RESTORATION OF OXIDATIVE PHOSPHORYLATION IN 'NON-PHOSPHORYLATING' SUBMITOCHONDRIAL PARTICLES BY OLIGOMYCIN

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In a previous paper (Lee et al., 1964a) we have shown that so-called 'non-phosphorylating' submitochondrial preparations from beef heart can carry out energy-dependent pyridine nucleotide transhydrogenation more efficiently with aerobically generated high-energy intermediates than with ATP as the source of energy. It was concluded that these particles are still capable of respiratory chain-linked energy-coupling, and that what makes them 'non-phosphorylating' is the lack of ability to transfer energy from primary high-energy intermediates to phosphorylate ADP to ATP. In this paper we wish to present evidence that such particles can also perform oxidative phosphorylation of ADP to ATP, as well as the reversal of this process, when assayed under suitable conditions. As will be shown, the restoration of these activities requires no externally added enzymes or 'coupling factors', but is achieved by the addition of appropriate amounts of the known inhibitor of oxidative phosphorylation, oligomycin (Lardy et at., 1958).

The present investigation was prompted by the previous observation (Lee et al., 1964a) that oligomycin stimulated the transhydrogenase reaction driven by aerobically generated high-energy intermediates in non-phosphorylating particles. Closer study of this effect revealed (Fig. 1) that the amount of oligomycin required to achieve maximal stimulation of the transhydrogenase reaction was much smaller than that required for maximal inhibition of the ATPase activity of the same particle preparation. This finding suggested that oligomycin may exhibit two effects on the particles (Fig. 2): an inhibition of the hydrolysis of a high-energy intermediate,

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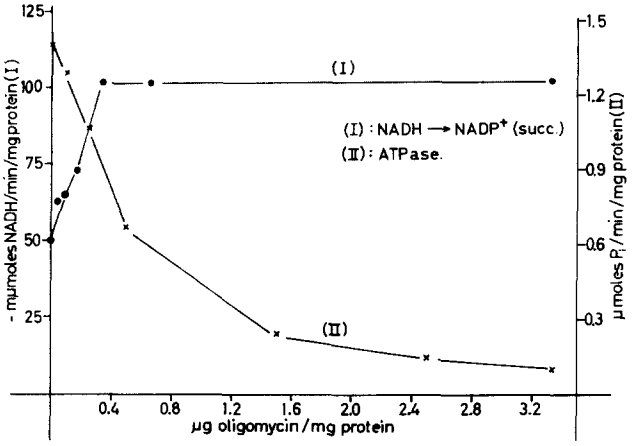

Fig. 1. Effect of oligomycin on ATPase and on succinoxidase-supported $NADP^+$ reduction by NADH. ATPase was assayed in a reaction mixture consisting of 180 mM sucrose, 50 mM Tris-acetate buffer, pH 7.5, 4 mM $MgSO_4$, particles (prepared in the presence of 2 mM EDTA, pH 8.6) containing 0.2 mg protein, and oligomycin as indicated. Reaction was started by the addition of 5 mM ATP. Final volume, 1 ml, temp., 30°C, time of incubation, 5 min. The reaction mixture for the transhydrogenase assay consisted of 180 mM sucrose, 50 mM Tris-acetate buffer, pH 7.5, 3.3 μM rotenone, 10 mM $MgSO_4$, 0.2 mM NADH, 0.15 mM $NADP^+$, 1 mM oxidized glutathione, an amount of glutathione reductase capable of oxidizing 0.5 μmoles of NADPH/min., particles containing 0.6 mg protein and oligomycin as indicated. Reaction was started by the addition of 5 mM succinate. Final volume, 3 ml, temp., 30°C.

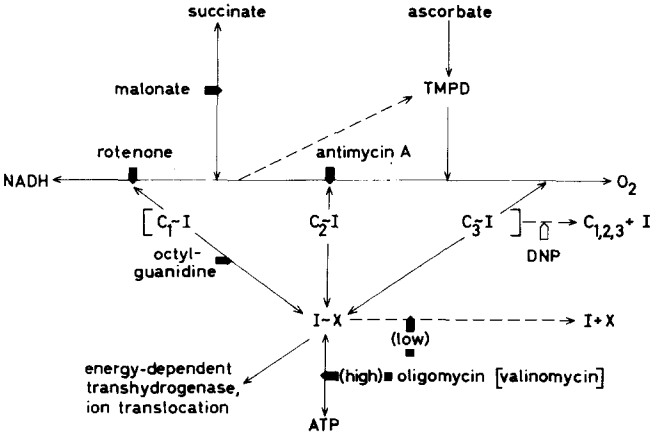

Fig. 2. Hypothetic scheme of the energy-transfer system linked to the respiratory chain. C_1, C_2, C_3 denote electron carriers at the energy-coupling sites of the respiratory chain; I and X denote hypothetic energy transfer carriers (for further definitions, see Ernster and Lee, 1964). Solid bars indicate site of action of inhibitors; open bar indicates site of stimulation by 2,4-dinitrophenol (DNP).

I~X, at low concentrations of oligomycin; and an inhibition of the interaction of the same intermediate with P_i and ADP, to yield ATP, at higher concentrations of oligomycin. If this interpretation were correct, it follows that low concentrations of oligomycin might enhance oxidative phosphorylation (or its reversal) in non-phosphorylating particles. The data presented below show that this indeed is the case.

As shown in Fig. 3, particles prepared in the presence of EDTA exhibited only a very low rate of ATP-supported succinate-linked NAD^+ reduction, in accordance with previous results (Lee et al., 1964a). When 0.2 μg of oligomycin per mg of particle protein was added, ATP induced a considerable rate of succinate-linked NAD^+ reduction. The reaction was completely inhibited by malonate and rotenone. As anticipated, the stimulation occurred only within a certain range of oligomycin concentration, above which the reaction was inhibited (Fig. 4). Oligomycin also stimulated the ATP-supported reduction of NAD^+ by ascorbate + N,N,N'N'--tetramethyl-p-phenylenediamine (TMPD), in a fashion analogous to the

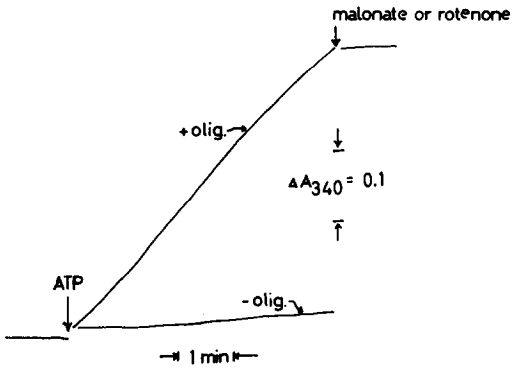

Fig. 3. Stimulation of ATP--supported succinate-linked NAD^+ reduction by oligomycin The reaction mixture consisted of 180 mM sucrose, 50 mM Tris-acetate buffer, pH 7.5, 1.6 mM KCN, 10 mM $MgSO_4$, 0.2 mM NAD^+, 5 mM succinate and particles (prepared in the presence of 2 mM EDTA, pH 8.5) containing 0.75 mg protein. Other additions: 3 mM ATP, 0.15 μg oligomycin, 1.6 mM malonate, and 1.6 μM rotenone. Final volume 3 ml, temp., 30°C.

succinate-linked NAD^+ reduction. It was also noticed (not shown) that, when the amount of oligomycin exceeded that needed for maximal stimulation, the rate of NAD^+ reduction by succinate or ascorbate + TMPD decreased with time, instead of being virtually constant as was the case at or below maximally stimulating oligomycin concentrations. This is consistent with the assumption

that, with increasing concentrations of oligomycin, the rate of NAD^+ reduction becomes increasingly limited by the generation of I~X from ATP, and consequently, more and more dependent on the equilibrium of the latter reaction. Similar though less striking effects were observed with valinomycin (McMurray & Begg, 1959), whereas octylguanidine (Pressman, 1963) as expected was in-

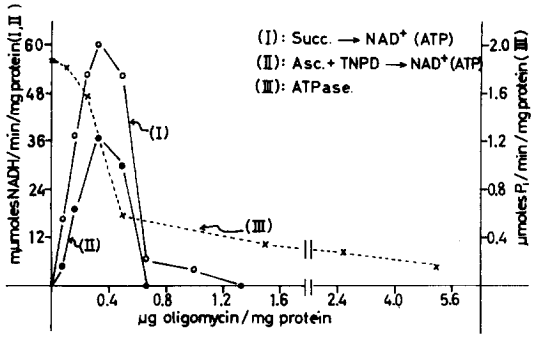

Fig. 4. Effect of varying amounts of oligomycin on ATPase and on the ATP--supported reduction of NAD^+ by succinate and by ascorbate + TMPD. Assay system for ATPase was the same as in Fig. 1. The reaction mixture for the ATP-supported reduction of NAD^+ by succinate and by ascorbate + TMPD consisted of 180 mM sucrose, 1.6 mM KCN, 10 mM $MgSO_4$, 0.2 mM NAD^+, particles (prepared in the presence of 2 mM EDTA, pH 8.9) containing 0.6 mg protein and oligomycin as indicated. In the case of succinate-linked NAD^+ reduction, 5 mM succinate was present; and in the case of ascorbate + TMPD--linked NAD^+ reduction, 2.5 mM ascorbate and 0.2 mM TMPD were present. 3 mM ATP was added to start the reaction. Final volume, 3 ml, temp., 30°C.

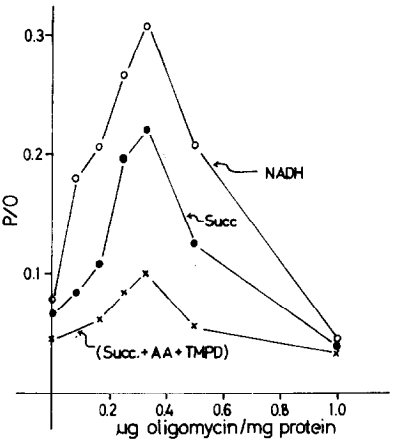

Fig. 5. Effect of varying amounts of oligomycin on oxidative phosphorylation with different substrates. The reaction mixture consisted of 180 mM sucrose, 50 mM Tris-acetate buffer, pH 7.5, 15 mM $MgSO_4$, 3 mM ATP, 15 mM glucose, 75 units hexokinase, 2 mM P_i^{32} (1.2×10^6 cpm/μmole), particles (prepared in the presence of 2 mM EDTA, pH 8.6) containing 0.6 mg protein and oligomycin as indicated. The substrate was either 0.2 mM NAD^+, 160 mM ethanol and 16 μg alcohol dehydrogenase; or 5 mM succinate; or 5 mM succinate, 3 μg antimycin A and 0.2 mM TMPD. Final volume, 3.1 ml, temp., 30°C. O_2 consumption was measured with a Clark electrode and the esterification of P_i was determined by the isotope distribution method (Lindberg & Ernster, 1955).

effective in replacing oligomycin, and even abolished the oligomycin-induced succinate-linked NAD^+ reduction.

In Fig. 5, data are summarized concerning the effect of oligomycin on the phosphorylating capacity of the particles prepared in the presence of

EDTA. Low concentrations of oligomycin enhanced the phosphate uptake with either NADH or succinate as substrate. The enhancement was about 4-fold in the case of NADH, and about 3-fold in the case of succinate. A significant, though less marked, increase in phosphate uptake was also obtained in the system where succinate oxidation was first blocked by antimycin A and then restored by TMPD, thus establishing a by-pass of electron transfer over the second energy-coupling site of the respiratory chain (Lee et al., 1964b). With ascorbate + TMPD as substrate, in the presence of antimycin A, oligomycin also gave rise to an increase in phosphate uptake, but this was less pronounced. In all cases, the amount of oligomycin causing an enhancement of phosphorylation was within the same range as in the case of the succinate- or ascorbate + TMPD-linked NAD^+ reduction (cf. Figs. 4 and 5).

Under the conditions employed above, oligomycin did not affect respiration when added in concentrations that stimulated phosphorylation maximally. In the absence of Mg^{++}, P_i and ADP, however, phosphorylation-stimulating concentrations of oligomycin greatly inhibited the respiration, which was restored partially by Mg^{++}, and completely either by Mg^{++} + P_i + ADP, or by DNP. These preliminary findings suggest that, when the hydrolysis of I~X is blocked by oligomycin, the particles exhibit a good extent of respiratory control, or, in other words, that there is only a slow hydrolysis taking place at the level of C~I (cf. Fig. 2). DNP most probably acts by promoting this hydrolysis (or by preventing C~I formation; cf. Ernster & Lee, 1964), whereas the effect of Mg^{++} may be due to an enhancement of I~X utilization for ion translocation, analogous to that found with intact mitochondria (Klingenberg, 1963).

For obtaining the effects of oligomycin as described above, it was found essential to maintain the pH of the EDTA-containing sonicating medium within the range of 8.5 to 9.0. At pH values below 8.5, the particles may retain a substantial capacity for both oxidative phosphorylation and ATP-supported succinate-linked NAD^+ reduction even when assayed in the absence of oligo-

mycin; at pH values above 9.0, the stimulating effect of oligomycin becomes less pronounced, especially as the stimulation of oxidative phosphorylation is concerned. Preliminary experiments with particles prepared in the presence of ammonia (Racker & Conover, 1963) have resulted in principally similar but much weaker effects, whereas those with Keilin-Hartree heart-muscle preparation (which likewise possesses the capacity for respiratory energy-coupling; Haas, 1964) have so far revealed no oligomycin-stimulated oxidative phosphorylation or ATP-supported succinate-linked NAD^+ reduction.

In conclusion, it is evident from the present results that so-called 'non-phosphorylating' submitochondrial particles prepared in the presence of EDTA possess the capacity, not only for respiratory energy-coupling, as concluded earlier (Lee et al., 1964a), but for the complete process of oxidative phosphorylation involving the three coupling sites of the respiratory chain. Of great importance may be the implications of the present results for the interpretation of the mode of action of soluble coupling factors' recently widely studied in different laboratories, primarily the cold-labile ATPase, called F_1, of Racker and associates (cf. Racker & Conover, 1963), and the three 'site specific' coupling factors of Green et al. (1963). The fact that oligomycin can restore phosphorylation in the same type of particles as do these factors, implies that the latter most probably do not act by restituting a missing enzyme species, but rather by facilitating the operation of one already present in the particles. Attempts to clarify these relationships are in progress.

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