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Review Article

MEMBRANE-ACTIVE ANTIMICROBIAL DRUGS – A REAPPRAISAL OF THEIR MODE OF ACTION IN THE LIGHT OF THE CHEMIOSMOTIC THEORY

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The so-called membrane-active antibacterial agents, which include cationic detergents, chlorhexidine, phenols in low concentrations, anilides and alcohols, have as their characteristic mode of action the ability to promote the leakage of small molecular weight material from the cytoplasm of the microbial cell. Purines, pyrimidine, ribose, amino acids and potassium are the molecular entities usually detected and evaluated in this reaction, and the phenomenon is attributed to a change in the integrity of the cytoplasmic membrane. Kuhn and Bielig (1940) were the first to suggest the physical effect, and Hotchkiss (1944) the biochemical effect. For recent summaries of the leakage phenomenon see Hugo (1976a, b).

Two major discoveries have been made in the last 8 years which have thrown significant new light on both the structure and function of biological membranes.

Since 1935, the membrane structure proposed by Danielli and Davson had held sway; it proposed that biological membranes consisted of a bimolecular layer of phospholipids overlayed on either side by a layer of protein (Fig. 1a). Thermodynamic considerations of lipid–protein–water interactions and solubilities led to the view that such a structure was not feasible. Singer and Nicolson (1972) suggested that a more likely structure, which still satisfied the basic analytical composition of membranes but which was thermodynamically valid, was given by the original concept of the phospholipid bimolecular leaflet, but in which proteins were inbedded. These proteins might reside in one half of the leaflet or span the complete membrane (Fig. 1b).

The second discovery, and one which has a much greater bearing on the problem under review, concerns the solution to the phenomenon of energy coupling. For many years biochemists had sought to explain how metabolic activity could drive such processes as active transport and oxidative and substrate level phosphorylation.

Mitchell (1966) formulated what is now known as the chemiosmotic theory and much subsequent work has done much to substantiate it. In brief, this theory proposes that the electron transport chain, whether it be in bacteria, mitochondria or chloroplasts, is fixed in a definite direction in the membrane. The proteins shown in Fig. 1b are fixed in the membrane and as depicted could fulfill this role. As a consequence of this the protons generating during the oxidation of substrate, leading eventually to the reduction of

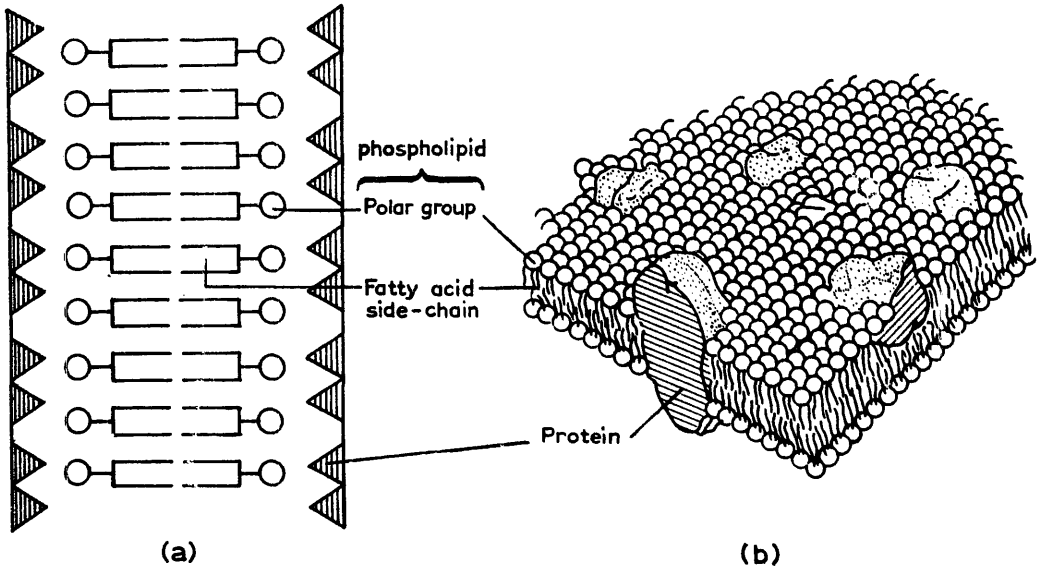

Fig. 1. a: Danielli Davson model. b: Singer model.

oxygen, would be transported from the interior to the exterior of the cell. In order to create a proton-motive force it is also a prerequisite that the membrane is impervious to the proton so that there is no immediate compensatory back flow. Such a situation would lead to acidification in the exterior of the cell and also a positive charge generation externally.

A proton-motive force may also be generated by the hydrolysis of adenosine triphosphate (ATP) by the enzyme adenosine triphosphatase (ATPase). This reaction explains the existence of a proton-motive force in anerobic bacteria and in facultative organisms metabolizing anerobically; in anerobic processes ATP is synthesized by what is known as substrate level phosphorylation (Decker et al., 1970). A diagram of the two proton-generating systems is shown in Fig. 2. Since the chemiosmotic hypothesis was formulated the weight of experimental evidence in its support is formidable and certainly outweighs evidence against it.

In order to quantify the extent of acidification, pH change, and change generation, the algebra of chemical thermodynamics was employed. The application of thermodynamic principles to non-equilibrium situations has been criticized in general terms by some biochemists, but Mitchell's treatment gives rise to numerical data which are supported by experimental findings.

In brief, the electrochemical potential of the proton, $\bar{\mu}_{H^+}$, is calculated, and is measured as the sum of two terms, one depending on the chemical concentration of H^+ and the other on the electrical charge difference contributed by the distribution of the positively charged proton across the cytoplasmic membrane.

Chemical potential is measured in Joules, but Joules can be converted to volts by dividing by the Faraday constant F. The factor $\bar{\mu}_{H^+}/F$ was called by Mitchell the proton-motive force denoted by p, and he showed that $\Delta p = \Delta\Psi - Z\Delta pH$ (all units electrical, millivolts). Z varies with temperature (pH is temperature-dependent) and has a value of

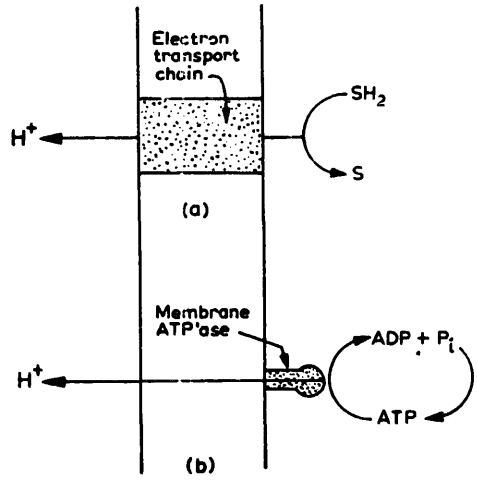

Fig. 2. Mitchells chemiosmotic theory. Generation of proton-motive force via (a) the electron transport chain and (b) hydrolysis of ATP.

61 at 37°C. For a full treatment of the theoretical derivation of this equation the publication of Mitchell (1968) should be consulted.

A recent review (Rottenberg, 1975) gives a very full account of the measurement of both ΔpH and $\Delta\Psi$.

In brief, ΔpH may be measured by a pH meter or by measuring the distribution of a weak acid, such as dimethyloxazolidinedione, aspirin or benzoic acid, across the membrane; $\Delta\Psi$ may be measured by the distribution of ions such as dibenzyldimethylammonium or triphenylmethylphosphonium by the application of the Nernst equation; ion distribution is measured spectroscopically. Since Rottenberg's review was published, Muratsugu et al. (1977) have described the construction of a dibenzyldimethylammonium electrode which enables the concentration of this ion, after distribution, to be measured directly.

It will be recalled that the proton-motive force ($\Delta\Psi - Z\Delta pH$) is said to be responsible for the synthesis of adenosine triphosphate as part of the process of oxidative phosphorylation.

It has been known almost from the beginning of this century that nitrophenols, especially 2,4-dinitrophenol (DNP), interfered with oxidative phosphorylation without inhibiting other metabolic processes. The name uncoupling agent was coined for DNP and later was applied to other compounds of like activity because they, like DNP, uncoupled oxidation from phosphorylation.

Mitchell (1961) showed that 2,4-dinitrophenol caused a back flow of protons across the bacterial and mitochrondrial membrane which could cause a partial or total collapse of the proton-motive force. It was thought that the molecular properties associated with the ability to promote uncoupling was that of a weak acid with lipid solubility. The molecule dissolved in the lipid bilayer of the membrane and acted as a proton-conductor by virtue of its ionizability. Since the discovery of this special property other substances, some of them used as antibacterial agents, have been found to act in a similar manner. Examples include Fentichlor (Hugo and Bloomfield, 1971; Bloomfield, 1974) and tetra-

chlorosalicylanilide (Hamilton, 1968). Harold and Papineau (1972) have shown that tetrachlorosalicylanilide will also discharge the Ψ component of the proton-motive force in *Streptococcus faecalis.*

Reviewing the above data it is possible to see a general pattern of behaviour. The nitrophenols short-circuit the membrane, as it were, causing a rapid back flow of protons into the cell and hence the collapse of Δp; they do not cause leakage of cellular constituents and are active at concentrations of 10^{-6} M.

Certain other phenolic compounds cause both leakage and collapse of Δp; it is often found that concentrations of 10^{-5} M cause Δp collapse whereas leakage is promoted by concentrations of the order of 10^{-4} M.

It seemed worthwhile to examine the effect of a membrane-active, non-phenolic substance such as a cationic detergent which possesses antibacterial activity and promotes leakage of cytoplasmic constituents. Such a compound is cetyltrimethylammonium bromide and Denyer and Hugo (1977) examined the effect of this extensively investigated compound on its ability to modify Δp.

Using *Staphylococcus aureus* it was shown that 18 μg/ml (5.3×10^{-5} M) cetrimide caused the discharge of the pH component of Δp; 18 μg/ml was also the bacteriostatic concentration and the concentration which caused the maximum leakage of material absorbing at 260 nm.

These results indicated that discharge of all or part of the proton-motive force was not the sole prerogative of uncoupling agents, and it became clear that this biochemical effect might be part of the general action of detergents.

From this it followed that studies on the mode of action of those antibacterial agents which show evidence of membrane activity by traditional techniques, i.e. leakage of cell constituents, must be extended to investigate whether components of the proton-motive force are modified. These experimental systems are more difficult to set up but to omit them and the results they may produce must always leave a doubt in the mind that the fundamental biochemical lesion has been missed.

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