sibly occur owing to partial relocation of this phosphatide to apolar sites formerly occupied by the 90% of major microsomal phospholipid components, which were hydrolysed. Such inhibition should be reversed not only by the phospholipid specifically required by the enzyme in question, but also by others which, re-occupying apolar sites, would reverse relocation of the minor phosphatides. If this hypothetical phenomenon of relocation occurs it may seriously hamper the correct assignment of specific phospholipid requirements to enzymes.

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The Use of Pure Phospholipases in the Study of Membrane Structure and Function

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The disposition of membrane components can be investigated by the use of phospholipases, proteolytic enzymes and group-specific labels. The general strategy to determine sidedness of membrane components can be outlined as described below, with the erythrocyte membrane as an example: the action of phospholipases, proteolytic enzymes and non-permeant labels towards the intact erythrocyte will provide information about those membrane components which are (at least partially) exposed on the exterior of the membrane, whereas effects on non-sealed 'ghosts' will in addition provide information about the components on the inner surface of the membrane.
Pure phospholipases have been shown to be useful tools in revealing asymmetrical distributions of phospholipids in erythrocyte membranes (Colley et al., 1973; Verkleij et al., 1973; Zwaal et al., 1973). In principle there are three possible results of incubation of erythrocytes with pure phospholipases. (i) No lipid hydrolysis; no haemolysis. (ii) Lipid hydrolysis; no haemolysis. Here, it is concluded that the phospholipids which are susceptible to the degradative action of the phospholipases are localized on the outside of the cell membrane. (iii) Lipid hydrolysis and haemolysis. In this case, the phospholipases penetrate towards the interior of the 'ghosts' produced by the haemolysis, resulting in a further degradation of the phospholipids at both sides of the cell membrane.

The effects of phospholipases towards erythrocyte membranes vary strongly with the type and origin of the phospholipase used. Thus, pure pancreatic phospholipase A$_2$ does not hydrolyse any phospholipid in the membrane of the intact cell, whereas the main glycerophospholipid classes are completely degraded in 'ghosts' (Roelofsen et al., 1971). However, phospholipases A$_2$ from sea-snake venom (Ibrahim & Thompson, 1965) and bee venom (Zwaal et al., 1973) have been shown to produce phosphatidylcholine breakdown in the membrane of the human erythrocyte without causing haemolysis. The most pronounced effect towards intact cells is observed with pure phospholipase A$_2$ from Naja naja venom, which produces a non-haemolytic degradation of 68% of the phosphatidylcholine of the intact human erythrocyte, without changing the freeze-fracture faces of the membrane as observed with freeze-etch electron microscopy (Verkleij et al., 1973). Phospholipase A$_2$ treatment of 'ghosts' produces complete breakdown of the glycerophospholipids and induces aggregation of particles on the freeze-fracture faces of the membrane.

Phospholipase C from Clostridium welchii produces degradation of approx. 70% of the phospholipids of the erythrocyte 'ghosts' (Lenard & Singer, 1968; Glaser et al., 1970). This degradation is accompanied with the appearance of dense droplets ('black dots') in the membranes as observed with phase-contrast microscopy (Coleman et al., 1970). Pure phospholipase C from Bacillus cereus, though not able to degrade phospholipids in intact cells, produces complete degradation of the main phospholipid classes in 'ghosts' with the exception of sphingomyelin, resulting in the release of 70% of the total lipid phosphorus (Roelofsen et al., 1971; Zwaal et al., 1971). Also in this case 'black dots' are formed, which have been identified with the diglycerides produced by the action of B. cereus phospholipase C, since the dots can be removed for the greater part by treatment with pancreatic lipase (Colley et al., 1973). Freeze-etching of phospholipase C-treated erythrocyte 'ghosts' shows a dramatic decrease in tangentially fractured membranes and large droplets with a diameter up to 1 µm are observed adhering to the membrane (Verkleij et al., 1973).

Sphingomyelinase from Staphylococcus aureus hydrolyses 80–85% of the sphingomyelin of human erythrocytes, without producing haemolysis of the cells (Colley et al., 1973). Freeze-etch electron microscopy shows that this action is accompanied by aggregation of membrane particles and the formation of small spheres (7.5–20 nm in diameter) on the outer fracture face with corresponding pits on the inner fracture face of the membrane (Verkleij et al., 1973). Treatment of 'ghosts' with this enzyme produces complete degradation of the sphingomyelin, and freeze-etching reveals the formation of droplets (100–300 nm in diameter) which are adherent to the membrane and not visible by phase-contrast microscopy.

When human erythrocytes are treated successively with phospholipase A$_2$ (N. naja) and sphingomyelinase (S. aureus) no haemolysis occurs, although the osmotic fragility is markedly increased. This combined action produces up to 48% degradation of the total phospholipid complement of the membrane. It is concluded that this phospholipid fraction, which contains 76% of the phosphatidylcholine, 82% of the sphingomyelin and 20% of the phosphatidylethanolamine, forms the outer monolayer of the human erythrocyte membrane. A similar suggestion has also been proposed by Bretscher (1972, 1973) on the basis of labelling studies with the relatively non-permeant label formylmethionyl-sulphone methyl phosphate. Since this label is unable to tag the choline-containing phospholipids, which form the majority of the outer membrane layer, this evidence
should be considered more indirect as compared with the results obtained with lipolytic enzymes.

In a wide range of mammalian erythrocytes, the sum of the choline-containing phospholipids (phosphatidylcholine and sphingomyelin) comprises 46–60% of the total membrane phospholipids (van Deenen & de Gier, 1964; Nelson, 1967). Whereas the ratio of phosphatidylethanolamine to phosphatidylserine is relatively constant in all these species, the ratio of phosphatidylcholine to sphingomyelin varies markedly. The most dramatic deviation is found in ruminant erythrocytes, where the choline-containing phospholipid fraction contains almost exclusively sphingomyelin and hardly any phosphatidylcholine. In this respect, it is of interest to mention that the phosphoglyceride molecules of intact ox and sheep erythrocytes are not accessible to phospholipases A2 and C, whereas sphingomyelinase is able to produce a considerable non-haemolytic breakdown of sphingomyelin in these cells (Colley et al., 1973). Moreover, a mixture of phospholipase C and sphingomyelinase, being strongly lytic towards a wide variety of mammalian erythrocytes, fails to produce haemolysis of ox and sheep erythrocytes. These results are taken to indicate that glycerophospholipids are essentially absent from the outer lipid layer of ruminant erythrocytes and that this layer consists predominantly of sphingomyelin. The presence of choline-containing phospholipids in the outer layer, almost to the exclusion of the other phospholipids, may account for the observed exchange of phosphatidylcholine and sphingomyelin between erythrocytes and plasma (Sakagami et al., 1965; Reed, 1968). Although ruminant erythrocytes differ strongly from those of other mammals in that they contain very little phosphatidylcholine, no such differences in the lipid composition are observed between various mammalian plasmas. Thus, the phospholipid composition of sheep plasma is virtually identical with that of human plasma, containing approx. 68% of phosphatidylcholine, 21% of sphingomyelin, 7% of lysosphatidylcholine and 4% of phosphatidylethanolamine. It has recently been discovered that a membrane-bound phospholipase A2, which is highly specific for phosphatidylcholine, is exclusively present in ruminant erythrocytes (R. F. A. Zwaal & P. Zahler, unpublished work). Moreover, this phosphatidylcholinase is localized at the exterior of the cell membrane, since it can be nearly completely inactivated by Pronase treatment of intact cells. Preliminary experiments may suggest that this enzyme plays a role (for some reason or another) in keeping phosphatidylcholine out of the erythrocyte membrane of ruminants, this in spite of the high phosphatidylcholine concentrations in the plasma.

The failure of pancreatic phospholipase A2 and phospholipase C from B. cereus to attack phospholipids in intact erythrocytes, although phospholipids are accessible to sphingomyelinase and phospholipases A2 from N. naja, sea-snake and bee venoms, leads to the suggestion that the substrate requirements are not fulfilled for the first group of enzymes, but that a proper enzyme–substrate complex can be formed with the second group of enzymes. Two possibilities can be considered in this context: (i) the phospholipids are not readily available on the outside but are shielded by proteins, and some phospholipases may be intrinsically able to disturb this shielding, leading to exposure of the phospholipids, and (ii) the phospholipids are directly available on the outside of the cell, but the ability of the different phospholipases to exert their action depends strongly on the packing of the lipids in the native membrane. The first possibility may be unlikely, since pretreatment of erythrocytes with proteolytic enzymes does not make the lipids available for pancreatic phospholipase A2 or phospholipase C (Roelofsen et al., 1971). The second possibility, however, seems to be supported by monolayer studies. Phospholipase A2 (N. naja) and sphingomyelinase (S. aureus), when injected under a monolayer of phosphatidylcholine and sphingomyelin respectively, are still able to degrade the lipids at surface pressures above 300 N/cm (R. A. Demel & R. F. A. Zwaal, unpublished work). On the other hand, pancreatic phospholipase A2 and B. cereus phospholipase C can only exert their action at surface pressures below 300 N/cm. It has been suggested that phospholipase C (B. cereus) acts only at the inner surface of the erythrocyte membrane (Low et al., 1973). Although this may be correct for normal erythrocytes, it is possible that this does not hold for hypo-osmotically swollen erythrocytes which can be
lysed by *B. cereus* phospholipase C (Woodward & Zwaal, 1972; Laster et al., 1972). Stretching of the membrane caused by hypo-osmotic swelling may lead to a concomitant decrease in surface pressure of the outer lipid layer, which may make the phosphatidylcholine available to phospholipase C. On the other hand, it is still possible that hypo-osmotically swollen erythrocytes may contain holes large enough for phospholipase C (mol.wt. 23000) to penetrate towards the cell interior, from where it can degrade the membrane from the inside, eventually leading to haemolysis. Further, caution should be exercised in extrapolating the results obtained with erythrocyte membranes to other isolated membrane preparations. A noteworthy deviation is observed with *Mycoplasma hominis* in that phospholipase C from *B. cereus* fails to produce hydrolysis of membrane phospholipids both in intact cells and in isolated membranes, whereas aqueous dispersions of the extracted phospholipids (which comprise predominantly phosphatidylglycerol) are readily attacked (Rottem et al., 1973). On the other hand, 40% of the membrane phospholipids can be hydrolysed after proteolytic digestion of the membranes, indicating that the phospholipids in the native membrane are inaccessible to phospholipase C owing to masking by proteins. This clearly demonstrates that the lipid-protein organization in *Mycoplasma* membranes is markedly different from that in erythrocyte membranes.

In addition to lipid asymmetry, studies with proteolytic enzymes and non-permeant labels strongly suggest the existence of an asymmetric protein distribution in erythrocyte membranes (see, for recent reviews, Bretscher, 1973; Juliano, 1973). Several functional aspects of membrane asymmetry have been discussed by Bretscher (1973). In relation to sidedness, I should like to touch on the Na\(^+\)+K\(^+\)-transporting ATPase (adenosine triphosphatase) system of erythrocyte membranes. That such a system must have an asymmetric alignment in the membrane may be clear from the observation that ATPase activity is stimulated by K\(^+\) at the outside and Na\(^+\) at the inside of the membrane (Whittam, 1967); thus, these ions only activate ATPase on that side of the membrane from where they are transported. Moreover, three lines of evidence exist which indicate that at least the active centre of the Na\(^+\)+K\(^+\)-stimulated ATPase is localized at the inside of the erythrocyte membrane: (i) only internal ATP can be utilized by the enzyme system; external ATP is not attacked (Whittam, 1967); (ii) lead phosphate, obtained by precipitation of the enzymically produced P\(_1\) with lead nitrate, is exclusively localized along the inner surfaces of the 'ghost' membranes (Marchesi & Palade, 1967a); (iii) Na\(^+\)+K\(^+\)-stimulated ATPase is not abolished by proteolytic digestion of intact cells (Martin, 1970), but is readily inactivated by trypsin treatment of 'ghosts' (Marchesi & Palade, 1967b). Roelofsen & van Deenen (1973) showed in studying the effects of pure phospholipases on transport ATPase that the Na\(^+\)+K\(^+\)-stimulated ATPase activity is contingent on a small fraction of the membrane phosphatidylserine. It is most likely that a proper orientation of this phosphatidylserine fraction on the membrane interior, at or near the active centres of transport ATPase molecules, is essential in maintaining this active-transport phenomenon in erythrocytes.

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GENETICS AND MOLECULAR ENZYMEOLOGY: a Colloquium organized on behalf of the Molecular Enzymology Group by R. N. Perham (Cambridge)

Genetic Approaches to Probing Enzyme Structure and Improving Enzyme Yield

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Advances in our understanding of the molecular genetics of bacteria and their viruses make it increasingly apparent that genetics can be a valuable tool to the enzymologist. Genetic technology can be used analytically, in studying the essential features of enzymatic function, or preparatively, as a means of facilitating the preparation and purification of enzymes.

Probing enzyme structure

There are now many examples in the literature of the ways in which genetic analyses can be used to elucidate essential features of enzymic function. Most simply, the effects of amino acid substitution on enzyme activity can often be studied by analysis of the altered enzyme from revertants of a single-site mutant. Revertants carrying one or more amino acid replacements can usually be distinguished from the wild-type revertant by simple phenotypic tests (Yanofsky et al., 1966). By this approach nine different amino acids have been substituted for a single glycine residue of the wild-type tryptophan synthetase α subunit of Escherichia coli (Berger et al., 1968). In E. coli systems, multiple amino acid substitution can readily be produced by the suppression of amber nonsense mutations with suppressors inserting different amino acids (reviewed by Hartman & Roth, 1973).

The phenomenon of ‘second-site reversion’ of mis-sense mutants can be used to yield information on the specific interactions between different regions of the folded polypeptide chain. Mis-sense mutations, resulting in a single amino acid substitution, can sometimes be reverted by a second mutational event that causes the replacement of an amino acid residue in a different region of the polypeptide chain. In the trpA gene of E. coli, which codes for the tryptophan synthetase α subunit, two independent mis-sense muta-