Equilibration of Reducing Equivalents among the Terminal Portions of the Mitochondrial Respiratory Chains*

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ABSTRACT: The functional interactions among terminal portions of mitochondrial respiratory chains have been investigated. The rates of oxidation of cytochromes were determined as a function of the number of carbon monoxide inhibited oxidases in the presence of antimycin A. All the cytochrome *a*'s and cytochrome *c*'s could be completely oxidized at all carbon monoxide concentrations used. The rate of oxidation of all the cytochrome *c* molecules was found to be closely proportional to the total concentration of non-CO-complexed cytochrome a_3's. This demonstrates rapid equilibration of reducing equivalents among respiratory chains on the oxygen side of the antimycin A block. The rates of oxidation of the cytochrome *a*'s in cytochromec extracted mitochondria in the presence of varying concentrations of CO consist of a fast phase and a slow phase. The amount of cytochrome *a* oxidized during the fast phase is directly proportional to the amount of cytochrome a_3 which is not complexed with CO.

The slow cytochrome *a* oxidation phase can be titrated back with cytochrome *c* to the fast oxidation rate of cytochrome *a* in normal mitochondria. This demonstrates that under these conditions cytochrome *c* is required for the rapid equilibration of reducing equivalents among cytochrome *a* molecules.

Keilin and Hartree (1939) were the first to observe a functional interaction among mitochondrial respiratory chains. They observed that cytochrome *a* could still be oxidized when most cytochrome a_3's were complexed with carbon monoxide. Other workers have considered the problem of interrespiratory chain electron flow and either made no statement as to the rate (Tyler *et al.*, 1965) or concluded that it must be slow (Vazquez-Colon and King, 1967; Lee *et al.*, 1965; Chance, 1965; Holmes, 1960). Nicholls (1965) and Kimelberg (1968) concluded from studies with cytochrome *c* extracted mitochondria and submitochondrial particles that rapid interchain electron flow occurs between cytochrome *b* and cytochrome *c*. Rapid electron flow rates between electron transport carriers of different respiratory chains have been shown to occur in *Haemophilus parainfluenza* by White and Smith (1964). They found no evidence in their studies that the respiratory system in these organisms is composed of several multienzyme complexes, each with a fixed proportion of respiratory pigments. Wohlrab and Jacobs (1967a,b) utilized copper-deficient rat liver mitochondria which have a deficiency only in cytochrome (aa_3) and suggested from steady-state studies with artificial electron donors that interchain electron flow rates among the terminal portions of the respiratory chains can be of the same order of magnitude as those along each chain. However, they were not able to decide at that time whether this fast interchain rate was only associated with copper-deficient mitochondria or with mitochondria in general. Preliminary direct kinetic studies with copper-deficient and copper-sufficient yeast cells which suggest rapid equilibration of electrons among cytochrome *c* molecules have been published (Wohlrab, 1969).

Direct kinetic evidence is presented in this communication which suggests that the rate of equilibration of reducing equivalents among the terminal portions of different respiratory chains in rat liver mitochondria is of the same order of magnitude as their rates of oxidation by intrachain electron flow.

Materials and Methods

Rat liver mitochondria were prepared in MSE[1] solution essentially according to the methods of Schneider (1948) and Lardy and Wellman (1952). Respiratory control ratios were routinely determined immediately before each experiment (Chance and Williams, 1955) and were found to be between 4.5 and 5.5. The substrate for the respiratory control assays was sodium succinate (pH 7.0, 10 mM). Rotenone (2 μM) was used to inhibit NAD-linked substrate oxidation.

The oxidation rates were determined with a regenerative flow apparatus (Chance *et al.*, 1967) which was mounted on a Johnson Foundation dual-wavelength spectrophotometer. The flow apparatus was calibrated using the pseudo-first-order reaction of horseradish peroxidase with hydrogen peroxide (Chance *et al.*, 1967). The mixing ratio of the flow apparatus was 1:65, which yields an oxygen concentration (using aerated buffer) of 3.7 μM O_2 after mixing.

Carbon monoxide gas was mixed in a volumetric gas mixer with nine volumes of helium gas. Degassed MSET medium was equilibrated with the CO–He gas mixture at about 1 atm in a tonometer yielding a 10% saturated CO solution (about 103 μM CO). This solution was transferred to a glass syringe and mounted as a second reservoir on the flow apparatus. The oxidation kinetics at various CO concen-

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[1] Abbreviations used are: MSE, 0.22 M mannitol, 0.07 M sucrose, and 200 μM Na_2EDTA (pH 7.2); MSET, MSE plus 10 mM Tris-Cl (pH 7.4).

trations were determined by injecting CO buffer into the closed system of the flow apparatus between oxygen mixings.

Cytochrome *c* was extracted from rat liver mitochondria by the method of Jacobs and Sanadi (1960). This procedure removes about 95% of the total cytochrome *c* from the mitochondria.

Cytochrome *c* (type VI) was purchased from Sigma Chemical Co. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was kindly donated by P. Heytler and was dissolved in ethanol. The concentration of mitochondrial respiratory chains is expressed in terms of their cytochrome *c* oxidase content. Mitochondrial[2] cytochrome *c* oxidase was determined from difference spectra of mitochondria that were solubilized with 2% sodium cholate and then reduced with 10 mM succinate. The difference extinction coefficient of ϵ_{mM} at 605–630 nm (reduced minus oxidized) of 26.4 mM^{-1} cm^{-1} (Van Gelder, 1966) was used for cytochrome *c* oxidase. The addition of dithionite to the reduced sample cuvet did not change the ΔA at 605–630 nm.

Mitochondrial cytochrome *c* was monitored at 550–540 nm and cytochrome *c* oxidase was monitored at 445–455 nm and 605–630 nm (Chance, 1965).

Cytochrome (a^{2+}, a_3^{2+}) refers to a cytochrome *c* oxidase with reduced cytochrome *a* and reduced cytochrome a_3. Cytochrome (a^{3+}, a_3^{3+}) refers to a cytochrome *c* oxidase with an oxidized cytochrome *a* and an oxidized cytochrome a_3.

P_{max} refers to the difference in optical density between the reduced state and the aerobic steady-state oxidation of mitochondria. P_t refers to the difference in optical density between the reduced state and the oxidized state of mitochondria at any given time (t) during the oxidation.

Results

Determination of the Equilibrium Constant between Mitochondrial Cytochrome a_3 and Carbon Monoxide. Rat liver mitochondria (0.15 μM cytochrome *c* oxidase) were suspended in MSET medium in the presence of 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone in a cylindrical glass vessel (100-ml volume) with a very small opening at the top to minimize gas equilibration with the atmosphere. The content of the vessel was stirred with a magnetic stirrer. The vessel was mounted on a Johnson Foundation dual-wavelength spectrophotometer. Sodium succinate (10 mM) was added to the vessel and the resulting optical density change from oxidized to reduced was measured at 445–455 nm. Then aliquots of carbon monoxide saturated MSET were added and the resulting optical density changes at 445–455 nm were monitored. The change in optical density between cytochrome (a^{2+}, $a_3^{2+}\cdot$CO) and cytochrome (a^{2+}, a_3^{2+}) at 445–455 nm when assigned about 55% of the change in optical density between cytochrome (a^{2+}, a_3^{2+}) and cytochrome (a^{3+}, a_3^{3+}) at 445–455 nm yielded the best straight-line fit on a plot of $(a_3^{2+}\cdot CO)/[a_{3tot}-(a_3^{2+}\cdot CO)]$ *vs.* $(CO)_{tot}$, where $(a_3^{2+}\cdot CO)$ is the concentration of the cytochrome $(a_3^{2+}\cdot CO)$ complex, a_{3tot} is the total cytochrome (a_3) concentration, and $(CO)_{tot}$ is the total carbon monoxide present. The slope of the resulting straight line yielded an equilibrium constant of 2.0 $\times 10^6$ M^{-1}. Cytochrome a_3 from normal, uncoupled rat liver mitochondria and from cytochrome *c* extracted mitochondria yielded the same equilibrium constant. This equilibrium constant compares favorably with the one for purified cytochrome *c* oxidase of 2.5 $\times 10^6$ M^{-1} (Gibson *et al.*, 1965).

Contribution of Mitochondrial Cytochrome a and a_3 to Optical Density Changes at 605–630 and 445–455 nm. To determine the contributions of cytochrome a_3 and cytochrome *a* to the difference (reduced minus oxidized) absorption spectrum of mitochondria, it is desirable for a given concentration of mitochondria to determine first the optical density difference between cytochrome (a^{2+}, a_3^{2+}) and cytochrome (a^{3+}, a_3^{3+}) at 605–630 and 445–455 nm and then to determine the optical density difference between cytochrome (a^{2+}, $a_3^{2+}\cdot$CO) and cytochrome (a^{3+}, $a_3^{2+}\cdot$CO). The latter difference in optical density yields the contribution of cytochrome (a^{2+}) minus cytochrome (a^{3+}) to the former difference. The result yields the contributions of cytochrome a_3 and cytochrome *a* to the optical density difference between cytochrome (a^{2+}, a_3^{2+}) and cytochrome (a^{3+}, a_3^{3+}) at 445–455 and 605–630 nm.

An attempt was made to do this experiment in the following way. The same vessel of the earlier experiment was used. However, this time rat liver mitochondria (about 0.15 μM cytochrome *c* oxidase) were added to the MSET medium which contained 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 10 mM sodium succinate and were allowed to reach anaerobiosis. Then about 0.40 μM antimycin A was added. Optical density changes were monitored alternatively at both 445–455 and 605–630 nm. Oxygenated MSET (0.05–0.1 ml) was added to the anaerobic suspension and the optical density changes were recorded. Upon having reached anaerobiosis again, a small aliquot of CO-saturated buffer was added which was followed by an aliquot of oxygenated buffer. Since the equilibrium constant between cytochrome a_3 and carbon monoxide was determined earlier, we now know the amount of CO which is needed to complex 50% of the cytochrome a_3. At that CO concentration, the change in optical density at 605–630 nm between the anaerobic mitochondria and the aerobic mitochondria is essentially identical (within 2 to 3%) with that in the absence of CO, while the optical density change at 445–455 nm is significantly smaller in the presence of CO than in the absence of CO. From these data it is concluded that the change in optical density between cytochrome (a_3^{2+}) and cytochrome (a_3^{3+}) contributes only 5% or less to the change in optical density between mitochondrial cytochrome (a^{2+}, a_3^{2+}) and cytochrome (a^{3+}, a_3^{3+}) at 605–630 nm. It should be noted that the above-mentioned optical density changes were corrected for swelling and dilution contributions by conducting a parallel experiment in the absence of CO, *i.e.*, only aliquots of oxygenated buffer were added to induce oxidation–reduction cycles repeatedly. Optical density changes of successive oxidation–reduction cycles decreased significantly, much more than was expected from sample dilutions. This fact makes this control very important.

Now we know that cytochrome *a* contributes more than 95% to the difference in optical density between reduced and oxidized mitochondrial cytochrome *c* oxidase at 605–630 nm. Let $\Delta A^{c}_{\lambda_2-\lambda_1}$ be the difference in optical density between reduced and oxidized mitochondrial cytochrome *c* oxidase at the two wavelengths λ_2 and λ_1 and in the presence of a

[2] Mitochondrial in this communication refers to an association with the mitochondrial membrane.

TABLE I: Carbon Monoxide Titration of Cytochrome Oxidation Kinetics in Mitochondria.[a]

CO (μM)	% Δ Optical Density at 605–630 nm[b]	% Cytochrome a_3 Which Is Not CO Complexed[c]	k_1 (sec^{-1})[d] 445–455 nm	605–630 nm	550–540 nm	Flow Mode
0	100	100	120	120	105	
0.88	97	46	76	70	48	Continuous flow
1.7	96	28	43	30	24	
4.3	96	12	9	9	8	
9.5	96	5	5	4.5	3.5	Stopped flow
18.1	94	5	2.5	2.5	2.0	

[a] Rat liver mitochondria were suspended at a concentration of 0.5 μM mitochondrial cytochrome *c* oxidase in MSET medium. The medium contained also 10 mM sodium succinate, 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and 10 nmoles of antimycin A/nmole of cytochrome *c* oxidase. Cytochrome oxidation changes were initiated by the injection of 3.7 μM O_2 into the anaerobic medium. [b] This is the change of optical density at 605–630 nm at a given CO concentration resulting from the addition of oxygen, expressed as per cent of the optical density change in the absence of CO. [c] These were calculated from the total cytochrome a_3 concentration, the total carbon monoxide concentration, and the CO–cytochrome a_3 equilibrium constant of 2×10^6 M^{-1}. [d] These are pseudo-first-order rate constants. The calculations of k_1 from continuous-flow data utilized the optical density change during the flow which here corresponded to about 20 msec after mixing.

concentration (*c*) of carbon monoxide. Then as the CO concentration becomes very large, $100 \times R$ approaches the per cent of cytochrome *a* which contributes to $\Delta A^{\circ}_{445-455\text{ nm}}$. *R* is given by

$$R = \frac{\Delta A^{c}_{445-455\text{ nm}}}{\Delta A^{c}_{605-630\text{ nm}}} \times \frac{\Delta A^{\circ}_{605-630\text{ nm}}}{\Delta A^{\circ}_{445-455\text{ nm}}}$$

At very high CO concentrations, *R* yielded a limiting value of about 0.45. This method is independent of the fact that the shift of the rate-limiting step in electron transport from the antimycin A site (low CO concentration) to cytochrome a_3 (high CO concentration) will change the per cent of cytochrome *a* which is oxidized during the steady state.

The same experiment was carried out for cytochrome *c* extracted mitochondria and within experimental errors the same contributions of cytochrome *a* and cytochrome a_3 to the difference in optical densities between reduced and oxidized mitochondria at 605–630 and 445–455 nm were found.

Titration of the Oxidation Kinetics of Mitochondrial Cytochrome c and Cytochrome a with Carbon Monoxide. Rat liver mitochondria (0.5 μM cytochrome *c* oxidase) were suspended in MSET medium in a regenerative flow apparatus which had been mounted on a Johnson Foundation dual-wavelength spectrophotometer. The medium also contained 10 mM sodium succinate, 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and 10 nmoles of antimycin A/nmoles of cytochrome *c* oxidase. The mitochondria were allowed to reach anaerobiosis before 3.7 μM oxygen was injected and the resulting optical density change was monitored at 605–630, 550–540, or 445–455 nm. Antimycin A induces a rate-limiting step in the electron transfer reactions which yields essentially 100% oxidized cytochromes on the oxygen side of the antimycin A block during the aerobic steady state. When carbon monoxide is added to the mitochondrial suspension, the rate of oxygen consumption changes very little, suggesting that at the levels of CO used, the rate-limiting electron flow step remains at the antimycin A site rather than shifting to the level of cytochrome a_3 which at very high concentrations of CO will become the rate-limiting step. Table I shows that the differences in optical density at 605–630 nm between reduced and oxidized mitochondria at various CO concentrations differ only very little from that in the absence of CO. This leads us to conclude that the rate-limiting step of electron transfer in the presence of our concentrations of CO remains at the antimycin A site.

Since we know the equilibrium constant between cytochrome a_3 and carbon monoxide and we also know the amount of cytochrome a_3 and CO present in the medium, we can calculate the amount of cytochrome a_3 which is complexed with carbon monoxide and correlate the rates of oxidation of cytochrome *a* and cytochrome *c* with the fraction of cytochrome a_3 which is not complexed with carbon monoxide.

Table I shows very clearly that the pseudo-first-order rate constant for cytochrome *c* oxidation is directly proportional to the amount of non-CO-complexed cytochrome a_3; the pseudo-first-order rate constant for cytochrome *a* oxidation also closely parallels the amount of non-CO-complexed cytochrome a_3 present. The kinetics of cytochrome *c* oxidation (Figure 1) when plotted on a first-order graph (Figure 2) show indeed that all the cytochrome *c* is oxidized as a single pool of reducing equivalents. The earliest measurement on the graph is during the mixing time of the flow apparatus, which in this case corresponds to about 20 msec. The first-order plot for optical density changes at 605–630 nm (cytochrome *a*) shows a small jump which could suggest that cytochrome *a* which is bound to a cytochrome a_3 which is not complexed with CO is oxidized faster than the other cytochrome *a*'s. This however is discussed later.

TABLE II: Carbon Monoxide Titration of Cytochrome Oxidation Kinetics in Cytochrome *c* Extracted Mitochondria.[a]

CO (μM)	% Δ Optical Density at 605–630 nm[b]	% Cytochrome a_3 Which Is Not CO Complexed[c]	% Cytochrome *a* and Cytochrome a_3 Which Are Rapidly Oxidized[d]	% Cytochrome *a* Which Is Rapidly Oxidized[e]	Slow Phase $t_{1/2}$ (msec) at[f]	
					445–455 nm	605–630 nm
0	100	100	100	100		
0.88	97	47	51	51	720	680
1.7	96.5	29	34	36	1200	940
4.4	96	12	16	15	1960	1840
4.4 + 0.32 μM cytochrome *c*	96	12			70 (10 sec^{-1})	70 (10 sec^{-1})

[a] Cytochrome *c* extracted rat liver mitochondria were suspended at a concentration of 0.61 μM cytochrome *c* oxidase in MSET medium. The medium also contained 10 mM sodium succinate, 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and 1 nmole of antimycin A/nmole of cytochrome *c* oxidase. Cytochrome oxidation changes were initiated by the injection of 3.7 μM O_2 into the anaerobic medium. [b] This is the change of optical density at 605–630 nm at the given CO concentration resulting from the addition of oxygen, expressed as per cent of the optical density change in the absence of CO. [c] These were calculated from the total cytochrome a_3 concentration, the total carbon monoxide concentration, and the CO–cytochrome a_3 equilibrium constant of 2×10^6 M^{-1}. [d] These were calculated from the fast-phase–slow-phase proportionality of Figure 3B (445–455 nm) as described in the text. [e] The same calculations as in footnote *d*, except that Figure 3A (605–630 nm) was used. [f] These are the slow-phase oxidation half-times as determined from Figure 3A, B.

The kinetics of the optical density changes at 445–455 nm show much more of a jump during the first 20 msec. This jump is expected because non-CO-complexed cytochrome a_3 is known to react with molecular oxygen with a half-time of approximately 5 msec (Table I).

Titration of the Cytochrome a Oxidation Kinetics of Cytochrome c Extracted Mitochondria with CO. The flow apparatus experiment was set up just like the experiment of the last section except that cytochrome *c* extracted mitochondria (0.61 μM cytochrome *c* oxidase) were utilized. The idea was to test whether indeed cytochrome *c* could be the cause of rapid equilibration of reducing equivalents between cytochrome *a* molecules in the presence of carbon monoxide. The per cent optical density changes (Table II) at 605–630 nm behaved very much like those of Table I. However this time

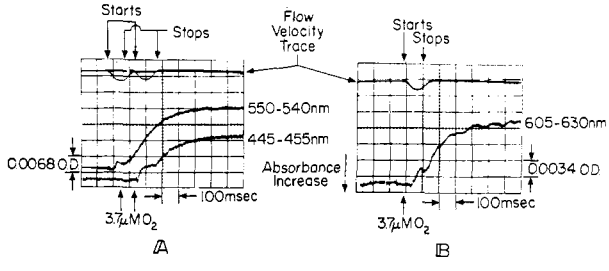

FIGURE 1: Oxidation kinetics of mitochondrial cytochromes in the presence of carbon monoxide. The regenerative flow apparatus contained MSET medium, rat liver mitochondria at a concentration of 0.54 μM cytochrome *c* oxidase, 10 mM sodium succinate, 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 5.4 μM antimycin A, and 4.3 μM CO. The optical path was 4 mm.

the optical density changes which resulted upon addition of 3.7 μM O_2 to the anaerobic mitochondrial suspensions consisted of two very different rates (Figure 3A,B). The extent of the fast phase diminished as the carbon monoxide concentration was increased. The rate of the fast phase is so much higher than that of the slow phase that one can assume that the fast component is essentially completely oxidized when the slow oxidation has only proceeded to a negligible extent.

We will attempt to correlate the extent of the fast-phase optical density changes with the amount of non-CO-complexed cytochrome a_3. In the absence of CO, the oxidation of cytochrome *a* and cytochrome a_3 in the presence of 3.7 μM O_2 goes to about 93% completion before the earliest measurement, which occurs here at about 20 msec. Since essentially all the optical density change resulting from oxidation of anaerobic mitochondria at 605–630 nm is due to cytochrome *a*, we can do the following calculation for the optical density changes at 605–630 nm: let A_s be the total optical density change of the slow phase and A_f be the total optical density change of the fast phase at any one CO concentration. Then

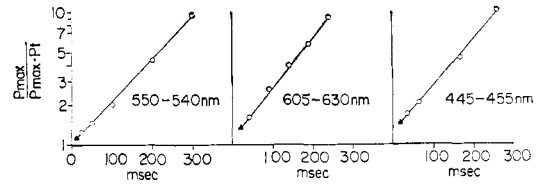

FIGURE 2: First-order kinetics plot of the oxidation rates of Figure 1.

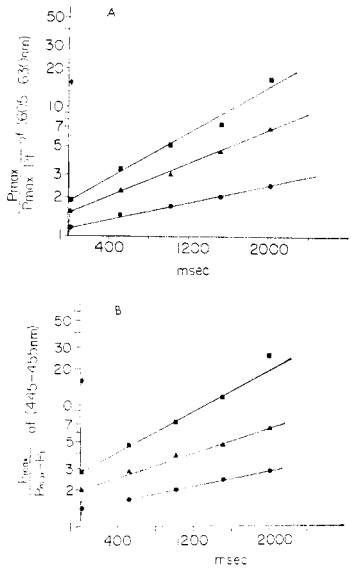

FIGURE 3: Oxidation rates of cytochrome *c* extracted mitochondria at various CO concentrations. The regenerative flow apparatus contained MSET medium, cytochrome *c* extracted rat liver mitochondria at a concentration of 0.61 μM cytochrome *c* oxidase, 10 mM sodium succinate, 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and 0.6 μM antimycin A. The oxidation kinetics were initiated by the injection of 3.7 μM O_2. The optical path was 4 mm: (◆) zero CO, (■—■) 0.88 μM CO, (▲—▲) 1.7 μM CO, (●—●) 4.4 μM CO. Observations done at (A) 605–630 nm and (B) 445–455 nm.

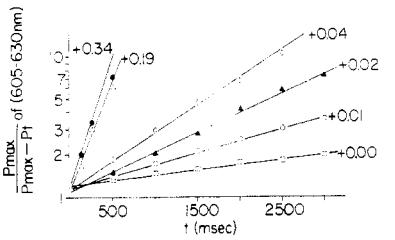

FIGURE 4: Titration of the oxidation kinetics of cytochrome *a* in cytochrome *c* extracted mitochondria in the presence of CO with cytochrome *c*. The regenerative flow apparatus contained MSET medium, cytochrome *c* extracted rat liver mitochondria at a concentration of 0.5 μM cytochrome *c* oxidase, 10 mM sodium succinate, 1.5 μM antimycin A, 3 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, and about 5 μM CO (enough to complex 80 to 90% of cytochrome a_3). The optical path was 6 mm. The oxidation kinetics were initiated by the injection of 3.7 μM O_2. The following amounts (final concentrations) were added: (□—□) no cytochrome *c*, (○—○) 0.01 μM, (▲—▲) 0.02 μM, (◇—◇) 0.04 μM, (△—△) 1.19 μM, and (●—●) 0.34 μM cytochrome *c*.

the per cent of the total cytochrome *a* which is oxidized during the fast phase (a_f) is

$$a_f = \frac{A_f}{A_s + A_f} \times 100 \times \frac{1}{0.93}$$

These values (a_f) are listed in column 5 of Table II.

A very similar calculation can be done of the total optical density changes at 445–455 nm, since the steady-state oxidation level at the CO concentrations used of cytochrome *a* (605–630 nm) remains essentially constant. Let k be the fraction of non-CO-complexed cytochrome a_3 molecules. We determined earlier that cytochrome *a* contributes about 45% to the change in optical density which occurs upon oxidation of antimycin A inhibited anaerobic mitochondria and that cytochrome a_3 contributes about 55% to this change. Let us assume that one cytochrome *a* gets rapidly oxidized for every cytochrome a_3 that gets rapidly oxidized. Then, if A_s and A_f have the same meaning as before except that they now refer to 445–455 nm, we can calculate k (at any given CO concentration) from

$$\frac{A_s + A_f}{A_s} = \frac{(0.55\,k + 0.45)}{(0.55\,k + 0.45) - 0.93\,k}$$

The values of 100 k are listed in Table II (column 4). In column 3 of Table II we listed the per cent of cytochrome a_3, which are non-CO complexed. This was calculated from the equilibrium constant between cytochrome a_3 and CO, the total cytochrome a_3 present, and the total CO concentration present. Columns 3, 4, and 5 of Table II agree very well, indicating that the a_3–CO equilibrium constants obtained by the three techniques are the same.

Titration of the Biphasic Cytochrome a Kinetics with Cytochrome c. Cytochrome *c* extracted rat liver mitochondria (0.5 μM cytochrome *c* oxidase) were added to the regenerative flow apparatus to MSET medium containing 10 mM sodium succinate, 1.5 μM antimycin A, and 3 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and about 5 μM CO (enough to complex 80–90% of cytochrome a_3) was then added to the closed volume. O_2 (3.7 μM) was injected into the anaerobic suspension and the optical density change at 605–630 nm was observed (Figure 4). Then small amounts of cytochrome *c* were injected into the closed system of the flow apparatus and after every cytochrome *c* injection, 3.7 μM O_2 was added to the anaerobic suspension. Figure 4 shows that while the extent of the fast phase remains essentially constant, the rate of the slow phase increases as more cytochrome *c* is added.

Discussion

The type of analysis described in this communication requires a very high degree of homogeneity of mitochondrial vesicles so that the oxidation rates indeed do reflect the behavior of redox systems of each mitochondrion. This is one reason why only preparations of intact rat liver mitochondria were used. They showed respiratory control ratios higher than 4.5 with succinate as electron donor before the addition of uncoupler.

The data presented in this communication show very clearly (Table I) that the pseudo-first-order rate constant of cytochrome *c* oxidation is directly proportional to the amount of cytochrome a_3 which is not complexed with CO. This implies that the cytochrome *c* molecules act as a rapidly equilibrating pool of reducing equivalents. In other words, the rate of equilibration of reducing equivalents among cytochrome *c* molecules appears to be as fast or faster than the rate of oxidation of the cytochrome *c* molecules by cytochrome *a*. A similar relationship almost holds also for cytochrome *a* oxidation; however, as indicated in Figure 2, there appears to be a fast-phase component. This could be interpreted as indicating that cytochrome *a*'s which are

associated with non-CO-complexed cytochrome a_3's are oxidized slightly faster than the cytochrome *a*'s which are associated with CO-complexed cytochrome a_3's.

The question then is: Does the rapid equilibration of electrons occur at the level of cytochrome *a* or at the level of cytochrome *c*? When cytochrome *c* was extracted, the cytochrome *a* molecules equilibrated their electrons only very slowly (Figure 3A,B). The rate of equilibration between cytochrome *a* molecules in the complete absence of cytochrome *c* molecules may indeed even be slower than is suggested in Figure 4 in the absence of added cytochrome *c*, since the salt extraction of mitochondria removes only about 95% of the total cytochrome *c* (Jacobs and Sanadi, 1960). Table I (also Figure 2) shows, however, that cytochrome *a* is oxidized faster than cytochrome *c* in the presence of CO. We conclude that in intact, uncoupled mitochondria cytochrome *c* is required for the rapid equilibration of reducing equivalents among the terminal portions of the respiratory chains. However, since cytochrome *a* is oxidized more rapidly than cytochrome *c* in the presence of CO, we conclude that cytochrome *c* causes the rapid transfer of electrons between cytochrome *a* molecules in a manner which requires no change in the redox state of cytochrome *c*.

Some theories (Chance *et al.*, 1968; Green *et al.*, 1965) in the past attributed a high mobility to cytochrome *c* in the mitochondrial membrane. It becomes thus of interest to titrate the slow equilibration among cytochrome *a* molecules with cytochrome *c*. Very low ionic strength medium was used to bind the cytochrome *c* as tightly to the mitochondrial membrane as possible. Figure 4 shows that the slow oxidation of cytochrome *a* molecules retains the first-order kinetic characteristics as the cytochrome *c* level is increased, even though the rate is higher in the presence of higher amounts of cytochrome *c*. This suggests that at low cytochrome *c* concentrations the cytochrome *c*'s are sufficiently mobile in this system to effect the many cytochrome *a* molecules equally. When a high concentration of cytochrome *c* is present, the rapid equilibration of reducing equivalents at the level of cytochrome *c* need not be due to a high degree of mobility, since cytochrome *c* molecules have been shown to equilibrate their electrons in solution with a second-order rate constant of 5×10^4 M^{-1} sec^{-1} (Kowalsky, 1965). The mitochondrial environment of cytochrome *c* may however affect this rate.

All the above studies were done in the presence of antimycin A. It is well known that cytochrome c_1 lies on the oxygen side of the antimycin A block (Keilin and Hartree, 1955; Chance, 1958; Estabrook, 1958) and the question arises, what role does cytochrome c_1 play in the equilibration of reducing equivalents among respiratory chains on the oxygen side of the antimycin A block? The present data are relevant to electron flow from cytochrome *c* to oxygen, and one can conclude only that in the absence of cytochrome *c*, the cytochrome c_1 cannot catalyze the transfer of electrons between cytochrome *a* molecules.

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References

Chance, B. (1958), *J. Biol. Chem. 233*, 1223.

Chance, B. (1965), *in* Oxidases and Related Redox Systems, King, T. E., Mason, H. S., and Morrison, M., Ed., New York, N. Y., Wiley, p 929.

Chance, B., DeVault, D., Legallais, V., Mela, L., and Yonetani, T. (1967), *in* Fast Reactions and Primary Processes in Chemical Kinetics, Claesson, S., Ed., Stockholm, Almqvist and Wiksell, p 437.

Chance, B., Lee, C. P., Mela, L., and DeVault, D. (1968), *in* Structure and Function of Cytochromes, Okunuki, K., Kamen, M. D., and Sekuzu, I., Ed., Tokyo, University of Tokyo, p 475.

Chance, B., and Williams, G. R. (1955), *J. Biol. Chem. 217*, 383.

Estabrook, R. W. (1958), *J. Biol. Chem. 230*, 735.

Gibson, Q. H., Palmer, G., and Wharton, D. C. (1965), *J. Biol. Chem. 240*, 915.

Green, D. E., Wharton, D. C., Tzagoloff, A., Rieske, K. S., and Brierley, G. P. (1965), *in* Oxidases and Related Redox Systems, King, T. E., Mason, H. S., and Morrison, M., Ed., New York, N. Y., Wiley, p 1032.

Holmes, W. F. (1960), Ph.D. Dissertation, University of Pennsylvania, Philadelphia, Pa.

Jacobs, E. E., and Sanadi, D. R. (1960), *J. Biol. Chem. 235*, 531.

Keilin, D., and Hartree, E. F. (1939), *Proc. Roy. Soc. (London) B127*, 167.

Keilin, D., and Hartree, E. F. (1955), *Nature 176*, 200.

Kimelberg, H. K. (1968), Ph.D. Dissertation, State University of New York at Buffalo, Buffalo, N. Y.

Kowalsky, A. (1965), *Biochemistry 4*, 2382.

Lardy, H. A., and Wellman, H. (1952), *J. Biol. Chem. 195*, 215.

Lee, C. P., Estabrook, R. W., and Chance, B. (1965), *Biochim. Biophys. Acta 99*, 32.

Nicholls, P. (1965), *in* Oxidases and Related Redox Systems, King, T. E., Mason, H. S., and Morrison, M., Ed., New York, N. Y., Wiley, p 774.

Schneider, W. C. (1948), *J. Biol. Chem. 176*, 259.

Tyler, D., Gonse, J., and Estabrook, R. W. (1965), *in* Oxidases and Related Redox Systems, King, T. E., Mason, H. S., and Morrison, M., Ed., New York, N. Y., Wiley, p 939.

Van Gelder, B. F. (1966), *Biochim. Biophys. Acta 118*, 36.

Vazquez-Colon, L., and King, T. E. (1967), *Arch. Biochem. Biophys. 122*, 190.

White, D. C., and Smith, L. (1964), *J. Biol. Chem. 239*, 3956.

Wohlrab, H. (1969), *Fed. Am. Soc. Exptl. Biol. Abstr.*, p 911.

Wohlrab, H., and Jacobs, E. E. (1967a), *Biochem. Biophys. Res. Commun. 28*, 991.

Wohlrab, H., and Jacobs, E. E. (1967b), *Biochem. Biophys. Res. Commun. 28*, 998.