2,3-butanedione) under conditions in which its enzyme activity is retained, exhibited a 50% reduction in surface binding to cultured fibroblast cells and in internalization into the cells. Significantly, when fibroblast membranes were used, a 90% reduction of binding was observed. Thus, here the reverse is true, the native membrane is more reactive [673].

The action of certain enzymes on surface membrane molecules may modify the reactivity of unperturbed molecules or the remaining segment of perturbed molecules. Therefore, limited trypsinization sometimes increases the extent of iodination when using the lactoperoxidase technique [474]. Neuraminidase treatment of cells exposes subterminal galactose or galactosamine residues and may interfere with their reactivity during the galactose oxidase-$NaB[^3H]H_4$ labeling [480,486].

Reagents have been divided in two classes: macromolecular and charged molecules (non-permeant) and liposoluble reagents (permeant). Extreme care should be taken in practical applications. Lactoperoxidase cannot penetrate the membrane. However, radioactivity has been found accompanying intracellular membrane subfractions when using the lactoperoxidase-$^{125}I^-$-labeling technique [435–437]. Iodination by formation of I_2 [256] or I^+ [255] was invoked to explain the unexpected labeling of molecules. I mentioned the possible formation of high molecular weight material [483] and the labeling of molecules by $NaB[^3H]H_4$ alone [484,485] when the galactose oxidase technique is being used. As concerns the ionic reagent, diazosulfanilate, which is reported to be non-permeant (see the successful examples of application given above [318,326,327,331]), this was found to penetrate the membrane of *A. laidlawii* [332]. The permeability of a reagent may be temperature dependent, as has been observed with *N*-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate) [383] and trinitrobenzenesulfonate [674] (see also the companion paper [2a]). It is noteworthy that a permeant reagent may apparently act in a non-permeant manner as exemplified by the action of dansyl chloride on erythrocytes [258].

In studying the action of mercurials on erythrocyte membrane, I reported their con-

comitant action on the permeability properties of the membrane. When examining the action of a chemical reagent, it should be ensured that changes in permeability do not occur during labeling. Enzyme assays [253,675] and exclusion or uptake of molecules [676–678] have been used for assessing the perturbation of membrane permeability. Similarly, when preparing membrane fractions, perturbations brought about in the membrane orientation should be carefully considered, since inside-out vesicles may be formed. Various methods I have examined may be used for assessing the comparative orientation of the original membrane and vesicles derived therefrom. In addition, let us consider electron-microscopic, cytochemical and cytoimmunological evidence presented to support the identical orientation of microsomes and endoplasmic reticulum. Thus, ribosomes are always located in both formations on the outer (cytoplasmic) surface; on the other hand, glucose-6-phosphatase [679] and cytochrome b_5 [680] have been located cytochemically and cytoimmunologically on the same side, in both formations.

Other remarkable observations have been made. Thus in the case of lactoperoxidase-catalyzed iodination, the inhibition of the enzyme by the membrane under study has been suggested [256]. In other methods, such as that of labeling using the transglutaminase technique, success was reported to depend on the source of the enzyme [507]. As we will see in the companion paper on the distribution of lipids, the specificity of many lipases depends on their origin.

The use of physical techniques often takes advantage of the asymmetric orientation or distribution of membrane proteins for the study of conformational perturbations rather than the establishment of the asymmetry. However, good use has been made of these techniques in asymmetry studies. As noted above, proteolysis changed the unequal distribution of particles appearing on freeze-fracture faces [637,638], as if this operation, eliminating part of the polypeptide chain, relieved some constraint due to the interactions of the eliminated segments so that the remainder could give rise to symmetrically distributed particles. However, in the light of recent work [639–643] (see also the companion paper [2a]), a new appraisal of the significance attributed to the particles might be worthwhile. The essential problem is now to delineate the examples in which proteins, lipids or both are involved.

Examples of studies concerning membrane proteins show, as a rule, their asymmetric orientation and/or distribution. Gap junctions, as studied, for example, using X-ray crystallographic and electron-microscopic analyses of junction plaques isolated from murine liver were found to be built up of units termed connexons in each of the pair of connected membranes. These units, considered to be formed of cylindrical assemblies of 'connexin' molecules, are arranged hexagonally. It is suggested that binding of the connexons, in symmetric pairs, at the center of the gap forms the junction structure, two seemingly identical connexons being connected in pairs and related by a two-fold axis of symmetry parallel to the plane of the junction at the center of the gap [681,682]. However, it should be borne in mind that in this case the symmetry concerns two connected membranes, and the possibility exists that the orientation of the molecules in each membrane is asymmetric. As mentioned above (see Introduction), oligomeric proteins may still have, a priori, a symmetric orientation of their subunits in each membrane. However, even in such situations, if they are encountered, a totally symmetric functioning is not guaranteed because of the environmental asymmetry and, conversely, a symmetric functioning cannot be taken necessarily as proof of a symmetric molecular arrangement unless shown otherwise.

As the general occurrence of the asymmetric orientation of transmembrane proteins is

beyond any doubt, one can wonder about its origin(s). I have recently discussed some aspects of this problem [2]. Additional comments seem appropriate. Known examples indicate that the insertion of these proteins mainly operates cotranslationally [15,683–695], as has been observed previously for secreted proteins (for a review, see Ref. 696), and more recently for a peripheral membrane protein, namely β_2-microglobulin [697]. A signal peptide [698,699], which for all but one [700–702] reported case is located on the N-terminus of the nascent chain, was suggested to bear the information necessary to direct the translating ribosome and to form a functional complex with receptors of the rough endoplasmic reticulum, the proteins of which would form a proteinaceous tunnel for the unidirectional translocation. A signal peptidase, located on the luminal side, removes the signal before the translation is completed [703]. Two proteins restricted to rough endoplasmic reticulum have been suggested to function as a receptor for ribosome binding [704]. However, recently, the 'membrane trigger hypothesis' was put forward and presented as an alternative in preference to the 'signal hypothesis' [6,705]. In the membrane trigger hypothesis, it is considered, in essence, that the thermodynamics of protein folding govern membrane assembly, the role of the signal peptide (or leader peptide) being to alter the folding pathway. What catalyzes the assembly in this hypothesis is the effect of the leader peptide on conformation. The asymmetric orientation of the protein is dictated by the primary sequence [6,705]. No protein transport system is envisaged and the ribosome-membrane interaction does not necessarily intervene. This is in fact a variant of views expressed previously in order to explain the insertion of integral membrane proteins [523,706]. It admits, in additions, a role for the leader sequence in the folding of the protein. Further in the same article [6], the author provides arguments against the necessity for participation of the leader sequence in protein assembly, since he admits and produces examples interpreted as proving that the isolated mature protein has the information necessary to be asymmetrically incorporated [6]. The author mentions some as yet unexplained facts such as the presence of proteins which are oriented in the membrane with their carboxyl terminus exposed externally or proteins that cross the membrane more than once and presents these facts as weaknesses in the signal hypothesis. Furthermore, a contradiction is seen between certain facts such as the cytoplasmic synthesis of the precursors of some chloroplast and mitochondrial proteins which are translocated post-translationally and the signal hypothesis. My opinion is that none of these and other data [6,705] can really weaken the signal hypothesis which in fact has been adapted recently so that it accounts for cases in which the proteins are cytoplasmically synthesized, generally (but not always) as precursors. These proteins comprise, in eucaryotic cells, those proteins which have to cross one or two membranes in order to appear either as a soluble protein in another compartment or as a membrane-linked protein. Examples of these proteins are the small subunit of the chloroplast stroma enzyme ribulose-1,5-biphosphate carboxylase [707–709], subunits IV–VII of the mitochondrial inner membrane cytochrome oxidase [327,710–715], α, β and γ subunits of yeast mitochondrial F_1-ATPase [716], two subunits of the mitochondrial cytochrome bc_1 complex, comprising cytochrome c_1 and subunit V [717,718], cytochrome *c* peroxidase which is an intermembrane compartment protein [718,719] and, the peroxisomal catalase and uricase [720]. In all these examples, the protein is believed to be endowed with a signal peptide and the presence of membrane receptors and translocators, by analogy with the case of secretory and integral membrane proteins, is suggested [696,721]. Similarly, in *Neurospora crassa*, some proteins of the inner mitochondrial membrane, mitochondrial ribosomes and the matrix compartment have been reported to be synthesized cytoplas-

mically, then transported to mitochondria [722–725]. Cytochrome *c* from yeast [718] and cytochrome *c* and adenine nucleotide transporter from *N. crassa* [725] have been shown not to be processed during transfer from the cytoplasm. In all cases, the signal hypothesis suggests the presence of a signal peptide, whether processed or not, and the presence of membrane receptor translocators. The signal hypothesis, the general validity of which remains to be demonstrated, does not deal with the driving force for the translocation of proteins in cases where the process is post-translational. However, work by Nelson et al. [718] and Schatz [726] indicates that the in vivo translocation of cytoplasmically produced precursors to the β and γ subunits of F_1-ATPase (regarding the matrix side), cytochrome c_1 and subunit V of the cytochrome bc_1 complex (regarding the intermembrane space) and cytochrome *c* peroxidase is energy-dependent and that the processing occurs during the translocation. Thus, there is a hint as to some sort of active transport. It would be useful to learn if the energy dependence of the translocation of proteins were a general phenomenon.

I wish, in closing this article on the asymmetric distribution and orientation of membrane proteins, to emphasize a few additional points. The first is that proteins of mitochondria, chloroplasts or peroxisomes, either membrane-associated or soluble, which are translocated post-translationally, have a particular character. They most often traverse at least one membrane and generally seem to form either soluble or membrane-associated complexes, membrane-associated complexes being, in most reported cases, formed of hetero oligomers (with the possible exception of transporters such as the ADP/ATP carrier which is formed of identical subunits). These proteins, either soluble or membranous, cannot be considered as genuine secretory proteins since they are liberated in the cytoplasm, then translocated to another cell compartment. In addition, the present evidence indicates that even those which are membrane-embedded are not the product of a direct cotranslational insertion in the membrane. This has the important corollary that their orientation should be dictated by factors other than those imposed by a direct insertion. Consequently, I propose: (a) to call these proteins migratory proteins and (b) as a working hypothesis that if most, if not all, secretory and integral transmembrane proteins are translocated, or membrane-inserted cotranslationally, most if not all the migrating proteins, either soluble or membrane-bound, are translocated or inserted post-translationally. This proposal * relieves in particular the constraint of orientation of membrane-embedded proteins of this category, i.e., they do not necessarily have to display their NH_2-terminus oriented remote from the cytoplasmic compartment, since forces involved in their translocation or in their complexation will play the major role. Mention seems appropriate, at this point, of the apparent location of intramitochondrial polysomes, which synthesize hydrophobic proteins (and, in particular, proteins I -III of the cytochrome oxidase in yeast). They are almost all membrane bound [727,728], as if even for integral proteins encoded by an organelle genome, also a cotranslational insertion could be envisaged if the product of the synthesis is not a migratory protein.

The second point, in correlation with the first, is that as previously remarked, if integral membrane proteins were cytoplasmically processed, their unique orientation with the NH_2-terminus exposed to the side opposite to the cytoplasm could not be easily explained [2]. Experimentally observed cases do not allow the conclusion that a unique 'natural' orientation of proteins in reconstitution assays is constantly favored [2] *. The

* Further elaboration by the author on this problem is in progress.

presence of migratory proteins relieves the constraint of orientation for a part of integral proteins. Furthermore, exceptions to the 'rule' of orientation are known even with proteins other than migratory proteins (see references contained in Refs. 2 and 6). More studies on the sidedness of integral membrane protein orientation are necessary to assess the extent of these 'exceptions' and, especially, the understanding of the exact mechanism(s) by which 'unexpected' orientation is established seems rewarding [2].

As yet, not only in eucaryotic but also in procaryotic cells, no evidence for the cytoplasmic release of integral proteins has been reported [15,683,691–695] and it is also admitted that periplasmic proteins are synthesized on membrane-bound ribosomes [729–733]. A case of post-translational insertion concerning the coliphage M_{13} coat protein in *E. coli* membrane has been described [6]. This case remains controversial; other authors find this protein to be cotranslationally inserted [685]. A particularly remarkable point is that for *E. coli* outer membrane matrix protein, no pool in the inner membrane could be found using pulse-chase experiments whereas for the lipoprotein a significant amount of the already processed chain could be found in the inner membrane. Therefore, here the processing does not accompany the final translocation of the protein and a mechanism for this translocation should be sought. Let us recall that lipopolysaccharides and phospholipids of the outer membrane are also synthesized in the cytoplasmic membrane and then translocated by an unknown mechanism [732,734,735]. However, the rate of translocation of the lipoprotein from the cytoplasmic membrane into the outer membrane was found to be faster than that of the lipopolysaccharides and phospholipids [694]. This was interpreted as confirming the previous observations that assembly of outer membrane phospholipids, lipopolysaccharides and proteins occurs independently [736–738].

The nature of the paths followed by proteins to their final destiny remains to be elucidated. The involvement of proteins essential in the signal hypothesis has found some support [704,739]. In addition, bacteria [740,741] and mitochondria [461,462,742,743] possess junction points between the outer and the inner membranes; the involvement of these points in the process of signal recognition and/or protein translocation also remains to be elucidated.

The membrane trigger hypothesis or similar ways suggested for the insertion of integral proteins into membranes [523,706] cannot constitute alternatives to the signal hypothesis; they may, at best, explain some particular cases and work in this line may shed light on the general problems presented by membrane-protein interactions. In fact, what tarnishes the significance of the membrane trigger hypothesis is that is is presented as an alternative to the signal hypothesis. That membrane-protein interactions occur cannot, of course, be denied and the possibility cannot be excluded that examples might be found in which a protein having two conformations will be present in both a soluble compartment and in membrane fractions, or depending on physiological or physiopathological conditions, prefer one or the other domain. In such cases, it would be of great value to determine the asymmetry of the protein orientation and in particular whether or not its NH_2-terminus is remote from the cytoplasmic side. The general validity of the signal hypothesis attributing only to discrete domains of protein sequences the critical role of directing all aspects of protein secretion, integration and sorting remains to be demonstrated.

The elucidation of the exact mechanism(s) by which migratory proteins are translocated to their ultimate location or the way(s) in which the eventual post-translationally integrated proteins are inserted will shed light on the nature of factor(s) involved.

It is of value to consider the genesis of proteins crossing the membrane more than once. The hypothesis of the presence in the sequence of these proteins of more than one

signal and stop sequences, stop sequences being those sequences in the transmembrane proteins that induce abrogation of translocation [688,721,744], remains to be proved.

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