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CYTOCHROMES *a* AND a_3

CATALYTIC ACTIVITY AND SPECTRAL SHIFTS *IN SITU* AND IN SOLUTION

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SUMMARY

1. The inhibitory and spectroscopic effects of azide on phosphorylating and non-phosphorylating systems have been studied. In non-phosphorylating particles succinate oxidase inhibition requires 10 times the concentration of azide needed to inhibit the oxidase itself.

2. Reduction of cytochrome *a* in non-phosphorylating particles oxidizing succinate occurs at low azide concentrations; inhibition supervenes only if sufficient azide is present to cause appreciable steady-state reduction of endogenous cytochrome *c*.

3. The previously reported shift in the α band of reduced cytochrome *a* from 605 to 600 mμ in presence of azide is now observed in all respiratory states of the mitochondrion, in Keilin–Hartree particles and in the isolated oxidase. It is concluded that this form of cytochrome *a* is neither a high-energy compound nor a complex with azide but the result of heme–heme interaction between reduced *a* and the classical oxidized a_3–azide complex.

4. The high sensitivity of mitochondrial respiration in ADP-stimulated State 3 to azide is not accompanied by an increased sensitivity of mitochondrial oxidase activity. The changes in steady-state reduction of cytochromes *c* and *a* parallel the inhibition of respiration. Relief of inhibition by uncouplers is not explained by changes in intramitochondrial azide concentration and does not involve the disappearance of cytochrome *a* with an α band at 600 mμ.

5. It is suggested that the relief of azide inhibition by uncouplers is due to a change in electron-transfer pathways permitting a direct reduction of a_3 by *c* and short-circuiting cytochrome *a*.

6. The spectrum of reduced a_3 is discussed and it is indicated that this cytochrome probably has no appreciable α band at 605 mμ but instead is characterized in the visible region solely by a broad band centered at 560 mμ.

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride.

INTRODUCTION

At least three problems confront the investigator of mammalian cytochrome oxidase. What is the nature of the oxidase components? What are the chemical relationships between these components during catalytic activity? How are these relationships modified in the mitochondrial membrane-bound enzyme? Some conventional answers to these questions are: that the oxidase is composed of two components, a and a_3, containing identical heme groups of which only a_3 reacts with O_2 and the inhibitors CO, cyanide and azide[1,2]; that a is oxidized by a_3 which in turn is oxidized by molecular oxygen[3,4]; and that the interactions between c and a *plus* a_3 are inhibited in the mitochondrion in the absence of acceptors or uncouplers[5].

Recently some doubt has been cast on these answers[6–8]. It has been shown that the spectrum of the reduced oxidase in the steady state ($a^{2+}a_3^{3+}$) is different in the presence of azide[6,7]. Moreover, in the intact mitochondrion, azide gives rise to an uncoupler-sensitive inhibition between cytochromes a and a_3[8]. Furthermore, contrary to their initial results[3], GIBSON AND GREENWOOD[9] now find no evidence for a net oxidation of a prior to the net oxidation of a_3 at low O_2 concentrations.

This paper describes experiments on the spectra and catalytic activity of the isolated oxidase[2], the non-phosphorylating Keilin–Hartree particles and phosphorylating rat-liver mitochondria. The results obtained will be used to support three conclusions about the oxidase. These conclusions are: (a) that cytochrome a_3 is not detectable in the anaerobic—aerobic difference spectra except at the Soret region; (b) that azide reacts with oxidized a_3 according to the suggestion of KEILIN AND HARTREE[1] and that the spectroscopic effects of azide on a are due to heme–heme interaction between cytochromes a and a_3; (c) that the effects of uncouplers on azide inhibition of mitochondrial respiration are due to a modification of electron-transfer pathways as between coupled and uncoupled systems, and not to reactions of azide, or uncouplers, or both, with high-energy forms of the oxidase.

METHODS AND MATERIALS

Rat liver mitochondria were prepared essentially by the method of MYERS AND SLATER[10]; Keilin–Hartree-type beef heart submitochondrial particles by the blendor modification of the classical method[11]; cytochrome oxidase by YONETANI'S[12] procedure. Sodium azide was obtained from British Drug Houses. Cytochrome c was Sigma Type III from horse heart. The initial suspension of Keilin–Hartree particles contained approx. 40 mg protein per ml.

Spectra were scanned in a Cary 14 spectrophotometer fitted with scattered transmittance accessory (room-temperature spectra) or in a Cary II spectrophotometer specially modified for low temperature (liquid-N_2 spectra)[13]. Steady-state measurements were made with an Aminco-Chance dual-beam spectrophotometer. O_2 uptake was monitored with a Clark oxygen electrode (YSI), or vibrating platinum–silver electrode when simultaneous O_2 uptake and differential spectrophotometric observations were made.

RESULTS

Azide inhibition of non-phosphorylating respiration

Fig. 1 shows the inhibition of cytochrome *c* oxidase and succinate oxidase activity by azide. As indicated previously[14], inhibition of succinate oxidase, located at the same site as that of cytochrome *c* oxidase activity, requires some 10 times the amount of azide for an equivalent effect. In Fig. 1, at pH 7.4, 30°, 1.4 mM azide is needed to inhibit the succinate oxidase activity by 50%, compared with 50–100 μM azide for an equivalent inhibition of cytochrome *c* oxidase[15]. A plausible explanation of this phenomenon is that the binding of the terminal oxidase by azide is compensated for by an increased reduction of *c* and possibly *a*. Thus, if the reducing system involved is normally 20% saturated with electrons, it will be necessary to reduce the amount of free oxidase (a_3) to 10% in order to observe 50% inhibition. The apparent K_i will then be ten times the actual K_i.

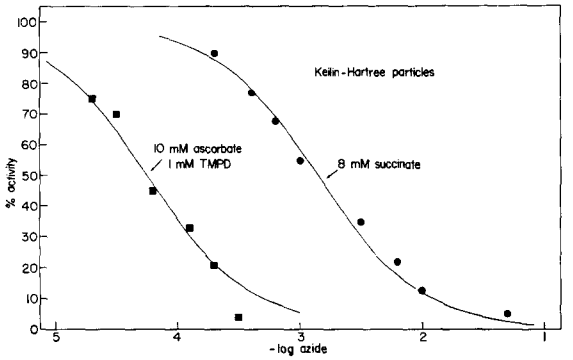


Fig. 1. Azide inhibition of succinate oxidase and cytochrome oxidase activity in Keilin–Hartree particles. Polarographic measurements on succinate oxidase with 8 mM succinate as substrate, and 60-fold dilution of particles in 0.065 M phosphate buffer (pH 7.4), 30°. Solid line is derived from mass law with $K_d = 1.4 \cdot 10^{-3}$ and $n = 1$. 100% activity was 1.5 μM $O_2 \cdot sec^{-1}$. Cytochrome oxidase assay employed 10 mM ascorbate, 1 mM TMPD, 27.4 μM cytochrome *c*, 1500-fold dilution of particles in 0.05 M potassium phosphate, 0.001 M EDTA (pH 7.3), 25°. Solid line is derived from mass law with $K_d = 5.6 \cdot 10^{-5}$ and $n = 1$. 100% activity was 1.2 μM $O_2 \cdot sec^{-1}$, corrected for auto-oxidation in the absence of enzyme.

In essence, this is the classical "excess oxidase" idea. An alternative explanation has been offered by PALMIERI AND KLINGENBERG[16], who suggest that the inhibition of respiration by azide is "uncompetitive", following Eqn. 1:

$$v = \frac{v_0 K_i}{I(v_0/v_{max})} + K_i \qquad (1)$$

Eqn. 1 predicts that I_{50} (the amount of inhibitor required to reduce the respiratory rate by 50%) will be equal to $K_i(v_{max}/v_0)$ where K_i is the inhibition constant for the electron-saturated system ($v_{max} = v_0$). For the succinate oxidase system ($v_0 = 20$ sec^{-1}), I_{50} should then be $5 \times K_i$ (for the oxidase reaction, $v_0 = v_{max} = 100$ sec^{-1}), instead of $10 \times K_i$ (above). Fig. 1, however, agrees with the present theory. Moreover, the idea of azide as an "uncompetitive" inhibitor is associated with the view[6,16] that it reacts with reduced cytochrome *a* rather than ferric a_3. Arguments against this conclusion are considered below.

Further support for the present concept is given by the steady-state reductions of cytochromes *a* and *c* in Keilin–Hartree particles. Fig. 2 shows that the inhibition of respiration (Fig. 1) parallels the changes in reduction of cytochrome *c*. Cytochrome *a* is markedly reduced at a very low azide concentration, indicating that cytochrome a_3 has already combined with the inhibitor, but a compensating increase in cytochrome *c* reduction occurs until some 90 % of the a_3 has been thus inhibited. At this level inhibition of respiration commences. The maximal steady-state reduction of 50 % at 444–460 mμ, even at high azide concentrations, supports the idea that a_3 contributes approximately half the absorption difference in the Soret region and that in the presence of azide it remains in the oxidized form.

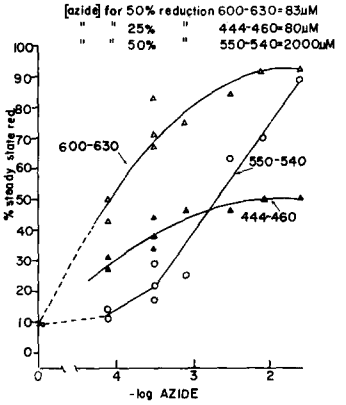
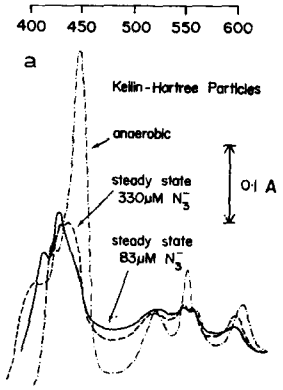


Fig. 2. Steady-state changes in azide-inhibited Keilin–Hartree particles. Rapid-scan measurements in a Cary 14 spectrophotometer using a 10-fold dilution of particles in 250 mM sucrose, 20 mM potassium phosphate (pH 7.4), 25°. Exogenous cytochrome *c* was added to a final concentration of 5.3 μM. Heme *a* concentration = 5.0 μM. Difference spectra, reduced *minus* oxidized with 17 mM succinate as substrate. Sucrose was present to facilitate Soret measurements.



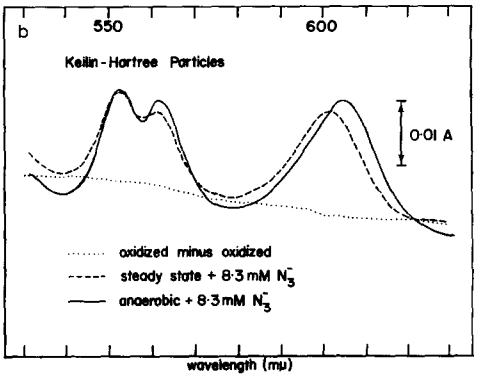


Fig. 3a. Effect of azide inhibition on the steady-state and anaerobic difference spectra of succinate-treated Keilin–Hartree particles. Conditions are the same as for Fig. 2. The anaerobic spectrum is identical in 330 and 83 μM NaN_3. Cary 14 spectra. Baseline (not shown) as in Fig. 3b.

Fig. 3b. Expanded spectra of the visible region in azide-inhibited succinate-treated Keilin–Hartree particles. A 15-fold dilution of particles in 0.065 M potassium phosphate, 0.001 M EDTA (pH 7.4), 6.7 mM succinate. High azide concentration gives almost total reduction in steady state. Difference spectra, reduced *minus* oxidized. 1 mμ·sec^{-1} scanning speed, 25°.

Spectroscopic effects of azide

The differential changes reflect overall spectroscopic changes. Fig. 3a shows the difference spectra of Keilin–Hartree particles, during succinate oxidase activity in presence of low and high azide concentrations, and after anaerobiosis. The shift in the absorption maximum of cytochrome *a* from 605 to 600 mμ, seen in mitochondria by WILSON[6], and the return to 605 mμ upon anaerobiosis, is shown here in uncoupled particles (this shift necessitates the use of the 600–630 difference as a measure of *a* reduction as in Fig. 2). Fig. 3b gives an expanded spectrum of the visible region in the presence of azide.

GILMOUR, WILSON AND LEMBERG[17] have recently shown that changes of this type are characteristic of cytochrome oxidase itself and are not dependent on the

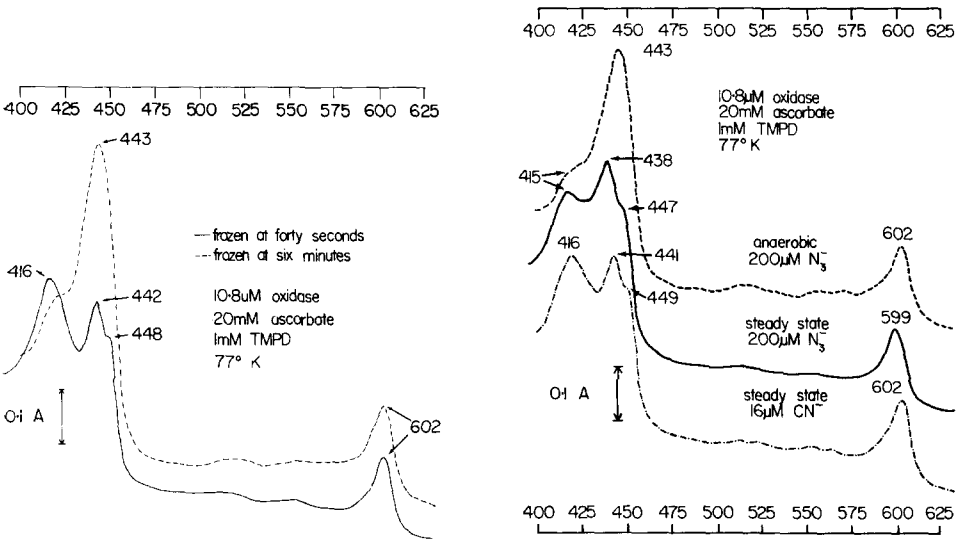


Fig. 4. Low-temperature absolute spectra of the uninhibited soluble cytochrome oxidase. 0.065 M potassium phosphate, 0.001 M EDTA (pH 6.6). At 77°K, cytochrome *a* is nearly 100% reduced in the steady state (40-sec spectrum). Note split Soret band[18,28] of a^{2+}, 416-mμ band (a_3^{3+}) and 443-mμ band (a^{2+}) after anaerobiosis. No change occurs at 602 mμ.

Fig. 5 Low-temperature absolute spectra of the azide- and cyanide-inhibited cytochrome oxidase. Conditions same as for Fig. 4. Note similarity of CN^--inhibited spectrum to uninhibited steady state (Fig. 4) and shift in α peak upon anaerobiosis in presence of azide.

intactness of the chain or the presence of energy-conserving reactions. Figs. 4 and 5 suggest that the spectroscopic shift requires cytochrome *a* to be reduced, cytochrome a_3 to be oxidized and combined with azide. If cytochrome a_3 is kept oxidized in the absence of azide, either in the uninhibited steady state (Fig. 4) or in the presence of cyanide (Fig. 5), the α band of *a* is at 602 mμ. If cytochrome a_3 is reduced, either in the absence (Fig. 4) or the presence (Fig. 5) of azide, the α band of *a* is also at 602 mμ.

The azide-induced spectral shift in the steady state is given in a room-temperature spectrum of the α band of soluble cytochrome oxidase in Fig. 6. No such shift is seen in the cyanide- or sulphide-inhibited steady states[17]. Temperature change affects the absolute position of the absorption bands but not the size of the azide shift. It can

now be seen that there is very little difference in the 590–620-mμ region between the cyanide-inhibited steady state and the anaerobic state.

Azide inhibition of phosphorylating systems

Increases in the steady-state reductions of cytochromes *a* and *c*, and the shift of the α band of *a* are also seen in intact mitochondria[6,18,19]. Mitochondria differ from the Keilin–Hartree particles, however, in that State 3 respiration is much more sensitive to azide inhibition than is uncoupled respiration[8]. Azide inhibition of Keilin–Hartree particles (Fig. 1) resembles the inhibition of uncoupled mitochondrial respiration. The responses of cytochromes *c* and *a* to azide inhibition of mitochondrial respiration were consequently examined in more detail.

Fig. 7 contrasts the increase of the steady-state reduction with increasing azide concentration, of cytochromes *a* (600–625 mμ) and *c*, for State 3 and the uncoupled state. In State 3 the steady-state reduction of *a* follows closely that of *c*. The concentrations of azide required to increase the steady-state reduction of *c* and *a* to 50% and decrease the respiration rate by 50% are the same. Upon the addition of an

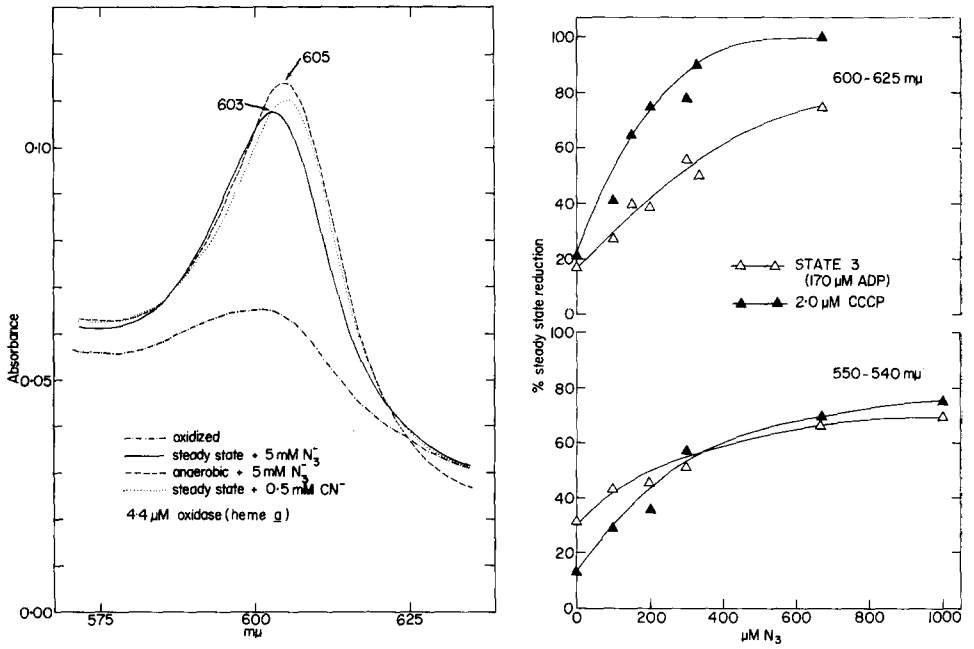


Fig. 6. Absolute spectra of the α peak of the uninhibited, azide- and cyanide-inhibited cytochrome oxidase at room temperature. 0.065 M potassium phosphate, 0.001 M EDTA (pH 6.7), 25°, oxidase reduced with 25 mM ascorbate *plus* 1 mM TMPD as substrate, in the absence of cytochrome *c*. Complete reduction checked by comparison of heme *a* concentration with that calculated from pyridine hemochromogen measurements[27].

Fig. 7. Steady-state changes in azide-inhibited rat liver mitochondria. 0.1 ml rat liver mitochondria (approx. 5 mg protein) in 3 ml 180 mM mannitol, 60 mM sucrose, 16 mM Tris-HCl, 10 mM potassium phosphate, 0.08 mM EDTA (pH 7.4), 25°, 16.7 mM succinate, 1.7 μM rotenone. Final concentration of heme *a* = 0.8 μM, cytochrome *c* = 0.8 μM. Changes in steady-state reduction followed in Aminco-Chance double-beam spectrophotometer, cytochrome *c* at 550–540 mμ, cytochrome *a* at 600–625 mμ.

uncoupler, the steady-state reduction of *a* is increased, that of *c* decreased. There is concomitant relief of inhibition, as originally shown by WILSON AND CHANCE[19]. The increased sensitivity of the steady-state reduction of cytochrome *a* to azide in the uncoupled state is such that the concentration of azide required to increase the steady-state reduction to 50 % is similar to the concentration required to inhibit the maximal activity of soluble cytochrome oxidase by 50 % (50–100 μM at pH 7.4). Thus, in the azide-inhibited State 3, the redox behavior of *a* mirrors that of *c*. Upon the addition of uncouplers, however, it appears directly related to the inhibition of a_3. Table I summarizes the kinetic parameters of azide inhibition in coupled and uncoupled mitochondria at pH 7.45.

TABLE I

SENSITIVITY OF MITOCHONDRIAL RESPIRATION AND STEADY-STATE REDUCTIONS TO AZIDE

Conditions as for Fig. 8.

	Turnover number uninhibited (electrons per heme a per sec)	*Azide conc. (μM) for:*		
		50 % inhibition of respiration	*50 % steady-state reduction*	
			600–625 mμ	*550–540 mμ*
State 3	16	300	250	250
Uncoupled	23	1400	90	250

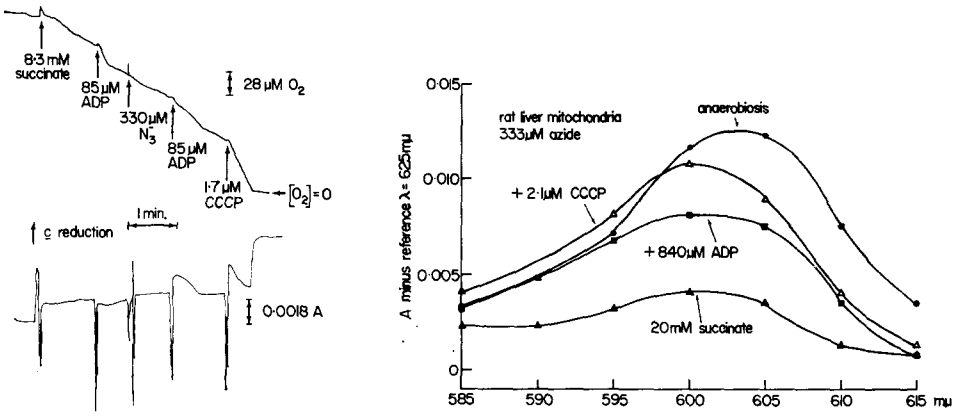


Fig. 8. Concurrent polarographic and spectroscopic changes in azide-inhibited rat liver mitochondria. 0.2 ml rat liver mitochondria (5 mg protein) in 6 ml. Reaction medium was 180 mM mannitol, 60 mM sucrose, 16 mM Tris–HCl, 10 mM potassium phosphate, 0.08 mM EDTA, 0.2 mM $MgCl_2$ (pH 7.45), 25°. Final concentration heme *a* = 0.4 μM, cytochrome *c* = 0.4 μM. 8.3 mM succinate, 2.1 mM rotenone. Respiration measured with vibrating platinum–silver electrode; optical changes as in Fig. 7.

Fig. 9. Effect of azide on the α peak of cytochrome *a* in rat liver mitochondria. 0.2 ml rat liver mitochondria (5 mg protein) in 3 ml reaction medium containing 280 mM mannitol. 80 mM sucrose, 50 mM potassium phosphate, 0.02 mM EDTA at pH 7.2, 25°, 20 mM succinate, 17 μM rotenone, 333 μM NaN_3. Aminco-Chance double-beam measurements. Reference wavelength, 625 mμ; measuring wavelength as indicated. Sequential additions made as in Fig. 8, except that azide was added first, then succinate, ADP and CCCP.

A concurrent polarographic and spectrophotometric tracing is shown in Fig. 8 for a single azide concentration. Upon the addition of carbonylcyanide *m*-chlorophenylhydrazone (CCCP) an initial overshoot is seen in the reduction of cytochrome *c*. No such "overshoot" was detected for cytochrome *a*. In agreement with previous observations[20], no effect on the P/O ratio, measured polarographically, is seen until relatively high azide concentrations are reached. At pH 7.45, the P/O ratio at 500 μM azide was 1.2 with succinate, the control value being 1.6. At azide concentrations higher than 500 μM the State 4 respiration rate is equal to or greater than the State 3 rate.

Complete spectra of the changes in the α peak of cytochrome *a* in azide-inhibited mitochondria are shown in Fig. 9. The position of the α peak of cytochrome *a* at 600 mμ, characteristic of azide inhibition, persists in State 4, State 3 and the uncoupled state. The λ_{max} immediately resumes its normal position upon the attainment of anaerobiosis. Thus the spectral effect of the uncoupler in the presence of azide is to increase the amount of the reduced 600-mμ form of cytochrome *a*.

DISCUSSION

Azide induces the formation of a new form of cytochrome a^{2+} with an absorption maximum at a shorter wavelength than the $a^{2+}a_3{}^{2+}$ type. The appearance of this form in uncoupled systems, Keilin–Hartree particles, and the soluble oxidase[17] as well as mitochondria in the presence of uncouplers argues against its interpretation as a possible azide-stabilized high-energy intermediate[8]. Further, its disappearance upon anaerobiosis (*i.e.* $a^{2+}a_3{}^{2+}$) in the presence of azide means it cannot simply be viewed as an a^{2+}–azide complex[6]. The interpretation adopted here is that the change in a^{2+} is a consequence of heme–heme interaction between $a_3{}^{3+}$-HN_3 and a^{2+}: possibly a conformational change in the environment of the heme of cytochrome a^{2+}, since the azide-induced a^{2+} spectral shift only disappears as $a_3{}^{2+}$ is formed.

The absence of a similar shift in a^{2+} in the cyanide- or sulphide-inhibited steady states implies that azide forms a complex with $a_3{}^{3+}$ different from that formed by these inhibitors. Azide also does not affect the rate of reduction at 444 mμ after anaerobiosis in Keilin–Hartree particles. Cyanide and sulphide slow this rate markedly (unpublished experiments).

The persistence of the azide-induced spectral shift in mitochondria uncoupled with CCCP contrasts with the marked relief of State 3 inhibited respiration by uncouplers, originally observed by WILSON AND CHANCE[19]. The spectroscopically detected effect of CCCP is to increase the steady-state concentration of "$a_{600}{}^{2+}$" and the concentration of azide required for 50 % steady-state reduction (Table I) is decreased to the amount required to inhibit the maximal activity of the oxidase by 50 %.

A possible explanation is to consider the sequence of Eqn. 2:

$$c \rightarrow a \rightarrow a_3 \rightarrow O_2 \qquad (2)$$

as obligatory only in State 3. The inhibited cytochrome at Site III may be $a^{3+} \sim I$, in the absence of acceptor. Relief of inhibition by ADP addition is prevented by $a_{600}{}^{2+}$ formation in the presence of azide, if $a_{600}{}^{2+}$ is also an inhibited form. In addition, we must assume $a_{600}{}^{2+}$ to be relatively stable; thus, the rate of the $a_{600}{}^{2+} \rightarrow a_{605}{}^{2+}$ transition must be slower than that of azide binding and dissociation (Eqn. 3):

$$a_3{}^{3+}\text{-}HN_3 \rightleftharpoons a_3{}^{3+} + HN_3 \qquad (3)$$

In the uncoupled state or in Keilin–Hartree particles an increase in the steady-state reduction of *c* overcomes the decrease of free a_3^{3+} due to its combination with azide. This is independent of a_{600}^{2+}. Conversely, in coupled systems in State 3, changes in steady-state reduction of *c* parallel changes in a_{600} (not a_{605}). In the uncoupled state we propose that cytochrome *a* does not reduce a_3 at a kinetically significant rate[21]. Cytochrome *c* must be responsible for the reduction of a_3 in such systems. Fig. 10 illustrates the proposed pathways.

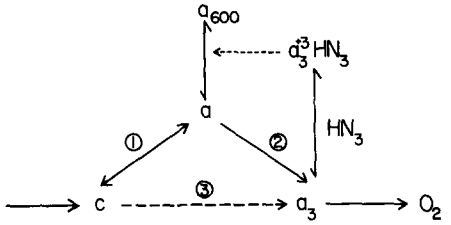


Fig. 10. Proposed pathways of electron transport. ⟶, electron-transfer reaction; ⇀↽, reversible equilibrium; ⟶ heme–heme interaction. (1), reduction of *a* by *c*, k = 50–100 sec^{-1}, independent of azide. (2), reduction of a_3 by *a*, fast for a_{605}, slow for a_{600}. (3), reduction of free a_3 by *c*, k = 50–100 sec^{-1} in uncoupled system, slow in coupled system.

PALMIERI AND KLINGENBERG[16] suggest that the relief of azide inhibition by uncouplers is associated with a release of accumulated azide from the mitochondrion. The high sensitivity of State 3 respiration is thus attributed to a higher internal mitochondrial azide concentration. The inhibition of the oxidase reflects a decline in the value of k in Eqn. 3:

$$v = k\,[c^{2+}]\,[a_3^{3+}] \tag{3}$$

If the inhibition is due simply to a binding of the oxidase during the intramitochondrial accumulation of azide, we may calculate the intramitochondrial azide concentration $[N_3^-]_i$ from Eqn. 4:

$$[N_3^-]_i = \left(\frac{v_0 C_i}{v_i C_0} - 1\right) K_i \tag{4}$$

where v_0 and v_i are the uninhibited and inhibited rates and C_0 and C_i the reductions of *c* in the uninhibited and inhibited systems, respectively. As both ratios, C_i/C_0 and v_0/v_i, increase concurrently with the increase in external azide concentration[19], the ratio $[N_3^-]_i/[N_3^-]_0$ must rise sharply with increasing $[N_3^-]_0$ and decreasing respiratory rate, the reverse of the situation expected for an energy-dependent carrier process.

The "reversed control" observed at certain azide concentrations[19] (where the State 4 rate exceeds the State 3 rate) also indicates a change in the mechanism of electron transfer rather than a change in inhibitor concentration. Further the similarity of the apparent K_i (in State 3) to K_i for isolated cytochrome oxidase is a coincidence in the view of PALMIERI AND KLINGENBERG[16], a necessary consequence of the present theory.

The theory that the oxidase is responding to high intramitochondrial azide concentrations would also predict increases in the steady-state reduction of cytochrome *a* at very low external azide concentrations; such changes do not occur, nor

does the mitochondrial oxidase prove exceptionally sensitive to azide when assayed with ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD). The very high sensitivity found in presence of valinomycin[16] is another matter. Neither the present results nor those of WILSON AND CHANCE[19] in any way militate against a role for the active azide uptake which doubtless occurs in this system simultaneously with the uptake of K^+.

The idea of WILSON AND GILMOUR[18], adopted by PALMIERI AND KLINGENBERG[16], that reduced *a* rather than oxidized a_3 reacts with azide is also hard to reconcile with certain experimental observations. Thus, the appearance of the 600-mμ form of *a* does not always parallel the azide inhibition (Figs. 7 and 9). SLATER[22] has observed small changes in the spectrum of ferric *a* and a_3 on the addition of azide in the inhibitory range. And VAN GELDER *et al.*[23] have demonstrated the appearance of an unusual paramagnetic signal (associated, therefore, with a_3^{3+}, but not present in totally oxidized enzyme) in the EPR spectrum of partially reduced oxidase in presence of azide (presumably $a^{2+}a_3^{3+}$-HN_3). This signal may be interpreted as a product of the interaction between a^{2+} and a_3^{3+}-HN_3 postulated here (Fig. 10). The change in the optical spectrum of a^{2+} is thus accompanied by a change in the EPR spectrum of a_3^{3+}-HN_3.

With GIBSON AND GREENWOOD[9] we conclude that in the isolated cytochrome oxidase and indeed all uncoupled systems, cytochrome *a* does not reduce cytochrome a_3. A quasi-independent role in the reduction of O_2 (ref. 9) attributed to *a* is not ruled out. With CHANCE[24] we interpose cytochrome *a* between cytochromes *c* and a_3 in the phosphorylating system. Unlike CHANCE, however, we see no reason to associate the spectral shifts in cytochrome *a* with "high-energy" forms. Though cytochrome *a* is evidently associated with the terminal phosphorylation, at present only kinetic evidence is available for that association.

The possibility that the spectrum of a^{2+} is modified by the state of a_3^{3+} seriously complicates analysis of the spectrum of a_3 by static difference spectra[26,27]. Fig. 6 suggests that the contribution of a_3 to the difference spectrum[25] at 605 mμ is 7.5 %. Such results are now questionable, however, in view of the shifts in the α peak of *a* with azide. The requirement for independence of the spectral properties of *a* and a_3 as a necessary assumption in the static method was mentioned by VANNESTE[26]. The same author calculated a contribution of a_3^{2+} at 602 mμ of not more than 10 %. Since that calculation involved the use of an $a^{2+}a_3^{2+}$-CO *minus* $a^{2+}a_3^{2+}$ difference spectrum, it implicitly assumes the absence of a_3^{2+}-CO $\rightarrow a^{2+}$ interaction. HORIE[27] also calculated absolute spectra on the basis of difference spectra, including $a^{2+}a_3^{2+}$ *minus* $a^{2+}a_3^{3+}$-CN^-. Both authors obtained a peak in the a_3^{2+} absolute spectrum at 565 mμ. HORIE reported an α peak for a_3^{2+} at 608 mμ; VANNESTE an α peak at 602 mμ. If the absorption changes in the 600-mμ region of these spectra are a consequence of variations in the spectrum of a^{2+} due to heme–heme interaction, then the true absorption of a_3^{2+} in the visible region is restricted to a broad absorption centered at 560 mμ (*cf.* hemoglobin at 559 mμ; carboxyhemoglobin at 570, 542 mμ, and a_3^{2+}-CO at 591, 548 mμ (ref. 27). The slowly reducible 605-mμ component[29,30], which may correspond to cytochrome a_3, represents a very small proportion of the 605-mμ absorption[30].

Thus cytochromes *a* and a_3 may have quite different reduced spectra. Ferrous cytochrome *a* is characterized by the split Soret band (maxima at 440 and 448 mμ)[28]

and by the hemochromogen-like band at 605 mμ. Ferrous cytochrome a_3 has a single Soret band at 445 mμ and perhaps a broad band at 560 mμ in the visible region.

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