From Walls to Membranes

The Sixteenth Marjory Stephenson Memorial Lecture

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It was with great joy that I received the invitation to present this year's Marjory Stephenson Memorial Lecture to pay tribute to one of the outstanding pioneers of microbial chemistry and I would like to thank the Society for this honour. For me it is a special privilege, as I was the last overseas student she accepted prior to her untimely death in the latter part of 1948. I have always regarded myself as being one of the very fortunate microbiologists to have known her and I have deeply regretted that it was not for a longer period of time. As an undergraduate in far-off Australia, her name was the hallmark for excellence in all matters microbiological and biochemical and Marjory Stephenson's *Bacterial Metabolism* was our 'bible'. It was cause for great excitement when I was awarded a CSIRO Studentship and accepted, as she wrote, 'for one of the two places reserved in our course for postgraduate students'. With bags and books packed, I embarked on the long journey from Sydney to Cambridge. In those pre-jet days the sea voyage to London took five weeks and it was that time of the year when many students and actors from Australia were heading off for the 'old country' to take up their scholarships and broaden their horizons. With such a mix of stage and science the voyage was rarely dull but we all eagerly awaited arrival and the experiences that lay ahead. The attractions and scientific exhilaration of Cambridge were so great that my stay became an extended one, thanks to Ernest Gale's hospitality as Director of the MRC Unit for Chemical Microbiology (later the Sub-Department of Chemical Microbiology, Department of Biochemistry) and his stimulus, enthusiasm and interest in the mysteries of the functioning of the bacterial surface. The brevity of my personal contact with Marjory Stephenson was fully compensated for by the enduring association with Ernest Gale and his colleagues in what was affectionately known as 'the bug hut'.

Interest in the bacterial cell surface goes all the way back to the birth of microbiology with the microscopic observations of Antony van Leeuwenhoek recorded in his letters to the Royal Society in 1676. Leeuwenhoek not only described the principal shapes of bacteria but also noted that he was unable to see the surface 'film' which held them together, thereby anticipating the existence of structural components of the cell responsible for their characteristic shape. Much of the ensuing evidence on the existence and nature of a cell wall or membrane in bacteria was indirect, fragmentary and inconclusive. About the mid to late 1940s there was a renewed interest in the bacterial surface, stimulated by transport studies such as those of Gale & Taylor (1947) on the accumulation of amino acids across the permeability barrier of Gram-positive bacteria and the direct visualization of walls and membranes in disrupted and bacteriophage-lysed cells by electron microscopy (Mudd & Lackman, 1941; Mudd *et al.*, 1942; Wyckoff, 1948). Indeed, the first Symposium held by this Society in 1949, 'The Nature of the Bacterial Surface', focused attention on the immunological, structural and functional aspects of this rapidly developing field of research. Significantly, in his introduction to the Symposium, Pirie (1949) said 'It is no longer necessary to apologize for the intrusion of biochemists into what used to be a purely bacteriological field.' In the span of some 34 years since this Symposium 'the intrusion of biochemists' has led to the remarkable development of our knowledge and understanding of the

biochemical properties and biosynthesis of the bacterial cell wall, outer membranes of Gram-negative bacteria and the plasma membranes of both Gram-positive and Gram-negative organisms. As a result of the intensive work carried out in many laboratories we now have a greater appreciation of the unique surface features of prokaryotic cells and their biological distinction from eukaryotic organisms as well as a fuller understanding of the biochemical processes that make them targets for the selective action of antibiotics. With the convergence of independent studies of cell wall isolation and chemical characterization, action of lytic enzymes, the discovery of muramic acid (Strange & Dark, 1956) and the biochemical basis of penicillin action, the 1950s saw the emergence of a whole new segment of chemical microbiology, laying the foundations for a broad understanding of the structure, functions and biosynthesis of bacterial walls, envelopes and membranes. In retrospect, it is amazing how Fleming's two separate sets of observations on lysozyme and penicillin action became inextricably intertwined in the emerging field of cell wall and membrane biochemistry, not forgetting, of course, the legacy he left us in the form of his isolate of *Micrococcus lysodeikticus*, now relegated by the taxonomists to the much less exotic designation, *M. luteus*.

Marjory Stephenson (1949) had great biochemical insight and, long before the details emerged, in her introductory remarks in the last edition of her *Bacterial Metabolism* she knew full well that 'the study of the action of chemotherapeutic agents and antibiotics is contributing unexpected information on intracellular synthetic mechanisms'. Nothing could have been closer to the mark for bacterial wall and membrane biosynthesis!

Cell walls

Although there had been attempts to define the chemical composition of bacterial cell walls as far back as the studies of Vincenzi (1887) with *Bacillus subtilis*, there was little firm evidence to suggest the precise nature of the walls. What evidence there was, was frequently based on qualitative tests and at best with conflicting results (see Knaysi, 1951, for an early review). That a direct attack on the problem of cell wall composition seemed feasible became evident from the use of the electron microscope in the examination of disrupted bacterial cells. Thus, Mudd & Lackman (1941) and Mudd *et al.* (1941) demonstrated that walls could be either partially or completely freed of cytoplasm by sonic disruption of bacteria. The spectacular electron micrograph of walls of *Staphylococcus aureus* shown by Dawson (1949) at the first Symposium of the Society clearly pointed the way to a new approach for subcellular fractionation, meeting the stringent criteria of homogeneity needed for analytical purposes. It will be recalled that Weibull (1948) was the first to isolate a morphological entity of the bacterial cell, the flagella, by differential centrifugation, utilizing electron microscopy as a criterion for establishing structural homogeneity. Similar approaches were developed for the isolation of homogeneous preparations of bacterial cell walls (Salton & Horne, 1951) for their subsequent chemical characterization (Salton, 1952*a*) and for study of their digestion by lysozyme (Salton, 1952*b*). Isolation of cell walls became a very popular hobby in the early 1950s and eventually resulted in the recognition of the unique bacterial wall polymers, the peptidoglycans, teichoic acids and teichuronic acids (Rogers *et al.*, 1980). The universality of peptidoglycan as the principal structural polymer of the cell wall of prokaryotes soon became apparent and the fine details of the chemistry and classification of peptidoglycans have been admirably documented by Ghuysen (1968) and Schleifer & Kandler (1972). The wall-less mycoplasmas constitute the exception, being completely devoid of peptidoglycan. The bacterial cell wall has been a reservoir of unique 'marker' molecules and muramic acid has for long been recognized as one of the principal 'hallmarks' of prokaryotic cells (with the above exception). The recent discovery of talosaminuronic acid instead of muramic acid in the pseudomurein of certain methanogenic and other Archaeobacteria (König & Kandler, 1979; Kandler, 1982) is a fascinating finding and points to the intrinsic diversity even for such 'universal' structures as the peptidoglycans.

The choice of *Micrococcus lysodeikticus*, now officially known as *M. luteus*, was in many respects a fortunate one for some of our early cell wall studies, although unfortunately its genetics have never been pursued very far. As seen in thin sections (Fig. 1) the wall of *M. lysodeikticus* is quite thick (about 40–60 nm) and relatively homogeneous in appearance; the

Fig. 1. Electron micrograph of a thin section of *Micrococcus lysodeikticus* showing the thick cell wall, rather amorphous in appearance, and the underlying plasma membrane and mesosomal vesicles.

isolated wall may account for 30–40% of the dry weight of the cell. Acid hydrolysis of the walls yielded a simple mixture of the amino acids alanine, glutamic acid, glycine and lysine, and hexosamine (Salton, 1953) and provided the first indication that the bacterial cell wall differed markedly from known structural polymers such as chitin, mucopolysaccharides and mucoproteins. As it turned out, the wall of *M. lysodeikticus* was somewhat unusual even for a Gram-positive organism, in that it was largely composed of peptidoglycan, which accounted for approximately 95% of the weight of the cell wall (Czerkawski *et al.*, 1963), was devoid of teichoic acid but contained about 5% of an associated polysaccharide composed of glucose and *N*-acetylmannosaminuronic acid (Perkins, 1963). Such polymers and related acidic polysaccharides in other bacterial walls have been classified chemically as the teichuronic acids (Janczura *et al.*, 1961). One other feature of the *M. lysodeikticus* wall was the low degree of peptide

substitution of the muramic acid residues, thus permitting the isolation of a disaccharide repeating unit of *N*-acetylmuramic acid and *N*-acetylglucosamine from lysozyme digests of the wall (Salton, 1956; Salton & Ghuysen, 1959; Perkins, 1960; Jeanloz *et al.*, 1963). Subsequent studies established a glycan backbone consisting of repeating disaccharide units and the head-to-tail mode of linkage of peptide moieties substituted on a proportion of the muramic acid residues (Schleifer & Kandler, 1967; Ghuysen, 1968). Thus, the peptidoglycan of *M. lysodeikticus* presented a relatively open mesh of glycan strands and a relatively low level of peptide cross-linking, in contrast to the highly cross-linked network of the peptidoglycan of *Staphylococcus aureus* cell wall with most of the muramyl residues substituted with peptides, possessing in addition covalently linked teichoic acid substituents (Ghuysen *et al.*, 1968).

Cell wall studies rapidly passed through the initial stages of determining the primary structures of peptidoglycans and teichoic acids (Baddiley, 1968, 1972; Ghuysen, 1968) and investigators turned their attention to the biosynthesis and assembly of the wall structures and the targets for wall inhibitor antibiotics (Strominger, 1970). Studies of the biosynthesis of the wall peptidoglycan revealed a unique set of enzymes in the bacterial cell involved in over 20 steps in the sequential synthesis of this heteropolymer and, moreover, established the specific target enzymes for a variety of wall inhibitors (Blumberg & Strominger, 1974). Clearly, much remains to be discovered about the regulation of wall synthesis and assembly, turnover, remodelling of the wall, its growth, extension and mechanisms of shape determination and the role of autolysins in these events, although substantial advances have been made in many of these areas of cell wall biochemistry during the past decade (Rogers *et al.*, 1980).

In the aftermath of the excitement generated by the elucidation of peptidoglycan structure and biosynthesis and that of the associated polymers, the complex Gram-negative envelope was relegated to the back bench for lack of suitable methods to dissect and characterize this multi-layered structure – but not for long! The early seventies witnessed a new and vigorous onslaught with the development of techniques to separate inner (cytoplasmic) membranes from outer membranes (Schnaitman, 1970; Osborn *et al.*, 1972). The pursuit of the outer membrane has been so energetic and productive that it has resulted in an abundance of reviews and a comprehensive monograph devoted solely to the outer membrane (Inouye, 1979). In contrast to the cell walls of Gram-positive organisms, the outer membranes of Gram-negative bacteria appear to play a more direct and vital role in the physiological and biochemical well-being of these organisms, being the site of specific receptors, pores, and transport and barrier systems.

The fact that the cell walls of Gram-positive organisms, at least as isolated for chemical and immunological characterization, were enzymically inactive, coupled with the ability of wall-less protoplasts to perform most of the biochemical functions of the intact cell (Weibull, 1953; McQuillen, 1960), including wall regeneration (Landman *et al.*, 1968; Elliott *et al.*, 1975), served to focus attention on the membrane as the site of many biochemical events. This, together with the absence of eukaryotic membranous organelles (e.g. mitochondria, endoplasmic reticulum, Golgi apparatus and lysosomes) suggested that the plasma (cytoplasmic) membrane, as the principal membrane system of the bacterial cell (apart from the mysterious mesosomes), would have to perform a multiplicity of functions including active transport, secretory, mitochondrial and biosynthetic functions. With all these functions packed into a single membrane system, the bacterial membrane therefore appeared to be an object worthy of closer scrutiny and for these diverse reasons we turned our attention to the membrane. At that time (in the early 1960s) the membrane system of *Micrococcus lysodeikticus* offered many attractions, with its wall so sensitive to lysozyme and the accessibility of its membrane to isolation, quantitative recovery and characterization (Salton & Freer, 1965). Moreover, the ability to form stable protoplasts by dissolution of the cell wall in osmotically suitable media proved to be a distinct advantage for studies of the asymmetry of the antigenic architecture of this bacterial membrane.

Bacterial plasma membranes

As in all cells the plasma membrane of the bacterial cell constitutes the boundary of the cytoplasmic compartment and possesses the characteristic profile seen in thin sections viewed in the electron microscope. Moreover, the principal fracture face seen on freeze-fracturing whole

bacterial cells is that of the plasma membrane and it exhibits a similar distribution of particles and pits on the two faces to that seen in other membranes (Salton & Owen, 1976). With the exception of the mycoplasmas (Razin, 1967), the plasma membrane of the bacterial cell forms the boundary between the cytoplasm and an external compartment or structure, the cell wall in Gram-positive bacteria, or the peripheral compartment (periplasmic region, peptidoglycan layer and outer membrane) of the Gram-negative cell envelope. Isolated plasma membranes of Gram-positive organisms and the corresponding 'inner' (plasma) membranes of Gram-negative bacteria are generally indistinguishable when seen in negatively stained preparations in the electron microscope. Both have an abundance of small, uniform 10 nm particles identified as the F_1 -ATPases and these membranes bear a striking resemblance to the inner mitochondrial membranes of eukaryotic cells. The outer membranes of Gram-negative bacteria are distinctly different in appearance and of course lack the F_1 -ATPase particles (Salton, 1978).

Ultrastructural studies have established the identity of the F_1 -ATPase particles by ferritin labelling with antibody conjugates specific for the purified ATPase of *M. lysodeikticus* (Oppenheim & Salton, 1973) and, moreover, demonstrated the membrane asymmetry, with the F_1 -ATPase localized on the protoplasmic, inner face of the plasma membrane. This site for the F_1 -ATPase on the protoplasmic side of the membrane was in accord with the function of the ATPase in Mitchell's (1968) chemiosmotic hypothesis and its topographical location on the membrane was further confirmed by immunochemical studies (Owen & Salton, 1977) and extended to the *Escherichia coli* membrane vesicle system by Owen & Kaback (1979).

Antigenic and biochemical architecture of Micrococcus lysodeikticus plasma membranes

Bacterial membranes are multifunctional structures performing a variety of biochemical functions and in this respect the plasma membranes of *M. lysodeikticus* are no exception. The respiratory chain components of this organism have been studied in detail by Gel'man *et al.* (1975) and Tikhonova (1974), and the F_1 -ATPase by Muñoz (1982) and Salton (1980). In addition, enzymes involved in the biosynthesis of cardiolipin and other membrane phospholipids, and the biosynthesis of lipomannan, peptidoglycan and teichuronic acid have been investigated in a number of laboratories (see review by Salton, 1980). The task of determining the topographical location of such enzymes would be a formidable undertaking. Although the ferritin-labelled antibody method provides an elegant way of determining the identity and site of specific membrane components such as the F_1 -ATPase, it requires the purification of the particular enzyme, the generation of monospecific antibody and the preparation of reactive ferritin-antibody conjugates (Oppenheim & Salton, 1973). The development of the high-resolution two-dimensional technique of crossed immunoelectrophoresis by Clarke & Freeman (1967), following Laurell's (1965) rocket method, has offered a new opportunity to probe the antigenic complexity and the asymmetry of the bacterial membrane by combining conventional antibody absorption techniques with immunoelectrophoretic analysis of complex mixtures of antigens such as those anticipated in bacterial membranes. This method has the added advantage of adaptability to the identification of immunoprecipitates as specific enzymes by zymogram staining procedures (Uriel, 1971; Owen & Smyth, 1977). Early attempts to resolve the complex mixtures of antigens in bacterial membranes by conventional agar gel-diffusion (Ouchterlony) or immunoelectrophoresis techniques had been disappointing, giving poor resolution and relatively small numbers of immunoprecipitate lines (Freimer, 1963; Fukui *et al.*, 1971; Kahane & Razin, 1969). The resolution of sonicated or detergent 'solubilized' membranes into only several immunoprecipitate lines (Fukui *et al.*, 1971) was particularly surprising considering the great variety of enzyme functions performed by bacterial membranes and the large number of polypeptides (generally ranging from about 50 to over 100) seen in the SDS-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of dissociated membranes. Accordingly, we turned to the immunochemical analysis of the membranes of *M. lysodeikticus* by the crossed immunoelectrophoresis method in order to probe the antigenic and, where possible, the biochemical architecture of this membrane system.

The suitability of crossed immunoelectrophoresis for studying membrane problems became evident from the studies of Johansson & Hjerten (1974) with Tween 20 soluble membrane

proteins of *Acholeplasma laidlawii* and Bjerrum's (1975) investigations of mammalian membrane proteins. The high molecular mass exclusion limits of agarose gels (approx. 10^8 daltons) and their compatibility with a variety of surface-active agents makes them ideal supporting media for studying detergent-solubilized membranes. Non-ionic detergents such as Triton X-100 minimize denaturation and generally optimize the solubilization of bulk membrane protein and a variety of membrane enzymes (Collins & Salton, 1979), two features important for the immunochemical analysis of membrane structures. With Triton X-100-soluble antigens of *M. lysodeikticus* plasma membranes, Owen & Salton (1975) were able to detect 17 distinct antigens by two-dimensional immunoelectrophoresis, using anti-membrane antibodies to form the immunoprecipitates in the second dimension of electrophoresis. Moreover five of the antigens were identified as specific enzymes, including two antigenically distinct NADH dehydrogenases, malate and succinate dehydrogenases and the F_0 - F_1 -ATPase complex. The fact that none of the immunoprecipitates stained for more than one enzyme activity argued strongly in favour of the detection of individual enzyme species and the absence of random multienzyme aggregates in the solubilized membrane preparations. In addition to the five enzymically active antigens, one other major membrane antigen was identified as the succinylated lipomannan. Because of the quantitative nature of antigen loading and peak heights or areas of immunoprecipitates in the crossed immunoelectrophoresis method, the lipomannan could be specifically identified by coelectrophoresis of the Triton X-100-solubilized membrane with purified lipomannan and quantified by affinoelectrophoresis (Owen & Salton, 1975, 1976). Thus, by combining rocket immunoelectrophoresis with zymogram staining, molecular species such as the various dehydrogenases and ATPase could be determined quantitatively in membrane extracts (Collins & Salton, 1979).

In addition to the electrophoretic analysis of membrane components with antibodies, the use of carbohydrate-specific lectins such as concanavalin A (Con A) either in the intermediate gel variation of the crossed immunoelectrophoresis method or in direct affinoelectrophoresis (Axelsen *et al.*, 1973) has permitted the identification of the lipomannan and several other antigens possessing reactive carbohydrate residues (Owen & Salton, 1975, 1976, 1977).

As pointed out by Axelsen *et al.* (1973), to obtain the maximum resolution of antigens in complex populations it is necessary to establish the immunoprecipitate patterns at both high and low antigen loadings and for different periods of electrophoretic separation of the antigens in the first dimension. The expanded analysis of the *M. lysodeikticus* membrane antigens resulted in the detection of an additional 10–11 immunoprecipitates, bringing the total number of distinct antigens to 27 (Owen & Salton, 1977). Although this number of antigens in a membrane performing so many diverse functions still appears to be rather lower than what one might expect, several factors have to be kept in mind. Limitations to the ability to detect the full array of antigens in a membrane may arise from weak antigenicity of some components and the need for prolonged immunization to reach high enough antibody titres for detection in this system. In addition, responses of the animal species being immunized and the individual responses of a given species to immunogens may all contribute to the failure to detect some antigens. It has also been suggested that some antigens may be deeply buried in the membrane and may, therefore, not be expressed under the usual conditions of immunization. Evidence supporting or against this possibility is scant, although in our studies with Triton X-100-solubilized membranes as immunogen we have observed immunoprecipitate patterns very similar to those found for whole membranes with no new prominent precipitates detectable (Fig. 2; C. Urban & M. R. J. Salton, unpublished results). One further problem relating to the limitation of the antigenic analysis by this procedure involves the extent of solubilization of membrane components and determination of just what fraction of the membrane is being recognized by antibodies in the immunoprecipitate patterns. Owen (1981) has suggested a figure of about 70% based on labelling of the membrane proteins. On the positive side of the balance, it is conceivable that the variety of proteins may be rather more limited in some membranes, e.g. the F_1 -ATPase of the *M. lysodeikticus* membrane accounts for about 10% of the total protein (Muñoz *et al.*, 1969). Despite all these limitations, the antigenic analysis of the membranes by these procedures has expanded our understanding of the complexity of the bacterial membrane and has given us the

Fig. 2. A comparison of the two-dimensional crossed immunoelectrophoretic patterns of immunoprecipitates obtained with Triton X-100 (4%) extracts of *Micrococcus lysodeikticus* membranes electrophoresed into antibodies generated to isolated membrane preparations in (a), and antibodies produced to Triton X-100-solubilized membranes as immunogen in (b). Note that no new major immunoprecipitates appear as a result of immunization with Triton X-100-solubilized membranes. Each plate contained 200 μ l of the respective antiserum and 45 μ g membrane protein (as Triton X-100 'solution') in each well for electrophoretic separation of antigens in the first dimension. The conditions of electrophoresis and staining were as described by Owen & Salton (1977).

opportunity of examining the asymmetry of the membrane structure in terms of identifiable molecular species.

Membrane asymmetry

From the early proposals of the bilayer model for biological membranes (Danielli & Davson, 1935) the possibility that these structures possessed asymmetry or 'sidedness' was recognized. The outer faces of the surface membranes of many eukaryotic cells have a variety of carbohydrate-rich components such as glycoproteins and macroglycolipids (Brady & Fishman, 1975; Marchesi, 1975; Gardas, 1976; Dejter-Juszynski *et al.*, 1978) and it is generally believed that they are not exposed at all on the inner or protoplasmic face of the membrane. Thus the occurrence of glycoproteins on the outer surface of mammalian cell membranes has suggested yet another characteristic feature of eukaryotic cells, and such components provide the cell surface with sugar-mediated receptor and recognition sites and a hydrophilic interface with the external environment or adjacent cells. The outer surfaces of both Gram-positive and Gram-negative bacteria are also rich in carbohydrate residues in the wall peptidoglycan-polysaccharide complexes of the former and the O-antigen polysaccharide chains of the lipopolysaccharides in the outer membranes of the latter. However, until recently there has been very little evidence indicating the nature of the outer face components of the protoplast (plasma) membranes of Gram-positive organisms or that of the less accessible inner (plasma) membranes of Gram-negative spheroplasts. Moreover, the occurrence of glycoproteins in prokaryotes, with a few possible exceptions, has been largely open to question. Earlier studies by Sleytr (1975) have provided evidence for the glycoprotein nature of the regular arrays of surface subunits of the fine-structured, outermost layer of two *Clostridium* spp. and recent investigations in Owen's laboratory (Doherty *et al.*, 1982) have demonstrated mannosylation of a limited number of *M. lysodeikticus* membrane proteins. So far, there is no evidence to suggest that either these putative glycoproteins or that of the *Halobacterium salinarum* envelope (Mescher & Strominger, 1976) possess the mannose-*N*-acetylglucosamine type of carbohydrate structure common to the majority of eukaryotic glycoproteins. The suggestion that the F_1 -ATPases of *M. lysodeikticus*, *E. coli* and chloroplasts are glycoproteins (Andreu *et al.*, 1976, 1978) has been questioned and will be discussed in a subsequent section of this presentation.

The ability to form stable protoplasts of a Gram-positive organism such as *M. lysodeikticus*

has provided an opportunity to probe the asymmetry of its membrane. Dissolution of the rigid cell-wall structure with lysozyme when performed in a medium containing a suitable osmotic stabilizer (e.g. sucrose) exposes the outer surface of the plasma or protoplast membrane and makes it directly accessible for reaction with antibodies, antibody–ferritin conjugates and other surface ‘markers’. Thus, by using ferritin-labelled antibody specific for the membrane F_1 -ATPase, no surface exposure of the F_1 could be detected when the conjugate was reacted with intact protoplasts of *M. lysodeikticus* (Oppenheim & Salton, 1973). Localization of wall and membrane lipoteichoic acids of lactobacilli had also been achieved with the ferritin–antibody labelling procedure (Wicken & Knox, 1975). With the accessibility of the membrane to reaction with antibodies, an alternative procedure for determining the location of cell-surface antigens, albeit not directly visualized by electron microscopy, became apparent through the combined use of conventional antibody absorption and analysis by crossed immunoelectrophoresis. Although antibody absorption studies have been the backbone of serology in the past, the resolution of the array of antigens involved has often been difficult and far from simple, requiring many cross-absorptions. With the resolution of the complex mixture of membrane antigens in the Triton X-100-solubilized membranes of *M. lysodeikticus* by the two-dimensional crossed immunoelectrophoresis system, it became a relatively simple task to determine by absorption studies which specific antigens were exposed on the outer surface of the intact protoplasts. Only those antibodies reacting with surface-exposed antigens on the protoplast surface would be removed from the anti-membrane antiserum and this would be reflected in the disappearance of these immunoprecipitates from the reference patterns established by crossed immunoelectrophoretic analysis of absorbed and unabsorbed antisera.

Thus Owen & Salton (1975, 1977) were able to establish that 12 of the 27 discrete antigens detectable in the membranes of *M. lysodeikticus* were exposed on the protoplast surface. One of the major antigens of the outer face of the membrane was identified as the membrane amphiphile, succinylated lipomannan. At least five of the 12 antigens detected on the surface reacted with Con A in affinoelectrophoretic studies. Five of the major membrane antigens identified by zymogram staining as two antigenically distinct NADH dehydrogenases, succinate and malate dehydrogenases and ATPase, were not detectable on the outer surface, since the peaks of these immunoprecipitates in the crossed immunoelectrophoretic analysis were unaffected by extensive absorption with protoplasts. As anticipated from our earlier studies, the ATPase was only accessible to antibodies when the inner face of the membrane was exposed. However, when isolated plasma membranes with both faces (outer and protoplasmic sides) exposed were absorbed against anti-membrane immunoglobulins, antibodies to the five identifiable enzymes, and indeed to all other antigens, were completely absorbed (Owen & Salton, 1977). Thus all antigens detectable in the Triton X-100-solubilized membranes were fully expressed on the surfaces of the isolated membranes. By using ^{59}Fe -labelled membranes and autoradiography of the immunoprecipitates in the crossed immunoelectrophoretic patterns of *M. lysodeikticus* (*M. luteus*) membranes, Crowe & Owen (1983) have detected at least nine antigens possessing bound iron, thus extending the total number of its membrane antigens to 32. One of the antigens previously found to be expressed on the outer face of the membrane (Owen & Salton, 1975) is believed to be a haem-containing ferroantigen (Crowe & Owen, 1983). It should also be recalled that Tikhonova *et al.* (1978) have also obtained evidence for a redox component(s) of the respiratory chain of *M. lysodeikticus* on the outer surface of the membrane, thus supporting a transmembrane organization of the chain.

Although the crossed immunoelectrophoresis–absorption studies of this and other bacterial membranes have been valuable in identification of surface antigens, there has been a paucity of information on the nature, functions and number of components of the outer layer of the membrane. As an approach to this problem Dr D. N. Ostrovsky and colleagues of the A. N. Bakh Institute of Biochemistry, Academy of Sciences of the U.S.S.R., Moscow, have studied proteolysis of *M. lysodeikticus* membranes to probe the arrangement of proteins in the outer layer (Simakova & Ostrovsky, 1980). In a collaborative study we have used immunochemical methods with labelling of the membranes by ^{14}C -labelled amino acids or $[^3\text{S}]\text{methionine}$ in an effort to gain further information on the nature and quantity of the outer layer membrane

proteins. The basic strategy used in these experiments was to react and saturate labelled protoplasts with anti-membrane antibodies, remove unreacted antibodies by washing, isolate the membranes and solubilize them in Triton X-100 and sequester the antigen-antibody complexes on *Staphylococcus aureus*-protein A (Pansorbin) or by binding to goat anti-rabbit gamma globulins (Ostrovsky *et al.*, 1981). The results of these experiments indicated that the antigens of the external layer or leaflet of the membrane accounted for about 5% of the total (labelled) membrane protein and consisted of five proteins having molecular weights of approximately 120000, 115000, 100000, 68000 and 16000, as determined by SDS-PAGE (Ostrovsky *et al.*, 1981). Two of the surface antigens were modified when protoplasts were exposed to trypsin and one of the two reacted with Con A. Of particular interest was the finding that the external antigens disappeared from the membranes when protoplasts were treated with snail gut enzymes (containing carbohydrases, phospholipases but no proteases – Holden & Tracey, 1950) as well as after exposure of growing *M. lysodeikticus* to the antibiotic cerulenin (100 µg ml⁻¹). All five antigens (16000–120000 mol. wt) disappeared from the SDS-PAGE profiles of membranes isolated from the cells exposed for about a mean generation time to cerulenin (Ostrovsky *et al.*, 1981). These results suggest that phospholipid degradation by the snail enzymes, or inhibition of membrane lipid biosynthesis with cerulenin, destabilizes the outer leaflet of the membrane with consequent shedding of external antigens. One other observation emerging from these studies and worthy of mention was the detection of lysozyme tightly bound to the membrane preparations. However, despite its presence in the membrane fractions it was not detectable as a 'membrane' antigen and appeared to affect the immunoprecipitate patterns only when added in large excess (Ostrovsky *et al.*, 1981). The possibility exists that the highly basic lysozyme firmly bound to the membrane could contribute to the stability of both protoplasts and isolated membranes, but this has never been critically determined.

The results of these various studies have been summarized in Table 1 and illustrate the asymmetric expression of the *M. lysodeikticus* membrane antigens (and enzymes) on outer and inner faces of the plasma membrane. It is perhaps significant to note that the outer face of this bacterial membrane is rich in components containing carbohydrate residues (lipomannan and other Con A-reactive antigens) and in this respect the membranes share a common feature with eukaryotic cell membranes.

The elegant studies of P. Owen & H. R. Kaback and their colleagues have contributed much to our knowledge of the antigenic architecture of the *E. coli* plasma membrane (see review by Owen, 1981). Moreover, their investigations have clearly established the right-side-out orientation for at least 95% of the vesicle population and that a minimum of dislocation or translocation of enzymes occurs on conversion to what are essentially sealed vesicles. Similar properties of asymmetry of this Gram-negative plasma (inner) membrane are evident, in that many of the dehydrogenases and ATPase are largely accessible to antibodies only when the vesicles are disrupted. Unlike the *M. lysodeikticus* isolated plasma membranes, which showed little evidence of contamination with cytoplasmic components, the *E. coli* vesicles, perhaps not surprisingly, exhibited entrapment of many cytoplasmic antigens.

One of the major remaining problems in determining the functional asymmetry of these

Table 1. *Asymmetric expression of M. lysodeikticus membrane antigens on the outer and protoplasmic faces of the plasma membrane*

Outer face	Inner (protoplasmic) face
Twelve antigens including lipomannan and five Con A reactive	F ₁ -ATPase
Five antigens detected in ¹⁴ C- or [³⁵ S]methionine-labelled membranes, mol. wt 16000–120000; two trypsin-sensitive	Two antigenically distinct NADH dehydrogenases
Redox (transmembrane?) component	Malate dehydrogenase
Haem-containing ferro-antigen	Succinate dehydrogenase

bacterial plasma membrane systems is the identification of transmembrane components with determinants (similar or dissimilar) expressed on both faces of the membrane as well as transmembrane complexes of multimeric proteins (e.g. F_0 segment of F_0 - F_1 -ATPase proton-translocating complex) with different subunit determinants exposed. The crossed immunoelectrophoresis-absorption method as applied to protoplasts and isolated plasma membranes in our studies could not be used for the direct identification of transmembrane components. It could, however, be used if homogeneous populations of sealed inside-out vesicles were available. Efforts to prepare vesicles of *M. lysodeikticus* meeting these stringent requirements for unambiguous absorption studies have not been successful, although it should be noted that Wientjes *et al.* (1979) have obtained excellent yields of inside-out vesicles of *Bacillus licheniformis*. However, alternative methods involving chemical and/or enzymic modification of membrane proteins (Carraway, 1975) could be used to probe their transmembrane organization and the results of such studies could now be interpreted in a more meaningful way in the light of the identification of the five outer layer, 16000–120000 mol. wt antigens of *M. lysodeikticus* membranes (Ostrovsky *et al.*, 1981). The recent separation of the F_0 - F_1 -ATPase complex of *M. lysodeikticus* membranes by Dr Young Sook Chung in our laboratory provides an opportunity of generating antibodies to the F_0 polypeptides and determining their exposure on the outer face and whether any of the F_0 subunits are organized in a transmembrane orientation.

Immunochemistry of the M. lysodeikticus F₁-ATPase

The universal importance of the F_1 -ATPases coupled to the membrane-embedded F_0 segment of the complex was recognized with the acceptance of the chemiosmotic hypothesis of Mitchell (1968) whereby the F_1 -ATPase can synthesize ATP against an electrochemical proton gradient or hydrolyse ATP to energize the membrane. The F_1 portions of the coupled complexes can be readily detached and they have been purified from mitochondria, chloroplasts and membranes from a wide variety of bacteria lacking these organelles (Downie *et al.*, 1979). With the recently reported exception of the *Lactobacillus casei* F_1 -like ATPase possessing six similar subunits of molecular weight 43000 (Biketov *et al.*, 1982), the purified F_1 -ATPases from all other sources are remarkably similar in that they possess five distinct subunits, the major α and β subunits, together with the γ , δ and ϵ subunits and their subunit molecular weights are very similar ranging from about 60000 to 10000 (Downie *et al.*, 1979).

In accord with the functional role of the F_1 -ATPase, it has been localized on the protoplasmic face of the bacterial plasma membrane (Oppenheim & Salton, 1973). Earlier investigations in our laboratory had shown that the purified F_1 -ATPase was a good immunogen and that antibodies inhibited its hydrolytic activity in a non-competitive manner (Whiteside & Salton, 1970). Moreover, antibodies to the *M. lysodeikticus* F_1 -ATPase were shown to cross-react and inhibit ATPase activity of related species and other bacterial species in the high GC (guanosine + cytosine) percentage range (Whiteside *et al.*, 1971). However, at that time there was no knowledge of the antigenic uniqueness of the subunits or the subunit-specificity of the antiserum. The development of the high-resolution technique of crossed immunoelectrophoresis and its associated intermediate gel and affinoelectrophoresis methods provided sensitive immunochemical approaches for the further characterization of the *M. lysodeikticus* F_1 -ATPase. Specifically, we wished to determine (1) whether, as suggested by Andreu *et al.* (1978), the F_1 -ATPase is a glycoprotein; (2) whether the individual subunits are antigenically unique; (3) the effects of subunit-specific antibodies on the hydrolytic activity of the F_1 -ATPase; (4) whether antibodies to native and denatured F_1 -molecules react only with topographical and unfolded polypeptide chain determinants respectively; and finally, (5) to what extent subunit determinants are conserved to give rise to cross-species and inter-generic immunological cross-reactions.

The suggestion that the F_1 -ATPase of *M. lysodeikticus* and indeed other F_1 -ATPases are glycosylated (Andreu *et al.*, 1976, 1978) was in conflict with the widely held view that glycoproteins, glycolipids and lipopolysaccharides have their hydrophilic carbohydrate domains orientated to the external face of biological membranes (Bretscher & Raff, 1975; Rothman & Lenard, 1977). Since we have shown that the F_1 -ATPase of *M. lysodeikticus* membranes

occupies an internal orientation towards the cytosol compartment of the cell, the suggestion that it may be a glycoprotein raised some basic questions about membrane asymmetry and architecture. We have accordingly examined this possibility using immunochemical and analytical procedures to determine whether there is good evidence for the covalent linkage of carbohydrate residues to the F_1 or any of its subunits. Even after extensive purification of *M. lysodeikticus* F_1 -ATPase to protein homogeneity by DEAE-Sephadex A25 chromatography (Huberman & Salton, 1979) carbohydrate contents varying from 2.7 to 10.8% were found (Lim & Salton, 1981). Con A-reactive components corresponding to the succinylated lipomannan were detected and separated from the ATPase in purified F_1 preparations by immunoelectrophoresis through agarose gels containing Con A. When the purified F_1 -ATPase was chromatographed on a column of Con A-Sepharose 4B, the carbohydrate components were removed without loss of the specific activity of the ATPase. The only sugar detectable by gas-liquid chromatography of the DEAE-Sephadex A25 purified F_1 -ATPase was mannose, but after chromatography on the Con A-Sepharose 4B column it was completely eliminated and no sugars could be detected by gas-liquid chromatography. No qualitative or quantitative changes in the subunit (α , β , γ , δ and ϵ) profiles were detectable when the SDS-PAGE gels were scanned by densitometry of the F_1 -ATPase before and after Con A-Sepharose 4B chromatography. We therefore concluded that there is no evidence of carbohydrate covalently linked to this F_1 -ATPase and that this membrane protein is not a glycoprotein (Lim & Salton, 1981). A similar conclusion that the F_1 -ATPases are not glycoproteins had also been reached by Nalin *et al.* (1979) on the basis of less direct analytical evidence with Schiff (PAS) staining of SDS gel profiles. The presence of carbohydrate in our F_1 -ATPase preparations was attributable to contamination with lipomannan, a membrane amphiphile which probably binds non-specifically to proteins and co-separates with the F_1 (Lim & Salton, 1981).

Antibodies have been invaluable as probes to study structural and functional properties of many proteins, especially multimeric proteins. Surprisingly, there appear to be very few instances where antibodies have been generated to all five F_1 subunits, the majority of the studies generally concentrating on antibodies to the α and β subunits. Smith & Sternweis (1982) have recently prepared antibodies to all five subunits of *E. coli* F_1 -ATPase but the α -antiserum reacted only with the denatured α chain. In order to evaluate reactivity with the native F_1 molecule and the antigenic uniqueness of the five subunits, we have generated antibodies to individual subunits of *M. lysodeikticus* F_1 excised from SDS-PAGE gels. As illustrated in the diagrammatic composite of immunoprecipitates of F_1 subunits in Fig. 3, all the subunits are both immunogenic and antigenically unique (Urban & Salton, 1983*a*). Moreover, we have confirmed that the subunit antibodies are monospecific by a variety of immunochemical

Fig. 3. The five distinct immunoprecipitates seen when SDS-dissociated F_1 -ATPase subunits are electrophoresed into their homologous subunit antisera are illustrated diagrammatically in this composite pattern. The anodal direction of electrophoresis is to the left in the first dimension and to the top in the second dimension. (Urban & Salton, 1983*a*; reproduced with permission of *Biochimica et biophysica acta*.)

methods and established that all SDS-dissociated subunit antibodies react with the native, purified F_1 -ATPase. In the course of examining the reactions of subunit-specific antibodies with the native F_1 by using the sensitive intermediate gel variant of the crossed immunoelectrophoresis method, we observed the formation of distinct immunoprecipitates in the intermediate gel zones containing either anti- ϵ and anti- δ antibodies (Fig. 4) in addition to the residual F_1 immunoprecipitate formed in the reference antibodies (anti-membrane, anti-purified F_1 or anti- $\alpha + \beta$ antibodies). This rather unexpected result suggested to us that interaction of δ or ϵ subunits with their respective antibodies destabilizes their association with the F_1 and results in the detachment of these subunits. The sequential detachment of δ and ϵ subunits, leaving an enzymically active α, β, γ -ATPase (by zymogram staining) was confirmed by subunit analysis of immunoprecipitates of F_1 labelled metabolically with ^{14}C -labelled algal protein hydrolysate or [^{35}S]methionine. Indeed, destabilization of subunit associations in multimeric proteins reacted with subunit-specific antibodies had been proposed by Bøg-Hansen *et al.* (1974) and our results have provided confirmation for this suggestion (Urban & Salton, 1983*a*). In addition, it will be recalled that Sternweis (1978) used anti- ϵ antibodies coupled to Sepharose to remove ϵ subunits to yield an α, β, γ complex from a δ -deficient F_1 -ATPase of an *E. coli* mutant. The recent demonstration by Boulain & Menez (1982) that specific antibody binding destabilized the neurotoxin-acetylcholine receptor complex provides further support to the suggestion that antibody may destabilize and detach certain subunits of multimeric proteins. The approaches we have used with the *M. lysodeikticus* F_1 may provide a new and novel way of studying complex multimeric proteins of biological interest and help to define the functions of specific subunits.

When tested for inhibitory activity against the ATPase, as shown in Table 2, all subunit-specific antisera were inhibitory (Urban & Salton, 1983*a*). Perhaps not surprisingly, the strongest inhibition of ATPase activity was seen with anti- F_1 , anti- α and anti- β antibodies. Since the present evidence suggests that the β subunits possess the catalytic sites, strong inhibition by antisera containing antibodies to β is readily understandable. Although inhibitory to a lesser extent, it is conceivable that interaction with the γ -, δ - and ϵ -specific antibodies may induce subtle conformational changes in the molecule and thereby modulate hydrolytic activity. Our results are in agreement with earlier studies showing inhibitory effects of anti- γ antiserum (Nelson *et al.*, 1973; Kanner *et al.*, 1975) and the recent study of Smith & Sternweis (1982) showing inhibition of *E. coli* ATPase activity with anti- β and anti- γ antibodies.

In studying the antigenic properties of this bacterial membrane F_1 and its subunits, it has been of interest to determine the types of domains being recognized by antibodies. Present models for protein antigens indicate that there are topographical regions or surface domains and that their antigenicity is dependent on the types of amino acids and their molecular conformations (Crumpton, 1974; Lerner, 1982). Recent evidence from the work of Lando *et al.*

Fig. 4. Electrophoresis of F_1 -ATPase sequentially through intermediate gels containing anti- ϵ subunit, anti- δ subunit antibodies and then into anti- $\alpha + \beta$ antibodies results in the detachment of ϵ and δ subunits and formation of respective ϵ and δ subunit immunoprecipitates and the heavy immunoprecipitate of the residual F_1 complex with anti- $\alpha + \beta$ antiserum (left hand slide, Coomassie blue staining). Zymogram staining (right-hand slide) shows that only the residual F_1 complex (α, β, γ subunits) immunoprecipitate retains ATPase activity. Data from Urban & Salton (1983*a*). (Reproduced with permission of *Biochimica et biophysica acta*.)

Table 2. Inhibition of *M. lysodeikticus* F_1 -ATPase activity by various subunit, purified F_1 -ATPase and membrane antibodies

The percentage inhibition values represent the means of a minimum of three determinations by each antibody used against 2–6 μ g purified F_1 -ATPase. Because of the latent character of this F_1 , antibody inhibition assays were performed in the presence and absence of trypsin, which unmasks the latent activity.

This table is modified from Urban & Salton (1983*a*).

Antibody	Percentage inhibition	
	+ Trypsin	– Trypsin
Anti- α/β	93	93
Anti- α	62	62
Anti- β	64	64
Anti- α + Anti- β	85	75
Anti- γ	37	38
Anti- δ	41	47
Anti- ϵ	40	53
Anti- <i>M. lysodeikticus</i> membrane	95	91
Anti-purified F_1 -ATPase	91	95
Anti-Triton X-100-solubilized membrane	96	96
Anti-SDS-denatured F_1 -ATPase	97	97

(1982) with sperm whale myoglobin has resolved two distinct antibody populations, one reacting with the native myoglobin and another reacting with peptide fragments obtained by cyanogen bromide cleavage. These studies and our investigations with the F_1 -ATPase support Sela's original suggestion (Sela *et al.*, 1967) that both sequential and topographical determinants can be expressed on a native protein molecule. By carrying out absorption studies we have found evidence for two distinct antibody populations to F_1 -ATPase, those reacting only with native F_1 and those reacting to the SDS-dissociated F_1 subunits. We have also shown that not only do antibodies generated to the individual unfolded subunits react with the native molecule but antibodies to the native F_1 react with SDS-denatured subunits and with cyanogen bromide fragments of the F_1 (Urban & Salton, 1983*b*). Thus the F_1 -ATPases may provide yet another biologically important protein model for the exploration of antigenic and functional domains.

The final aspect of our work on the immunochemistry of F_1 -ATPases relates to their cross-reactivity. Although our earlier work based on ATPase inhibition and precipitate formation by gel diffusion indicated cross-reactivity of *M. lysodeikticus* F_1 -ATPase antibodies with other bacterial F_1 -ATPases, the greatest cross-reactivities were largely confined to those organisms in the high GC percentage range (Whiteside *et al.*, 1971). At that time we had little knowledge of the determinants being recognized by our antisera. Recent evidence has focused more and more attention on the β subunits of the F_1 -ATPases and cross-reactions have been observed between β -subunits of *E. coli*, yeast and rat liver mitochondrial F_1 -ATPase and Swiss chard chloroplast F_1 -ATPase (Rott & Nelson, 1981). These results suggest that of all the F_1 subunits, the catalytic β subunit may have been conserved to the greatest extent during the evolution of energy-transducing ATPases. Indeed, the recent discovery of the striking sequence homology between the β subunits of *E. coli* and bovine F_1 -ATPases and the human bladder carcinoma oncogene product, the p21 protein (Gay & Walker, 1983) opens an exciting new chapter in the study of the evolution and fundamental similarities of this important polypeptide. In this context, it will be most intriguing to discover whether the new F_1 -like ATPase of *L. casei* (Biketov *et al.*, 1982) possesses only the β subunit equivalents and whether they have extensive sequences of amino acid homologies with other β subunits in their polypeptide chains. Recent studies in our laboratory by Dr Carl Urban have also established, with the highly sensitive intermediate gel technique of crossed immunoelectrophoresis, cross-reactivities of F_1 -ATPases of widely separated bacterial species including *E. coli*, *Haemophilus influenzae* and *Neisseria gonorrhoeae* with one another and with the Gram-positive *M. lysodeikticus* F_1 , and evidence of cross-reactivity with beef-heart mitochondrial F_1 . Extension of these approaches to the other subunits

of the F_1 -ATPases may lead to a further understanding of evolutionary divergence of these universally occurring H^+ -ATPases.

In summary, the organism *Micrococcus lysodeikticus*, which we chose originally for detailed studies of wall composition, structure and degradation by lysozyme, proved to be an equally interesting object for the study of the structure–function relationships of its membrane. Its cell wall turned out to be one of the simplest of most Gram-positive bacteria, consisting essentially of 95% peptidoglycan and about 5% associated teichuronic acid (*N*-acetylmannosaminuronic acid–glucose polymer). The wall could be completely degraded by hen egg-white lysozyme into relatively small molecular weight products. The ability to selectively remove the wall with lysozyme permitted the formation of stable protoplasts and greatly facilitated both the isolation and characterization of the plasma membranes and study of its antigenic and biochemical asymmetry. The membrane of this strict aerobe is especially rich in the energy-transducing F_0 – F_1 -ATPase complex and the F_1 segment has proved to be a valuable antigenic and biochemical marker in determining the asymmetric distribution of membrane components and in studies of the antigenic properties of a complex multimeric protein. The arrangement of some of the molecular species in this typical prokaryotic plasma membrane is depicted diagrammatically in the model presented in Fig. 5.

Marjory Stephenson was born just two years after Christian Gram developed his Gram staining procedure in 1884. This divided the vast number of bacterial species and indeed many biochemists and microbiologists into two worlds, the true blue Gram-positives and the pink Gram-negatives. As one of the great and devoted biochemists, Marjory Stephenson's interests in matters biochemical spanned both worlds, having worked with both Gram-positive and Gram-negative bacteria. Some of the basic physiological and biochemical differences between the two Gram worlds had already emerged prior to her death. Had she still been alive today, almost 100 years since her birth and the introduction of the Gram stain, she would have shared our great enthusiasm for the role microbial biochemistry has played in unravelling the exciting molecular genetics of our present era and the complexities of these small, unicellular prokaryotic organisms and the striking biochemical differences and similarities of the Gram-positive and Gram-negative worlds.

Fig. 5. Diagrammatic representation of the asymmetry of *Micrococcus lysodeikticus* plasma membrane with the membrane-bound succinylated lipomannan (LM) and its negatively charged groups ($-\text{COOH}$) on the outer face of the membrane. The F_1 -ATPase segment of the F_0 – F_1 complex appears on the inner (protoplasmic) face and a transmembrane F_0 segment, together with other membrane dehydrogenases (e.g. malate, succinate, NADH), is anchored by lipid of the bilayer.

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