In 1969 Azzi and co-workers concluded that the fluorescence changes of ANS which accompanied its reaction with mitochondria 'indicated structural changes of the mitochondrial membrane associated with energy conversion' (Azzi et al., 1969). Azzi (1969) reported a 20% decrease in the amount of dye bound after adding succinate; from this, coupled with the fact that a positively charged dye, auramine-O, showed increased binding on energization, he deduced that 'the interaction between dyes and the energized membrane is electrostatic' and that 'it is probably important in the mechanism of ATP synthesis that an asymmetrical charge distribution is associated with energy conservation in the mitochondrial membrane'. Nordenbrand & Ernster (1971) extended Azzi's studies, differentiating the non-energized and energized ANS binding and fluorescence changes by several different kinetic indices; they suggested that the energy-dependent reaction reflected 'a charge separation in a special locus ... distinct from non-energy-dependent binding sites', and that the energized state was to be considered as a 'heterogeneous concept ... rather than a homogeneous entity such as that defined by a bulk chemical or electrical potential across the mitochondrial membrane'.

It was, however, a more homogeneous view of the mitochondrial energy transduction mechanism, in the form of the chemiosmotic hypothesis, which gained the authority of a near consensus during the ensuing decade, a view that held no explicit function for the fixed-charge changes during respiratory activity is it right to assume that transmembrane potentials are at any time, even during permeant cation exchange, the primary mediating force in energy transduction. (ii) To show that an artificially energizing process has also been assumed by Kell (1979), which they were nonetheless used, or which were interpreted with a free inferential logic which left much to be desired. Archbold et al. (1975, 1979) attempted to draw attention to these remarkable lacunae in the arguments of a major hypothesis, and two examples will be mentioned here in general terms. (i) It is not correct to infer from the proton movements in the cation-exchange reactions of an oxygen-pulse experiment that bulk-phase transmembrane forces will be operating in synthesis; nor, indeed, in the light of the fixed-charge changes during respiratory activity is it right to assume that transmembrane potentials are at any time, even during permeant cation exchange, the primary mediating force in energy transduction. (ii) To show that an artificially imposed $\Delta \mu_\text{H}^+$ can lead to synthesis (e.g. Thayer & Hinkle, 1975) provides no proof of the in vivo mechanism of synthesis; such experiments require the use of respiratory-inhibited particles and are therefore carried out in the absence of the fixed-charge development which is intrinsic to normal oxidative phosphorylation; they demonstrate no more that the energy input for ATP synthesis may be presented successfully on an experimental basis in more than one form.

Two consequences may be expected to follow from an increase in the negative surface charge of the coupling membrane: an increase in the surface electrical field giving an enlargement of the diffuse double layer, and an increase in the concentration of cations retained within the diffuse double layer. The retentive force will be electrostatic and exerted chiefly on $\text{Ca}^{2+}$ and $\text{H}^+$. It is important to note that as soon as we cease to regard the protonic force as a generalized, dissipative bulk-phase property, and see it as localized and non-dissipative, the surface area involved in energy transduction may become a mere fraction of the total membrane.

Abbreviation used: ANS, 1-anilino-8-naphthalene-sulfonic acid.

Some implications of fixed-charge formation during electron-transport-chain activity

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surface area (Nordenbrand & Ernst, 1971; Archbold et al., 1976). The electrical activity will be concentrated at special foci related to the topographical arrangement of the electron-transport chains and not spread uniformly over the whole surface; the picture will be of a dam, with sluice-gates open at intervals.

The claim of the coulombic hypothesis (Malpress, 1981a,b, 1982) is that the primary mediating force in ATP synthesis is the electrostatic potential of protons ($H^+$) in these selective areas of the diffuse double layer (Fig. 1). It rests principally on four types of evidence: (i) the increase in the negative fixed-charge presence during respiratory chain activity (approx. 15% for site-1 and site-2 substrates; approx. 6% for site-3 substrates); (ii) the re-interpretation of the processes underlying oxygen-pulse experiments, consequent upon (i); (iii) a more critical evaluation of experiments hitherto regarded as supporting the chemiosmotic hypothesis; and (iv) the clarification which the hypothesis brings to a number of problems which have appeared in the literature and proved intractable to a chemiosmotic solution, for example the wide variation of $\Delta G_p : \Delta \mu_{H^+}$ ratios encountered under different experimental conditions.

The electrochemical forces of the chemiosmotic hypothesis are seen as 'phantom' parameters, completely non-existent until they are called into being by the very procedures designed for their measurement: permeant ion distributions, or spectrophotometric methods calibrated by diffusion potentials. The hidden conversion of $\Psi_{H^+}$ into $\Delta \mu_{H^+}$ which these measurements entail will be subject to losses characteristic of the system in use and may be represented by the form:

$$\Delta \mu_{H^+} = f \Psi_{H^+},$$

where $f$ is a conversion factor always less than 1. It is the resultant of all the forces acting upon a proton in the diffuse double layer when the conversion is taking place, for example in any 'rationale' for measuring $\Delta \Psi$ (Fig. 2). The heterogeneity of the fixed-charge distribution has so far precluded any estimate of the surface potential ($\Psi$) in the delineated areas of electron-transport chain activity and we shall assume that the mediating $\Psi_{H^+}$ values are commensurate with the needs of the phosphate potential. With this datum and the $\zeta$-potentials calculated from electrophoretic mobility studies (Archbold et al., 1980b; Malpress, 1981c) we can construct energy profiles for the diffuse double layer under both energized and non-energized conditions (Malpress, 1982). We may also calculate for any given permittivity, the distance between a surface fixed charge and a translocated proton that will determine a coulombic force equal to that fraction of the phosphate potential suited...
to any given H⁺:P ratio. If the ratio is 3 and the permittivity 20, the distance is approximately 0.3 nm. Unlike the electrochemical parameters, \( \psi_H \) values do not depend on a cumulative ion effect, one proton alone at the right distance from a fixed charge will be at the potential required for synthetic activity. On these terms the rate of synthesis is determined by the number of charge–charge interactions and is therefore closer to the views of Williams (1978).

The coulombic hypothesis is a ‘local energized proton’ interpretation of mitochondrial energy transduction, and is therefore closer to the views of Williams (1978) than to those of Mitchell (1979). But in many respects it may be radically differentiated from Williams’ prototype model, notably in its acceptance of the evidence for transmembrane proton pump activity as opposed to intermembrane proton release, in its explicit involvement of newly generated negative fixed-charges and of the diffuse double layer, by the electrostatic mechanism which it sees as the first step in the utilization of the energized protons in contrast to the kinetic ‘hydrating-dehydrating’ process of the prototype view (Fig. 3), and in its interpretation of Ca²⁺ movements.

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A structural basis for mosaic protonic energy coupling

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The chemiosmotic concept of Mitchell (1961) has proved to be of great predictive and practical value in the analysis of free-energy transduction. In order to make maximal use of the predictive value, we have incorporated this concept in a mosaic non-equilibrium description of oxidative phosphorylation (Westerhoff & Van Dam, 1982). This description relates the rates of different processes in mitochondrion (or bacteria) to their driving forces (free-energy differences), as well as to the activities and kinetic properties of the enzymes involved.

Some experimental tests of the mosaic non-equilibrium thermodynamic description gave results that fitted with a simple model of chemiosmotic energy transduction in mitochondria, with \( H^+ \) ions as the central coupling agent. For instance, the prediction that the dependence of the rate of phosphorylation on the rate of oxygen uptake should be linear and steeper in the presence of a protonophore than in its absence, could be verified (Van Dam et al., 1978).

However, some predicted relations did not stand up to experimental test. An important anomaly is the finding that in State 4, i.e. at the maximal degree of phosphorylation of the adenine nucleotides, the ratio \( \Delta G_p/\Delta H_p \) is not independent of \( \Delta H_p \) (Wischmann et al., 1975; Azzone et al., 1978, Westerhoff et al., 1981). The ratio is consistently found to increase at low values of \( \Delta H_p \). Such a finding cannot be explained by the presence of slips in the proton pumps, since this would actually lead to a decrease in the measured ratio.

A second anomaly is found when so-called double-inhibitor titrations are performed: under some conditions partial inhibition of the oxidative proton pump does not lead to the expected diminished sensitivity of phosphorylation to an inhibitor of the ATPase or vice versa (Baum et al., 1971; Hitchens & Kell, 1982; Venturoli & Melandri, 1982; Westerhoff et al., 1983). Respiratory chain and ATPase complexes behave as if they are functionally coupled in such a way that energy delivered by one complex is available to another complex only. In terms of the proton as energy-transducing agent, there is no equilibration throughout the bulks phases inside or outside the mitochondria.

A physical counterpart of the functional compartmentalization may be seen in the model of the mitochondrial cristae as proposed by Sjöstrand (1978). According to this model, the cristae membranes are closely apposed. We now specify that protons pumped to the (virtual) outside cannot escape, except to a neighbouring complex. We have obtained evidence that there is indeed a correlation between the “localized” behaviour of the protons and the putative dilation of the cristae, for instance by comparing mitochondria isolated in the presence or absence of a high molecular weight osmotic support, such as polyvinylpyrrolidone. The surprising conclusion that the localized protons are in contact with the external medium is schematically depicted in Fig. 1.

In order to combine these different aspects in a coherent picture of energy transduction, a new model was proposed: mosaic protonic coupling (Westerhoff et al., 1983; cf. H. V. Westerhoff, B. A. Melandri, G. Venturoli, G. F. Azzone & D. B. Kell, unpublished work, 1988). This model allows us to make a better quantitative description in terms of mosaic non-equilibrium thermodynamics. As an example we have calculated the expected variation of \( \Delta G_p/\Delta H_p \) with \( \Delta H_p \), when the proton permeability of the mitochondrial inner