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Control of Adenine Nucleotide Exchange in Mitochondria by Cations and Protons*

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ABSTRACT: The influence of cations and protons on the translocation of ADP and ATP in rat liver and heart mitochondria at 0–5° has been examined. In rat liver mitochondria at pH 7.1, 20 mM K^+ decreases the $K_{m,app}$ of ATP from 219 to 15 μM, and of ADP from 48 to 10 μM. The removal of the outer membrane by digitonin treatment has no effect on the K_m and V_{max} values of the exchange in the presence or absence of K^+. At low concentrations of ATP or ADP and at a pH above 7, there is an absolute dependence of the exchange upon cations, whereas increasing the concentration of ADP or ATP competitively removes the activating effect of K^+. As the pH is increased in a K^+-free medium from pH 6.0 to 8.0, the translocation of ADP and ATP is decreased 15 to 58%, respectively. There is little effect of pH on the adenine nucleotide translocation in the presence of 20 mM KCl. The adenine nucleotide exchange is stimulated by 10 mM $Tris^+$, Na^+, Mg^{2+}, and Li^+, to nearly the same extent as K^+. At low concentrations, divalent cations are more effective in stimulating the translocation of ATP than are monovalent cations. There is a $K_{m,app}$ of 0.02 and 0.87 mM for Ca^{2+} and Mg^{2+}, respectively, whereas K^+ or Na^+ yield a half-maximal activation at about 2 mM. The exchange of ATP was stimulated to the same extent by (Na)Cl$^-$, NO_3^-, ClO_3^-, and CH_3COO^-, but was inhibited by the SO_4^{2-} anion. Gramicidin, which increases the passive cation permeability, stimulates the ATP exchange in the presence of Na^+, but does not increase the binding affinity for Na^+. The binding of atractyloside, a nonpenetrating trivalent anion, is dependent on cations. In the presence of K^+, the amount of atractyloside required for half-maximal inhibition of the respiratory control decreases from 0.7 to 0.08 nmole per mg. In addition to the cation requirement for the adenine nucleotide translocase, there is an oligomycin-dependent increase in the intramitochondrial ATP:ADP ratio at 0° by K^+, Na^+, and Mg^{2+}. The data suggest that the primary effect of cations occurs at the binding between the adenine nucleotide anion and the membrane, rather than directly upon the translocation process.

It is generally accepted that adenine nucleotides are carried across the mitochondrial membrane by an enzyme, found in the inner membrane, called an adenine nucleotide translocase (Pressman, 1958; Klingenberg and Pfaff, 1966; Meisner, 1970). The translocation of adenine nucleotides across the mitochondrial membrane is characterized by (a) its specificity for ADP and ATP (Pfaff *et al.*, 1965; Pfaff and Klingenberg, 1968), (b) inhibition by atractyloside (Chappell and Crofts, 1965b; Bruni, 1966; Klingenberg and Pfaff, 1966) or bongkrekic acid (Henderson and Lardy, 1970; Klingenberg *et al.*, 1970), and (c) relative independency toward pH and cations (Pfaff and Klingenberg, 1968). The latter point is rather puzzling, in view of the work of Lardy and Wellman (1953) and others (Cereijo-Santalo, 1968; Amons *et al.*, 1968), showing a cation dependency of the uncoupler-activated ATPase activity and a stimulation of oxidative phosphorylation by K^+ in fresh mitochondria. If adenine nucleotides must first be translocated across the membrane before reaching the site of phosphorylation (Chappell and Crofts, 1965b; Pfaff *et al.*, 1969), it is possible that the exchange of adenine nucleotides, and not the phosphorylation of ADP, may be sensitive to cations. In fact, a variable but small effect of cations on the adenine nucleotide exchange has been noted (Pfaff and Klingenberg, 1968; Pfaff *et al.*, 1969). Recently, Meisner and Wenner (1970) showed that Na^+ increases the exchange of ATP in digitonin particles, and the addition of gramicidin further increases the exchange. This paper presents evidence that there is a dependence of cations and protons on the exchange of ATP and ADP in rat liver and heart mitochondria.

Methods

Rat liver mitochondria were isolated as previously described (Meisner, 1970). Rat heart mitochondria were isolated in 0.3

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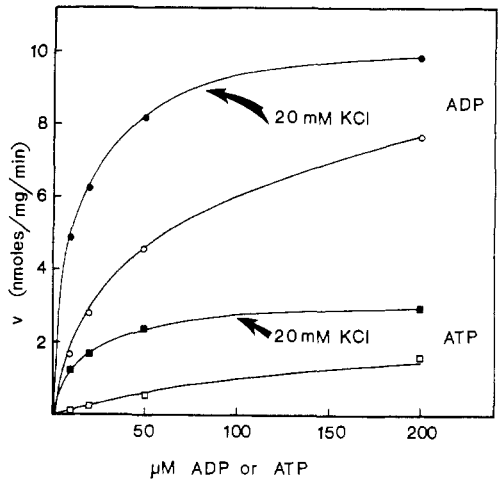

FIGURE 1: Activation of the adenine nucleotide exchange in rat liver mitochondria by K^+. Labeled mitochondria (1.26 mg of protein) were added to 0.26 M sucrose, 0.2 mM Tris-EGTA, 5 mM Tris-TES, ±20 mM KCl, and Tris-ADP or Tris-ATP added to initiate the exchange. Final pH, 7.1; temperature, 5°.

M sucrose–2 mM EDTA–20 mM triethanolamine buffer (pH 7.2) and the minced ventricular muscle homogenized in Nagarse (0.5 mg/g wet weight). Mitochondria from heart and liver were washed once in 0.3 M sucrose, and labeled for 15 min, 0°, with 1 uCi of [^{14}C]ADP or [^{14}C]ATP, and 0.5 mM carrier. The labeled mitochondria were washed twice, and suspended in 0.3 M sucrose to a final concentration of about 25 mg/ml.

To measure the exchange of ADP or ATP, prelabeled mitochondria were added to the incubation medium (for composition, see figures) at 0–10°, in a final volume of 1.0 ml, and 1 min later the back-exchange initiated with Tris-ADP or Tris-ATP. At 15 sec, the exchange was stopped with 10–50 μM atractyloside, the mitochondria immediately separated from the medium in an Eppendorf microcentrifuge, and the radioactivity in the supernatant counted (Pfaff and Klingenberg, 1968; Meisner, 1970). Controls were incubated under identical conditions in the presence of atractyloside, and the unspecific leakage of ^{14}C label (between 12 and 16% for liver and between 21 and 25% for heart mitochondria) subtracted from the total exchange. In this manner, only the exchange is measured, and not the specific and unspecific binding of adenine nucleotides (Weidemann *et al.*, 1970). The rate of exchange (nanomoles of adenine nucleotide per milligram of protein per minute) refers to the sum of the intramitochondrial ADP plus ATP (Pfaff *et al.*, 1969), as measured enzymatically (Lamprecht and Trautschold, 1958).

NaATP and NaADP were converted into the Tris salt by passing through a Dowex 50 column and neutralizing to pH 7.2 with Tris. The final concentration was verified by measuring the OD_{260}, using an extinction coefficient of 1.54×10^6 cm^2/mole. All cations were added as the chloride salt.

Atractyloside was the kind gift of Dr. A. Bruni, Padova, Italy.

Results

Pfaff and Klingenberg (1968) have shown that certain cations, particularly K^+ and Mg^{2+}, moderately stimulate the exchange of ADP and ATP, and have attributed the relative ineffectiveness to the normal impermeability of the membrane to cations and H^+. The results in Figure 1, however, clearly show that K^+ stimulates the exchange of ADP and ATP in rat liver mitochondria in the presence of 0.2 mM Tris-EGTA[1] and 5 mM Tris-TES (pH 7.1). The enhancement of the exchange by K^+ is most noticeable at the lowest concentrations of ADP or ATP, and, as illustrated by the common intersect in the Lineweaver–Burk plots of Figure 2a,b, can be overcome by increasing the adenine nucleotide levels. The V_{max} is not altered by KCl, being 3.0 and 9.6 nmoles per mg per min for ATP and ADP, respectively, whereas the binding affinity of the adenine nucleotides is greatly affected. The $K_{m,app}$ for ADP is decreased by 20 mM KCl from 48 to 10 μM, and that of ATP from 219 to 15 μM. It should be pointed out that the K_m's in the presence of K^+ are higher than the values of 1.3 μM (ADP) and 2.5 μM (ATP) obtained by Pfaff *et al.* (1969). However, these authors used protein concentrations of 0.07 mg/ml, which yields smaller K_m's by lowering the endogenous pool of ADP and ATP.

Heidrich *et al.* (1970) have recently shown that the mitochrondrial inner membrane–matrix preparation exhibits a completely different migration in free-flow electrophoresis than does the purified outer membrane, and concluded that the outer membrane is the "sole determinant" of surface charge. If so, inner membrane preparations might be qualitatively different with respect to the cation dependency of the adenine nucleotide exchange. As Figure 3 demonstrates, however, there is no significant difference in the binding affinity or the maximum velocity of ADP exchange in digitonin-treated inner membrane preparations compared to intact mitochondria (Figure 2). If differences in surface charge do exist, they are too subtle to be recognized by measurements of the exchange kinetics of the ADP anion.

Although Amons *et al.* (1968) have concluded that the dinitrophenol-stimulated ATPase activity is absolutely dependent on cations, the following results suggest that the primary effect of cations may be on the adenine nucleotide translocation. Figure 4, in which heart mitochondria were incubated with 0.5 mM Tris-TES (pH 7.1), and 20 μM Tris-ADP or ATP, shows that 20 mM K^+ gives a maximum enhancement of the adenine nucleotide exchange. (The activity coefficients of K^+ are somewhat lower, but will not be considered here.) When the total monovalent cation concentration is measured by including $Tris^+$, as shown by the dotted lines, there appears to be an absolute requirement for cations at these low levels of ADP and ATP. A reciprocal plot of the rate of exchange *vs.* the concentration (Figure 5) reveals a $K_{m,app}$ for K^+ of 2.31 and 1.45 mM in the presence of 20 μM ATP and ADP, respectively.

The question whether cations influence the binding of the adenine nucleotides to the membrane, or the transport through the membrane, was approached in two ways. If cations affect the transport through the membrane, gramicidin, which increases the passive cation permeability (Henderson *et al.*, 1969), should lower the K_m of the cation. As Figure 6 shows, gramicidin stimulates the ATP exchange, but does not increase the binding affinity for Na^+, as revealed by the rather uniform 35–45% stimulation at all levels of Na^+. A more direct means was to measure the effect of cation on the binding of atractyloside. Atractyloside, a trivalent anion at neutral pH (Bruni, 1966), is bound to the adenine nucleotide translocase

[1] Abbreviations used are: EGTA, ethylene glycol tetraacetic acid; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; mCCP, *m*-chlorocarbonyl cyanide phenylhydrazone; MES, 2(*N*-morpholino)-ethanesulfonic acid; MOPS, morpholinopropanesulfonic acid.

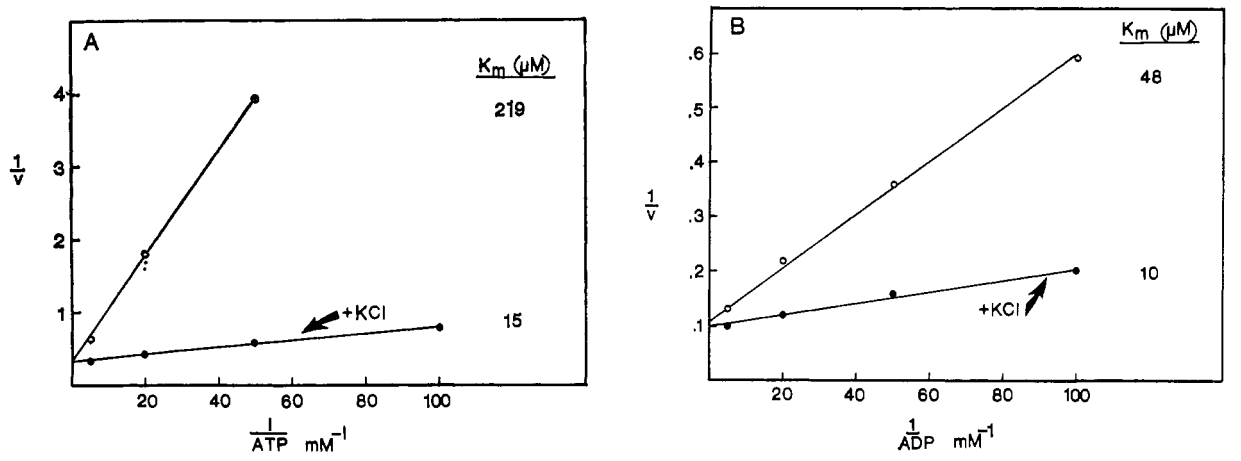

FIGURE 2: The dependence of the adenine nucleotide exchange on K^+. The Lineweaver–Burk plots are taken from the data presented in Figure 1.

on the outer surface of the inner membrane (Meisner and Klingenberg, 1968; Vignais and Vignais, 1970), and should not be influenced by Na^+ or K^+, if changes in membrane permeability are involved. In Figure 7, the respiratory control ratio is used as an indicator of the binding of atractyloside to the membrane (Bruni, 1966), and is lowered considerably by addition of 20 mM K^+ to the medium. Apparently, the absence of cation renders the membrane more negatively charged, and decreases the affinity toward atractyloside. This effect can be overcome by increasing the atractyloside concentration, in an analogous manner to the decrease of the cation dependence of the exchange by increasing the extramitochondrial concentration of ADP or ATP. These results are taken as evidence that the binding of anions, rather than the transport through the membrane, is affected by cations.

The general nature of the stimulation of adenine nucleotide exchange by cations is revealed in Table I, where the effect of a variety of mono- and divalent cations on the exchange of 10 μM Tris-ATP is considered. While K^+ is most effective and stimulates ATP exchange sevenfold, other cations such as $Tris^+$, Li^+, and Mg^{2+} also enhance the exchange of ATP considerably. The stimulation of the ATP exchange by a wide variety of cations is further evidence that these ions are not being translocated through the inner membrane accompanying the ADP or ATP exchange.

The dependence of the exchange of ATP on the concentration of Mg^{2+} and Ca^{2+} is depicted in Figure 8. From a comparison with Figure 4, it is obvious that divalent cations, in particular Ca^{2+}, are more effective than monovalent cations in stimulating the ATP exchange. Reciprocal $1/v$ *vs.* $1/S$ plots give a $K_{m,app}$ of 0.87 and 0.020 mM for the activation by Mg^{2+} and Ca^{2+}, respectively. Since the stimulation of the ATP exchange by divalent cations is dependent upon the protein

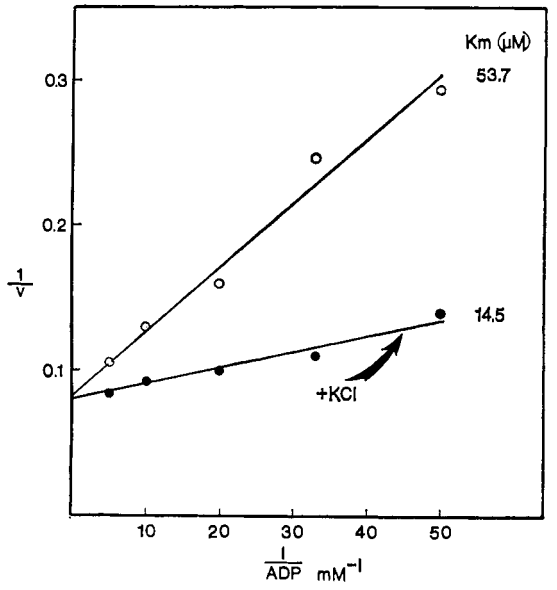

FIGURE 3: Dependence of ADP exchange on K^+ in digitonin-treated inner membrane particles. Digitonin particles were prepared as previously described (Hoppel and Cooper, 1969; Meisner, 1970), and 2.16 mg of protein incubated at 5° with 0.26 M sucrose, 0.2 mM EGTA, 1 mM Tris-TES, and when indicated, 20 mM KCl. The exchange was initiated with Tris-ADP, and stopped at 15 sec with 10 μM atractyloside. Final pH, 7.25.

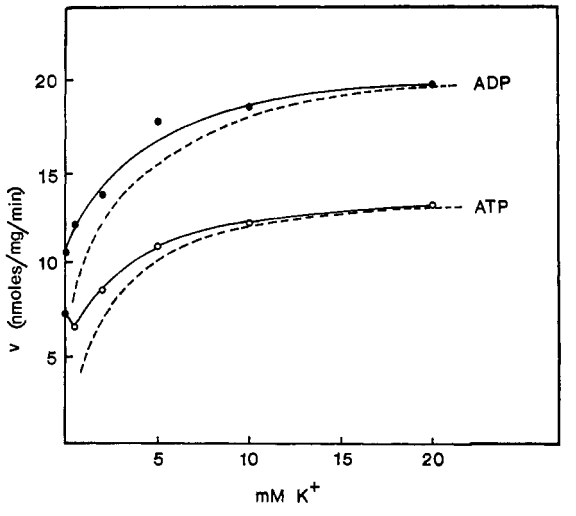

FIGURE 4: Effect of K^+ on the exchange of ATP and ADP in rat heart mitochondria. Labeled mitochondria (1.02 mg) were added to 0.26 M sucrose, 0.2 mM Tris-EGTA, 0.5 mM Tris-TES, ± KCl, and the exchange initiated with 20 μM Tris-ADP or Tris-ATP. Solid lines represent the [K^+], dotted lines the total [cation], using 3× [EGTA] + 1.4× [TES]. Final pH. 7.1, temperature, 5°.

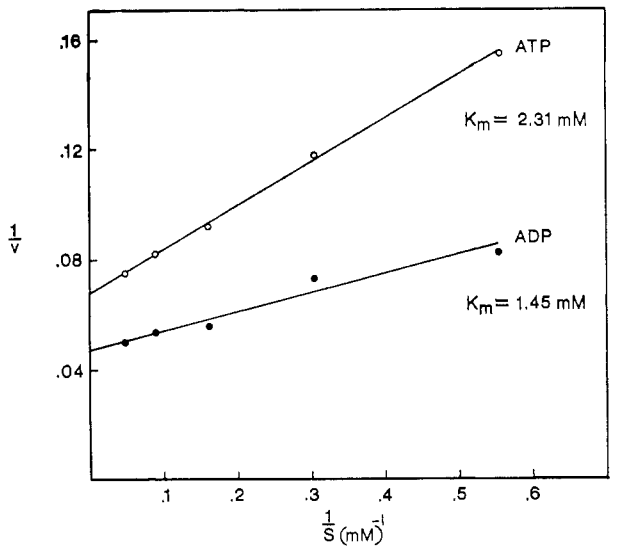

FIGURE 5: Lineweaver–Burk plots from Figure 4 of $1/V$ *vs.* 1/total cation concentration. The K_m values represent apparent binding affinities of total cations in the presence of 20 μM ADP or ATP.

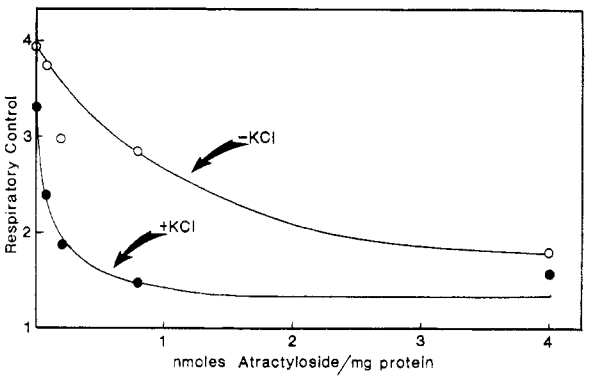

FIGURE 7: Effect of K^+ on the binding of atractyloside to rat liver mitochondria. Mitochondria (2.52 mg) were added to 1 ml of medium containing 0.2 M sucrose, 2 mM Tris-TES (pH 7.6), ±20 mM KCl, atractyloside as indicated, and incubated for 2 min at 0°. The suspension was centrifuged for 45 sec at 0°, and washed once with medium minus atractyloside. The degree of binding of atractyloside was determined by polarographically measuring state III–state IV respiration at 30° in 0.2 M sucrose, and 4 mM triethanolamine (pH 7.2), 2 mM $MgCl_2$, 2 mM P_i, 3 mM succinate, and ±0.3 mM ADP. Final volume, 0.65 ml.

concentration (H. Meisner, unpublished data), the K_m of activation is better expressed as 780 and 11 nmoles per mg for Mg^{2+} and Ca^{2+}.

In studying the stimulation of the adenine nucleotide exchange by cations, the chloride salt has been used in all cases. As Table II shows, however, other anions such as chlorate, nitrate, and bromide can be substituted for chloride, using Na^+ as the cation, with nearly the same degree of stimulation. The exception appears to be Na_2SO_4, which does not increase the ATP exchange.

The increase of the adenine nucleotide exchange with cations suggests that protons may have a similar effect, and at a low pH, should eliminate the enhancement of the exchange by cations. This is supported by the data presented in Figure 9, showing that as the concentration of protons is decreased in a potassium-free medium, the exchange of ATP and ADP in rat heart mitochondria is reduced. When 20 mM K^+ is present, however, raising the pH either increases the ADP exchange or does not reduce the ATP exchange as much. At pH 6.15, there is no effect of K^+ on the initial exchange rate of ADP and ATP.

Uncoupling agents have been shown to selectively stimulate the exchange of ATP, and not affect the exchange of ADP (Pfaff and Klingenberg, 1968). That this effect of uncoupler on the ATP exchange is dependent on cations is shown in a typical experiment presented in Table III. Oligomycin was added to prevent breakdown of ATP. The addition of KCl plus mCCP enhanced the exchange of ATP to 5.0 nmoles per mg per min, which is greater than the exchange with either KCl (1.7) or mCCP (1.4) alone. As expected, while the ADP exchange is enhanced by KCl from 2.7 to 3.7 nmoles per mg per min, the further addition of mCCP does not increase the exchange rate appreciably. The ADP exchange in the presence of mCCP is reduced from 2.7 to 1.5 nmoles per mg per min, which perhaps reflects the anionic nature of the uncoupler.

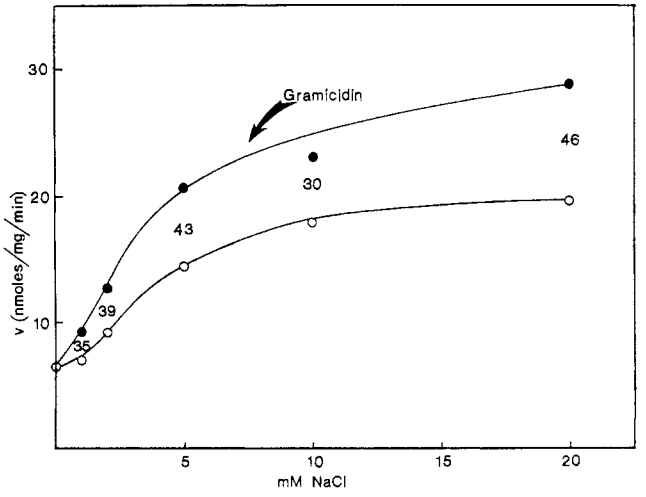

FIGURE 6: Effect of gramicidin on the Na^+-dependent ATP exchange in rat heart mitochondria. Labeled mitochondria (1.08 mg) were added to 0.26 M sucrose, 0.5 mM Tris-TES, and 0.2 mM Tris-EGTA, ± NaCl, and when indicated, 0.5 μg of gramicidin. The exchange was initiated with 20 μM Tris-ATP, and stopped at 10 sec with 10 μM atractyloside. Final pH, 7.1; temperature, 5°.

TABLE I: Effect of Cations on the ATP Exchange in Rat Heart Mitochondria.[a]

Additions	v (nmoles per mg per min)
	1.13
Li^+	6.82
Na^+	6.75
K^+	7.37
Cs^+	6.91
$Tris^+$	5.87
Mg^{2+}	5.78
Ca^{2+}	6.00

[a] Labeled rat heart mitochondria (0.97 mg) were added to 0.26 M sucrose, 0.5 mM Tris-TES, 0.2 mM Tris-EGTA, and ±10 mM cation as the chloride salt. The exchange was initiated with 10 μM Tris-ATP, and stopped with 10 μM atractyloside. Final $pH_{25°}$, 7.1.

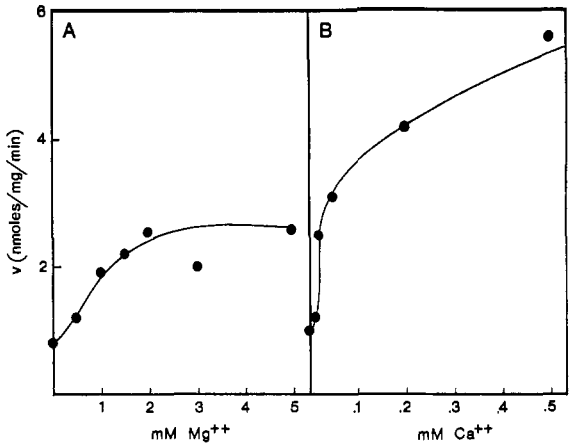

FIGURE 8: Effect of Mg^{2+} and Ca^{2+} on the exchange of ATP in rat heart mitochondria. Labeled mitochondria (1.12 mg in A, 1.75 mg in B) were incubated at 0° in 0.26 M sucrose, 1 mM Tris-TES, Ca^{2+}, or Mg^{2+} as indicated, and the exchange initiated with 10 μM Tris-ATP. Final pH, 7.6.

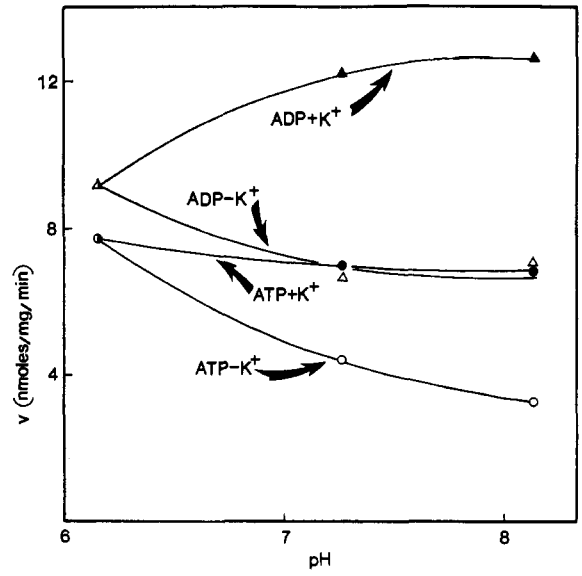

FIGURE 9: Effect of pH on the exchange of ADP and ATP in rat heart mitochondria. Labeled mitochondria (1.44 mg) were incubated at 5° with 0.26 M sucrose, 0.2 mM EGTA, 1 mM buffer, and when indicated, 20 mM KCl. The exchange was initiated with 10 μM Tris-ADP or Tris-ATP, and stopped at 10 sec with 10 μM atractyloside. Final pH was 6.15, 7.26, and 8.13.

It is concluded that K^+ provides the positive surface charges on the membrane that attract the adenine nucleotide and perhaps also the mCCP anion.

In addition to the stimulatory effect of cations on the adenine nucleotide exchange, it is possible that there is a separate effect on the oxidative phosphorylation system (Lardy and Wellman, 1953; Cereijo-Santalo, 1968; Amons *et al.*, 1968). Table IV shows the effect of cations and protons on the endogenous ADP and ATP levels in rat liver mitochondria under conditions of the adenine nucleotide exchange. When the pH is increased from 6.0 to 7.6, there is an increase in the ATP:ADP ratio in the presence of K^+ from 0.42 to 0.64, and ($-K^+$) from 0.28 to 0.31. If pH is maintained constant at 7.6, K^+ increases the ATP:ADP ratio from 0.31 to 0.64 (expt 1) or from 1.14 to 1.59 (expt 2), and oligomycin blocks this effect. Experiment 2 shows that Na^+ and Mg^{2+} are also effective, raising the ATP:ADP ratio to 1.36 and 1.27, respectively.

Although these data confirm the well-known stimulatory effect of K^+ and other cations on oxidative phosphorylation, the increase in the ADP and ATP exchange by K^+ in the presence of oligomycin (Table III) makes it unlikely that the primary effect of cation is to increase the intramitochondrial ATP levels, and thus possibly the exchange of exogenous adenine nucleotide. That the ratio of ATP:ADP has no effect on the rate of exchange has been demonstrated by Pfaff and Klingenberg (1968), who concluded that the exchange was governed by the sum of ADP plus ATP.

Discussion

Effect of Cations and Protons on Adenine Nucleotide Translocation. This study has shown that protons, as well as a variety of cations, stimulate the exchange of ADP and ATP in isolated rat liver and heart mitochondria, provided that low concentrations (less than 20 μM) of adenine nucleotides are used. The small or negligible effect of cations in previous studies (Pfaff and Klingenberg, 1968; Pfaff *et al.*, 1969) may be attributed to the high levels of ADP or ATP employed (0.2–0.5 mM),

TABLE II: Effect of Anions on the Exchange of ATP in Rat Heart Mitochondria.[a]

Additions	v (nmoles per mg per min)
	3.1
Cl^-	5.8
CH_3COO^-	4.6
ClO_3^-	5.4
NO_3^-	5.5
SO_4^{2-}	2.8
Br^-	5.1
HCO_3^-	5.1

[a] Labeled mitochondria (0.95 mg) were incubated at 5° in a sucrose–MOPS–EDTA medium (pH 7.7) and 20 mM anion (Na^+) as indicated. The exchange was initiated with 20 μM Tris-ATP, and stopped at 15 sec with 10 μM atractyloside.

TABLE III: Dependence of the Uncoupler-Stimulated ATP Exchange in Rat Liver Mitochondria on K^+.[a]

Additions	v (nmoles per mg per min) ATP	ADP
	0	2.7
KCl	1.7	3.7
mCCP	1.4	1.5
KCl + mCCP	5.0	4.3

[a] Labeled rat liver mitochondria (1.6 mg) were incubated at 5° with 0.26 M sucrose, 2 mM Tris-TES, 0.2 mM Tris-EGTA, 10 μg of oligomycin, and, when indicated, 1 μM mCCP, and 20 mM KCl. The exchange was initiated with 50 μM Tris-ADP or Tris-ATP, and stopped at 20 sec with 10 μM atractyloside. Final pH, 7.6.

TABLE IV: Effect of Cations and pH on ADP and ATP Levels in Rat Liver Mitochondria.[a]

	Additions	pH	nmoles/mg ADP	nmoles/mg ATP	ATP:ADP
Expt 1		6.0	6.15	1.69	0.28
	K^+	6.0	5.66	2.40	0.42
		7.6	6.24	1.94	0.31
	K^+	7.6	5.10	3.25	0.64
Expt 2		7.6	3.54	4.03	1.14
	K^+	7.6	3.85	6.11	1.59
	K^+ + oligomycin	7.6	3.85	4.29	1.11
	Na^+	7.6	3.76	5.10	1.36
	Mg^{2+}	7.6	4.02	5.10	1.27

[a] Mitochondria (expt 1, 3.0 mg; expt 2, 1.85 mg) were incubated at 0° in 0.26 M sucrose, 2 mM Tris-MES or TES buffer (pH 6.0 or 7.6), and when indicated, 20 mM K^+ or Na^+, 2 mM Mg^{2+} ,and 2.5 μg of oligomycin in a total volume of 0.2 ml. After 1 min, 100 μl of 20% perchloric acid was added, the supernatant was neutralized, and the adenine nucleotides were measured enzymatically.

which, as we have seen, competitively remove the activating effect of cations. Similarly, the negligible effect of pH on the adenine nucleotide translocase originally observed (Pfaff and Klingenberg, 1968) is undoubtedly due to the presence of cations and the high levels of adenine nucleotides employed. This is supported by recent studies (Weidemann *et al.*, 1970) carried out between 1 and 20 μM ADP, minus cation, showing a 90% decrease in the amount of ADP specifically bound to the carrier sites upon raising the pH from 7.0 to 8.0.

The wide diversity of positive charges that can stimulate the translocation, coupled with experimental evidence that the mitochondrial membrane is normally impermeable to protons and cations (Chappell and Crofts, 1965a; Mitchell, 1966) makes it likely that the binding of adenine nucleotides to the membrane, rather than the translocation process, is primarily affected by the positive charges. This interpretation is reinforced by the cation-dependent binding of atractyloside, a nonpenetrating anionic inhibitor of the adenine nucleotide exchange, and by the failure of gramicidin to decrease the concentration of Na^+ necessary to half-maximally stimulate the ATP exchange. The translocation of adenine nucleotides may thus be considered to consist of a cation- and proton-dependent binding, followed by a cation-independent translocation.

Effect of Cations on Phosphorylation. In addition to the cation requirement of the adenine nucleotide translocase, the present data show that extramitochondrial Mg^{2+}, Na^+, and K^+ increase the endogenous levels of ATP, confirming other experiments that point toward a dependence of oxidative phosphorylation and ATPase activity on K^+ and other cations in intact mitochondria (Lardy and Wellman, 1953; Cereijo-Santalo, 1968; Amons *et al.*, 1968). Assuming that extramitochondrial ADP and ATP must first be translocated across the mitochondrial membrane before interacting with the phosphorylation sites (Chappell and Crofts, 1965b; Heldt *et al.*, 1965), and that the membrane is impermeable to cations and protons (Chappell and Crofts, 1965a; Mitchell, 1966), the oligomycin-sensitive stimulation of the phosphorylation of endogenous ADP to ATP most likely represents an indirect effect of cations on the oxidative phosphorylation system. This is supported by work with phosphorylating submitochondrial particles, which show a low permeability toward cations, and no respiratory stimulation upon addition of cations (Beyer *et al.*, 1969; Chance and Mela, 1967). The submitochondrial particles, which are thought to be turned "inside out" (Lee and Ernster, 1966; Chance and Mela, 1967), and are atractyloside insensitive (Low *et al.*, 1963; Brierley and O'Brien, 1965), would be expected to show a stimulation of respiration if the oxidative phosphorylation system were accessible to cations.

Control by Cations in Vivo. The *in vivo* importance of intracellular ions in the control of the adenine nucleotide translocase must remain an open question, but the low concentration of Ca^{2+} (20 μM) needed to activate the exchange suggests that this ion may have a physiological role. Furthermore, there is evidence that intracellular ions are not in a simple aqueous electrolyte solution, and that the osmotically active concentration may be considerably less than suspected (Czeisler *et al.*, 1970; Ling, 1960). Czeisler *et al.* (1970) and Cope (1970) have recently found that only 30–40% of the Na^+ in frog skeletal muscle is detectable by nuclear magnetic resonance as being osmotically active, the rest probably being bound to cellular polyelectrolyte macromolecules. Although microelectrode studies by Lev (1964) have indicated that the activity of K^+ in frog muscle is greater than Na^+, this has not been upheld by nuclear magnetic resonance studies (Damadian, 1969; Cope and Damadian, 1970).

The susceptibility of the membrane to the ionic environment has been shown by measurements of the conductivity of different types of artificial membranes in the laboratories of Lehninger (Hopfer *et al.*, 1970) and Eisenman (McLaughlin *et al.*, 1970). This work indicates that the charge a phospholipid bilayer carries can change the electrostatic attraction of the membrane toward ions by many orders of magnitude. In this manner, small changes in the extramitochondrial cation concentration may have a noticeable effect on the rate of the adenine nucleotide translocation.

Acknowledgments

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neo-Inositol in Mammalian Tissues. Identification, Measurement, and Enzymatic Synthesis from Mannose 6-Phosphate*

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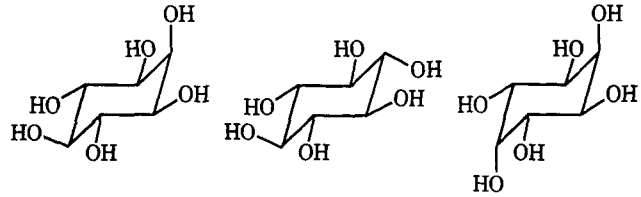
ABSTRACT: *neo*-Inositol has been isolated from calf brain and identified, as its trimethylsilyl ether and as its acetate ester, by gas chromatography and by combined gas chromatography–mass spectrometry. *neo*-Inositol has been found to be present in micromolar amounts in the brain, heart, kidney, testis, and spleen of the rat, but absent from liver. We have shown that the same enzyme preparation which converts D-glucose 6-phosphate into L-*myo*-inositol 1-phosphate is capable of transforming D-mannose 6-phosphate to a substance which, by mass spectrometry, as its trimethylsilyl ether ester is an inositol phosphate. By analogy with the cyclization of D-glucose 6. phosphate this product should be L-*neo*-inositol 1-phosphate. We have found that this enzyme preparation displays typical Michaelis–Menten kinetics with D-mannose 6-phosphate giving a K_m value of 5.6×10^{-3} as compared to a K_m of 6.2×10^{-3} with D-glucose 6-phosphate. A comparison of the V_{max} values for the two products using the same enzyme preparation shows *myo*-inositol to be formed at a rate about 200-fold faster than *neo*-inositol.

During a study in which we identified *myo*-inosose-2 by the gas chromatography of a lyophilized and trimethylsilylated extract of calf brain an unknown substance was found to have been inadvertantly isolated along with the inosose. A mass spectrum of the unknown was obtained at the same time that trimethylsilyl- (Me_3Si) *myo*-inosose-2 was identified by combined gas chromatography–mass spectrometry (Sherman *et al.*, 1968). The unknown substance has now been identified as *neo*-inositol.



myo-inositol *scyllo*-inositol *neo*-inositol

neo-Inositol hexaphosphate has been recognized as a soil constituent where it is found accompanied by *myo*-inositol hexaphosphate and *scyllo*-inositol hexaphosphate (Cosgrove, 1963; Cosgrove and Tate, 1963).

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