Acid-Base Titration across the Membrane System of Rat-Liver Mitochondria

CATALYSIS BY UNCOUPLERS

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1. Pulsed acid-base titrations of suspensions of rat-liver mitochondria under anaerobic equilibrium conditions show fast and slow titration processes. 2. The fast process is the titration of the outer aqueous phase of the mitochondria, which is continuous with the suspension medium, and the slow process can be identified with the titration of the inner aqueous phase of the mitochondria, which is separated from the outer aqueous phase by the non-aqueous osmotic barrier or M phase of the cristae membrane system. 3. The buffering power of the outer and inner phases have been separately measured over a range of pH values. 4. The rate of titration of the inner aqueous phase under a known protonmotive force across the M phase has been characterized by an effective proton conductance coefficient, which, near pH 7 and at 25°, is only $0.45 \mu\text{mho}/\text{cm}^2$ of the M-phase membrane. 5. The low effective proton conductance of the M phase will account quantitatively for the observed respiratory control in state 4, assuming that oxidoreduction and phosphorylation are coupled by a circulating proton current as required by the chemiosmotic hypothesis. 6. The addition of 2,4-dinitrophenol (or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) at normal uncoupling concentrations causes a large increase in the effective proton conductance of the M phase of the cristae membrane. 7. The increase of the effective proton conductance of the M phase by 2,4-dinitrophenol (or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) will account quantitatively for the short-circuiting effect of the uncoupling agent on the proton current and for the observed rise of the rate of respiration to that characteristic of state 3 or higher.

The chemiosmotic hypothesis of oxidative and photosynthetic phosphorylation (Mitchell, 1961*a*, 1963*a*, 1966*b,c*) is based on four main postulates that are susceptible to experimental verification. It is strategically economical to examine one postulate at a time, since the falsification of any one of them would probably provide sufficient grounds for rejecting the hypothesis as a whole.

According to the chemiosmotic hypothesis, the coupling between oxidoreduction and phosphorylation in mitochondrial respiratory-chain phosphorylation is due to the circulation of a proton current between inner (I) and outer (O) aqueous phases separated by a relatively ion-impermeable non-aqueous phase (M) in the coupling or cristae membrane. The reversible adenosine triphosphatase and the respiratory chain, which are situated in the cristae membrane, are supposed to be the main catalytic carrier systems through which the proton current flows (respectively inwards and outwards) during oxidative phosphorylation, and the M

phase of the cristae membrane must have a relatively low permeability to H^+ ions (presumably as H_3O^+ ions) and to OH^- ions to account for the observed efficiency of coupling. We have accordingly sought to show, by means of controlled acid-base titrations, whether suspensions of rat-liver mitochondria behave as three-phase systems with respect to the distribution of H^+ and OH^- ions, and whether there is experimental evidence that the osmotic barrier or M phase of the cristae membrane has a low permeability to H^+ and OH^- ions as postulated.

Gilby & Few (1958) observed that, when unbuffered suspensions of *Micrococcus lysodeikticus* were titrated stepwise with hydrochloric acid, the initial rapid fall of the pH of the suspension medium, measured by a glass-electrode system, was followed by a slower rise to an equilibrium value. The slowness of the completion of the titration was attributed to the limitation of the rate of passage of H^+ and OH^- ions between the unbuffered suspension

medium and the cytoplasmic buffers by the plasma membrane of the cells. Mitchell (1961b, 1963b) confirmed that acid-base titration across the plasma membrane of non-metabolizing anaerobic suspensions of *M. lysodeikticus* in 0.1M-potassium chloride at 25° appeared to be no greater than about 20 μg . ions of H^+ /sec.g. dry wt. of cells, when the external pH was 5.0 and the internal pH was near 7.0. Further, it was shown (Mitchell, 1963b) that titration of the cell contents occurred very rapidly when *n*-butanol was added at a concentration (5%, v/v) sufficient (see Mitchell, 1953) to lyse the plasma membrane of the cells. It was also found that the uncoupling agents 2,4-dinitrophenol, azide and dicoumarol catalysed the acid-base titration of the cell contents in anaerobic cell suspensions. These observations were extended to anaerobic suspensions of rat-liver mitochondria in a medium containing 150mM-potassium chloride, 25mM-sucrose, 5mM-magnesium chloride and 2mM-EDTA (Mitchell, 1961b, 1963b). The results suggested that, although comparatively fragile, the membrane system of mitochondria is not normally very permeable to H^+ or OH^- ions, and that 2,4-dinitrophenol, azide and dicoumarol catalyse acid-base titration through the membrane system in the same range of concentration as that in which they are effective as uncouplers of oxidative phosphorylation.

The present paper describes a more sophisticated and quantitative study of acid-base titration of rat-liver mitochondria, and confirms and extends the earlier findings. Some of this work has been discussed in a colloquium (Mitchell & Moyle, 1967).

MATERIALS AND METHODS

Reagents. CFCCP* and valinomycin respectively were gifts from Dr P. G. Heytler and Dr J. C. MacDonald. Triton X-100, glycylglycine and sucrose (as caster sugar) were respectively obtained from Lennig Chemicals Ltd. (London, W.C. 1), Hopkin and Williams Ltd. (Chadwell Heath, Essex) and Tate and Lyle Ltd. The KCl was A.R. grade, and contained up to $6.5\text{Na}^+ / 10^3\text{K}^+$ and up to $7.5 (\text{Ca}^{2+} + \text{Mg}^{2+}) / 10^5\text{K}^+$. All solutions were made up in double-glass-distilled water. The 250mM-sucrose solution referred to throughout the paper was bubbled with a stream of oxygen-free nitrogen (British Oxygen Co. Ltd.) to remove at least 98% of the dissolved oxygen and sufficient of the carbon dioxide to bring the pH to 6.3 before use.

Isolation of mitochondria. Male Wistar rats of 350–400g. weight (obtained from A. Tuck and Son Ltd., Rayleigh, Essex) were starved overnight for isolation of liver mitochondria on the following morning. For the standard preparation, two rats were stunned and immediately decapitated and allowed to bleed thoroughly. The livers were excised and placed on a stainless-steel plate in the cold room at 4°, where the rest of the preparation was done, with the exception of centrifugation on an MSE High Speed 18

refrigerated centrifuge, operated at 4°. The livers (approx. 8g. weight each) were cut into 2g. portions, slit into ribbons with scissors and rinsed rapidly in 250mM-sucrose, and each portion was placed in a 50ml. polypropylene centrifuge tube (MSE no. 59635) containing 20ml. of 250mM-sucrose. The liver tissue was disintegrated by a smooth spherical-ended glass pestle rotating at 1500rev./min., having a diameter 0.3mm. less than the bore of the tubes. The pestle was inserted into each tube, pressed to the bottom and withdrawn almost to the top of the tissue suspension. Each tube was given six strokes of this disintegration procedure in rapid succession to produce a homogenate. The bulk of the cell debris was removed from the homogenate by centrifuging at 300g for 10min. in an MSE Angle Rotor no. 69181. The supernatant, containing the mitochondria and microsomes, was transferred to clean capped tubes and centrifuged at 12000g for 10min. The resulting supernatant, containing most of the microsomes, was poured off and discarded, and the sedimented mitochondria were dispersed in 60ml. of 250mM-sucrose contained in two 50ml. centrifuge tubes, by using a glass pestle. A small quantity of cell debris and red blood corpuscles was removed by centrifuging at 300g for 10min. The resulting supernatants were transferred to clean tubes, and the top of the sedimented material was rapidly washed off with a small quantity of 250mM-sucrose and added to the supernatant material. This material was centrifuged at 12000g for 10min., and the supernatant, containing microsomes, was discarded. A small quantity of 'fluffy layer', present on the top of the sedimented mitochondria, was washed off with 250mM-sucrose and discarded. After redispersion of the mitochondria in 60ml. of 250mM-sucrose, they were again centrifuged at 12000g for 10min., and the almost clear supernatant was discarded.

The sedimented mitochondria were finally dispersed in 250mM-sucrose to give a total volume of approx. 8.5ml., containing approx. 60mg. of mitochondrial protein/ml. This stock mitochondrial suspension was stored at 4° for 1hr. before use, and meanwhile the protein concentration was determined by the method of Itzhaki & Gill (1964).

Description and use of the reaction cell and electrodes. The titrations were done at 25° in a thermostatically controlled cylindrical glass cell of 6ml. capacity containing an H^+ -sensitive glass electrode (standard laboratory electrode of W. G. Pye Ltd., Cambridge), a saturated-KCl junction leading to a calomel half-cell, a Clark oxygen electrode (Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio, U.S.A.) and a magnetically driven glass stirrer. The cell could be closed by a glass piston, but a shallow groove in the piston, parallel to the axis of the cell, permitted the insertion of a fine glass needle so that nitrogen could be bubbled in to flush oxygen from the cell, and reagents could be added from calibrated micro-syringes. Oxygen from the air was excluded by a flow of nitrogen continuously directed at the top of the piston.

At the beginning of each experiment with anaerobic mitochondria, the oxygen was flushed from the suspension medium in the cell with oxygen-free nitrogen while the glass piston was at the top of the cell. When the oxygen-electrode trace showed that the medium was virtually free of oxygen, the cold anaerobic mitochondrial suspension (0.6ml.) was introduced to give a total volume of 6ml., the piston was lowered on to the surface of the suspension, the pH was adjusted to the appropriate range with standard anaerobic

* Abbreviation: CFCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

acid or alkali (see below) and 5–10 min. was allowed for equilibration before the pH measurements were begun.

Glass- and oxygen-electrode circuitry and recording. The oxygen-electrode circuitry was similar to the conventional system recommended by the makers of the electrode, and the output was fed into a strip-chart recorder (Honeywell-Brown Electronik, from Honeywell Controls Ltd., Brentford, Middlesex) or an X-Y recorder with time base (XY/t Auto Plotter, from Bryans Ltd., Mitcham, Surrey), having a sensitivity corresponding to at least 10 cm./mv and a pen speed of at least 10 cm./sec.

The H⁺-sensitive glass electrode and KCl junction/calomel half-cell reference system were connected to an E.I.L. Vibron Electrometer (33B-2) and pH Measuring Unit (C33B-2) (Electronic Instruments Ltd., Richmond, Surrey), the output of which drove recorders similar to those used for the oxygen-electrode measurements, giving a sensitivity variable up to 10 m./pH unit. At the highest amplification, the 'noise' level corresponded to about 0.0002 pH unit or about 2 mm. on the chart. Control experiments in which pulses of HCl and KOH were injected into the reaction vessel, containing various aqueous media over a range of pH values without added mitochondria, showed that, under the standard conditions described in this paper, there was an initial delay of 0.8 sec. between injection of acid or alkali and the commencement of the response of the glass-electrode system. This delay was affected by the rate of stirring and can probably be attributed to turbulent diffusion across the boundary layer on the glass electrode. The time-constant of the glass-electrode system, measured from the end of the delay time, was very dependent on the cleanliness of the surface of the glass electrode and on the presence of acid-base buffer. The electrode was cleaned as a routine by rubbing with paper tissue soaked with a 1% (w/v) solution of Pyroneg [Diversey (U.K.) Ltd., London, W. 1] and rinsing with dilute KCl solution and with water. The freshly cleaned glass electrode had an almost pH-independent time-constant of 0.95 sec. at 25° between pH 5.5 and 8 in a medium containing 150 mm-KCl and 3.3 mm-glycylglycine. In the absence of glycylglycine, the time-constant increased about tenfold. When a rapid response of the pH-recording system was required, we therefore normally included 3.3 mm-glycylglycine buffer in the mitochondrial suspension medium.

There was generally a pH drift in anaerobic mitochondrial suspensions that was attributable to slow metabolic activity in the mitochondria. This drift was sufficiently slow and constant to permit pH extrapolation over time-intervals up to 5 min. As a routine, the experimental pH traces showing pulsed acid-base titrations were corrected for pH drift by replotting as a set of points measured from the base line given by the extrapolation of the pH recorded over an interval of time (1–5 min.) before or after the injection of the acid or alkali pulse.

Acid-base titration with oxygen-free solutions. The standard acid and alkali solutions used for the titrations were respectively 50 mN-HCl and 50 mN-KOH made up in 100 mm-KCl. The solutions were freed from air by evacuation in Thunberg tubes and flushed with oxygen-free nitrogen. The oxygen-free standard acid and alkali were drawn into calibrated all-glass titrating syringes [Agla micrometer syringes (Burrroughs Wellcome and Co., London, N.W. 1) fitted with fine glass needles made in our Laboratories] under an atmosphere of nitrogen.

Virtually oxygen-free inhibitor solutions. Valinomycin and CFCCP were respectively made up as 0.225 mm and 1 mm solutions in ethanol. The solutions were freed from air by bubbling with a stream of ethanol-saturated oxygen-free nitrogen, and were drawn into dry calibrated micro-syringes that had been flushed with oxygen-free nitrogen.

Definition of pH. We have adopted the operational definition of pH given by MacInnes (1939), which, in the present studies, is practically equivalent to that based on H⁺ ion activity, [H⁺] × f_{H^+} , the symbol f_{H^+} representing the abstract H⁺ ion activity coefficient, which is approximately equal to the mean ionic activity coefficient (see Robinson & Stokes, 1959):

$$pH = -\log[H^+] \times f_{H^+} \quad (1)$$

The value of f_{H^+} has been taken as 0.75 in the 150 mm-KCl medium of the present studies, from Tables of mean activity coefficients of HCl and KCl solutions given by Robinson & Stokes (1959).

Computation of buffering capacities in pulsed acid-base titrations. The buffering capacity of the mitochondrial suspension was almost independent of pH within a given range of 0.1 pH unit over most of the titration range (see Figs. 6 and 7). It was therefore generally permissible to equate the virtually instantaneous pH changes of the outer medium (ΔpH_o) caused by the injection of a pulse of acid or alkali (within a range of 0.1 pH unit) with the amount of H⁺ ion (ΔH_o^+) effectively added to or removed from the phase in rapid equilibrium with the mitochondrial suspension medium (called the outer phase). It was thus possible to describe an outer-phase buffering power (B_o) defined by:

$$\left(\frac{\partial H^+}{\partial pH}\right)_w + B_o = -\frac{\partial(H_o^+)}{\partial(pH_o)} \approx -\frac{\Delta H_o^+}{\Delta pH_o} \quad (2)$$

where the first term on the left represents the change of free H⁺ ion content of the outer phase with pH, or the capacity of this watery medium (subscript w) to contain free H⁺ ions, given by:

$$\left(\frac{\partial H^+}{\partial pH}\right)_w = -2.30[H^+] \times f_{H^+} \quad (3)$$

Eqn. (3) is obtained by differentiating eqn. (1), the factor 2.30 being $\ln[H^+]/\log[H^+]$. In the absence of added buffer, B_o represents the buffering capacity of the outer phase contributed by the mitochondria only (B_o^m). In the presence of added buffer:

$$B_o = B_o^m + B_o^b \quad (4)$$

where B_o^b is the buffering power of the added buffer. When time was allowed to permit acid-base equilibration between the outer phase and any other phase equilibrating relatively slowly with respect to H⁺ and OH⁻ ions, it was possible to equate a total buffering power, B_T , with the sum of the outer-phase buffering power and an inner-phase buffering power, or:

$$B_T = B_o^m + B_o^b + B_T^i \quad (5)$$

Relationship between concentration of buffer group and buffering power at the pK. It can readily be shown that the concentration of a buffer group gives a buffering power at the pK corresponding approximately to:

$$\frac{\partial H^+}{\partial pH} = -2.30 \times \frac{[\text{buffer group}]}{4} \quad (6)$$

No correction is made for activity coefficients in this equation.

Calculation of effective proton conductance coefficient. It is useful to define a conductance coefficient of the coupling membrane for protons, corresponding to the effective rate of proton translocation (i.e. the gross rate of translocation of H^+ ions one way or of OH^- ions the other way or both) under a known protonmotive force. In accordance with general principles (reviewed by Teorell, 1949), we assume that the effective rate of proton translocation across the membrane by diffusion is governed by a linear law of the type:

$$\left(\frac{\partial(\rightarrow H^+)}{\partial t}\right)_{\text{obs.}} = -(k_1[H^+]_M + k_2[OH^-]_M)\Delta\vec{p} \quad (7)$$

where $(\partial(\rightarrow H^+)/\partial t)_{\text{obs.}}$ is the observed rate of proton translocation, k_1 and k_2 are velocity constants, $\Delta\vec{p}$ is the protonmotive force, and $[H^+]_M$ and $[OH^-]_M$ represent respectively the effective concentrations of H^+ and OH^- ions subject to the protonmotive force (see Mitchell, 1967). The effective concentration of H^+ and OH^- ions in the osmotic barrier (phase M) can be taken as approximately constant coefficients when the pH values in both phases I and O are near to a given value. It is convenient to define the composite coefficient of eqn. (7) as the effective proton conductance, C_M , given by:

$$C_M = k_1[H^+]_M + k_2[OH^-]_M \quad (8)$$

The composite coefficient, C_M , can be described in units of $\mu\text{g.ions of } H^+/\text{sec.g. of mitochondrial protein unit protonmotive force}$, or in the normal electrical units used to define electric conductance, as shown below. The protonmotive force (see Mitchell, 1966b,c) is related to the pH difference across the membrane ($\Delta\vec{pH}$) and to the membrane potential in mv ($\Delta\vec{E}$) at 25° by the equation:

$$\Delta\vec{p} = \Delta\vec{E} - 59\Delta\vec{pH} \quad (9)$$

The conductance coefficient (for a given quantity of mitochondria or area of membrane) in units of $\mu\text{g.ions of } H^+/\text{sec.pH unit}$, (C_M)_{Chem.}, is related to the conductance coefficient in units of $\mu\text{mhos (reciprocal megohms)/sec.}$, (C_M)_{Electr.}, by the relationship:

$$(C_M)_{\text{Electr.}} = (C_M)_{\text{Chem.}} \times \frac{F^2}{2.30RT} \quad (10)$$

where F is the faraday, R is the gas constant and T is the absolute temperature. Hence, at 25° :

$$(C_M)_{\text{Electr.}} = (C_M)_{\text{Chem.}} \times 1.64 \times 10^6 \quad (11)$$

Mitchell (1966a) has calculated that the area of the cristae membrane is $4 \times 10^5 \text{ cm.}^2/\text{g. of protein}$, and it is therefore possible to convert the conductance coefficient/g. of mitochondrial protein into the coefficient/cm.² membrane area by multiplying by the factor 2.5×10^{-6} .

When a pH difference is the only component of the protonmotive force, we can represent the first-order effective rate of proton translocation across the membrane by the equation:

$$\left(\frac{\partial(\rightarrow H^+)}{\partial t}\right)_{\text{obs.}} = C_M \Delta\vec{pH} \quad (12)$$

where $\Delta\vec{pH}$ represents the unequilibrated pH difference across the membrane. The relationship between $(\rightarrow H^+)$ and $\Delta\vec{pH}$ during effective proton translocation across the membrane is defined by the buffering-power differential:

$$B = -\frac{\partial(\rightarrow H^+)}{\partial(\Delta\vec{pH})} \quad (13)$$

When H^+ ions are added to phase O and taken from phase I only by translocation outwards through phase M:

$$\Delta(\rightarrow H^+) = \Delta H_O^+ = -\Delta H_I^+ \quad (14)$$

Also:

$$\Delta\vec{pH} = \text{pH}_O - \text{pH}_I \quad (15)$$

In the pH range between 6 and 8, the first term on the left of eqn. (2) can be neglected, and:

$$\frac{\partial \text{pH}_O}{\partial H_O^+} = -\frac{1}{B_O} \quad (16)$$

$$\frac{\partial \text{pH}_I}{\partial H_I^+} = -\frac{1}{B_I} \quad (17)$$

Therefore:

$$\frac{1}{B} = \frac{1}{B_O} + \frac{1}{B_I} \quad (18)$$

It follows that:

$$C_M = \frac{B}{t_i} \ln 2 \quad (19)$$

and:

$$\left(\frac{\partial(\rightarrow H^+)}{\partial t}\right)_{\text{obs.}} = \Delta\vec{pH} \frac{B}{t_i} \ln 2 \quad (20)$$

where t_i stands for the time for half equilibration and $\ln 2 = 0.69$.

To make it possible to obtain the value of B in the experiments with glycylglycine in the suspension medium, the buffering power of this medium, B_O^0 , has been measured over a range of pH values (Fig. 1). In this case, the buffering

Fig. 1. Buffering power, B_O^0 of 3.3mm-glycylglycine in 150mm-KCl between pH 5.5 and 8.0 at 25° , measured by using acid and alkali pulses giving pH steps not greater than 0.05 pH unit, and applying eqns. (2) and (4).

power is described in $\mu\text{g.ions of H}^+/\text{pH unit ml.}$, and must be multiplied by the number of ml. of medium containing 1 g. of mitochondrial protein to bring it to the same units as B_T^m and B_O^m .

EXPLANATION OF RESULTS

Pulsed acid-base titrations of mitochondria without added buffer. Fig. 2(A) shows a pulsed acid titration of anaerobic rat-liver mitochondria (5.53 mg. of protein/ml.) in unbuffered 150 mM-potassium chloride-25 mM-sucrose at 25° in the range pH 7.1-7.0. At zero time, a pulse of 2.0 $\mu\text{l.}$ of oxygen-free 50 mM-hydrochloric acid in 100 mM-potassium chloride was added to the 6 ml. of mitochondrial suspension. The black circles (●) show the course of the recorded pH changes in the normal mitochondrial suspension, corrected for pH drift by our routine procedure. The open circles (○) of Fig. 2(B) show the corresponding pH changes observed when Triton X-100 (final concentration 0.1%) had been added to the mitochondrial suspension 10 min. before the pulse of hydrochloric acid was added.

Fig. 2. Time-course of the pH of the outer phase during pulsed acid titrations of anaerobic mitochondria (5.53 mg. of protein/ml.) in 150 mM-KCl-25 mM-sucrose at 25°. The initial pH_0 was 7.09 ± 0.01 , and 2.0 $\mu\text{l.}$ of oxygen-free 50 mM-HCl in 100 mM-KCl was injected at zero time. In (A) (●) there was no addition, but in (B) (○) Triton X-100 (final concn. 0.1%) was added 10 min. before the acid pulse. The line through the points in (A) is given by eqn. (21), and the values of pH_0^{α} and pH^{ω} (upper and lower broken lines respectively) are obtained from Fig. 3(a). The line through the points in (B) is also given by eqn. (21) for the special case that $\text{pH}_0^{\alpha} = \text{pH}^{\omega} = \text{pH}_0$. In both (A) and (B) the points approach the theoretical line according to eqn. (22).

In the normal mitochondrial suspension, the pulsed titration appears to consist of a fast and a slow process. The time (t) course of the pH recording during the slow process is accurately described by the relationship:

$$\ln \left(\frac{\text{pH}^{\omega} - \text{pH}_0^{\alpha}}{\text{pH}^{\omega} - \text{pH}_0} \right) = k_3 t \quad (21)$$

Eqn. (21) has been used to draw the continuous curve of Fig. 2(A), which extrapolates to pH_0^{α} at zero time. The value of pH_0 at equilibrium, pH^{ω} , shown by the lower broken line in Fig. 2(A), was selected to give a straight line in the plot of $\ln(\text{pH}^{\omega} - \text{pH}_0)$ against t in Fig. 3(a). The reaction of the acid pulse with the outer phase is, of course, much faster than the rate of response of our pH-measuring system. This is borne out by the fact that, as shown in Fig. 3(b), the initial part of the time-course of the recording of Fig. 2(A) follows the relationship:

$$\ln \left(\frac{\Delta \text{pH}_0}{\text{pH}_0^{\text{obs}} - \text{pH}_0} \right) = k_4 t \quad (22)$$

where pH_0 stands for the pH of the suspension medium described by eqn. (21), and pH_0^{obs} stands for the apparent value given by the glass-electrode system. According to Fig. 3(b), the time for half equilibration, t_1 , in the initial, electrode-dominated, pH response is 6.2 sec., corresponding to the glass-electrode time-constant of 8.5 sec. under these conditions. The initial pH response in the Triton X-100-treated mitochondrial suspension of Fig. 2(B), and in the other acid- and alkali-pulse experiments described in this paper, can similarly be shown to follow the relationship of eqn. (22), the velocity constant, k_4 , being characteristic of the glass electrode. We shall not, in this paper, pay further attention to the initial electrode-dominated pH response except inasmuch as its effects must be seen not to interfere with the pH measurements during the relatively slow titration reactions in which we are mainly interested.

Fig. 3(a) shows that the t_1 in the slow titration of the normal mitochondria was 95 sec. in this typical experiment. The slow hydrochloric acid titration did not represent a once-for-all reaction, such as might have resulted from the unfolding of tertiary structures, for it could be obtained repeatedly in the same mitochondrial suspension by readjusting the pH to the original value with potassium hydroxide between hydrochloric acid pulses. Further, the slow titration obtained with pulses of potassium hydroxide was shown in other experiments to be kinetically indistinguishable from that obtained in the corresponding experiments with hydrochloric acid pulses.

Since, over a small pH range, H^+ and OH^- ion concentrations are virtually proportional to pH,

Fig. 3. pH_0 measurements of Fig. 2(A) plotted semi-logarithmically. (a) Plot of $\ln(\text{pH}^\omega - \text{pH}_0)$ against time. The straight line through the points is represented by eqn. (21). (b) Plot of $\ln(\text{pH}_0^{\text{obs}} - \text{pH}_0)$ against time, where the value of pH_0 is given by the straight line through the points in (a). The straight line through the points in (b) is represented by eqn. (22).

the data of Figs. 2 and 3 show that the kinetics of the slow titration are first-order with respect to H^+ or OH^- ions. The slow titration process may therefore correspond to the titration of an inner phase separated from the outer phase by a phase of low permeability to H^+ and OH^- ions. If this were the case, the buffering power of the outer phase of the mitochondria, B_0^m should be given approximately by $B_0^m = -\Delta\text{H}_0^+/\Delta\text{pH}_0^\omega$, where ΔH_0^+ is the quantity of acid added/g. of mitochondrial protein, and ΔpH_0^ω is the virtually instantaneous change of pH given by extrapolating the slow titration (Fig. 2A) to zero time. The sum of the buffering powers of the outer and inner phases, $B_0^m + B_I^m$, is correspondingly given by:

$$B_0^m + B_I^m = -\Delta\text{H}_0^+/\Delta\text{pH}_0^\omega \quad (23)$$

where ΔpH_0^ω is the change of external pH during the course of the slow titration. In this experiment, B_0^m and B_I^m were respectively 35.5 and 18.3 $\mu\text{g.ions of H}^+/\text{pH unit/g. of protein}$.

The presence of a membrane-lytic agent, such as Triton X-100, should cause the inner phase to coalesce with the outer phase, and the virtually instantaneous titration should correspond to the buffering power $B_0 + B_I$. It is evident from Fig. 2(B) that the pulsed titration in the presence of Triton X-100 lacks the slow titration process

corresponding to the inner-phase titration. However, in the presence of Triton X-100, the equilibrium pH was 0.0095 unit higher than in its absence, showing that the total buffering power was increased by 11.0 $\mu\text{g.ions of H}^+/\text{pH unit/g. of protein}$ under the conditions of this experiment. Titration of the suspension medium in the presence and absence of Triton X-100 showed that, at pH 7, the Triton X-100 itself gave a total buffering power of 6.3 $\mu\text{g.ions of H}^+/\text{pH unit/181 ml. of medium}$ (equivalent to the volume occupied by 1 g. of mitochondrial protein in the experiment of Fig. 2). As there was no ready explanation for the remaining discrepancy of 4.7 $\mu\text{g.ions of H}^+/\text{g. of protein}$, alternative methods of hastening acid-base equilibrium between the inner and outer phase were sought.

Fig. 4 shows the effects of valinomycin (10 $\mu\text{g./g. of protein}$), CFCCP (1 μM) and valinomycin + CFCCP on a pulsed acid titration under similar conditions to the experiments of Fig. 2. Valinomycin alone has a possibly significant initial effect, the t_i being 86 sec. compared with the value of 92 sec. in the normal control experiment. The presence of CFCCP partially abolished the slow titration reaction, and the combination of valinomycin and CFCCP completely abolished the slow titration reaction.

Fig. 4. Time-course of the pH of the outer phase during pulsed acid titrations of anaerobic mitochondria (5.89 mg. of protein/ml.) in 150 mM-KCl-25 mM-sucrose at 25°: (●) no addition; (○) in the presence of 10 μg. of valinomycin/g. of protein; (■) in the presence of 1 μM-CFCCP; (□) in the presence of 10 μg. of valinomycin/g. of protein+1 μM-CFCCP. The initial pH_0 and acid pulse were as in Fig. 2.

The equilibrium pH in the presence of the titration catalyst, valinomycin + CFCCP, corresponds to $B_T^a + B_T^p = 54.6$ μg.ions of H^+ /pH unit.g. of protein in the experiment of Fig. 4, compared with the equilibrium value of 53.8 μg.ions of H^+ /pH unit.g. of protein corresponding to Fig. 2(A). Thus the value of B_T^p observed from the virtually instantaneous acid titration in the presence of valinomycin + CFCCP is within experimental error of that obtained from the equilibrium titration of untreated mitochondria.

It was observed that 50 μM-2,4-dinitrophenol exerted a similar catalytic effect to 1.0 μM-CFCCP on the attainment of acid-base equilibrium.

The results of our observations on untreated mitochondria, and on mitochondria treated with valinomycin and CFCCP or 2,4-dinitrophenol, are consistent with the existence of an inner and outer titrating mitochondrial phase, separated by a membrane of low ion permeability; but the results of the observations described above on the mitochondria treated with Triton X-100 appear to be only partially consistent with this interpretation.

Fig. 5. Time-course of total mitochondrial buffering power, B_T^a , at 25° in 150 mM-KCl-25 mM-sucrose: (○) in the presence of 0.1% Triton X-100, and correcting for the buffering power of the Triton X-100 in the absence of mitochondria, by using eqn. (5); (■) or (□) in the presence of 10 μg. of valinomycin/g. of mitochondrial protein together with 1 μM-CFCCP or 50 μM-2,4-dinitrophenol respectively. The measurements were made, with acid and alkali pulses, as in the experiments of Fig. 1.

Fig. 6. Total mitochondrial buffering power, B_T^a , as a function of pH; the methods and conditions corresponding to those of Fig. 5 were used: (■) in the presence of 0.1% Triton X-100; (●) in the presence of 10 μg. of valinomycin/g. of protein+1 μM-CFCCP.

Observations on the time-course of the effect of Triton X-100, however, resolve this discrepancy.

Fig. 5 shows the dependence of the total mitochondrial buffering power, B_T^a , estimated from the virtually instantaneous acid-pulse titration of mitochondrial suspensions to which Triton X-100 (final concentration 0.1%) had been added, on the length of time for which the Triton X-100 had been present before the injection of the pulse of acid. The value of B_T^a increases with time, and extrapolates at zero time to a value close to that given by valino-

mycin+CFCCP and valinomycin+2,4-dinitrophenol, measured on the same batch of mitochondria. The values of B_T^m obtained by using valinomycin+CFCCP or valinomycin+2,4-dinitrophenol are time-independent.

Fig. 6 shows the total mitochondrial buffering power estimated in Triton X-100-treated and valinomycin+CFCCP-treated mitochondrial suspensions between pH 5 and 9. The value of B_T^m

Fig. 7. Outer mitochondrial buffering power, B_O^m (●), measured as a function of pH; and inner mitochondrial buffering power, B_I^m (○), estimated as a function of pH by subtracting the values of B_O^m from the values of B_T^m determined in the presence of valinomycin+CFCCP in the experiments of Fig. 6.

obtained from the Triton X-100-treated mitochondria is similar to that given by the valinomycin+CFCCP-treated mitochondria at pH 8.5. Below pH 8.5, however, the B_T^m values obtained with Triton X-100 are considerably greater than those obtained with valinomycin+CFCCP. The larger buffering powers found in the Triton X-100-treated mitochondria can probably be attributed to the exposure of additional titratable groups resulting from the breaking of the tertiary structure of protein molecules and lipid complexes by the detergent activity of Triton X-100, assisted by charge repulsion at low pH. The measurements of B_T^m obtained with valinomycin+CFCCP have been used to compute B_I^m by difference from measurements of B_O^m in Fig. 7.

Rate constants for effective proton translocation in buffered mitochondrial suspensions. The slow titration reaction shown in the semi-logarithmic plot of Fig. 8(a) was obtained by equilibrating the mitochondrial suspension at pH 7.5 for 10 min., adding valinomycin (10 μg./g. of protein) under anaerobic conditions and then injecting a pulse of hydrochloric acid (2 μg.ions of H^+) to bring the pH into the range 7.2–7.3. The suspension medium in these experiments was 150 mM-potassium chloride–25 mM-sucrose, and was buffered with 3.3 mM-glycylglycine to decrease the time-constant of the glass-electrode system as described in the Materials and Methods section. For clarity, we have omitted the part of the pH trace that occurs during the first 20 sec. after the addition of the hydrochloric acid

Fig. 8. Time-course of acid-pulse titration of anaerobic mitochondria (6.28 mg. of protein/ml.) at 25° in 150 mM-KCl–25 mM-sucrose, containing 3.3 mM-glycylglycine and 10 μg. of valinomycin/g. of mitochondrial protein. The results are plotted as $\ln(pH^w - pH_0)$ against time as in Fig. 3(a). The mitochondria were equilibrated at approx. pH 7.5 before 40 μl. of 50 mM-HCl in 100 mM-KCl was injected at zero time. (a) No addition. (b) With addition of 3 μl. of 1.0 mM-CFCCP at the arrow, 30 sec. after the HCl pulse.

pulse, so that only the subsequent slow titration reaction is shown. In the experiment of Fig. 8(b), the arrow indicates the addition of $0.5 \mu\text{M}$ -CFCCP 30 sec. after the hydrochloric acid pulse. As in Fig. 2, the time-course of the pH recording is accurately described by eqn. (21), the value of pH^w (the pH at equilibrium) being selected to give a straight line in both the semi-logarithmic plots of Fig. 8. In the experiments of Fig. 8, the values of t_i for the valinomycin- and valinomycin + CFCCP-treated mitochondria were respectively 78 and 5.1 sec. In experiments similar to those of Fig. 8, but with 0.1 mM -2,4-dinitrophenol in place of $0.5 \mu\text{M}$ -CFCCP, and pulsing mitochondria equilibrated at pH 6.95 with potassium hydroxide ($1.5 \mu\text{g}$. ions of OH^-) to bring the pH into the range 7.2–7.3, the values of t_i for the valinomycin- and valinomycin + 2,4-dinitrophenol-treated mitochondria were respectively 77 and 6.4 sec. The t_i values observed with acid and alkali pulses did not differ significantly. At an outer pH of 7.2–7.3, seven experiments each with valinomycin- and valinomycin + CFCCP-treated mitochondria and eight experiments with valinomycin + 2,4-dinitrophenol-treated mitochondria gave values for t_i with standard errors respectively of 80 ± 5 , 5.0 ± 0.3 , and 7.3 ± 0.5 sec. The value of t_i obtained in duplicate alkali-pulse experiments with valinomycin-treated mitochondria in the presence of 1 mM -EDTA was 75 sec.

The t_i of the slow titration of normal and valinomycin-treated mitochondria was measured in various outer pH ranges in duplicate experiments under similar conditions to those of Fig. 8, but with pulses of alkali. The results (in sec.) (valinomycin values in parentheses) were as follows: at pH_0 5.2–5.3, $t_i = 35$ (25); at pH_0 6.2–6.3, $t_i = 80$ (55); at pH_0 7.2–7.3, $t_i = 75$ (77); at pH_0 8.2–8.3, $t_i = 95$ (95).

DISCUSSION AND CONCLUSIONS

Observations on the osmotic and permeability properties of mitochondria reviewed by Lehninger (1960, 1962, 1964) and work described in two recent symposia (Tager, Papa, Quagliariello & Slater, 1966, 1967) indicate that the water-soluble solutes are distributed between two main morphologically defined mitochondrial compartments, as originally suggested by Werkheiser & Bartley (1957). These compartments correspond to the region inside the cristae membrane, and the region between the cristae membrane and the outer mitochondrial wall defined in electron-microscope studies (see Palade, 1956; Hackenbrock, 1966). As the mitochondrial wall is relatively pervious to solutes of low molecular weight (Werkheiser & Bartley, 1957; Malamed & Recknagel, 1959; Share, 1960; Parsons, 1966), the morphologically defined outer aqueous compartment is effectively continuous with the sus-

pension-medium phase. Judged from measurements of the sucrose- and sodium chloride- or potassium chloride-impermeable volume of 'heavy' rat-liver mitochondria (Amoore & Bartley, 1958; Birt & Bartley, 1960; Bartley, 1961; Getz, Bartley, Stirpe, Notton & Renshaw, 1962; Bartley & Enser, 1964), the aqueous phase inside the cristae membrane contains about 1 ml. of water/g. of mitochondrial protein; and the osmotic and electron-microscopic studies referred to above show that the volume or dry weight of the region outside the osmotic barrier in the cristae membrane and bounded by the outer surface of the mitochondrial wall is of the same order of magnitude as that inside the osmotic barrier of the cristae membrane.

According to our observations, the total buffering power, B_T^m , of rat-liver mitochondria at pH 7 is $53 \mu\text{g}$. ions of H^+ /pH unit. of mitochondrial protein. No other measurements of total mitochondrial buffering power are available for comparison. However, it is noteworthy that the buffering power of egg albumin at pH 7 calculated from the data of Cannan, Kibrick & Palmer (1941) is $56 \mu\text{g}$. ions of H^+ /pH unit. of protein. Within the range pH 5–9, 55–70% of the titration of acidic and basic groups in the mitochondria occurs rapidly, but 30–45% of the titration occurs slowly with first-order kinetics in H^+ or OH^- ions or both. The slow titration is reversible and gives t_i values of about 1 min. at 25° at neutral pH. The extent, the reversibility and the kinetics of the slow titration leave little doubt that it involves (and is rate-limited by) the translocation of H^+ or OH^- ions or both through the osmotic-barrier component of the cristae membrane that separates the titratable groups of the inner phase from the suspension medium. The non-aqueous M phase of the coupling membrane of the chemiosmotic hypothesis would thus correspond to the region of the cristae membrane that acts as the osmotic barrier for diffusion of hydrophilic low-molecular-weight solutes generally. The alternative possibility that the M phase might have corresponded to the surface of micelles or molecules distributed within the mitochondria may be ruled out in view of the high proportion of the total buffering power involved in the slow titration.

The relationship between the aqueous phases, O and I, the non-aqueous phase, M, and the main morphological features of mitochondria are illustrated diagrammatically in Fig. 9. This diagram emphasizes the important function of the M phase in defining the physical limits of the aqueous phases O and I. The titratable groups of phase I, for example, would belong to all the substances (including diffusible solutes) confined within the inner margin of the M phase, and would even include the titratable groups of the coupling factor F_1 of the adenosine-triphosphatase system and parts of the respiratory

Fig. 9. Diagram of the relationship between the inner and outer aqueous phases (I and O), the non-aqueous osmotic barrier phase (M), and the main morphological features of rat-liver mitochondria.

carriers and other proteins that belong to the fabric of the cristae membrane, but project inside the non-aqueous osmotic barrier component that we describe as the M phase. Similar considerations apply to the acid-base content and physical limits of phase O. The relatively low rate of acid-base equilibration across the M phase shows that this phase does not contain many proton-donating or accepting groups that are freely available from both phases O and I. Titratable groups attached to phase M, which are accessible only from phase O or only from phase I, would effectively belong to one or other of the aqueous phases, and should not be regarded as residing in the non-aqueous phase M. It is conceivable that certain titratable groups might be mobile across the M phase when protonated or when deprotonated, but not in both forms. In that case, the M phase could possess an intrinsic buffering power that would be related to a stoichiometric passage of hydrogen atoms from one side of the membrane to the other. Part of the buffering power that we have attributed to phase O could possibly be due to such an intrinsic buffering power of phase M.

Observations on the uptake of Ca^{2+} and the associated liberation of H^+ in respiring mitochondrial suspensions have led Chance & Mela (1966a) to state that '30 $\mu\text{moles/mg. protein of buffer capacity is observed in the mitochondrial membrane in the resting state (State 4) \dots$ '. The units in which this 'buffer capacity' is expressed, and the method of measuring it, suggest that Chance & Mela (1966a) intended to refer to a quantity of titrating groups in the membrane, rather than to a buffering power as defined here (and see Mitchell & Moyle, 1965; Mitchell, 1966b,c). Eqn. (6) shows that the buffering

power of 30 μmoles of titrating groups/g. of mitochondrial protein at the pK would correspond to about 17 $\mu\text{g.ions}$ of $\text{H}^+/\text{pH unitg.}$ of protein. Since the pK of the acidic groups of phospholipids or proteins reacting with Ca^{2+} would be expected to be several units acid to $\text{pH} 7$, their buffering power should be at least an order of magnitude less than 17 $\mu\text{g.ions}$ of $\text{H}^+/\text{pH unitg.}$ of protein near $\text{pH} 7$.

The pH -buffering power curves of Figs. 6 and 7 show that the buffering powers of phases O and I are relatively high at acid and at alkaline pH values, and are minimal in the neutral range. Our earlier titrations of rat-liver mitochondria, employing Triton X-100 to obtain the total buffering power (Mitchell & Moyle, 1965), gave an inner-phase buffering power of 130 $\mu\text{M-H}^+/\text{pH unit}$ for mitochondria at a concentration of 6 $\text{mg. of protein/ml.}$ near $\text{pH} 7.15$, corresponding to a B_T° value of about 22 $\mu\text{g.ions}$ of $\text{H}^+/\text{pH unitg.}$ of protein. The present results show that this estimate was high because of side effects of Triton X-100 on the total number of available titratable groups. It should be borne in mind, however, that contamination of our mitochondrial preparations with other particulate material, and lysis or leakiness of some of the mitochondria, will tend to increase the observed value of B_T° and depress B_T° . Chance & Mela (1966b) have estimated that the buffering power of the site of adsorption of bromothymol blue is about 166 $\mu\text{M-H}^+/\text{pH unit}$ in rat-liver mitochondrial suspensions at a concentration of 5 $\text{mg. of protein/ml.}$ near $\text{pH} 7.4$, corresponding to a B_T° value of 33 $\mu\text{g.ions}$ of $\text{H}^+/\text{pH unitg.}$ of protein. This is very much greater than the buffering power of the inner phase estimated by our titrations (see Fig. 7).

We have defined the rate of acid-base titration across the M phase in terms of the effective rate of proton translocation from one aqueous phase to the other (see Mitchell, 1966b,c). However, the rate of effective proton translocation is not attributable only to the permeation of H^+ ions (presumably mainly as H_3O^+ ions) and OH^- ions through the M phase, but must be attributed partly to other reactions, such as the exchange of H^+ for K^+ , between the outer and inner phases. The driving force on effective proton translocation is defined as the protonmotive force ($\Delta\vec{p}$) given in terms of the membrane potential ($\Delta\vec{E}$) and the pH differential (ΔpH) by eqn. (9). Since we are not able to measure the membrane potential independently of $\Delta\vec{p}$ and ΔpH , it is possible to estimate the driving force on proton translocation only when $\Delta\vec{E}$ is virtually eliminated. As valinomycin is a specific catalyst for the equilibration of K^+ ions across natural and artificial non-aqueous membranes (Chappell &

Crofts, 1966; Chappell & Haarhoff, 1967), it would be expected to collapse the membrane potential across the M phase to a relatively small, constant, value in the 150mM-potassium chloride medium.

We have previously described (see Mitchell, 1966b,c, 1967; Mitchell & Moyle, 1967) the mechanism by which the slow titration reaction is catalysed by valinomycin+CFCCP or valinomycin + 2,4-dinitrophenol. This can be summarized as follows: (i) the proton-conductor (2,4-dinitrophenol or CFCCP) catalyses the permeation of H^+ ions across the phase separating the outer and inner phases of the mitochondria; (ii) the K^+ -conductor (valinomycin) collapses the membrane potential that would otherwise result from and restrain process (i). Our observations thus confirm the general conclusion that valinomycin is a specific catalyst of K^+ translocation. We have made use of this property of valinomycin to obtain a relationship between the rate of effective proton translocation and the pH difference across the M phase established by the pulsed titration of the mitochondrial suspensions with acid or alkali under various conditions.

As shown in the Materials and Methods section, the first-order kinetics of the decay of the apparent pH difference across the M phase in the valinomycin-treated mitochondria can be described by eqns. (12) and (20).

$$\left(\frac{\partial(\rightarrow H^+)}{\partial t}\right)_{obs.} = \Delta p H \frac{B}{t_i} \ln 2 = \Delta p H C_M \quad (24)$$

where $\Delta p H$ means the displacement of pH across the M phase from equilibrium, C_M is the effective proton conductance coefficient and B is the buffering-power differential across the M phase, defined by eqn. (18).

The presence of valinomycin has a marginal effect on the t_i value of the decay of $\Delta p H$ near pH 7, and it can be inferred that the increase in the permeability of the M phase to H^+ or OH^- ions resulting from the presence of valinomycin in the neutral pH range is very small compared with the increase in the permeability to K^+ ions observed by Chappell & Crofts (1966). Using the data of Fig. 1 and Figs. 7 and 8 to obtain the value of B and the values of t_i , we calculated that the effective conductance of the M phase to protons near pH 7.2, defined by C_M , is $0.110 \pm 0.006 \mu g.ions$ of H^+ /sec.pH unitg. of mitochondrial protein. Since the presence or absence of valinomycin does not have a significant effect on C_M near pH 7.2 in normal rat-liver mitochondria, it is probable that the natural rate of charge leakage through the M phase is sufficient to prevent the development of a rate-controlling membrane potential at this slow rate of proton

translocation. The value of C_M is increased respectively to 1.76 ± 0.12 and $1.21 \pm 0.06 \mu g.ions$ of H^+ /sec.pH unitg. of mitochondrial protein in the presence of $0.5 \mu M$ -CFCCP and $0.1 mM$ -2,4-dinitrophenol, provided that valinomycin is present. Presumably the valinomycin is required for the measurement of these comparatively high values of C_M because the rate of flow of charge (as protons) across the membrane would build up a significant membrane potential if discharged only through the natural ion conductance of the M phase.

One can calculate from the observed values of C_M whether the controlled state of respiration in state 4 could be rate-limited by the effective proton flux through the membrane, and whether the release from the controlled state to state 3 by CFCCP or 2,4-dinitrophenol could be accounted for quantitatively in terms of the catalysis of the rate-limiting proton translocation reaction by the proton-conducting uncouplers. According to the chemi-osmotic hypothesis (Mitchell, 1966b,c) the closed cyclic property of the proton flux requires that the flux through the respiratory chain $(\partial(\rightarrow H^+)/\partial t)_{or}$ should be equal to the total return flux: through the reversible adenosine triphosphatase (h/d), by diffusion (D) and by exchange (X), as described by the respective terms of the following equation:

$$\left(\frac{\partial(\rightarrow H^+)}{\partial t}\right)_{or} + \left(\frac{\partial(\rightarrow H^+)}{\partial t}\right)_{h/d} + \left(\frac{\partial(\rightarrow H^+)}{\partial t}\right)_D + \left(\frac{\partial(\rightarrow H^+)}{\partial t}\right)_X = 0 \quad (25)$$

The translocation rate that we have measured at known protonmotive force might correspond only to the diffusional term in eqn. (25), but it is likely that it includes part of the exchange term also. At all events, the value of $(\partial(\rightarrow H^+)/\partial t)_{obs.}$ calculated from our effective conductance coefficient, C_M , by using eqn. (12), should not exceed the sum of the diffusion and exchange terms in eqn. (25). The rate of respiration is supposed to be limited in state 4 because the second term of eqn. (25) is small in the absence of phosphate acceptor, and the total back flow of protons represents leakage through the membrane via the diffusion and exchange reactions. We can calculate the flux of protons through the respiratory-chain system, $(\partial(\rightarrow H^+)/\partial t)_{or}$, from the rate of respiration and from the $\rightarrow H^+/O$ quotient with a given substrate; and we should find that the corresponding value of the back flow of protons calculated from $C_M \Delta p$ in state 4 does not exceed the respiration-driven outward flux. In state 3, or in the uncoupled state, the rate of respiration is not supposed to be limited by the proton flux, but by other reactions. In the uncoupled state, therefore,

Table 1. *Effective rates of proton translocation through the M phase of rat-liver mitochondria in states 3 and 4*

The upper two lines of numbers show the rates of proton translocation in states 3 and 4 ($\mu\text{g. ions of H}^+/\text{sec. g. of protein}$) calculated from the values of the rates of respiration ($\mu\text{g. atoms of O/sec. g. of protein}$) and the proton-translocation quotients measured previously by Mitchell & Moyle (1967). The lower three lines show the rates of proton translocation in normal and uncoupler-treated mitochondria calculated from the measured values of C_M ($\mu\text{g. ions of H}^+/\text{sec. pH unit g. of protein}$) and the values of $\Delta\vec{p}$ (pH units) estimated by Mitchell (1966b,c) to correspond to states 3 and 4.

	Substrate or uncoupler	State 3 (or uncoupled state)	State 4
$Q_{O_2} \times \frac{\rightarrow H^+}{O} = \left(\frac{\partial(\rightarrow H^+)}{\partial t} \right)_{O_2}$	Succinate	$1.00 \times 4 = 4.0$	$0.288 \times 4 = 1.15$
	β -Hydroxybutyrate	$0.53 \times 6 = 3.2$	$0.108 \times 6 = 0.65$
$C_M \times \Delta\vec{p} = \left(\frac{\partial(\rightarrow H^+)}{\partial t} \right)_{\text{obs.}}$	No addition		$0.110 \times 4.5 = 0.50$
	CFCCP	$1.76 \times 3.7 = 6.5$	
	2,4-Dinitrophenol	$1.21 \times 3.7 = 4.5$	

we should expect the value of the diffusional proton flux calculated from the state 4 value of $\Delta\vec{p}$ to exceed the proton flux due to respiration in state 3. Further, since the uncouplers are expected to depress the value of $\Delta\vec{p}$ below that characteristic of state 3 when present at uncoupling concentrations, the value of the diffusional proton flux calculated from the state 3 value of $\Delta\vec{p}$ should also exceed the proton flux due to respiration in state 3.

Table 1 shows the respiration-driven proton fluxes calculated from respiration rates and $\rightarrow H^+/O$ quotients observed during succinate and β -hydroxybutyrate oxidation at 25° in state 3 and in state 4, under conditions similar to those used in the experiments reported here (Mitchell & Moyle, 1967). It

has been estimated that the values of $\Delta\vec{p}$ in states 4 and 3 would be expected to correspond to about 270 mV and 220 mV or to 4.5 and 3.7 pH units respectively (Mitchell, 1966b,c). Using eqns. (9) and (12) we have calculated the diffusional proton fluxes shown in Table 1 from our values for the coefficients C_M and the values of $\Delta\vec{p}$ in states 4 and 3. As required by the chemiosmotic hypothesis, the value of $C_M \Delta\vec{p}$ is less than $(\partial(\rightarrow H^+)/\partial t)_{O_2}$ in state 4, but the value of $C_M \Delta\vec{p}$ for the mitochondria uncoupled with CFCCP or 2,4-dinitrophenol is greater than $(\partial(\rightarrow H^+)/\partial t)_{O_2}$ in state 3.

We can conclude that: (a) the permeability of the coupling membrane to H^+ (and OH^-) ions is low enough to account for the observed tightness of coupling between oxidoreduction and phosphorylation in terms of the circulation of a proton current as required by the chemiosmotic hypothesis; (b) the uncoupling activity of CFCCP and 2,4-dinitrophenol is quantitatively accounted for in terms of the observed activity of these reagents as catalysts of proton translocation. Our observations do not

support the suggestion of Chance & Mela (1966a) that the mitochondrial membrane is freely permeable to H^+ ions. The value of the effective proton conductance, C_M , in normal mitochondria near pH 7.2 and at 25° is only $0.45 \mu\text{mho/cm.}^2$ [see eqns. (10) and (11)]. This is the lowest natural membrane ion conductance known to us, the usual range being $10\text{--}1000 \mu\text{mhos/cm.}^2$ (Maddy, Huang & Thompson, 1966). Only in the artificial 'black' lipid membranes have ion conductances in the region of $0.1 \mu\text{mho/cm.}^2$ been recorded previously (Huang, Wheeldon & Thompson, 1964; Maddy *et al.* 1966).

The fact that CFCCP or 2,4-dinitrophenol causes only a limited acid-base equilibration across the M phase unless valinomycin is also present shows that the main reaction catalysed by CFCCP or 2,4-dinitrophenol is proton translocation, and not the exchange of H^+ ions against other cations. The observations of Chappell & Haarrhoff (1967) and of Bielawski, Thompson & Lehninger (1966) and unpublished work of A. D. Bangham (personal communication) on the effects of CFCCP and 2,4-dinitrophenol on the permeability of artificial lipid membranes to H^+ and other ions support our interpretation of the mechanism of action of the uncoupling agents of the 2,4-dinitrophenol type (Mitchell, 1961b, 1963a,b, 1966b,c, 1967; Mitchell & Moyle, 1965, 1967). It is not yet possible to decide, however, whether the proton-conducting uncoupling agents carry protons across the M phase by diffusing one way as the free acid and the other way as the anion, or whether the protons may be conducted across the π -orbital systems of molecules of uncoupler that are adsorbed and relatively immobile in the M phase.

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REFERENCES

- Amoore, J. E. & Bartley, W. (1958). *Biochem. J.* **69**, 223.
- Bartley, W. (1961). *Biochem. J.* **80**, 46.
- Bartley, W. & Enser, M. B. (1964). *Biochem. J.* **93**, 322.
- Bielański, J., Thompson, T. E. & Lehninger, A. L. (1966). *Biochem. biophys. Res. Commun.* **24**, 948.
- Birt, L. M. & Bartley, W. (1960). *Biochem. J.* **76**, 328.
- Cannan, R. K., Kibrick, A. & Palmer, A. H. (1941). *Ann. N.Y. Acad. Sci.*, **41**, 247.
- Chance, B. & Mela, L. (1966a). *Nature, Lond.*, **212**, 369.
- Chance, B. & Mela, L. (1966b). *J. biol. Chem.* **241**, 4588.
- Chappell, J. B. & Crofts, A. R. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 293. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
- Chappell, J. B. & Haahrhoff, K. (1967). In *Biochemistry of Mitochondria*, p. 75. Ed. by Slater, E. C., Kaniuga, Z. & Wojtezak, L. London: Academic Press (Inc.) Ltd.
- Getz, G. S., Bartley, W., Stirpe, F., Notton, B. M. & Renshaw, A. (1962). *Biochem. J.* **83**, 181.
- Gilby, A. R. & Few, A. V. (1958). *Biochim. biophys. Acta*, **30**, 421.
- Hackenbrock, C. R. (1966). *J. Cell Biol.* **30**, 269.
- Huang, C., Wheeldon, L. & Thompson, T. E. (1964). *J. molec. Biol.* **8**, 149.
- Itzhaki, R. F. & Gill, D. M. (1964). *Analyt. Biochem.* **9**, 401.
- Lehninger, A. L. (1960). *Pediatrics, Springfield*, **26**, 466.
- Lehninger, A. L. (1962). *Physiol. Rev.* **42**, 467.
- Lehninger, A. L. (1964). *The Mitochondrion*, pp. 132-231. New York: W. A. Benjamin Inc.
- MacInnes, D. A. (1939). *The Principles of Electrochemistry*, pp. 258-259. New York: Reinhold Publishing Co.
- Maddy, A. H., Huang, C. & Thompson, T. E. (1966). *Fed. Proc.* **25**, 933.
- Malamed, S. & Recknagel, R. O. (1959). *J. biol. Chem.* **234**, 3027.
- Mitchell, P. (1953). *J. gen. Microbiol.* **9**, 273.
- Mitchell, P. (1961a). *Nature, Lond.*, **191**, 144.
- Mitchell, P. (1961b). *Biochem. J.* **81**, 24P.
- Mitchell, P. (1963a). *Symp. biochem. Soc.* **22**, 142.
- Mitchell, P. (1963b). In *Cell Interface Reactions*, p. 33. Ed. by Brown, H. D. New York: Scholar's Library.
- Mitchell, P. (1966a). In *Regulation of Metabolic Processes in Mitochondria*, p. 65. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
- Mitchell, P. (1966b). *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, pp. 1-192. Bodmin, Cornwall: Glynn Research Ltd.
- Mitchell, P. (1966c). *Biol. Rev.* **41**, 445.
- Mitchell, P. (1967). *Advanc. Enzymol.* **29**, 33.
- Mitchell, P. & Moyle, J. (1965). *Nature, Lond.*, **208**, 147.
- Mitchell, P. & Moyle, J. (1967). In *Biochemistry of Mitochondria*, p. 53. Ed. by Slater, E. C., Kaniuga, Z. & Wojtezak, L. London: Academic Press (Inc.) Ltd.
- Palade, G. E. (1956). In *Enzymes: Units of Biological Structure and Function*, p. 185. Ed. by Gaebler, O. H. New York: Academic Press Inc.
- Parsons, D. F. (1966). *Proc. 7th Canad. Cancer Res. Conf., Ontario* (in the Press).
- Robinson, R. A. & Stokes, R. H. (1959). *Electrolyte Solutions*, 2nd ed., pp. 223-252. London: Butterworths Scientific Publications.
- Share, L. (1960). *Biochim. biophys. Acta*, **38**, 154.
- Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. (Eds.) (1966). *Regulation of Metabolic Processes in Mitochondria*. Amsterdam: Elsevier Publishing Co.
- Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. (Eds.) (1967). *Mitochondrial Structure and Compartmentation* (in the Press). Bari: Adriatica Editrice.
- Teorell, T. (1949). *Annu. Rev. Physiol.* **11**, 545.
- Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.