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# RESPIRATORY CONTROL IN SUBMITOCHONDRIAL PARTICLES OBTAINED BY SONICATION

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SUMMARY

1. Uncoupling agents markedly stimulate oxidation of NADH and succinate by particles obtained from sonication of heavy beef-heart mitochondria. Such respiratory stimulation is demonstrable in the complete absence of factors or agents affecting the phosphorylation sequence itself.

2. The respiratory control thus revealed is most prominent at the NADH–flavin coupling site but is also present at the cytochrome *b* region coupling site.

3. Uncoupler concentrations inducing maximal respiratory rates exceed those abolishing the phosphorylative capacity by one order of magnitude or more.

4. The addition of glucose, hexokinase and ADP prior to that of uncoupler reduces the uncoupler-induced respiratory stimulation.

5. A respiratory stimulation initiated by $Ca^{2+}$ is additive to the uncoupler-nduced effect both in the NADH and succinate oxidase systems.

## INTRODUCTION

Submitochondrial particles obtained by sonic irradiation exhibit a certain degree of respiratory control if precautions are taken to decrease high-energy intermediate splitting reactions. A respiratory stimulation caused by uncouplers has been demonstrated after addition of oligomycin[1,2] or dicyclohexylcarbodiimide[3], in the presence of specific factors[4] or after a pretreatment with *o*-phenanthroline[5,6]. An influence on the respiratory rate by phosphate acceptor was shown in the *o*-phenanthroline system.

The investigation reported here has been performed with particles prepared by sonic irradiation of heavy beef-heart mitochondria in the presence of $MgCl_2$ and ATP (ref. 7). P/O ratios of 2.5 with NADH, 1.8 with succinate and 0.6 with ascorbate and tetramethyl-*p*-phenylenediamine as the substrate, respectively, can generally be demonstrated with these particles. However, respiratory control revealed either by a respiratory stimulation induced by uncouplers, by the presence of phosphate ac-

Abbreviation: CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

ceptor or by a respiratory inhibition by oligomycin in higher concentrations has usually been negligible.

A gradual improvement of the quality of mitochondria constituting the material for particle preparation and a refinement of methods for respiratory measurements[8] have now made it possible to demonstrate this control mechanism without the need of extra additions.

## MATERIALS AND METHODS

### *Materials*

Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was a gift from Dr. P. G. HEYTLER, E.I. Du Pont de Nemours and Co., Wilmington, Del., U.S.A. Hexokinase was bought from Boehringer and Soehne GmbH, Mannheim, Germany and other enzymes and fine chemicals from Sigma Chemical Co., St. Louis, Mo., U.S.A.

### *Methods*

Preparation of beef-heart mitochondria and the sonic irradiation of the heavy fraction followed by differential centrifugation giving rise to the submitochondrial particulate fraction was performed essentially as described earlier[7]. Minor modifications of the original preparation procedure and a few changes in the external conditions have affected the quality of mitochondria in a favourable way. The biological material is now obtained from a supplier who avoids the radial cuts for veterinary examination which formerly opened the hearts before transportation to the laboratory. The homogenizing procedure with the Ultra-Turrax used at 20000 rev./min has been slightly extended to 1.15 min. The centrifugation data are unchanged except that sedimentation of mitochondria in the GSA rotor is now done in the Servall RC-2B centrifuge, which by its faster acceleration considerably decreases the total preparation time. Homogenization of mitochondria in the Potter–Elvehjem homogenizer at the different steps of the procedure is now done at a slower speed, about 400 rev./min. The mitochondrial preparation is finally stored in 10-ml aliquots with 40 mg mitochondrial protein per ml at —25°. The particles prepared were kept at 4° and used no later than 24 h after preparation. Protein content was determined according to GORNALL, BARDAWILL AND DAVID[9]. Rates of respiration were measured polarographically and recorded both as the integrated and differentiated function, essentially according to CHAPPELL AND CROFTS[8]. The thermostable electrode chamber used at 30° had a final volume of 3.0 ml. The basic medium contained 50 mM glycylglycine buffer (pH 7.5), 3 mM $MgCl_2$, 0.167 M sucrose, 3.33 mM phosphate buffer (pH 7.5), 2 mM ATP, 20 mM glucose, dialyzed hexokinase in excess and 0.33 mg of submitochondrial particle protein per ml.

For measuring NADH oxidase, the following substrates were used: 0.66 mM $NAD^+$, 0.07 mg of crystalline alcohol dehydrogenase powder (EC 1.1.1.1) per ml, 38 mM ethanol and 2 mM semicarbazide; for measuring succinate dehydrogenase 3.3 mM sodium succinate was used. Phosphate esterification was determined by the $^{32}P$ distribution method as described by LINDBERG AND ERNSTER[10]. A decrease in a previously employed magnesium concentration[11] has favourably affected the P/O ratios, especially when NADH is used as substrate. The influence of magnesium has been elaborated by LEE AND ERNSTER[1,2].

## RESULTS

The stimulation of respiration caused by various concentrations of 2,4-dinitrophenol in the NADH and succinate oxidase systems, respectively, is demonstrated in Fig. 1. The maximal respiratory stimulations are obtained by concentrations of the uncoupler far exceeding those required to obtain uncoupling in intact rat-liver mitochondria[12]. The NADH oxidase stimulation has a maximum at a final concentration of $10^{-3}$ M dinitrophenol. The respiratory increase of the succinate oxidase systems has more of a plateau-like pattern; concentrations above $10^{-4}$ M dinitrophenol do not give a substantial increase in respiratory activity. In both systems a decline in stimulation is obtained by concentrations higher than $10^{-3}$ M dinitrophenol. Fig. 2 demonstrates the influence of substrate concentration, in this case of succinate, on the stimu-

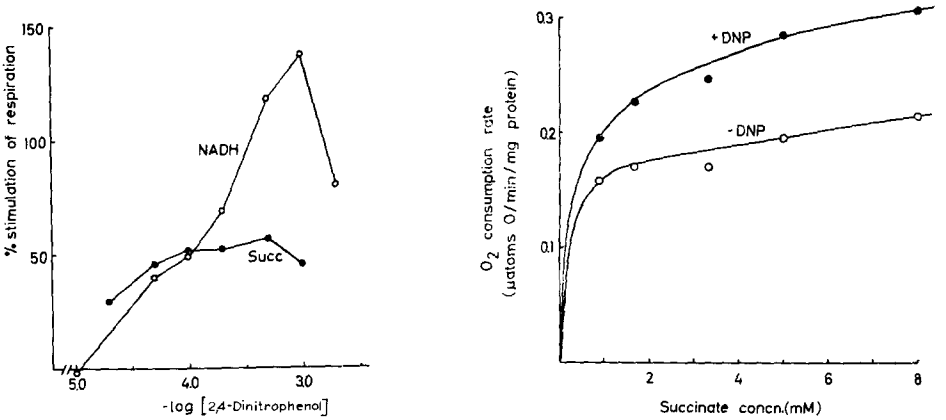


Fig. 1. Stimulation of NADH (○—○) and succinate oxidase systems (●—●) by 2,4-dinitrophenol. Standard incubation medium containing 0.33 mg particle protein per ml at 30°. 0.66 mM $NADH^+$ (kept reduced with alcohol dehydrogenase and ethanol) or 3.3 mM succinate used as substrate. Control rates: with NADH as the substrate, 0.234 μatom O/min per mg protein; with succinate 0.146 μatom O/min per mg protein.

Fig. 2. Dinitrophenol-stimulated succinate oxidase systems. Standard incubation medium at 30°. Succinate concentration varied as indicated in the figure. ●—●, $5 \cdot 10^{-4}$ M 2,4-dinitrophenol (DNP) added; ○—○, no 2,4-dinitrophenol.

TABLE I

THE EFFECT OF 2,4-DINITROPHENOL ON NADH OXIDASE

Standard incubation medium at 30°. NADH (added as $NAD^+$ at the concentrations indicated) was kept reduced by alcohol dehydrogenase and ethanol.

| *NADH concn.* (*M*) | *Rate of respiration* (*μatom O/min per mg protein*) | | *Dinitrophenol concn.* (*M*) |
|---|---|---|---|
| | *− DNP* | *+ DNP* | |
| $1.67 \cdot 10^{-3}$–$3 \cdot 10^{-5}$ | 0.193 | 0.343 | $10^{-3}$ |
| $3.3 \cdot 10^{-6}$ | 0.139 | 0.132 | $10^{-4}$ |
| | 0.139 | 0.095 | $3.3 \cdot 10^{-4}$ |
| | 0.139 | 0.088 | $6.7 \cdot 10^{-4}$ |
| | 0.139 | 0.076 | $10^{-3}$ |

lation of respiration caused by a constant amount of uncoupler. Oxygen consumption is stimulated by the presence of the uncoupler at all concentrations of substrate.

The stimulatory pattern obtained when dinitrophenol is added to the NADH oxidase is quite different (Table I). A variation of the NADH concentration between $3 \cdot 10^{-5}$ M and $1.67 \cdot 10^{-3}$ M does not affect the respiratory rate and the uncoupler-induced respiratory stimulation also remains constant. This picture is changed, however, when the amount of substrate becomes limiting for the respiratory rate. Dinitrophenol then starts to inhibit the respiration and the inhibition is proportional to the concentration of uncoupler added. A decrease in dinitrophenol concentration below what is optimal when high NADH concentrations are used, diminishes the inhibition.

During a decrease in respiratory rate brought about by a titration of the rotenone-sensitive site, the NADH oxidase stimulation caused by dinitrophenol gradually declined (Fig. 3). It was not possible, however, to obtain the inhibitory pattern caused by the uncoupler at low NADH concentrations as shown above.

Since the experiments described so far were performed without $Mg^{2+}$ added, it was of interest to investigate whether the $Mg^{2+}$ concentration affected the degree of uncoupler stimulation, as was reported in the oligomycin-induced system[1]. The submitochondrial particles used are routinely prepared in the presence of $Mg^{2+}$ and suspended in a sucrose medium containing this cation. Hence the influence of EDTA was checked simultaneously. Fig. 4 shows that the NADH oxidase activity is enhanced by an increase in $Mg^{2+}$ concentration. The additive stimulation by a constant concentration of uncoupler was enhanced in a similar way up to a level where the access of substrate probably became rate limiting. Less expected was the effect of EDTA.

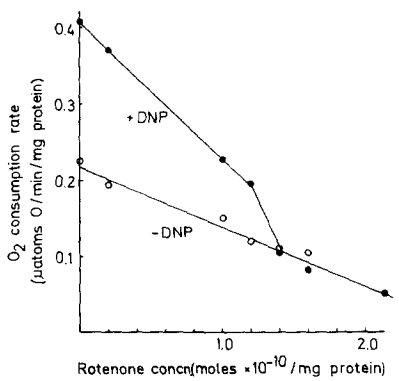


Fig. 3. Dinitrophenol-stimulated NADH oxidase system in the presence of rotenone. Standard incubation medium at 30°. NADH (0.66 mM), kept reduced by alcohol dehydrogenase and ethanol, used as substrate. Rotenone concentration varied as indicated in the figure. ●—●, 2,4-dinitrophenol (DNP) added at a final concentration of $5 \cdot 10^{-4}$ M; ○—○, no 2,4-dinitrophenol.

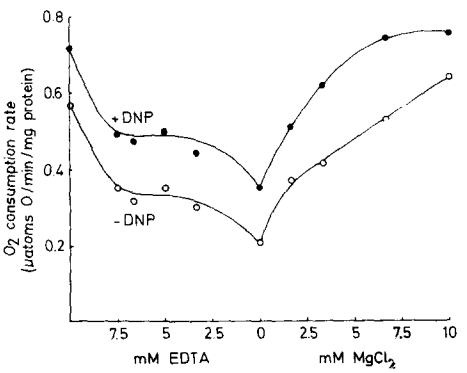


Fig. 4. Effect of $Mg^{2+}$ or EDTA on dinitrophenol-stimulated NADH oxidase system. Standard incubation medium at 30°. $MgCl_2$ or EDTA added as indicated in the figure. ●—●, 2,4-dinitrophenol (DNP) added at a final concentration of $5 \cdot 10^{-4}$ M; ○—○, no 2,4-dinitrophenol.

The stimulatory pattern suggests that an inhibitory component is titrated by EDTA and that it is possible to overcome this inhibition also by $Mg^{2+}$.

In Fig. 5 is demonstrated how the succinate oxidase system is affected by

various concentrations of $Mg^{2+}$ or EDTA and the extent to which the oxidase is stimulated by a constant amount of CCCP under these conditions. In contrast to the dramatic stimulation of the NADH oxidase system obtained in the presence of high EDTA concentrations, there is only a little stimulation when succinate is used as substrate.

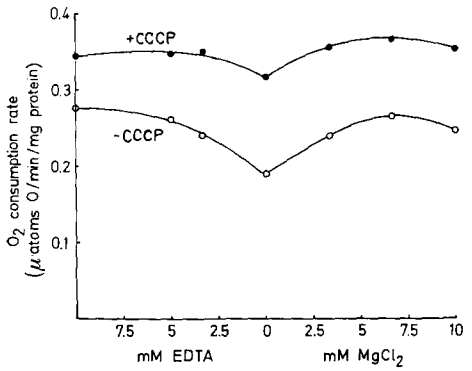


Fig. 5. Effect of $Mg^{2+}$ or EDTA on CCCP-stimulated succinate oxidase system. Standard incubation medium at 30°. $MgCl_2$ or EDTA added as indicated in the figure. ●—●, CCCP added at a final concentration of $5 \cdot 10^{-6}$ M; ○—○, no CCCP.

The augmentation of NADH oxidase respiration caused by ADP, hexokinase and glucose above the level reached by the $Mg^{2+}$ addition is minor as shown in Table II. The acceptor system contributes to a further stimulation of about 10%. With these constituents added the dinitrophenol stimulation is markedly decreased. It was not possible to demonstrate any influence of the phosphate acceptor on the rate of succinate oxidase system.

TABLE II

THE EFFECT OF $MgCl_2$, PHOSPHATE ACCEPTOR OR DINITROPHENOL ON NADH OXIDASE

Conditions as in Table I, with 0.33 mM NADH and, as phosphate acceptor, 20 mM glucose and hexokinase. Additions as indicated in the table.

| *Additions* | *Respiratory rate* (*$\mu$atom O/min per mg protein*) |
|---|---|
| None | 0.214 |
| $MgCl_2$ (3.3 mM) | 0.362 |
| $MgCl_2$ + phosphate acceptor | 0.400 |
| $MgCl_2$ + $5 \cdot 10^{-4}$ M dinitrophenol | 0.504 |
| $MgCl_2$ + phosphate acceptor + $5 \cdot 10^{-4}$ M dinitrophenol | 0.428 |

The respiratory rates are only slightly suppressed by oligomycin (Table III). The optimal effect is obtained at a $Mg^{2+}$ concentration of 3.3 mM, where oligomycin decreased both the NADH and the succinate dehydrogenase activity by about 20%. The increase in respiratory rate initiated by dinitrophenol, when added to the oligomycin-inhibited system, was more prominent when $Mg^{2+}$ was omitted from the medium. This is especially the case in the NADH oxidase system.

TABLE III

THE EFFECT OF OLIGOMYCIN ON RESPIRATION IN SONICATED PARTICLES

Standard incubation medium at 30°. $MgCl_2$ added in concentrations indicated. Where present, the concentration of oligomycin was 3 μg per mg protein and that of dinitrophenol $5 \cdot 10^{-4}$ M.

| *Substrate* | *$MgCl_2$ concn. (mM)* | *Respiratory rate (μatom O/min per mg protein)* | | |
|---|---|---|---|---|
| | | *— Oligomycin* | *+ Oligomycin* | *Oligomycin + DNP* |
| Succinate | — | 0.170 | 0.161 | 0.265 |
| | 3.3 | 0.227 | 0.189 | 0.302 |
| | 6.6 | 0.233 | 0.218 | 0.340 |
| NADH | — | 0.145 | 0.137 | 0.305 |
| | 3.3 | 0.281 | 0.133 | 0.315 |
| | 6.6 | 0.428 | 0.363 | 0.441 |

A marked stimulation of NADH oxidase by $CaCl_2$ in submitochondrial particles has recently been demonstrated by LOYTER, CHRISTIANSEN AND RACKER[13]. The particles used in their investigation seem essentially to lack the capacity of uncoupler-induced respiratory stimulation in the presence of Factor $F_1$.

The stimulatory effect of $CaCl_2$ and CCCP on the NADH and succinate oxidase systems of our preparation of submitochondrial particles is demonstrated in Fig. 6. The oxygen consumption is shown here as the differentiated function. The NADH oxidase rate was enhanced by 200% by the addition of 5 mM $CaCl_2$, the optimal

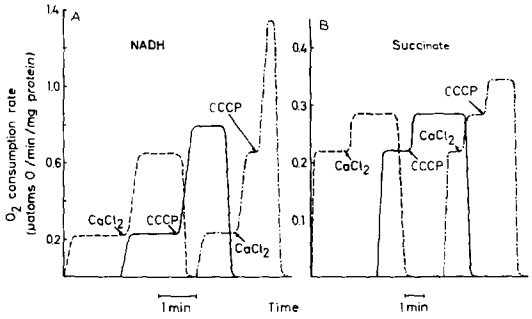


Fig. 6. Effect of $CaCl_2$ and CCCP on NADH (A) and succinate oxidase systems (B). Conditions as in Fig. 1 except that the final succinate concentration was increased to 5 mM; $CaCl_2$ and CCCP added to final concentrations of 5 mM and $10^{-5}$ M, respectively, when indicated. The figure shows the differentiated functions of the oxygen consumption measured essentially according to CHAPPELL AND CROFTS[8]. Reactions started by the addition of $NAD^+$ or succinate. Each half of the figure represents three separate experiments where the effect of $CaCl_2$, of CCCP and of $CaCl_2$ and then CCCP were tested.

concentration for obtaining this effect. The $Ca^{2+}$-initiated respiratory stimulation of the succinate oxidase system was definitely less prominent, but was consistent over a number of experiments. The $Ca^{2+}$ effect and the uncoupler-induced increase in respiration were additive. Dinitrophenol gives qualitatively the same results as CCCP.

Fig. 7 shows the effect of various concentrations of CCCP added to the NADH oxidase system and acted upon by a constant amount of $CaCl_2$. The respiratory

stimulation caused by carbonyl phenylhydrazone was more pronounced than the dinitrophenol stimulation (Fig. 1). The optimal concentration of CCCP enhanced the respiratory rate by 275 %, whereas dinitrophenol stimulated 135 %. There was no significant difference in the extent of succinate oxidase stimulation exerted by the two uncouplers, although there was a definite optimal value in the case of CCCP.

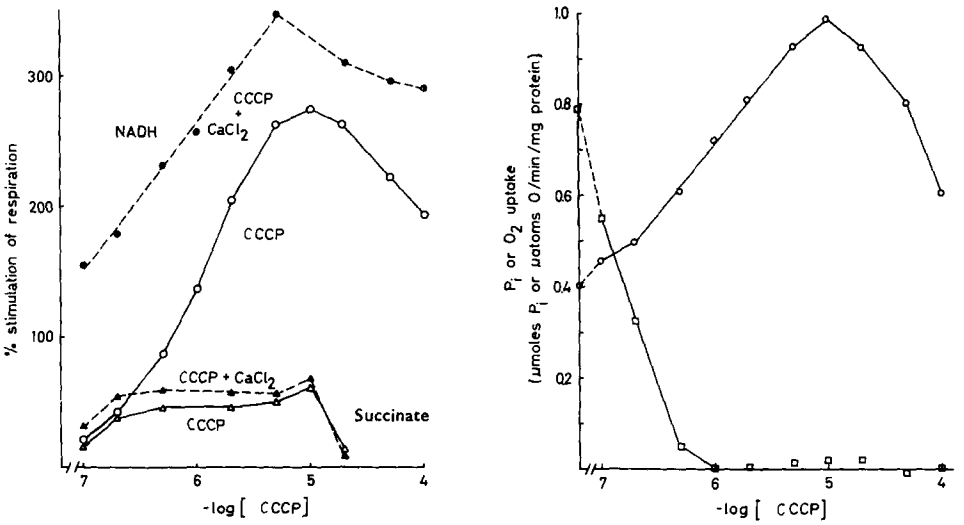


Fig. 7. NADH oxidase system stimulated by CCCP and $CaCl_2$. Standard incubation medium at 30°. NADH (final concentration, 0.66 mM) kept reduced by alcohol dehydrogenase and ethanol. CCCP was added at final concentrations indicated. After the stimulation was obtained, $CaCl_2$ was added at a final concentration of 5 mM and further stimulation recorded. Control rates: 0.262 μatom O/min per mg protein with NADH and 0.189 μatom O/min per mg protein with succinate as the substrate, respectively.

Fig. 8. The effect of CCCP on oxidative phosphorylation with NADH as the substrate. Standard incubation mixture containing in addition dialyzed hexokinase (EC 2.7.1.1), and 3 mM $MgCl_2$. $^{32}P$ was added to the phosphate buffer. The substrate was 0.66 mM NADH, kept reduced with ethanol and alcohol dehydrogenase. □, μmoles $P_i$ esterified; ○, μatoms O consumed.

Fig. 7 further demonstrates that with NADH as substrate, $Ca^{2+}$-stimulated respiration markedly at all concentrations of the uncoupler. An increase in uncoupler-induced respiration was followed by a decreased $Ca^{2+}$ stimulation. The NADH and succinate systems differ in that in the NADH oxidase, uncoupler concentrations higher than optimal for respiratory stimulation are accompanied by an increased $CaCl_2$ stimulation, whereas $Ca^{2+}$ has no effect in the corresponding situation in the succinate oxidase system.

The effect of CCCP under complete conditions for oxidative phosphorylation with NADH as the substrate is demonstrated in Fig. 8. As can be seen from the figure, 50 % of the phosphorylative capacity is eliminated at less than $2 \cdot 10^{-7}$ M CCCP, while the respiration is stimulated to 14 %. Phosphorylation is completely abolished at $10^{-6}$ M of the uncoupler, while 50 % of the maximal respiratory stimulation is obtained. The difference between the concentration eliminating the phosphorylation and the concentration for optimal respiratory stimulation is sometimes even larger than in this particular experiment. The same tendency can be demonstrated with

2,4-dinitrophenol where 70% of the phosphorylation is extinguished at $2 \cdot 10^{-4}$ M, while the respiratory stimulation is only about 5% of the maximal.

## DISCUSSION

The degree of respiratory stimulation demonstrated in our preparations of submitochondrial particles far exceeds those reported previously in systems where additions of phosphorylation inhibitors[1-3], factors[4,13] or *o*-phenanthroline[5,6] were a prerequisite for such a stimulation. The exception is the dicyclohexylcarbodiimide-induced respiratory control recently demonstrated by BEYER, CRANKSHAW AND KUNER[3], where the NADH oxidase stimulation induced by carbonylcyanide *p*-trifluoromethoxylhydrazone is of the same order of magnitude as the one found with CCCP in our system. The succinate oxidase stimulation after dicyclohexylcarbodiimide addition is more prominent than the one we could demonstrate. The fact that phosphorylation inhibitors or factors are not needed for a demonstration of a respiratory stimulation in our submitochondrial preparation might indicate the presence of an endogenous respiratory control which is not generally found in other preparations.

From the data given it is evident that the highest degree of uncoupler-stimulated respiration is obtained when NADH is used as the substrate. This indicates a more prominent respiratory control in the phosphorylation site of the NADH–flavin region than elsewhere. The fact that the degree of respiratory stimulation in the succinate dehydrogenase is about the same with CCCP or dinitrophenol as the uncoupler may indicate that the lipophilic character of the uncoupler is of less importance in this site. A demonstration of a decreased phosphorylative capacity followed by a respiratory stimulation in the cytochrome oxidase region was not possible with either CCCP or dinitrophenol. These criteria were, on the other hand, fulfilled by relatively high concentrations of piericidin A in a system where electrons are mediated from ascorbate *via* tetramethyl-*p*-phenylenediamine to the cytochrome oxidase region as reported elsewhere[14]. Thus it must be deduced that in these sonicated particles a certain degree of respiratory control is present at all three coupling sites along the respiratory chain. Three different modes of uncoupler action on submitochondrial systems may be discussed according to effective concentrations.

Uncoupler concentrations which abolish the phosphorylative capacity in these particles affect the respiratory rate to a minor degree. This situation is evident when the action of CCCP is tested on oxidative phosphorylation with NADH as the substrate (Fig. 8). The same tendency can be demonstrated with dinitrophenol and also when succinate is used as the substrate. There may be a slight difference between dinitrophenol concentrations abolishing the phosphorylation in intact mitochondrial systems and when submitochondrial particles are used. BRIERLEY, MURER AND BACHMANN[15] have reported a 50% decrease in phosphorylative capacity of intact mitochondria at $2 \cdot 10^{-5}$ M dinitrophenol, a concentration at which 80% of the capacity is still intact in the present submitochondrial particles from the same kind of material.

Concentrations of uncouplers which cause a maximally stimulated respiration exceed those abolishing the phosphorylative capacity as seen from different figures and tables above. This fact, to some extent, may explain why the degree of stimulation demonstrated here is much more prominent than that generally observed in the

presence of factors or phosphorylation inhibitors. VAN DAM[16] has briefly reported uncoupler concentrations inducing maximal respiratory rates in rat-liver mitochondria with succinate as the substrate. The dinitrophenol concentration he reports coincides approximately with those found in our investigation. It should be noted that these uncoupler concentrations also coincide with the concentrations causing an increased hydrolysis of ATP in submitochondrial systems preincubated for the reversed electron transport, as reported elsewhere[14,17]. The observation that the extent of uncoupler-induced respiratory stimulation decreased in the presence of phosphate acceptor system (Table II) seems to indicate that normally there is a change of equilibrium in the phosphorylation sequence during the enhanced respiration. This change is counteracted when glucose, hexokinase and ADP are present.

Concentrations of the uncouplers higher than the optimal for respiratory stimulation are of the same order of magnitude as those that initiate respiratory inhibition in intact mitochondrial systems[16]. A similar effect can be obtained with the present material when the initial respiratory rate is limited by the amount of NADH added (Table I). Obviously the effect is not primarily related to a decrease in respiratory rate, since a partial rotenone inhibition could not substitute for the substrate limitation. It has been argued that the respiratory inhibition of uncouplers in intact mitochondrial systems is mainly due to an inhibition of the energy-linked substrate transport (*cf.* ref. 18). Although the level of substrate has a relevance in the present particulate systems, the view of inhibited substrate transport is more difficult to apply here. It can be assumed that in these particles such a transport is of minor importance since the inner mitochondrial membrane with its enzymic sites is believed to be turned inside out during preparation and hence faces added substrate in the surrounding medium[1,19].

As demonstrated in Figs. 6 and 7 the effect on respiration of $Ca^{2+}$ is additive to that of uncouplers. The enhancement of respiration caused by $Ca^{2+}$ as well as by uncouplers is most prominent when NADH is the substrate but it also indisputably occurs in the succinate oxidase system. No conclusion can be drawn from these experiments whether the site of action of $Ca^{2+}$ is identical to that of CCCP and dinitrophenol. It has been argued[13] that since the presence of uncouplers does not diminish the extent of $Ca^{2+}$ accumulation in submitochondrial particles, the site of action of $Ca^{2+}$ should be considered more proximal to the respiratory chain than that of uncouplers. The findings, demonstrated in Fig. 6, that the uncoupler-induced respiration is unchanged in extent when the agent is added to the $Ca^{2+}$-stimulated systems, make this conclusion less probable. It seems, however, somewhat questionable to discuss the $Ca^{2+}$ effect as an influence on the respiratory control as classically defined. The enhancement in respiratory activity initiated by $Ca^{2+}$ is indubitable, but this stimulation is not followed by a simultaneous decrease in phosphorylative capacity[13]. We have been unable to demonstrate either a consistent decrease in phosphate esterification with $Ca^{2+}$ present or a $Ca^{2+}$-initiated stimulation of ATPase in these particles.

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REFERENCES


1 C. P. LEE AND L. ERNSTER, in J. M. TAGER, S. PAPA, E. QUAGLIARELLO AND E. C. SLATER, *Regulations of Metabolic Processes in Mitochondria*, Elsevier, Amsterdam, 1966, p. 218.
2 C. P. LEE AND L. ERNSTER, *European J. Biochem.*, 3 (1968) 391.
3 R. E. BEYER, D. L. CRANKSHAW AND J. M. KUNER, *Biochem. Biophys. Res. Commun.*, 28 (1967) 758.
4 E. RACKER AND T. E. CONOVER, *Federation Proc.*, 22 (1963) 1088.
5 R. A. BUTOW AND E. RACKER, in A. SAN PIETRO, *Non-heme Iron Proteins*, The Antioch Press, Yellow Springs, 1965, p. 383.
6 R. A. BUTOW AND E. RACKER, *J. Gen. Physiol.*, 49 (1965) 149.
7 H. LÖW AND I. VALLIN, *Biochim. Biophys. Acta*, 69 (1963) 361.
8 J. B. CHAPPELL AND A. R. CROFTS, *Biochem. J.*, 95 (1965) 378.
9 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
10 O. LINDBERG AND L. ERNSTER, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 3, Interscience, New York, 1956, p. 1.
11 H. LÖW, I. VALLIN AND B. ALM, in B. CHANCE, *Energy-linked Functions of Mitochondria*, Academic Press, New York, 1963, p. 5.
12 H. C. HEMKER, *Biochim. Biophys. Acta*, 63 (1962) 46.
13 A. LOYTER, R. O. CHRISTIANSEN AND E. RACKER, *Biochem. Biophys. Res. Commun.*, 29 (1967) 450.
14 I. VALLIN AND H. LÖW, *European J. Biochem.*, 5 (1968) 402.
15 G. P. BRIERLEY, E. MURER AND E. BACHMANN, *Arch. Biochem. Biophys.*, 105 (1964) 89.
16 K. VAN DAM, *Biochim. Biophys. Acta*, 131 (1967) 407.
17 I. VALLIN, in preparation.
18 K. VAN DAM AND E. C. SLATER, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 2015.
19 B. CHANCE AND L. MELA, *J. Biol. Chem.*, 242 (1967) 830.