Respiration-Driven Proton Translocation in Rat Liver Mitochondria

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1. Pulses of acidity of the outer aqueous phase of rat liver mitochondrial suspensions induced by pulses of respiration are due to the translocation of H^+ (or OH^-) ions across the osmotic barrier (M phase) of the cristae membrane and cannot be attributed to the formation (with acid production) of a chemical intermediate that subsequently decomposes. 2. The effective quantity of protons translocated per bivalent reducing equivalent passing through the succinate-oxidizing and β -hydroxybutyrate-oxidizing spans of the respiratory chain are very close to 4 and 6 respectively. These quotients are constant between pH 5.5 and 8.5 and are independent of changes in the ionic composition of the mitochondrial suspension medium provided that the conditions permit the accurate experimental measurement of the proton translocation. 3. Apparent changes in the $\rightarrow H^+/O$ quotients may be induced by conditions preventing the occurrence of the usual backlash; these apparent changes of $\rightarrow H^+/O$ are attributable to a very fast electrically driven component of the decay of the acid pulses that is not included in the experimental extrapolations. 4. Apparent changes in the $\rightarrow H^+/O$ quotients may also be induced by the presence of anions, such as succinate, malonate and phosphate, or by cations such as Na^+ . These apparent changes of $\rightarrow H^+/O$ are due to an increase in the rate of the pH-driven decay of the acid pulses. 5. The uncoupling agents, 2,4-dinitrophenol, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and gramicidin increase the effective proton conductance of the M phase and thus increase the rate of decay of the respiration-driven acid pulses, but do not change the initial $\rightarrow H^+/O$ quotients. The increase in effective proton conductance of the M phase caused by these uncouplers accounts quantitatively for their uncoupling action; and the fact that the initial $\rightarrow H^+/O$ quotients are unchanged shows that uncoupler-sensitive chemical intermediates do not exist between the respiratory-chain system and the effective proton-translocating mechanism. 6. Stoichiometric acid-base changes associated with the activity of the regions of the respiratory chain on the oxygen side of the rotenone- and antimycin A-sensitive sites gives experimental support for a suggested configuration of loop 3.

According to the chemiosmotic hypothesis, the respiratory-chain system of the cristae membrane of mitochondria couples the flow of reducing equivalents along the chain to the translocation of protons from one side of the membrane to the other (Mitchell, 1961, 1966*a,b*). It has been suggested that the respiratory chain is folded into three so-called oxidoreduction loops corresponding approximately in position to the three coupling sites of the chemical coupling hypothesis. Each loop is supposed to consist of one hydrogen and one electron carrier, so that two hydrogen atoms pass outwards and two electrons pass inwards, releasing two protons on the outer surface and taking in two protons on the inner surface of the membrane as each bivalent reducing equivalent is conducted through each oxidoreduction loop. This arrange-

ment of the respiratory chain system has been inferred from proton translocation to oxygen quotients ($\rightarrow H^+/O$) of approx. 6 and 4 with β -hydroxybutyrate and succinate as substrate respectively (Mitchell & Moyle, 1965, 1967*b*), and from proton translocation to electron pair quotients ($\rightarrow H^+/2e$) of approx. 2 for each loop in partial reactions, with ferrieyanide as oxidant and tetramethyl-*p*-phenylenediamine as reductant (Mitchell & Moyle, 1967*b*).

The object of the present work was to improve the precision of measurement of $\rightarrow H^+/O$ quotients for β -hydroxybutyrate and succinate oxidation, and to explore, in more detail than before, the optimum conditions required for such measurements. We hoped that this work would help to explain the inability of certain others to confirm

our earlier findings (see e.g. Chance & Mela, 1966a, b, c, d; Tager, Veldsema-Currie & Slater, 1966; Chappell & Haarhoff, 1967).

In accordance with a simplifying convention (Mitchell, 1966a, b) we shall use the phrase 'effective proton translocation' to describe the translocation of H^+ ions one way or the translocation of OH^- ions the opposite way or both.

Two main conditions have to be satisfied to permit the accurate measurement of $\rightarrow H^+/O$ quotients (Mitchell & Moyle, 1965): (i) the $\rightarrow H^+$ must correspond to a known amount of oxygen consumed; (ii) the measurements of $\rightarrow H^+$ must be done in such a way as to minimize the back flow or exchange of H^+ ions and of OH^- ions across the cristae membrane. We have satisfied condition (i) by injecting known small volumes of air-saturated saline as pulses into strictly anaerobic mitochondrial suspensions. Owing to the high affinity of cytochrome oxidase for oxygen [apparent K_m less than $0.5 \mu M$ (Longmuir, 1957; Chance & Schindler, 1965)] the pulses of respiration terminate abruptly.

Condition (ii) is more difficult to satisfy than condition (i). We have shown (Mitchell & Moyle, 1967c) that the osmotic barrier (or M phase) of the cristae membrane of rat liver mitochondria has a low effective proton conductance, and that the time for half equilibration in acid-base titrations across the M phase is about 100 sec. near pH 7 at 25° in a potassium chloride-glycylglycine medium. However, according to the chemiosmotic hypothesis a membrane potential component of the total protonmotive force across the M phase should build up during respiration-driven proton translocation, and, if there were no leakage of charged particles across the M phase, the protonmotive force should reach some 250 mV when only about $1 \mu g.$ ion of H^+ had been translocated/g. of mitochondrial protein (Mitchell, 1966a, b). On the other hand, one would expect that mitochondrial suspensions would contain a certain small number of charged particles or dipoles that would be mobile across the M phase, so that a backlash corresponding to the translocation of a few $\mu g.$ ions of H^+ would have to be taken up before the back pressure corresponding to the membrane potential began to reach high values (Mitchell, 1966a, b; Mitchell & Moyle, 1967a, b). We concluded, on this basis, that condition (ii) could best be satisfied by using the smallest oxygen pulses per g. of mitochondrial protein consistent with the accurate measurement of the corresponding pH displacements with a sensitive glass-electrode system. Condition (ii) also requires that anions and cations that may participate in the effectively proton-coupled translocation systems, originally postulated by Mitchell (1961), and recently experimentally identified (Chappell & Crofts, 1966; Heldt, 1966; Klingenberg & Pfaff, 1966; Mitchell,

1967b), should be excluded or that their effects should be minimized.

MATERIALS AND METHODS

The method of isolating the mitochondria, the description of the pH measuring and recording equipment, the methods of computing the relationship between proton translocation and change of pH, and equations for computing the effective proton conductance of the M phase have been described previously (Mitchell & Moyle, 1967c). Only relevant additional materials and methods, and variations of the earlier techniques, are described here.

Reagents. Oligomycin, antimycin A and sodium β -hydroxybutyrate were obtained from Sigma (London) Chemical Co. Ltd. (London, S.W. 6). Rotenone was obtained from British Drug Houses Ltd. (Poole, Dorset). Gramicidin (a mixture of gramicidins A, B and C, predominantly A) was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.). Succinic acid and malonic acid were obtained from Hopkin and Williams Ltd. (Chadwell Heath, Essex), and converted into the potassium salts with A.R.-grade KOH. Various inorganic salts were A.R. grade.

Atomic absorption spectrophotometry. The calcium ion content of the mitochondrial suspensions was estimated by the method of David (1959) with an atomic absorption spectrophotometer (Evans Electroselenium Ltd., Halstead, Essex).

Oxygen pulse titrations with air-saturated saline. Known quantities of oxygen were introduced into the anaerobic mitochondrial suspension by injecting pulses of air-saturated 150 mM-KCl solution from a glass syringe fitted with a micrometer head [Trevan's micrometer head; Baird and Tatlock (London) Ltd., Chadwell Heath, Essex] that permitted a predetermined quantity of KCl, up to 0.1 ml., to be discharged within 0.1 sec. The air-saturated 150 mM-KCl was equilibrated in a water bath at 25° in a stoppered conical flask equipped with a centre cup containing 2 N-KOH and a filter-paper wick for absorption of CO_2 . The quantity of oxygen dissolved in 150 mM-KCl at 25° in equilibrium with water vapour-saturated air was taken as $470 \mu g.$ atoms/l. (*International Critical Tables*, 1928; *Handbook of Chemistry and Physics*, 1962-63; Chappell, 1964), when the barometric pressure, at 50% relative humidity, was 760 mm. Hg. The relative humidity of our laboratory was controlled at approx. 50%, and the quantity of oxygen in the 150 mM-KCl was estimated from the pressure given by a Kew-type barometer, assuming a linear relationship between total atmospheric pressure and the oxygen content of the KCl solution. Oxygen pulses in 0.1 or 0.05 ml. of 150 mM-KCl were used except where indicated in the text. In some experiments, oxygen-saturated saline was used in place of air-saturated saline. The concentration of oxygen was then taken to be $2.55 \text{ mg. atoms/l.}$ in place of $470 \mu g.$ atoms/l.

The displacements of the pH of the outer phase (ΔpH_o) obtained in the pulsed oxygen titrations were measured in terms of $\rightarrow H^+/O$ quotients by calibrating the pH scale with a pulse of a standard solution of oxygen-free 50 mM-KOH or 50 mM-HCl in 100 mM-KCl (see Mitchell & Moyle, 1967c). Thus a given ΔpH_o , within a range of 0.1 unit, could be converted into the equivalent quantity of protons ($\pm \Delta H^+$) appearing in or disappearing from the suspension medium.

Oxygen-free inhibitor solutions. Oligomycin, antimycin

A, gramicidin and rotenone were made up as 0.36%, 0.036%, 0.036% and 400 μM solutions respectively in ethanol. The solutions were freed from air and drawn into calibrated micro-syringes as previously described for other inhibitors (Mitchell & Moyle, 1967c).

Substitution of other cations for K^+ . In experiments requiring the substitution of Na^+ or choline ions for K^+ the appropriate salts were used for the saline required for the oxygen pulses and for adjustment of the pH, so that the ionic composition of the medium remained almost unchanged. To avoid leakage of K^+ ions into the system from the KCl junction of the reference electrode, the KCl junction was formed in a small polytetrafluoroethylene tap situated about 2 cm. from the reaction vessel, and connected to the bottom of the reaction vessel by a 1 mm.-bore capillary tube filled with the suspension medium. This arrangement gave unusually stable pH traces, the noise level being less than 0.0001 pH unit.

Definition of pH. The pH index in these studies was as defined by Mitchell (1967a).

Correction for response time of the pH-recording system. As shown previously, the response of the pH-recording system was approximately exponential, and in the present work the t_1 was 0.65 sec. at 25° and 1.5 sec. at 5° in 150 mm-KCl containing 3 mm-glycylglycine near pH 7.

In the previous paper (Mitchell & Moyle, 1967c) it was assumed that the glass-electrode response did not affect the measurements of the rates of proton translocation computed from plots of the logarithm of the observed pH displacement ($\ln \Delta\text{pH}_0^{\text{obs.}}$) against time, even when the rates of change of pH were comparatively high; and it was also assumed that for slow rates of pH change [as in Figs. 2 and 3 and eqn. (21) of that paper] the pH-measuring electrode would be almost in equilibrium with the outer aqueous phase. In the present studies it was required, not only to measure fast rates of pH change, but also to obtain precise extrapolated initial ΔpH_0 values from the corresponding semi-logarithmic plots of the decay of ΔpH_0 . It was therefore necessary to use a more exact method of pH correction and extrapolation than before.

The exponential response of the glass-electrode system is described by the relationship:

$$\frac{d\text{pH}_0^{\text{obs.}}}{dt} = k_1(\text{pH}_0 - \text{pH}_0^{\text{obs.}}) \quad (1)$$

where k_1 is a velocity constant characteristic of the electrode. When the outer pH displacement (ΔpH_0) at time t changes exponentially from an initial displacement (ΔpH_0^i) according to the relationship:

$$\Delta\text{pH}_0^t = \Delta\text{pH}_0^i e^{-k_2 t} \quad (2)$$

it follows that:

$$\frac{\Delta\text{pH}_0^{\text{obs.}}}{\Delta\text{pH}_0^i} = \frac{k_1}{k_1 - k_2} \cdot \frac{1 - e^{-(k_1 - k_2)t}}{1 - e^{-k_1 t}} \quad (3)$$

When $(k_1 - k_2)t \geq 5$, eqn. (3) can be simplified to the approximate form:

$$\frac{\Delta\text{pH}_0^{\text{obs.}}}{\Delta\text{pH}_0^i} = \frac{k_1}{k_1 - k_2} \quad (4)$$

and substituting in the t_1 values for the electrode and for the ΔpH_0 decay, written as $(t_1)_e$ and $(t_1)_{\Delta\text{pH}}$ respectively, we obtain:

$$\ln \Delta\text{pH}_0^{\text{obs.}} - \ln \Delta\text{pH}_0^i = -\ln \left(1 - \frac{(t_1)_e}{(t_1)_{\Delta\text{pH}}} \right) \quad (5)$$

Eqn. (5) shows that, when $(t_1)_e = 0.65$ sec., the plot of $\ln \Delta\text{pH}_0^{\text{obs.}}$ against time is parallel to that of $\ln \Delta\text{pH}_0^i$ from a time corresponding to about 6.5 sec. after the commencement of the decay of ΔpH_0 ; and that the displacement between the two plots is generally constant and equal to $-\ln[1 - 0.65/(t_1)_{\Delta\text{pH}}]$. When, for example, $(t_1)_{\Delta\text{pH}}$ is 65 sec., the pH error given by $\ln(\Delta\text{pH}_0^{\text{obs.}}/\Delta\text{pH}_0^i)$ corresponds to less than 1% difference between $\Delta\text{pH}_0^{\text{obs.}}$ and ΔpH_0^i . On the other hand, when, for example, $(t_1)_{\Delta\text{pH}}$ is 6.5 sec., the pH error corresponds to approximately a 10% difference between $\Delta\text{pH}_0^{\text{obs.}}$ and ΔpH_0^i . In the case where $(t_1)_{\Delta\text{pH}}$ varies slowly during an experiment it is permissible to calculate the pH error from the slope of $\Delta\text{pH}_0^{\text{obs.}}$ at any given time.

In all the plots of $\ln \Delta\text{pH}_0^i$ against time in this paper the experimental points taken from the $\text{pH}_0^{\text{obs.}}$ recordings have been corrected for the instrumental error of the glass-electrode system as described here.

Extrapolation of ΔpH_0 to give initial proton translocation values. The $\rightarrow\text{H}^+/\text{O}$ quotients are estimated by extrapolating the value of ΔpH_0^i back to a certain time after injecting the oxygen pulse. It was previously shown that the rate of oxygen utilization during the respiratory pulse corresponds approximately to the state 3 rate (Mitchell & Moyle, 1967a,b), in conformity with earlier findings (Chance, Schoener & De Vault, 1965). The initial value of ΔpH_0^i from which the effective proton displacement subsequently decays is therefore taken to occur at a time δt after the injection of the oxygen, given by:

$$\delta t = \frac{1}{2} \frac{N_{\text{O}_2}}{Q_{\text{O}_2}} \quad (6)$$

where N_{O_2} is the amount of oxygen in the pulse per unit weight of mitochondria and Q_{O_2} is the rate of state 3 respiration per unit weight of mitochondria. In practice δt corresponds to 0.75 sec. in most of the experiments described in this paper.

Notation for temporal changes and spatial differences and translocations. When an intensity factor, such as pH, changes during an interval of time in a given phase (e.g. phase O), we write, for example, ΔpH_0 to denote the temporal pH change.

When an intensity factor, such as pH, has a different value on either side of the M phase at any given time, we write, for example, ΔpH to denote the spatially significant pH difference across the M phase.

When a component, such as the H^+ ion, is translocated through the M phase, we write, for example, $\rightarrow\text{H}^+$ to denote the quantity translocated.

EXPLANATION OF RESULTS

Substrate and inhibitor characteristics of respiration-driven pH displacements. Fig. 1 shows pulsed respiration-driven displacements of the observed outer pH ($\text{pH}_0^{\text{obs.}}$) in mitochondrial suspensions equilibrated anaerobically in the usual 150 mm-potassium chloride medium containing 3.3 mm-glycylglycine buffer in the pH_0 range 7.2–7.3 at 25°. In the absence of added substrate (Fig. 1a) the

initial respiration-driven change of pH_0^{obs} occurred sluggishly and in two stages, giving $\rightarrow\text{H}^+/\text{O}$ quotients generally less than 4. The second stage of the change of pH_0^{obs} diminished in speed and extent when a series of respiratory pulses was induced in the same mitochondrial suspension. In the presence of rotenone (25 $\mu\text{g./g.}$ of protein) alone (Fig. 1b), the two-stage characteristic of the change of pH_0^{obs} was more pronounced than in the absence of rotenone, and the second stage was inhibited by 2mM-malonate (Fig. 1c). When both rotenone and antimycin A (20 $\mu\text{g./g.}$ of protein) were present (Fig. 1d), the first stage of the pH_0^{obs} change was abolished; but, when CFCCP* (1 μM) was present (Fig. 1e), the normal increase of acidity of the first stage of the respiration-driven reaction was

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

replaced by a decrease of acidity of approximately the same time-course.

When 2mM-succinate or 2mM- β -hydroxybutyrate was present as substrate (Figs. 1f and 1j), the change of pH_0^{obs} was fast, and the $\rightarrow\text{H}^+/\text{O}$ quotients were close to 4 and 6 respectively. With succinate as substrate, the presence of rotenone (25 $\mu\text{g./g.}$ of protein) had no significant effect on the change of pH_0^{obs} (Fig. 1g), but, with β -hydroxybutyrate as substrate, both the rate and the extent of the acidification were diminished by rotenone (Fig. 1k). The rate of displacement of pH_0^{obs} from the initial value and the apparent value of $\rightarrow\text{H}^+/\text{O}$ were diminished by the presence of antimycin A (20 $\mu\text{g./g.}$ of protein) with succinate as substrate in presence of rotenone (Fig. 1h) or with β -hydroxybutyrate as substrate (Fig. 1l). Oligomycin (1 mg./g. of protein) had no effect on the respiration-driven

Fig. 1. Time-course of changes of observed pH_0 induced by pulsed respiration in suspensions of rat liver mitochondria (5.65 mg. of mitochondrial protein/ml.) preincubated anaerobically in 6 ml. of 150mM-KCl-25mM-sucrose-3.3mM-glycylglycine at 25° in the pH_0 range 7.2-7.3. Intervals of 20 sec. are marked on horizontal axes. Increase in acidity of the outer phase is plotted upwards. Pulses of 150mM-KCl containing 47 and 23.5 m $\mu\text{g.}$ -atoms of O were injected in (a)-(i) and in (j)-(m) respectively. Additions to suspension medium: (a) none; (b) rotenone (25 $\mu\text{g./g.}$ of mitochondrial protein); (c) rotenone (25 $\mu\text{g./g.}$ of protein) and 2mM-malonate; (d) rotenone (25 $\mu\text{g./g.}$ of protein) and antimycin A (20 $\mu\text{g./g.}$ of protein); (e) rotenone (25 $\mu\text{g./g.}$ of protein), antimycin A (20 $\mu\text{g./g.}$ of protein) and 1 μM -CFCCP; (f) 2mM-succinate; (g) 2mM-succinate and rotenone (25 $\mu\text{g./g.}$ of protein); (h) 2mM-succinate, rotenone (25 $\mu\text{g./g.}$ of protein) and antimycin A (20 $\mu\text{g./g.}$ of protein); (i) 2mM-succinate, rotenone (25 $\mu\text{g./g.}$ of protein) and oligomycin (1 mg./g. of protein); (j) 2mM- β -hydroxybutyrate; (k) 2mM- β -hydroxybutyrate and rotenone (25 $\mu\text{g./g.}$ of protein); (l) 2mM- β -hydroxybutyrate and antimycin A (20 $\mu\text{g./g.}$ of protein); (m) 2mM- β -hydroxybutyrate and oligomycin (1 mg./g. of protein).

Fig. 2. Effect of succinate, malonate and inorganic phosphate on the time-course of changes of observed pH_0 induced by pulsed respiration in rat liver mitochondria. Experimental conditions were as for Fig. 1. Intervals of 20 sec. are marked on horizontal axes. Increase in acidity of the outer phase is plotted upwards. In (a)–(e), the medium contained 6.91 mg. of mitochondrial protein/ml. and rotenone (25 $\mu\text{g.}/\text{g.}$ of protein). Pulses of 150 mM-KCl containing 47 $\mu\text{g.}$ atoms of O were injected. Additions to suspension medium: (a) 20 μM -succinate; (b) 0.1 mM-succinate; (c) 0.5 mM-succinate; (d) 2 mM-succinate; (e) 8 mM-succinate. In (f)–(o), the medium contained 6.52 mg. of mitochondrial protein/ml. and 2 mM- β -hydroxybutyrate. Pulses of 150 mM-KCl containing 23.5 $\mu\text{g.}$ atoms of O were injected. Additions to suspension medium: (f) none; (g) 0.1 mM-malonate; (h) 0.5 mM-malonate; (i) 2 mM-malonate; (j) 7.5 mM-malonate; (k) 0.1 mM-phosphate; (l) 0.5 mM-phosphate; (m) 2 mM-phosphate; (n) 5 mM-phosphate; (o) 5 mM-phosphate and oligomycin (1 mg./g. of mitochondrial protein).

change of $\text{pH}_0^{\text{obs.}}$ with either succinate (Fig. 1i) or β -hydroxybutyrate (Fig. 1m) as substrate.

Stoichiometric proton movements associated with oxidoreduction of respiratory-chain components. Further experiments on the respiration-driven changes of $\text{pH}_0^{\text{obs.}}$ in the absence of added substrate showed that the change of $\text{pH}_0^{\text{obs.}}$ representing the first stage of the respiratory pulse was proportional to the quantity of mitochondria and independent of the quantity of oxygen when the pulse contained 0.5–10 $\mu\text{g.}$ atoms of oxygen/g. of mitochondrial protein. Seven experiments gave a mean value of $1.81 \pm 0.076 \mu\text{g.}$ ions of H^+ /g. of mitochondrial protein for the effective quantity of protons liberated in this initial reaction period. When antimycin A and CFCCP were present, the effective proton uptake was similarly related to the quantity of mitochondria. Seven experiments gave a mean value of $0.32 \pm 0.023 \mu\text{g.}$ ion of H^+ /g. of mitochondrial protein.

Effects of anions on ΔpH_0 decay. Figs. 2(a)–2(e) show a set of pulsed respiration-driven $\text{pH}_0^{\text{obs.}}$

displacements in the usual potassium chloride-glycylglycine medium containing 25 $\mu\text{g.}$ of rotenone/g. of mitochondrial protein and various concentrations of succinate at 25° in the pH_0 range 7.2–7.3. Fig. 2 also shows corresponding sets of respiration-driven $\text{pH}_0^{\text{obs.}}$ displacements with 2 mM- β -hydroxybutyrate as substrate, no rotenone, and with the addition of various concentrations of malonate (Figs. 2f–2j) and phosphate (Figs. 2k–2n). Fig. 2(o) shows that the effect of phosphate is oligomycin-insensitive. The rate of decay of the $\text{pH}_0^{\text{obs.}}$ displacements obtained with succinate as substrate, or with β -hydroxybutyrate as substrate in the presence of malonate or phosphate, are dependent on succinate, malonate or phosphate concentration. When the decay of $\Delta\text{pH}_0^{\text{obs.}}$ in the experiments of Fig. 2 is plotted as $\ln \Delta\text{pH}_0^{\text{obs.}}$ against time it can be seen that the anions succinate (Fig. 3) and malonate (Fig. 4) catalyse the decay of $\Delta\text{pH}_0^{\text{obs.}}$ but do not change the quantity of protons initially translocated through the membrane, characterized by the $\rightarrow\text{H}^+/\text{O}$ quotient. With phosphate, it is not

Fig. 3. Effect of succinate on decay of $\Delta p\text{H}_0$. Plots of $\ln \Delta p\text{H}_0^i$ against t shown in curves (b)–(e) correspond to plots (b)–(e) of the experiments described in Fig. 2. Values of $\Delta p\text{H}_0^i$ were derived from the values of $\Delta p\text{H}_0^{\text{obs}}$ as explained in the Materials and Methods section. Oxygen was injected at zero time, and the arrow indicates the time to which $\ln \Delta p\text{H}_0^i$ should be extrapolated to give the $\rightarrow \text{H}^+/\text{O}$ quotient.

Fig. 5. Effect of inorganic phosphate on decay of $\Delta p\text{H}_0$. Plots of $\ln \Delta p\text{H}_0^i$ against t shown in curves (f) and (k)–(o) correspond to plots (f) and (k)–(o) of the experiments described in Fig. 2. Derivation and symbols are as given in Fig. 3.

Fig. 4. Effect of malonate on decay of $\Delta p\text{H}_0$. Plots of $\ln \Delta p\text{H}_0^i$ against t shown in curves (f)–(j) correspond to plots (f)–(j) of the experiments described in Fig. 2. Derivation and symbols are as given in Fig. 3.

evident whether the plot of the decay of $\ln \Delta p\text{H}_0^i$ against time should extrapolate to an $\rightarrow \text{H}^+/\text{O}$ quotient of 6 at 25° (Fig. 5). When, however, this

experiment is done at 5° , it can be seen, as shown in Fig. 6, that phosphate does not significantly depress the initial $\rightarrow \text{H}^+/\text{O}$ quotient below 6.

It was observed in experiments with succinate and malonate, corresponding to Figs. 3 and 4, that the presence of valinomycin ($10 \mu\text{g./g.}$ of mitochondrial protein) had only a marginal effect that could probably be attributed to a slight swelling of the mitochondria. On the other hand, Fig. 6 shows that valinomycin substantially decreased the initial rate of decay of $\Delta p\text{H}_0^i$, catalysed by phosphate, but increased the total extent of the initial relatively rapid depression of $\Delta p\text{H}_0^i$ by phosphate.

Effect of Na^+ ions on $\Delta p\text{H}_0$ decay. Fig. 7 shows the effect of changing the Na^+/K^+ ratio on the respiration-driven change of $p\text{H}_0$ in the usual potassium chloride–glycylglycine medium in the $p\text{H}_0$ range 7.2–7.3, with 2mm- β -hydroxybutyrate as substrate. The Na^+ ion, like certain anions, catalyses the decay of $\Delta p\text{H}_0^i$ at 25° (Figs. 7a–7d), but has little effect at 5° (Fig. 7e). The respiratory pulse at 5° terminates in 5sec., and the plot of $\ln \Delta p\text{H}_0^i$ is accordingly extrapolated to 2.5sec. in this case, whereas the time of extrapolation is 0.75sec. after the commencement of the respiratory pulse at 25° . As with the anions, the semi-logarithmic plots indicate that Na^+ does not depress the $\rightarrow \text{H}^+/\text{O}$ quotient, but hastens the decay of $\Delta p\text{H}_0^i$.

Dependence of $\rightarrow \text{H}^+/\text{O}$ quotients on $p\text{H}_0$ and on

Fig. 6. Effect of inorganic phosphate, (A) in the absence of valinomycin and (B) in the presence of $10\text{ }\mu\text{g.}$ of valinomycin/g. of protein, on decay of ΔpH_0 at 5° . Experimental conditions were otherwise as for Fig. 1. The suspension medium contained an amount of mitochondria corresponding to 5.1 mg. of protein/ml. and $2\text{ mM-}\beta$ -hydroxybutyrate. Pulses of 150 mM-KCl containing $23.5\text{ }\mu\text{g.}$ atoms of O were injected at zero time. Additions to suspension medium: (a) none; (b) 0.1 mM-phosphate ; (c) 0.5 mM-phosphate ; (d) 2 mM-phosphate ; (e) 5 mM-phosphate . Derivation and symbols for plots of $\ln\Delta\text{pH}_0'$ against t are as given in Fig. 3.

ionic composition of the outer phase. The respiration-driven pH_0' displacements, with β -hydroxybutyrate as substrate, measured at various pH_0 regions in media containing 150 mM-potassium chloride (Fig. 8A) or 150 mM-sodium chloride (Fig. 8B) contaminated with about 0.5 mM-K^+ from the mitochondria, show that the catalysis of the decay of $\Delta\text{pH}_0'$ by Na^+ ions is strongly pH-dependent. At acid pH_0 values, the decay of $\Delta\text{pH}_0'$ also appears to be catalysed by K^+ ions. However, the $\rightarrow\text{H}^+/\text{O}$ quotient is practically constant between $\text{pH } 5.5$ and 8.5 . Similar results have been obtained in experiments with succinate as substrate in place of β -hydroxybutyrate. With either succinate or β -hydroxybutyrate as substrate, the replacement of the 150 mM-potassium chloride by 250 mM-sucrose , the replacement of K^+ by choline^+ , or the addition of 5 mM-magnesium chloride to the 150 mM-potassium chloride medium, did not change the $\rightarrow\text{H}^+/\text{O}$ quotients from values close to 4 and 6 respectively at 25° . With the replacement of K^+ by choline^+ , and in the presence of valinomycin ($10\text{ }\mu\text{g.}$ /g. of mitochondrial protein), the t_4 of the

decay of $\Delta\text{pH}_0'$ in the pH_0 range $7.2\text{--}7.3$ at 25° with β -hydroxybutyrate as substrate was $130 \pm 7\text{ sec.}$ (four values).

Catalysis of ΔpH_0 decay by uncouplers. The catalysis of the decay of the respiration-driven pH displacement by the classical uncoupler CFCCP in the usual potassium chloride-glycylglycine medium is illustrated in Fig. 9. In these experiments, the substrate was β -hydroxybutyrate, and the effects of valinomycin ($10\text{ }\mu\text{g.}$ /g. of protein) and of gramicidin ($100\text{ }\mu\text{g.}$ /g. of protein) are shown for comparison. Gramicidin and CFCCP catalyse the decay of $\Delta\text{pH}_0'$, but valinomycin retards the decay of $\Delta\text{pH}_0'$ under the conditions of these experiments. The plots of $\ln\Delta\text{pH}_0'$ against time (Fig. 9) show that neither CFCCP, gramicidin nor valinomycin significantly affect the stoichiometry of the initial respiration-driven effective proton displacement, even when the catalysed rate of decay of $\Delta\text{pH}_0'$ approaches ten times the uncatalysed decay rate. For decay rates exceeding ten times the uncatalysed rate, an apparent depression of the $\rightarrow\text{H}^+/\text{O}$ quotient occurs; however, this effect is attributable

Fig. 7. Effect of K^+/Na^+ ratio on decay of ΔpH_0 . The suspension medium contained 25 mM-sucrose and 3.3 mM-glycylglycine with the addition of: (a) 150 mM-KCl; (b) 135 mM-KCl and 15 mM-NaCl; (c) 15 mM-KCl and 135 mM-NaCl; (d) and (e), 150 mM-NaCl. Temperature: (a)–(d), 25°; (e), 5°. Basic experimental conditions were as for Fig. 1. The suspension medium contained an amount of mitochondria corresponding to 6.99 mg. of protein/ml. and 2 mM- β -hydroxybutyrate. Pulses of 150 mM-saline containing 23.5 μ g.atoms of O were injected at zero time. Derivation and symbols for plots of $\ln \Delta pH_0$ against t are as given in Fig. 3. The arrow indicates the time (0.75 sec.) to which $\ln \Delta pH_0$ is extrapolated in plots (a)–(d). At 5° (plot e), $\ln \Delta pH_0$ is extrapolated to 2.5 sec.

to a very fast electrically driven decay of ΔpH_0^i during the first 5 sec. after the injection of oxygen, and before commencement of the effective recordings of ΔpH_0 in the present experiments. Similar results have been obtained with succinate as substrate, and with 2,4-dinitrophenol in place of CFCCP.

Fig. 10 describes an experiment in which CFCCP (final concn. 0.5 μ M) was injected 15 sec. after the pulse of oxygen. This and many other similar measurements (23 in all) showed that the time for half decay (t_1) of ΔpH_0^i in the pH_0 range 7.2–7.3 at 25°, with 2 mM- β -hydroxybutyrate as substrate, was 73 ± 12 sec. in normal mitochondria, and that it was decreased to 2.7 ± 0.2 sec. (four values) in the presence of 0.5 μ M-CFCCP and to 3.8 ± 0.2 sec. (two values) in the presence of 50 μ M-2,4-dinitrophenol. When valinomycin was present in the mitochondrial suspension (10 μ g./g. of mitochondrial protein)

the t_1 of the ΔpH_0^i decay was increased to 87 ± 5 sec. (six values) in the normal mitochondria, and fell to 3.2 ± 0.2 sec. (seven values) on the addition of 0.5 μ M-CFCCP and to 8.7 ± 0.3 sec. (two values) on the addition of 50 μ M-2,4-dinitrophenol.

Effect of extent of respiratory pulses on $\rightarrow H^+/O$ quotients. In Figs. 11 and 12 we have assembled the values of apparent $\rightarrow H^+/O$ quotients obtained from several experiments in the usual potassium chloride-glycylglycine medium in the pH_0 range 7.2–7.3 at 25° with succinate (rotenone present) or with β -hydroxybutyrate (rotenone absent) as substrate, with oxygen pulses ranging from 0.06 to 20 μ g.atoms of oxygen/g. of mitochondrial protein. With succinate as substrate, the apparent $\rightarrow H^+/O$ quotients approximated closely to 4 from the lowest oxygen pulse to pulses corresponding to 7 μ g.ions of H^+ translocated/g. of mitochondrial protein. The mean value of $\rightarrow H^+/O$ over this range of oxygen pulse was 3.99 ± 0.22 . The more the oxygen pulses exceeded the quantity corresponding to 7 μ g.ions of H^+ translocated/g. of mitochondrial protein, the more the apparent $\rightarrow H^+/O$ quotients with succinate as substrate fell below 4. When β -hydroxybutyrate was used as substrate, the $\rightarrow H^+/O$ quotients approximated closely to 6 from pulses of 0.3 to 1.5 μ g.atoms of oxygen/g. of mitochondrial protein. The mean value of $\rightarrow H^+/O$ within this range was 5.92 ± 0.27 . When the quantity of oxygen in the pulses was increased, the threshold above which the apparent $\rightarrow H^+/O$ quotients began to fall below 6 with β -hydroxybutyrate as substrate was the same as was observed with succinate as substrate when expressed in terms of the quantity of protons translocated per g. of mitochondrial protein (i.e. 7 μ g.ions of H^+ /g. of protein), but not when expressed as a quantity of oxygen reduced. When the oxygen pulses were diminished from about 0.3 towards about 0.06 μ g.atom of oxygen/g. of mitochondrial protein the $\rightarrow H^+/O$ quotients were diminished from values near 6 to values near 4.

Time-course of ΔpH_0 and $\rightarrow H^+/O$ with respiratory pulses of long duration. Fig. 13(a) shows that, when the oxygen pulses with succinate as substrate exceeded amounts corresponding to the translocation of 12 μ g.ions of H^+ /g. of mitochondrial protein, a 'ceiling' was reached from which the pH_0 'rebounded' at about the same moment as the concentration of oxygen in the suspension medium reached zero. Fig. 13(b) also shows that, when 18 μ g.ions of Ca^{2+} were added/g. of mitochondrial protein, the 'ceiling' height of ΔpH_0^i increased from 10 to 29 μ g.ions of H^+ /g. of mitochondrial protein. The presence of 0.2 mM-EDTA, on the other hand (Fig. 13c), inhibited the progress of ΔpH_0^i towards the usual 'ceiling'.

Effects of EDTA and valinomycin on $\rightarrow H^+/O$

Fig. 8. Effect of pH on decay of $\Delta p\text{H}_0$, (A) in usual suspension medium containing 150mM-KCl and (B) in suspension medium in which 150mM-NaCl replaced 150mM-KCl. Experimental conditions were as for Fig. 1. The suspension medium contained an amount of mitochondria corresponding to 6.70mg. of protein/ml. and 2mM- β -hydroxybutyrate. Pulses of 150mM-saline containing 23.5m μ g.atoms of O were injected at zero time. Regions of $p\text{H}_0$: (A), (a) 8.0; (b) mean of values at 6.8, 7.1 and 7.4, which do not differ by more than normal experimental error; (c) 6.0; (d) 5.5; (B), (a) 8.5; (b) 7.8; (c) 7.1; (d) 6.8. Derivation and symbols for plots of $\ln \Delta p\text{H}_0$ against t are as given in Fig. 3.

Fig. 9. Effect of uncouplers on decay of $\Delta p\text{H}_0$. Experimental conditions were as for Fig. 1. The suspension medium contained an amount of mitochondria corresponding to 6.40mg. of protein/ml. and 2mM- β -hydroxybutyrate. Pulses of 150mM-KCl containing 23.5m μ g.atoms of O were injected at zero time. Additions to the suspension medium: (a) none; (b) 0.02 μ M-CFCCP; (c) 0.05 μ M-CFCCP; (d) 0.1 μ M-CFCCP; (e) valinomycin (10 μ g./g. of protein); (f) gramicidin (100 μ g./g. of protein). Derivation and symbols for plots of $\ln \Delta p\text{H}_0$ against t are as given in Fig. 3.

quotients. Fig. 14 illustrates the inhibitory effect of 0.2mM-EDTA on respiration-driven $p\text{H}_0$ displacements (0.65 μ g.atom of oxygen/g. of mitochondrial protein) with succinate as substrate, and shows the reversal of this effect by valinomycin. Results closely analogous to those of Figs. 13 and 14 were obtained with β -hydroxybutyrate as substrate in place of succinate. The quantity of endogenous calcium in the mitochondrial preparations used in these experiments has been shown by atomic absorption spectrophotometry to range between 10 and 12 μ g.ions of Ca^{2+} /g. of mitochondrial protein.

It was found that the extent of the backlash in experiments corresponding to those of Figs. 11–13 was not changed by washing the mitochondria in 1mM-EDTA or 1mM-ethylenedioxibis(ethylene-amino)tetra-acetic acid as the tris salt in 250mM-sucrose at pH 7, provided that the chelating agent was subsequently thoroughly removed by washing the mitochondria in 250mM-sucrose alone. However, it was also found that this washing procedure with chelating agent did not significantly depress the calcium content of the mitochondrial preparations. Other variations of this washing procedure in which, for example, 150mM-potassium chloride and 50mM-glycylglycine were present, or in which

Fig. 10. Effect of CFCCP on decay of $\Delta p\text{H}_0$. Experimental conditions were as for Fig. 1. The suspension medium contained an amount of mitochondria corresponding to 6.28 mg. of protein/ml. and 2 mM- β -hydroxybutyrate. A pulse of 150 mM-KCl containing 23.5 μg .atoms of O was injected at zero time. At the time indicated by the arrow, CFCCP to give a final concentration of 0.5 μM was injected. Derivation for plots of $\ln \Delta p\text{H}_0'$ against t is as given in Fig. 3.

Fig. 11. Dependence of the apparent $\rightarrow \text{H}^+/\text{O}$ quotient for succinate oxidation by rat liver mitochondria on the quantity of oxygen injected. Measurements were made at 25° in the $p\text{H}_0$ range 7.2–7.3 in the presence of 2 mM-succinate and rotenone (25 μg ./g. of mitochondrial protein) in 150 mM-KCl–25 mM-sucrose–3.3 mM-glycylglycine. To obtain total H^+ translocation corresponding stoichiometrically to an oxygen pulse, the value of the quantity of oxygen must be multiplied by the $\rightarrow \text{H}^+/\text{O}$ quotient.

Fig. 12. Dependence of the apparent $\rightarrow \text{H}^+/\text{O}$ quotient for β -hydroxybutyrate oxidation by rat liver mitochondria on the quantity of oxygen injected. Measurements were made at 25° in the $p\text{H}_0$ range 7.2–7.3 in the presence of 2 mM- β -hydroxybutyrate in 150 mM-KCl–25 mM-sucrose–3.3 mM-glycylglycine. To obtain total H^+ translocation corresponding stoichiometrically to an oxygen pulse, the value of the quantity of oxygen must be multiplied by the $\rightarrow \text{H}^+/\text{O}$ quotient.

Fig. 13. Time-course of changes of observed $p\text{H}_0$ during extended pulses of respiration by suspensions of rat liver mitochondria (5.6 mg. of protein/ml.) in the presence of 2 mM-succinate and rotenone (25 μg ./g. of protein), and the effect of addition of EDTA and Ca^{2+} ions. Experimental conditions were as for Fig. 1. Pulses of 150 mM-KCl containing 16.2 μg .atoms of O/g. of mitochondrial protein were injected into the anaerobic suspensions. Additions to the suspension medium: (a) none; (b) 18.0 μg .ions of Ca^{2+} (as CaCl_2)/g. of protein; (c) 0.2 mM-EDTA.

a period of anaerobiosis was induced, were not successful in lowering the calcium content of the mitochondrial suspension or changing the extent of the backlash.

DISCUSSION AND CONCLUSIONS

Cause of the $p\text{H}_0$ changes. The transient acidification of the suspension medium caused by a pulse

Fig. 14. Time-course of changes of observed pH_O induced by pulsed respiration by suspensions of rat liver mitochondria (6.23 mg. of protein/ml.) in the presence of 2 mM-succinate and rotenone (25 $\mu\text{g.}$ /g. of protein), and the effect of addition of EDTA and valinomycin. Experimental conditions were as for Fig. 1. Pulses of 150 mM-KCl containing 23.5 $\mu\text{g.}$ atoms of O were injected into the anaerobic suspensions. Additions to the suspension medium: (a) none; (b) valinomycin (10 $\mu\text{g.}$ /g. of protein); (c) 0.2 mM-EDTA; (d) 0.2 mM-EDTA and valinomycin (10 $\mu\text{g.}$ /g. of protein). Intervals of 20 sec. are marked on the horizontal axes.

of respiration might be attributed to the effective outward translocation of protons and their subsequent diffusion back across the mitochondrial membrane system, or might be attributed to the net synthesis of a labile acidic substance that subsequently decomposes. We concluded previously (Mitchell & Moyle, 1965) that acidification is due to outward proton translocation for the following reasons: (i) the respiration-driven pH_O changes were abolished by the membrane-lytic agent Triton X-100; (ii) when, immediately after the respiration-driven pH_O displacement, the appropriate quantity of alkali was added to the suspension medium to bring the pH_O to approximately the same value as the inner pH (pH_I), pH_O subsequently remained constant. The present work provides additional support for our previous conclusion: (iii) the kinetics of the decay of the respiration-driven pH_O displacements described here correspond, under appropriate conditions, to the kinetics of decay of a difference of pH (ΔpH) across the M phase, measured in acid-base titrations (Mitchell & Moyle, 1967b). For example, with valinomycin-treated mitochondria to minimize the development of the membrane potential component (ΔE) of the protonmotive force across the M phase, at pH 7.2–7.3 and 25°, the t_1 values for the decay of ΔpH_O after a pulse of β -hydroxy-

butyrate oxidation compare with the t_1 values for the decay of ΔpH measured in the acid-base titrations (shown in parentheses) as follows: valinomycin only present, 87 ± 5 sec. (80 ± 5 sec.); valinomycin and 100 μM -2,4-dinitrophenol present, 6.7 ± 0.3 sec (7.3 ± 0.5 sec.); valinomycin and 0.5 μM -CFCCP present, 3.2 ± 0.2 sec. (5.0 ± 0.3 sec.). These observations confirm our earlier conclusion that the effective proton conductance of the M phase is normally 0.4–0.5 $\mu\text{mho/cm.}^2$, and also indicate that the respiratory system translocates protons through the M phase between the same pair of aqueous phases as that defined by means of the pulsed acid-base titrations (Mitchell & Moyle, 1967c).

Relationship between inner and outer pH changes and the pH difference across the M phase. Having thus established, beyond any reasonable doubt, that the pulses of acidification of the suspension medium that accompany the respiratory pulses are due to the outward translocation of H^+ ions (or inward translocation of OH^- ions), one can legitimately calculate, from the observed change of pH of the outer phase, the change of pH of the inner phase and the pH difference generated across the M phase.

When the pH values are between 5 and 9 under the conditions of the present experiments, the change of concentration of free H^+ and OH^- ions with pH can be neglected [see eqn. (2) in Mitchell & Moyle (1967c)], and eqns. (7)–(9) define the outer-phase and inner-phase buffering powers, B_O and B_I , and the buffering power differential, B (Mitchell, 1966a,b):

$$\frac{d\text{pH}_O}{d(\rightarrow\text{H}^+)} = -\frac{1}{B_O} \quad (7)$$

$$\frac{d\text{pH}_I}{d\text{H}_I^+} = -\frac{1}{B_I} \quad (8)$$

$$\frac{d(\Delta\text{pH})}{d(\rightarrow\text{H}^+)} = -\frac{1}{B} \quad (9)$$

Since, by definition:

$$\Delta\text{pH} = \Delta\text{pH}_O - \Delta\text{pH}_I \quad (10)$$

and:

$$\Delta(\rightarrow\text{H}^+) = \Delta\text{H}_O^+ = -\Delta\text{H}_I^+ \quad (11)$$

it follows that:

$$\frac{1}{B} = \frac{1}{B_O} + \frac{1}{B_I} \quad (12)$$

As the values of B , B_O and B_I do not change rapidly with pH, the following approximate equation is applicable within a given small pH range:

$$\Delta(\rightarrow\text{H}^+) = -B_O\Delta\text{pH}_O = B_I\Delta\text{pH}_I = B\Delta\text{pH} \quad (13)$$

The value of B_I^m in the pH_I range 7.2–7.5 is 13 $\mu\text{g.ions}$ of H^+/pH unitg. of protein, but the values of B_O and B depend on the concentration of the mitochondrial suspension and the substrate present in the usual potassium chloride–glycylglycine medium. In practice, B_O is measured in each experiment from the virtually instantaneous pH change (ΔpH_O^0) obtained by extrapolating the pH change of the suspension medium to zero time in calibrations with standard acid or alkali (Mitchell & Moyle, 1967c). The value of B_O can also be obtained from the following formula:

$$B_O = B_O^m + \frac{(B_O^b + B_O^s)}{[m]} \quad (14)$$

where B_O^m is the mitochondrial outer-phase buffering power, $[m]$ is the concentration of mitochondria (in g. of protein/ml.), and B_O^b and B_O^s are the buffering powers of the glycylglycine buffer and substrate (in $\mu\text{g.ions}$ of H^+/pH unitml.) respectively. In the pH_O range 7.2–7.3, B_O^m is 33 $\mu\text{g.ions}$ of H^+/pH unitg. of protein, B_O^b is 0.69 $\mu\text{g.ion}$ of H^+/pH unitml. (Mitchell & Moyle, 1967b), and B_O^s is 0.027 and 0.008 $\mu\text{g.ion}$ of H^+/pH unitml. for 2mM-succinate and 2mM- β -hydroxybutyrate respectively. At the usual mitochondrial concentration corresponding to 6.0mg. of protein/ml., B_O is 153 and 149 $\mu\text{g.ions}$ of H^+/pH unitg. of protein with succinate and β -hydroxybutyrate respectively as substrate. The corresponding value of B is 12 $\mu\text{g.ions}$ of H^+/pH unitg. of protein within experimental error. Hence, under our usual experimental conditions, ΔpH_I is about 12 times ΔpH_O and ΔpH is about 13 times ΔpH_O .

Comparison with indicator method. It has been observed that under conditions approximating to those of our pulsed respiration experiments (Chance & Mela, 1966d), or when state 4 respiration is transiently released to state 3 by the injection of bivalent cation salts into suspensions of rat liver mitochondria (Chance & Mela, 1966b,c), the pH change indicated by bromothymol blue at a concentration of about 3 μM is opposite to that indicated by bromocresol purple or by a glass electrode. These indicator studies corroborate our general conclusion that the pH changes of the inner and outer phases are opposite in direction. As yet, however, their quantitative significance is open to doubt (see Mitchell, 1966a,b).

Exact stoichiometry of $\rightarrow\text{H}^+/\text{O}$ quotients. When the respiratory pulses translocate about 5 $\mu\text{g.ions}$ of $\text{H}^+/\text{g.}$ of mitochondrial protein, and the $\rightarrow\text{H}^+/\text{O}$ quotients are obtained by extrapolating the decay of ΔpH_O , the values fall less than 2% short of the numbers 6 and 4 with β -hydroxybutyrate and succinate respectively as substrate. The values remain unchanged when the membrane system is

made permeable to K^+ ions by valinomycin. There is little or no effect of pH in the pH_O range 5.5–8.5, and the ionic composition of the suspension medium does not appear to affect the actual $\rightarrow\text{H}^+/\text{O}$ quotients. We conclude that the stoichiometry of the proton-translocating oxidoreduction system corresponds closely to 2 protons and 4 protons translocated per bivalent reducing equivalent traversing loop 1 (or site 1) and loops 2 and 3 (sites 2 and 3) respectively.

As the stoichiometry of proton translocation through the M phase is not depressed when the M phase is made permeable to K^+ ions by valinomycin, the translocation of the protons could not possibly be the secondary consequence of K^+ ion translocation.

Other estimates of $\rightarrow\text{H}^+/\text{O}$ quotients. Chappell & Haarhoff (1967) have cited measurements of $\rightarrow\text{H}^+/\text{O}$ quotients, obtained by methods similar to ours by Mr P. J. F. Henderson and Mr B. H. Robinson, that are said to throw some doubt on our observations. They used 4mM-succinate as substrate (without rotenone), and the mitochondria (isolated in 250mM-sucrose containing 1mM-EDTA) were suspended in 145mM-potassium chloride containing only 0.4mM-glycylglycine buffer at 30°. The $\rightarrow\text{H}^+/\text{O}$ quotients obtained from the observed maximum values of ΔpH_O varied unsystematically between 0.8 and 1.2 in nine experiments in which different amounts of oxygen and different concentrations of mitochondria were used. When various amounts of a calcium salt were added to the EDTA-containing mitochondrial suspensions (more than sufficient to titrate the EDTA of the mitochondrial preparation) the $\rightarrow\text{H}^+/\text{O}$ quotients varied unsystematically between 1.9 and 5.6, but gave a mean value (computed by us) of 3.64 ± 0.74 . They also observed that the low $\rightarrow\text{H}^+/\text{O}$ quotients given by EDTA-treated mitochondria were increased by valinomycin or gramicidin. These measurements appear to have been subject to large experimental errors. Nevertheless they agree qualitatively with our findings.

The addition of a small quantity of a calcium (Chance, 1963, 1965; Rossi & Lehninger, 1964; Brierley, Murer & O'Brien, 1964), strontium (Carafoli, 1965) or manganese (Chappell, Cohn & Greville, 1963) salt to mitochondria respiring in state 4 induces a change of the steady respiratory state to that corresponding to state 3 and back, and a certain quantity of H^+ ions is liberated into the suspension medium during the respiratory transition. Attempts have been made to measure $\rightarrow\text{H}^+/\text{O}$ quotients in such experiments by comparing the amounts of oxygen consumed and of H^+ ions liberated during the apparent state 4–state 3–state 4 transition. Rossi & Azzone (1965) observed that, if the total amount of oxygen (not the extra

amount) consumed during the respiratory transition caused by the addition of calcium chloride to rat liver mitochondrial suspensions was related to the amount of H^+ ions liberated, $\rightarrow H^+/O$ quotients near 4 and 6 with succinate and glutamate respectively as substrate were obtained. However, $\rightarrow H^+/O$ quotients of 6 or 7 were obtained for succinate oxidation if the extra oxygen consumed (rather than the total) was related to the H^+ ions liberated. Further, it was observed by Rossi, Bielawski & Lehninger (1966) and by Carafoli, Gamble, Rossi & Lehninger (1967) that $\rightarrow Ca^{2+}/O$ quotients obtained during experiments on Ca^{2+} uptake by respiring rat liver mitochondria at low salt concentration were consistent with the translocation of $2H^+$ /electron pair traversing each oxidoreduction loop or site. But at high salt concentration and at high pH values the $\rightarrow H^+/2e$ quotient per oxidoreduction loop could apparently rise from the normal value of 2 to a value as great as 5. The steady-state-transition method of obtaining $\rightarrow H^+/O$ quotients is open to a fundamental objection (see Mitchell, 1967b). It may be permissible to establish stoichiometric relationships between the variables that change when a system is disturbed from a steady state and returns to that same steady state. But there is no theoretical justification for relating the variables such as oxygen consumed and H^+ ejected during the respiratory transition from state 4 through state 3 to another respiratory state, when the system fails, in fact, to return to state 4. In particular, the system contains extra bivalent cation salt after the transition, and this and other related differences (e.g. failure to return to state 4 respiratory rate) presumably account for changes of H^+ ion location that are not directly connected with a state 4-state 3-state 4 transition or with the changes of proton translocation rate that accompany the respiratory transitions. In spite of the obvious difficulties of interpretation, however, it has been argued that this work is consistent with the fact that the 'normal' $\rightarrow H^+/2e$ quotient per oxidoreduction loop or site is 2.0 (Carafoli *et al.* 1967). We attribute the so-called abnormal $\rightarrow H^+/O$ quotients, not to 'flexibility' of the stoichiometry of the respiratory-chain-linked proton translocation reaction, but to errors of interpretation.

Is loop 1 operative? Tager *et al.* (1966) have suggested that the NAD-linked oxidation of β -hydroxybutyrate is inoperative under the conditions of our measurements. We have measured the changes of difference spectra characteristic of NADH oxidation and reduction during pulsed oxidation of β -hydroxybutyrate and have shown that, with oxygen pulses of about $1 \mu g.$ atom of oxygen/g. of mitochondrial protein, there is a virtually complete cycle of oxidation and reduction of the equivalent

amount of the endogenous NADH (Mitchell & Moyle, 1967a). It is significant that, when the amount of oxygen in the pulses was only about $0.1 \mu g.$ atom/g. of mitochondrial protein, little or no NADH oxidation could be detected (P. Mitchell & J. Moyle, unpublished work). Fig. 12 shows that the $\rightarrow H^+/O$ quotient with β -hydroxybutyrate as substrate approaches that with succinate as substrate when the amount of oxygen in the pulses is diminished below about $0.3 \mu g.$ atom/g. of mitochondrial protein. We attribute this to the fact that the mitochondrial respiratory chain contains a total reducing capacity of about 2μ equiv. on the oxygen side of NADH, and, with oxygen pulses approaching only one-tenth of this quantity, the presence of traces of substrates, such as succinate, connecting directly with loop 2 would react fast enough to siphon off the oxidizing potential that would otherwise pull reducing equivalents from NADH through loop 1.

Causes of loss or decay of pH_O displacement. The relevance of our measurements of $\rightarrow H^+/O$ quotients (Mitchell & Moyle, 1965) to the chemiosmotic hypothesis has been questioned by Chance & Mela (1966a,b,c,d) and by Chappell & Haarhoff (1967) because pH changes corresponding to relatively small $\rightarrow H^+/O$ quotients are observed under certain experimental conditions. However, one would expect to be able to measure the number of protons translocated across the respiratory-chain system by a respiratory pulse only under conditions minimizing the decay of the asymmetry of proton distribution (Mitchell & Moyle, 1965; Mitchell, 1966a,b).

The observations of the present paper confirm that there are two distinct types of decay of ΔpH that influence estimates of $\rightarrow H^+/O$ quotients. These appear to correspond to the ΔE -driven ΔpH decay and the ΔpH -driven ΔpH decay described semi-quantitatively on the basis of the chemiosmotic model (Mitchell, 1966a,b; Mitchell & Moyle, 1967b). More recent theoretical work (Mitchell, 1968) indicates that the extent of the ΔE -driven ΔpH decay depends on the ratio of the total ion conductance to the effective proton conductance of the M phase ($n = C_M/C_M$), and that a proportion $1/n$ of the total pH displacement should undergo the ΔE -driven decay reaction. The t_i of the ΔE -driven decay would normally be expected to be about 1 sec., and would thus be 99% complete within about 7 sec. The apparent $\rightarrow H^+/O$ quotients extrapolated only for the ΔpH -driven decay of ΔpH_i^0 , as in this paper, should therefore be $100(n-1)/n$ per cent of the actual quotients.

ΔE -driven ΔpH_O decay. When the $\rightarrow H^+/O$ quotients are measured in the presence of 0.2 mM-EDTA, they are depressed to some 60% of the normal values. We attribute this fact to the

depression of the total ion conductance of the M phase by the EDTA so that the total ion conductance of the membrane ($\bar{C}_M$) is about $1.25 \mu\text{mhos/cm}^2$ or n is approx. 2.5 (see Mitchell & Moyle, 1967c). When valinomycin ($10 \mu\text{g./g.}$ of mitochondrial protein) is added to the EDTA-treated mitochondrial suspension, the $\rightarrow \text{H}^+/\text{O}$ quotient returns to the normal value. We infer that the high K^+ ion conductance of the M phase, induced by valinomycin, restores the value of $\bar{C}_M$ to not less than $50 \mu\text{mhos/cm}^2$ so that n would be not less than 100 and $100(n-1)/n$ would be at least 99%. The fact that, as normally isolated in 250mm-sucrose, rat liver mitochondria equivalent to 1g. of protein contain about $10 \mu\text{g.ions}$ of Ca^{2+} , and that the extent of the backlash can be increased by $19 \mu\text{g.ions}$ of H^+ when $18 \mu\text{g.ions}$ of Ca^{2+} are added (Fig. 13), suggests that the M phase may have a high conductance to calcium ions in a form corresponding to Ca^+ . The compensating ion translocation could be due to coupled K^+ ion translocation but could not be due to coupled H^+ ion or OH^- ion translocation. We have been unable to remove the calcium from rat liver mitochondria by washing in EDTA or ethylenedioxybis(ethylene-amino)tetra-acetic acid solutions under various conditions. There therefore appears to be some doubt as to whether the small endogenous calcium ion content of the mitochondria should be regarded as free (see also Chance, 1965). The calcium could be bound to some anionic membrane component that effectively neutralizes one of the two positive charges of the Ca^{2+} ions, or to some zwitterionic component without overall ionic valency change. The backlash effect, in which the values of $\bar{C}_M$ and n must be high for a limited translocation of charge through the M phase, might be partly due to the migration of the calcium complex to the inner side of the M phase under the influence of the membrane potential in state 4. But, to account quantitatively for most of the backlash, all of the calcium complex would have to be present on the outer surface of the M phase in state 5 if it were mobile effectively as Ca^+ , whereas the calcium complex would have to be about equally distributed across the M phase in state 5 if it were mobile effectively as Ca^{2+} . Dr Brian Chappell has recently informed us that certain artificial phospholipid membranes appear to have a relatively high permeability to calcium ions.

ΔpH -driven ΔpH_0 decay. The potent catalytic effect of succinate, malonate, phosphate and Na^+ ions on the decay of ΔpH_0 illustrates how the ionic composition of the mitochondrial suspension medium may influence the apparent magnitude of the respiration-driven proton displacement. However, the apparent $\rightarrow \text{H}^+/\text{O}$ quotients obtained by extrapolation of the decay of ΔpH_0 are not affected

by the ionic composition of the suspension medium, provided that the pH-measuring system can respond fast enough to record the course of the changes of ΔpH_0 . The kinetic features of the catalysis of the decay of ΔpH_0 by the anions succinate, malonate and phosphate (Figs. 3-6) imply that the anions are translocated with H^+ ions or against OH^- ions, as observed by Chappell & Crofts (1966) and Chappell & Haarhoff (1967). Similarly, Na^+ ions catalyse the decay of ΔpH_0 , by a process that can be identified as the translocation of Na^+ with OH^- or against H^+ ions (Fig. 7). The fact that, in each case, the velocity of ΔpH_0 decay is first accelerated by the presence of the specific anion or cation, but is later decelerated, indicates that a concentration difference of the specific anion is created across the M phase during the accelerated period of proton translocation, and that this subsequently subsides and reverses the direction of the associated proton translocation. Plots (not shown) of the reciprocal of the catalysed rate of proton translocation against the reciprocal of the concentration of succinate or malonate at 25° , and phosphate at 5° , are approximately linear and indicate Michaelis-Menten type kinetics with half-maximal reaction velocities at total anion concentrations ($\text{pH } 7.2-7.3$) close to 1.0 mM . The large effect of the presence of valinomycin on the catalysis of ΔpH_0 decay by phosphate may possibly indicate that phosphate translocation is coupled, not only to the translocation of H^+ ions (or OH^- ions), but also to the translocation of K^+ ions.

With the Na^+ ion-catalysed translocation, the secondary decelerated period of the proton translocation (Fig. 7) does not appear to terminate with a rate lower than that in the control when the whole of the K^+ of the 150 mM -potassium chloride medium is replaced by Na^+ . This may be due either to experimental error in the relatively small terminal ΔpH_0 values measured in this case, or to the fact that the proton conductance of the membrane is slightly higher in 150 mM -sodium chloride than in 150 mM -potassium chloride. However, when only 10% of the K^+ ions are replaced by Na^+ ions, the secondary decelerated period of proton translocation is slower than in the 150 mM -potassium chloride control. Both the anion and cation catalysis of ΔpH_0 decay exhibit high temperature coefficients; and, with the Na^+ -catalysed decay of ΔpH_0 , measurements with an Na^+ -sensitive electrode have confirmed that Na^+ ions pass out of the mitochondria as H^+ ions pass in. The fact that the $t_{\frac{1}{2}}$ of decay of ΔpH_0 in 150 mM -choline chloride containing 3.3 mM -glycylglycine and valinomycin ($10 \mu\text{g./g.}$ of mitochondrial protein) in the pH_0 range $7.2-7.3$ at 25° is $130 \pm 7 \text{ sec.}$ (four values), corresponding to a C_M value of only $0.27 \mu\text{mho/cm}^2$, suggests that K^+ ions may participate in a

slow H^+-K^+ exchange through the M phase, possibly via the same antiporter system as that used by Na^+ . In the normal potassium chloride-glycylglycine medium at 25° the $t_{\frac{1}{2}}$ of decay of ΔpH_0 in the presence of valinomycin is 87 ± 5 sec., corresponding to C_M $0.39 \mu\text{mho/cm}^2$, whereas at 5° the $t_{\frac{1}{2}}$ of decay of ΔpH_0 is 171 sec. (Fig. 6), corresponding to C_M $0.20 \mu\text{mho/cm}^2$. The biphasic decay of ΔpH_0^i in the 150 mM-potassium chloride medium at pH_0 values near 6.0 and 5.5 and at 25° (Fig. 8A) suggests that an H^+-K^+ antiporter is operative in the M phase at acid pH_0 .

Do uncoupler-sensitive coupling intermediates exist? The $\rightarrow H^+/O$ quotients are not correspondingly depressed when the effective proton conductance of the M phase is increased about tenfold (i.e. to about $5 \mu\text{mhos/cm}^2$) by the presence of CFCCP, 2,4-dinitrophenol or gramicidin (Fig. 9). It follows that the uncouplers could not be hydrolysing any hypothetical coupling intermediate between the respiratory chain and the proton-translocating mechanism. The suggestion of Chappell & Crofts (1965, 1966), Lardy, Connelly & Johnson (1964) and Tager *et al.* (1966) that such intermediates exist is therefore purely speculative. The present data reinforce our previous conclusion, based on pulsed acid-base titrations (Mitchell & Moyle, 1967*b,c*), that the uncoupling effect of CFCCP and 2,4-dinitrophenol can be quantitatively accounted for by the increase in the value of C_M that these reagents specifically induce. The apparent fall of the $\rightarrow H^+/O$ quotient by 8% in the presence of $0.1 \mu\text{M}$ -CFCCP is attributable to the fast electrically driven ΔpH_0 decay. The effective proton conductance of the M phase corresponds to about $5 \mu\text{mhos/cm}^2$ under these conditions, and the factor $100(n-1)/n$ would be 92% if the total ion conductance of the M phase were about $60 \mu\text{mhos/cm}^2$.

Proton translocation associated with ubiquinone oxidation by oxygen and proton uptake associated with oxygen reduction by cytochromes. When the transfer of reducing equivalents down the respiratory chain is blocked by rotenone, oxygen pulses in excess of the capacity of the available part of the respiratory chain cause the translocation of $1.81 \pm 0.076 \mu\text{g.ions}$ of $H^+/\text{g.}$ of mitochondrial protein. This may be attributed to the oxidation of ubiquinone through loop 3, since the quantity of ubiquinone is known to be very approximately $1 \mu\text{mole/g.}$ of mitochondrial protein (Redfearn, 1960). When the respiratory chain is further blocked by antimycin A, and when (and only when) CFCCP is also added to permit the detection of the withdrawal of protons by the reduction of oxygen on the inner side of the M phase, oxygen pulses in excess of the capacity of the available part of the respiratory chain cause the uptake of

$0.32 \pm 0.023 \mu\text{g.ion}$ of $H^+/\text{g.}$ of mitochondrial protein. This may be attributed to the oxidation of cytochrome oxidase and of cytochrome *c*. The precise quantity of the cytochromes present in rat liver mitochondria equivalent to 1 g. of protein appears to be rather a matter of conjecture, but there is very approximately $0.2 \mu\text{g.}$ electron equivalent of cytochrome oxidase ($0.1 \mu\text{mole}$ of cytochromes $a + a_3$ and $0.1 \mu\text{g.}$ equiv. of Cu) and $0.2 \mu\text{g.}$ electron equivalent (or μmole) of cytochromes $c + c_1$ (Redfearn, 1960; Van Gelder, 1966; Van Gelder & Muijsers, 1966). Since the H^+ ion uptake accompanying the oxidation of the cytochromes is not observed unless CFCCP is present, the site of reduction of oxygen by cytochrome oxidase must presumably be on the inner surface of the M phase. These observations provide some experimental support for the arrangement of loop 3 suggested by Mitchell (1966*a,b*).

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