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On the Locus of Action of Na^+ at Site I of Oxidative Phosphorylation

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Abstract

The effect of octylguanidine on the Na^+ stimulated oxygen uptake of rat liver mitochondria and bovine heart submitochondrial particles and on the Na^+ induced efflux of K from the mitochondria has been examined. The results indicate that the action of Na^+ is inhibited by octylguanidine, but that the degree of inhibition depends on the concentration of the cation. Apparently, a competition exists between Na^+ and octylguanidine for a common site. Octylguanidine, but not oligomycin, at certain concentrations restores in mitochondria incubated with Na^+ the capacity to respond to uncouplers. A competitive effect between monovalent cations and octylguanidine has been observed in submitochondrial particles oxidizing NADH.

Introduction

Rat liver mitochondria are partially uncoupled and loose their respiratory control when they are incubated with Na^+, EDTA, and inorganic phosphate, but not when they are incubated with K^+ in the same conditions [1]. As revealed by studies with arsenate, this detrimental action of Na^+ is most probably due to the alteration of a reaction that lies before the entrance of inorganic phosphate into the oxidative phosphorylation sequence [2]. These results indicate that Na^+ profoundly influence the oxidative phosphorylation reaction and thus, a study of its site and mechanism of action is of some importance.

In this work, the location of the site of action of Na^+ has been studied in reference to the locus of action of octylguanidine, an inhibitor of

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Site I of oxidative phosphorylation [3, 4]. Octylguanidine was chosen because the detrimental action of Na^+ is counteracted competitively by K^+ [5], a cation which has been shown to be necessary or to increase the rate of formation of the energized state at Site I of oxidative phosphorylation [5-7].

The use of guanidine in the study of oxidative phosphorylation was introduced by Hollunger [8] and since that time, several investigators have found that the effect of several guanidine derivatives in inhibiting the energy transfer reactions depends on the length of the alkyl substituents [3, 9, 10]. In particular, octylguanidine inhibits the ADP stimulated respiration and phosphorylation, but not the 2,4-dinitrophenol associated respiration [3, 8]. This effect of alkyl guanidines is more easily evidenced at Site I of oxidative phosphorylation [3, 4, 8], the site where the effect of monovalent cations, both, in mitochondria and in submitochondrial particles is more easily evidenced [5-7].

Methods

Rat liver mitochondria were isolated in 0.25 M sucrose and 1 mM EDTA, pH 7.4, according to the method of Schneider and Hogeboom [11]. The experimental techniques employed to test the effect of octylguanidine on mitochondrial respiration are described under individual experiments. When mitochondria are incubated in 100 mM NaCl, 1 mM EDTA, 10 mM H_3PO_4 (adjusted to pH 7.3 with Tris base), 10 mM glutamate, 20 mM Tris-HCl (pH 7.3) and 50 mM sucrose for 3 min at room temperature, their K^+ content falls from approximately 100 mM to 15 mM or less [5, 6]. The effect of octylguanidine on this extrusion of K^+ from the mitochondria was assayed as follows: mitochondria from approximately 4 g of rat liver were incubated in the above described conditions with and without octylguanidine in a final volume of 12.5 ml. The suspension was diluted to approximately 40 ml with sucrose-EDTA and centrifuged. The pellet was washed with 40 ml of sucrose EDTA and centrifuged again. The resulting mitochondrial pellet was suspended in sucrose-EDTA and K^+ was determined in the perchloric acid extract. This technique has been described previously [5]. Oxygen uptake was measured either polarographically (Yellow Springs Instrument Co.) or manometrically according to the conventional Warburg technique.

EDTA submitochondrial particles were prepared as described by Lee *et al.* [12] from beef heart mitochondria which had been prepared according to Löw and Vallin [13] or from rat liver mitochondria prepared in sucrose EDTA. Bovine heart mitochondria were stored at -4° for at least 24 h in sucrose EDTA before preparing the EDTA particles.

Results

Sodium ions in combination with EDTA and inorganic phosphate stimulate oxygen uptake to an extent which approaches or equals the activation of respiration induced by 2,4-dinitrophenol [1]. The results of the experiment outlined in Fig. 1 show that the Na^+ stimulated oxygen uptake is inhibited by octylguanidine in a concentration dependent

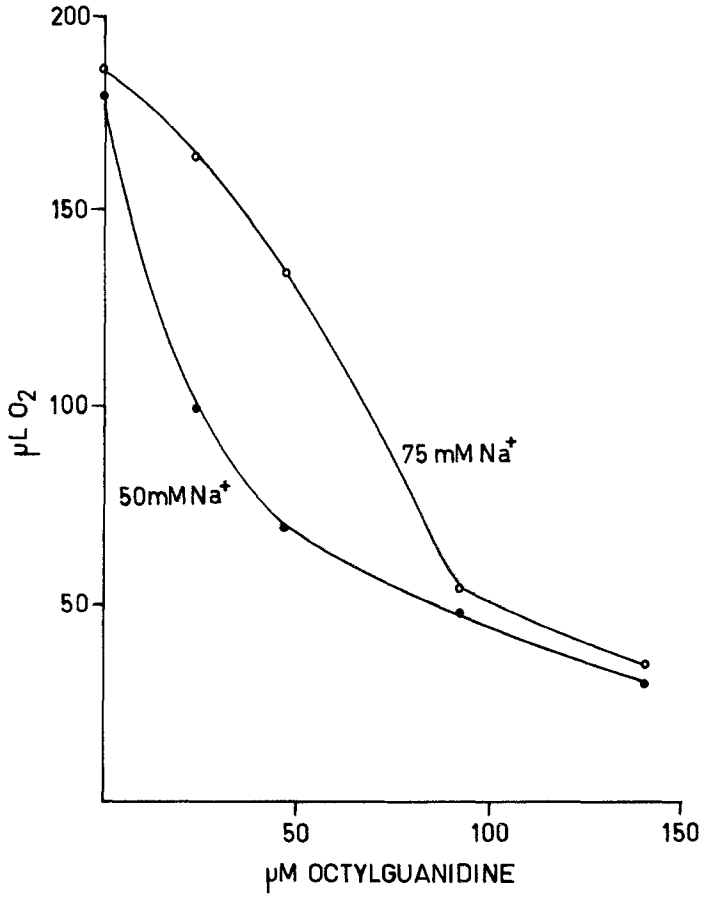

Figure 1. Effect of octylguanidine on the Na^+ stimulated oxygen uptake of fresh mitochondria. Oxygen uptake was measured manometrically in 3.0 ml of incubation mixture which contained 75 mM NaCl or 50 mM NaCl as indicated, 10 mM H_3PO_4 (adjusted to pH 7.3 with Tris base), 10 mM glutamate, 1 mM EDTA, 50 mM sucrose and the indicated concentrations of octylguanidine. The first reading was made after 7 min of thermoequilibrium at 25°. Each vessel contained 18 mg of mitochondrial protein. The oxygen uptake in 24 min of incubation is expressed.

process. However, the inhibition induced by octylguanidine also depends on the concentration of NaCl in the mixture; as shown in Fig. 1, 50 and 75 mM NaCl stimulated oxygen uptake to the same extent, but the degree of inhibition induced by several concentrations of octylguanidine was lower at the higher concentration of NaCl.

The effect of a fixed concentration of octylguanidine on the mitochondrial oxygen uptake stimulated by various concentrations of NaCl is shown in Fig. 2. At relatively low concentrations of Na^+, octylguanidine inhibits the Na^+ stimulated oxygen uptake by about 70%, while at 100 mM NaCl, the same concentration of octylguanidine inhibits the Na^+ stimulated oxygen uptake by approximately 50%.

In the experiment of Fig. 3, mitochondria were incubated with octylguanidine in the absence of Na^+ and after approximately 17 min of exposure of the mitochondria to octylguanidine sodium ions were added. After a lag period, the addition of Na^+ induced an increase in the slope of the respiratory rate. On the other hand, the addition of octylguanidine to mitochondria whose respiration had been stimulated by Na^+ produced a strong inhibition of oxygen uptake (Fig. 3). Nevertheless, it should be observed that the final respiratory rate is independent of the order of addition of Na^+ and octylguanidine. This

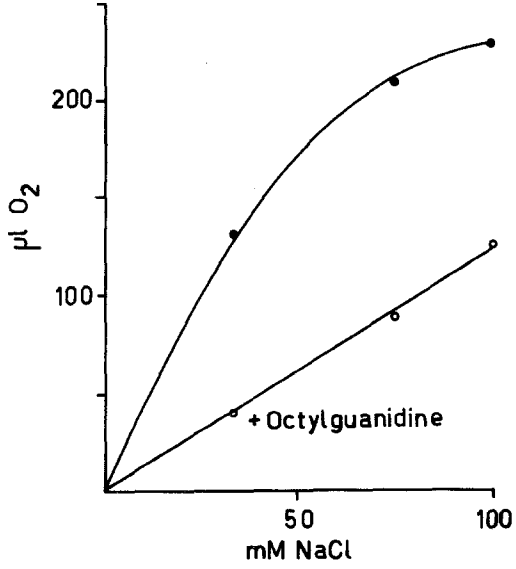

Figure 2. Effect of octylguanidine on mitochondrial oxygen uptake at various concentrations of NaCl. Oxygen uptake was measured as in Fig. 1 except that the incubation mixture contained the indicated concentration of NaCl and 47 μM octylguanidine where indicated. Each vessel contained 22 mg of mitochondrial protein. The oxygen uptake in 20 min of incubation is expressed.

experiment suggests that a competition exists between octylguanidine and Na^+, the former being a more effective agent and that, regardless of the order of addition of Na^+ and octylguanidine, the mitochondria respond to a different proportion of octylguanidine and Na^+.

In respect to the inhibition of the Na^+ stimulated oxygen uptake by octylguanidine, it should be mentioned that Na^+ inhibit the stimulation of oxygen uptake by dinitrophenol [1]. Also it has been reported that octylguanidine fails to affect the final rate of the dinitrophenol stimulated oxygen uptake [3, 8]. That is, on mitochondrial respiration, the three following effects have been observed: first, octylguanidine inhibits the action of Na^+; second, Na^+ preclude the action of dinitrophenol [1], and third, the final rate of mitochondrial respiration activated by dinitrophenol is insensitive to octylguanidine [3, 8].

In the experiment of Fig. 4, different concentrations of octylguanidine were added to mitochondria incubated with Na^+ so as to produce different rates of Na^+ stimulated respiratory activity. Subsequently, dinitrophenol was added in order to test the ability of octylguanidine to restore the sensitivity to dinitrophenol in mito-

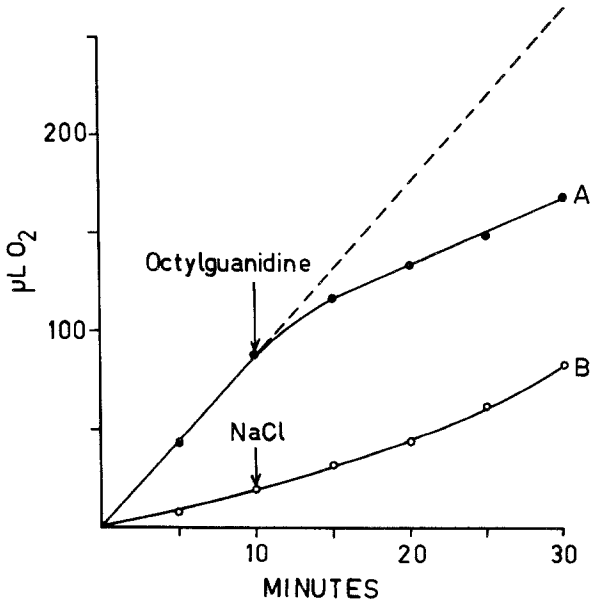

Figure 3. Effect of octylguanidine and Na^+ on the oxygen uptake of mitochondria. In A the mitochondria were incubated as in Fig. 1 with 75 mM NaCl and 94 µM octylguanidine was added where indicated. In B, the mitochondria were incubated with 94 µM octylguanidine from the beginning of the experiment, 75 mM NaCl was added where indicated. The dashed line indicates the oxygen uptake with no octylguanidine added. Each vessel contained 17 mg of mitochondrial protein.

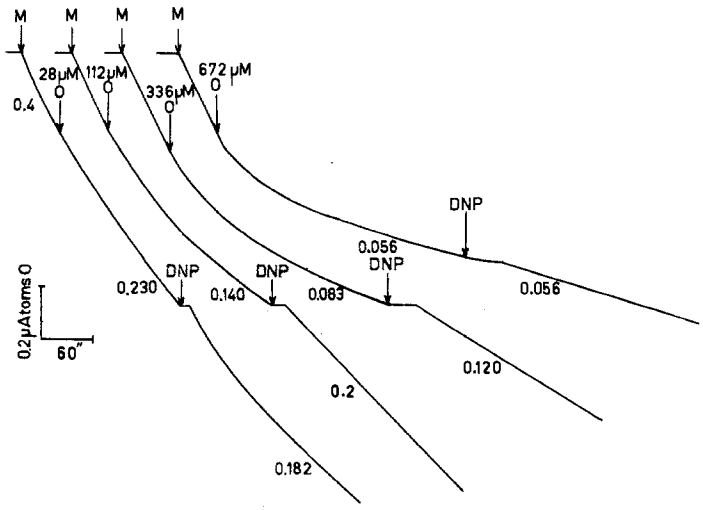

Figure 4. Effect of octylguanidine and 2,4-dinitrophenol on the oxygen uptake of mitochondria incubated with Na^+. Oxygen uptake was measured polarographically in 5.0 ml of incubation mixture with the same final concentrations as in Fig. 1, NaCl was 100 mM. The reaction was started by adding the mitochondria (8.0 mg). Other additions were octylguanidine (O) at the indicated concentration and 10^{-4} M 2,4-dinitrophenol (DNP). The numbers on the side of the trace indicate the rate of respiration (μatoms O/min) at the point where the numbers have been drawn.

chondria which fail to respond to uncouplers. The results show that octylguanidine, at certain concentrations, indeed restores the sensitivity to dinitrophenol. However, octylguanidine fails to restore the sensitivity to dinitrophenol when the inhibition of the Na^+ stimulated respiration is not complete or when extremely high concentrations of octylguanidine are employed. This experiment indicates that octylguanidine simultaneously inhibits the action of Na^+ and induces a sensitivity to dinitrophenol. However, when extremely high concentrations of octylguanidine are employed, even though the action of Na^+ is completely reversed, dinitrophenol is unable to overcome the guanidine effect. The latter effect of octylguanidine has been discussed by Pressman [3].

Effect of Octylguanidine on the Na^+ induced K^+ Release

Sodium ions besides inducing loss of respiratory control also produce the extrusion of K^+ from the mitochondria [5, 6, 14]. Accordingly, the octylguanidine induced sensitivity to dinitrophenol could be due either to a displacement of Na^+ by octylguanidine or, to an inhibition of the leakage of K^+ from the mitochondria which according to earlier data

seems to be a necessary component of the phosphorylation reaction at Site I [5-7]. Therefore, it was considered necessary to study whether octylguanidine also inhibited the extrusion of K^+ from the mitochondria.

The results of Fig. 5 show that octylguanidine inhibits the Na^+ induced release of K^+ from the mitochondria and that this inhibition is proportional to the concentration of octylguanidine. Also it was found that increasing concentrations of Na^+ progressively overcome the inhibiting action of octylguanidine (Fig. 6).

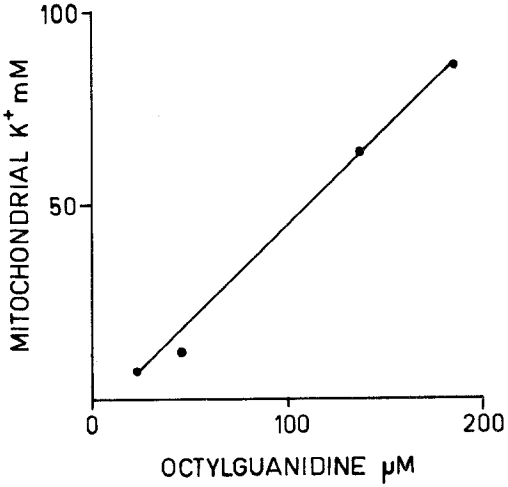

Figure. 5. Effect of octylguanidine on the Na^+ induced K^+ efflux in mitochondria. The K^+ content of mitochondria incubated as described under methods with the indicated concentrations of octylguanidine was assayed. The K^+ content of mitochondria in which the incubation step was omitted was 88 mM.

The latter results suggest that the octylguanidine induced sensitivity to dinitrophenol could be due to an inhibition of the release of K^+ from the mitochondria. However, in mitochondria which have been depleted of K^+ and in which, Na^+ also induce high rates of respiration [5, 6], octylguanidine still produced a lowering of the respiratory rate and induced a certain sensitivity to dinitrophenol similar to that observed in fresh mitochondria (Fig. 4) (this experiment is not shown). Thus, the effect of octylguanidine is not due to an inhibition in the release of K^+, but rather to an inhibition of the detrimental action of Na^+.

Comparison Between the Action of Octylguanidine and Oligomycin in Mitochondria Incubated with Na^+

The action of alkyl substituted guanidines has been compared to that of oligomycin [15]. The latter blocks almost instantaneously the ADP

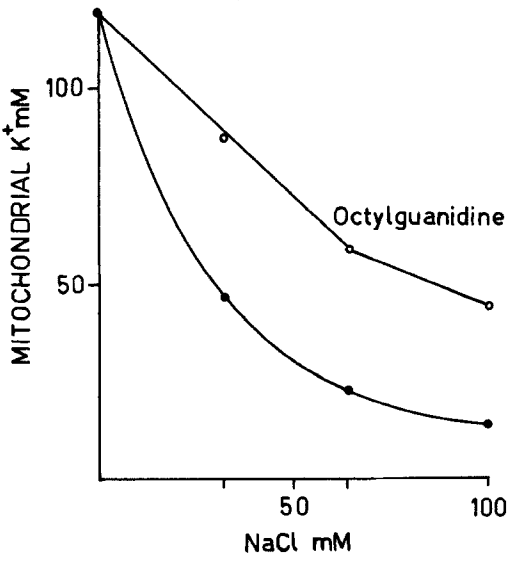

Figure 6. Effect of octylguanidine on the efflux of K^+ induced by various concentrations of Na^+. Mitochondria were incubated as described under Methods with the indicated concentrations of NaCl in the absence and presence of 80 μM octylguanidine.

stimulated oxygen uptake, while the action of the guanidines is rather gradual [3]. Also it has been shown that the reversal of the alkyl guanidine inhibited respiration by dinitrophenol takes place gradually, while on the other hand, the effect of uncouplers on an oligomycin inhibited respiration may be qualified as instantaneous [3]. Figure 7 shows another difference between octylguanidine and oligomycin. Oligomycin, in agreement with previous data [1], partially inhibited the Na^+ stimulated oxygen uptake. However, the further addition of dinitrophenol did not affect the final respiratory rate. These findings contrast with those of Fig. 4 in which the octylguanidine inhibited respiration was partially reversed by dinitrophenol.

Effect of Octylguanidine on the Respiratory Activity of Submitochondrial Particles

Recently Christiansen *et al.* [16] and Pinto *et al.* [7] reported that salts of monovalent cations increase the respiratory rate of submitochondrial particles oxidizing NADH, but not succinate. These findings were explained on the assumption that monovalent cations including Na^+ are either necessary for or increase the rate of formation of the energized state at Site I [7]. Table I shows that the inhibition by

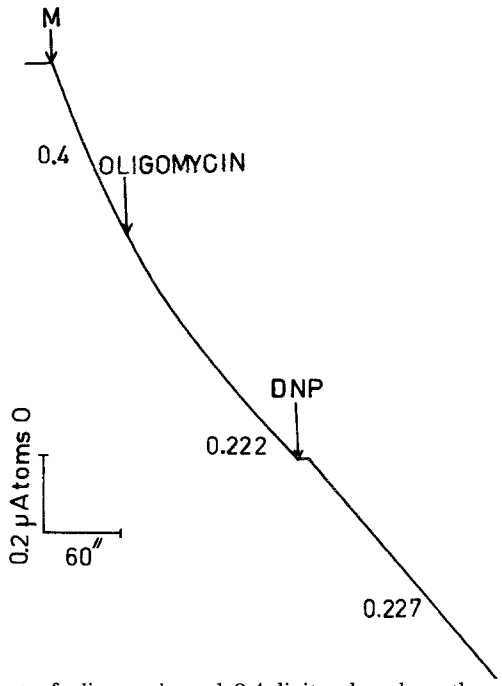

Figure 7. Effect of oligomycin and 2,4-dinitrophenol on the oxygen uptake of mitochondria incubated with Na^+. The incubating conditions were as in Fig. 4. Other additions were: oligomycin (10 μg) and 10^{-4} M 2,4-dinitrophenol (DNP). The numbers on the side of the trace indicate the rate of oxygen uptake (μatoms 0/min) at that particular point. Each vessel contained 8 mg of mitochondrial protein.

TABLE I. Effect of octylguanidine on the oxygen uptake of submitochondrial particles incubated with and without NaCl

Octylguanidine	natoms of oxygen consumed in the first 2 min of incubation	
	−NaCl	NaCl
−	528	1080
10 μM	276	876
20 μM	276	744
40 μM	192	366
100 μM	168	160

Rat liver submitochondrial particles were incubated in 2 mM Tris-HCl (pH 7.4), 0.75 mM NADH and the indicated concentrations of octylguanidine, also 50 mM NaCl was added where indicated. The final volume was 3.0 ml. Respiration was started by the addition of NADH. The respiratory rates expressed are those obtained in the first 2 min after the addition of NADH.

octylguanidine of submitochondrial particle respiration in the presence of NADH is concentration dependent. However, it should be mentioned that the inhibition of respiration is not instantaneous; rather, after the addition of octylguanidine, there is a slow decline in the respiratory rate until finally a steady state is reached. Both the final rate and the time required to reach the final respiratory rate depend on the concentration of octylguanidine. In the presence of KCl or NaCl, submitochondrial particles respire at a faster rate [7, 16] (Table I). In these conditions, octylguanidine also inhibits oxygen uptake with the characteristics described above, but below 40 μM octylguanidine, the respiratory rate is higher in the presence of KCl or NaCl. Also it should be mentioned that submitochondrial particles derived from bovine heart mitochondria respire also at a faster rate in the presence of octylguanidine when NaCl is included in the mixture (data not shown).

A Lineweaver-Burk plot of submitochondrial particle respiration at several concentrations of NaCl in the absence and presence of octylguanidine is presented in Fig. 8. A relatively low concentration of octylguanidine was employed in order to avoid significant declines in the respiratory rates which could complicate the calculation. Also it should be mentioned that the initial respiratory rates were used to calculate the velocity of submitochondrial particle respiration. The results outlined in Fig. 8 are highly suggestive that octylguanidine inhibits competitively the action of Na^+.

Discussion

It has been reported that Na^+ exert a detrimental action on oxidative phosphorylation [1], most probably by acting on a reaction that lies between the electron transport chain and the site of action of 2,4-dinitrophenol [2]. This work has been an attempt to localize and indirectly characterize the site of action of Na^+ with the use of octylguanidine. The locus of action of octylguanidine has been the subject of several studies and there is a more or less general agreement that octylguanidine is an inhibitor of Site I of oxidative phosphorylation [3, 4, 8, 17-19].

As far as the possible interrelation between Na^+ and octylguanidine the following observations indicate that Na^+ and octylguanidine act on a common site:

1. The effect of octylguanidine is due to an inhibition of the Na^+ effect and not to an inhibition of the Na^+ induced release of K^+ from whole mitochondria.
2. In whole mitochondria, the final rate of respiration depends on the ratio between Na^+ and octylguanidine.

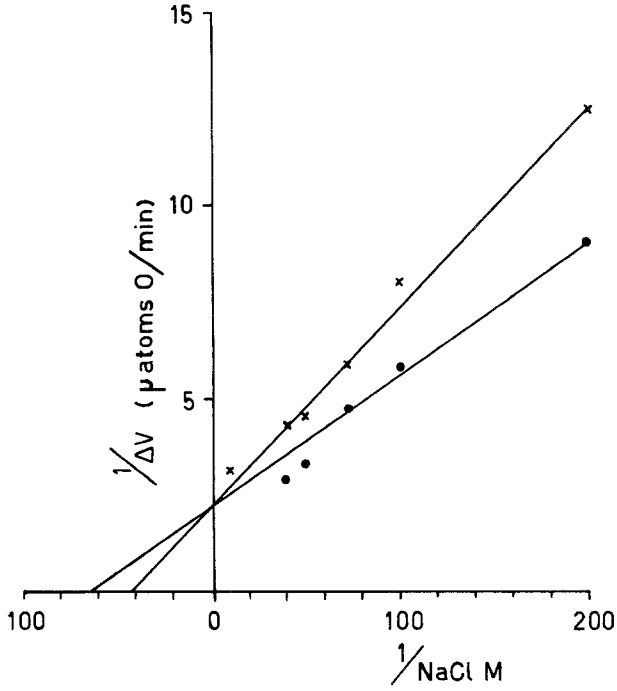

Figure 8. Lineweaver-Burk plot for Na^+ in submitochondrial particle respiration in the absence and presence of octylguanidine. Rat liver submitochondrial particles (1.5 mg of protein) were incubated with 0.75 mM NADH, 2 mM Tris-HCl (pH 7.3) and the indicated concentrations of NaCl. Octylguanidine 10 μM was added where indicated. The final volume was 3.0 ml. The rate of respiration was calculated from the time required to consume the initial 0.36 μatoms of 0 after the addition of NADH.

3. Octylguanidine, but not oligomycin, restores the capacity of Na^+ treated mitochondria to respond to 2,4-dinitrophenol.
4. In submitochondrial particles, Na^+ and octylguanidine act on an apparent competitive form.

However, in this apparent competitive action between Na^+ and octylguanidine, it should be taken into consideration that Na^+ exerts its action in the mM range, while octylguanidine affects mitochondrial respiration at μM concentrations. This large difference in concentration most probably depends on the greater degree of lipophilicity of octylguanidine. Indeed, Pressman [3] demonstrated on a concentration basis, that the degree of effectiveness of several guanidine derivatives augmented as the length of the alkyl substituent increased.

Nevertheless, the question remains as to why octylguanidine and Na^+ have different effects on mitochondria even if they act on the same locus. Furthermore, not only octylguanidine inhibits the action of Na^+, but also Na^+ may reverse the action of octylguanidine, both in mitochondria and in submitochondrial particles.

Apparently, the common factor between Na^+ and octylguanidine is their positive charge and it should be through this common factor, the form in which one competes with the other. According to this rationale, the different response of the mitochondria to octylguanidine and Na^+ must be due to the different size or electronic configuration of the agents involved.

If this conclusion is correct, the hitherto reported experimental findings may provide the basis for an explanation as to why Na^+ and K^+ produce entirely different effects on mitochondria at Site I of oxidative phosphorylation; the former produces a complete loss of respiratory control [1, 2], while the latter is necessary for higher State 3/State 4 ratios [5, 6]. That is, the different effects of Na^+ and K^+ at Site I should be due to their different size or to their different electron inducing effects.

Another question arises and this is in respect to Mitchell's chemiosmotic hypothesis [20]. Briefly, this hypothesis implies that the driving force for the synthesis of ATP in oxidative phosphorylation is the pH gradient and the membrane potential that are established across the coupling membrane during electron transport. If it is considered that ATP is formed at three sites of the electron transport chain, then it is difficult to explain according to the Mitchell hypothesis why the action of K^+ is limited to Site I, both in whole mitochondria and in submitochondrial particles. The building of the pH gradient and membrane potential should occur at the three sites, and not merely be limited to one or two sites, since the transport of electrons through the complete respiratory chain is a phenomenon that occurs in a single membrane.

As judged by the different sensitivity to Na^+ and K^+, at least in the reaction that leads to the formation of the energized state, Site I would be different to Sites II and III in respect to the mechanism of synthesis of ATP. At the present state of our knowledge, it is not possible to decide which mechanism is the one involved in the formation of the energized state at Site I. However, the fact that the same locus at this Site in mitochondria and submitochondrial particles has a characteristic response pattern to Na^+, octylguanidine, and most probably K^+ suggests that a structure with a locus for a positive charged ion is involved in the process. This locus should also be in a very intimate association with lipophilic structure(s) in order to explain the data which has accumulated on octylguanidine [3, 4, 8, 10, 17].

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