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Steady-State Kinetics of the Overall Oxidative Phosphorylation Reaction in Heart Mitochondria. Determination of the Coupling Relationships Between the Respiratory Reactions and Miscellaneous Observations Concerning Rate-Limiting Steps

Clinton D. Stoner[1]

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Abstract

The linear sequence of steps involved in the oxidation of extramitochondrial succinate by O_2 in bovine heart mitochondria was examined by a steady-state kinetic method to determine whether or not freely diffusible intermediates occur between the various inhibitor-sensitive steps. The kinetic method is based on the facts (1) that if two inhibitor-sensitive steps within a sequence are linked by a freely diffusible intermediate, inhibition of one will make the other less rate limiting in the overall reaction and thus will increase the amount of inhibitor of the other step required for half-maximal inhibition of the overall reaction, and (2) that if the two steps are not linked in this manner, inhibition of one will make the other more rate limiting and thus will decrease the amount of inhibitor of the other required for half-maximal inhibition. These two types of "coupling relationships" between steps were designated as "sequential" and "fixed," respectively. The results indicate the existence of freely diffusible intermediates (sequential coupling relationships) between the succinate transport and succinate dehydrogenase reactions, between the succinate dehydrogenase and cytochrome bc_1 reactions, and between the cytochromes bc_1 and aa_3 reactions. Uncoupling respiration from phosphorylation results in the coupling relationship between the bc_1 and aa_3 reactions becoming partially fixed. This change is accompanied by marked decreases in the degrees to which the bc_1 and aa_3 reactions limit the overall reaction and appears to account for the large uncoupler-induced releases of inhibition at the levels of the bc_1 and aa_3 reactions observed previously by others. It is suggested that cytochrome *c* is the freely diffusible intermediate between the bc_1 and aa_3 reactions and that the uncoupler-induced changes occur as a result of formation of functional and highly efficient supercomplexes between cytochrome *c* and the cytochromes bc_1 and aa_3 complexes.

[1]Department of Surgery, College of Medicine, Ohio State University, 1645 Neil Avenue, Columbus, Ohio 43210.

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Key Words: Mitochondria; oxidative phosphorylation; steady-state kinetics; rate-limiting step; coupling relationship; succinoxidase; specific inhibition.

Introduction

In a previous study of this nature (Stoner and Sirak, 1979), two types of coupling relationships between individual reactions of the overall oxidative phosphorylation reaction were observed, an individual reaction being a reaction within the overall reaction that is linked to others by freely diffusible intermediate reactants. The coupling relationships were designated as sequential and nonsequential and concern the manner in which two individual reactions affect each other in regard to how much they limit the rate of the overall reaction. In the sequential case a change in the degree to which one is rate limiting results in an oppositely directed change in the degree to which the other is rate limiting, whereas in the nonsequential case a change in the degree to which one is limiting has no apparent affect on the other in this regard. Which of these relationships exists between individual reactions appears to depend on how the reactions are coupled.

The sequential relationship is characteristic of individual reactions linked in simple sequence and its mechanism can readily be seen by examining simple model reactions of the type

$$A \overset{E_1}{\rightleftharpoons} I_1 \overset{E_2}{\rightleftharpoons} I_2 \overset{E_3}{\rightleftharpoons} I_3 \overset{E_4}{\rightleftharpoons} P \qquad (1)$$

In this reaction a substrate A is converted to a product P by way of four enzyme-catalyzed reactions linked by the freely diffusible intermediate reactants I_1, I_2, and I_3. The system is assumed to be free of allosteric interactions and the concentrations of the substrate and product are assumed to be maintained constant by continuous flux of A in and P out. The existence of the sequential coupling relationship between the individual reactions can be seen by considering what would happen if, for instance, the E_3-catalyzed reaction were caused to be more rate limiting such as by decreasing the effective concentration of E_3 by adding a specific inhibitor of this enzyme. It is clear that the concentrations of the intermediate reactants would adjust during the approach to the new steady state such that in the new steady state the E_3-catalyzed reaction would be operating farther from thermodynamic equilibrium and all the other individual reactions would be operating closer to thermodynamic equilibrium and thus would be less rate limiting in the overall reaction.

As was pointed out previously (Stoner and Sirak, 1979), sequential coupling relationships would exist between the individual reactions of mul-

tienzyme reactions of the above type even if the individual reactions were linked by "transfer type" intermediates (coenzymes) rather than by the relatively simple "accumulation type" intermediates shown. Thus the intermediates could just as well be electron carriers or hydrogen carriers such as those involved in the overall oxidative phosphorylation reaction. Consequently, the individual reactions of linear sequences of such reactions within the overall oxidative phosphorylation reaction should exhibit coupling relationships among themselves similar to those expected among the individual reactions of overall reaction (1). Furthermore, one should be able to test for the presence or absence of freely diffusible intermediates between various inhibitor-sensitive steps within linear sequences of the overall oxidative phosphorylation reaction. The primary purpose of this study was to conduct such tests on the sequence

$$S_{out} \overset{DCC}{\rightleftharpoons} S_{in} \overset{SDH}{\rightleftharpoons} Q \overset{bc_1}{\rightleftharpoons} c \overset{aa_3}{\rightleftharpoons} H_2O \qquad (2)$$

in which S_{out} and S_{in} are extramitochondrial succinate and intramitochondrial succinate, respectively, DCC is the dicarboxylate carrier, SDH is succinate dehydrogenase, Q is ubiquinone, bc_1 is the cytochrome bc_1 complex, c is cytochrome c, and aa_3 is the cytochrome aa_3 complex. Other reactants and enzymes known or thought to be involved in the oxidation of extramitochondrial succinate have been omitted for clarity and the cytochrome aa_3 step has been assumed to be reversible from a theoretical standpoint. The sequence is in the same form as overall reaction (1) and assumes that intramitochondrial succinate, ubiquinone, and cytochrome c are freely diffusible intermediates.

The general approach was to determine the effects of specifically inhibiting one step within the above sequence on the sensitivities of the other steps to specific inhibitors. It was used originally by Nijs (1967) and Baum *et al.* (1971) and is based on the fact that if two steps within the sequence are linked by a freely diffusible intermediate, inhibition of one will make the other less rate limiting in the overall reaction and will consequently increase the amount of inhibitor of the other reaction required to achieve a particular degree of inhibition of the overall reaction. On the other hand, if two steps within the sequence are not connected by a freely diffusible intermediate, inhibition of one will result in the other being more rather than less rate limiting and the amount of specific inhibitor of the other required to achieve a particular degree of inhibition of the remaining activity of the overall reaction will decrease rather than increase. For convenience, this coupling relationship between steps will henceforth be referred to as "fixed." In all the studies of the above type, phosphorylating and uncoupled mitochondria were compared to give some idea as to how energy conservation and linkage to the energy-consuming steps involved in the phosphorylation of extramitochondrial ADP

affect the relationships between the individual reactions of overall reaction (2).

With the exception of cytochrome *c* there is little question that the intermediate reactants of overall reaction (2) are freely diffusible. That intramitochondrial succinate functions in this manner is clearly expected from the facts that succinate normally enters the mitochondrion largely by way of the dicarboxylate carrier and that only intramitochondrial succinate is oxidized (Chappell and Haarhoff, 1967; Chappell and Robinson, 1968; Palmieri *et al.*, 1971). In the case of ubiquinone, Klingenberg and Kröger (1970) and Kröger and Klingenberg (1970, 1973) have shown by a number of methods that this carrier distributes electrons more or less randomly among the respiratory chain dehydrogenases and cytochrome chains of individual mitochondria and thus that it functions as a freely diffusible intermediate.

Owing to its relatively large size and low concentration, cytochrome *c* functioning in this manner seems much less likely. However, like ubiquinone, it is confined to a small space within the mitochondrion and forms complexes with both its reductase and its oxidase. According to current concepts it is confined largely to the outer surface of the inner membrane through electrostatic bonding to phospholipids and is free to diffuse among and form complexes with the bc_1 and aa_3 complexes (Chance, 1974; Nicholls, 1976; Roberts and Hess, 1977; Hochman *et al.*, 1982). Although the bc_1 and aa_3 complexes are likely also freely diffusible in the plane of the membrane, their transmembrane disposition and relatively large size might be expected to result in their diffusion being very slow relative to that of cytochrome *c* (Schneider *et al.*, 1980; Sowers and Hackenbrock, 1981; Hackenbrock, 1981).

Cytochrome *c* functioning as a freely diffusible intermediate has been suggested by a number of observations. For example, in early studies on CO-inhibited mitochondria, Chance (1965) obtained evidence for the distribution of electrons among respiratory chains at the cytochrome level, and Wohlrab (1970) demonstrated this distribution to be dependent on cytochrome *c*. Such distribution was indicated also in the previous steady-state kinetic study (Stoner and Sirak, 1979) by the observation that the coupling relationships of the antimycin- and cyanide-sensitive reactions to the P_i and AdN[2] transport reactions differ. In the present study this problem was tackled more directly by determining the coupling relationship between the respiratory chain reactions that cytochrome *c* links.

[2]Abbreviations used: AdN, adenine nucleotide; HQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; $I_{0.5}$, concentration of inhibitor required for 50% inhibition; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

Materials and Methods

Isolated bovine heart mitochondria were generously donated by G. P. Brierley and his associates. They were obtained according to the Nagarse procedure described by Jung *et al.* (1977) and were approximately one day old when used. Prior to use they were washed once and resuspended at 70–90 mg protein/ml in 250 mM sucrose + 5 mM K-Pipes (pH 7.0). To gain some idea as to the condition of these mitochondria relative to freshly isolated mitochondria, preparations obtained on five successive workdays were evaluated immediately after isolation and again after 24 hours of storage in capped vials at 0°C. Except for the absence of $MgCl_2$, the test medium was as described below for the assay of succinoxidase activity. The respiratory control ratios, ADP/O's, and State 3 respiratory activities (±SD) obtained in this evaluation were 4.1 ± 0.8, 1.60 ± 0.09, and 0.50 ± 0.10 μatom O/min/mg protein, respectively, with the freshly isolated mitochondria, and 3.6 ± 0.7, 1.64 ± 0.04, and 0.44 ± 0.10 μatom O/min/mg protein, respectively, with the one-day-old preparations.

Mitochondrial oxygen consumption in the incubation mixtures was monitored under rapid stirring in a closed, thermostated (30°C) reaction chamber (Stoner and Sirak, 1973). In the assays of succinoxidase activity the standard incubation mixture (8 ml) contained 140 mM sucrose, 10 mM glucose, 5 mM K-Pipes (pH 6.5), 5 mM K-phosphate (pH 6.5), 10 mM K-succinate, 1 mM $MgCl_2$, 0.1 mM K-EGTA, 0.25 mg mitochondrial protein/ml, and 4 nmols rotenone/mg mitochondrial protein. The standard phosphorylating system contained in addition 500 μM ADP and 2.5 units of yeast hexokinase/ml, and the standard uncoupled system contained in addition 0.5 μM S-13. The concentration of hexokinase in the phosphorylating system was saturating under the conditions of high ADP concentration employed, and thus the hexokinase reaction was likely virtually nonlimiting (Stoner and Sirak, 1979). The concentration of S-13 in the standard uncoupled system was 5- to 10-fold higher than that needed for maximum stimulation of succinate respiration and was slightly inhibitory relative to that giving maximum stimulation. In the assays of ascorbate + TMPD oxidation, the conditions were as described above except that antimycin (0.5 nmol/mg protein) was added rather than rotenone, the oxidizable substrate was 7.5 mM K-ascorbate + 0.75 mM TMPD, and the concentration of mitochondria was 0.05 mg protein/ml. The presence of other constituents of the incubation mixtures, deviations from the standard conditions outlined above, and details concerning length of preincubation period and order of addition of constituents to the incubation mixture are given in the figure legends.

Antimycin, myxothiazol, and UHDBT were quantitated spectrophotometrically in ethanol using absorbance coefficients ($mM^{-1} \times cm^{-1}$) of 4.8 at

320 nm for antimycin (Strong *et al.*, 1960), 10.5 at 313 nm for myxothiazol (Gerth *et al.*, 1980), and 12.2 at 287 nm for UHDBT in ethanol containing 0.1 mM acetic acid (Trumpower and Haggerty, 1980). Stock TMPD solutions (0.25 M) were prepared by dissolving the hydrochloride salt in water while taking the pH to 5.0 with KOH. Ascorbic acid (20 mM) was included to maintain the TMPD in its relatively stable reduced state. The autooxidation rate of TMPD prepared in this manner and stored at low temperature is very low and remains so for at least several days of storage.

TMPD was obtained from Eastman Chemical Company (Rochester), phenylsuccinic acid from Aldrich Chemical Company (Milwaukee), and yeast hexokinase (Type F-300), succinic acid, ascorbic acid, Pipes, ADP, rotenone, antimycin, and HQNO from Sigma Chemical Company (St. Louis). S-13, nigericin, and valinomycin were obtained as gifts from P. C. Hamm (Monsanto Company, St. Louis), R. L. Harned (Commercial Solvents Corporation, Terre Haute), and L. C. Vining (Dalhousie University, Halifax, Nova Scotia), respectively. 3′-Hexyloxycarboxin was donated by G. A. White (Agriculture Canada Research Center, London, Ontario). Myxothiazol and UHDBT were obtained from J. S. Rieske (Ohio State University), who received them as gifts from W. Trowitzsch (Gesellshaft für Biotechnologische Forschung, Braunsweig) and B. Trumpower (Dartmouth Medical School), respectively. Funiculosin and mucidin were donated by P. Bollinger (Sandoz, Ltd., Basel) and J. Šubík (Food Research Institute, Bratislava), respectively.

Results

Preliminary Studies

A number of preliminary studies were conducted to determine which inhibitors might be best for the purposes of this study. The primary requirement was that the inhibitors be very specific. Since all the experiments were to involve the simultaneous use of two inhibitors and comparison of phosphorylating and uncoupled mitochondria, other requirements were that interactions between the inhibitors and changes in mitochondrial energy status have little or no affect on the binding of the inhibitors. Tightly bound inhibitors are the most likely to meet these requirements, and consequently inhibitors of this type were favored in cases where there was a choice.

In the case of the first reaction of the succinoxidase sequence, the succinate transport reaction, a number of inhibitors of relatively low affinity are known (Chappell and Robinson, 1968; Palmieri *et al.*, 1971), the one used here being phenylsuccinate. This inhibitor competes for the dicarboxylate carrier with succinate and other dicarboxylates but is not transported (Chap-

pell and Robinson, 1968; Palmieri *et al.*, 1971). To enhance its effectiveness, the concentration of succinate was decreased to 5 mM in all the experiments in which it was used. This had little effect on the maximal (uninhibited) rate of respiration because 5 mM succinate was nearly saturating in respect to the overall reaction under the conditions employed and likely also was nearly saturating in respect to the succinate transport reaction.

An analog of carboxin, 3′-hexyloxycarboxin, was used to inhibit the succinate dehydrogenase step, the second reaction of the succinoxidase sequence. The carboxins and related compounds are the most effective succinate dehydrogenase inhibitors known (White, 1971; White and Thorn, 1975) and bind at the same site as the iron chelator thenoyltrifluoroacetone (Mowery *et al.*, 1977; Ramsey *et al.*, 1981). Unlike the latter inhibitor, however, the carboxins appear to be highly specific for the succinate dehydrogenase reaction and appear not to uncouple respiration from phosphorylation. The 3′-hexyloxy analog was found to equilibrate very rapidly with its site of inhibition, making it feasible to determine inhibition curves by successive additions of the inhibitor.

In the case of the third reaction in the succinoxidase sequence, the cytochrome bc_1 reaction, a number of excellent inhibitors are available, the best known and the one that is most tightly bound being antimycin. The antimycin-sensitive step is relatively nonlimiting in the overall oxidative phosphorylation reaction (Stoner and Sirak, 1979), a consequence of which is that a high proportion of the antimycin-binding sites must be occupied to decrease the rate by one-half. Owing to this and the very high affinity of antimycin binding, the $I_{0.5}$ for antimycin is ordinarily only slightly lower than the concentration giving maximal inhibition. Because increases in the $I_{0.5}$ within this small range are unimpressive and difficult to measure accurately, antimycin was not the inhibitor of choice in experiments requiring detection of decreases in the degree to which the bc_1 step limits the overall succinoxidase reaction. Myxothiazol, a relatively new specific inhibitor of the bc_1 step (Thierbach and Reichenbach, 1981), was used in most cases. Although this inhibitor is bound only slightly less tightly than antimycin, it was generally possible to detect increases in its $I_{0.5}$ in a reliable and reproducible manner. However, it has the disadvantage relative to antimycin of equilibrating slowly with its site of inhibition, making relatively long incubation periods necessary.

Studies by Thierbach and Reichenbach (1981), Becker *et al.* (1981), and Meinhardt and Crofts (1982) suggest that antimycin and myxothiazol bind at different sites in the bc_1 complex. In view of this and of the possibility that ubiquinone links steps within the bc_1 reaction in addition to linking the dehydrogenase reactions with the bc_1 reaction (Mitchell, 1976), it was of interest to determine whether or not a freely diffusible intermediate exists

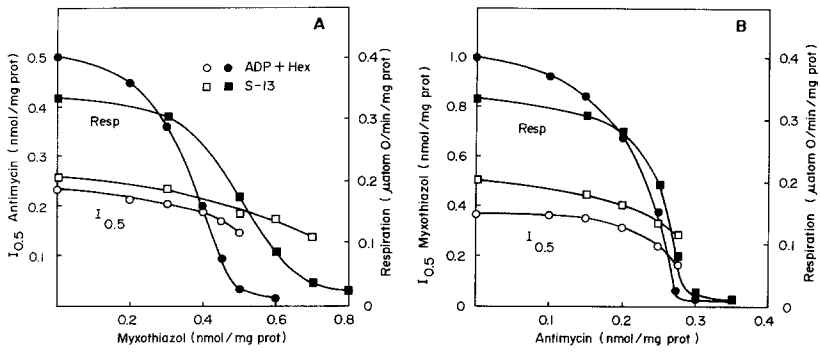

Fig. 1. Coupling relationship between the antimycin- and myxothiazol-sensitive steps of the overall succinoxidase reaction. The mitochondria were preincubated for 4 min in the presence of the inhibitors at the concentrations indicated and of all the constituents of the standard incubation media (see Materials and Methods) except succinate. The average rate of respiration during the 5th minute of incubation following the addition of 10 mM succinate was taken to be the steady-state rate.

between the antimycin- and myxothiazol-sensitive steps. As shown in Fig. 1, inhibition of the overall succinoxidase reaction by one of these inhibitors decreases the $I_{0.5}$ of the other, indicating the existence of a fixed coupling relationship between the steps and thus the absence of a freely diffusible intermediate between them. Tests of this nature were made for a number of other inhibitor combinations (antimycin vs. UHDBT, HQNO, funiculosin, and mucidin; myxothiazol vs. UHDBT, HQNO, and mucidin), and in all cases a fixed coupling relationship between the inhibitor-sensitive steps was indicated.

The inhibitor of choice for the fourth reaction of the succinoxidase sequence, the cytochrome aa_3 reaction, was cyanide. Although azide is a much less troublesome inhibitor of this step, there is some uncertainty as to its specificity (Bogucka and Wojtczak, 1966), and its inhibitory action at the aa_3 level is released somewhat by uncoupling under conditions where a similar release of cyanide inhibition is not observed (e.g., under conditions of ascorbate + TMPD oxidation). Nevertheless, all the experiments of this study involving the use of cyanide were done also with azide and, except for the difference noted above, the results obtained with azide were qualitatively identical to those obtained with cyanide.

As is well known, the principal difficulty in using cyanide as an inhibitor of the aa_3 reaction is slow equilibration of the inhibitor with its binding site. Erecińska *et al.* (1972) and Wilson and Fairs (1974) have shown the equilibration rate to be strongly decreased by ATP in an oligomycin- and uncoupler-sensitive process, but attributed the effect to interaction of the ATPase with the aa_3 complex upon binding ATP. Observations made in the present study suggest that this effect of ATP was actually due to energization

of the mitochondria. Thus the amount of time required for equilibration with cyanide was found to be much longer in energized mitochondria than in deenergized mitochondria. This can be seen from Fig. 2, which shows the effects of respiratory activity and mitochondrial energy status on the extent of respiratory inhibition by 3 μM cyanide. Ascorbate was used as the oxidizable substrate to avoid complications from other reactions as much as possible. The mitochondria were preincubated in the absence of TMPD for periods of time ranging from 1 to 12 min. During this time oxygen uptake was ascorbate dependent and very slow. The rate after TMPD addition was followed through the 8th minute, and average rates in the presence of 3 μM cyanide are given for the 1st, 2nd, 4th, and 8th minutes as percent of the rate observed under the same conditions except for the absence of cyanide.

It can be seen that in the course of preincubation in the absence of

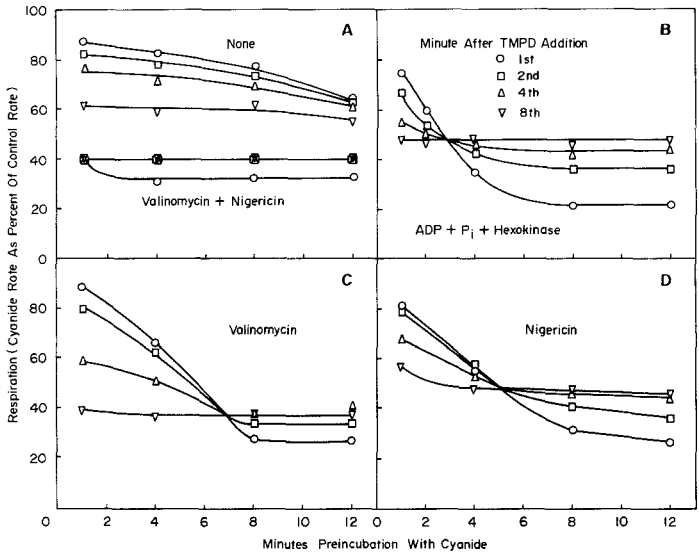

Fig. 2. Effect of incubation period and mitochondrial energy status on cyanide inhibition in mitochondria oxidizing ascorbate + TMPD. The compositions of the incubation mixtures were standard except that K-Pipes was substituted for K-phosphate in all but the phosphorylating system (B) and that valinomycin (0.1 μM) + nigericin (0.1 μM) was used as the uncoupler. The ascorbate was added initially and 3 μM cyanide (KCN) was added 1 min after the mitochondria. The TMPD was added after the indicated minutes of preincubation with the cyanide. Respiration rates are presented as the rate in the presence of cyanide as percent of the rate observed under identical conditions except for the absence of cyanide. The average control rates in μatom O/min/mg protein were 0.70, 1.22, 0.84, 0.74, and 1.22 for "None," "ADP +P_i + Hexokinase," "Valinomycin," "Nigericin," and "Valinomycin + Nigericin," respectively. The control rate in the absence of TMPD was ascorbate dependent and was in all cases approximately 0.05 μatom O/min/mg protein.

TMPD, a crossover occurs in the direction in which the extent of cyanide inhibition changes in approaching the equilibrium degree of inhibition. Presumably this is due to cytochrome aa_3 having a higher affinity for cyanide in the oxidized form than in the reduced form (Nicholls and Kimelberg, 1972; Wilson *et al.*, 1972) and to a relatively large proportion of the aa_3 being in the oxidized form during the preincubation period. The amount of time it takes to reach the crossover point is an indication of the rate of cyanide equilibration with cytochrome aa_3 during the preincubation period. As can be seen, the energy status has a large effect on this rate. Figure 2A shows that under the conditions employed where the mitochondria were most energized (i.e., "None"), equilibration was so slow that crossover was not reached within the 12-min preincubation period, whereas under conditions of uncoupling ("Valinomycin + Nigericin"), equilibration was relatively very fast and crossover was reached within approximately 1 min. As might be expected, deenergization of the mitochondria through phosphorylation of ADP was somewhat less effective than uncoupling (Fig. 2B).

To gain some idea as to the relative importance of the $\Delta\psi$ and ΔpH components of the protonmotive force that presumably gives the energized state, the individual effects of valinomycin and nigericin were determined (Figs. 2C and 2D). Both of these agents increased the equilibration rate very noticeably, the increase due to nigericin being relatively large. Since both valinomycin and nigericin increased the control rate of respiration (see legend to Fig. 2), the effects of these agents could have been due in part to uncoupling. However, since nigericin increased the control rate relatively little while increasing the rate of cyanide equilibration to a relatively large extent and is known to decrease ΔpH without decreasing $\Delta\psi$ (Jackson *et al.*, 1968), it seems likely that the large increase in rate of cyanide equilibration due to the uncoupling action of valinomycin + nigericin (Fig. 2A) was due more to dissipation of the ΔpH component of the protonmotive force than to dissipation of the $\Delta\psi$ component.

The experiment of Fig. 2 also gives some indication of the rate of cyanide equilibration in mitochondria respiring in the presence of ascorbate + TMPD. In some cases the rate during ascorbate + TMPD oxidation can clearly be seen to differ from the rate during the preincubation period. For example, in the case of the mitochondria preincubated in the absence of energy-dissipating agents (Fig. 2A), the rate of equilibration with cyanide after TMPD addition appears to have been considerably greater than the rate before TMPD addition. Since one could expect the mitochondria to be more energized in the presence of TMPD than in its absence, it appears that the relationship between rate of cyanide equilibration and mitochondrial energy status is far from simple. Fortunately, the primary goal of the experiment was merely to determine the preincubation period necessary for cyanide equilibration under

different conditions of mitochondrial energy status. The information obtained in this regard proved to be reliable and very useful in subsequent studies on the overall succinoxidase reaction.

Relationship of the Succinate Transport Reaction to the Succinate Dehydrogenase, Cytochrome bc_1, and Cytochrome aa_3 Reactions

According to the succinoxidase sequence of overall reaction (2), the coupling relationship of the succinate transport reaction to all the others in the sequence should be sequential. If such is the case, one could expect specific inhibition of the succinate transport reaction to result in all the other reactions becoming less rate limiting and thus in the $I_{0.5}$'s for specific inhibitors of these other reactions increasing. Furthermore, since coupling relationships are reciprocal, one could expect specific inhibition of any one of the other steps to result in the succinate transport reaction becoming less rate limiting and thus in the $I_{0.5}$'s for specific inhibitors of this reaction increasing. Using phenylsuccinate, 3'-hexyloxycarboxin, myxothiazol, and cyanide as the inhibitors of the succinate transport, succinate dehydrogenase, bc_1, and aa_3 reactions, respectively, this is what has been observed (Figs. 3–5).

In all cases the observed coupling relationship was the same regardless of whether the mitochondria were phosphorylating or uncoupled. This was expected because there is nothing to suggest that a change in mitochondrial energy status alters the mechanism by which the succinate transport reaction is linked to the respiratory chain reactions. On the other hand, differences in

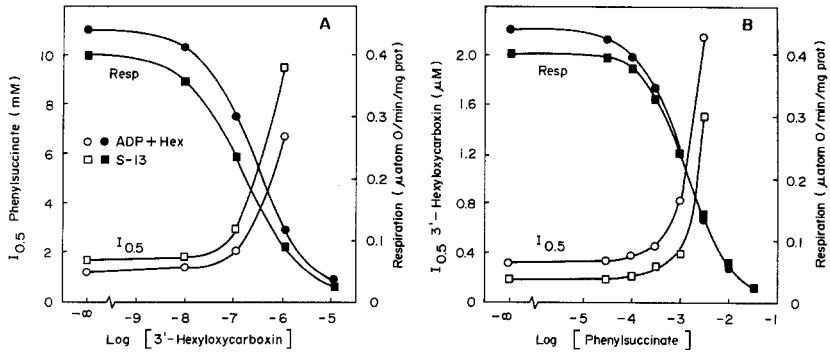

Fig. 3. Coupling relationship between the succinate transport and succinate dehydrogenase reactions of the overall succinoxidase reaction. The mitochondria were preincubated for 2 min in the presence of phenylsuccinate at the concentrations indicated and of all the constituents of the standard incubation media except ADP + hexokinase (phosphorylating system) and S-13 (uncoupled system). The steady-state rates of respiration in the two systems at the various levels of 3'-hexyloxycarboxin were determined by making successive additions of the inhibitor at intervals of 1.5 min after adding ADP + hexokinase and S-13. The concentration of succinate was 5 mM.

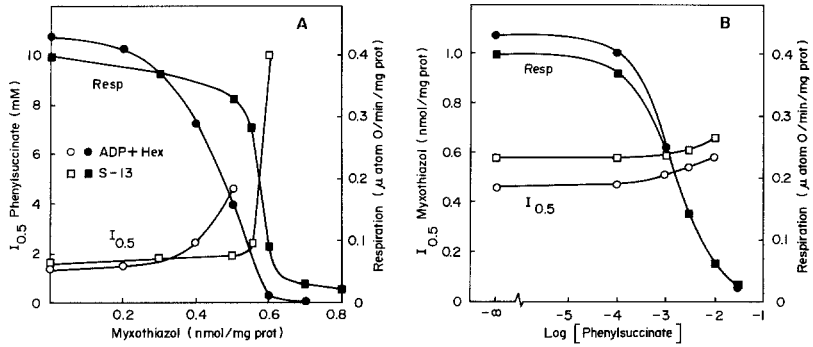

Fig. 4. Coupling relationship between the succinate transport and cytochrome bc_1 reactions of the overall succinoxidase reaction. The mitochondria were preincubated for 3 min in the presence of the inhibitors of these reactions at the concentrations indicated and of all the constituents of the standard incubation media except ADP + hexokinase (phosphorylating system) and S-13 (uncoupled system). The average rate of respiration during the 3rd minute of incubation following the addition of ADP + hexokinase and S-13 was taken to be the steady-state rate. The concentration of succinate was 5 mM.

$I_{0.5}$ values can certainly be expected because of differences in the two systems in regard to the rate of the overall reaction and to the number of individual reactions involved. Figures 3–5 show that the relatively low overall reaction rate observed in the uncoupled system is associated with a decrease in $I_{0.5}$ for the succinate dehydrogenase inhibitor (Fig. 3B) and with increases in the $I_{0.5}$'s for the inhibitors of the other three steps in the succinoxidase sequence. These

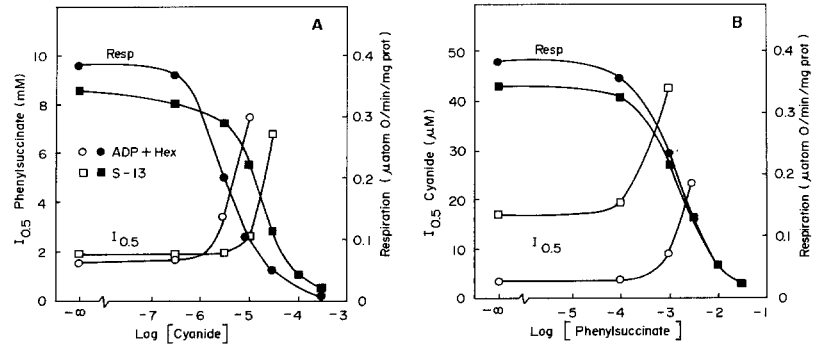

Fig. 5. Coupling relationship between the succinate transport and cytochrome aa_3 reactions of the overall succinoxidase reaction. Prior to the addition of cyanide, the mitochondria were preincubated for 1 min in the presence of phenylsuccinate at the concentrations indicated and of all the constituents of the standard incubation media except succinate. After the addition of cyanide, the mitochondria were preincubated either for an additional 3 min (phosphorylating system) or an additional 1 min (uncoupled system). The average rate of respiration during the 4th minute of incubation following the addition of 5 mM succinate was taken to be the steady-state rate.

changes indicate that uncoupling resulted in the succinate dehydrogenase reaction becoming more rate limiting and in all the others becoming less rate limiting. Since they were accompanied by a decrease in the rate of the overall reaction, it appears at first glance that uncoupling resulted in inhibition at the level of the succinate dehydrogenase reaction and that all the other individual reactions became less rate limiting as a result. However, as will be pointed out below, the bc_1 and aa_3 reactions became much less rate limiting than can be anticipated from the small uncoupler-induced decrease in overall reaction rate. In addition, evidence will be presented indicating that uncoupling results in inhibition also at the level of the succinate transport step.

Relationship of the Succinate Dehydrogenase Reaction to the Cytochromes bc_1 and aa_3 Reactions

As was noted above, Klingenberg and Kröger (1970) and Kröger and Klingenberg (1970, 1973) have provided extensive evidence indicating that ubiquinone links the dehydrogenases of the respiratory chain with the cytochrome chain and that within individual mitochondria it diffuses freely among the dehydrogenases and cytochrome complexes between which it transports electrons. Consequently there was no surprise whatever in obtaining results consistent with there being a freely diffusible intermediate between the succinate dehydrogenase and bc_1 reactions (Fig. 6) and between the succinate dehydrogenase and aa_3 reactions (Fig. 7). Nevertheless, because the approach of the present study differs from those used by Kröger and Klingenberg, it was important that these experiments be done. In addition, the

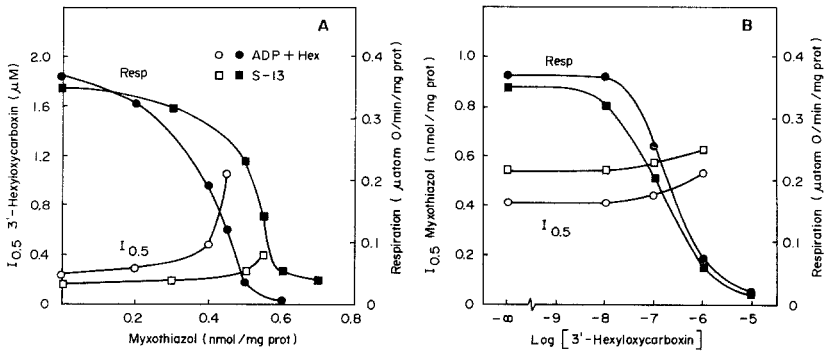

Fig. 6. Coupling relationship between the succinate dehydrogenase and cytochrome bc_1 reactions of the overall succinoxidase reaction. The mitochondria were preincubated for 3 min in the presence of the inhibitors at the concentrations indicated and of all the constituents of the standard incubation media except ADP + hexokinase (phosphorylating system) and S-13 (uncoupled system). The average rate of respiration during the 5th minute of incubation following the addition of ADP + hexokinase and S-13 was taken to be the steady-state rate.

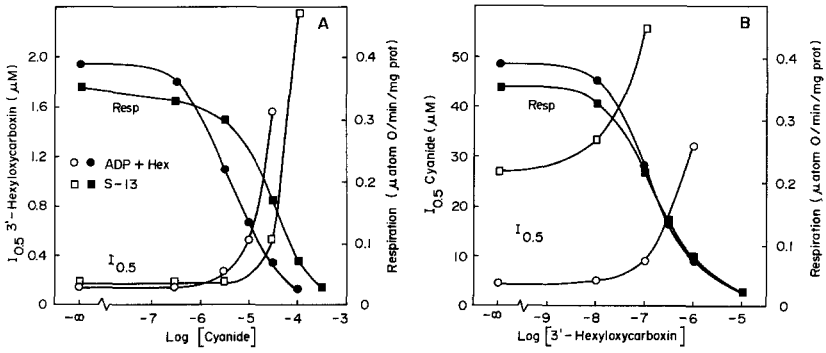

Fig. 7. Coupling relationship between the succinate dehydrogenase and cytochrome aa_3 reactions of the overall succinoxidase reaction. Prior to the addition of cyanide the mitochondria were preincubated for 1 min in the presence of 3'-hexyloxycarboxin at the concentrations indicated and of all the constituents of the standard incubation media except succinate. After the addition of cyanide the mitochondria were preincubated either for an additional 3 min (phosphorylating system) or for an additional 1 min (uncoupled system). The average rate of respiration during either the 5th minute (phosphorylating system) or the 3rd minute (uncoupled system) of incubation following the addition of 10 mM succinate was taken to be the steady-state rate.

experiments have value in that they serve as checks on the experiments involving the use of phenylsuccinate. Thus, since the reactions of the cytochrome chain are separated from both the succinate transport reaction and the succinate dehydrogenase reaction by a freely diffusible intermediate, half-maximal inhibition of the overall reaction by specific inhibitors of these reactions should increase the $I_{0.5}$'s for specific inhibitors of the cytochrome reactions approximately to the same extent. The results of Figs. 4–7 are in generally good agreement with this expectation, particularly in view of the fact that the different experiments represent different mitochondrial preparations.

It may be noted that whereas uncoupling decreased the $I_{0.5}$ for the succinate dehydrogenase inhibitor in the experiments of Figs. 3 and 6, it increased the $I_{0.5}$ for this inhibitor in the experiment of Fig. 7. This indicates that in contrast to what was indicated in the other experiments, uncoupling resulted in the succinate dehydrogenase reaction being less rate limiting in the experiment of Fig. 7. Since, as in the other experiments, uncoupling in the experiment of Fig. 7 resulted in a decrease in the rate of the overall reaction, there is a question as to which of the individual reactions was inhibited by uncoupling in this experiment.

The inhibition of succinoxidase activity by uncoupling has often been observed and occurs by at least three mechanisms: (1) matrix condensation due to uncoupler-induced efflux of intramitochondrial ions (Nicholls and Lindberg, 1972); (2) accumulation of intramitochondrial oxalacetate due to

an uncoupler-induced decrease in the level of intramitochondrial ATP (Papa *et al.*, 1969); and (3) uncoupler-catalyzed dissipation of energy required for accumulation of succinate (Harris *et al.*, 1967; Quagliariello and Palmieri, 1968; Papa *et al.*, 1969). The first is likely to be quite unspecific as to which of the individual reactions is inhibited, whereas the second is specific for the succinate dehydrogenase reaction and the third for the succinate transport reaction. The first can be ruled out in respect to the present study because care was taken to assure that the tonicity of the suspension medium was optimal for succinoxidase activity in both the phosphorylating and uncoupled systems. The results of this study (Figs. 3–7) suggest that inhibition occurs by both of the other two mechanisms and that inhibition by the second is ordinarily the most pronounced.

That at least some of the uncoupler-induced inhibition observed in the present study was due to oxalacetate accumulation is indicated by the observations that the activity of the uncoupled mitochondria could be increased by treatments that increase the level of intramitochondrial ATP (e.g., adding ATP and decreasing the level of uncoupler) and decreased by treatments that decrease the level of intramitochondrial ATP (e.g., adding ADP + hexokinase and increasing the level of uncoupler). Previous studies with liver mitochondria (Quagliariello and Palmieri, 1968) and kidney mitochondria (Papa *et al.*, 1969) have indicated that oxalacetate inhibition of succinate oxidation in uncoupled mitochondria is prevented more or less completely by rotenone. The discrepancy between the previous and the present studies was likely not due to the use of insufficient rotenone in the present

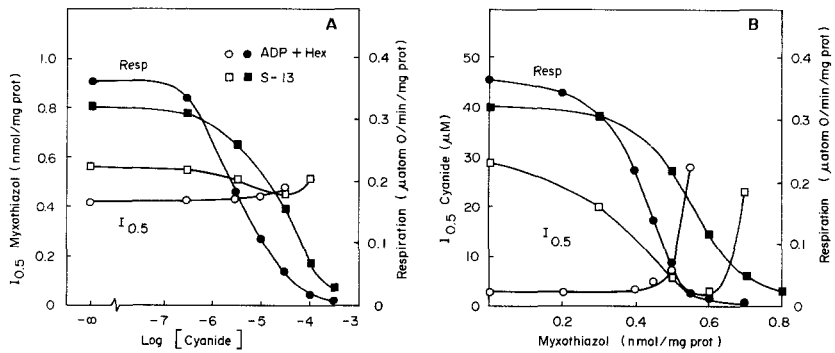

Fig. 8. Coupling relationship between the cytochromes bc_1 and aa_3 reactions of the overall succinoxidase reaction as indicated by myxothiazol and cyanide inhibition. The mitochondria were preincubated for 1 min prior to cyanide addition and for 3 min after cyanide addition in the presence of myxothiazol at the concentrations indicated and of all the constitutents of the standard incubation mixtures except succinate. The average rate of respiration during the 5th minute of incubation following the addition of 10 mM succinate was taken to be the steady-state rate.

study because doubling the level of rotenone in the present study had no effect on the overall reaction rate. The above previous studies also indicated that uncoupler-induced inhibition at the level of the succinate transport step can be largely avoided by using 10 mM succinate. With the exception of the experiment of Fig. 7, the results of the present study in this case seem to be in fairly good accord with the previous ones.

Relationship Between the Cytochromes bc_1 and aa_3 Reactions

Figure 8 presents the results of an experiment designed to determine the coupling relationship between the bc_1 and aa_3 reactions using myxothiazol and cyanide as the specific inhibitors. It can be seen that in contrast to the other individual reaction pairs examined, a difference here in coupling relationship, depending on whether the mitochondria are phosphorylating or uncoupled, is indicated. In the case of phosphorylating mitochondria, the coupling relationship does not differ qualitatively from that of the other reaction pairs examined, indicating that a freely diffusible intermediate links the bc_1 and aa_3 reactions. In the case of uncoupled mitochondria, however, it appears that two types of coupling relationships exist simultaneously, a fixed relationship in addition to the sequential relationship observed in the case of phosphorylating mitochondria. Thus, as the extent of inhibition of one reaction is increased, the $I_{0.5}$ for the inhibitor of the other reaction initially decreases substantially and then increases substantially. In each case the $I_{0.5}$ for the inhibitor decreases

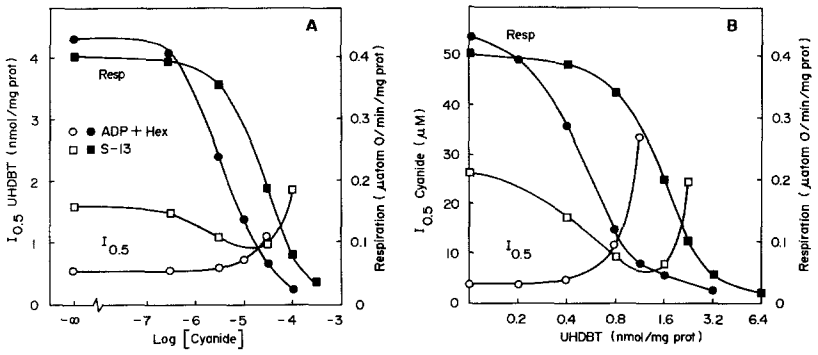

Fig. 9. Coupling relationship between the cytochromes bc_1 and aa_3 reactions of the overall succinoxidase reaction as indicated by UHDBT and cyanide inhibition. Prior to the addition of cyanide the mitochondria were preincubated for 1 min in the presence of UHDBT at the concentrations indicated and of all the constitutents of the standard incubation media except succinate. After the addition of cyanide, the mitochondria were preincubated either for an additional 3 min (phosphorylating system) or an additional 1 min (uncoupled system). The average rate of respiration during either the 4th minute (phosphorylating system) or 3rd minute (uncoupled system) of incubation following the addition of 10 mM succinate was taken to be the steady-state rate. Note the use of an unusual exponential scale on the abscissa in part B.

approximately to the level of the lowest $I_{0.5}$ observed under conditions of phosphorylation before increasing, suggesting a complete or nearly complete reversal of whatever is responsible for the large uncoupler-induced decreases in the degrees to which the bc_1 and aa_3 reactions limit the overall reaction.

As was observed also in other cases, the qualitative aspects of the results summarized in Fig. 8 do not depend on the choice of specific inhibitors. Owing to the relatively complex nature of the coupling relationships between the bc_1 and aa_3 reactions and to the fact that changes in the $I_{0.5}$ for myxothiazol are small, this is demonstrated here (Fig. 9) in the case of the bc_1 step by showing the kind of results obtained with UHDBT as the bc_1 inhibitor. As can be expected from the relatively low affinity of UHDBT binding, the $I_{0.5}$ values for this inhibitor and the magnitudes of the changes in these values due to changes in the degree to which the bc_1 reaction limits the overall reaction are relatively large. Consequently, resolution and reproducibility of changes in $I_{0.5}$ are relatively good with UHDBT.

Discussion

On the Coupling Relationships Between the Respiratory Reactions

The results of this study suggest the presence of freely diffusible intermediates between all the individual reactions depicted as such in the succinoxidase sequence of overall reaction (2). Uncoupling of respiration from phosphorylation apparently alters this situation somewhat in regard to the coupling between the bc_1 and aa_3 reactions, as indicated by the coupling relationship between these steps becoming partially fixed. The change in coupling relationship is accompanied by marked decreases in the degrees to which the bc_1 and aa_3 reactions limit the overall reaction. The relationship between these reactions is such that the uncoupler-induced decrease in the degree to which one is rate limiting can be reversed more or less completely by inhibition at the level of the other (Figs. 8 and 9), suggesting that whatever causes the decreases also brings about the change in coupling relationship.

The uncoupler-induced decreases in the degrees to which the bc_1 and aa_3 reactions were limiting were indicated by large increases in the $I_{0.5}$'s of specific inhibitors of these reactions. The increases in $I_{0.5}$ do not appear to have been due to decreases in binding affinity. Thus, as can be seen from Fig. 2, in the case of cyanide inhibiton of the aa_3 reaction, uncoupling appears to increase rather than decrease the affinity of the aa_3 complex for cyanide, suggesting that the uncoupler-induced decrease in the degree to which the aa_3 step limited the overall succinoxidase reaction was even greater than indicated by the increase in $I_{0.5}$ for cyanide. In the case of bc_1 inhibitors, five were tested in

regard to the effect of uncoupling on $I_{0.5}$ and in each case the same kind of result was obtained.

Since uncoupling was associated with slight inhibition of the overall succinoxidase reaction at the levels of the succinate transport and succinate dehydrogenase reactions, at least some of the uncoupler-induced increases in the $I_{0.5}$'s for inhibitors for the bc_1 and aa_3 steps can be attributed to this. However, the increases could not have been due entirely to this inhibition because the degrees of inhibition at the succinate transport and succinate dehydrogenase steps required to increase the $I_{0.5}$'s of the bc_1 and aa_3 inhibitors to the extent seen are much greater than the observed degrees of inhibition due to uncoupling. Thus, in the experiments of Figs. 4 and 6, uncoupling decreased the rate of the overall reaction by an average of 6%, whereas with the inhibitors of the succinate transport and succinate dehydrogenase steps it was necessary to decrease the rate by an average of 83% to increase the $I_{0.5}$ for myxothiazol to the extent that it increased as a result of uncoupling. In the experiments of Figs. 5 and 7, the rate was decreased by an average of 10% by uncoupling, and with the inhibitors it was necessary to decrease the rate by an average of 72% to increase the $I_{0.5}$ for cyanide to the extent that it increased as a result of uncoupling.

Since the bc_1 and aa_3 reactions drive the energy-consuming steps involved in phosphorylation, it might be supposed that the removal of this burden through uncoupling was responsible for the large uncoupler-induced decreases in the degrees to which the bc_1 and aa_3 reactions were limiting. Whether or not such a supposition would be correct depends on how the energy-consuming steps are linked to the respiratory reactions and on the mechanism and extent of uncoupling. Present evidence indicates that the reactions are coupled chemiosmotically essentially as postulated by Mitchell (1966). According to Mitchell the reactions are linked by freely diffusible intermediates ($\Delta\psi$ and ΔpH), and uncouplers function by catalyzing a reaction that consumes these intermediates. If these ideas are correct, uncoupling in the present study involved the substitution of a proton transport reaction catalyzed by S-13 for the P_i transport, ATP synthase, AdN transport, and hexokinase reactions and the substitution could be expected to have resulted in the bc_1 and aa_3 reactions being less limiting only if the concentration of S-13 used was not high enough to make the S-13 reaction less limiting than the reactions it replaced. That the concentration of S-13 was high enough for this is indicated by studies on mitochondria oxidizing ascorbate + TMPD showing that the rate of oxygen uptake is higher in the uncoupled system than in the phosphorylating system, on the average by a factor of 1.2. Consequently one might expect the uncoupling to have resulted in the bc_1 and aa_3 reactions becoming more limiting rather than less limiting. This assumes of course that Mitchell's postulates are correct. Preliminary studies on the coupling relationships of the

respiratory reactions to the energy-consuming steps by the methods of the present study clearly indicate them to be correct in the cases of coupling sites I and III, but not in the case of site II, where fixed coupling relationships of the bc_1 reaction to the energy-consuming steps are clearly indicated. However, a fixed coupling relationship is observed even between the bc_1 and hexokinase reactions, suggesting that the fixed relationships come about by allosteric mechanisms rather than by coupling mechanisms that are fundamentally different from those at the other sites.

Regardless of whether or not uncoupling of the energy-consuming reactions from the bc_1 and aa_3 reactions decreased the degrees to which the respiratory reactions were rate limiting, such uncoupling by itself would not explain the associated conversion of the coupling relationship between the bc_1 and aa_3 reactions from a wholly sequential to a partially fixed one. This conversion suggests that a freely diffusible intermediate links these reactions but that uncoupling either partially eliminates the need for the intermediate or to some extent allows it to become fixed to the bc_1 and aa_3 complexes such that it is functional but not freely diffusible. If cytochrome *c* is the intermediate in question, the latter possibility is the most likely because electron transfer between the bc_1 and aa_3 complexes in mitochondria is absolutely dependent on cytochrome *c* regardless of mitochondrial energy status. That cytochrome *c* is in fact the freely diffusible intermediate is indicated by preliminary studies on the effect of cytochrome *c* concentration on the degrees to which the bc_1 and aa_3 steps limit the overall succinoxidase reaction. These studies show that increasing the concentration of cytochrome *c* in mitochondria depleted of cytochrome *c* by the method of Jacobs and Sanadi (1960) results in both the bc_1 and aa_3 steps becoming less rate limiting. If cytochrome *c* were to bind to and thus directly affect the activity of only one of the complexes, say the aa_3 complex by forming with it a complex capable of accepting electrons either directly from the bc_1 complex or from an intermediate linking the bc_1 and aa_3 reactions, it would in effect be a component of the aa_3 complex and an increase in its concentration in cytochorome *c*-deficient mitochondria would thus make only the aa_3 reaction less limiting. The resulting increase in the rate of the overall reaction would then result in all the other steps, including the bc_1, becoming more rate limiting. Other preliminary studies of this nature indicate that the large uncoupler-induced increases in the $I_{0.5}$'s of inhibitors of the bc_1 and aa_3 reactions are strongly dependent on cytochrome *c* and that the coupling relationship between these reactions in uncoupled mitochondria becomes more fixed and less sequential as the concentration of cytochrome *c* is increased.

A plausible explanation for the observations to date seems to be that uncoupling results in some of the bc_1 and aa_3 complexes becoming linked by cytochrome *c* into functional supercomplexes. Owing to the elimination of

association–dissociation reactions between cytochrome *c* and the bc_1 and aa_3 complexes and steps involving the diffusion of cytochrome *c* between the two complexes, these supercomplexes could be extraordinarily efficient in transporting electrons and thus their formation, even in small amount, could account for the large uncoupler-induced decreases in the degrees to which the bc_1 and aa_3 steps limit the overall succinoxidase reaction. Their apparent absence in phosphorylating mitochondria could be due to the relatively high electrochemical potential difference that might be expected to exist across the inner membranes of these mitochondria, preventing conformational changes necessary to the formation of the supercomplexes.

The formation of the functional supercomplexes implies the existence of different binding sites on cytochrome *c* for the bc_1 and aa_3 complexes. The existence of these sites has been suggested also by a number of previous studies, the most convincing of which are the antibody studies of Smith *et al.* (1973), the affinity labeling studies of Erecińska *et al.* (1975, 1980), and the membrane fusion studies of Schneider *et al.* (1980). Like the present work, the studies of Schneider *et al.* (1980) provide strong support for the existence of the supercomplexes in uncoupled mitochondria. The existence of the supercomplexes is supported also by the studies of Chiang and King (1979) showing that complexes consisting of cytochromes c_1 and *c* and the cytochrome aa_3 complex in the ratio of 1:1:1 form upon mixing isolated preparations of these cytochromes. On the other hand, studies involving differential modification of lysyl residues on cytochrome *c* (Speck *et al.*, 1979; Rieder and Bosshard, 1980) suggest that there is considerable overlap of the reductase and oxidase binding sites on cytochrome *c* and thus that formation of the supercomplexes is unlikely. However, these studies were done with isolated reductase and oxidase complexes, with which the binding domains could have been unusually large owing to unusually good induced fits.

The large uncoupler-induced decreases in the degrees to which the bc_1 and aa_3 steps limit the overall succinoxidase reaction have been noticed and studied previously as uncoupler-induced releases of specific inhibition at these steps. In the case of the bc_1 step, Howland (1963, 1968) and Howland *et al.* (1973) conducted extensive studies with a number of bc_1 inhibitors and showed in all cases that the inhibition in phosphorylating mitochondria can be largely reversed by uncouplers over a narrow concentration range of the inhibitor, the concentration range being extremely narrow in the case of antimycin (Howland, 1968). The reversal was suggested to be due to the uncouplers dissipating energy required for accumulation of the inhibitors within the mitochondria.

In the case of the aa_3 step, uncoupler-induced releases of inhibition by azide (Bogucka and Wojtczak, 1966; Wilson and Chance, 1967; Palmieri and Klingenberg, 1967; Nicholls and Kimelberg, 1968) and hydroxylamine (Wilson and Brooks, 1970) have been noted and the release of azide inhibition has

been extensively studied. A number of explanations for the release of azide inhibition have been offered (see Nicholls and Kimelberg, 1972), the one given the most attention being that of Palmieri and Klingenberg (1967). These workers attributed the release to efflux of azide accumulated in the matrix, an explanation that is based on the assumption that N_3^- is the inhibitory species and, as Mitchell and Moyle (1970) have pointed out, one that is thus not consistent with strong indications (Stannard and Horecker, 1948; Wever *et al.*, 1973) of the inhibitory species being HN_3.

Miscellaneous Observations Concerning Rate-Limiting Steps

As is evident from the foregoing, a matter of considerable importance to the present study is the relative degrees to which the individual reactions of a multienzyme reaction limit the rate of the overall reaction. This subject is difficult to deal with and is currently plagued by confusion and controversy, particularly as it pertains to respiratory control in mitochondria (for recent reviews, see Vignais and Lauquin, 1979; La Noue and Schoolwerth, 1979; Williamson, 1979; Hansford, 1980; Ferguson and Sorgato, 1982; Erecińska and Wilson, 1982). In fact there appears to be so much misunderstanding in this area that the present communication is likely to be misunderstood by a large proportion of those who bother to read it. Therefore, some further discussion appears to be in order.

Some of the trouble seems to come from misunderstandings as to what determines the degrees to which the individual reactions of a multienzyme reaction limit the rate of the overall reaction in the steady state. As was pointed out in the previous study of this nature, the degree to which a particular individual reaction is limiting is determined by the effective concentration of the enzyme in relation to the effective concentrations of the enzymes of all the other individual reactions, and the effective concentration of a particular enzyme is determined by its actual concentration, its potential activity, and everything else in and about the system that affects its activity. From this it is apparent that virtually any change at virtually any step within a multienzyme reaction will affect all the individual reactions in regard to how much they limit the overall reaction. This point has been emphasized previously by Waley (1964) and by Kacser and Burns (1973, 1979).

One of the things that determines the effective concentration of an enzyme is the thermodynamic equilibrium of the reaction it catalyzes. A serious problem in this regard has arisen from the classification of individual reactions into "near-equilibrium" and "nonequilibrium" categories as though the degree of disequilibrium at which an individual reaction is operating were, like the thermodynamic equilibrium, intrinsic to the reaction. This has resulted in the confusion of reactions operating close to thermodynamic equilibrium("near-equilibrium reactions") with those having equilibrium constants near unity and of reactions operating far from thermodynamic

equilibrium ("nonequilibrium reactions") with those having equilibrium constants much greater than unity (e.g., see Newsholme and Crabtree, 1979). Since the degree of disequilibrium at which an individual reaction is operating is an indicator of how much it is limiting, this confusion misleads one to believe that reactions with equilibrium constants near unity are the least limiting and that those with equilibrium constants much greater than unity are the most limiting. Actually the thermodynamic equilibrium is only one of a number of things that determines how much an individual reaction is limiting, and from the Haldane relationship one might expect individual reactions with equilibriums relatively favorable to the overall reaction to be relatively nonlimiting.

Another source of difficulty seems to be the harboring by some of a feeling that there is invariably a particular individual reaction, "the rate-limiting step," that controls the overall reaction somewhat as the weakest link determines the strength of a chain. From a theoretical standpoint virtually all the individual reactions can be expected to limit the overall reaction to some extent (Waley, 1964; Kacser and Burns, 1973, 1979; Heinrich and Rapoport, 1974; Groen *et al.,* 1982), and the most efficient use of enzymes occurs when the individual reactions are equally limiting (Waley, 1964; Stoner and Sirak, 1979). Nevertheless, it is reasonable to suppose that one of the individual reactions will ordinarily be more limiting than any of the others and henceforth this reaction will be referred to as the principal rate-limiting step and will be assumed to be the one meant by others who have referred to "the rate-limiting step" or "the rate-controlling step" without specifying exactly what was meant.

A subject of primary concern in studies on respiratory control has been identification of the principal rate-limiting step in the overall oxidative phosphorylation reaction under the steady-state conditions that normally exist *in vivo* (i.e., conditions of high extramitochondrial [ATP]/[ADP] and of high concentrations of P_i and Krebs cycle substrates). Some of the investigators who have concerned themselves with this problem have noted that the rate of the overall reaction under these conditions changes primarily in response to changes in extramitochondrial [ATP]/[ADP] and have concluded therefrom that the AdN transport reaction is the principal rate-limiting step (e.g., see Davis and Lumeng, 1975; Küster *et al.,* 1976). Since extramitochondrial ADP and ATP influence the overall mitochondrial respiratory process primarily as substrate and product, respectively, in the AdN transport reaction, there is no question that this step is one through which mitochondrial respiration is to a large extent controlled under such conditions. However, this does not mean that the AdN transport reaction is the one that is most limiting. The principal rate-limiting step would be the one that is operating farthest from thermodynamic equilibrium and thus the one at which a particular degree of change of enzyme concentration would have the largest effect on the rate of the overall reaction.

Wilson *et al.* (1973, 1974, 1977), Owen and Wilson (1974), and Wilson (1980) have contended that the principal rate-limiting step is the cytochrome aa_3 reaction. However, they apparently came to this view on the assumption that nearly irreversible reactions cannot or do not operate near thermodynamic equilibrium. In view of the very favorable thermodynamic equilibrium of the cytochrome aa_3 reaction, one might expect it to be relatively nonlimiting. The observation that the cytochrome *a* of intact mitochondria becomes only slightly reduced upon initiation of State 3 respiration (Chance and Williams, 1956) seems to be in good accord with this expectation.

It should be noted that under the conditions of high [ATP]/[ADP] that normally exist *in vivo,* all the individual reactions of the overall oxidative phosphorylation reaction would be operating fairly close to thermodynamic equilibrium and thus none of them would be very rate limiting *per se.* Consequently the matter of which step is the most limiting under these conditions might not be very important. Therefore, in considering the mechanism of respiratory control under such conditions it might be more relevant to concern oneself rather with the principal rate-limiting substance, as was originally done by Chance and Williams (1956), or with the principal extramitochondrial ATP-consuming step (Hansford, 1980). Another possibility in this regard is the proton leak reaction (Groen *et al.,* 1982).

Groen *et al.* (1982) have considered the proton leak reaction to be the principal rate-limiting step in State 4 mitochondria. They apparently came to this view out of a need which arose as a result of their belief in the hypothesis of Kacser and Burns (1973, 1979) and Heinrich and Rapoport (1974) that the individual reactions of a multienzyme reaction can be assigned "control strengths" which give the relative degrees to which the reactions limit the overall reaction and which approach zero as the reactions approach thermodynamic equilibrium. Now if it were true that these control strengths approach zero as the individual reactions approach equilibrium, one could expect the sum of the control strengths to approach zero as the overall reaction approaches equilibrium. Since by definition the control strengths must always add up to one, this hypothesis is obviously not entirely correct. Assuming that control is transferred to an interfering leak reaction as the overall reaction approaches equilibrium is also obviously not correct because it is possible to come up with multienzyme reactions that do not have leak reactions.

Some of the current confusion concerning the mechanism of respiratory control in mitochondria has come from attempts to determine the principal rate-limiting step from the shapes of respiratory inhibition curves without properly taking into account that the shape of an inhibition curve depends greatly on the method of plotting and on how tightly the inhibitor is bound. Furthermore, determination of inhibition curves for only one or two individual reactions has in some cases been deemed adequate. Determination of the principal rate-limiting step would require consideration of all the individual

reactions and there are only two known methods whereby it could be done unequivocally. One would be to determine the degree of disequilibrium at each step and would be extremely difficult (Hansford, 1980). The other is more feasible and is based on the fact that in multienzyme reactions consisting of two or more individual reactions, K_m's can be obtained for the catalytic components as well as for the substrates (Hearon, 1952; Waley, 1964; Stoner and Sirak, 1979). It would involve determination of the concentrations and K_m's of all the catalytic components under the specific conditions of interest. This method was employed in the previous study of this nature to determine the relative degrees to which the antimycin-, aurovertin-, and carboxyatractyloside-sensitive steps limit the overall oxidative phosphorylation reaction. The results emphasize the importance of specifying the conditions of substrate concentration and of knowing the coupling relationships between the individual reactions in such studies.

Confusion and controversy have come also from failures to recognize that changes in the degrees to which individual reactions limit a multienzyme reaction can occur without an appreciable change in the rate of the overall reaction. That this can in fact occur is clearly evident from the observations of the present study indicating that uncoupling respiration from phosphorylation can result in marked changes of this nature in the overall succinoxidase reaction. Such changes are likely accompanied by changes in the redox states of the electron carriers (e.g., see Davis and Blair, 1977) and in the levels of ΔpH and $\Delta\psi$ and could account for the discrepancies between phosphorylating and uncoupled mitochondria in regard to the relationship between Δp and respiration rate noted by Padan and Rottenberg (1973) and by others (see Ferguson and Sorgato, 1982).

In studies on the relationships between the rate of the overall oxidative phosphorylation reaction and the concentrations of substrates and intermediate reactants, confusion has resulted also from failures to establish and maintain a specific thermodynamic base. In the case of single-enzyme reactions this is ordinarily achieved by selecting conditions such that the concentrations of the substrates are in effect constant and the concentrations of the products are in effect zero during the period of measurement. In the case of thermodynamically unfavorable reactions it is often necessary to remove the products as fast as they are formed by linking the reaction to one that is thermodynamically favorable and nonlimiting. If this is not done, the results are likely to be misleading as a result of an inhibitory amount of product building up during the period of measurement. Since the phosphorylation of extramitochondrial ADP in the overall oxidative phosphorylation reaction is quite unfavorable thermodynamically, it is important in kinetic studies on this reaction to link the ATP-forming steps to one that is thermodynamically favorable and nonlimiting such that the concentration of

ADP is constant and that of ATP near zero throughout the period of measurement and throughout the range of substrate concentrations employed. In contrast to the single-enzyme case, the need for this might not be readily apparent from the rate of the overall reaction because conditions may be such that limitation occurs primarily at the level of substrate uptake or oxidation and the rates of respiration and ATP formation might in consequence be virtually constant over a considerable range of [ATP]/[ADP] (e.g., see Davis and Blair, 1977). It is possible that the poor correlation between rate of ATP synthesis and Δp noted recently by Zoratti *et al.* (1982) resulted from their failure to take this into account.

In linking the AdN transport step to a thermodynamically favorable ADP regeneration reaction, it is important to take into account the fact that the degrees to which these reactions limit the overall reaction will depend greatly on the concentration of AdN employed in linking them (Stoner and Sirak, 1979). If this is not done, misleading results can be obtained (e.g., see Jacobus *et al.*, 1982).

Another source of confusion has been the failure to take into account that apparent K_m's of substrates of multienzyme reactions are not entirely analogous to those of single-enzyme reactions. In multienzyme reactions, apparent K_m's of both substrates and catalytic components depend greatly on the degrees to which the individual reactions in which the substrates and catalytic components are directly involved are rate limiting in the overall reaction (Stoner and Sirak, 1979). In a number of steady-state kinetic studies on the overall oxidative phosphorylation reaction as catalyzed by submitochondrial particles (Schatz and Racker, 1966; Kayalar *et al.*, 1976; Schuster *et al.*, 1977; Hatefi *et al.*, 1982), this was not taken into account and consequently the interpretations given are likely incorrect.

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