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INTERACTION BETWEEN UNCOUPLERS AND SUBSTRATES IN RAT-LIVER MITOCHONDRIA

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SUMMARY

1. The inhibition of the dinitrophenol-stimulated ATPase in rat-liver mitochondria by excess uncoupler is kinetically competitive with respect to ATP.

2. Competitive inhibition of substrate oxidation by excess uncoupler is most marked when substrate and uncoupler have a net charge of the same sign.

3. In the absence of an energy-generating system dinitrophenol is accumulated by mitochondria like other acidic compounds.

4. Dinitrophenol accumulation is competitively inhibited by anionic compounds and not affected by cationic compounds.

5. It is proposed that an acidic uncoupler enters the mitochondrion in exchange for endogenous anions and that the uncoupler can leave the mitochondrion as the uncharged acid. In the presence of an energy-generating system, the endogenous anions are possibly hydroxyl ions. These movements, together with an intramitochondrial energy-dependent splitting of water, can explain both the uncoupling and inhibitory action of uncouplers as well as the importance of lipid solubility of anionic uncouplers.

INTRODUCTION

Excessive concentrations of uncouplers of oxidative phosphorylation inhibit both mitochondrial respiration and the uncoupler-induced ATPase. This inhibitory effect was explained by HÜLSMANN[1] and by HEMKER[2] by assuming that the uncoupler forms a complex with one of the factors involved in oxidative phosphorylation, thereby preventing optimal operation of the phosphorylating pathway.

Later experiments by WENNER[3] showed that, at least for the case of succinate oxidation, the inhibition can be overcome by addition of more substrate. This observation was confirmed and extended by WILSON AND MERZ[4] who demonstrated that the inhibition of succinate oxidation by several uncouplers is kinetically of the competitive type. At the same time, VAN DAM[5] observed that in the inhibited state the respiratory carriers are highly oxidized, indicating that the inhibition occurs at a

Abbreviation: TMPD, tetramethyl-*p*-phenylenediamine.

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very early stage of the interaction between the substrate and the mitochondrion. He proposed that the inhibition is due to an inhibition of the penetration of the substrate into the mitochondria.

This proposition was supported by the observation that the accumulation of substrates by mitochondria is inhibited by high concentrations of uncouplers[6–9]. Based on these and other observations, a mechanism of uncoupling was advanced by VAN DAM AND SLATER[10]. According to this mechanism, uncouplers are transported actively into the mitochondrion, but differ from other anionic compounds in that they can diffuse out passively in the undissociated form.

One of the main predictions of this mechanism is that uncoupler anions taken up *via* an exchange–diffusion carrier should be kept out of the mitochondria competitively by other anions. Therefore, uncouplers should become less effective in the presence of other penetrating anions, including substrates[11–13]. Also, it would be expected that the competition occurs only with uncouplers and substrates with a net charge of the same sign.

The validity of these predictions was tested by measuring the kinetic behaviour of the dinitrophenol-induced ATPase and the charge specificity of the inhibition of substrate oxidation by uncoupler.

More direct proof for the interaction between uncoupler and substrate at the level of the mitochondrial membrane was sought by studying the accumulation of ^{14}C-labelled dinitrophenol by mitochondria and the influence of various compounds on this process (*cf.* VAN DAM AND TSOU[9]). The binding of other uncouplers to mitochondria has been described by others[14–16].

METHODS

Rat-liver mitochondria were isolated by the method of HOGEBOOM[17] as described by MYERS AND SLATER[18]. Protein was determined with the biuret method as modified by CLELAND AND SLATER[19].

ATPase experiments were carried out with a sensitive pH recording system, containing an Ingold 203-M5 pH electrode and an Electronic Instruments Ltd. pH meter attached to a Philips PR2100/A21 recorder. The experiments were confirmed using conventional phosphate determinations, but the reproducibility of this method was poorer because of the lower sensitivity.

The oxidation of succinate and of tetramethyl-*p*-phenylenediamine (TMPD) (*plus* ascorbate) was measured with a Clark oxygen electrode (Yellow Springs Instruments).

The accumulation of ^{14}C-labelled 2,4-dinitrophenol (a gift from Dr. J. L. HOWLAND) was measured with the centrifugation–filtration technique of WERKHEISER AND BARTLEY[20], as modified by HARRIS AND VAN DAM[21]. The concentration of dinitrophenol in the matrix space was calculated by correction for the amount expected to be present in the sucrose-permeable space of the mitochondria[9]. Samples taken after 30, 60, 90 or 120 sec incubation gave essentially the same values for intramitochondrial dinitrophenol concentration, and usually the average of the four samples was used to calculate the accumulation.

The uncouplers lauric acid and laurylamine (both obtained from British Drug Houses) were used in ethanolic solutions.

RESULTS

ATPase experiments

The 2,4-dinitrophenol-induced ATPase of rat-liver mitochondria was measured at different ATP and uncoupler concentrations (Fig. 1). In agreement with the results of HEMKER[2] higher concentrations of dinitrophenol have an inhibitory effect. The experiment in Fig. 1 demonstrates that this inhibition is stronger at low ATP concentration. A Lineweaver–Burk plot of the values obtained at inhibitory uncoupler concentrations shows that the inhibition by dinitrophenol is competitive with respect to ATP (Fig. 2). The K_i for dinitrophenol is 0.11 mM.

Oxidation experiments

Both the oxidation of succinate and of TMPD (*plus* ascorbate), measured in the absence of phosphate acceptor, are stimulated by low concentrations of lauric acid or laurylamine. The inhibition by higher uncoupler concentrations, however, becomes

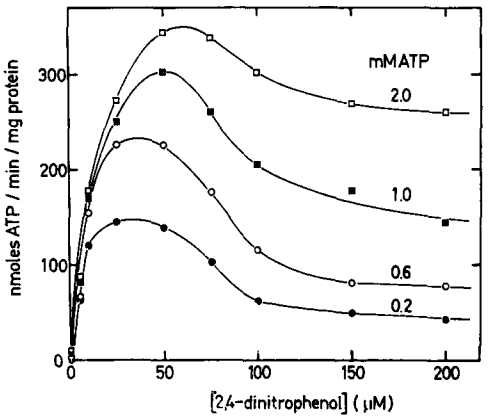

Fig. 1. Effect of varying ATP and dinitrophenol concentrations on the dinitrophenol-induced ATPase of rat-liver mitochondria. Rat-liver mitochondria were incubated at 25° at a concentration of 0.65 mg protein/ml in a medium containing 50 mM sucrose, 50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA and varying amounts of ATP and 2,4-dinitrophenol. The final pH was approx. 7.0. The ATPase reaction was followed by measuring H^+ production with a pH meter.

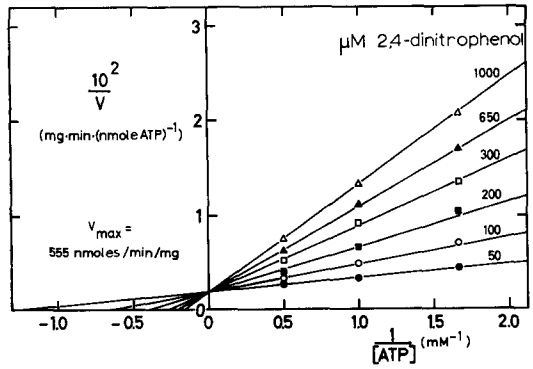

Fig. 2. Competitive inhibition by dinitrophenol of dinitrophenol-induced ATPase. Reaction conditions as described in Fig. 1.

more prominent and of the competitive type with the pairs succinate and lauric acid *or* TMPD and laurylamine (Fig. 3). In the other combinations, there was relatively little effect of uncoupler. Thus, substrate utilization is affected most when substrate and uncoupler have a net charge of the same sign.

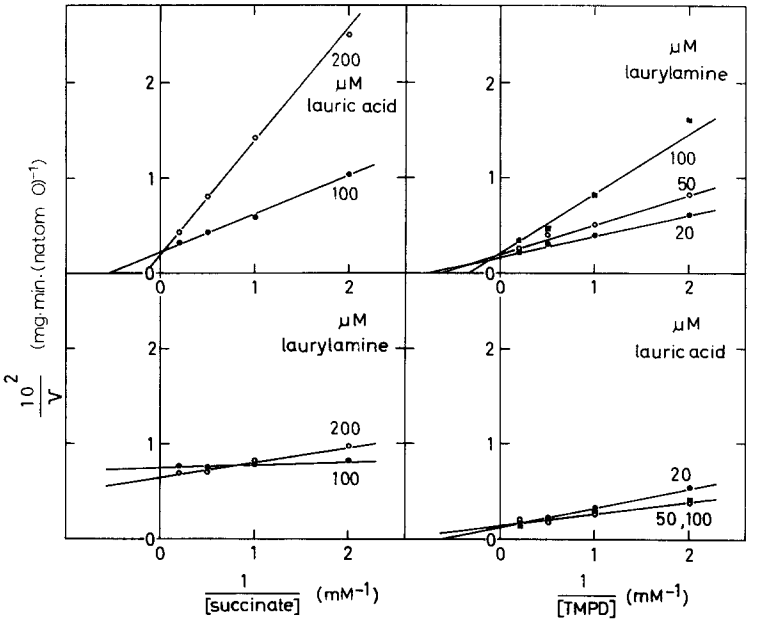

Fig. 3. Effect of lauric acid and laurylamine on the oxidation of succinate and TMPD. O_2 consumption was monitored with a Clark oxygen electrode at room temperature in a medium containing 50 mM sucrose, 50 mM Tris–HCl, 50 mM KCl, 5 mM phosphate buffer, 6 mM $MgCl_2$, 2 mM EDTA, 1 μg/ml rotenone, 3.2 mg (succinate oxidation) or 1.6 mg (TMPD oxidation) mitochondrial protein and, in the case of TMPD oxidation, 6 mM ascorbate. The final pH was 7.4 and the volume 3 ml.

Dinitrophenol-uptake experiments

As is the case with the accumulation of substrates[6,7,9] the intramitochondrial dinitrophenol concentration was much higher than that in the surrounding medium. Samples collected at 30, 60, 90 or 120 sec after starting the reaction with mitochondria always gave the same intramitochondrial concentration, so that the internal steady-state concentration is reached within 30 sec. The mean values at different incubation times were usually used for the calculations.

A typical example of the relationship between intra- and extramitochondrial dinitrophenol concentration is given in Fig. 4. Dinitrophenol uptake shows typical saturation characteristics in that there is a straight-line relationship in a plot of the reciprocal values of intra- and extramitochondrial concentrations, in which respect dinitrophenol behaves as a typical anionic substrate (*cf.* ref. 9). Thus, the mitochondrion has a limited capacity for the uptake of the uncoupler. The affinity constant (K_m for uptake) derived from the Lineweaver–Burk plot is 0.19 mM and the maximal intramitochondrial concentration is 4.0 mM (average of 14 experiments). It may be noted that these values are approximately one order of magnitude smaller than those for the accumulation of the substrates tested[9].

The effect of different compounds on the accumulation of dinitrophenol is listed in Table I. It can be concluded that all anionic compounds, including substrates and uncouplers, are more or less inhibitory. Pyruvate and glutamate are very weak inhibitors. Neutral or positively charged compounds do not affect the accumulation to a significant extent. Increasing the tonicity by 60 mosM does not affect the accumulation.

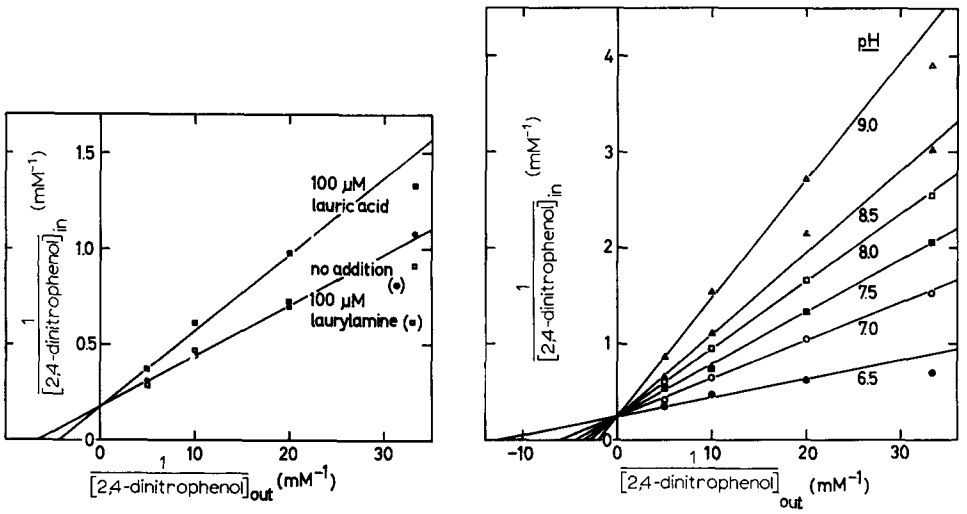

Fig. 4. Accumulation of dinitrophenol by rat-liver mitochondria and the effect of lauric acid and laurylamine on this process. Rat-liver mitochondria were incubated at room temperature at a concentration of 2.32 mg protein/ml in a medium containing 50 mM sucrose, 50 mM Tris–HCl, 50 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 µg/ml rotenone and varying concentrations of dinitrophenol. The final pH was 7.4. The medium contained 0.05 µC per ml 2,4-dinitro[^{14}C]phenol (uniformly labelled) and 0.2 µC per ml ^3H_2O. From the incubation volume of 1 ml, 0.2-ml samples were withdrawn and centrifuged through silicone[21]. ●, no competitor added; ■, 100 µM lauric acid added; □, 100 µM laurylamine added.

Fig. 5. Competitive inhibition of dinitrophenol accumulation by OH^-. Incubation conditions as described in Fig. 4.

Atractyloside and valinomycin both inhibit competitively, whereas the inhibitors antimycin and oligomycin (either in the presence or absence of an energy donor: succinate and ATP, respectively) have little effect. The difference between cationic and anionic uncouplers is further illustrated in Fig. 4. The accumulation of dinitrophenol is inhibited competitively by lauric acid, but not by the structurally related laurylamine. The accumulation is strongly dependent on the pH of the medium (Fig. 5). The Lineweaver–Burk plot shows that the maximal intramitochondrial dinitrophenol concentration is independent of the pH. Therefore, the OH^- behaves as a typical competitive inhibitor with respect to dinitrophenol. However, the K_i for OH^- as calculated from the change in slope of the lines is not constant.

DISCUSSION

The results presented show that dinitrophenol competes with anionic substances in general, a conclusion that can be drawn both from the effects on the ATPase and

TABLE I

EFFECT OF VARIOUS COMPOUNDS ON 2,4-DINITROPHENOL ACCUMULATION IN RAT-LIVER MITOCHONDRIA

Rat-liver mitochondria were incubated as described under Fig. 4. Inhibitors were added to a final concentration as indicated.

Compound	*Inhibitor concentration (mM)*	*Competitive inhibition*	K_i *(mM)*
ATP	1	+	5.6
ADP	1	+	8.3
Phosphate	1	+	12.5
Succinate	5	+	8.8
Pyruvate	10	+	20
Glutamate	10	+	20
Citrate	10	+	10
Glutamine	10	−	
Ornithine	10	−	
4-Octyl-2,6-dinitrophenol	0.1	+	0.13
Lauric acid	0.1	+	0.18
Laurylamine	0.1	−	
KOH	0.01	+	0.002*
KCl	30**	−	
Atractyloside	0.18	+	0.4***
Oligomycin (+ 5 mM ATP)	0.5 μg/mg	−	
Antimycin (+ 5 mM succinate)	0.5 μg/mg	−	
Valinomycin	0.1 μg/mg	+	0.17 μg/mg

* KOH was tested at different concentrations (*cf.* Fig. 5) and the K_i was calculated by comparison of the uptake with that at pH 6.5; the K_i varied from 0.1 to 2.0 μM between pH 7.0 and 9.0.

** KCl concentrations higher than 30 mM were slightly inhibitory.

*** Concentrations of atractyloside higher than 0.18 mM gave no greater inhibition.

the oxidation of substrates, and from the accumulation experiments. Thus, the proposition that dinitrophenol is transported into the mitochondrion in its anionic form[10] is supported.

As was shown by VELDSEMA-CURRIE AND SLATER[12], added anions can diminish the ATPase activity induced by sub-optimal concentrations of dinitrophenol. Since the inhibitory effects of different compounds are additive when more carrier systems are involved, the conclusion was drawn that dinitrophenol acts competitively on each of the different specific carrier systems[12].

In the oxidation as well as the accumulation experiments the competitive interaction between substrate and uncoupler occurs only when both compounds have a net charge of the same sign. The magnitude of the net charge probably need not be the same, although possibly a more highly charged ion may prove a more effective competitor (*cf.* also HARRIS[22]).

The effect of several compounds on dinitrophenol accumulation (Table I) requires some additional comments. If uncouplers can enter the mitochondrion through a number of different carrier systems, the inhibitory effect of single substrates will be relatively small, since they will be able to block only one of the carriers (*cf.* ref. 12). Therefore, both dinitrophenol-induced reactions and accumulation of dinitrophenol will be relatively insensitive to addition of single substrates. The uncouplers octyl-

dinitrophenol and lauric acid, on the other hand, are presumably also able to act on all carrier systems and will thereby inhibit accumulation of dinitrophenol much more strongly.

Since atractyloside is known to inhibit the adenine nucleotide translocation[23,24], we expected that it would have the same effect on dinitrophenol accumulation as ADP or ATP. Indeed, very high concentrations of atractyloside competitively inhibit dinitrophenol accumulation. At these high concentrations atractyloside also inhibits the dinitrophenol-stimulated oxidation of succinate (R. KRAAYENHOF, unpublished experiments).

The fact that the inhibition of dinitrophenol accumulation by valinomycin is also of the competitive type is surprising. During the accumulation the mitochondria are in an energy-poor state (with rotenone present) and one would expect the combination of valinomycin *plus* uncoupler to release the endogenous potassium[25], leading to an expected decrease in accumulation (*cf.* ref. 9). It is not quite clear why at very high dinitrophenol concentrations this process should be prevented.

Generalizing from the example of dinitrophenol we propose that uncoupling lipophilic weak acids follow the general pattern of anion exchange during their accumulation in mitochondria[9]. In the absence of an energy-generating system the ratio $[\text{anion}]_{in}/[\text{anion}]_{out}$ is the same for all permeating acidic substances, including uncouplers. The most notable difference between uncoupler and other acids is the much lower K_m and maximal uptake of the former. It is suggested that, under conditions where energy is generated inside the mitochondrion, uncouplers can cycle across the mitochondrial membrane at the expense of energy. The way in which we visualize this cycling process is depicted in Fig. 6. The first step is an exchange of the phenolate anion for an endogenous OH^- on one of the available carrier systems. This step is not energy-requiring in itself, although the OH^- as depicted is derived from a water molecule that is split at the expense of a high-energy bond. The phenolate ion, once inside, reacts with the proton also derived from the splitting of the water molecule and forms the undissociated phenol, which can diffuse outwards passively. This latter step is only possible if the undissociated phenol has a sufficiently high lipid solubility to cross the phospholipid region of the membrane[2].

The net result of the cycle is an outward transport of a water molecule at the expense of energy, catalysed by the uncoupler. Presumably the water molecule diffuses back again into the mitochondrion to maintain the osmotic balance.

The continuous operation of the cycle is dependent on a continuous splitting of

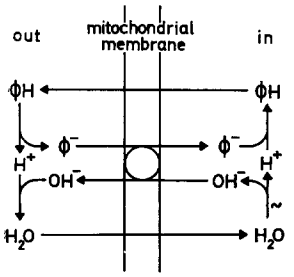

Fig. 6. The proposed mechanism of uncoupling by lipid-soluble weak acids. ΦH represents the uncoupler acid.

water at the cost of energy, generated either from ATP or from respiration. It is important to note that in our mechanism, in contrast to that of MITCHELL[26], the water splitting occurs at one side of the mitochondrial membrane. Of course, we have to assume the presence of a chemical or physical barrier to prevent the recombination of the H^+ and OH^- formed before the operation of the OH^-–anion exchange. However, we believe that the hypothesis that this barrier is the mitochondrial membrane itself is difficult to reconcile with several experimental observations (*cf.* also SLATER[27]).

The present mechanism differs from the one proposed by VAN DAM AND SLATER[10] in the following points. Firstly, we no longer believe that the actual inward movement of the anion requires energy from ATP or oxidizable substrate, but that this movement is always balanced by an outward movement of another anion. Secondly, to provide a continuous source of anions for uncoupler movement, we postulate the presence of a system that can split water at the expense of energy. Under some conditions the carrier system may be limiting, under other conditions, the passive outward diffusion. The finding of HEMKER[2] that nitrophenols become more efficient uncouplers at low pH suggests that, at least under his conditions, the rate of outward diffusion was limiting because of the low H^+ concentration.

It has been found that many uncouplers have an effect on purified enzymes, for instance β-hydroxybutyrate dehydrogenase[28] and malate dehydrogenase[29]. Also, it was shown by WEINBACH AND GARBUS[14] that uncouplers interact strongly with mitochondrial proteins. On the other hand, there is a clear correlation between the efficiency of uncouplers in mitochondria and their ability to decrease the resistance of artificial pure phospholipid membranes[30,31]. We feel that in our mechanism both these phenomena are accommodated: the uncoupler–protein interaction may be the basis of the binding of the uncoupler to the carriers and the charge-conducting property may be related to the passive diffusion of the undissociated uncoupler through the mitochondrial membrane.

It has been argued that the measurements of dinitrophenol accumulation give information only about the factors determining an equilibrium and may not necessarily be related to the actual rate of the uncoupler movement[32]. In recent experiments, using a different technique and working at lower temperatures, we have been able to measure the actual rate of the second half of the accumulation process. The results of these preliminary experiments indicate that there is a close correlation between the rate of entry of dinitrophenol and the final concentration reached in equilibrium. We feel, therefore, justified in using the equilibrium measurements as support for our mechanism.

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REFERENCES

1 W. C. HÜLSMANN, *Over het Mechanisme van de Ademhalingsketenphosphorylering*, M. D. Thesis, Klein Offset Drukkerij, Amsterdam, 1958.

2 H. C. HEMKER, *Biochim. Biophys. Acta*, 81 (1964) 1, 9.
3 C. E. WENNER, *Federation Proc.*, 24 (1965) 544.
4 D. F. WILSON AND R. D. MERZ, *Arch. Biochem. Biophys.*, 119 (1967) 470.
5 K. VAN DAM, *Biochim. Biophys. Acta*, 131 (1967) 407.
6 E. J. HARRIS, K. VAN DAM AND B. C. PRESSMAN, *Nature*, 213 (1967) 1126.
7 E. QUAGLIARIELLO, F. PALMIERI AND M. CISTERNINO, *Boll. Soc. Ital. Biol. Sper.*, 43 (1967) 297.
8 E. QUAGLIARIELLO AND F. PALMIERI, *European J. Biochem.*, 4 (1968) 20.
9 K. VAN DAM AND C. S. TSOU, *Biochim. Biophys. Acta*, 162 (1968) 301.
10 K. VAN DAM AND E. C. SLATER, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 2015.
11 A. FONYO, *Biochim. Biophys. Acta*, 81 (1964) 196.
12 R. D. VELDSEMA-CURRIE AND E. C. SLATER, *Biochim. Biophys. Acta*, 162 (1968) 310.
13 R. KRAAYENHOF AND K. VAN DAM, *Abstr. 5th Meeting Federation European Biochem. Socs., Prague, 1968*, p. 52.
14 E. C. WEINBACH AND J. GARBUS, *J. Biol. Chem.*, 240 (1965) 1811.
15 H. LEES, *Biochim. Biophys. Acta*, 131 (1967) 310.
16 J. L. HOWLAND, *Biochem. J.*, 106 (1968) 317.
17 G. H. HOGEBOOM, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 16.
18 D. K. MYERS AND E. C. SLATER, *Biochem. J.*, 67 (1957) 558.
19 K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
20 W. C. WERKHEISER AND W. BARTLEY, *Biochem. J.*, 66 (1957) 79.
21 E. J. HARRIS AND K. VAN DAM, *Biochem. J.*, 106 (1968) 759.
22 E. J. HARRIS, in S. PAPA, J. M. TAGER, E. QUAGLIARIELLO AND E. C. SLATER, *The Energy Level and Metabolic Control in Mitochondria*, Adriatica Editrice, Bari, in the press.
23 A. KEMP, JR. AND E. C. SLATER, *Biochim. Biophys. Acta*, 92 (1964) 178.
24 M. KLINGENBERG AND E. PFAFF, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, BBA Library, Vol. 7, Elsevier, Amsterdam, 1966, p. 180.
25 C. MOORE AND B. C. PRESSMAN, *Biochem. Biophys. Res. Commun.*, 15 (1964) 562.
26 P. MITCHELL, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, BBA Library, Vol. 7, Elsevier, Amsterdam, 1966, p. 65.
27 E. C. SLATER, *European J. Biochem.*, 1 (1967) 317.
28 P. JURTSHUK, JR., I. SEKUZU AND D. E. GREEN, *J. Biol. Chem.*, 238 (1963) 3595.
29 R. T. WEDDING, C. HANSCH AND J. R. FUKUTO, *Arch. Biochem. Biophys.*, 121 (1967) 9.
30 J. BIELAWSKI, T. E. THOMPSON AND A. L. LEHNINGER, *Biochem. Biophys. Res. Commun.*, 24 (1966) 948.
31 V. P. SKULACHEV, in S. PAPA, J. M. TAGER, E. QUAGLIARIELLO AND E. C. SLATER, *The Energy Level and Metabolic Control in Mitochondria*, Adriatica Editrice, Bari, in the press.
32 K. VAN DAM, in S. PAPA, J. M. TAGER, E. QUAGLIARIELLO AND E. C. SLATER, *The Energy Level and Metabolic Control in Mitochondria*, Adriatica Editrice, Bari, in the press.