REVIEW LETTER

# ISOLATION AND STUDY OF FUNCTIONAL MEMBRANE PROTEINS

## Present status and future prospects

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## 1. Introduction

The purpose of this review letter is to summarize recent attempts to label and isolate membrane proteins, in particular, substrate transporters, and also several drug receptors and enzymes. It is assumed that the transport or receptor system has already been studied with respect to substrate specificity, inhibitors, kinetics etc., before efforts are begun to isolate the proteins involved in the activity. Attempts to isolate these specific proteins may be divided into two types:

1) The protein is specifically labelled in situ, solubilized, and then purified.

2) Alternatively, the membrane is solubilized, and the protein identified and purified subsequently.

## 2. In situ labelling

The theoretical and practical aspects of active site labelling of purified soluble proteins have been discussed [1–3]. One major point to be emphasized here is that the criteria for site specificity have been satisfied to varying degrees for a number of different enzymes, but in most cases there is no direct evidence that labelling has occurred exclusively at the presumed site. The second point is that a particular membrane transporter or receptor will probably account for less than 1% of the total cellular protein. Therefore, it is especially relevant to note that specific labelling of a single protein has yet to be demonstrated when this protein is a minor component of a mixture.

Tables 1, 2 and 3 list the types of membrane systems in which labelling has been attempted with either specific or non-specific covalently-bound inhibitors. Several of these are discussed in the text, followed by some general comments on in situ labelling of membrane proteins.

*Abbreviations:*

ACh : acetylcholine
ADP : adenosine diphosphate
ATP : adenosine triphosphate
ATPase : adenosine triphosphatase
Carb : carbamylcholine
DFP : diisopropylfluorophosphate
DNP-Lys: $\epsilon$-*N*-(2,4-dinitrophenyl)-lysine
FDNB : fluoro-2,4-dinitrobenzene
NEM : *N*-ethylmaleimide
SDS : sodium dodecyl sulfate
The Roman numerals refer to the compounds in tables 1–4.

### 2.1. *The β-galactoside permease in E. coli*

This permease (M protein) was identified by using a double-labelling technique, with $^{3}H$- and $^{14}C$-NEM (I), which involved substrate protection of uninduced and induced cells, and control studies with mutant strains lacking permease activity [4]. The usual non-metabolizable transport substrate, methyl-1-thio-β-D-galactoside (II), did not protect the active sites from being labelled, whereas β-D-galactopyranosyl-1-thio-β-D-galactoside protected these sites and caused a 2.5 fold decrease in incorporated tritium counts, suggesting the presence of at least two classes of sugar binding sites on the M protein [5, 6]. The membrane was solubilized with Triton X-100 or with SDS, and the labelled fraction contained five major and numerous minor protein bands; 59% of the radio-

Table 1
Affinity labelling of membrane transporters.

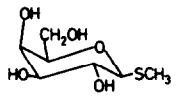
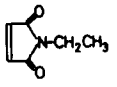
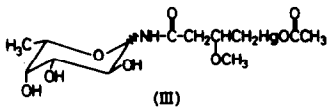
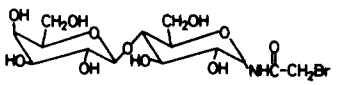
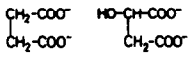
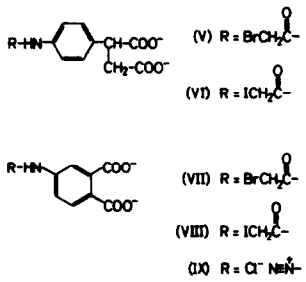
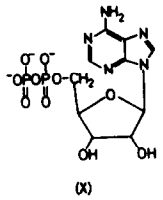
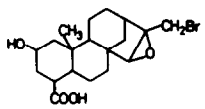
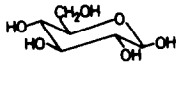
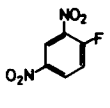
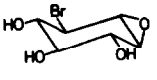
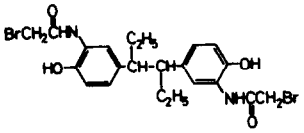
| Transport system | Substrate | Labelling reagent | Comments | Refs. |
|---|---|---|---|---|
| Lactose permease *Escherichia coli* |  (II) |  (I) | See text. | 4 |
| | |  (III) | *N*-(4-Acetoxymercuri)-3-methoxybutyryl-L-fucosylamine (III) inhibited sugar uptake at $10^{-3}$ M. | 8 |
| | |  (IV) | *N*-Bromoacetyl-α-lactosylamine (IV) inhibited at $10^{-4}$ M. Nature of inhibition not known. | |
| Dicarboxylate transporter, rat liver mitochondria |  Succinate Malate |  (V) R = $BrCH_2C$– (VI) R = $ICH_2C$– (VII) R = $BrCH_2C$– (VIII) R = $ICH_2C$– (IX) R = $Cl^-$ $N\equiv N$– | See text | 11 |
| Adenine nucleotide transporter, rat liver mitochondria |  (X) |  (XI) | Atractyligenin is a competitive inhibitor of ADP (X) translocation, but not known whether inhibitor and substrate react at same sites. Epoxide (XI) is competitive inhibitor at $2.5 \times 10^{-4}$ M. C15-OH and C16-methylene groups of atractyligenin not essential for activity. | 11 |
| Glucose transporter, human red cells |  β-D-Glucose |  FDNB | Inhibition by FDNB known to be dependent upon square of inhibitor concentration. One aliquot of cells incubated with 1 mM FDNB for 10 min, and 2nd aliquot with 10 mM FDNB for 1 min, upon assumption that 2nd aliquot would be preferentially labelled at carrier site. Total of $3 \times 10^5$ DNP residues (4.5 nmoles DNP residues per ml packed cells), thought to be specific for carrier; isolated mainly as derivative of cysteine, plus small amounts of lysine and histidine. No direct proof that carrier was labelled. | 15 |

Table 1 (continued)

| Transport system | Substrate | Labelling reagent | Comments | Refs |
|---|---|---|---|---|
| Monosaccharide transporter, rabbit red cell |  D-Xylose |  (XII)  (XIII) | Substrate analogue (XII) and competitive inhibitor (XIII) failed to inhibit xylose transport. | 16, 17 |

activity was located in one band, which did not correspond to any of the major protein bands [7]. Some of the labelling reagent was recovered as *S*-succinyl-cysteine, confirming that the reactive residue in the M protein was a cysteine. The molecular weight of the major labelled band was between 29,000 and 31,000 daltons and contained no phospholipid. In fully induced cells, the M protein represented between 2–4% of the protein in the particulate fractions, and about 0.35% of the total protein [13].

Another approach to labelling the *lac* permease involved the use of a number of derivatives of D-glucopyranosylamine, D-galactopyranosylamine, L-fucosylamine and α-lactosylamine [8]. The *N*-4-acetoxymercuri-3-methoxybutyryl derivatives (III) of the first three of these gave 100% inhibition of sugar uptake at $10^{-4}$ M, whilst *N*-bromoacetyl-α-lactosylamine (IV) gave 100% inhibition at $10^{-3}$ M. No further data are available at the present time.

Stein and his colleagues [9] attempted to label the *lac* permease not with an inhibitor but by comparing the incorporation into protein of phenylalanine in induced and uninduced populations, using $^{14}C$ and $^{3}H$ isotopes respectively [10]. Enrichment of a protein in $^{14}C$ over $^{3}H$, indicating that the protein may be a product of the *lac* operon, was found in fractions corresponding to β-galactoside transacetylase and β-galactosidase, as well as two other fractions. One of the latter fractions was membrane bound, had a molecular weight of 30,000–40,000 daltons and accounted for 12% of the protein. It may correspond, in part, to the M protein. The fouth labelled fraction was cytoplasmic (soluble), had a low molecular weight, and may represent either a biosynthetic precursor or a degradation product of the operon. This most interesting approach requires an inducible transporter and, therefore, cannot be used for the majority of mammalian membrane systems.

### 2.2. *Dicarboxylate transport system in rat liver mitochondria*

We have used substrate analogues which are non-penetrating competitive inhibitors. The non-penetrability restricted by about 80% the amount of protein with which the labelling reagent could interact. Phthalate and 2-phenylsuccinate specifically inhibited the dicarboxylate porter with inhibitory constants ($K_i$) of about $1 \times 10^{-3}$ M and $2 \times 10^{-4}$ M, respectively [11].

We have synthesized several affinity-labelling substrate analogues: *p*-bromoacetamidophenylsuccinate (V), *p*-iodoacetamidophenylsuccinate (VI), 4-bromoacetamidophthalate (VII) and 4-iodoacetamidophthalate (VIII). When these compounds were tested at high concentrations and immediately after addition to the mitochondria, they were as good competitive inhibitors as were their parent compounds. However, they proved to be relatively slow alkylating reagents, requiring from 6 to 24 hr of incubation for significant irreversible effects. The amount of inhibition was variable, and usually there were effects upon other transport systems and other mitochondrial functions [11]. Furthermore, iodoacetate itself occasionally gave comparable effects. The more reactive *N*-diazo salts derived from 3- and 4-aminophthalate (IX) gave

Table 2
Affinity labelling of membrane receptors.

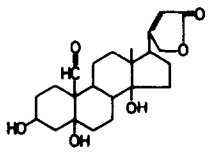
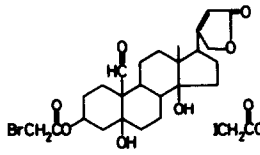
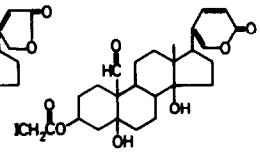
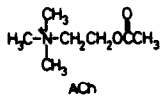
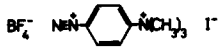
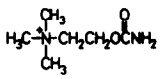
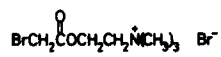
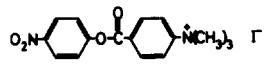
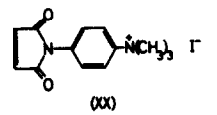
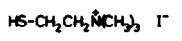
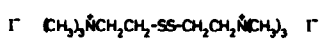
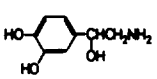
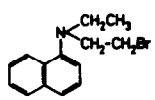
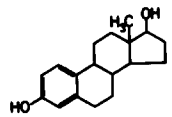
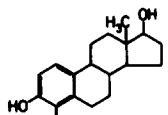
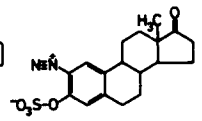
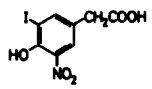
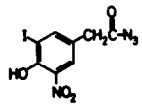
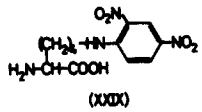
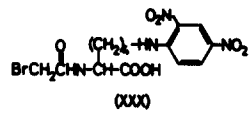
| Receptor system | Physiological ligand | Labelling reagent | Comments | Refs. |
|---|---|---|---|---|
| ATPase, Na, K-stimulated ouabain inhibited, brain microsomes |  (XIV) |  (XV)  (XVI) | Enzyme already solubilized with lubrol and purified 25 fold before labelling. Little or no inhibition with bromoacetate analogue until $5 \times 10^{-4}$ M, and then rapid increase in inhibition up to 100% at $10^{-3}$ M. Substrate protection difficult to assess. Much non-specific labelling. | 18, 19 |
| Acetylcholine receptor, electroplax excitable membrane |  ACh |  (XVII) | Very narrow effective concentration range, between $10^{-4}$ and $10^{-3}$ M. Very short incubation time. Reversed by incubation of tissue with $10^{-2}$ M histidine for 19 hr. | 23 |
| |  Carb |  (XVIII)  (XIX) | Requires prior reduction of disulphide bond. See text. | 27 |
| | |  (XX) | Much non-specific labelling. See text. | 28, 29 |
| | |  (XXI)  (XXII) | Cholinethiol = 2-mercaptoethyltrimethylammonium iodide (XXI), and bis-cholinethiol (XXII) cause several thousand fold increase in rate of recovery following treatment of tissue with dithiothreitol. | 30 |
| Noradrenaline α-receptor, rat vas deferens |  Noradrenaline |  (XXIII) | Reactive species is ethyleniminium ion. Large amount of non-specific uptake by tissues, apparently unrelated to α-receptors; no decrease in uptake by prior incubation with protective reagents. | 31, 32 |

Table 2 (continued)

| Receptor system | Physiological ligand | Labelling reagent | Comments | Refs. |
|---|---|---|---|---|
| Estrogen receptor, rat uterus |  (XXIV) |  (XXV)  (XXVI) | Mercurial analogue competes for estrogen binding sites on tissues as well as on proteins such as egg albumin, glutamate dehydrogenase, and pyruvate kinase. 2-Diazoestrone sulfate (XXVI) studied only with soluble proteins. Mercurial (XXV) is bound non-covalently, and may therefore migrate during isolation procedures. | 33–35 |
| Antigen receptor on antibody-synthesizing cells, mouse spleen |  (XXVII) |  (XXVIII) | 4-Hydroxy-3-iodo-5-nitrophenylacetic acid = NIP (XXVII). 4-Hydroxy-3-iodo-5-nitrophenacetyl azide = NIP azide (XXVIII) specifically inhibits antibody synthesis to NIP at $7 \times 10^{-6}$ M, but at $10^{-4}$ M, responses are inhibited to other antigens as well as to NIP. | 36 |
| |  (XXIX) |  (XXX) | α-*N*-Bromacetyl-ε-*N*-(2,4-dinitrophenyl)-lysine = BADL (XXX) has significant inhibitory effects upon response to DNP-Lys (XXIX) at $5 \times 10^{-6}$ M. Also inhibits rabbit anti-DNP antibodies. | 37 |

almost complete inhibition of osmotic swelling within 15–30 min and at concentrations about 100-fold lower than the haloacetamido compounds, but they too affected other mitochondrial activities. In addition, similar effects were shown by *N*-diazo-*p*-aminobenzoate, although the *p*-aminobenzoate itself caused only slight competitive inhibition at concentrations 100 fold higher.

### 2.3. *Adenine nucleotide transport system*

We have synthesized epoxybromo-atractyligenin (XI) which is as good a competitive inhibitor of ADP (X) transport as atractyligenin itself. The epoxide ring is accessible and therefore is a potential nucleophile, but in preliminary experiments the compound has not been a satisfactory affinity label [11].

### 2.4. *Sugar transporters in red cells*

A unique method was used in an attempt to label the glucose transporter in human red cells. Langdon and Sloan [12] proposed that the sugar carbonyl formed a Schiff base (imine) with carrier amino groups and they attempted to trap this imine by reduction with $NaBH_4$. Incorporation of $^{14}$C-glucose was demonstrated with intact red cells, red cell ghosts and adipose cells, 50% of the $^{14}$C being located

in one protein fraction. Label could also be trapped in certain proteins, including hemoglobin, but with kinetics which were different from that in cells. LeFevre [13] detected errors in the original kinetic analyses and also reported unpublished experiments with 1,5-anhydro-D-glucitol, which lacks a carbonyl function but has a very high affinity for the carrier. Further investigations showed that the C-1 carbonyl group was not incorporated in a Schiff base to any significant extent, and that the original results were probably not related to a major pathway for glucose transport [14].

Cooper et al. [16] have recently tried to label the monosaccharide transporter from rabbit red cells. 6-Bromo-deoxyconduritol-β-epoxide (XII), which had previously been used by Legler [17] to label the active centre of β-glucosidases A and B, had no inhibitory activity in the transport assay, and in fact caused a 15% increase in xylose uptake. Stilboestrol and hexoestrol, at $10^{-4}$ M, competitively inhibit xylose transport to the levels of 50 and 60%, respectively. Cooper et al. [16] prepared 3,3′-dibromoacetamidohexoestrol (XIII), but this potential alkylating reagent caused no significant reversible or irreversible inhibition.

### 2.5. *Ouabain-sensitive Na,K-dependent ATPase from brain microsomes*

A solubilized enzyme preparation was studied with a variety of inhibitors derived from strophanthidin (XIV). Strophanthidin-3-bromoacetate (XV) caused irreversible inhibition when high concentrations ($10^{-3}$ M) and prolonged incubation were used [18]. Bromoacetate itself gave little inhibition under these conditions. A much larger number of strophanthidin molecules were taken up by the enzyme preparation than the number of enzyme active sites as estimated from the amount of glutamyl-γ-phosphate (a known intermediate for this reaction). Ninety percent of the non-specific labelling could be eliminated by prior incubation with non-analogue haloacetate compounds whilst competitive substrate analogues had no protective effects. This work is presently being extended using a more potent inhibitor, hellebrigenin-3-iodoacetate (XVI) [19].

### 2.6. *Acetylcholine receptor*

A great deal of information is available about this complex system in the electroplax electric organ, but for the present purposes, it is sufficient to note that several classes of ACh receptor sites can be distinguished functionally from the esterase site of acetylcholinesterase, and from the acetylcholine-triggered 'ionophore' responsible for ion transport [20]. Receptor activity has been demonstrated in vesicular fragments derived from the membrane [21]. One affinity labelling analogue, *p*-(trimethylammonium) benzene diazonium tetrafluoroborate (XVII) [22], has been shown to block a receptor site [23] as well as at least two classes of sites on the enzyme (the anionic site of the active centre and a second non-catalytic anionic site) [24–26]. Several alkylating inhibitors have been synthesized: bromoacetylcholine bromide (XVIII), *p*-nitrophenyl ester of *p*-carboxyphenyltrimethylammonium iodide (XIX) [27] and 4-(*N*-maleimide)-phenyltrimethylammonium iodide (XX) [28, 29]. These reagents had virtually no irreversible effects unless the tissue was treated with a reducing agent prior to their addition. Their non-specific counterparts, e.g., bromoacetate or NEM, were much less effective as inhibitors [29]. Recent studies showed that the amount of alkylating reagent incorporated into the tissue greatly exceeded the estimated number of receptor sites [29].

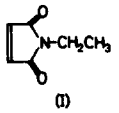
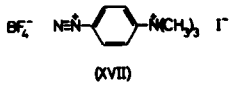
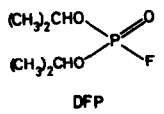
### 2.7. *Noradrenaline α-receptor*

This drug receptor has been extensively studied in the rat vas deferens [31, 32]. *N*-(2-Bromoethyl)-*N*-ethyl-1-naphthylamine (XXIII), a competitive antagonist of noradrenaline known to form covalent bonds with tissue proteins was adsorbed in large quantities, and showed little specificity for the α-receptor. Prior treatment of tissue with a reversible antagonist did not result in a detectable decrease in amount of inhibitor taken up by the tissues. Experiments with a variety of amino compounds showed no correlation between their protective effects and their sympathomimetic activities. It was concluded that the tissue had a high binding capacity for the 2-halogenoethylamines and that these were largely unrelated to specific receptor sites.

### 2.8. *Estrogen receptor of rat uterus*

4-Mercuri-17-β-estradiol (XXV), a competitive inhibitor of 17-β-estradiol (XXIV) [33], showed estrogen activity in rat uterus tissue preparations

Table 3
Labelling of membrane-associated enzymes.

| Enzyme | Labelling reagent | Comments | Refs |
|---|---|---|---|
| Adenosine triphosphatase, sarcoplasmic reticulum |  (I) | Amount of radioactivity incorporated into membrane preparation decreased in proportion to amount of ATP used for protection. Between 1 and 8 sulphydryl groups protectable, depending of ATP concentration. When 1 SH group protected per $10^5$ g protein, most of radioactivity confined to 1 band, corresponding to major protein band. Total number of counts with protection was 40% that without protection. No direct evidence that labelled band corresponded to ATPase. | 38 |
| Acetylcholinesterase, human red cell |  (XVII) | Enzyme highly purified and crystallized before labelling. Inhibitor blocks anionic site of active centre plus non-catalytic anionic site. | 22, 23 |
| |  DFP | Label is predominantly in single band with MW = 90,000 daltons in SDS-acrylamide. In presence of substrate, amount of labelling reduced by 80%. | 39 |

[34] which was prevented by prior treatment with sulphydryl reagents. Binding of the mercurial to soluble proteins from uterine tissue was inhibited by unlabelled 17-β-estradiol. The concentration of the mercurial required for maximal stimulation of induced enzyme activities was five times as high as that of the 17-β-estradiol; the reason for this difference is not known.

2.9. *Antigen receptors*

Two studies have recently been made of labelling of cell-bound antigen receptors [36, 37] but the results are too preliminary for final assessment.

2.10. *Membrane-bound enzymes*

Two other membrane enzymes have been labelled (table 3).

2.11. *Miscellaneous techniques and reagents*

Several physical techniques have recently been used to study other aspects of membrane structure or function (table 4). They require the use of reagents which are designed to localize in different areas within the membrane by virtue of the chemical properties of the non-probe portion of the molecule [43–49]. In addition, substrate analogues may be synthesised which incorporate either a fluorescent probe, a spin label radical, or a function suitable for nuclear magnetic resonance studies. Such reagents will probably be more useful in studies on isolated transporter or receptor proteins in model membrane systems, than on the intact biological membranes.

2.12. *General comments on in situ labelling*

In each of the above examples the labelling was found to be more or less non-specific. It is ironic that the labelling of the lactose permease, with NEM, was at least as specific as that of any of the other systems. This raises the question of whether it is worth the effort to synthesize substrate analogues, although it is true that in some cases non-specific inhibitors are relatively ineffective. On the other hand, since the purpose of labelling is only to identify a particular protein, a moderate amount of non-specific labelling might be acceptable, provided that it is distributed randomly in contrast to the specific labelling which ought to be in a single protein.

They are probably a variety of reasons for the non-specific labelling. One of these is that the majority of potentially reactive amino acid side chains do not be-

Table 4
Other reagents and techniques for studying membranes.

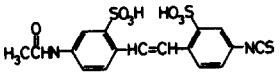
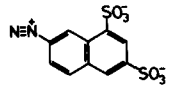
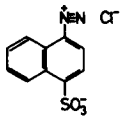
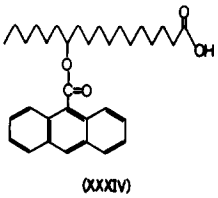
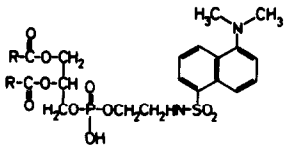
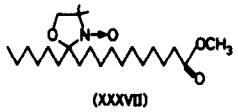
| Membrane function or location | Reagent | Comments | Refs |
|---|---|---|---|
| Membrane proteins whose amino acid side chains are exposed to internal or bulk phase solvent |  (XXXI) | 4-Acetamido-4′-isothiocyanostilbene-2,2′-disulphonic acid (XXXI). Non-pentrating. Binds to protein. Reacts completely after 5 min, with maximum uptake of about $5 \times 10^5$ moles per red cell. Labelled residues not identified. | 40 |
| |  (XXXII) | 7-Diazonium-1,3-napthylene-disulphonate (XXXII). Non-penetrating, fluorescent. Used to demonstrate the sulphate binding protein is accessible to solvent. Inhibits sulphate binding, sulphate transport and β-galactoside transport. | 41 |
| |  (XXXIII) | Naphthalene-1-diazonium-4-sulphonate (XXXIII). Non-penetrating and fluorescent. Will be used to determine whether any protein species are accessible from both inside and outside of membrane. | 42 |
| Mobility and chemical environment of membrane lipids and proteins |  (XXXIV)  (XXV)  (XXXVI) | Measurements of fluorescence quanta yields and emission spectra for probe in liposomes compared with known solvent mixtures provides information on proximity, rotational mobility and polarity of probe, and of its environment within membrane. Fluorescent substrate analogues also can be synthesized. 12-(9-Anthroyl)-stearic acid (XXXIV), *N*-dansyl-phosphatidylethanolamine (XXXV), and *N*-octadecyl-naphthyl-2-amine-6-sulphonate (XXXVI) localize in the hydrocarbon, glycerol, and aqueous interfacial regions, respectively. | 43 |
| Mobility and orientation of membrane lipids and proteins |  (XXXVII) | The 4′,4′-dimethyloxazolidine nitroxide of methyl stearate (XXXVII), or cholestane nitroxide incorporated into phospholipid spherules and relaxation rates of nitroxide label measured by paramagnetic resonance. Nitroxide radical may theoretically be incorporated into substrate analogues, for study of transport process. | 44–48 |

Table 4 (continued)

| Membrane function or location | Reagent | Comments | Refs |
|---|---|---|---|
| Interaction between membrane proteins and small molecules. | Proton magnetic resonance or $^{19}F$ magnetic resonance | Interaction of adrenaline with adipose cell membrane studied by measuring relaxation of drug protons. Membrane protons not distinguishable. | 49 |

long to the protein to be labelled. Although the affinity between the specific reagents and the transporter or receptor binding site should increase the rate and possibly the extent of labelling at this site compared with other sites, the odds against this in most cases may simply be too high. In systems such as the dicarboxylate transporter, the relatively high $K_m$ makes the situation even more unfavourable. Another point is that in some cases there are several proteins with an affinity for a particular ligand and each of these may be labelled. For example, in rat liver inner mitochondrial membranes, there are several carriers and at least one enzyme (succinate dehydrogenase) with binding sites for dicarboxylate anions. Finally, the possibility should be kept in mind that membrane proteins may be intimately linked, structurally and functionally, to one another in such a way that inhibition of one species in the membrane may be reflected in the activities of the other species as well. Although Changeux and colleagues [20] have proposed that cooperative effects may be involved in certain normal membrane functions, there is little experimental evidence in support of this possibility. However, in the case of the soluble complex of phosphorylase *a* and alanine aminotransferase from rabbit muscle, competitive inhibitors of one enzyme have a marked inhibitory effect on the activity of the other member of the complex, whereas these 'crossed' effects are not seen when the isolated enzymes are tested with the same inhibitors [50]. Thus it may be necessary to isolate the labelled component(s) and characterize them with respect to structure and/or function before evaluating the specificity of the labelling.

Because of the technical difficulties usually encountered in purifying membrane proteins, the fastest way of estimating the number of labelled components may be to do a preliminary fractionation of the solubilized proteins, and then subject the labelled fraction to enzymatic digestion, high voltage electrophoresis, and radio-autography. Specific labelling will be indicated by one or a small number of major peptides with a unique amino acid sequence, whereas a non-specific reaction will result in a large number of minor peptides.

There are several possible ways to increase the specificity of labelling:

a) Substrate protection: Although this was effective for the lactose permease, it has not been very successful for most of the other membrane systems.

b) Photoactivated affinity labels: These reagents are useful in situations where there is a sufficiently high affinity between ligand and binding site to allow non-specifically adsorbed label to be removed before the inhibitor is activated. The type of reagents presently available are diazoketones [51] and aryl azides [52]. A possible advantage of the latter compounds is that they are activated at wavelengths greater than 400 nm and may have less effects upon aromatic amino acids and other cellular constituents. These reagents have not yet been used for cellular or membrane systems.

c) Optimizing incubation conditions: Specific labelling will be favoured by the use of a low molar ratio of inhibitor to active site, and short duration of incubation. Although these conditions are likely to give less than 100% inhibition and a corresponding reduction in number of sites labelled, this may be relatively unimportant in return for the increased specificity.

A final difficulty inherent in affinity labelling is that it usually, although not invariably, inactivates the labelled protein. This makes it difficult to study the biological activity of the isolated protein and to use it in reconstitution experiments. It is sometimes possible to reactivate inhibited proteins, for example, deacylation of serine hydroxyl residues in certain proteases [53] and thiolytic cleavage of dini-

trophenyl residues [54, 55], but it is not certain that this will be feasible with all labelling reagents or all proteins.

In conclusion, we feel that the usefulness of affinity labelling in the isolation of membrane proteins has yet to be proved. Furthermore, the technical difficulties and inherent shortcomings are sufficiently great to warrant the use of supplementary or alternative approaches. The remainder of this review is devoted to consideration of one such approach.

## 3. Solubilization of membrane followed by purification

The use of osmotic shock in bacteria has resulted in isolation of large and growing family of proteins which have specific high affinity binding sites (usually one per protein) for transported molecules such as galactose and amino acids [56, 57]. There is a great deal of indirect evidence implicating the binding proteins in bacterial membrane transport. This evidence was carefully evaluated by Kaback [57] who concluded that whilst these proteins may in some cases be necessary for transport, there is no evidence that they are sufficient.

Osmotic shcok has recently been used by Lehninger's group in an attempt to solubilize the energy-dependent $Ca^{2+}$ transport system which they have described in rat liver mitochondria [58, 59]. The preliminary results with the mitochondrial system are very reminiscent of those with bacterial binding proteins, and we think that the reservations presented by Kaback [57] apply to the mitochondrial systems. It is likely that there are other proteins involved in transport which are intimately bound within membranes and which have not yet been obtained in soluble, active form. Examples of such proteins are the M protein of the lactose operon [7], and the family of Enzymes II in the phosphoenolpyruvate-dependent phosphotransferase system [60]. A third example of this type of protein may be the cholinergic receptor of the electroplax, which has recently been solubilized using 1% deoxycholate [61]. The solubilized receptor activity and that seen in isolated electroplax membrane or vesicular membrane fragments [21] are very similar to one another with respect to the dissociation constants for several agonists and antagonists, and the irreversible inhibition by α-bungarotoxin [62]. In addition, the receptor activity and the catalytic activity due to acetylcholinesterase appear to be on separable proteins, as indicated by ultracentrifugation and selective heat denaturation [62].

An inherent difficulty with this approach is that most of the activities associated with membranes cannot be assayed once the membrane has been disrupted. The solubilized proteins can be tested only for the ability to bind substrates or inhibitors. Even when the various kinetic properties of the soluble and membrane activities coincide, it is difficult to unequivocally relate this binding to the biological activity in question. Some uncertainty must remain until the protein can be shown to mediate the activity itself.

The types of binding assays (equilibrium dialysis, ion exchange, etc.) used for high affinity systems are not likely to be useful for systems with affinities of the order of $10^{-3}$ M. For this reason, we are preparing affinity chromatographic reagents which can be coupled with the amino-analogues of dicarboxylate anions for the assay and purification of the mitochondrial dicarboxylate transporter. This technique is especially promising because it has been used successfully to purify β-galactosidase using a relatively weak competitive inhibitor, *p*-aminophenyl-β-D-thiogalactopyranoside, $K_i$ about $10^{-3}$ M [63, 64]. The principles of affinity chromatography have been discussed by others [64–66]. It appears that the optimal linkage for a given protein–ligand system can be determined only by trial and error, although it has been suggested that the length and possibly the nature of the hydrocarbon chain between the insoluble matrix and the ligand is especially critical for weakly binding systems [64]. The major problem with this approach (apart from the detection and identification of a small amount of specifically bound protein) is the solubilization of the membrane. The technique as usually carried out requires biologically active proteins in aqueous solution. Therefore, most of the techniques used for solubilizing membranes, which denature the proteins, cannot be used. On the other hand, less drastic techniques may not be adequate for solubilizing transport proteins, and techniques may have to be developed for the extraction, purification and assay of these proteins in a hydro-

Table 5
Techniques used for isolation of membrane proteins.

| Technique or reagent | Protein and source | Comments | Refs |
|---|---|---|---|
| Sonication | Coupling factor $F_0$ (oligomycin-sensitivity conferring-factor), beef heart mitochondria | Isolated from submitochondrial particles previously treated with trypsin, EDTA, and urea. Particulate. | 70 |
| Osmotic shock | High affinity binding proteins, Gram negative bacteria | Proteins released are probably peri-cytoplasmic or loosely bound to membrane rather than integral components of membrane. | 56 |
| Distilled water | 'Structural' protein from human red cell ghosts | Membrane completely solubilized only if ghosts have been thoroughly deionized. | 69 |
| Low ionic strength | ATPase, *Str. faecalis* | Enzyme solubilized by repeated washes with 1 mM tris HCl buffer in absence of $Mg^{2+}$. | 71 |
| High ionic strength | Protein from human red cell ghosts | 0.8 M NaCl solubilizes about 40% of membrane protein. | 67 |
| Chelating agents | 'Spectrin', human red cell ghosts | 5 mM EDTA, low ionic strength buffer, plus β-mercaptoethanol. | 68 |
| Extraction of acetone powder with neutral or alkaline aqueous buffers | Succinate dehydrogenase, beef heart mitochondria | Enzyme isolated in presence of succinate (or certain reducing agents) retains activity in succinate oxidase (respiratory chain) and phenazine methosulphate assays, whilst in absence of substrate, only latter activity retained. Mechanism of substrate effect not known. | 72 |
| Lubrol | ATPase, brain microsomes | Large amount of lubrol adheres to solubilized proteins. | 19 |
| Triton X-100 | Acetylcholinesterase, electroplax electric organ | 0.5% w/w detergent solution for 48 hr at 4°. Sonication prior to detergent treatment. Enzyme partially inactivated. | 73 |
| Deoxycholate | ACh receptor, electroplax electric organ | 1% w/w detergent solution. Acetylcholinesterase also solubilized. | 61 |
| Alkaline borohydride | Polypeptide with phytohaemagglutinin receptor activity, human red cell ghosts | Multiple enzyme digestions also required. | 74 |
| Enzyme digestion | Protein with sucrase-isomaltase activity, plus carrier-like sugar binding site, hamster small intestine | Trypsin, in presence of 5 M urea also used. | 75 |

Table 6
Organic solvents for solubilizing membranes.

| Solvent | Comments | Refs |
|---|---|---|
| Acetic acid | 80% aqueous solution gives almost complete solubilization of red cell membrane. | 77 |
| 2-Chloroethanol, ethylcellosolve, methylcellosolve, formamide, dimethylformamide, diethylene glycol monobutyl ether | 9:1 v/v solvent: water, pH brought to 2 with HCl; solubilization of 95% of membrane protein; all solvents miscible with water; interact with hydrogen bonds, and have high lipid solubility; proteins retain some secondary structure, but effect upon biologic activity is unknown. | 77–79 |
| Pyridine | 33% aqueous pyridine dissolves red cell membranes; proteins tend to aggregate even in presence of β-mercaptoethanol. | 80 |
| Butanol | See text. | 76 |
| Urea or guanidine | Produce random polypeptide chains if disulphide bonds have been reduced and alkylated; most proteins denatured, but in some cases, reversibly. | 67, 81 |
| Sodium dodecyl sulphate | SDS binds to all proteins in constant proportion w/w: 0.4 and 1.4 g SDS/g protein at SDS monomer concentrations of $5–8 \times 10^{-4}$ M, and $> 8 \times 10^{-4}$ M respectively; polypeptide chains not completely random. | 82 |
| | Use of SDS resulted in isolation of low molecular weight proteins (6500–10,000 daltons), in yields of 30–90% of total protein, from red cells, mitochondria, retinal rods, and sarcoplasmic reticulum. | 83, 84 |

phobic environment. This problem will be discussed in the final section.

### 3.1. *Solubilization of membrane proteins*

There are two broad approaches to this problem. The first involves the application of techniques used for aqueous systems. It is clear that a variable, and sometimes large, amount of protein can be solubilized using fairly mild conditions, such as high ionic strength [67], chelating agents [68] and even distilled water [69]. We have shown that large amounts of protein can be solubilized from rat liver mitochondria using either 0.8 M NaCl or 1 mM EDTA [11]. These techniques may be even more valuable when combined with additional physical or chemical techniques. Note that in most of the examples listed in table 5, several techniques have been used sequentially.

Many membrane proteins are difficult to solubilize under these conditions [7], others tend to aggregate irreversibly [67], and still others are unstable in aqueous solutions [76]. Furthermore, these difficulties may occur even in the presence of detergents and other denaturing reagents [67]. Nevertheless, solubilization and fractionation may be considerably facilitated by the use of reagents such as ionic detergents, urea, guanidine, and organic solvents (table 6). We have been able to separate solubilized mitochondrial proteins into 5 or 6 major fractions using anion exchange chromatography with 8 M urea and a linear salt gradient [11]. Under these conditions, most proteins will undergo irreversible denaturation, and consequently the usefulness of this technique for our purposes is limited.

The recent work of Strominger's group has indicated the possibility of extracting membrane proteins directly into organic solvents without denaturation [76]. In principle this is a very sensible way of handling such proteins, although it may not always be successful [7]. Strominger's results will be given in some detail because of the interest and novelty of the approach. The enzyme, an ATP-dependent C-55 isoprenoid alcohol phosphokinase from the membrane

of *Staphylococcus aureus*, has the following properties: 1) extractable in active form into acidic butanol at room temperature, from which it precipitates reversibly at $-15^{\circ}$; 2) stable in organic solvents such as acetone, heptane, ether and benzene, but not chloroform–methanol (1:1); 3) insoluble in water and relatively unstable in aqueous buffers; 4) undergoes 50% increase in activity upon heating in butanol at $100^{\circ}$ for 20 min; 5) enzyme activity requires either phosphatidyl glycerol and/or cardiolipin, but this requirement may be partially satisfied by certain detergents [76, 85].

It will be necessary to screen a variety of such organic solvents in order to assess the general usefulness of this approach. Proteins solubilized in this way can be fractionated in ways analogous to those used for lipids and other non-polar molecules. This requires the development of hydrophobic derivatives of dextran or acrylamide beads and other supporting materials [86], such as Sephadex LH 20. The feasibility of detecting reactions which are largely ionic or electrostatic in non-polar solvents is uncertain, although such reactions are known to occur [87]. It may be possible to synthesize affinity columns which can be used in non-polar systems.

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