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# ON THE NATURE OF ELECTRON AND ENERGY TRANSPORT IN MITOCHONDRIA

## I. MULTIPLE INHIBITION OF MITOCHONDRIAL RESPIRATION

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SUMMARY

1. A series of eight classical respiratory-chain inhibitors was studied. The slopes of State-3 respiratory rate *versus* dose plots are convex for antimycin, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO), rotenone and sulfide, and concave for malonate, Amytal, cyanide and azide.

2. Plots of ADP:O ratio *versus* dose indicate uncoupling effects at higher concentrations of antimycin, HOQNO, cyanide and azide. On the other hand, sulfide and rotenone have no effect on the phosphorylating efficiency. Malonate increases the ADP:O ratio.

3. Two inhibitors can be combined in such a way that the total inhibition should be equal to the inhibition caused by the single inhibitors if each inhibitor affects respiration independently (additivity of inhibition). In practice, however, antagonism and synergism are also found.

4. Additivity of combined inhibition occurs where both inhibitors act on the same enzyme.

5. Antagonism is observed where the two inhibitors act on different enzymes of the same chain.

6. Synergism is found where the two inhibitors act on enzymes in different branches of a forked chain. This turns into normal additivity when the electron flow through both branches is made equal.

7. The results are compatible with the hypothesis that respiratory enzymes are arranged in chains. The possibility that the chains may be cross-linked or branched is discussed.

## INTRODUCTION

The general organization of the enzymatic pathways involved in mitochondrial respiration is more or less known but many details are still missing. Reducing equivalents are transferred 'downhill' from succinate or NAD-linked substrates toward

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oxygen through the flavoproteins and the cytochromes, and the liberated energy equivalents are chemically bound into ATP at 3 different phosphorylating sites.

Diverging theories have been proposed by various authors concerning the detailed arrangements, the organization and the framework of the electron-transferring enzymes (*cf.* CHANCE and co-workers[1–4], KLINGENBERG AND KROELER[5], GREEN AND LESTER[6], MITCHELL[7], LEHNINGER AND WADKINS[8,9]).

In the present study we have used a new kinetic tool, *i.e.* multiple inhibition, in an attempt to clarify the functional arrangement of the electron-transferring enzymes in mitochondria. The results will be discussed in terms of the generally accepted theory of respiratory enzymes arranged in chains, but also in the light of the more recent hypothesis of branched chains.

## MATERIAL AND METHODS

Intact rat-liver mitochondria were prepared as described by CHANCE AND HAGIHARA[10,11] for pigeon-heart mitochondria except that no proteinase was used. In this way sufficiently high respiratory control ratios could be obtained: greater than 6 (usually 8–10) for succinate + glutamate or malate + glutamate as substrates; greater than 5 (usually 6–7) for succinate alone, and greater than 3 (usually 4–5) for β-hydroxybutyrate.

The reaction mixture (2.5 ml final vol.) contained 20 mM Tris–HCl buffer, 210 mM mannitol, 70 mM sucrose, 2 mM $MgCl_2$, 10 mM KCl, 5 mM $Na_2HPO_4$–$KH_2PO_4$ and about 5.0 mg mitochondrial protein (determined by the biuret method). The final pH was 7.4.

The inhibitors rotenone, antimycin and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) were added in ethanolic solutions. The final concentration of ethanol did not exceed 3 %. The other inhibitors were added as aqueous solutions.

The respiratory rates, which are all initial ones, were estimated by the polarographic method, using either a stationary platinum electrode[12] or a Clark oxygen electrode (Yellow Springs Instrument Co.). The reaction temperature was 22°, and there was magnetic stirring.

ADP:O ratios were estimated as described by CHANCE AND WILLIAMS[3].

## RESULTS

In a series of preliminary experiments the relationship between respiratory rate and concentration of inhibitors used singly was determined. Two types of curves were obtained when State-3 respiration was measured. An S-shaped curve (Fig. 1A) was obtained with the inhibitors antimycin (half-maximal concn., 31 nmoles per g protein), HOQNO (360 nmoles/g protein), rotenone (37 nmoles/g protein) and sulfide (10 μM). A concave curve (Fig. 1B) was obtained with malonate (400 μM), Amytal (150 μM), cyanide (30 μM) and azide (300 μM). The substrate was succinate + glutamate (both 6 mM) except with rotenone and Amytal, where glutamate + malate (both 6 mM) and 6 mM malonate were used.

The ADP:O ratio as a function of concentration of the inhibitors used singly was also checked. As indicated by Fig. 2, an uncoupling effect (decreasing phosphorylating activity at higher concentrations of inhibitor) was found (with succinate as

substrate) with antimycin (MYERS AND SLATER[13], HEMKER[14]), HOQNO (HOWLAND[15]), cyanide (SLATER[16]) and azide (LOOMIS AND LIPMANN[17]). On the other hand, malonate increased the ADP:O ratio with succinate as substrate. There was no effect of sulfide (with succinate as substrate) or of rotenone (with β-hydroxybutyrate as substrate). Amytal[18], which was not investigated in this respect, is known to have uncoupling properties at higher concentrations.

We can combine two inhibitors in such a way that the total inhibition of State-3 respiration should theoretically be equal to the same level of inhibition as caused by the single inhibitors in separate experiments, *e.g.* 40 %, if each inhibitor affected

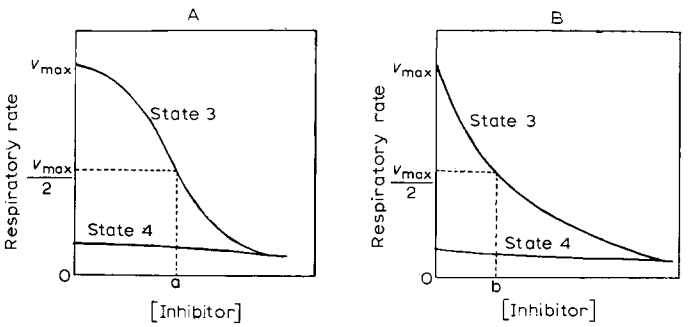


Fig. 1. Effect of concentration of inhibitors (added singly) on respiration. Somewhat idealized respiratory rate *versus* dose plots of the two observed classes of inhibitors. Experimental conditions as described in MATERIAL AND METHODS.

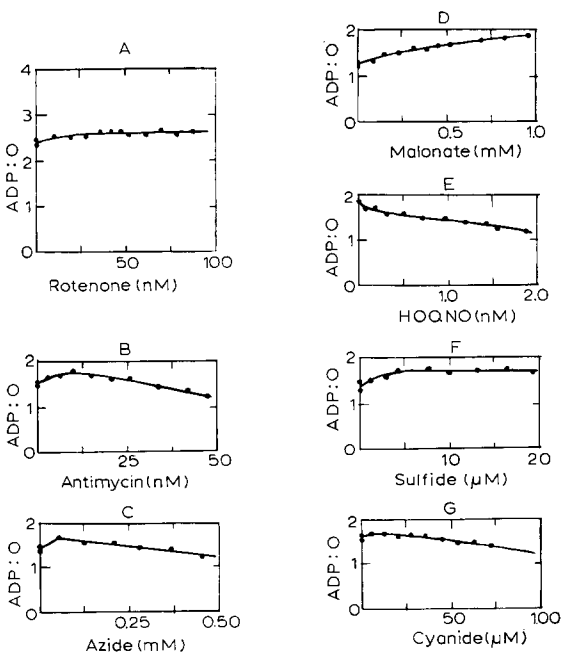


Fig. 2. Effect of concentration of inhibitors (added singly) on the ADP:O ratios. Experimental conditions as described in MATERIAL AND METHODS. The substrate was succinate (6 mM) except in Fig. 2A where 6 mM β-hydroxybutyrate (+6 mM malonate) was used.

TABLE I

INHIBITION TO BE EXPECTED FROM A COMBINATION OF TWO INHIBITORS, ASSUMING THE INHIBITORS TO ACT INDEPENDENTLY

| *Inhibitor 1* ($\mu M$) | *Inhibition* (%) | *Inhibitor 2* ($\mu M$) | *Inhibition* (%) | *Inhibition** (%) |
|---|---|---|---|---|
| $a$ | 40 | 0 | 0 | 40 |
| $0.75 \times a$ | 30 | $0.25 \times b$ | 10 | 40 |
| $0.50 \times a$ | 20 | $0.50 \times b$ | 20 | 40 |
| $0.25 \times a$ | 10 | $0.75 \times b$ | 30 | 40 |
| 0 | 0 | $b$ | 40 | 40 |

* Expected when both inhibitors present.

respiration independently. Let us assume that we need $a$ $\mu$M Inhibitor 1 or $b$ $\mu$M Inhibitor 2 for 40 % inhibition of the respiration. Table I shows the types of combination that can be made. Fractions of a certain amount $a$ of Inhibitor 1 are substituted for by corresponding fractions of an amount $b$ of Inhibitor 2, producing the same inhibition. Because it is difficult to obtain exactly the same degree of inhibition, *e.g.* 40 %, for the two inhibitors used singly, the endpoints usually differ somewhat. However this does not affect the principle of the method. By plotting the respiratory rates as a function of the proportions of concentrations of the two inhibitors, combined as in Table I, we should theoretically find straight lines if the two inhibitors act independently. This is called additivity.

When the two inhibitors produce an effect that is less than expected on the basis of their individual performances, antagonism is commonly assumed to occur. When the combined effect is greater than expected on the basis of the individual performances, synergism occurs.

These are the three cases[19] which are possible in practice and which indeed cover all the different combinations we have studied.

*Additivity*

This is found to occur when both inhibitors act on the same enzyme: (1) antimycin + HOQNO (in the region of cytochrome *b*); (2) cyanide + azide; and (3) cyanide + sulfide (both on cytochrome oxidase). The results for the combination antimycin + HOQNO, using succinate as substrate, are shown in Fig. 3.

We did not study the couple Amytal + rotenone, both acting between NAD and flavoprotein, but we may anticipate that this combination will behave in the same manner.

*Antagonism*

This may be inferred when the respiratory rate plotted as described above gives convex curves. This occurs where the two inhibitors are acting on different enzymes in the same chain and is found with: (1) antimycin + azide (the region of cytochrome *b* and cytochrome oxidase, respectively); (2) antimycin + sulfide (the region of cytochrome *b* and cytochrome oxidase, respectively); (3) antimycin + rotenone (the region of cytochrome *b* and the NAD side of flavoprotein, respectively); (4) antimycin + Amytal (the region of cytochrome *b* and the NAD side of flavo-

protein, respectively); (5) HOQNO + sulfide (the region of cytochrome *b* and cytochrome oxidase, respectively); (6) malonate + azide (succinate dehydrogenase and cytochrome oxidase, respectively); (7) rotenone + azide (the NAD side of flavoprotein and cytochrome oxidase, respectively); and (8) rotenone + cyanide (the NAD side of flavoprotein and cytochrome oxidase, respectively).

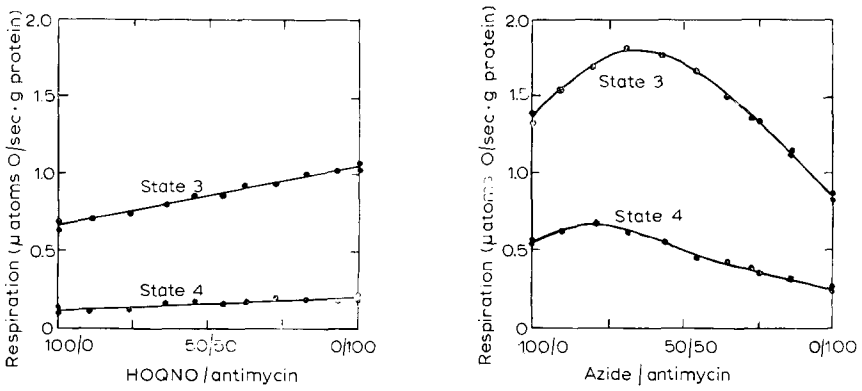


Fig. 3. Additivity of inhibition of respiration by antimycin and HOQNO. Experimental conditions as described in MATERIAL AND METHODS. The substrate was succinate (4 mM). State-3 respiration was measured in the presence of 200 $\mu$M ADP. The reaction mixture contained 3.5 mg mitochondrial protein. The ratios HOQNO/antimycin represent percent of maximum concentration of HOQNO used (398 nM)/percent of maximum concentration of antimycin used (31.6 nM). The rate of respiration in the absence of inhibitor was 1.49 and 0.24 $\mu$atoms O per sec·g protein, in State 3 and State 4, respectively.

Fig. 4. Antagonism between antimycin and azide in their effects on respiration. Experimental conditions as described in MATERIAL AND METHODS. The substrate was succinate (4 mM). State-3 respiration was measured in the presence of 200 $\mu$M ADP. The reaction mixture contained 3.62 mg mitochondrial protein. The ratios azide/antimycin represent percent of maximum concentration of azide used (360 $\mu$M)/percent of maximum concentration of antimycin used (44 nM). The rates of respiration in the absence of inhibitors in State 3 and State 4 were 2.54 and 0.45 $\mu$atoms O per sec·g protein, respectively.

The results for the combination antimycin + azide are shown in Fig. 4. The substrate used was succinate except with the combinations antimycin + rotenone, antimycin + Amytal, rotenone + azide and rotenone + cyanide, where it was $\beta$-hydroxybutyrate, malonate being also present to eliminate endogenous respiration. With malonate + azide, rotenone was added to inhibit oxidation of endogenous NAD-linked substrates.

*Synergism*

When the respiratory rate plotted as described above gives a concave curve a synergistic action of the two inhibitors is indicated. This is found where the two inhibitors act on enzymes in different branches of a forked chain, *e.g.* (1) malonate + rotenone (succinate dehydrogenase and the NAD side of flavoprotein, respectively); (2) malonate + Amytal (succinate dehydrogenase and the NAD side of flavoprotein, respectively). The results for malonate + rotenone are shown in Fig. 5A. The substrate in both experiments was a combination of succinate and $\beta$-hydroxybutyrate.

The ADP:O ratios plotted in the same way also gave concave curves.

However this synergism (concave plots) of both the respiratory rates and the ADP:O ratios can be changed into simple additivity (linear plots) by reducing the relatively fast oxidation of succinate to the level of that of β-hydroxybutyrate, by adding extra malonate (Fig. 5B).

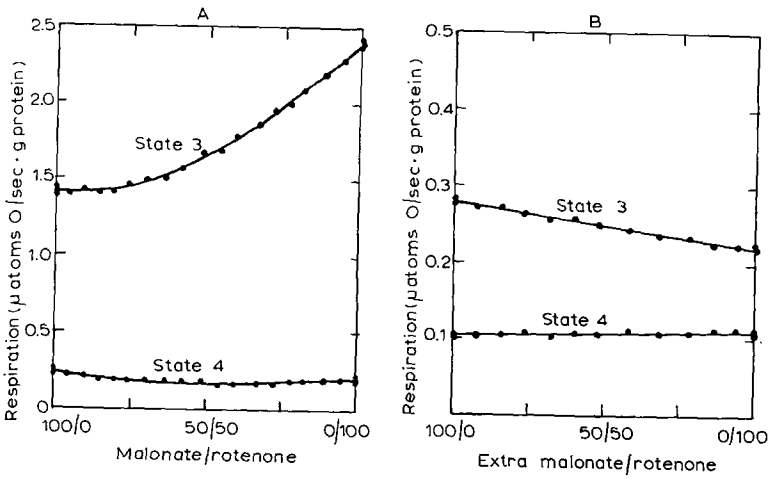


Fig. 5. Synergism between malonate and rotenone in their effects on respiration. Reaction conditions as described in MATERIAL AND METHODS. The ratios malonate/rotenone represent percent of maximum concentration of malonate used/percent of maximum concentration of rotenone used. A. The substrate was succinate (4 mM) + β-hydroxybutyrate (4 mM). State-3 respiration was measured in the presence of 200 μM ADP. The reaction mixture contained 8.25 mg mitochondrial protein. The maximum concentrations of the inhibitors were: malonate, 0.32 mM; rotenone, 44 nM. The rates of respiration in the absence of inhibitor in State 3 and State 4 were 2.3 and 0.37 μatoms O per sec·g protein, respectively. B. The substrate was succinate (4 mM) + β-hydroxybutyrate (4 mM). In addition 0.4 mM malonate was present. State-3 respiration was measured in the presence of 200 μM ADP. The reaction mixture contained 8.5 mg mitochondrial protein. The maximum concentrations of the inhibitors were: extra malonate, 0.4 mM; rotenone, 40 nM. The rates of respiration in the absence of inhibitor in State 3 and State 4 were 0.46 and 0.13 μatoms O per sec·g protein, respectively.

## DISCUSSION

### *Additivity*

In all combinations where the two inhibitors act on the same enzyme, additivity was observed. In other words the combined effect of two such inhibitors is a simple summation of their separate effects. Each inhibitor affects respiration independently. This implies that an enzymic molecule inhibited at a particular site by the first inhibitor cannot be further attacked by the second inhibitor. If two inhibitors act on the same enzyme but on different sites one would expect antagonism. This was not observed with any of the combinations of inhibitors acting on the same enzyme.

### *Antagonism*

This was observed only where the two inhibitors act on different enzymes of the same chain. At first sight, antagonism is to be expected, if we assume that the two inhibitors act at random and attack either different chains or the same chain.

The experiment with rotenone + azide may be taken as an example. The rotenone-sensitive reaction is faster when the ratio rotenone:azide is 50:50 than when it is 100:0. Similarly the azide-sensitive reaction is faster when the ratio rotenone:azide is 50:50 than when 100 % azide is present. Therefore respiration is faster when the ratio rotenone:azide is 50:50 than when it is 100:0 or 0:100.

However when we consider the specific sites of action of the two inhibitors the situation becomes more complicated. It is known that azide and cyanide have a greater affinity for the reduced form of cytochrome oxidase than for the oxidized form[20,21]. ESTABROOK[22] has reported that rotenone acts preferentially with the oxidized form of the respiratory carrier with which it combines. When a certain inhibitor acts on an enzyme somewhere in the respiratory chain, the enzymes before the point of attack, *i.e.* on the substrate side of the chain, become more reduced; and the ones following the point of attack, *i.e.* on the oxygen side, become more oxidized (see KEILIN[23], SLATER[24] and CHANCE AND WILLIAMS[25]).

If rotenone reacted with the respiratory chain before azide, it would make the cytochrome oxidase in the same chain 'more oxidized'. Therefore azide, preferring the reduced form of cytochrome oxidase, will combine with this enzyme in a chain uninhibited by rotenone. If, on the other hand, azide were to react with a respiratory chain before rotenone, the carriers on the substrate side of the site of inhibition would become more reduced. Therefore rotenone, having a greater affinity for the oxidized chains, would attack a chain uninhibited by azide. In both examples one would expect additivity of the combined inhibition. In spite of this, antagonism is observed with rotenone + azide and with rotenone + cyanide. A possible explanation of the lack of additivity is that the enzyme chains are branched[26–28]. Branching makes the oxidized part of the inhibited chains more or less accessible to electrons from uninhibited chains and makes the reduced part of the inhibited chains more or less leaky for electrons towards uninhibited chains. The need for a second inhibitor, because of its affinity for one of the oxido–reduction forms, to react preferentially with enzymes belonging to uninhibited chains, leading to complete additivity, is therefore considerably decreased.

*Synergism*

An explanation of the synergistic effect of two inhibitors is (mentioned above), that they inhibit on different branches of a forked chain. It is known that, with low concentrations of succinate, the oxidation of succinate also includes malate oxidation; with low concentrations of NAD-linked substrates the oxidation of malate may include endogenous substrate[18]. This may be the explanation of synergism in the combined inhibition by malonate + rotenone and by malonate + Amytal. If this would be so, the synergism would not be due to any particular characteristic of the forked chains which would of itself be expected to lead to additivity of the combined inhibition. This, indeed, it does when we make the electron flow through both arms of the fork about equal by adding extra malonate.

This study also introduces the possibility of estimating the point of attack of new inhibitors of the respiratory chain by simply combining them with already known ones.

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