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Oxygen-Pulse Curves in Rat Liver Mitochondrial Suspensions

SOME OBSERVATIONS AND DEDUCTIONS

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1. The inference, implicit in the chemiosmotic hypothesis, that protons move into the bulk phase during ATP synthesis was investigated. 2. Incubation of rat liver mitochondria in the presence of the cation exchanger CM-Sephadex C-50 caused alkalinization in the medium, though total ATP synthesis remained unchanged. The addition of *N*-ethylmaleimide prevented the alkalinization, but there was still no indication of protons passing into the medium. The expected proton movement [Mitchell & Moyle (1967) *Biochem. J.* **105**, 1147–1162] was readily detected when given as an equivalent acid pulse. 3. Analysis of ΔH^+ decay curves after O_2 pulses ($3\,\mu$g-atoms of O/g of protein) indicated the presence of fast and slow components of decay, with first-order rate constants (k) of $0.24\,s^{-1}$ and $0.032\,s^{-1}$. The fast decay was finite and was eliminated in the presence of *N*-ethylmaleimide. 4. These observations are interpreted as evidence for the development or unmasking of fixed charges on the outer surface of the mitochondrial inner membrane during energization and for the existence of proton-retentive electrical fields (ρ-zones) on this surface. The charge concentration is calculated as about 1 charge/$10\,nm^2$. 5. A cycle of changes in a single fixed-charge molecule is proposed which mediates both Ca^{2+} uptake and the first step in the utilization of the ρ-zone protonmotive force, Δp^{ρ}.

Oxygen-pulse experiments have provided basic data for the development of the chemiosmotic hypothesis of mitochondrial energy transduction (Mitchell & Moyle, 1965, 1967*b*). They indicated that proton translocation across the osmotic barrier of the inner membrane was, in all probability, a primary event, and they led to estimates of the H^+/P stoicheiometry for the coupling process (Mitchell & Moyle, 1968; Brand *et al.*, 1976*a*,*b*,*c*; Brand & Lehninger, 1977; Moyle & Mitchell, 1977*a*). According to the hypothesis the movement of protons into the outer bulk phase on energization gives rise to a protonmotive force (Δp), part electrical, part chemical, which constitutes the driving force for ATP synthesis (Mitchell, 1966, 1968). The relationship is formulated as:

$$\Delta p = \Delta\psi - Z\Delta pH$$

where $\Delta\psi$ represents the membrane potential and ΔpH the pH gradient, both measured across the inner membrane from the outer to the inner bulk phase. Z is the conversion factor $2.3\,RT/F$, which has a value of 60 at 30°C when the potentials are expressed in mV.

When measured over a range of experimental conditions the steady-state values of the two partial terms in this relationship were found to vary widely, but an inverse relationship between the terms preserved a constant, or relatively constant, total protonmotive force (Mitchell & Moyle, 1969; Nicholls, 1974). However, the measurements of $\Delta\psi$ and ΔpH on which these estimates of Δp were based depend on the assumption that the protonmotive force is correctly assessed as a bulk-phase parameter, an electrochemical potential existing between the outer, extramitochondrial solution and the mitochondrial matrix.

Oxygen-pulse experiments appear to validate this view, for protons are seen to enter the medium. But the conditions of such experiments are highly artificial. Before an oxygen pulse can be given there must be a period of anaerobiosis during which ions, especially Ca^{2+} and phosphate, leak from the mitochondria into the surrounding medium (Thomas *et al.*, 1969; Brand *et al.*, 1976*a*,*b*). Proton movement into the medium when the pulse is given is directly related to the electrophoretic charge-compensating re-uptake of this leaked Ca^{2+}, but when no further counter-movement of Ca^{2+} is possible the transfer of protons changes from an electroneutral to an electrogenic process. This type of movement, characteristic of proton transfer during synthesis, will cause an increase in $\Delta\psi$ rather than ΔpH. Mitchell (1968) has calculated that the translocation of 1 ng-ion of H^+/mg of mitochondrial protein under these conditions would be sufficient to give a mem-

brane potential of 250mV, a value approaching the requirement for synthesis in State 4 (Chance & Williams, 1955).

It seems to have been accepted that the electrogenic movement of protons in this way is still a movement into the bulk phase, and that the protonmotive force is still properly measured and studied as a bulk-phase parameter when ATP synthesis, and not cation counter-movement, is taking place. But we know of no experimental evidence which is unambiguously in support of this contention, which is, in our view, purely inferential. Since this is a matter central to the chemiosmotic argument we have made some attempts, reported in this paper, to test its truth. Preliminary reports have been given elsewhere (Archbold *et al*, 1974, 1975*a*,*b*,*c*, 1976).

Experimental

Materials

Dowex 50 (X8; 20–50 U.S. mesh; Na^+ form) was obtained from BDH Chemicals, Poole, Dorset, U.K. CM-Sephadex (C-50 cross-linking) and SE-(sulphoethyl)-Sephadex (C-50 cross-linking) were purchased in the Na^+ form from Pharmacia, Uppsala, Sweden. Luciferase, ADP and ATP (disodium salt) were from Boehringer, Mannheim, Germany; rotenone and Ruthenium Red were from BDH Chemicals; oligomycin, *N*-ethylmaleimide and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO, U.S.A.; O_2-free N_2 and O_2 (medical grade) were supplied by British Oxygen Co., London, S.W.19, U.K. All other chemicals used were from Hopkin and Williams, Chadwell Heath, Essex, U.K., and were of analytical grade.

Deionized water was prepared by passing glass-distilled water through an Elgastat Portable Deioniser (Elga Group, Bucks., U.K.) until the effluent had a resistance exceeding 4MΩ/cm; it was used in the preparation of all aqueous solutions.

Preparation of cation-exchangers

Dowex 50 (X8; Na^+ form) was crushed in a ball-mill for several days. The original particles were spherical with a mean diameter about 0.5mm; the ground resin was sharply angular with particle sizes of 0.005mm or less. Resin in the K^+ form was obtained by passing a large excess of 0.1M-KCl slowly by upward displacement through columns containing 5g of Dowex 50 (X8; Na^+ form) until the eluent and effluent K^+ concentrations were the same. After washing with deionized water until the effluent was free from K^+, the preparations were dried in air and ground in the same way as the Na^+ form. Samples of all preparations were characterized by elution and complete cation exchange with 0.1M-HCl, followed by assay of the effluent by flame photometry (Table 1).

Similar methods were used for preparing CM-Sephadex C-50 in the K^+ form, from the Na^+ form pre-equilibrated in water. When exchange and washing were complete excess water was removed by suction and the preparation was shrunk by addition of ethanol solutions of increasing strength up to 95% (v/v). Excess ethanol was then removed and the exchanger washed with ether and dried at room temperature.

Use of the Sephadex exchangers was complicated by their uptake of 'included water' from aqueous solutions. It was found that when the dried samples were swollen in the presence of added phosphate (15mg of exchanger equilibrated in 3ml of 0.25M-sucrose solution containing 1 μmol of phosphate), the phosphate did not diffuse into the included volume. This observation provided a ready method for obtaining apparent included volumes of the dextran exchangers by measuring the change in phosphate content of the equilibrating solutions before and after swelling had taken place (Table 1).

When used in mitochondrial experiments all Sephadex preparations were equilibrated overnight in the volume of medium to be used in parallel control experiments, plus an amount equal to the included volume. The swollen exchangers were subsequently transferred directly to the incubation vessels as suspensions, thus ensuring a strict comparability between control and test experiments.

Preparation of rat liver mitochondria

Mitochondria were isolated from fresh rat liver tissue by the method of Schneider (1948) as modified by Johnson & Lardy (1967). All stages were carried

Table 1. *Ion contents (±S.D.) and apparent included volumes of cation exchangers*

Exchanger	Form	Ion content (μequiv./10mg)	Included volume (ml/15mg)
Dowex 50 (X8)	Na^+	31.2±1.3 (20)	—
Dowex 50 (X8)	K^+	34.2±0.9 (6)	—
CM-Sephadex C-50	Na^+	36.8±1.8 (6)	0.7
CM-Sephadex C-50	K^+	39.0±2.6 (4)	0.7
SE-Sephadex C-50	Na^+	19.8±3.4 (6)	0.65

out at 0–4°C. The final pellet of washed mitochondria was suspended in ice-cold 0.25M-sucrose solution to give a stock preparation containing 60mg of mitochondrial protein/ml.

Mitochondrial protein was determined by a biuret method (Dittebrandt, 1948), by using a standard reference curve prepared for bovine serum albumin.

Incubations and measurements

The basic medium used in both oxygen-electrode (total volume 3ml) and oxygen-pulse studies (total volume 6ml) was a solution of 0.25M-sucrose containing 2mM-sodium succinate at pH6.7–7.0. Rotenone, dissolved in ethanol, was added at the rate of 1μg/3ml of incubation, except in O_2-pulse decay analysis experiments. Mitochondria were present at a concentration of 5mg of mitochondrial protein/ml of incubation. In studies involving the use of cation-exchangers, succinate and, when required, phosphate additions were made by using the same cation as that present on the exchanger.

The basic medium satisfied the need for a minimal exogenous cation content, which was essential for exchange studies; it supported active synthesis in the presence of added phosphate and ADP, and routinely permitted respiratory-control-index (State 3/State 4) values greater than 3.5. Respiration was measured at 30°C with a Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) coupled to a Vitatron recorder (Fisons Scientific Apparatus, Loughborough, Leics., U.K.).

O_2-pulse experiments were carried out in a cylindrical glass vessel of 10ml capacity placed in a water bath thermostatically controlled at 30°C. A pH-sensitive micro-combination glass electrode (type 003-11-306/E2; Activion Glass Ltd., Kinglassie, Fife, Scotland, U.K.) was used to monitor pH changes in the medium; it was connected to an EIL pH-measuring unit (C-33-B) and Vibron Electrometer (Electronic Instruments Ltd., Richmond, Surrey, U.K.) the output from which drove a Vitatron recorder. Mixing was by a Rank immersible magnetic stirrer driving a glass-coated follower bar. Mitochondria were added to media pre-saturated with O_2-free N_2, and anaerobic conditions were maintained throughout experiments by passing N_2 over the surface of the mitochondrial suspensions. O_2 pulses were given after a 3min anaerobic period, as injections of O_2-saturated 0.25M-sucrose, by using a Repette (Jencons Scientific Ltd., Hemel Hempstead, Herts., U.K.) or a Hamilton syringe (Hamilton Bonaduz A.G., 7402 Bonaduz, Switzerland). When CM-Sephadex (Na^+ or K^+ form) was present in the medium, O_2 was given as a H_2O_2 pulse in the presence of added catalase; this change was made because of small 'spike' pH responses in media containing this exchanger (but no mitochondria), when large (400μl) sucrose-solution pulses were injected; the effect seems to have been purely physical and was eliminated when the same amount of O_2 was given as a small (50μl) diluted H_2O_2 pulse.

ATP assay

Samples (approx. 2ml) were withdrawn from incubations by suction through a coil of fine-bore glass tubing (internal diameter 3mm; length 30cm) leading to a small test tube, both immersed in a boiling-water bath. After 1min the sample was removed from the bath and centrifuged at 20000g for 10min. The supernatant was assayed by luciferase: assay medium and ATP sample were mixed in a standard cell, path length 0.5cm, to give a final concentration of 4mM-$MgSO_4$ and 20mM-glycylglycine at pH8, in a volume of 1.6ml. With the cell in position in the spectrophotometer and the shutter closed, a portion of luciferase (about 0.1mg) was added. The contents of the cell were rapidly mixed and the light-emission was measured by opening the shutter after a standard period of 4s, timed by metronome. An RCA IP28 photomultiplier coupled to a Philbrick/Nexus 100a amplifier was used as a current-to-voltage converter and the light-emission was recorded on a Honeywell Electronik 194 recorder. A double-reciprocal plot of flash height versus ATP concentration was linear under these conditions (Lemasters & Hackenbrock, 1973) and a calibration graph was routinely obtained for each experiment. The variation in the activity of luciferase preparations precluded the use of a definitive calibration. ATP, like phosphate, did not penetrate the included volume of Sephadex exchangers and the assay was applicable without modification for incubations in which these exchangers were used.

Calcium assays

Total mitochondrial calcium was extracted by the method of Carafoli & Lehninger (1971) and analysed by atomic absorption spectrophotometry (Perkin–Elmer model 403). The calcium content of anaerobic incubation supernatants was determined by using the EGTA–Ruthenium Red quench solution introduced by Reed & Bygrave (1974). Quench solution (0.75ml) was added to incubations at stated intervals to give final quenching concentrations of 2mM-EGTA and 3μM-Ruthenium Red. The quenched solutions were centrifuged for 10min at 20000g and the supernatants analysed by atomic absorption spectrophotometry. Ruthenium Red concentrations were obtained by measuring the A_{533} of a stock solution and comparing the calculated molar absorption with that given by a pure sample (Fletcher *et al.*, 1961).

Analysis of pH-decay curves

The aim of these analyses was to determine whether the pH-decay curves after an O_2 pulse followed a single exponential course or whether they were more complicated. Measurements were taken from the recording charts as percentages over convenient time intervals (full chart span = 100%). The chart speed most frequently used (4cm/min) permitted readings at 3.75 and 7.5s with an accuracy of (reading) ± 0.1%. Readings were normally limited to a period not exceeding 45s; beyond this time most readings were less than 5%. Baseline drift was in most cases absent or negligible; but in some experiments (not reported here), for example in the presence of calcium ionophores, the drift was too large for a confident analysis to be made by these methods.

With different systems (small and large pulses; the presence or absence of *N*-ethylmaleimide, or added Ca^{2+}) the curves presented a number of problems in analysis. Three methods were finally used. They will be referred to as Methods I, II and III and are identified in Fig. 1.

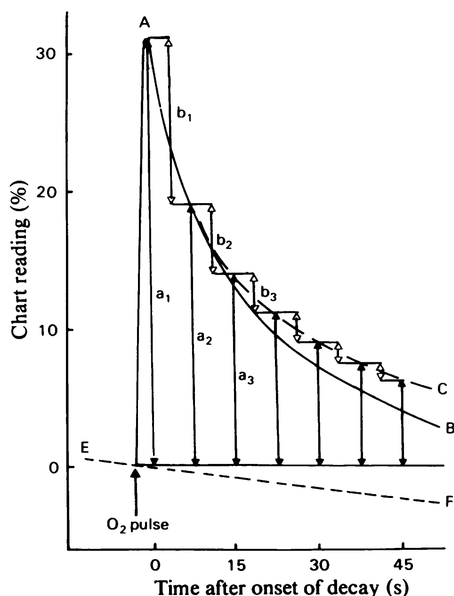


Fig. 1. *Methods used in curve analysis*
AB is the recorded curve, and AC the curve corrected for the baseline drift, EF. a_1, a_2 etc. are readings for Method I, and b_1, b_2 etc. readings for Method II. The readings for Method III are obtained in the same way as for Method II by using the uncorrected curve, AB. Approximate equivalencies of the chart readings are: chart reading 30%; ΔpH in medium, −0.10; ΔH^+ in medium, 0.2μequiv. Additional intermediate points were also used at the start of many decay curves.

Method I. This used direct chart readings (% of full span) corrected if necessary for any drift at the time when the O_2 pulse was injected. A computer program gave the intercept and fitted points for the best single exponential curve which could be derived from the input data. [Data: ΔH^+ recorded as chart reading (%); data points, 7 or 8; time, 30 or 37.5s.]

Method II. This used the readings from Method I to give decay rates for successive intervals. These values were taken as the mean decay rates for each interval and assigned to the interval mid-points. A semi-logarithmic plot of the data, assuming a single exponential decay, gave ln(intercept) and slope estimates from an unweighted least-squares linear-regression analysis. {Data: $\ln[(\Delta H^+)_t-(\Delta H^+)_{t+1}]$, ΔH^+ measured as chart readings (%); data points, 6 or 7; time (interval mid-points), 26.25 or 33.75s.}

Method III. This was similar to Method II. Decay rates were read directly from the chart without baseline corrections and the time-span was extended. {Data: $\ln[(\Delta H^+)_t-(\Delta H^+)_{t+1}]$; data points 5 or 6; time (interval mid-points), 41.25s.} Comparable kinetic analyses have been applied in a more sophisticated form to the anaerobic proton release from submitochondrial particles by Papa *et al.* (1973).

The analyses were directed towards the demonstration of any fast exponential decay occurring in the first few seconds after the onset of anaerobiosis. This was done by comparing the intercepts and slopes given when all the data points were used with those given when either the first, or in some cases the first two data points, were omitted.

The presence of small 'lag' periods (<2s) at the peak of some curves led to a probable underestimate of the first data point in such cases. No attempt was made to extrapolate to what might have been a truer value, for this would have involved a pre-judgement of the question at issue and the correction would have been different depending on whether an initial fast phase was present or not. Any successful demonstration of a fast phase was therefore dependent on its continuing for a period beyond the 'lag' phase.

Results

Total Ca^{2+} and anaerobic Ca^{2+} release

Total Ca^{2+} determined for six separate mitochondrial preparations was 14.7 ± 1.8μg/30mg of mitochondrial protein.

When mitochondria were added to anaerobic media from the ice-cold stock suspension, the corrected Ca^{2+} concentrations in the supernatant after immediate quenching were already high (12μg/30mg of mitochondrial protein) and showed negligible change over a further 12min anaerobic period. The progressive course of Ca^{2+} leak could be followed

after an O_2 pulse had been given just sufficient for the re-uptake of this released Ca^{2+}; when anaerobic conditions returned it was found that about 5μg of Ca^{2+} was released in the first minute, 9μg after 3min and 10μg after 5min. These results indicate that in the present series of experiments there would be about 10–12μg of Ca^{2+} free in the incubations when an O_2 pulse was injected.

Effects of added cation exchangers

When an extended O_2 pulse (200–400μl of O_2-saturated 0.25M-sucrose solution, or an equivalent amount of H_2O_2 in the presence of catalase) was given to mitochondria incubated anaerobically for 3min in media containing increasing amounts of CM-Sephadex C-50 (Na^+ form) (see the Experimental section), the customary acidification caused by Ca^{2+}/proton exchange was progressively diminished until, when 20–30mg of exchanger/30mg of mitochondrial protein was used, there was an alkalinization of the outside solution (Fig. 2). This action, which follows the removal of 'leaked' Ca^{2+} from the suspensions by the exchanger, and the consequent absence of Ca^{2+} uniport movement across the inner membrane, is paralleled by other procedures which eliminate the possibility of Ca^{2+} uptake, for example the addition of EGTA to the suspensions (Thomas *et al.*, 1969) to chelate the ion (Fig. 3) or Ruthenium Red to prevent Ca^{2+}-carrier function; both treatments increase the pH of the medium after pulsing. The alkalinization in the presence of Sephadex was itself eliminated by adding *N*-ethylmaleimide to the media before pulsing (Fig. 4). This result indicates that the alkalinization involves the *N*-ethylmaleimide-sensitive phosphate/hydroxyl carrier, working either alone or as a part of substrate (succinate) transport.

When ADP and phosphate (17μM; pH7) were added to incubations containing Sephadex, ATP

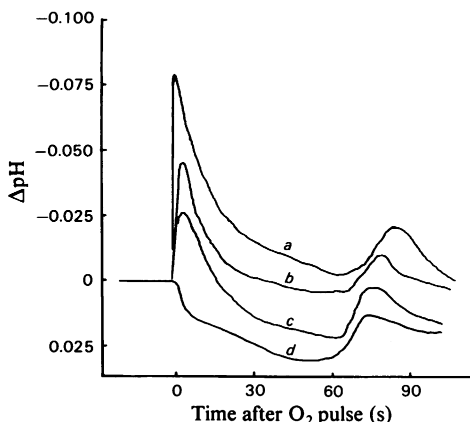


Fig. 2. *O_2-pulse curves given by rat liver mitochondrial suspensions anaerobically preincubated in the presence of increasing amounts of CM-Sephadex C-50*

A pulse of 50μl of H_2O_2 (diluted 1:250) was given in the presence of catalase (5μl) to rat liver mitochondrial suspensions containing 30mg of mitochondrial protein, after a 3min anaerobic preincubation period; curve *a*, without addition; curves *b*, *c*, *d*, with 5, 10 and 30mg of added pre-swollen CM-Sephadex. The incubations had a total volume of 6ml (without the included volume of the exchanger) and contained 2mM-sodium succinate and 1μg of rotenone. The secondary peak in each curve marks the end of the aerobic period and re-acidification due to the hydrolysis of endogenous ATP.

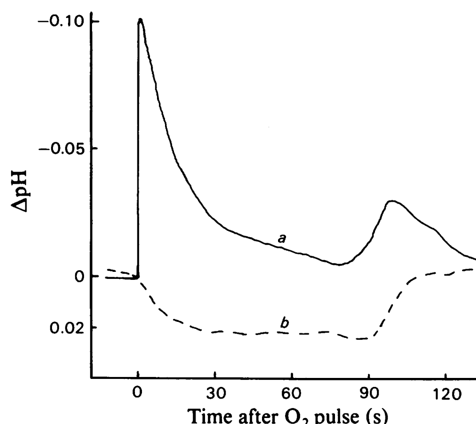


Fig. 3. *O_2-pulse curves given by rat liver mitochondrial suspensions preincubated in the presence and absence of EGTA*

A pulse of 400μl of O_2-saturated 0.25M-sucrose solution was given to rat liver mitochondrial suspensions preincubated anaerobically for 3min. Curve *a*, without addition; curve *b*, in the presence of 1.5μmol of EGTA (pH7). Basic system was as in Fig. 2.

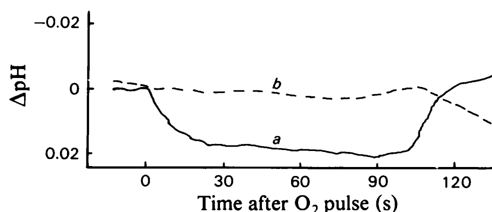


Fig. 4. *Effect of N-ethylmaleimide on the O_2-pulse curve given by rat liver mitochondria anaerobically preincubated in the presence of CM-Sephadex C-50*

System was as in Fig. 2. Curve *a*, control with 30mg of CM-Sephadex; curve *b*, with 30mg of CM-Sephadex (Na^+ form) and *N*-ethylmaleimide (30nmol/mg of mitochondrial protein).

synthesis after pulsing was at first accelerated relative to control incubations without Sephadex; at a later stage it was slowed, but total synthesis in experimental and control incubations always reached the same value (Fig. 5). This pattern was closely followed when EGTA or Ruthenium Red was responsible for eliminating Ca^{2+} uptake. The explanation of the 'cross-over' synthesis curves seems to be that in control systems the priority of Ca^{2+} uptake at first delays synthesis; later, because of the link between Ca^{2+}, phosphate and substrate anion transport, there is a greater immediate availability of intra-mitochondrial phosphate and succinate for synthetic activity. More slowly these concentrations increase in the incubations with Sephadex and synthesis ultimately reaches the control value.

Fig. 6 shows chart recordings, from four different mitochondrial preparations, of the pH changes in mitochondrial suspensions preincubated anaerobically for 3min in the presence of CM-Sephadex, when given either an O_2 pulse or a pulse of 30nmol of HCl. The acid pulse represents the pH change that might be expected to follow the O_2 pulse, on the basis of the calculation that the electrogenic transport of 1ng of H^+/mg of mitochondrial protein into the bulk phase is required to give a protonmotive force sufficient for synthesis (Mitchell, 1968).

Fig. 7 gives results from a similar series of O_2-pulse and acid-pulse experiments in which the cation exchanger was Dowex 50 (X8; Na^+ form). The results show differences in detail compared with those in Fig. 6. The acidification of the outer solution was

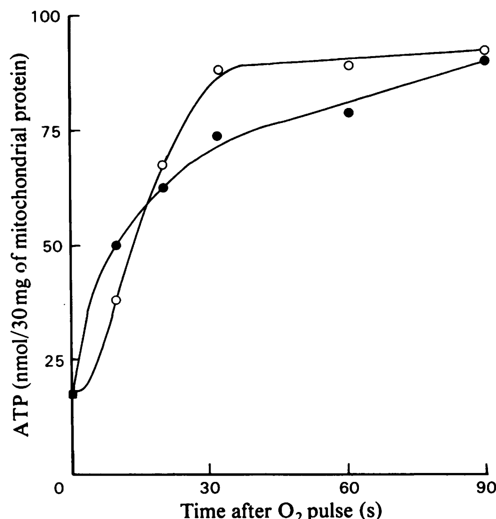


Fig. 5. *ATP synthesis in rat liver mitochondrial suspensions after an O_2 pulse given in the presence or absence of CM-Sephadex*

A pulse of 80μl of H_2O_2 (diluted 1:250) in the presence of catalase was given to rat liver mitochondrial suspensions preincubated anaerobically as in Fig. 2. Sodium phosphate (17μM) and ADP (100nmol) were present during the preincubation period; ○, control; ●, plus 30mg of CM-Sephadex (Na^+ form). Samples (2ml) were removed by suction, and inactivated in a boiling-water bath for 1min. They were then cooled, centrifuged and the supernatants assayed for ATP by using luciferase.

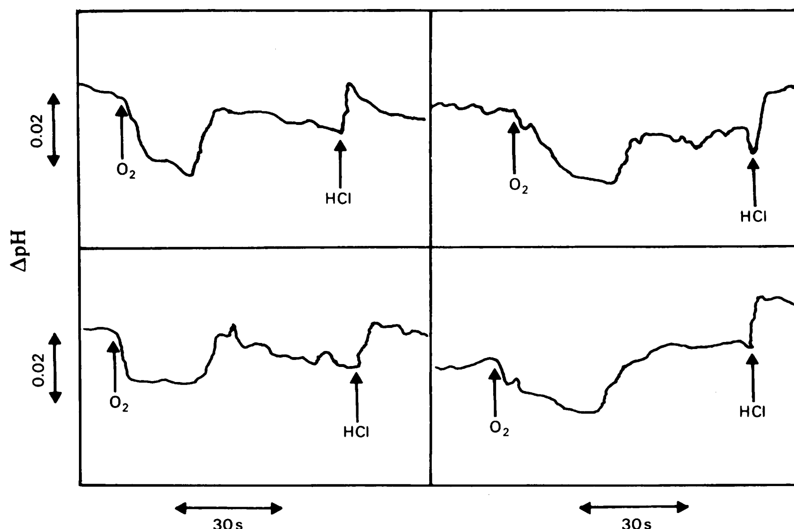


Fig. 6. *pH changes in the medium caused by O_2 pulses and acid pulses given to rat liver mitochondrial suspensions containing CM-Sephadex*

O_2 pulses (100μl of O_2-saturated 0.25M-sucrose solution) or acid pulses (30nequiv. of HCl injected as 30μl of N_2-saturated 1mM-HCl) were given to anaerobic rat liver mitochondrial suspensions containing 30mg of pre-swollen CM-Sephadex (Na^+ form). Conditions were as in Fig. 2. The four recordings are from different mitochondrial preparations.

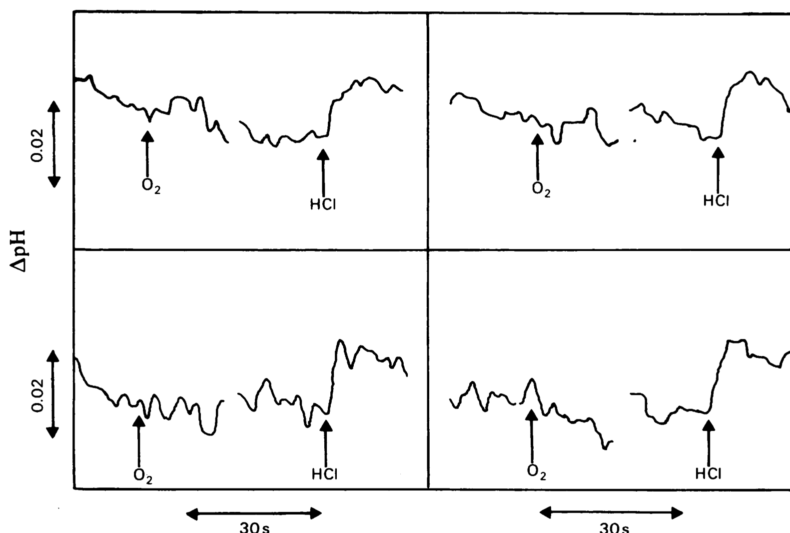


Fig. 7. *pH changes in the medium caused by O_2 pulses and acid pulses given to rat liver mitochondrial suspensions containing Dowex* 50 (*X*8)

O_2 pulses and acid pulses (as in Fig. 6) were given to anaerobic rat liver mitochondria in the presence of 30mg of Dowex 50 (X8; Na^+ form). The four recordings are from different mitochondrial preparations.

again eliminated, but no alkalinization was caused. Further, the addition of *N*-ethylmaleimide or oligomycin to the medium in the presence of Dowex produced a slow acidification after an O_2 pulse. The evidence suggests that the sulphonic acid groups on the Dowex resin cause a more complicated response than the weakly acidic carboxymethyl groups of CM-Sephadex. This view is reinforced by two further observations: firstly, that ATP synthesis in the presence of Dowex, although showing the 'cross-over' pattern with the curves from control incubations, fails to reach the final extent of ATP production given by the controls (Archbold *et al.*, 1975*a*); and secondly that the sulphonic acid form of the dextran exchanger, SE-Sephadex, also showed anomalous behaviour in O_2-pulse studies, lowering but never fully eliminating the initial acidification.

Cation exchangers were initially used in these studies because it was thought they would have the advantage of being completely extramitochondrial in their action. It now appears that this may not be true for exchangers with sulphonic acid groupings; on the other hand CM-Sephadex seems uncomplicated in its action, permitting full synthetic activity and exerting its influence indirectly through changes confined to the bulk phase.

O_2-pulse decay curves

Four kinds of decay were analysed: after small (40–50μl) or large (200–400μl) pulses of O_2-saturated 0.25M-sucrose; and after the same pulses given in the presence of 30nmol of *N*-ethylmaleimide/mg of mitochondrial protein. Pulse curves from each group are shown in Fig. 8.

The general principle of analysis is shown graphically in Figs. 9 and 10, with data from an actual curve from the series given in Table 3. When comparisons were needed for chi-square tests best-fit exponential curves (Method I, Fig. 9) or regression lines (Methods II and III, Fig. 10) were obtained for all data points, and for all points minus the first point; since the intercept obtained by using all points is greater in the given examples, they would score '+' in the chi-square test. When the best quantitative estimates of fast and slow exponential decays were required (Fig. 10) it was usually necessary to obtain the slow decay by eliminating the first two data points. Fig. 10 includes the calculated plot for a fast phase and the summation plot which more accurately defines the data.

Small O_2 pulses (*Fig.* 8*a*). These pulses are just sufficient for the uptake of all, or nearly all, the leaked Ca^{2+}; anaerobiosis therefore supervenes at the peak of acidification and the ΔpH decay curve is produced anaerobically. As we have indicated above, Ca^{2+} is leaked under these conditions, and we have confirmed the observation of Brand *et al.* (1976*a*,*b*) that phosphate is also released into the medium. Experiments carried out in sucrose medium alone, with no added succinate, gave decay rates commensurate with those in which succinate was present. Unlike the

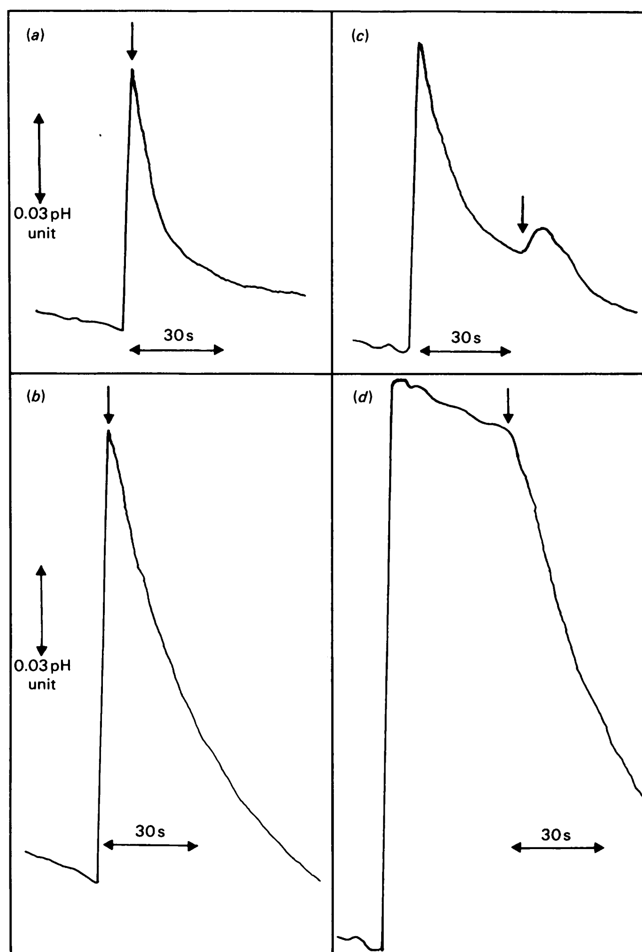


Fig. 8. *O_2-pulse curves under various conditions*
Basic system was as in Fig. 2 without rotenone. Curve (*a*), small O_2 pulse (40μl of O_2-saturated 0.25M-sucrose solution); curve (*b*), small pulse in the presence of *N*-ethylmaleimide (30nmol/mg of mitochondrial protein); curve (*c*), large O_2 pulse (200μl of O_2-saturated 0.25M-sucrose solution); curve (*d*), large pulse in the presence of *N*-ethylmaleimide (30nmol/mg of mitochondrial protein). In each case the approximate point of re-established anaerobiosis is indicated by the arrow. The time scales mark the measured periods. The examples are characteristic, but not from the same mitochondrial preparation.

system of Mitchell & Moyle (1967*b*), therefore, anion$^-$/OH^- exchange seems to have played a negligible part in causing the alkalinization in the present experiments. We conclude that the decay arm of the ΔpH curve after a small O_2 pulse is caused by the leak of Ca^{2+} and phosphate into the medium, balanced by an inward proton movement ensuring the electroneutrality of the exchange.

In accord with this view, an O_2 pulse given during the alkalinization phase caused an immediate acidification which, when the experiment was carried out in the presence of *N*-ethylmaleimide, restored the ΔpH to its original maximum value. Results from this series are given in Table 2.

The lack of agreement between the results from Methods I and II and from Method III could be due to the longer time-span used in Method III. If the data represented a composite curve, Method III would involve an increased proportion of any slower component and the indication of biphasicity would be more evident. It seemed reasonable to suppose that any fast decay, if present at all, would derive from a specific and quantitatively finite source (see the Discussion section). If therefore Ca^{2+} was added

to the incubations before pulsing, a point might be reached at which the fast decay would be maximal and constant, but the slow decay would be relatively increased. Under such conditions Methods I and II might also show the presence of fast and slow components.

Table 3 gives the results from a series of experiments in which Ca^{2+} in the medium was increased by about 50% by adding 6 μg of Ca^{2+}, as $CaCl_2$, to the incubations before giving a small O_2 pulse. The additions allowed longer periods for curve analysis (52.5s by Method I and 48.75s by Method II), and clear indications of biphasicity were found. Since the rate constant for the fast component ($k = 0.24\,s^{-1}$) was greater than that for the composite curve without added Ca^{2+} ($k = 0.097\,s^{-1}$), some slow component must have been present in the latter case, and it was of interest to know what amounts of slow component would give this decrease. Fig. 11 shows the variation in k given by an increasing admixture of the slow component to a system in which the contribution of the fast component was equivalent to a constant chart reading of 32.3%; this value is equal to antilog(intercept) for the fast decay curve derived from the mean values of the experiments in which Ca^{2+} was added to the medium before pulsing (see Fig. 10 for the treatment of an individual case with data points). The variation in k also depends on the limiting point adopted for the decay curve: for decay to a 1% chart reading [ln(reading) = 0], $t = 69.5$ s, k(composite) =

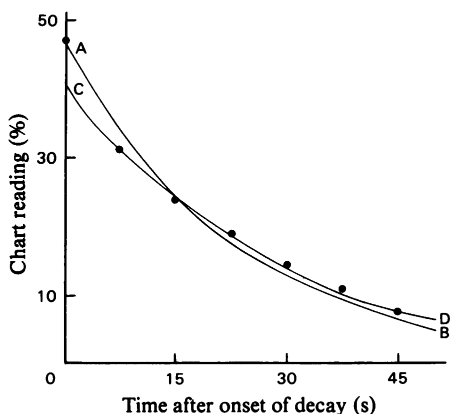


Fig. 9. *Curve analysis (Method I)*
AB is the best single exponential curve obtained by using all data points. CD is the best single exponential curve obtained by using all data points except the first (intercept) point. Chi-square test: (+) intercept A > intercept C; (−) intercept A < intercept C.

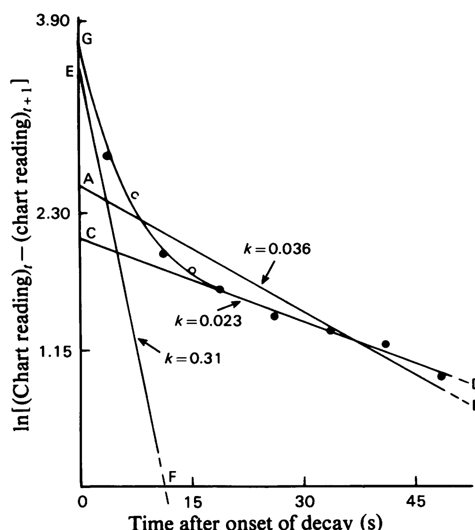


Fig. 10. *Curve analysis (Method II)*
AB is the best-fit regression line through all data points. CD is the regression line for the slow component of decay obtained from data points at $t > 15$ s. EF is the regression line for the fast component of decay, obtained by calculation from curve CD and four data points ($t = 3.75$–15 s). GD is the summation curve (CD+EF). ●, Data points; ○, additional data points used in obtaining curve EF. t, $t+1$, refer to successive times, normally at 7.5 s intervals.

Table 2. *Anaerobic ΔH^+ decay after a small O_2 pulse*

A pulse of 40–50 μl of O_2-saturated 0.25 M-sucrose solution (approx. 0.1 μg-atom of O) was given to rat liver mitochondria, containing 30 mg of mitochondrial protein, suspended in 6 ml of anaerobic 0.25 M-sucrose solution containing 2 mM-sodium succinate. n, Number of curves analysed (number of separate mitochondrial preparations). Intercept distribution: (+), intercept from all data points > intercept from all points minus the first data point; (−), intercept from all data points < intercept from all points minus the first data point. k, First-order rate constant (±S.D.) Abbreviation: N.S., not significant ($P > 0.1$).

Method	n	Intercept distribution (+)	Intercept distribution (−)	Chi-square test (P)	Mean k (s^{-1})	$t_{\frac{1}{2}}$ (s)
I	12 (10)	4	8	N.S.	0.101 ± 0.017	6.9
II	12 (10)	6	6	N.S.	0.097 ± 0.019	7.1
III	15 (12)	13	2	<0.01	0.085 ± 0.007	8.2

Table 3. *Anaerobic* ΔH^+ *decay after a small* O_2 *pulse given in the presence of added exogenous* Ca^{2+}
Conditions and abbreviations were as in Table 2; 6 μg of Ca^{2+} as $CaCl_2$ was added to the mitochondrial suspension during the pre-pulse anaerobic period.

Method	*n*	Intercept distribution (+)	Intercept distribution (−)	Chi-square test (*P*)	Decay (% of total)	Mean *k* (s^{-1})	$t_{\frac{1}{2}}$ (s)
I	12 (9)	12	0	⩽0.01	—	0.041 ± 0.009	16.9
II	12 (9)	12	0	⩽0.01	—	0.044 ± 0.008	14.1
II (fast component)	12 (9)	—	—	—	77.8	0.24 ± 0.10 (Range: 0.11–0.43)	2.9
II (slow component)	12 (9)	—	—	—	22.2	0.032 ± 0.008 (Range: 0.023–0.048)	21.7

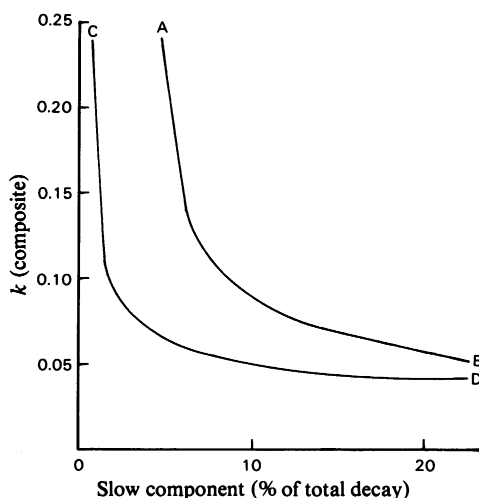


Fig. 11. *Analysis of the effect on the composite first-order rate constant, k, of increasing the proportion of the slow component of* ΔpH *decay while keeping the fast component constant*
The fast component is equal to a chart reading of 32.3%; the slow component varies from 0.2 to 9.2%. AB and CD give the relationships for decay to chart reading of 1.0 and 0.1% (see the text).

0.054; for decay to 0.1% [ln(reading) = −2.3], $t = 141.2$ s, k(composite) = 0.043. In either case, however, the amount of slow component required to decrease k from 0.24 to 0.097 is less than 10% of the total Ca^{2+} movement.

From this we conclude that in the small pulse experiments, when no Ca^{2+} was added (Table 2), decay was preponderantly due to a fast component, and that analysis by Methods I and II was not sufficiently sensitive to detect the complexity of the decay curve, but that when Ca^{2+} was added and the slow decay raised to 22.2% of the total decay, these Methods conformed with Method III to give clear evidence of biphasicity.

Mitchell & Moyle (1967*a*,*b*), using rat liver mitochondria in lightly buffered KCl/sucrose media and preincubation periods longer than those used in the present study, put forward the suggestion that ΔpH decay in the medium was due to a fast, electrically driven component ($t_{\frac{1}{2}} = 1$ s), and a slow, transmembrane-ΔpH-driven component with a time characteristic ($t_{\frac{1}{2}} = 87$ s) similar to that of acid-pulse decay ($t_{\frac{1}{2}} = 80$ s). Movements of protons rather than of Ca^{2+} were thus seen as the primary cause of anaerobic decay. This could not be true for our system, where there is no measurable electrical component caused by protons in the outer bulk phase, and where the pH-decay after an acid-pulse in anaerobic conditions was very slow ($t_{\frac{1}{2}} > 300$ s).

Small O_2 *pulses in the presence of N-ethylmaleimide* (*Fig.* 8*b*). In this series the inhibition of phosphate transport by *N*-ethylmaleimide caused an initial acidification, after an O_2 pulse, greater than that in the corresponding series without *N*-ethylmaleimide, and led to anaerobic decay curves which were due to uncomplicated Ca^{2+}/proton exchange. The decay showed no evidence of biphasic properties and the rate constants by all methods of analysis (Table 4) resembled the value for the slow-component decay given in Table 3. In these experiments the addition of exogenous Ca^{2+} to the media caused no change in k values. The results suggest that although Ca^{2+} uptake is unaffected by *N*-ethylmaleimide, the fast phase of the anaerobic decay is dependent on the presence of intact phosphate-carrier transport.

Large O_2 *pulses* (*Fig.* 8*c*). Large pulses give a decay curve under aerobic conditions. Despite their close similarity therefore, curves (*a*) and (*c*) in Fig. 8 are quite distinct and result from completely different activities. This is made clear by the effects of added *N*-ethylmaleimide on the characteristics of the two curves (Figs. 8*b* and 8*d*), and by the fact that in the absence of exogenous substrate the aerobic decay curve was eliminated. We consider that the ΔH^+ decay after a large O_2 pulse is therefore due to phosphate–hydroxyl exchange linked to succinate uptake (Chappell & Crofts, 1966; Chappell & Haarhoff, 1967; Mitchell & Moyle, 1967*a*,*b*; Papa *et al.*, 1970).

The decay gave evidence of biphasicity when analysed by Method III (Table 5), but this was not confirmed by the other Methods. In two experiments in which 6μg of Ca^{2+} was added to the medium, the rate constant for ΔH^+ decay fell to about 80% of the control values, a much smaller decrease than that found in the parallel experiments with small O_2 pulses (Tables 2 and 3). However, the factors influencing aerobic decay are potentially more complicated than those operating in the anaerobic state; for example the cross-over curves for ATP synthesis clearly indicate the presence of secondary effects induced by changes in external Ca^{2+} concentrations (Fig. 5). At this stage therefore we consider that the anion movements determining these aerobic decay curves offer no useful insights into the nature of primary proton transport.

Large O_2 pulses in the presence of N-ethylmaleimide (*Fig. 8d*). The Ca^{2+}/proton exchange responsible for the anaerobic decay in this series showed a very low rate constant and clear evidence of an initial lag period when analysed by all methods (Table 6). First-order analysis gave correlation coefficients much lower than in the other series examined (mean value −0.82), and it seems probable that decay in this series approached zero-order kinetics. These observations suggest that Ca^{2+} release in these experiments originated either from the matrix or from a membrane association of relatively low lability, and that the passage of the ion through the membrane, or from the matrix into the membrane, was a rate-limiting factor. The addition of exogenous Ca^{2+} to the media before pulsing slightly increased k values, but not sufficiently to equal the carrier rate constant ($0.032\,s^{-1}$) indicated by small-pulse experiments.

Discussion

The investigations reported here centre on the observations made when mitochondrial suspensions were incubated in the presence of cation-exchange resins (Figs. 6 and 7). The use of a dextran exchanger in the carboxymethyl form seems to have provided an uncomplicated extramitochondrial action which removed leaked Ca^{2+} from the outer solution and eliminated cation-compensated proton movements,

Table 4. *Anaerobic ΔH^+ decay after a small O_2 pulse given in the presence of N-ethylmaleimide*
Conditions and abbreviations were as in Table 2, with addition of 0.9μmol of *N*-ethylmaleimide in each experiment.

Method	n	Intercept distribution (+)	Intercept distribution (−)	Chi-square test (P)	Mean k (s^{-1})	$t_{\frac{1}{2}}$ (s)
I	12 (11)	7	5	N.S.	0.027 ± 0.008	25.7
II	12 (11)	6	6	N.S.	0.035 ± 0.008	19.8
III	37 (21)	23	14	N.S.	0.029 ± 0.004	23.9

Table 5. *Aerobic ΔH^+ decay after a large O_2 pulse*
A pulse of 200–400μl of O_2-saturated 0.25M-sucrose solution (approx. 0.4–0.8μg-atom of O) was given to rat liver mitochondria containing 30mg of mitochondrial protein suspended in 6ml of anaerobic 0.25M-sucrose solution containing 2mM-sodium succinate. Other details and abbreviations were as in Table 2.

Method	n	Intercept distribution (+)	Intercept distribution (−)	Chi-square test (P)	Mean k (s^{-1})	$t_{\frac{1}{2}}$ (s)
I	15 (13)	10	5	N.S.	0.081 ± 0.014	8.6
II	15 (13)	10	5	N.S.	0.084 ± 0.013	8.3
III	31 (24)	27	4	<0.01	0.078 ± 0.010	8.9

Table 6. *Anaerobic ΔH^+ decay after a large O_2 pulse given in the presence of N-ethylmaleimide*
Conditions and abbreviations were as in Table 5, with the addition of 0.9μmol of *N*-ethylmaleimide in each experiment. k values in this series were calculated after omitting the first (lag) data point (see the Results section).

Method	n	Intercept distribution (+)	Intercept distribution (−)	Chi-square test (P)	Mean k (s^{-1})	$t_{\frac{1}{2}}$ (s)
I	5 (5)	0	5	0.025	0.019 ± 0.006	36.5
II	5 (5)	0	5	0.025	0.012 ± 0.003	57.8
III	29 (16)	1	28	<0.01	0.017 ± 0.006	40.8

without interfering with electron-transport or oxidative-phosphorylation processes. The only modifying action that this exchanger had on the mitochondria themselves was shown in the marginal rate changes in synthesis (Fig. 5), which we believe arise from small differences in phosphate or substrate availability when Ca^{2+} uniport activity is in abeyance. Moyle & Mitchell (1977*b*) have presented evidence for a symport action of combined Ca^{2+} and phosphate uptake, and although this has not been confirmed (Chappell *et al.*, 1978), an indirect influence of Ca^{2+} uniport on the rate of phosphate uptake through phosphate–hydroxyl exchange could still underlie this cross-over effect.

The retention of full synthetic capacity was unaccompanied by the appearance of any manifest proton ejection, though calculation has shown that 1 ng of H^+ should be translocated for every mg of mitochondrial protein present (Mitchell & Moyle, 1967*b*; Mitchell, 1968). An equivalent acid pulse given under identical conditions was easily detected (Figs. 6 and 7). Various explanations might be given for this observation: that the value for proton translocation under steady-state, synthesizing conditions is an overestimate; or that an actual acidification is being masked by some other transport activity involving H^+ or OH^- movement. But even if either or both of these explanations were true, the chemiosmotic view that protons move into the bulk phase during synthesis and develop a bulk-phase protonmotive force remains a claim based purely on inference.

Our own explanation (Archbold *et al.*, 1974, 1975*a*,*b*,*c*, 1976) has been that the presence of protons in the medium is only associated with counter-cation movements, and that when permeant cations, other than protons, have reached a steady state and synthesis begins, proton movements are from that moment restricted absolutely to the surface of the inner membrane. Their retention within space-charge (ρ-zone) areas results from the continued masking and unmasking of negative fixed charges on the membrane surface during energization, the protons being held within the electrical field of these charges. The effective protonmotive force is Δp^{ρ}, which is greater than Δp. When Ca^{2+} is present in the medium its uptake involves the neutralization of these negative fields, and protons are in consequence free to disperse into the bulk phase. This would be a transient, 'saturated' process, quantitatively defined at all times during Ca^{2+} uptake by the fixed charge capacity of the membrane. Only when this electrophoretic Ca^{2+} uptake is complete will the fixed charges exert a retentive influence on translocated protons.

These views derive support from the theoretical studies of Mauro (1962) and Coster (1965, 1973) on the properties of fixed-charge membranes, and from subsequent demonstrations of negative fixed-charge development in mitochondria on energization (Kamo *et al.*, 1976; Aiuchi *et al.*, 1977; Quintanilha & Packer, 1977). It can be easily shown that if the ρ-zone interpretation of proton movement is correct, the rationales for both $\Delta\psi$ and ΔpH must lead to incorrect values for the effective protonmotive force. The artificiality of the values (Mitchell & Moyle, 1969; Nicholls, 1974) results from the fact that proton concentrations which refer only to the ρ-zone are interpreted in terms of changes taking place in the bulk phase. According to the ρ-zone view the membrane potential ($\Delta\psi$) measured from medium to matrix should be a typical Nernst potential (Archbold *et al.*, 1974). Kinnally *et al.* (1978) have confirmed this by direct measurement in giant mitochondria; however, these authors then relate the changes that they find in the fluorescence of membrane-potential-sensitive dyes to K^+-diffusion potentials in the presence of valinomycin. In this way their calibration, too, is in terms of $\Delta\psi$ rather than $\Delta\psi^{\rho}$ and must, in our view, lead to underestimated values of the membrane potential which is contributing to the protonmotive force.

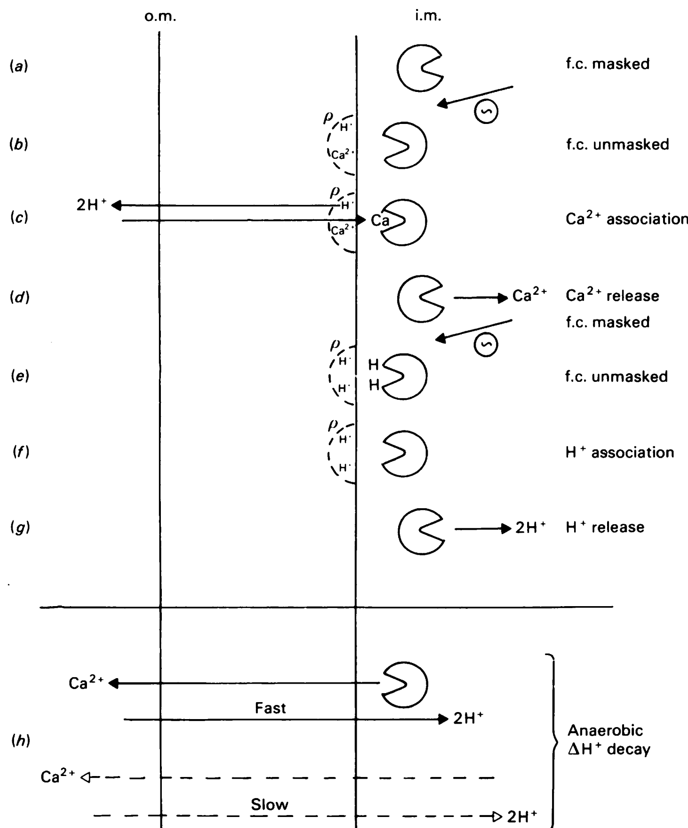
In order to test the ρ-zone interpretation further we analysed O_2-pulse decay curves with the following considerations in mind: (i) when an O_2 pulse is given to an anaerobic suspension of mitochondria there will be two forms of Ca^{2+} uptake: by electrostatic association with the negative fixed charges of energization, and by a conventional Ca^{2+} facilitated transport carrier. The first will be very fast; the second relatively slow. (ii) If anaerobiosis intervenes during the process of Ca^{2+} uptake the fixed charges will disappear and there will be two forms of Ca^{2+} release: a limited fast release of Ca^{2+} at that moment in association with the fixed charges, and a slow release of Ca^{2+} through the carrier. (iii) The extent of the fast release will be a measure of the fixed-charge capacity of the inner membrane. (iv) If anaerobiosis occurs after a steady state has been reached in cation movement, and when ATP synthesis has begun, Ca^{2+} release will be solely through the slow carrier system. (v) Ca^{2+} uptake by fixed-charge association is a rapid 'flow-through' mechanism, since fixed-charge renewal must be a continuing process kinetically determined by, and linked to, electron transport and proton translocation.

The results obtained by our analyses lend support to these considerations. First, both fast and slow ΔpH-decay rates have been shown (Table 3; Fig. 10), which we interpret as being caused by fast and slow modes of Ca^{2+} release. Secondly the composite k value for decay curves after a small O_2 pulse (mean 0.097; Table 2) is only reconcilable with the rate constants for fast and slow decay on the basis of a preponderance of the fast process (>90%; Fig. 11).

Thirdly, the addition of exogenous Ca^{2+} to the system before pulsing causes a fall in the composite rate constant (0.044; Table 3) owing to increased decay through the slow carrier system (22.2% of the total movement); this would not have happened if any significant increase in fixed-charge association, and therefore in the fast decay on anaerobiosis, had been possible.

There appear to be good grounds therefore for accepting the intercept value for fast decay, 32.3% of the chart span (Table 3), as representing saturation or near-saturation by Ca^{2+} of the fixed-charge capacity of the inner membrane. It is equivalent to an acid pulse of 150–200ng of H^+ in our basic system containing 30mg of mitochondrial protein. If we take the area of the inner mitochondrial membrane as $40m^2/g$ of mitochondrial protein (Mitchell, 1968) we obtain an estimate for the energized membrane of 1 charge/10.0–13.3nm^2. This is in close agreement with the surface charge of $-1.2\mu C/cm^2$ in State 4 given by Kamo *et al.* (1976) on the basis of cytopherometer experiments.

Scheme 1 (steps *a–e*) shows the cycle of changes that fixed-charge molecules might undergo during



Scheme 1. *Representation of the changes taking place in latent fixed-charge molecules on energization, and during anaerobic decay supervening during the phase of Ca^{2+} uptake*

(*a*) is a latent or masked fixed-charge molecule; on energization, (*b*) the negative fixed charges are unmasked and the developed electrical fields retain the translocated protons in space-charge areas (ρ-zones) at the membrane surface. (*c*) Ca^{2+} if present in the medium is preferentially taken up within the field of the fixed charges. Its transient, flow-through association with the fixed-charge molecules restores them to the masked form, (*d*); in this process Ca^{2+} exchanges with protons, which move out of the ρ-zones into the medium, (*c*). If Ca^{2+} is absent from the medium, or in a steady state across the membrane, protons will pass first into the ρ-zones (*e*) and then into fixed-charge association, (*f*). This represents the first stage in the utilization of the ρ-zone protonmotive force, Δp^{ρ}, in synthesis (Archbold *et al.*, 1976). The fixed-charge molecules will again be restored to their masked form by the proton association and the cycle will be repeated, (*g*). (*h*) represents the events after anaerobic decay supervening during Ca^{2+} uptake. Ca^{2+} release from fixed charge association into the medium is fast and quantitatively limited; slow release of ionic Ca^{2+} takes place through the carrier system. These forms of release are reflected in corresponding fast and slow proton uptake (see Fig. 10). Abbreviations: f.c., fixed charge; ρ, ρ-zone; i.m., inner membrane; o.m., outer membrane.

respiratory-dependent Ca^{2+} uptake interpreted in the light of the results given in the present paper. The molecular changes that must be involved in fixed-charge expression are promoted by energization and reversed by Ca^{2+} association; when Ca^{2+} uptake is complete the cycle of changes becomes the pattern for the ensuing uptake of protons from the ρ-zones into the energy-transduction pathway (Scheme 1, steps *e*, *f*, *g*). The paths of ΔH^+ decay after a small O_2 pulse are also shown (*h*). Phosphate would seem to have a role in the orientation of Ca^{2+} release within the membrane under anaerobic conditions (see the Results section).

A recognition of the importance of the development of membrane fixed charges in energy transduction is not part of the orthodox chemiosmotic view, but the evidence for their production and for their involvement in the energy-transduction processes of mitochondria is accumulating (Higuti *et al.*, 1978). Their presence, if confirmed, must have profound effects on many inner-membrane surface relationships, above all on the production of highly localized space-charge areas, and we believe that they should be a considered factor in any mechanistic interpretation of proton movements in relation to ATP synthesis.

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