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INHIBITION OF MITOCHONDRIAL RESPIRATION BY LOSS OF INTRA-MITOCHONDRIAL K^+

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SUMMARY

1. A system is described in which intra-mitochondrial K^+ concentration can be manipulated by the use of dinitrophenol and valinomycin.

2. As mitochondria lose K^+ a striking inhibition of O_2 consumption occurs with both succinate and DPN-linked substrates, but not if tetramethyl-*p*-phenylenediamine + ascorbate serves as substrate. Respiration can be re-activated by adding K^+ to the medium.

3. Results are discussed in terms of sensitivity of electron transport or substrate dehydrogenation to intra-mitochondrial K^+ content.

INTRODUCTION

More than 30 years have elapsed since DICKENS AND GREVILLE[1] reported that K^+ stimulates respiration in brain slices. This has since been verified by several investigators[2–5], although the mechanism of stimulation has remained obscure. A similar K^+-stimulated respiration has been observed in mitochondria prepared from brain tissue[6]. This is not generally true for liver or kidney mitochondria. If, however, agents which alter K^+ permeability are added to isolated mitochondria from liver or kidney their rates of respiration are also enhanced by K^+ (refs. 7–9).

There are several possibilities as to the nature of the K^+-sensitive site. Ouabain, which has been shown to inhibit Na^+–K^+ transport across cell membranes, inhibits the stimulation of respiration by K^+ observed in isolated tissue slices[10,11]. This fact, coupled with known dependence on Na^+ for the K^+ effect[1,4] suggests the Na^+–K^+-activated ATPase, first described by SKOU[12], may be important. Ouabain has been shown to inhibit this enzyme system[13]. However, Na^+–K^+-activated ATPase and the inhibitory effect of ouabain are not observed in isolated mitochondria[14], thus limiting this mechanism to the cellular level.

PRESSMAN AND LARDY[15], and more recently KRALL, WAGNER AND GOZANSKY[16] have shown that both oxidation and phosphorylation are stimulated by K^+. The data of PRESSMAN was obtained in the presence of a phosphate-acceptor system, hence ADP was not rate-limiting. They suggest that one of the phosphorylative reactions is the K^+-sensitive site. The results could be equally well explained if K^+ stimulated electron transport. A third possibility is that the K^+-stimulated respiration is a conse-

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

quence of ion transport, a process thought to require intermediates of oxidative phosphorylation[17,18]. This transport 'uncoupling' phenomena would tend to obscure any direct stimulation of electron transport or dehydrogenation by K^+, a point which must be considered as a fourth possibility for the site of K^+ action.

It is the purpose of this paper to report evidence indicating sensitivity of either electron transport or substrate dehydrogenation to changes in intra-mitochondrial K^+ content. As it was desired to study effects of K^+ on the electron transport chain directly, without interfering effects operating at the level of oxidative phosphorylation, it was decided to study a dinitrophenol-uncoupled system. Then, by conferring K^+ permeability on the normally K^+ impermeable mitochondrial membrane, internal K^+ concentration should be responsive to changes in the K^+ concentration of the incubating medium. Valinomycin was chosen as the agent for altering K^+ permeability primarily for its apparent specificity[8,19].

METHODS

Mitochondria were prepared from the livers of male Wistar rats by a modification of the method of SCHNEIDER[20], employing 0.37 M sucrose–0.05 mM EDTA as the homogenization medium. The twice washed mitochondrial pellet was suspended in sufficient 0.37 M sucrose to given a protein concentration of about 25 mg/ml. Protein was determined by the biuret method[21].

Incubations were carried out in a medium containing 225 mM mannitol, 75 mM sucrose, 5 mM Tris phosphate, 1 mM $MgCl_2$, and 1–5 mM of the appropriate substrate. This medium is hereafter referred to as MST-phosphate medium. In some experiments 5 mM Tris chloride replaced Tris phosphate, and in this case the medium is designated as MST-chloride medium. O_2 consumption was monitored with a vibrating platinum electrode, and light scattering simultaneously in an Aminco–Chance dual wavelength spectrophotometer using a wavelength pair of 500 and 520. Some experiments were carried out in an apparatus which monitors H^+, K^+, and O_2 concentrations, fluorescence, and light scatter simultaneously. This equipment was designed and constructed in the electronics shop of the Johnson Research Foundation, University of Pennsylvania. Incubations were at room temperature (20°), and about 4 mg of mitochondrial protein were used per ml of incubation medium.

In experiments following $^{14}CO_2$ production, mitochondria equivalent to 2.5 mg protein were incubated in medium containing 37.5 mM Tris chloride (pH 7.4), 0.75 mM DPN, 7.5 mM substrate, 0.1 mM dinitrophenol, 0–80 mM KCl, 0.2 μC ^{14}C-labeled substrate, and sucrose to 250 mosM. Total vol. was 1.0 ml. Incubations were for 1 h at 30°. Valinomycin, when present, was used at a concn. of 0.1 μg/ml. CO_2 was trapped in hyamine, and the radioactivity of the hyamine determined in a Packard liquid scintillation spectrometer.

[1,4-$^{14}C_2$]Succinate, [5,6-$^{14}C_2$]isocitrate, [1-^{14}C]pyruvate, uniformly ^{14}C-labeled malate, and α-[5-^{14}C]ketoglutarate were purchased from New England Nuclear Corporation.

RESULTS

Addition of valinomycin to rat-liver mitochondria respiring in K^+-free MST-phosphate medium causes no detectable change in the rate of O_2 consumption. Subse-

quent addition of 10 mμmoles KCl leads to a burst of respiration which is sustained until the medium becomes anoxic. Conversely, if the KCl is added first no change occurs, but adding valinomycin subsequently causes increased respiration to anoxia (Fig. 1a). Under no conditions will K^+ or valinomycin alone stimulate respiration, although the two together always do so, if respiration coupled with phosphorylation is occurring. These results are in agreement with those of PRESSMAN[8], and OGATA AND RASMUSSEN[7], and are consistent with the hypothesis that valinomycin allows K^+ to permeate the mitochondrial membrane where it is transported into the matrix space by an energy-requiring process.

In order to test our initial hypothesis that intra-mitochondrial K^+ content could be changed in the presence of a combination of dinitrophenol and valinomycin, the following experiment was performed. Extra-mitochondrial K^+ concentration was monitored in an MST-phosphate medium suspension of mitochondria with a potassium electrode (Beckman No. 39137). Succinate served as substrate, and valinomycin and dinitrophenol were added. Results are shown in Fig. 1b. Note that either valinomycin or dinitrophenol may be added with little or no change in K^+ content of the medium. If both agents are present, however, K^+ leaks out of the mitochondria. If K^+ is present in the medium, leakage is inhibited to an extent depending on the amount present, until at initial K^+ concentration above 10 mM, the changes become too small to detect by this method. In cases with K^+ present in the medium, dinitrophenol must be added first, as adding valinomycin first will cause mitochondrial accumulation of K^+ and stimulated respiration, due to the well-known active transport process. On the basis of these data it seems apparent that intra-mitochondrial K^+ levels parallel external levels when both valinomycin and dinitrophenol are present.

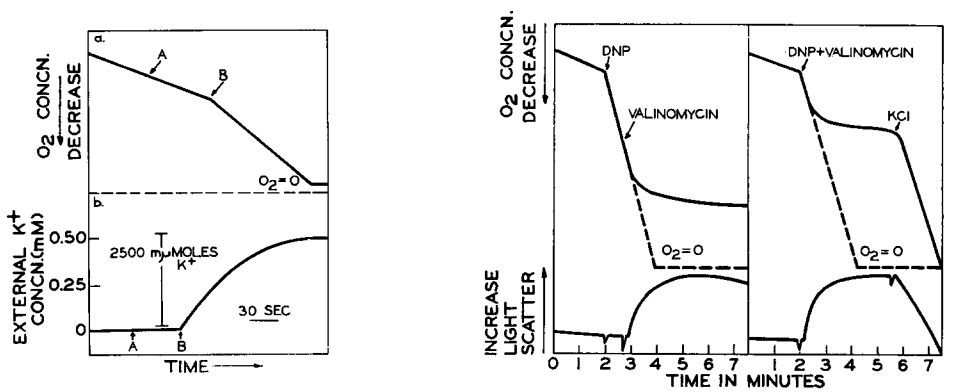


Fig. 1. a. Effects of K^+ and valinomycin on O_2 consumption by rat-liver mitochondria. Incubation medium was MST-phosphate + succinate, and mitochondria equivalent to 4 mg protein were used in a 3.0-ml volume at 25°. Addition at Point A is either 1.5 mM potassium or 0.1 μg/ml valinomycin, and at Point B, the component not added at A. b. Effects of dinitrophenol and valinomycin on K^+ efflux from isolated mitochondria. An upward deflection represents increased K^+ in the medium. Mitochondria equivalent to 20 mg protein were incubated in 5 ml MST-phosphate medium + succinate at 25°. At Point A either 0.1 mM dinitrophenol or 0.1 μg/ml valinomycin was added, and at Point B, the component not added at A.

Fig. 2. Effect of dinitrophenol (DNP) and valinomycin on O_2 consumption and light scattering of mitochondria incubated in 3.0 ml MST-phosphate medium + malate at 25°. Increased light scattering is recorded as an upward deflection and represents contraction of the mitochondria. KCl to a final concn. of 13.3 mM was added at the point indicated. Concentration of dinitrophenol was 0.1 mM; of valinomycin, 0.1 μg/ml. – – – –, O_2 consumption in presence of dinitrophenol alone.

Fig. 2 shows the rates of O_2 consumption of mitochondria treated with dinitrophenol and valinomycin in K^+-free MST-phosphate medium with succinate as substrate. As illustrated, dinitrophenol in sufficient quantity to cause complete uncoupling (10^{-4} M), stimulated O_2 consumption until the medium became anoxic. If valinomycin was added after the dinitrophenol, there was a short lag period followed by an almost complete inhibition of respiration. If valinomycin and dinitrophenol were added together, a lag of the same time interval occurred, again followed by the inhibited state. Adding valinomycin prior to dinitrophenol did not shorten the lag time, indicating a certain time interval is required for sufficient K^+ to be lost for inhibition to occur. KCl (13 mM) added after respiration was inhibited caused stimulation of O_2 uptake, often to the rate observed with dinitrophenol alone. Similar results were obtained with malate, glutamate, α-ketoglutarate, citrate, β-hydroxybutyrate, and a combination of citrate and malate as substrates.

When identical experiments were performed with Tris chloride in place of Tris phosphate, the results obtained were the same when DPN-linked substrates were used, but differed slightly when succinate served as substrate. In the latter case the rate at which inhibition was attained was markedly slowed. Potassium electrode experiments showed that the initial rate of K^+ loss did not differ significantly (last line of Table II) in the two media, although loss during the final stages was much more rapid in the presence of phosphate. Also, there was a greater spontaneous loss of K^+ from mitochondria incubated in phosphate, than from those in chloride medium. However, to obtain the same degree of inhibition the total loss of K^+ was the same as shown in Table III. Similarly, increasing the pH of the suspending medium led to a gradual increase in both the time to initiate inhibition and the time to achieve complete inhibition of respiration. This data is shown in Fig. 3, and also represents decreases in rate of K^+ loss as pH increases.

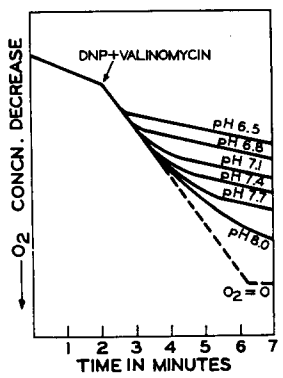
In order to determine if K^+ loss from mitochondria was inhibitory over longer periods of time, experiments were carried out with radioactive substrates, and the rate of $^{14}CO_2$ production measured in the presence of dinitrophenol and varying levels of K^+, either with or without valinomycin. Typical results are shown in Fig. 4. Note that CO_2 production in the control group (dinitrophenol only) increased only slightly as external K^+ concentration increased. On the other hand, there was a striking increase in CO_2 production as K^+ concentration increased in the K^+-permeable group (dinitrophenol + valinomycin). At a K^+ concentration of about 40 mM or slightly above, there is no difference in decarboxylation rates. Endogenous K^+ levels of liver mitochondria are also thought to be approx. 40 mM (ref. 22). It is noteworthy that even at a K^+ concentration of 30 mM a significant degree of inhibition was observed (Fig. 4). Above 40 mM K^+ there is a slight but consistent stimulation of CO_2 production. The results illustrated were obtained with isocitrate as substrate, but entirely indentical results were obtained with α-ketoglutarate, succinate, L-malate, and pyruvate.

The possibility remained that substrate was lost from the mitochondria along with K^+, and this accounted for the observed inhibition of O_2 consumption and CO_2 production. This question was answered by increasing substrate concentration at a fixed level of K^+. Data are recorded in Table I. The constant ratio of

$$\frac{CO_2 \text{ production}-\text{permeable}}{CO_2 \text{ production}-\text{impermeable}}$$

as substrate concentration increased 4-fold indicates this alternative is not correct.

Another alternative existed in that mitochondrial integrity might be damaged sufficiently by K^+ leakage to disrupt the electron transport chain and cause inhibition (*i.e.* severe swelling might be occurring). Instead, mitochondria were observed to contract as K^+ was lost as shown in Fig. 2, and to regain their initial size after K^+ was added.



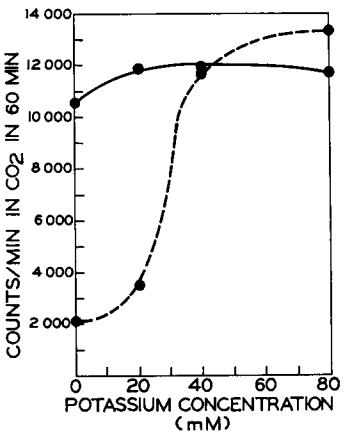


Fig. 3. Effect of pH on the inhibition of mitochondrial oxygen consumption by dinitrophenol (DNP) and valinomycin. Incubation medium was MST-chloride with glutamate and malate as substrates. Concentration of mitochondrial protein was 4 mg/ml. – – – –, O_2 consumption in presence of dinitrophenol only.

Fig. 4. $^{14}CO_2$ production from [5,6-$^{14}C_2$]isocitrate as a function of K^+ concentration. The incubation medium was as described in the text. K^+ was added as the chloride, and sufficient sucrose added to maintain osmolarity at 250 mM. ———, values obtained when mitochondria were treated only with 0.1 mM dinitrophenol. – – – –, values obtained in presence of both dinitrophenol and 0.1 μg/ml valinomycin.

TABLE I

EFFECT OF INCREASING SUBSTRATE CONCENTRATION ON DEGREE OF INHIBITION OF MITOCHONDRIAL CO_2 PRODUCTION DURING K^+ LOSS

Incubation medium was as described in the text with 20 mM K^+ added. L-[1,4-$^{14}C_2$]Malate served as substrate.

Substrate concn. (mM)	*Counts/min in CO_2 in 60 min*		*Ratio*
	Dinitrophenol	*Dinitrophenol + valinomycin*	*Dinitrophenol + valinomycin / Dinitrophenol*
7.5	5748	3500	0.61
11.3	5697	3767	0.66
15.0	6486	4019	0.62
30.0	8312	4765	0.57

On several occasions oxygen electrode experiments indicated respiratory inhibition when only dinitrophenol had been added, especially if the ratio of dinitrophenol to mitochondrial protein was high. These data suggested that at high concentrations of dinitrophenol sufficient K^+ might have been lost to inhibit respiration. When this concept was tested using the potassium electrode the results shown in Table II were

obtained. A slow rate of K^+ release occurred at dinitrophenol concentrations in the range where O_2 consumption is normally stimulated, but at higher concentrations K^+ loss approached the rate observed with both dinitrophenol and valinomycin, especially in phosphate medium. It is interesting that both CHAPPELL[25] and CHANCE, WILLIAMS AND HOLLUNGER[26] have shown that higher concentrations of uncouplers inhibit O_2 uptake but neither attribute the inhibition to loss of K^+. Furthermore,

TABLE II

EFFECT OF INCREASING UNCOUPLER CONCENTRATION ON INITIAL RATES OF K^+ LOSS FROM MITOCHONDRIA

Dinitrophenol concn.	*mμmoles K^+ lost per min*	
	MST-phosphate medium	*MST-chloride medium*
5 μM	17.3	
10 μM	25.0	10.9
15 μM	32.5	
20 μM	58.4	15.3
30 μM		35.0
100 μM	178.0	65.0
50 μM + 0.1 μg valinomycin per ml	224.0	205.0

TABLE III

COMPARISON OF RESPIRATORY INHIBITION WITH SUCCINATE AS SUBSTRATE IN PHOSPHATE AND CHLORIDE MEDIA

	MST-chloride medium	*MST-phosphate medium*
Time after dinitrophenol + valinomycin for inhibition of respiration (min)	4.55	1.2
K^+ lost before inhibition occurs (mμmoles)	175	160

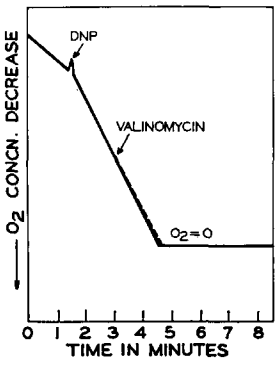


Fig. 5. Effect of dinitrophenol (DNP) and valinomycin on mitochondrial O_2 consumption in MST-phosphate medium with TMPD + ascorbate (3.3 mM) as substrate. – – – –, rate in presence of 0.1 mM dinitrophenol and 0.1 μg/ml valinomycin. ———, rate in presence of only dinitrophenol.

the concentration of dinitrophenol required for optimal K^+ loss is in the same range as CHAPPELL reports for inhibition of O_2 uptake.

Finally, O_2 uptake studies were conducted using ascorbate and tetramethylphenylenediamine (TMPD) as electron donors to the terminal portion of the electron transport chain[23,24]. This system, in contrast to the others, is not sensitive to K^+ loss (see Fig. 5).

DISCUSSION

The above results are consistent with the concept that mitochondrial electron transport is sensitive to intra-mitochondrial K^+ content. Contributions to the observed effects from possible other K^+-sensitive sites at the level of phosphorylation have been circumvented by the use of an uncoupler. The site of sensitivity seems to be at a point prior to cytochrome *c* as evidenced by the lack of inhibition in the TMPD–ascorbate system which donates electrons at this point. Assuming only one sensitive site, the data also indicates that the region between cytochromes *b* and *c* is responsible, as inhibition was obtained with succinate as well as with the DPN-linked substrates. The data in no way excludes more than one sensitive site, and it is possible that all the mitochondrial dehydrogenases are K^+ sensitive. If the latter were true, one would expect DPN to be relatively more oxidized after K^+ loss when DPN-linked substrates are used. When pyridine nucleotide fluorescence was measured it was found that greater reduction of the nucleotides occurred if valinomycin and dinitrophenol were given together, but increased oxidation if valinomycin was added after the uncoupler. Clearly, these results do not allow a definitive answer as to the locus of the K^+-sensitive site.

The possibility exists that the observed effects are actually due to changes in intra-mitochondrial H^+ concentration rather than K^+, as H^+ usually moved in opposition to K^+ (also see ref. 7). The experiment of Fig. 3 in which a 10-fold decrease in H^+ concentration caused only a slight decrease in the rate at which respiratory inhibition was attained dispels this alternative. Even in this situation, the maximal inhibition of respiration was correlated with the extent of K^+ loss, *i.e.* the rate at which inhibition took place was a function of the rate of K^+ loss, but maximal inhibition occurred when the extent of K^+ loss was equal. It is possible, however, that other ions aid in mediating the K^+ effect as indicated by the slower rate of inhibition with succinate as substrate in the absence of phosphate. However, in this case too the extent of K^+ loss was correlated with the extent of inhibition. These data are all consistent with the view that the internal concentration of K^+ is the controlling factor, and not the rate of K^+ movement across the membrane.

The fact that high concentrations of dinitrophenol alone may cause K^+ loss with concomitant respiratory inhibition is particularly interesting. The phenomenon of respiratory inhibition as uncoupler concentration increases is quite general in that any uncoupler exhibits this effect[27]. That such inhibition is due to loss of intra-mitochondrial K^+ seems quite possible on the basis of the present data.

Recently GRAVEN, ESTRADA-O AND LARDY[28] reported a similar inhibition of respiration when mitochondria lose K^+. There are several important differences between that data and the present results. Respiratory inhibition was observed for the DPN-linked substrates, malate, pyruvate, glutamate and α-ketoglutarate, but

they reported no inhibition of respiration with succinate or β-hydroxybutyrate, even though K^+ was lost. This fact is in direct contrast with the present results, but may be due in part to differences in the method used to induce K^+ loss. Those authors used Nigericin, rather than dinitrophenol, in combination with valinomycin. Nigericin is not an uncoupler of oxidative phosphorylation, but inhibits the ATP–P_i exchange reaction, and ion transport. The difficulties of interpretation characteristic of a system with possible K^+-sensitive sites at the levels of phosphorylation as well as electron transport are thus encountered, as mentioned at the beginning of this paper. Also, they reported no inhibition if a combination of malate and citrate were used as substrate, while marked inhibition was observed with these substrates in our system. Lastly, they found a K^+ concentration greater than 30 mM was required for a significant reversal of inhibition in O_2 uptake studies, whereas 13 mM K^+ was sufficient for nearly maximal reversal in our studies (although higher concentrations were required in experiments spanning longer time intervals—see Fig. 4). In summary it appears that GRAVEN and co-workers may be studying effects of K^+ either at the phosphorylative site, or at the level of substrate oxidative enzymes, as they suggest.

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