Energy-Dependent Effects on the Oxidation–Reduction Midpoint Potentials of the *b* and *c* Cytochromes in Phosphorylating Submitochondrial Particles from Pigeon Heart†

J. Gordon Lindsay,* P. Leslie Dutton, and David F. Wilson

ABSTRACT: A new procedure for the preparation of phosphorylating submitochondrial particles from pigeon heart mitochondria is described. The biochemical properties of these particles indicate that they are very similar to those prepared by more conventional techniques. Redox titrations of the *b* cytochromes in these particles reveal the presence of a third *b*-type cytochrome which is not detectable in intact pigeon heart mitochondria. It has a λ_{max} in the α band at approximately 558 nm and an $E_{m7.2}$ of +120 mV. The midpoint potentials of cytochromes b_K, b_T, and $c + c_1$ are the same as observed in intact mitochondria being $+30 \pm 10$, -30 ± 10, and $+235 \pm 10$ mV, respectively, at pH 7.2. In contrast to the situation in pigeon heart mitochondria, in submitochondrial particles only 40–50% of the cytochrome b_T complement assumes a more positive midpoint potential ($E_{m7.2} = +240$ mV) in the presence of ATP. Addition of ATP has no effect on the midpoint potentials of the other *b* cytochromes or of cytochromes $c + c_1$. These results are discussed in terms of current ideas on the organization of the respiratory chain in intact mitochondria and submitochondrial particles.

Isolated pigeon heart mitochondria contain high levels of the various cytochrome components, have a low rate of endogenous substrate respiration and remain tightly coupled for relatively long periods of time (1–2 days). These properties make them particularly suitable for a wide variety of biochemical studies. In this laboratory pigeon heart mitochondria have been employed extensively in kinetic studies of the respiratory chain components (Chance and Erecinska, 1971; Boveris *et al.*, 1972) and in problems related to the mechanism of energy conservation (Wilson and Dutton, 1971).

A systematic survey of the midpoint potentials of the individual cytochromes has recently been carried out by Wilson and Dutton (Dutton, 1970; Dutton *et al.*, 1970). Their technique has been to perform simultaneous oxidation–reduction potential–absorbance titrations using dyes to mediate between the membrane-bound cytochromes and the platinum electrode. Studies of this type in pigeon heart mitochondria have revealed the presence of two *b*-type cytochromes. These have been termed cytochromes b_K and b_T having oxidation–reduction midpoint potentials at pH 7.2 ($E_{m7.2}$)[1] of $+30 \pm 10$ and -30 ± 10 mV, respectively. In the presence of ATP cytochrome b_T assumes a midpoint potential of +240 mV while the midpoint of cytochrome b_K is unaltered (Wilson and Dutton, 1970). The two *b* cytochromes have also been defined on the basis of their differing kinetic behavior (Chance *et al.*, 1970) and spectral properties (Sato *et al.*, 1971). The approximate λ_{max} for the α-band of reduced b_K is at 561–562 nm and that of cytochrome b_T is at 564–565 nm at room temperature.

In summary, therefore, the low-potential, long-wavelength *b* component is energy dependent (E_m becoming more positive with increasing phosphate potential) and may be the primary energy-transducing component at site II of the mitochondrial respiratory chain.

In this communication we have extended our original observations to a study of phosphorylating submitochondrial particles. The first section of this communication is devoted to a description of the properties of submitochondrial particles isolated from pigeon heart mitochondria using a new gas pressure device. The employment of PH-SMP for this study has enabled us to make a direct comparison to pigeon heart mitochondria in which the thermodynamic parameters of the cytochromes are already well defined. The second part of the paper lays emphasis on the spectral properties of the *b* cytochromes in the presence and absence of ATP and on the quantitation of the ATP effects on the midpoint potentials of the *b* and *c* cytochromes. A previous report (Dutton *et al.*, 1971) has shown that the behavior of the *b* and *c* cytochromes in coupled beef heart submitochondrial particles is qualitatively similar to that observed in intact mitochondria.

Materials and Methods

Pigeon heart mitochondria were prepared by the method of Chance and Hagihara (1963). The basic procedure for preparation of PH-SMP involves disruption of the mitochondria by extrusion from the outlet valve of a Yeda press (Yeda Research and Development Corp., Rehovot, Israel) under a pressure of 2,000 psi of nitrogen. Details of the press and its operation have been reported previously by Shneyour and Avron (1970). The advantages of this method appear to be (a) the employment of an inert gas, *e.g.*, nitrogen or argon and (b) the use of a constant controlled pressure to bring about disruption.

Pigeon heart mitochondria (approximately 5 mg of protein/ml) were suspended for 10 min at 0–4° in a hypotonic medium

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[1] Abbreviations used are: SMP and PH-SMP, submitochondrial particles and submitochondrial particles from pigeon heart, respectively; PMS, phenazine methosulfate; MS-MOPS buffer is 0.225 M mannitol–0.05 M sucrose containing 0.05 M morpholinopropanesulfonate buffer adjusted to the indicated pH with KOH; $E_{m7.2}$ is the midpoint potential of an oxidation–reduction couple at pH 7.2; *n* is the number of electrons transferred in the oxidation–reduction reaction.

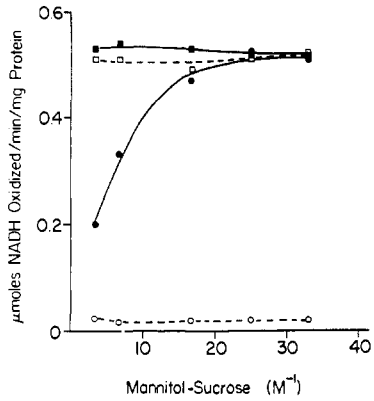

FIGURE 1: NADH oxidase activity of pigeon heart mitochondrial preparations after disruption at various mannitol–sucrose concentrations. The mitochondria were disrupted as described in Materials and Methods using various dilutions of 0.3 M mannitol–sucrose to regulate the tonicity of the medium. Control samples were treated in an identical manner except that they were not passed through the Yeda press. After disruption all samples were made 0.3 M with respect to mannitol–sucrose concentration to prevent osmotic breakage of intact mitochondria. The assay mixture consisted of MS-MOPS buffer adjusted to pH 7.4 with KOH, 5 mM $MgSO_4$, 0.33 mM NADH, and 0.2 mg of particle protein. The temperature was 20° and the final assay volume, 3 ml. NADH disappearance was followed by measuring the decrease in absorbance at 340 nm. (●) Pressure disrupted mitochondria; (○) control mitochondria; (■) pressure disrupted mitochondria treated with 0.5% sodium cholate; (□) control mitochondria treated with 0.5% sodium cholate.

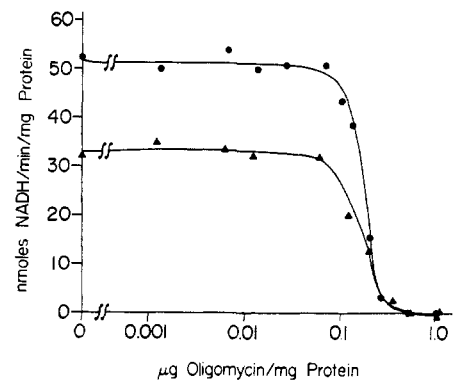

FIGURE 2: The effect of oligomycin on the rate of reversed electron transport in PH-SMP. The assays were performed in MS-MOPS buffer (pH 7.4) using the conditions described by Lee and Ernster (1965). For ascorbate–PMS-linked reduction of NAD^+, ascorbate was present at a final concentration of 5 mM and PMS at 3 μM. There was 0.4 mg of particle protein/assay and the temperature was 20°. (●) ATP-supported succinate-linked reduction of NAD^+; (▲) ATP-supported ascorbate–PMS-linked reduction of NAD^+.

consisting of 0.023 M mannitol–0.007 M sucrose (0.03 M mannitol–sucrose), 3 mM $MgSO_4$, 5 mg/ml of crystalline bovine serum albumin, and 100 μM NaEDTA in 5 mM morpholinopropanesulfonate buffer (pH 7.4). Immediately before disruption ATP was added to give a final concentration of 3 mM. The mitochondria were then disrupted by extrusion from the outlet valve of the press under a pressure of 2000 psi of nitrogen, the outlet valve being regulated to give a flow rate of 2–5 drops/sec. Disrupted material was centrifuged at 105,000*g* for 20 min and the pellet resuspended in a solution of 0.22 M mannitol, 0.05 M sucrose, 2 mM $MgSO_4$, and 5 mM morpholinopropanesulfonate buffer (pH 7.4) and recentrifuged as before. The particles were finally suspended in the above medium in the absence of Mg^{2+} at 15–20 mg of protein/ml. The yield was 40–50% of the original mitochondrial protein.

The procedures and equipment for the determination of the oxidation–reduction midpoint potentials of the cytochromes have previously been described in detail (Dutton, 1970; Dutton *et al.*, 1970).

The redox potential of the system was made more electronegative with a freshly prepared dilute solution of dithionite and more electropositive with a 100 mM solution of potassium ferricyanide.

All reagents employed were of the highest grade available commercially.

Results

Preparation of PH-SMP. The degree of breakage of pigeon heart mitochondria at various mannitol–sucrose concentrations was estimated by measuring the NADH oxidase activity of the disrupted preparations (Figure 1). Intact mitochondria are impermeable to NADH and therefore exhibit little or no NADH oxidase activity (Ernster, 1959). Under isotonic conditions only about 40% of the mitochondria were broken after passage through the press and it was necessary to lower the mannitol–sucrose concentration to 0.05 M or less before breakage was complete. The amount of breakage at a given tonicity was found to be highly reproducible from batch to batch.

Control mitochondria which were subjected to hypotonic conditions but not passed through the press showed no increase in NADH oxidase activity. Indeed treatment of the mitochondria with 2000 psi of nitrogen without subsequently extruding them *via* the needle valve did not cause their disruption. To check that breakage was complete both intact mitochondria and disrupted preparations were treated with 0.5% sodium cholate (Figure 1). The maximal levels of NADH oxidase activity obtained were identical with those found for mitochondria disrupted at low tonicity using the Yeda press, indicating that breakage was 100% under these conditions.

A spectral comparison of PH-SMP and pigeon heart mitochondria (not shown) reveals that there is a 20–30% loss of cytochrome *c* during preparation of the particles; otherwise the spectra are very similar. Thus cytochrome *c* depletion of these SMP is much less than for the corresponding sonicated preparations where about 80% of the cytochrome *c* is removed during isolation of the particles.

Energy-Linked Functions of PH-SMP. As shown in Figure 2, PH-SMP will catalyze ATP-supported succinate or ascorbate–PMS-linked reduction of NAD^+. The rates of reversed electron transport compare favorably with those of other phosphorylating SMP preparations (Löw and Vallin, 1963; Lee and Ernster, 1965). Magnesium ions are specifically required for the reaction. The reaction rate was independent of Mg^{2+} concentration in the range of 1–20 mM. In fresh preparations oligomycin did not stimulate either the succinate- or the ascorbate–PMS-mediated reactions while both reactions were inhibited by amounts of oligomycin above 0.1 μg of oligomycin/mg of protein.

In 1-day old PH-SMP (Figure 3) the rates of reversed electron transport are 50–75% of their original values and these can be restored to their previous levels by addition of 0.03–0.05 μg of oligomycin/mg of protein. The stimulating effects

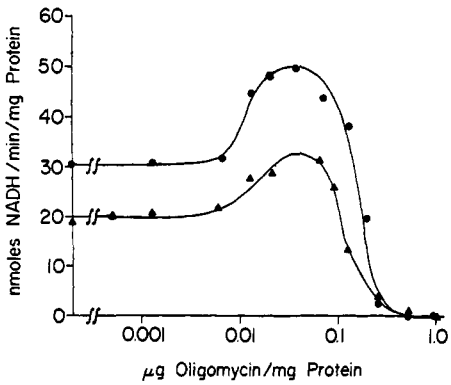

FIGURE 3: The effect of oligomycin on the rate of reversed electron transport in PH-SMP aged for 24 hr at 0–4°. The assay procedures were as described for Figure 2. (●) ATP-supported succinate-linked reduction of NAD^+; (▲) ATP-supported ascorbate–PMS-linked reduction of NAD^+.

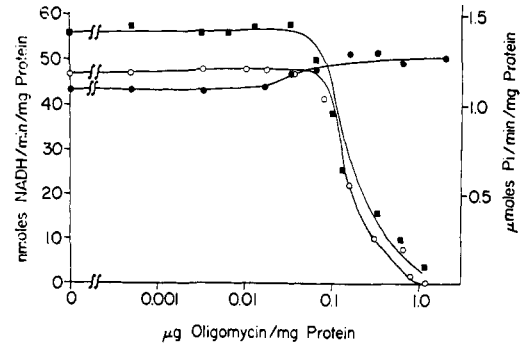

FIGURE 4: The effects of oligomycin on the succinooxidase or ATP supported reduction of $NADP^+$ by NADH in PH-SMP. The transhydrogenase assay was performed at 20° in MS-MOPS buffer (pH 7.4) using the conditions of Lee and Ernster (1965). The course of inhibition of oligomycin on the ATPase activity is included for comparison. The ATPase assay was carried out at 30° as described by Lee and Ernster (1965). (●) Succinooxidase-driven transhydrogenase reaction; (○) ATP-driven transhydrogenase reaction; (■) ATPase activity.

of oligomycin in aged PH-SMP suggest that the reduction in energy-linked function may be due to a loss of coupling factors from the particles during storage (Racker and Horstmann, 1967). The amount of oligomycin required to restore maximal activity is considerably less than that reported for EDTA-treated SMP from beef heart (Lee and Ernster, 1965) which are completely dependent on the addition of oligomycin to catalyze ATP formation. Presumably in aged PH-SMP there is only a small loss of coupling factors and the amount of oligomycin required to restore full activity is correspondingly less. In both fresh and aged SMP levels of oligomycin above 0.1 μg/mg of protein are inhibitory to the ATP-driven reversal of electron transport. This value is close to that found for other SMP preparations (Löw and Vallin, 1963; Lee and Ernster, 1965).

In common with other SMP systems, PH-SMP support an energy-linked reduction of $NADP^+$ by NADH—the so-called transhydrogenase reaction. The reaction can be driven by high-energy intermediates formed by substrate oxidation in nonphosphorylating preparations or by ATP in coupled SMP (Lee and Ernster, 1965). A comparison of the succinate and ATP-driven reactions in fresh PH-SMP is shown in Figure 4. There is little or no stimulation of either reaction by low concentrations of oligomycin. However, the ATP-supported reduction of $NADP^+$ by NADH is sensitive to oligomycin in agreement with the known action of the inhibitor on the mitochondrial ATPase. Oligomycin does not inhibit the transhydrogenase reaction when it is driven by nonphosphorylated high-energy intermediates generated by succinate oxidation, a reaction which does not require ATP formation. The course of inhibition of the mitochondrial ATPase by increasing amounts of oligomycin is also shown in Figure 4 to indicate its close correlation with the inhibition of the ATP-driven energy-linked reactions.

P:O Ratios in PH-SMP. The phosphorylation capacity of PH-SMP was measured by estimation of the P:O ratios utilizing succinate and NADH as substrates (Figure 5). The values obtained were 0.7–0.8 for succinate oxidation and 1.0–1.2 for NADH oxidation, in close agreement with reported values for MgATP particles prepared by sonication procedures from beef heart mitochondria. Phosphorylation in PH-SMP (like reverse electron transport) was dependent on the presence of Mg^{2+} but was independent of the Mg^{2+} concentration in the range 1–20 mM Mg^{2+}. In contrast, in oligomycin-treated EDTA particles from beef heart, oxidative phosphorylation was found to be most efficient in the presence of 2 mM Mg^{2+} and was progressively inhibited at higher concentrations (Lee and Ernster, 1966). A possible explanation for this difference centers on the ability of Mg^{2+} to prevent the oilgomycin-induced respiratory control in EDTA particles, suggesting that Mg^{2+} may be antagonistic to the action of oligomycin in restoring oxidative phosphorylation to these SMP.

Redox Titrations in Coupled PH-SMP. Potentiometric titrations of the *b* cytochromes in intact pigeon heart mitochondria have revealed the presence of only two components: cytochrome b_K ($E_{m7.2}$ = +30 mV) and cytochrome b_T ($E_{m7.2}$ = −30 mV). Figure 6A shows the results of a similar experiment carried out with PH-SMP and part B of this figure resolves the titration into three components. In addition to cytochrome b_K ($E_{m7.2}$ = +40 mV) and cytochrome b_T ($E_{m7.2}$ = −30 mV), a third component is seen which has an $E_{m7.2}$ of +120 mV. Using the wavelength pair 561.5–575 nm this component accounts for 15–25% of the total

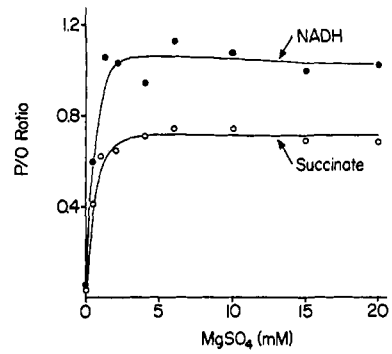

FIGURE 5: P:O ratios in PH-SMP using succinate and NADH as substrates. Assays were performed at 25° using the following reaction medium: 0.5 M mannitol, 50 mM Tris-acetate buffer (pH 7.5), 1 mM ATP, 30 mM glucose, 100 μM NaEDTA, 1.0 mg/ml of bovine serum albumin, 15 mM [^{32}P]P_i (1.5×10^7 cpm/μmole, 75 units of hexokinase, and 0.5 mg of particle protein in a final volume of 2 ml. The substrate was either 0.2 mM NAD^+, 50 mM ethanol, and 20 μg of alcohol dehydrogenase (10 mM semicarbazide-HCl present) (●) or 5 mM sodium succinate (○). Oxygen consumption was measured with a Clark electrode and esterification of P_i was determined by the isotope distribution technique of Conover *et al.* (1963).

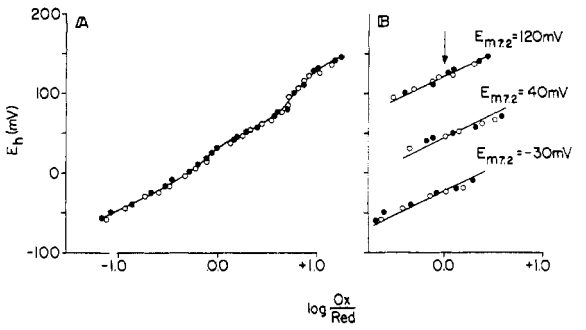

FIGURE 6: The course of oxidation–reduction of the *b* cytochromes in PH-SMP. The SMP (2.6 mg of protein/ml) were suspended under an atmosphere of argon (<1 ppm of O_2) in MS-MOPS buffer (pH 7.2). The following redox mediators were employed: 20 μM diaminodurol, 40 μM each of PMS, phenazine ethosulfate, and duroquinone, 6 μM pyocyanine and 15 μM 2-hydroxy-1-4-naphthoquinone. The measuring wavelengths were 561.5–575 nm. Part A shows the logarithm of oxidized to reduced cytochrome plotted for the total absorbance change from +300 to −180 mV. Both oxidative (○) and reductive (●) titrations were performed. In part B the curve is resolved arithmetically into three components; theoretical $n = 1$ lines are drawn through the points.

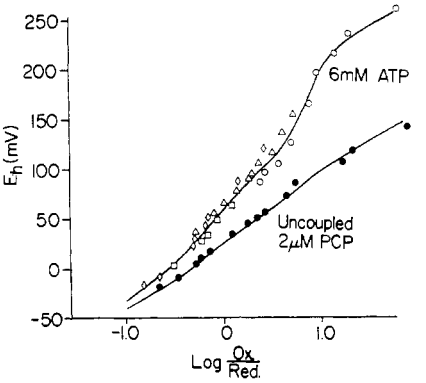

FIGURE 8: The effect of ATP on the oxidation–reduction midpoint potentials of the *b* cytochromes in PH-SMP. The SMP (2.4 mg of protein/ml) were employed in the same system as described in Figure 6 except that 6 mM $MgSO_4$ was added and the titrations were performed in the presence of 6 mM ATP (open symbols). Individual titrations were limited to 6 min. To cover the entire potential span from +300 to −180 mV several part titrations were carried out, each one covering a separate but overlapping potential range (open symbols). The closed circles were taken after uncoupling the ATP titration with 2 μM pentachlorphenol.

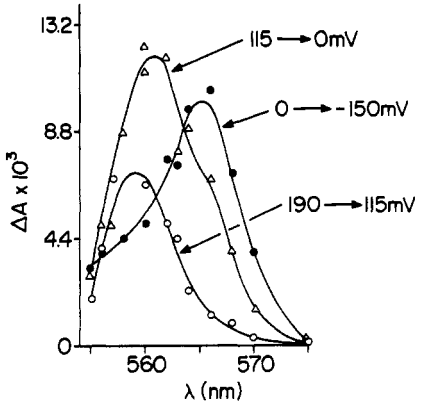

FIGURE 7: Spectra of the *b* cytochromes in PH-SMP. The SMP (2.4 mg of protein/ml) were maintained under the conditions described in Figure 6. The absorbance change occurring from +190 to +115 mV is cytochrome *b* with $E_{m7.2}$ + 120 mV (○); from +115 to 0 mV is mostly cytochrome b_K with $E_{m7.2}$ +40 mV (△) and from 0 to −150 mV is mainly due cytochrome b_T with $E_{m7.2}$ −30 mV (●). The absorbance changes are plotted as a function of wavelength using 575 nm as reference.

absorbance change. It seems unlikely that the appearance of this cytochrome in PH-SMP is an artifact of the isolation procedure as a similar high-potential *b* cytochrome has been detected in intact beef and chicken heart mitochondria and in beef heart SMP (Dutton *et al.*, 1970).[2] Rather it appears that failure to detect the third *b* cytochrome in pigeon heart mitochondria is accounted for by the presence of an interfering component which causes a decreased absorbance change in the titration as the redox potential of the system is reduced from 150 to 100 mV; on occasions a net decrease in absorbance was in fact observed over this potential range. The absence of this "masking" component in PH-SMP is possibly due to its soluble nature. Dye control experiments, performed in the absence of SMP, but otherwise employing the conditions of Figure 6, indicate that this additional component is not an artifact arising from a contribution to the absorbance by one of the redox mediators. The titration curve is also found to be independent of the concentration of added mediators (a fourfold range is routinely used).

Spectral Analysis of the b Cytochromes in PH-SMP. Figure 7 reveals the spectra of the three *b* cytochromes in PH-SMP as separated on a potential basis. The spectrum generated in the range of 0 to −150 mV is that of reduced cytochrome b_T as confirmed by its λ_{max} at 565 nm and a shoulder at 557 nm. Spectral analysis of the component reduced from +115 to 0 mV reveals that it is principally due to cytochrome b_K with a λ_{max} at 561 nm. The high potential *b* component (+190 to 115 mV) is also shown to be a *b*-type cytochrome having a λ_{max} of the reduced form at approximately 558 nm. Its relation to the electron-transport chain or the processes of energy conservation is unknown at present.

ATP-Induced Effects on the b Cytochromes. In Figure 8 the course of oxidation–reduction of the *b* cytochromes in PH-SMP is shown in the presence of 6 mM ATP. Under these conditions about 20% of the total absorbance is accounted for by a component which titrates with an $E_{m7.2}$ of +230 mV, characteristic of the high-energy form of cytochrome b_T. This component is not present if the titration is performed in the presence of 2 μM pentachlorophenol. The midpoint potentials of cytochrome b_K and the new *b* cytochrome appear to be unaffected by the presence of ATP, although resolution of these two components is difficult under these conditions. Only about half of the cytochrome b_T still titrates at −30 mV suggesting that approximately 50% has shifted to the high potential form.

Spectral analysis of the individual species (Figure 9) confirms these observations. The ATP-induced high-potential cytochrome *b* (reduced at +190 mV) has a λ_{max} in the reduced form at 565 nm, consistent with the idea that it is cytochrome b_T. For comparison the spectrum of the remaining b_T (0 to −150 mV) which does not respond to the addition of ATP is included. The spectrum generated from +190 to 0 mV is as expected for a combination of cytochrome b_K and the new *b* cytochrome (($E_{m7.2}$ +120 mV). Thus, in coupled PH-SMP it appears that only 40–50% of the cyto-

[2] J. G. Lindsay, P. L. Dutton, and D. F. Wilson, unpublished data.

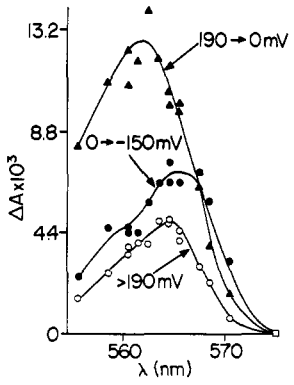

FIGURE 9: Spectra of the *b* cytochromes in PH-SMP in the presence of ATP. Experimental conditions were as described in Figure 8. The redox potential was set at +190 mV and 6 mM ATP added. The ATP induced reduction at this potential (○) is the high-energy form of cytochrome b_T ($E_{m7.2}$ +240 mV). Reduction from +190 to 0 mV (▲) represents principally cytochrome b_K but also contains a contribution from the cytochrome *b* with $E_{m7.2}$ +120 mV. Absorbance changes from 0 to −180 mV represent principally cytochrome b_T (●) which is not affected by the presence of ATP.

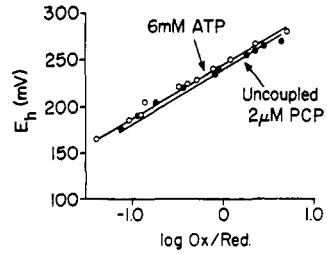

FIGURE 10: The effect of ATP on the oxidation–reduction midpoint potentials of cytochromes $c + c_1$ in PH-SMP. The experimental conditions were as described in Figure 6. The open circles represent a reductive titration carried out in the presence of 6 mM ATP. The closed circles represent a reductive titration after the effect of ATP had been uncoupled with 2 μM pentachlorophenol. Theoretical n = 1 lines are drawn through the points.

chrome b_T complement is "energizable" by the presence of ATP.

Titrations of cytochromes $c + c_1$ (550–540 nm) in the presence and absence of ATP indicate that ATP does not significantly affect the midpoint potentials of these cytochromes (Figure 10). The $E_{m7.2}$ for these cytochromes was +235 ± 10 mV, identical with the value previously obtained for intact pigeon heart mitochondria. Hinkle and Mitchell (1970) have estimated the midpoint potentials of cytochromes $c + c_1$ to be 250 mV in rat liver mitochondria. This value is also unaltered by the addition of ATP.

Discussion

Shneyour and Avron (1970) have reported that chloroplasts prepared from *Euglena gracilis* using the Yeda press retain a higher capacity for ATP synthesis than preparations isolated by more conventional procedures. Their data suggest that this technique may prove generally applicable to the preparation of subcellular fractions with high biological activity. Two factors appear to be important in obtaining consistently good preparations: (1) the maintenance of a constant pressure during breakage allowing controlled disruption of the material and (2) the employment of inert gases to bring about disruption.

The data presented in this paper indicate that the technique has been successfully adapted to the preparation of phosphorylating SMP from pigeon heart mitochondria. Their ability to carry out oxidative phosphorylation and other associated energy-linked functions compares favorably to previous data for MgATP particles prepared by sonication. In many respects the properties of the two types of preparation indicate that their overall morphology is very similar. For instance, the high NADH oxidase activity of PH-SMP and the fluorescence changes observed on energization in the presence of 8-anilino-1-napthalenesulfonate (not shown) are also typical of sonicated particles, indicating the "inside-out" nature of PH-SMP with respect to intact mitochondria. PH-SMP have proved particularly useful in redox titration studies. The good yields obtained and the high cytochrome content of the particles have simplified our comparative studies in relation to previous work with pigeon heart mitochondria. Anaerobic sampling techniques have also allowed redox titrations of PH-SMP to be followed by electron paramagnetic resonance spectroscopy at liquid helium temperatures. In this manner it has been possible to identify signals associated with some of the individual heme groups and to obtain midpoint potentials in close agreement with those obtained by optical methods (Wilson *et al.*, 1972).

Redox Titrations. Redox titrations of the *b* cytochromes in PH-SMP have revealed the presence of a third *b*-type cytochrome in addition to the two previously recognized components, cytochrome b_K ($E_{m7.2}$ = +30 mV) and cytochrome b_T ($E_{m7.2}$ = −30 mV). Spectral analysis of the individual species confirms that cytochrome b_K has a λ_{max} at 561–562 nm and cytochrome b_T at 565–566 nm. The new component is a high potential *b* cytochrome ($E_{m7.2}$ = +120 mV) which has a λ_{max} at about 558 nm and accounts for 15–25% of the total absorbance using the wavelength pair 561.5–575 nm. Failure to detect this component in pigeon heart mitochondria appears to be the result of spectral interference by an unknown component which is lost during preparation of the particles.

The principal difference between these results and those obtained with intact mitochondria is that only 40–50% of the cytochrome b_T is "energized" by the addition of ATP. However, the midpoint potential shift is the same as that observed in intact mitochondria (−30 to +240 mV). Confirmation of these findings has been achieved by an analysis of the ATP effects on the midpoint potentials of cytochromes *a* and a_3 in PH-SMP.[2] Again only 50% or less of the total cytochrome a_3 ($E_{m7.2}$ +350 mV) is shifted to a lower midpoint potential ($E_{m7.2}$ approximately +130 mV) in the presence of ATP. The midpoint potential of cytochrome *a* ($E_{m7.2}$ +220 mV) is the same in both pigeon heart mitochondria and PH-SMP and is not greatly affected by ATP.

In summary, therefore, these findings verify (1) the ATP-induced changes on the midpoint potentials of cytochromes b_T and a_3, (2) the insensitivity of the midpoint potentials of cytochromes b_K, $c + c_1$ and *a* to a state of energization induced by ATP, (3) the long-wavelength nature of cytochrome b_T in the presence or absence of ATP and (4) identify a third *b* component ($E_{m7.2}$ +120 mV) with a reduced λ_{max} in the α-band at 558 nm whose function is unknown.

The observation that only 40–50% of cytochromes b_T and a_3 respond to ATP addition in PH-SMP is consistent with other studies on SMP prepared by sonication procedures. Chance *et al.* (1970) have demonstrated that only about

50% of the respiratory chain components are accessible to oxidation by the ferricyanide anion or by a cytochrome *c* peroxidase–H_2O_2 complex. In addition, it has been reported that SMP contain both phosphorylating and nonphosphorylating respiratory chains (Lee *et al.*, 1967, 1969). These results have been interpreted to suggest that membrane "scrambling" occurs as a consequence of the intense cavitation during sonication of the mitochondria (Chance *et al.*, 1970). Our data, indicating that a similar behavior is observed in PH-SMP prepared by a very different technique, appears to render the membrane "scrambling" hypothesis less tenable. Further studies should be directed towards an understanding of the structural organization of SMP in terms of the interactions of individual membrane fragments following disruption of the mitochondria.

In connection with the midpoint potential shifts of cytochromes b_T and a_3 in response to the addition of ATP, Hinkle and Mitchell (1970) have reported that cytochrome a_3 (and to a lesser extent cytochrome *a*) assume more negative midpoint potentials on energization of rat liver mitochondria. Mitchell (1968) has previously postulated that an ATP-induced membrane potential will cause an apparent change in the midpoint potential of any cytochrome not in direct electrical contact with the outer aqueous phase. Thus, it is supposed that there is a net negative charge on the inside of the mitochondrial vesicle during ATP hydrolysis. This would tend to favor oxidation of any component situated on the inside of the inner membrane, *e.g.*, cytochrome a_3 which might then assume an apparently more negative midpoint under these conditions. The data of Hinkle and Mitchell are consistent with this idea although the ATP-induced shifts measured by them are small (40–50 mV) compared to the changes observed for cytochromes b_T and a_3.

In general, the results presented in this communication cannot be explained readily on the basis of apparent midpoint potential changes generated by an ATP-induced transmembrane potential. The polarity of this potential is postulated to be reversed in SMP which are "inside-out" and the rates of interaction of the redox mediators with the respiratory chain components are drastically altered. The demonstration that the ATP-induced midpoint potential changes are quantitatively the same as observed in pigeon heart mitochondria would suggest (a) that these changes are not the result of imposed membrane potentials and (b) the measured midpoint potentials are not dependent on the rates of interaction of the various dyes with the individual cytochromes. In mitochondria, cytochrome a_3 but not b_T responds as expected if these components are not in direct electrical contact with the outer aqueous phase. However, the unaltered behavior of these components in "inside-out" SMP suggests that, under our conditions, (*i.e.*, employing redox mediators to promote a state of equilibrium between the membrane-bound cytochromes and the bulk aqueous phase) an ATP-induced membrane potential is not relevant to the chemical events of the energy conservation mechanism. Furthermore, in neither preparation is their any effect of ATP on the midpoint potentials of cytochromes b_K, $c + c_1$, or *a*.

It should be added that while efforts have been made to eliminate the possibility that apparent midpoint potential shifts are a result of "reversed electron flow," this possibility cannot be entirely ruled out. Recent studies on the solubilized succinate–cytochrome *c* reductase (Wilson *et al.*, 1971) and isolated b–c_1 complexes (Rieske, 1971), however, have provided independent evidence for a direct role of cytochrome b_T in energy transduction at site II of the mitochondrial respiratory chain. Preliminary data also indicate that the high-energy reduced form of cytochrome a_3 may have slightly differing spectral properties.[2] In this connection, Wikström and Saris (1967) have previously reported an energy-linked alteration in the spectrum of reduced a_3.

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Studies on the Role of Mg^{2+} and the Mg^{2+}-Stimulated Adenosine Triphosphatase in Oxidative Phosphorylation†

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ABSTRACT: When heart mitochondria were incubated with NAD-linked substrates at pH 7.4, added $MgCl_2$ markedly reduced the respiratory control and P:O ratios. The magnitude of the effect of Mg^{2+} on the P:O ratio was a direct function of the ratio of ATP:ADP present in the suspending medium. Added Mg^{2+} stimulated "state 4" respiration in a transcient and biphasic manner. This response was completely blocked by oligomycin or atractyloside, and markedly reduced by aurovertin, an ADP trap (phospho*enol*pyruvate *plus* pyruvic kinase), or by the absence of inorganic phosphate. The amount of oxygen consumed during the initial rapid phase of respiration was proportional to the amount of adenine nucleotide present, but was independent of the concentrations of added Mg^{2+} or mitochondrial protein. The rapid phase of Mg^{2+}-stimulated respiration was accompanied by an immediate and marked dephosphorylation of ATP to ADP, followed by a much more gradual increase in the ratio of ATP to ADP in the suspending medium. In contrast, the intramitochondrial ATP:ADP ratio was unaffected by Mg^{2+}. The Mg^{2+}-ATPase was blocked by oligomycin but was only partially arrested by aurovertin and was insensitive to atractyloside. It is concluded that all of the effects of Mg^{2+} reported herein are mainly, if not wholly, accounted for by the Mg^{2+}-stimulated ATPase activity which ensues simultaneously with oxidative phosphorylation. This activity appears to be regulated, in turn, by the ATP:ADP ratio. This ATPase, which is in all respects inert when no Mg^{2+} is added, and which functions as an "external" ATPase in the presence of Mg^{2+}, is probably identical with the ATPase-coupling factor of oxidative phosphorylation which is present as a contaminant of fully intact mitochondria. The failure of uncouplers to reverse the inhibition of the oxidation of α-oxoglutarate or glutamate by mitochondria incubated in the absence of Mg^{2+} is interpreted to be due not to inhibition of nucleoside diphosphate kinase or of succinyl-CoA synthetase, since the activities of the isolated enzymes were not affected by oligomycin. Rather, it is suggested that depletion of extramatrix Mg^{2+} is responsible for failure of nucleoside diphosphate kinase to furnish GDP required for respiration under these conditions.

Early reports of studies on isolated heart sarcosomes (see *e.g.*, Cleland and Slater, 1953; Maley and Plaut, 1953; Chance and Baltschefsky, 1958; Harman and Fiegelson, 1952) described them as "loosely coupled" due to the presence of adenosine triphosphatase (ATPase) activities. These preparations differed from "tightly coupled" mitochondria in that rapid respiration was not dependent on the presence of added ADP. Packer (1957, 1958) later reported that heart sarcosomes in sucrose containing EDTA were tightly coupled, if they were incubated in a medium without added magnesium ion. He also reported that the measurable endogenous ATPase of these sarcosomes was very low, and that it was markedly stimulated by Mg^{2+} or Ca^{2+} (Packer, 1958). The phosphorylation efficiency (ADP:O ratio) was decreased when Mg^{2+} was added.

One of us (Davis, 1965a) reported that acetate is oxidized rapidly by tightly coupled heart sarcosomes prepared in EDTA, but that if Mg^{2+} and 2,4-dinitrophenol were included in the incubation mixture, acetate was not oxidized. These results were taken as indicative that the Mg^{2+}-activated mitochondrial ATPase prevented acetate activation. There is convincing evidence that bound Mg^{2+} is absolutely required to sustain oxidative phosphorylation (see, *e.g.*, Kielley and Bronk, 1957; Pullman *et al.*, 1960; Lee and Ernster, 1966). However, the energy-coupling mechanism is very efficient in intact mitochondria which are prepared in the presence of a chelating agent and are incubated in the absence of added divalent metal ions. The demonstrable endogenous ATPase is quite low in these mitochondrial preparations and is markedly stimulated by added Mg^{2+}.

Davis (1965b) reported that uncouplers of oxidative phosphorylation failed to reverse the inhibition by oligomycin of α-oxoglutarate or glutamate oxidation by heart mitochondria prepared in sucrose containing EDTA, and incubated in the absence of added Mg^{2+}. The latter prevented, but did not reverse this unexpected effect. No satisfactory explanation has been given for this observation.

The oligomycin-sensitive portion of the ADP–ATP ex-

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