BBA 45633

ON THE NATURE OF ELECTRON AND ENERGY TRANSPORT IN MITOCHONDRIA

II. MULTIPLE ACTIVATION OF MITOCHONDRIAL RESPIRATION

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(Received August 2nd, 1967)

SUMMARY

1. Combinations of uncoupling agents with various electron transfer inhibitors suggest that State 4 is only an endogenously inhibited State 3.

2. Combinations of two uncouplers provide a tool for detecting differences in their behavior. It is concluded that dicoumarol may act only on phosphorylation Sites 2 and 3, and dinitrophenol and dibromophenol on all three of them.

3. Experiments with combinations of: (1) uncoupler *plus* ADP, (2) uncoupler *plus* substrate, and (3) substrate *plus* ADP, suggest a strong competition between substrate, uncoupler and ADP for a common receptor system.

4. It is concluded that at least two hypotheses can explain the results. Hypothesis 1, taking into account the mechanisms of oxidative phosphorylation suggested by CHANCE[2] and SLATER[3], places the point of interaction of substrate, uncoupler and ADP at the junction between electron and energy transfer chains, and Hypothesis 2, based on the observations of VAN DAM and co-workers[15–17] and the suggestions of VAN DAM AND SLATER[20], places it in the active-transport mechanism of the membrane itself.

INTRODUCTION

Many details are missing in our knowledge of the structural and functional organization of mitochondria. In the previous paper[1] the results of a study were reported in which respiratory inhibitors were used in an attempt to elucidate this organization. In the present paper attention has been paid to the interactions between physiological and non-physiological inhibitory and stimulatory forces in the mitochondria.

The kinetic method of combining various substances has again been used. These substances were mostly ones that activate respiration, like substrate, phosphate *plus* phosphate acceptor, and uncouplers; but inhibitors were also used.

The results obtained are discussed in the light of the mechanism proposed by

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CHANCE[2] and SLATER[3] for the interactions of electron and energy transfer in mitochondria, and in the light of recent findings of a competition between substrate anions and uncouplers for entry into the mitochondria.

MATERIAL AND METHODS

The method of preparation of rat-liver mitochondria, the reaction mixture employed and the polarographic apparatus used, were the same as described in the previous paper[1]. The uncouplers used were in aqueous solution.

It should be pointed out that in this paper respiration caused by addition of uncouplers is not called a State 3 but a certain form of State 4, as distinct from the notation in CHANCE's[4] original paper. State 3 refers only to the active state caused by physiological agents such as ADP + P_i, without regard to the presence or absence of either inhibiting or uncoupling agents.

RESULTS

Five different series of experiments were carried out, in each of which the interaction of only two parameters was considered. The relative concentrations of these two parameters were systematically varied and the kinetics of multiple inhibition and/or activation were studied.

Uncoupler plus inhibitor

In an extensive series of experiments, an attempt was made to compare the so-called endogenous inhibition, supposed by CHANCE and co-workers[5–7] to occur at the level of the three phosphorylation sites by means of the high-energy intermediates I_a, I_b and I_c, with the usual exogenous type, *i.e.*, the inhibition exerted by externally added inhibitors.

In order to vary the degree of endogenous inhibition we used uncouplers, assuming that when there is no uncoupler (0 %) present and in the absence of ADP, the effective endogenous inhibition will be maximal (100 %) and that it will be minimal (0 %) when the concentration of uncoupler is high enough (100 %).

In this respect endogenous and exogenous inhibitors were combined in the same way as two external inhibitors (see previous paper[1]). Dicoumarol and dinitrophenol were used (to control the degree of endogenous inhibition) in combination with malonate, Amytal, rotenone, antimycin, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, cyanide, azide and sulfide. Three different substrate systems were used: succinate *plus* glutamate, glutamate *plus* malate (*plus* malonate) and succinate alone (*plus* rotenone).

When the respiratory rates are plotted against the systematically varied ratios of inhibitor and uncoupler, essentially the same curves were obtained for all combinations studied. Fig. 1 is a typical example, in which dicoumarol was used to vary the extent of endogenous inhibition and azide was used as an exogenous inhibitor. Thus 100 % azide: 100 % dicoumarol means 100 % exogenous inhibition: 0 % endogenous inhibition; when neither azide nor dicoumarol are present, there is 0 % exogenous and 100 % endogenous inhibition. For State 4, which is relevant because of the absence of ADP, we find again an antagonism similar to that found with two

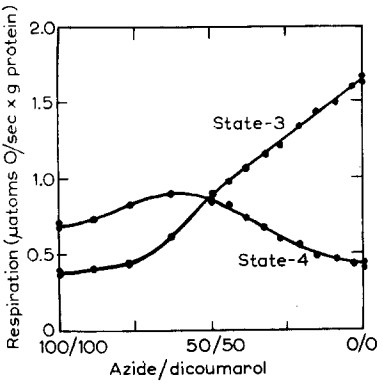

Fig. 1. Effect of decreasing concentrations of dicoumarol *plus* azide on respiration in the absence and presence of phosphate and phosphate acceptor. Experimental conditions as described in METHODS. The substrate was 4 mM glutamate + 4 mM malate in the presence of 8 mM malonate. State 3 respiration was measured in the presence of 200 μM ADP. The reaction mixtures contained 5 mg mitochondrial protein. The ratios azide: dicoumarol represent % of maximum concentration of azide used (640 μM): % of maximum concentration of dicoumarol used (3.2 μM).

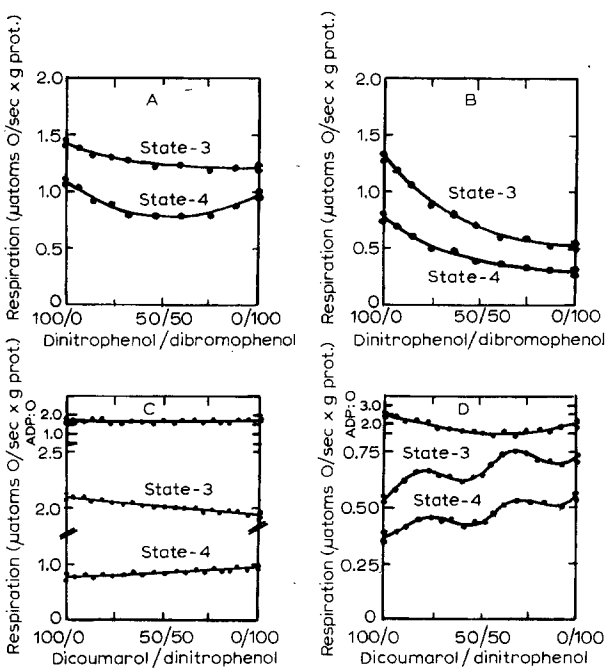

Fig. 2. Effect of combinations of dinitrophenol *plus* dibromophenol and dicoumarol *plus* dinitrophenol on respiration in the absence and presence of phosphate *plus* phosphate acceptor. Experimental conditions as described in METHODS. The substrate was 10 mM succinate (+ 2 μM rotenone) in Figs. 2A and C, and 6 mM glutamate + 6 mM malate (+ 8 mM malonate) in Figs. 2B and D. State 3 respiration was measured in the presence of 200 μM ADP. The reaction mixtures contained the following amounts of mitochondrial protein: 5.75 mg in A, 5 mg in B, 4.25 mg in C and 4.36 mg in D. The ratios dinitrophenol: dibromophenol and dicoumarol: dinitrophenol represent % of maximum concentration of dinitrophenol used: % of maximum concentration of dibromophenol used, and % of maximum concentration of dicoumarol used: % of maximum concentration of dinitrophenol used, respectively. The maximum concentrations (μM) of the uncouplers were: A. Dinitrophenol 9.6; dibromophenol 32. B. Dinitrophenol 12.8; dibromophenol 32. C. Dicoumarol 2.4; dinitrophenol 6.4. D. Dicoumarol 2.13; dinitrophenol 9.6.

external inhibitors acting on different enzymes in the respiratory chain (*cf.* ref. 1). The situation is more complicated when ADP is present (State 3); at higher concentrations of uncoupler *plus* inhibitor, the rate of respiration is smaller in the presence of ADP than in its absence.

Uncoupler plus uncoupler

Only the interactions of dinitrophenol *plus* dibromophenol and dinitrophenol *plus* dicoumarol were examined.

When the combined action of dinitrophenol *plus* dibromophenol is plotted in the same way as was done for a combination of two inhibitors[1] no marked difference between the oxidation of succinate and of glutamate *plus* malate was found (Figs. 2A and B). For both oxidation pathways there is a slight antagonism, *i.e.* the combined effect is less than would be expected on the basis of the individual performances.

Considering, however, the combination dinitrophenol *plus* dicoumarol, one sees a marked difference between the two pathways (Figs. 2C and D); with succinate, there is a perfect additivity, whereas with glutamate *plus* malate (*plus* malonate) there is a double antagonism, *i.e.* two maxima are found.

Uncoupler plus ADP

Fig. 3 shows a typical plot of respiratory rate *versus* concentration of dinitrophenol for three different levels of ADP (State 3 rates) and with glutamate *plus* malate (*plus* malonate) as substrate. Essentially the same phenomena have been observed for systems with dicoumarol and succinate (*plus* rotenone) and with dicoumarol and glutamate *plus* malate (*plus* malonate).

The inhibitory effect of ADP at higher concentrations of uncoupler (see Fig. 1) is again visible: at a certain concentration of dinitrophenol, which is greater for smaller

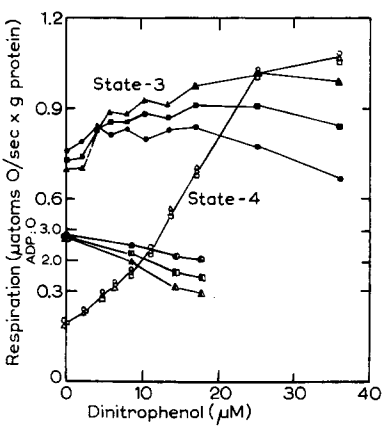

Fig. 3. Effect of dinitrophenol concentration on the rate of respiration and the ADP:O ratio at different levels of phosphate *plus* phosphate acceptor. Experimental conditions as described in METHODS. The substrate was 6 mM glutamate + 6 mM malate (+ 8 mM malonate). State 4 respirations were measured before the additions of ADP (State 3). For each level of dinitrophenol considered, three experiments were carried out, *i.e.* with three different levels of ADP (μM): 200 (▲, △, ◭), 600 (■, □, ◧) and 1200 (●, ○, ◐), respectively. In each experiment three parameters were measured: (1) State 4 respiration, before addition of ADP (note that, for each level of dinitrophenol, the three State 4 rates coincide) (△, □, ○); (2) State 3 respiration, after addition of ADP (▲, ■, ●); (3) the ADP:O ratios (see CHANCE[3]) (◭, ◧, ◐).

levels of ADP, the State 3 rates become smaller than the corresponding common State 4 rates. We call attention to the fact that this decrease of State 3 rates starts before that of State 4, which corresponds to the known inhibition provoked by high concentrations of uncoupler[8–19].

The State 3 rates which initially increase with increasing amounts of ADP when there is little or no uncoupling, gradually decrease with increasing levels of ADP at higher uncoupling. The phosphorylating efficiency, as measured by the ADP: O ratios, decreases, of course with increasing concentrations of dinitrophenol, but this decrease is smaller for higher levels of ADP.

Uncoupler plus substrate

Studying the respiratory rate as a function of concentration of the uncoupler at various levels of exogenous substrate gives essentially the same curves for glutamate *plus* malate (*plus* malonate) as for succinate (*plus* rotenone), with either dicoumarol or dinitrophenol, respectively. Fig. 4 is a typical example.

We can vary the effect of substrate on both States 3 and 4 by either varying the concentrations of substrate, as shown in Fig. 4A for glutamate *plus* malate (*plus* malonate), or by adding an inhibitor, *i.e.* rotenone, to an excess of the same substrate, as shown in Fig. 4B.

State 3 rates initially increase with higher concentrations of uncoupler and the maximum is reached much faster for lower concentrations of substrate or higher concentrations of inhibitor. Further addition of ADP results in the already observed inhibition of State 3 rates, which now become smaller than the corresponding State 4 rates.

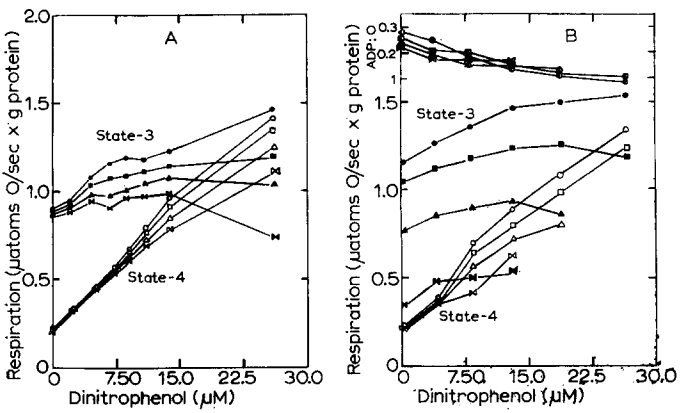

Fig. 4. Effect of dinitrophenol concentration on the rate of respiration (and the ADP:O ratio) at different levels of substrate. Experimental conditions as described in METHODS. A. For each level of dinitrophenol four experiments were done, *i.e.* with four different levels (mM) of glutamate *plus* malate (+ 8 mM malonate): 10 (○, ●), 4.8 (□, ■), 3.2 (△, ▲), and 1.6 (▽, ▼), respectively. In each experiment two parameters were measured: (1) State 4 respiration, before addition of ADP (○, □, △, ▽); (2) State 3 respiration, after addition of ADP, 200 μM (●, ■, ▲, ▼). The reaction mixtures contained 7.25 mg mitochondrial protein. B. Four experiments were done for each concentration of dinitrophenol, *i.e.* with four different concentrations (nM) of rotenone: 0 (○, ●, ◐); 20 (□, □, ◧), 40 (△, ▲, ◭), and 60 (▽, ▼, ⊗), respectively. In each experiment there was 6 mM glutamate + 6 mM malate (+ 8 mM malonate) and the following parameters were measured: (1) State 4 respiration, before addition of ADP (○, □, △, ▽); (2) State 3 respiration, after addition of 200 μM ADP (●, ■, ▲, ▼); (3) the ADP:O ratios (◐, ◧, ◭, ⊗). The reaction mixtures contained 7 mg mitochondrial protein.

These State 4 rates also increase with higher concentrations of uncoupler but the increase is slower for smaller concentrations of 'effective' substrate. The classical inhibitory action of the uncoupler, which, however, does not take place under our reaction conditions in State 4, may therefore be anticipated to occur also more quickly at lower 'effective' concentrations of substrate, analogously to what happens in State 3.

At low concentrations of uncoupler we find the greatest phosphorylating efficiency, *i.e.* the highest ADP:O ratios, for the highest levels of effective substrate. However, at higher concentrations of uncoupler we find the relatively highest efficiency for the lowest effective substrate concentrations.

ADP plus substrate

In separate measurements on the oxygraph we had observed a small but definite decrease in State 3 rates, when we went from normal to very high additions of ADP. This was the case with an excess of substrate, both with succinate and NAD-linked substrates.

On the other hand in experiments with 10–100 times less exogenous substrate, we noticed an increase in State 3 rates when we went from small to larger additions of ADP (P. NIJS, unpublished results).

Therefore we tried to assemble these results, which all seemed to point in the same direction, in one set of experiments, where we omitted any uncoupler and studied the effect on State 3 of different ratios of exogenous substrate: ADP. Fig. 5 is a typical example with glutamate *plus* malate (*plus* malonate) as substrate. To obtain different effective concentrations of substrate, we either varied the concentrations (Fig. 5A) or added rotenone to an excess of substrate (Fig. 5B).

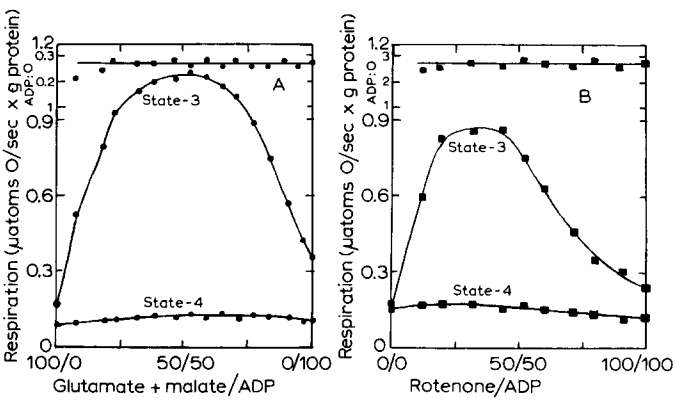

Fig. 5. Effect of combinations of substrate *plus* ADP and of inhibitor *plus* ADP on respiration and phosphorylation efficiency. Experimental conditions as described in METHODS. An excess of malonate (8 mM) was present in each experiment. The denominator ADP of the abcissas does not apply for the State 4 rates, measured in the indicated conditions but before addition of ADP. A. The ratios glutamate *plus* malate: ADP represent % of maximum concentration of glutamate *plus* malate (both 6.4 mM) used: % of maximum concentration of ADP used (640 μM), taking into account that 0% of substrate corresponds in this case to a minimum concentration of substrate (0.08 mM). The reaction mixtures contained 6 mg mitochondrial protein. B. The substrate was 6.4 mM glutamate + 6.4 mM malate. The ratios rotenone: ADP represent % of maximum concentration of rotenone used (50 nM): % of maximum concentration of ADP used (640 μM). The reaction mixtures contained 5.5 mg mitochondrial protein.

The course of the State 3 rates observed in both Figs. 5A and B seems at first sight quite normal because both substrate and phosphate acceptor are necessary for State 3 respiration. The phosphorylation efficiency, however, remains remarkably constant in spite of the fact that the rate of oxygen uptake first increases and then, after reaching a maximum, decreases again. The State 4 rates, preceding the observed State 3 rates in both Figs. 5A and B, also remain almost constant during the operation.

DISCUSSION

The experimental results, with the exception of those discussed in the first two sections, can be explained according to at least two hypotheses, shown in Figs. 6A and B.

The first hypothesis is based on the mechanisms of oxidative phosphorylation suggested by CHANCE[2] and SLATER[3]. The second is that suggested by VAN DAM AND SLATER[20] and is based on the observations of VAN DAM and co-workers[15–17], WILSON and co-workers[18,19] and WENNER[14].

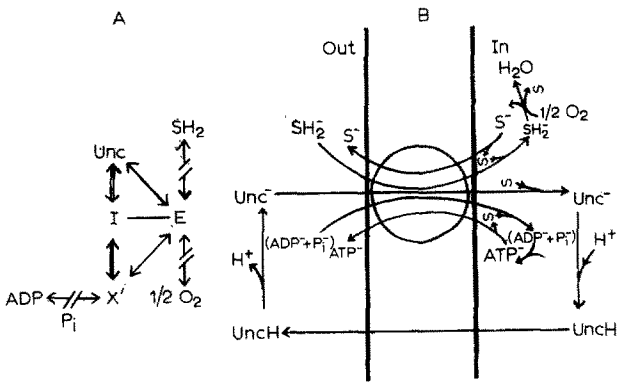

Fig. 6. Scheme of interactions of substrate, uncouplers, phosphate and phosphate acceptor. A. *Hypothesis 1*. Interactions of substrate (SH_2), uncoupler (Unc), and intermediate X' (acting for ADP + P_i) at the junction of electron transfer (symbol: carrier E) and energy transfer chains (symbol: intermediate I). B. *Hypothesis 2*. Interactions of the anionic forms of substrate (SH_2^-), product (S^-), phosphate *plus* phosphate acceptor (P_i^- + ADP^-), uncoupler (Unc^-), and ATP^- at the level of the active-transport mechanism in the membrane. The uncoupler in its weak acid form is represented by UncH and energy by $\sim$.

In fact the fundamental difference between the two hypotheses lies in the problem of the exact location of the observed phenomena and most of the arguments for the existence of real interactions between substrates, uncouplers and phosphate acceptors demonstrated in support of Hypothesis 1, can equally well be used for Hypothesis 2.

Before dealing with the two hypotheses we want to discuss the first two sections. With CHANCE[5–7] we assume that the endogenous intermediates I_d, I_b, and I_c, representing the first members of the energy transfer pathways, are blocking most of the mitochondrial respiration during the resting state (State 4) by formation of high-energy complexes with neighbouring enzymes of the electron transport chain. In this respect it was logical to compare this endogenous inhibition to an external one. What

we found was always a pronounced antagonism such as for a combination of two external inhibitors acting on different enzymes in the same chain (see ref. 1 and Fig. 1).

Therefore we believe that: (1) the three *I* intermediates act presumably as real inhibitors in a way essentially comparable to external inhibitors; (2) there is no common site of action for the resulting effect of endogenous inhibitors and any of the external inhibitions so far studied: the endogenous inhibition therefore should be a 'multisite' one. Indeed, we especially concentrated on combinations of dinitrophenol with a mixture of Amytal, antimycin and azide, because these inhibitors behave to some extent as uncouplers[21–24], suggesting that their sites of action may lie very close to the phosphorylation sites, but we never succeeded in finding linear plots, *i.e.* additivity.

As a general conclusion we assume that the resting state is only an endogenously inhibited active state (see also ref. 25).

Considering Section *Uncoupler plus uncoupler* and Figs. 2, we see no marked difference between the effect on respiration of the combination of dinitrophenol *plus* dibromophenol for succinate and for NAD-linked oxidations (Figs. 2A and B). The couple dinitrophenol *plus* dicoumarol, however, shows an important difference in it (Figs. 2C and D). The reason for this is unknown. One possible explanation of the results is to assume that dicoumarol acts on only the last two phosphorylation sites whereas dinitrophenol (or dibromophenol) acts on all three sites, as suggested by PRESSMAN[26]. This could be tested by studying the oxidation of NADH by fumarate (*cf.* ref. 27), that is, using a system in which only the first phosphorylation site is involved.

Hypothesis 1

Let us assume that the observed interactions of substrates, uncouplers and phosphate acceptor take place at the junction of electron and energy transfer pathways. This is shown in Fig. 6A.

Electron flow goes from any oxidizable substrate, SH_2, through the respiratory chain to oxygen. *E* is a respiratory carrier, situated near one of the phosphorylation sites. Energy flow goes from *E* through the intermediates *I* and *X'* to ADP + P_i. *I* is one of the intermediates I_d, I_b or I_c, discussed before. The intermediate *X'*, which we assume to be mobile and lipophilic, such as an uncoupler, may either coincide with *X* (*cf.* CHANCE[2] and SLATER[3]) or be of a different nature.

Complexing of *I* with *E*, both supposed to lie in a lipid environment (probably the cristae and the inner mitochondrial membrane), results in the resting state (State 4) and this can be counteracted by reaction with one of two kinds of lipophilic substances: (1) *X'*, acting only after addition of ADP + P_i, and leading to the physiologically active state (State 3); (2) uncouplers, giving rise to another, unphysiologically active state.

It is well known that uncoupled and ADP-stimulated systems usually do not have the same respiratory maxima[28–31]: uncoupled systems normally have a higher respiration. Assuming now that both uncoupler and ADP act on the same receptor (directly or indirectly), which in this hypothesis is the intermediate *I* and in the second one the active anion transport system, one can kinetically explain the State 3 curves of Fig. 3 by a competitive dualism in their action, as pointed out by ARIENS[32]. He finds theoretical dose–response curves for combinations of a compound of higher

intrinsic activity (uncouplers) with one of lower intrinsic activity (ADP + P_i), which are completely analogous to our State 3 curves in Fig. 3.

As one can see in Figs. 4A and B, there is a competition between electron flow and inhibitory action of the uncoupler: we are able to partially release the weakened respiration in both State 4 and 3, if we increase in some way the electron flow: a progressive lifting up of the increasingly declining curves of both State 4 (uncoupler alone present) and State 3 (ADP and uncoupler present) is caused by either increasing the concentration of substrate (Fig. 4A) or by decreasing the external inhibitor (Fig. 4B).

On the other hand, the competition between electron flow and auto-inhibitory action of ADP + P_i is not unequivocally proven by the course of the State 3 curves in Figs. 5A and B. It is, however, quite remarkable that the phosphorylation efficiency (see the ADP:O curves on the same figure) remains strictly constant in spite of the fact that the respiration decreases at high levels of ADP and at concentrations of substrate, which are still able to give the full State 4 electron flow. Moreover, looking back at Figs. 4A and B, we notice that additions of high levels of ADP to partially or fully uncoupled State 4 systems, hinder in some way the stimulatory action of the uncoupler and give rise to an inhibition of respiration: State 3 rates become smaller than State 4 rates. Fig. 1 also shows that additions of ADP to a system with relatively high concentrations of uncoupler *plus* inhibitor give rise to State 3 rates which are smaller than the corresponding State 4 rates.

For all these reasons we believe that, in analogy with uncouplers, also ADP + P_i, used in excess, has an auto-inhibitory action on respiration, which can be partially overcome by greater electron flow.

Hypothesis 2

We can as well assume that the observed interactions of substrate, phosphate *plus* phosphate acceptor, and uncouplers take place at the level of the membrane, separating the external from the internal medium of the mitochondria, as shown in Fig. 6B.

Since inhibition by uncouplers of substrate oxidation occurs only with anionic substrates (see ref. 17), since it is kinetically of the competitive type (see ref. 19), and since uncouplers are weak acids, it also appears reasonable to relate the inhibitory effect to a competition between anions for entry into the mitochondria. In this light we can also explain the results in Figs. 4A and B.

Moreover one can extend this view to the action of ADP + P_i. Both are anions and the site of their inhibitory interactions with uncouplers and substrates, as observed in Figs. 3 and 5, respectively, can equally well be transferred from the junction of respiratory and energy-conserving chains to the place of entry of the other anionic species into the mitochondria. The argumentation based on ARIENS' kinetics[32] remains valuable.

To summarize, we would have a competition between the anionic substrates, uncouplers, phosphate and phosphate acceptor for the presumably active-transport mechanism in the mitochondrial membrane. As represented in Fig. 6B, the net effect of this set of transfer processes would be an exchange of product S^- for substrate SH_2^- and of ATP^- for $ADP^- + P_i^-$ without consumption of energy if the steady state is

reached. Only the net effect of the transport of uncouplers would be a dissipation of energy (see ref. 20).

The data available at the present time do not allow one to distinguish between the two hypotheses.

Finally we think our results show that the kinetic method of multiple activation and/or inhibition of complicated enzyme systems is a useful tool in studies of biological chain reactions. There is, however, one restriction: one has to know the exact site(s) of action of at least one of the two members of the combination in order to explain the results properly (see also ref. 33).

ACKNOWLEDGEMENTS

The author is very grateful to Dr. B. CHANCE for many helpful suggestions and for his hospitality in allowing him to make use of the facilities of the Johnson Research Foundation, and to Dr. J. M. TAGER for his help with the final preparation of the manuscript. He also wishes to thank the U.S. Public Health Service for the award of an International Post-doctoral Fellowship (I F05-TW-701-01).

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