specific activity is eight- to ten-fold lower than that in liver microsomes. This enzyme can be utilized therefore as a marker for microsomal contamination in mitochondria. Rotenone-insensitive NADH-cytochrome *c* reductase has been found to be present in both mitochondrial and microsomal fractions at approximately the same specific activity, under conditions where glucose 6-phosphatase specific activity in mitochondria was at least 20-fold lower than in microsomes. Mitochondria could oxidize α -oxoglutarate, succinate, pyruvate, malate, citrate and isocitrate and could not oxidize β -hydroxybutyrate. Glutamate was oxidized only very slowly. P/O ratios obtained with these substrates were very close to those reported in the literature for this tissue (Cammer & Estabrook, 1967). Respiratory control ratios were as high as 5.

By swelling in hypo-osmotic medium, osmotically shrinking and sonication of mitochondria followed by continuous density-gradient centrifugation it was possible to separate the outer from the inner mitochondrial membranes. As enzyme markers of the two membranes, rotenone-insensitive NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase were followed respectively. The distribution pattern of cytochrome *P*-450 in the gradient follows essentially that of succinate-cytochrome *c* reductase, indicating that the pigment is localized in the inner rather than in the outer mitochondrial membrane. Similar results have been obtained by subfractionating mitochondria by means of differential rather than isopycnic centrifugation.

These findings open the possibility that hydroxylations taking place inside the mitochondrion make use of intramitochondrial NADPH, under the control of energy-conservation mechanisms of the respiratory chain, via the energy-linked transhydrogenase. In the endoplasmic reticulum, on the other hand, hydroxylation of steroids makes use of NADPH produced in the cytoplasm by the pentose phosphate pathway under the control of adrenocorticotrophin. The inner mitochondrial membrane would provide a barrier between the two control mechanisms.

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The Long-Wavelength Absorption Band of Cytochrome *c* Oxidase

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The broad absorption band of oxidized cytochrome *c* oxidase centred at 830nm, first noticed by Griffiths & Wharton (1961) and since identified with cupric copper in the enzyme by Wharton & Tzagoloff (1964), has been used by a number of workers (Gibson & Greenwood, 1965; Van Gelder & Slater, 1966) to investigate the role of copper. In all the investigations to date little attention has been paid to the state of the protein, and we have therefore been concerned to look at conditions that affect the 830nm band and to try to relate these to protein structure through the use of circular dichroism and ultracentrifugal analysis.

Pig heart cytochrome *c* oxidase as prepared by Yonetani's (1960) method yields a material of *s* value 10.5S at 20°C. It possesses a broad absorption band at 830nm that is optically active. The circular dichroism has been measured throughout the visible region and found to be in agreement with the measurements made by Urry & Van Gelder (1968) for the Soret band. When treated with cyanide at alkaline pH (Wharton & Tzagoloff, 1964) to remove copper, it was found that the structural integrity of the oxidase was completely abolished. Not only was the 830nm band bleached, but also the dichroism throughout the range 500–830nm.

Further investigation showed that alkaline pH alone caused a diminution of the *s* value from 10.5S to 5.8S, indicating that the prepared material had split into two. For complete transition from 10.5S to 5.8S material a pH of at least 10 is required.

Dialysis of the 5.8S material back to neutrality leads to partial non-specific reassociation to form aggregates of various *s* values. In particular a 16.2S unit, presumably a trimeric form, may be discerned. The 5.8S material may be separated from the aggregated protein by centrifugation at 27000g for 30min. It retains the 830nm band and dichroism in this region both at pH7.4 and pH10.4. There are however, significant changes in the electron-spin-resonance spectrum of the copper and in the dichroic spectrum of the haem as compared with the native material. Treatment of the 10.5S or 5.8S preparation at neutral pH with a high concentration of cyanide (approx. 0.5M) leaves the 830nm band and the ultracentrifuge behaviour unmodified.

This information suggests: (i) that as the subunits are dispersed by treatment with alkali and reassociate non-specifically, it is possible that electrostatic forces are the major factors contributing to the aggregated subunits' stability; (ii) that internal changes occur

in the subunits on dissociation as evidenced by the dichroism and electron-spin-resonance measurements.

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Cytochrome *c* Redox Potentials as a Function of the Energy State in Chromatophores

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Spectroscopic assay of *c*-type cytochrome (α -peak 551nm) in the Ga mutant of *Rhodospseudomonas spheroides* and potentiometric analysis (Caswell & Pressman, 1968) of wild-type *Rhodospirillum rubrum* in the potential range of cytochrome c_2 have been employed to monitor electron flow in chromatophores. Both methods showed that *c*-type cytochromes were oxidized on illumination. The rate and extent of oxidation were increased and reduction in the dark was slower in the presence of antimycin A, consistent with the site of inhibition suggested by Nishimure (1963). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone decreased cytochrome *c* oxidation, indicating at least one coupling site before cytochrome *c*. Subsequent addition of antimycin raised cytochrome *c* oxidation to a value less than that in the presence of antimycin alone, suggesting some 'leak' through the inhibited site.

Addition of ADP during illumination in the presence of Mg^{2+} and phosphate at pH 7.6 showed cycles of reduction in the potentiometric recording that were inhibited by oligomycin.

Valinomycin added before illumination, especially in KCl medium, inhibited the initial rate of cytochrome *c* oxidation and also, though less, the extent. In KCl media valinomycin greatly slowed the reduction in the dark. When added during illumination valinomycin produced transient lowering of cytochrome *c* oxidation. Nigericin had little effect on the initial rate of cytochrome *c* oxidation, but slightly lowered the steady-state value in the presence of K^+ . When both antibiotics were present simultaneously in the presence of K^+ , the steady-state

oxidation rate was lowered to that obtained on the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. We have previously discussed effects of these agents on photophosphorylation and the protonmotive force (Jackson, Crofts & von Stedingk, 1968; Jackson & Crofts, 1969).

Synergistic uncoupling of electron flow and photophosphorylation were observed with actin or, at higher concentrations, with valinomycin and NH_4Cl . This combination collapses the electrochemical potential of an H^+ gradient because NH_3 is able to cross the membrane (Crofts, 1968) and equilibrate the chemical H^+ gradient and NH_4^+ -antibiotic is able to cross the membrane and equilibrate the electrical potential (see also Cockrell & Racker, 1969; McCarty, 1969). A synergism explicable in similar terms was observed with nigericin, together with Triton X-114 at low concentrations that render the chromatophore membrane permeable to small ions (Crofts & Jackson, 1969).

These observations suggest that the membrane-potential component of the protonmotive force (Mitchell, 1966) might control electron flow.

Measurement of initial rates of H^+ uptake with Bromocresol Purple and electron flow through the coupling sites by subtraction of initial rates of cytochrome *c* changes in the presence and absence of antimycin yielded values of $H^+/2e \approx 4$ over a range of light-intensities and in the presence and absence of valinomycin. This is compatible with two phosphorylation sites of adenosine triphosphatase type II (Mitchell, 1966) in the chromatophore cyclic electron-transport pathway.

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