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AZIDE INHIBITION OF MITOCHONDRIAL ELECTRON TRANSPORT

I. THE AEROBIC STEADY STATE OF SUCCINATE OXIDATION

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SUMMARY

The azide inhibition of the succinate oxidase activity of rat-liver mitochondria is specific for active (State 3) respiration with no observable inhibition of resting (State 4) respiration. In the range of azide concentrations which inhibit State 3 to rates less than those of State 4, a negative control of respiration by ADP and inorganic phosphate is observed. The inhibition is specific for a site between cytochromes *a* and a_3, causing a crossover between these two cytochromes with cytochrome *a* becoming reduced and cytochrome a_3 remaining highly oxidized. Trapped steady-state difference spectra at liquid nitrogen temperatures show that the reduced cytochrome *a* in the azide-inhibited system has an α band at 596 mμ, 6 mμ displaced from its usual position at 602 mμ.

The azide inhibition is released by uncouplers of oxidative phosphorylation such that the uncoupled respiration requires up to ten times as much azide as does coupled (State 3) respiration for comparable inhibition. The release of inhibition by uncouplers occurs with no change in the steady-state concentration of reduced cytochrome a_{593} and the increased respiration is attributed to an increased rate of oxidation of the cytochrome a_{596}. This cytochrome is postulated to be either an intermediate in electron transport and energy conservation reactions or an azide compound of such an intermediate.

INTRODUCTION

Azide was introduced as an inhibitor of mitochondrial electron transport by KEILIN in 1939 (refs. 1, 2). He observed that when azide and substrate were added to a cytochrome-containing particulate preparation from heart muscle, the cytochromes *a*, *c* and *b* became reduced and the oxygen uptake was inhibited. Cytochrome a_3 was observed to remain oxidized, and it has been generally assumed that the azide inhibits by forming an oxidized cytochrome a_3 azide compound. However, MINNAERT[3] and YONETANI[4], working with the Keilin–Hartree heart-muscle preparation and isolated soluble cytochrome *c* oxidase, respectively, were unable to assign a particular component for the azide-binding site.

Abbreviations: TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

In intact tissue[5–8] azide is able to inhibit active respiration but has little effect on the resting respiration. ARMSTRONG AND FISHER[8] found an azide-insensitive respiration in Fundulus and Salmon embryo hearts and an associated azide-insensitive beat frequency. A selective inhibition of the respiration associated with nerve transmission with no affect on the resting respiration of nerve fibers was observed by BRINK *et al.*[6]. In a similar manner the State 3 respiration of intact mitochondria (active) is inhibited by azide but the State 4 respiration (resting) is not[9,10].

In separate studies on oxidative phosphorylation, azide has been reported to uncouple oxidative phosphorylation[11,12] and to inhibit the uncoupler-stimulated ATPase activity[13,14] but not the ADP–ATP exchange reaction[15].

The present paper is part of an intensive investigation of the effect of azide on intact mitochondrial electron transport and oxidative phosphorylation which was undertaken in order to gain insight into the mechanism of electron transport and the coupling of electron transport to energy conservation reactions. A preliminary account of this work has been published[10].

METHODS AND MATERIALS

Rat-liver mitochondria were prepared in 0.22 M mannitol, 0.075 M sucrose and 200 μM EDTA by the method of CHANCE AND HOLLUNGER[16] except when the mitochondria were to be used for Ca^{2+} uptake experiments. In those preparations the EDTA was omitted during the washing procedure.

All assays were carried out in a 3.0-ml reaction chamber and used a 0.22 M mannitol, 0.075 M sucrose, 15 mM tris(hydroxymethyl)aminomethane (MST) medium. Unless otherwise stated the succinate concentration was 10 mM. The ethylenediaminetetraacetate (EDTA) concentration, inorganic orthophosphate (P_i) concentration and pH are given in the figure and table legends. The oxygen concentration was measured polarographically at 23° using a vibrating platinum electrode assuming the air-saturated medium contained 250 μM O_2. The cytochromes were measured simultaneously by a Johnson Foundation double-beam (dual-wavelength) spectrophotometer. The liquid nitrogen temperature spectra were run on a Johnson Foundation wavelength scanning spectrophotometer. This instrument is a modification of the instrument designed and developed by CHANCE and co-workers[17,18] and adapted for use at liquid nitrogen temperatures by ESTABROOK[19,20]. In the present work a Bausch and Lomb diffraction grating monochromater was used as a source of monochromatic light. Under the experimental conditions the effective band width of the monochromatic light was 1.7 mμ. The peak positions in the spectra were reproducible to 10 Å and the wavelength was calibrated to $\pm$ 10 Å.

The sample cuvettes were those described by BONNER[21] with a 2-mm copper insert instead of a 3-mm aluminum insert. Samples were frozen by the trapped steady-state technique of CHANCE AND SPENCER[22] in which the samples are injected into cuvettes precooled to liquid nitrogen temperatures. This technique gives a uniform rapid freezing process which results in very reproducible low-temperature spectral enhancements.

Practical grade sodium azide was obtained from Matheson Coleman and Bell. Sigma grade adenosine 5′-diphosphate (ADP), reagent grade EDTA and tris(hydroxymethyl)aminomethane were obtained from Sigma Chemical Company. The 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB) was the generous gift of Dr. R. B.

BEECHEY of Shell Research, Ltd., Milstead Laboratory of Chemical Enzymology, Kent, Great Britain. The carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a generous gift of Dr. P. G. HEYTLER, Central Research Department, E. I. duPont de Nemours and Co., Wilmington, Del. The 4-isooctyl-2,6-dinitrophenol was from a sample generously supplied by Prof. E. C. SLATER and crystallized by Dr. B. C. PRESSMAN.

EXPERIMENTAL RESULTS

The effect of azide on the respiration and redox levels of the cytochromes

Fig. 1A illustrates the inhibition of mitochondrial succinate oxidation by azide at pH 6.7. The State 3 respiration is markedly inhibited with half-maximum inhibition in this case occurring at about 60 μM azide. Azide inhibition is pH dependent[1,3,4] and more azide is required at higher pH values. The State 4 respiration is much less sensitive and remains azide-insensitive until the azide concentration exceeds 300 μM and then it is inhibited to the same level as the State 3 respiration. This 'inhibition of State 4' may be due to the very low P/O ratio of mitochondria in the presence of high azide concentration[11,12]. The rotenone-inhibited mitochondria under the assay conditions used here were in State 2 in the presence of azide when the succinate was added and were unable to phosphorylate completely their endogenous ADP; thus, they never attained a true State 4.

The steady-state levels of reduction of the respiratory carriers were determined at the same time as the oxygen uptake with a double-beam spectrophotometer–oxygen electrode combination. Fig. 1B demonstrates the azide dependence of the steady-state level of cytochrome *c* reduction as a function of azide concentration. By direct comparison of Figs. 1A and 1B, the relationship of the rate of oxygen uptake and

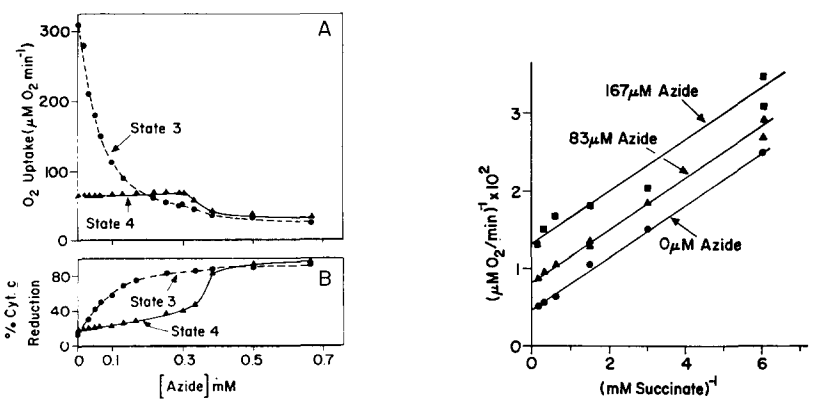


Fig. 1A, B. The azide inhibition of rat-liver mitochondria. The mitochondrial suspension contained 2.2 mg protein/ml (pH 6.9). MST medium contained 9 mM orthophosphate, 200 μM EDTA and 70 μM Mg^{2+}. 3 μM rotenone was added prior to the substrate which was 2 mM glutamate and 6.7 mM succinate. The oxygen uptake and cytochrome *c* reduction (550–540 mμ) were measured simultaneously. States 4 and 3 refer to before and after the addition of 600 μM ADP, respectively.

Fig. 2. Lineweaver–Burk plot of the azide inhibition. The mitochondrial suspension was 2 mg protein/ml in MST medium–P_i medium (pH 7.2) containing 10 mM orthophosphate. 3 μM rotenone, 2 mM glutamate and 200 μM ADP was added and then the reactions started with succinate.

cytochrome *c* reduction can clearly be seen. Plots similar to Fig. 1B may be made for cytochrome *a* (605–575 mμ or 605–630 mμ). In a typical experiment at pH 6.9, the azide concentrations for half-maximal effect would be 40 μM for inhibition of respiration (State 3), 40 μM for cytochrome *a* reduction and 70 μM for cytochrome *c* reduction. When cytochromes $a + a_3$ were measured at 445–460 mμ, approximately one-half of the optical change was titrated parallel to the cytochrome *a* α band and one-half occurred only after anaerobiosis. The cytochrome a_3 must, therefore, be oxidized in the azide-inhibited mitochondria in the aerobic steady state.

Type of inhibition

The azide inhibition of State 3 respiration at pH 7.2 is the uncompetitive type as shown by the approximately parallel lines of the Lineweaver–Burk plots in Fig. 2. When the apparent inhibitor constants were calculated from the intercepts of the lines at the ordinate using the equations for uncompetitive inhibition this value was found to be slightly dependent on the azide concentration, *i.e.*, decreasing with increasing azide concentration. This concentration dependence is consistent with the observation that for most mitochondrial preparations, the State 3 respiration rate with ADP + P_i is somewhat less than the rate in the presence of uncouplers. Low concentrations of azide would then inhibit less than expected for the system with maximum flux.

The ADP/O ratio

Azide has previously been reported by LOOMIS AND LIPMANN[11] and SLATER[12] to uncouple oxidative phosphorylation, while CHANCE AND WILLIAMS[9] did not find a significant decrease in the ADP/O ratios with azide concentrations which gave 50 % inhibition of State 3 respiration with succinate as substrate. All of these reports are consistent with the data in Table I. As shown, the ADP/O ratio for succinate oxidation is relatively independent of the azide concentration until the State 3 respiration is inhibited to about 25 % of the control value. In view of the uncompetitive nature of the azide inhibition, it is not possible to extrapolate these data directly to other substrates which have different maximal rates of oxidation.

TABLE I

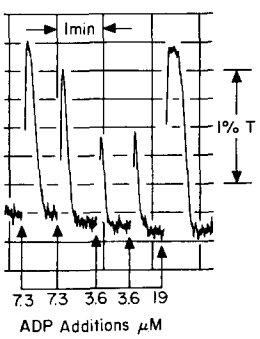
THE EFFECT OF AZIDE ON THE ADP/O RATIOS OF RAT-LIVER MITOCHONDRIA

The State 4 respiration of 52 μM O_2/min was unaffected by azide at the concentrations used in this experiment. The assay medium was pH 6.7 and contained 3 mg protein/ml.

Azide conc. (μM)	*State 3 respiration* (μM O_2/min)	*ADP/O ratio*
0	300	1.8
16	190	1.8
32	125	1.6
48	87.5	1.4
64	75	1.5
82	72	1.3
128	45	0.9
144	37	0.7

The State 4 to 3 transition in the presence of azide as induced by ADP, P_i or Ca^{2+}

CHANCE AND WILLIAMS[9] had previously used ADP to titrate the appearance of reduced cytochrome *a* in the State 4 to 3 transition of mitochondria in the presence of azide. They observed very low concentrations (less than 12 μM) of ADP required for half-maximal effects. A double-beam tracing of such an experiment is shown in Fig. 3. In this experiment the addition of small amounts of ADP resulted in the appearance of reduced cytochrome *a* as measured either at 605–575 mμ or 445–460 mμ. This reduced cytochrome *a* was then reoxidized as the ADP was phosphorylated. This appearance of reduced cytochrome *a* required both P_i and ADP, or divalent cations such as Ca^{2+}, and the extent of reduction was dependent on the concentration added, as shown in Fig. 4 for ADP and Ca^{2+}. Routine values for half-maximal effects at azide concentrations which inhibit State 3 respiration to less than State 4 respiration for mitochondrial suspensions containing 2–2.5 mg protein/ml were of the order of 5 μM ADP (in the presence of 9 mM P_i), 6–8 μM Ca^{2+} (with or without P_i when the medium contained acetate) and 25 μM P_i (in the presence of 500 μM ADP). It must be emphasized that these values are dependent on the azide concentration as previously reported[9], and in the present experiments the azide concentrations selected were the maximum which still permitted a State 4 to 3 transition as measured by the change in reduction of cytochrome *a*. Therefore, they represent maximal values only and are probably not true binding constants.



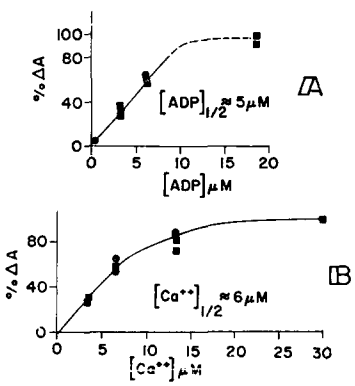


Fig. 3. The effect of ADP additions on the redox level of cytochrome *a* in azide-inhibited mitochondria. The mitochondrial suspension contained 2.3 mg protein/ml in MST medium (pH 7.1) with 10 mM orthophosphate and 660 μM azide. The wavelength settings were 445–455 mμ.

Fig. 4A, B. The relationship between the concentration of added Ca^{2+} and ADP on the magnitude of the change in reduction of cytochrome *a*. The changes are expressed as percent of the maximum achieved by larger additions measured as shown in Fig. 3. The medium used was MST acetate (pH 7.1) with 9 mM orthophosphate added for Fig. 4A.

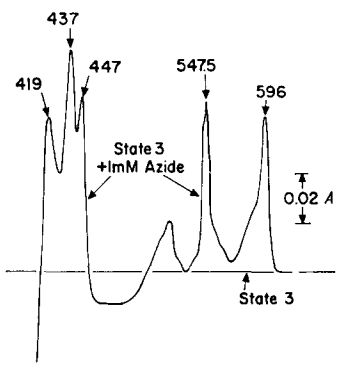
The crossover in the respiratory chain on addition of azide

The site of inhibition of State 3 respiration was tested by the crossover theorem. As shown in Fig. 5 the difference spectrum at 190° of mitochondria in State 3 *minus* that of mitochondria in State 3 *plus* azide was obtained by the trapped steady-state technique of CHANCE AND SPENCER[22]. The site of interaction is between cytochromes *a* and a_3 as shown by the reduction of cytochrome *a* (Soret maxima at 437 and 447 mμ and α mazimum at 596 mμ) while a_3 remains highly oxidized (see also ref. 23). The

reduction of cytochrome a_3 occurs only after anaerobiosis as seen by the appearance of an absorption band at 443 mμ (ref. 23).

The interaction of uncouplers and azide

The release of azide inhibition by uncouplers of oxidative phosphorylation has been previously reported[10]. When the uncompetitive inhibitor constant was calculated from Lineweaver–Burk plots of the azide inhibition in the presence of uncouplers, the inhibitor constant increased from 92 to 372 μM as the dicoumarol concentration was increased from 0 to 40 μM. A kinetically less satisfactory but experimentally more manageable presentation is shown in Fig. 6. Since the succinate concentrations used (16–30 mM) were much greater than the K_m for succinate in State 3 (1–1.6 mM), the rates of oxygen uptake in the absence of azide are reasonable approximations of the kinetic maximal rate (v_{max}), and the azide concentrations for half-maximal effect are approximately inhibitor constants. The Dixon plots (the reciprocal of the respiration rate against inhibitor concentration) of azide inhibition in the presence of the uncoupler (FCCP) are linear, but the slope decreases with increasing uncoupler concentration. The azide concentration required for half-maximal inhibition changed from 110 μM in the presence of ADP *plus* P_i to 820 μM when 0.67 μM FCCP was added.



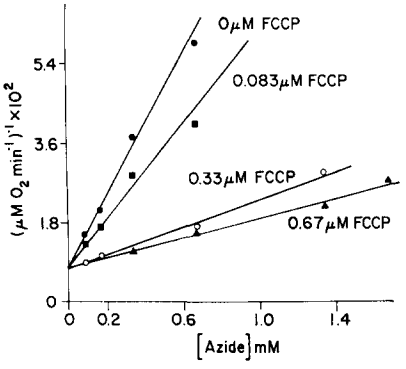


Fig. 5. The effect of azide on the redox levels of mitochondria in State 3. Rat-liver mitochondria at 15 mg/ml were suspended in MST medium (pH 7.3) with 10 mM orthophosphate. The reference sample was frozen after addition of 3 μM rotenone, 12 mM succinate and 2 mM ADP. The measure sample was then frozen after the addition of 1 mM azide and the spectrum represents the State 3 mitochondria in the presence of azide *minus* State 3 mitochondria.

Fig. 6. The release of azide inhibition by uncouplers. Rat-liver mitochondria at 2.4 mg protein/ml were suspended in MST medium (pH 7.3) with 10 mM orthophosphate. 3 μM rotenone was added before the substrate which was 16 mM succinate. In all the assays the ADP concentration was 600 μM.

This release of azide inhibition is a general property of uncouplers as shown in Table II. The azide concentrations for half-maximal effect were determined from Dixon plots. The results are normalized and presented as the ratio of the azide concentration for half-maximal effect in the presence of uncoupler to that in the absence of uncoupler. Three classes of uncouplers are represented: FCCP is a substituted phenylhydrazone of carbonylcyanide, TTFB is a substituted benzimidazole, and the rest are substituted phenols. In each case, the uncoupler was effective in the concentration range used to uncouple oxidative phosphorylation.

TABLE II

REVERSAL OF AZIDE INHIBITION BY VARIOUS UNCOUPLERS

The assay medium was MST medium (pH 7.3) containing 10 mM orthophosphate. The column on the right is the ratio of the azide concentration required for half-maximal inhibition in the presence of uncoupler to that in its absence.

Uncoupler	*Uncoupler concn.* (μM)	*Azide concn. + uncoupler* / *Azide concn.*
FCCP	0.43	4.7
FCCP	1.7	8.9
TTFB	1.9	4.6
TTFB	4.9	7.0
Dicoumarol	10.8	3.3
Dicoumarol	21.6	3.9
Pentachlorophenol	6.6	1.4
Dinitrophenol	52.0	1.3
Dinitrophenol	208.0	3.3

To what degree the azide inhibition can be released by uncouplers is a question which is difficult to answer. The direct addition of high concentrations of uncouplers to the assay introduces the possibility of interference by side reactions of the uncouplers such as their direct inhibition of respiration[24]. The uncoupler FCCP, at concentrations well above those used for uncoupling phosphorylation (3.4 μM *vs.* 0.35 μM for maximal stimulation of respiration), gives a ratio of 10 for the azide concentration for half-maximal effect in the presence of uncoupler to that in its absence without measurably increasing the intercept of the Dixon plot. The electron transport in the presence of uncoupler is, therefore, still inhibited by azide but has a greatly decreased sensitivity to the inhibitor.

The crossover for the release of azide inhibition by uncouplers

The inhibition by azide and its release by uncouplers made the system amenable to test for the inhibitory site by the crossover theorem as shown in Table III. The

TABLE III

THE EFFECT OF AZIDE AND DICOUMAROL ON THE STEADY-STATE REDOX LEVELS OF THE CYTOCHROMES OF RAT-LIVER MITOCHONDRIA

The MST medium contained 2.2 mg protein/ ml, 10 mM orthophosphate, and 600 μM ADP with final pH 7.2. The measuring wavelengths were 605–575 mμ, 550–540 mμ and 561–575 mμ for cytochromes *a*, *c*, and *b*, respectively. State 3 respiration rate is expressed as μM O_2/min.

Azide concn. (μM)	$\pm$ *Dicoumarol* (*33* μM)	*State 3 respiration*	% *Reduction*		
			a	*c*	*b*
0	—	236	13	19	28
0	+	233	13	14	<5
83	—	153	51	40	43
83	+	198	63	46	<5
167	—	99	70	60	66
167	+	167	79	64	8
500	—	40	90	78	67
500	+	115	90	79	19

addition of 33 μM dicoumarol to an azide-inhibited system containing excess ADP (400 μM) caused an abrupt oxidation of cytochrome *b* while cytochromes *c* and *a* became somewhat more reduced. In the control experiment in the absence of azide the dicoumarol caused an oxidation of both cytochromes *b* and *c*. The change in the dicoumarol effect of cytochromes *c* from oxidation in the absence of azide to reduction in its presence is evidence for a weak site of azide inhibition between substrate and cytochrome *c*. In addition, increased flux occurs with a minimal change in the steady-state reduction of the cytochromes *c* and *a*. A 3-fold increase in the electron flux without a concomitant increase in the steady-state reduction of these cytochromes can best be interpreted as an increased rate of oxidation of reduced cytochrome *a*.

DISCUSSION

The classical concept of azide inhibition of electron transport is that of KEILIN in which heme-binding agents such as azide react with cytochrome a_3 to form an inhibitory compound. Azide should then be very similar to cyanide as an inhibitor since cyanide reacts with cytochrome a_3 either in the reduced or oxidized form to give an inhibitory cyanide compound the reduced form of which is readily oxidized[4]. This analogy is poor even in non-phosphorylating systems such as isolated cytochrome oxidase[3,4] in which azide and cyanide inhibition differ as to inhibitor type (non-competitive and uncompetitive with respect to ferrocytochrome *c*, respectively) and pH dependence of the inhibition.

The comparison is even less satisfactory in phosphorylating systems where the azide inhibition is reversed by uncouplers of oxidative phosphorylation, is uncompetitive with respect to succinate, and is characterized by its ability to induce reversed control of electron transport by ADP and P_i. Cyanide differs in all of these points.

Oligomycin[25,26] resembles azide in that neither is able to inhibit State 4 respiration and in the fact that their inhibition of State 3 is released by uncouplers. However, the differences are much greater than the similarities. When azide is added to State 3 mitochondria, the cytochromes (with the exception of a_3) become reduced while oligomycin causes only a return to State 4 levels of reduction. The addition of Ca^{2+} has no effect on an azide-inhibited State 3 whereas in the oligomycin-inhibited system it causes a State 4 to State 3 transition while the ion is being accumulated[26,27].

Azide inhibition causes a reduction of cytochrome *a* while cytochrome *a* remains highly oxidized, indicating that the reduction of cytochrome a_3 by cytochrome *a* is inhibited. This observation, combined with the well-known ability of azide to form compounds with hemes and the reactivity of cytochrome a_3 toward cyanide and CO, remains the only evidence for the formation of an oxidized cytochrome a_3 azide compound which is responsible for the inhibition of respiration.

The azide inhibition is highly specific for the site between cytochromes *a* and a_3 since the azide concentration required for half-maximal inhibition is, within the experimental error, identical to that required for half reduction of cytochrome *a* in the aerobic steady state. Although the overall mechanisms of phosphorylation and electron transfer of cytochrome oxidase *in situ* remain obscure, they must contain certain partial reactions. The donation of electrons to the cytochrome oxidase must give rise to a kinetic equivalent to the enzyme–substrate complex which then becomes an inhibitory form in the presence of azide. The azide would thus be an uncompetitive

inhibitor of electron flow. This inhibitory form must be of the form, reduced cytochrome *a*–oxidized cytochrome a_3, in order to be consistent with the observed redox levels of these cytochromes in the azide-inhibited steady state. We have previously stressed the role of cytochrome *a* in oxidative phosphorylation[10]. The evidence presented here for a spectrally distinct cytochrome *a* (a_{596}) which is found in the presence of azide and converted to cytochrome a_{602} on reduction of cytochrome a_3, suggest that a_{596} is either an intermediate in the energy conservation reactions of a product or the reaction of azide with an intermediate in oxidative phosphorylation and electron transport.

The original observation by CHANCE AND WILLIAMS[29] was that the transition from State 4 to State 3 was accompanied by an oxidation of cytochrome *c* and a reduction of cytochrome *a*. This crossover in the high to low energy states was interpreted in terms of the crossover theorem to mean that the inhibited form of the carrier in State 4 (carrier-I) was either reduced cytochrome *c* or oxidized cytochrome *a*. The present observations are 2-fold: first, that reduced cytochrome *a* can exist in two spectrally distinct forms, and second, that uncouplers increase the rate of oxidation of reduced cytochrome *a* in the azide-inhibited state.

The energy conservation reactions are such that 2 electrons flow through a phosphorylation site for each ADP phosphorylated, and yet the cytochromes are nominally one-electron carriers. The inhibition of both the oxidation and reduction of cytochrome *a* by inhibitors of the energy conservation reactions and the existence of two forms of reduced cytochrome *a* are strong evidence that the 2 electrons are transferred sequentially and that the inhibitors can preferentially inhibit one or the other transfer. It is reasonable to postulate that electron transfer in coupled electron transport occurs not by two-electron transfer as has been suggested[30,31], but by an ordered pair of single electron transfers.

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