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# EVIDENCE FOR THE ELECTROGENIC NATURE OF THE ATP-ADP EXCHANGE SYSTEM IN RAT LIVER MITOCHONDRIA

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## SUMMARY

A transient decrease in the fluorescent intensity of the dye, 3,3'-dipropylthiodicarbocyanine iodide was seen upon the addition of ATP to rat liver mitochondria which had been pre-treated with $2 \cdot 10^{-6}$ M rotenone and 3.3 $\mu$g oligomycin/ml. This decrease which is indicative of a hyperpolarization (internal more negative) was half maximal at $2 \cdot 10^{-5}$ M ATP and was not seen with either ADP or AMP. Atractyloside, bongkrekic acid, ADP and to a lesser extent AMP inhibited the decrease observed with ATP. The characteristics of inhibition by these compounds were similar to those observed in experiments where either transport or binding of adenine nucleotides was measured. The addition of ADP after ATP led to a transient increase in fluorescent intensity indicative of depolarization. This increase was also blocked by atractyloside and bongkrekic acid. The evidence presented supports the hypothesis that the exchange of ADP and ATP via an adenine nucleotide exchange mechanism is electrogenic.

## INTRODUCTION

The studies of Klingenberg and his associates (ref. 1 and see review in ref. 2) have suggested that the exchange of ADP and ATP via a translocation mechanism in the mitochondrion is electrogenic. Evidence for the electrogenic nature of this exchange (presumably of $ATP^{4-}$ for $ADP^{3-}$) is based on the observation that in the presence of appropriate ionophores movements of cations can be recorded which parallel the exchange of nucleotides [3, 4]. Hence, when $ATP_{external}^{4-}$ exchanges with $ADP_{internal}^{3-}$ there is an uptake of $H^+$ in the presence of uncouplers or of $K^+$ in the presence of valinomycin. The electrogenic behavior of the exchange may account for the asymmetry observed in the adenine nucleotide exchange system under certain metabolic conditions. In the coupled state, kinetic studies indicate that ADP is favored over ATP for entrance, although ATP and ADP compete equally well for exit [1]. This asymmetry is lost when the mitochondria are uncoupled. According to Klingenberg [1] the large negative (internal) membrane potential developed across the inner membrane in the coupled state would inhibit the electrogenic $ATP_{external}^{4-}$/

$ADP_{internal}^{3-}$ exchange but not electroneutral exchanges or the exchange of $ADP_{external}^{3-}$ for $ATP_{internal}^{4-}$. Since the potential across the inner membrane of the mitochondrion would drop towards zero under the influence of uncouplers or of $K^+$ plus valinomycin, the exchange of $ATP_{external}^{4-}$ for $ADP_{internal}^{3-}$ should not be inhibited in the uncoupled state. Hence, Klingenberg [2] has pointed out that the electrogenic nature of the exchange would be a substantial factor in the regulation of the ratio of ATP/ADP between the inner and outer spaces of the mitochondrion, favoring a higher ratio of ATP/ADP outside than inside. The regulation of this ratio would have an influence on the phosphorylation potential of ATP.

Since the fluorescent intensity of the dye, 3,3'-dipropylthiodicarbocyanine iodide, was shown to be sensitive to the membrane potential in mitochondria in a previous report [5], the same method was employed subsequently to study the electrogenic nature of this ATP-ADP exchange mechanism.

## METHODS

Livers were removed from rats of the Wistar strain and the mitochondria isolated using the technique described by Kennedy and Lehninger [6]. Protein content of the mitochondrial preparations was determined by the method of Lowry et al. [7].

The methods used to record fluorescence have been described previously [5, 8]. The mitochondrial suspensions used in these experiments contained 0.5 mg of mitochondrial protein per 3 ml of a medium containing 0.225 M sucrose, 0.036 M NaCl and 0.02 M triethanolamine buffered to pH 7.4 with NaOH in most cases or to 7.0 where noted. The fluorescent dye 3,3'-dipropylthiodicarbocyanine iodide, kindly supplied by Dr. Alan Waggoner of Amherst College, was used at a final concentration of $1.5 \cdot 10^{-6}$ M. Stock solutions of dye (0.5 mg/ml), rotenone (0.003 M) and oligomycin (5 mg/ml) were added as solutions in ethanol; atractyloside (0.0012 M), ATP (0.02 M), ADP (0.02 M) and AMP (0.09 M), as aqueous solutions buffered to pH 7.4 with Tris-(hydroxymethyl) aminomethane. Bongkrekic acid was obtained from Dr. W. Berends of the University of Delft as a solution (12.5 mg/ml) in 2 M $NH_4OH$. From this solution, a stock solution of bongkrekic acid containing 1.25 mg/ml was prepared in 10 mM sodium phosphate buffer at pH 7.4. Experiments with bongkrekic acid were carried out at 25 °C; and all others reported below, at room temperature (approximately 21 °C). Other details are given in the figure legends and text.

## RESULTS

The intensity of fluorescence of the dye attained a constant level 1–2 min after its addition to suspension of rat liver mitochondria. When $2 \cdot 10^{-6}$ M rotenone was added to inhibit endogenous metabolism, there was an increase in fluorescent intensity of approx. 10 %. Rotenone was added to all suspensions of mitochondria prior to the addition of dye and any other reagents. Following the addition of ATP (final concentration equals 100 $\mu$M) there was a rapid decrease in fluorescence which occurred in 2 phases: a very rapid decrease followed by a slower decline to a new level which was maintained for several minutes. The change recorded here is comparable to the decrease reported previously with hamster liver mitochondria [5].

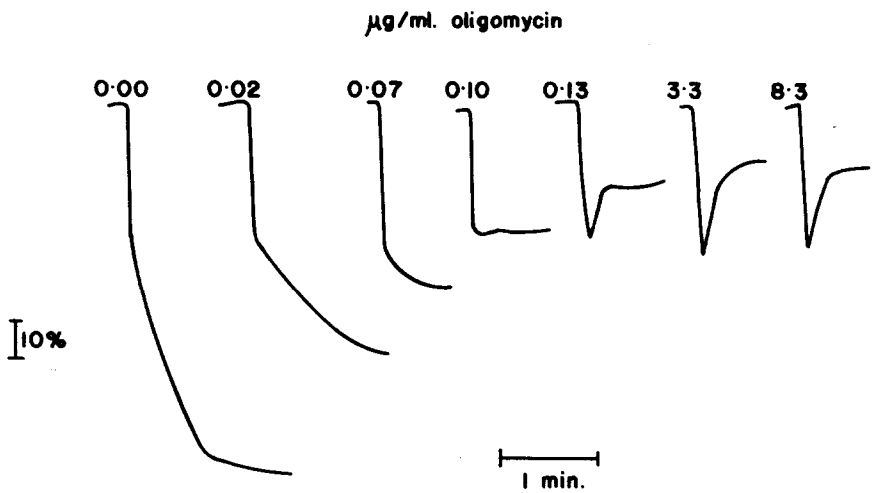


Fig. 1. Relative changes in fluorescent intensity of 3,3′-dipropylthiocarbocyanine iodide (final concentration, $1.5 \cdot 10^{-6}$ M) of suspensions of rat liver mitochondria (0.16 mg protein/ml suspension) in a medium containing 0.225 M sucrose, 0.036 M NaCl, $2 \cdot 10^{-6}$ M rotenone and 0.02 M triethanolamine buffered to pH 7.4 with NaOH upon the addition of ATP (final concentration, $10^{-4}$ M) in the presence of varying amounts of oligomycin. Oligomycin added 2 min before the start of the record.

The decrease obtained with ATP may be reversed almost completely within 30 s by the addition of 3.3 µg/ml of oligomycin, an inhibitor of mitochondrial ATPase. (Studies have demonstrated, however, that the 2,4-dinitrophenol-stimulated ATPase activity is not completely inhibited by this concentration of oligomycin; Laris, unpublished observations.) Although this concentration of oligomycin could reverse the effect of ATP on fluorescence almost completely, the initial rapid drop in fluorescence was still observed upon the addition of ATP to mitochondria pre-treated with a range (0.02–8.3 µg/ml) of concentrations of oligomycin (Fig. 1). The initial phase seen with ATP appeared, therefore, to be insensitive to oligomycin. The slower decline, however, was very sensitive to oligomycin and was diminished with increasing oligomycin concentrations (Fig. 1). With higher concentrations of oligomycin, the slow decline was eliminated and the fluorescence rose following the initial rapid phase to a new level which was generally lower than that seen prior to the addition of ATP. The nature of the initial rapid change in fluorescent intensity dependent on ATP but insensitive to oligomycin is explored in the remainder of this study.

*Dependence on ATP concentration*

The magnitude of the decrease in fluorescent intensity recorded upon the addition of ATP to mitochondria pretreated with 3.3 µg oligomycin/ml varied with the ATP concentration. As shown in Fig. 2, with increasing ATP concentration the percentage change in fluorescence increased and approached a maximum. Since apparent saturation was observed with ATP, the data were plotted (Fig. 3) as substrate/velocity vs. substrate, where the velocity was equated to the initial percent change in fluorescent intensity. At $2 \cdot 10^{-5}$ M ATP, the change in fluorescent intensity was half maximal.

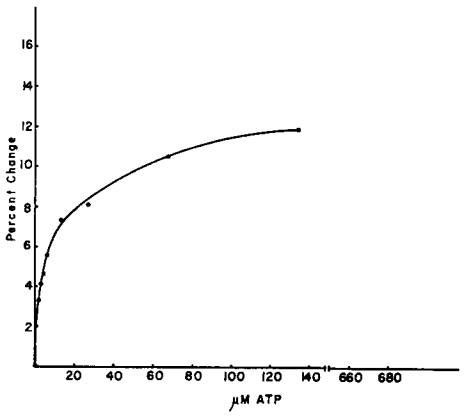


Fig. 2. Percentage decrease in fluorescent intensity of 3,3-dipropylthiocarbocyanine iodide (final concentration, $1.5 \cdot 10^{-6}$ M) in suspensions of rat liver mitochondria upon addition of varying amounts of ATP. The medium contained $2 \cdot 10^{-6}$ M rotenone and 3.3 μg/ml oligomycin. For other details see Fig. 1.

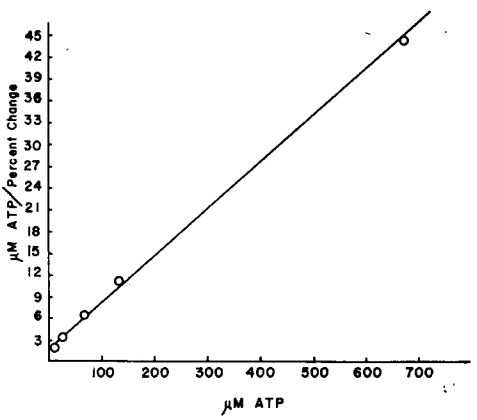


Fig. 3. Plot of the data from Fig. 2, where the ordinate represents ATP concentration/percent decrease in fluorescent intensity vs. ATP concentration.

*Addition of atractyloside*

Although the addition of atractyloside, a specific inhibitor of the adenine nucleotide exchange system of mitochondrial membranes [9], had no influence on the level of fluorescent intensity, it did markedly inhibit the ATP dependent-oligomycin insensitive decrease in fluorescent intensity. The relationship between inhibition of the ATP dependent-oligomycin insensitive change in fluorescent intensity and the logarithm of atractyloside concentration is given in Fig. 4. A 50 % reduction was seen with $1.5 \cdot 10^{-6}$ M atractyloside when $1 \cdot 10^{-4}$ M ATP was used. With $3.3 \cdot 10^{-5}$ M ATP, 50 % inhibition occurred with $5 \cdot 10^{-8}$ M atractyloside.

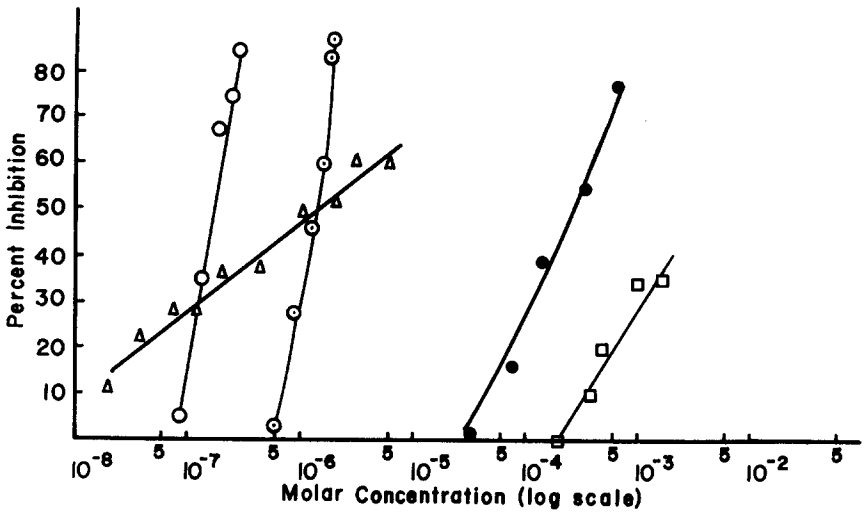


Fig. 4. Percentage inhibition of change (decrease) in fluorescent intensity of 3,3-dipropylthiocarbocyanine iodide upon the addition of $10^{-4}$ M ATP versus log concentration of atractyloside (△), ADP (●), AMP (□), bongkrekic acid (○) in media at pH 7.4 or bongkrekic acid (○) in media at pH 7.0. The medium contained $2 \cdot 10^{-6}$ M rotenone and 3.3 μg/ml oligomycin. Bongkrekic acid was added at 25 °C, 1.5 min prior to ATP. In all other cases, the inhibitor was added at 20 °C, less than 20 s prior to ATP. For other details, see Fig. 1 and text.

*Addition of bongkrekic acid*

The addition of bongkrekic acid, another inhibitor of the adenine nucleotide exchange system [10, 11], in concentrations up to $6 \cdot 10^{-5}$ M, had no influence on the level of fluorescent intensity. When mitochondria were incubated at 25 °C with bongkrekic acid for 1.5 min before the addition of ATP (final concentration, $1 \cdot 10^{-4}$ M), inhibition of the ATP dependent-oligomycin insensitive change in fluorescent intensity was observed (see Fig. 4). The inhibitory effect of bongkrekic acid was markedly influenced by pH (Fig. 4), e.g., 50% inhibition was observed with $1.5 \cdot 10^{-6}$ M bongkrekic acid at pH 7.4 and with $1.5 \cdot 10^{-7}$ M bongkrekic acid at pH 7.0. The influence of bongkrekic acid was also shown to be very sensitive to temperature. When mitochondria were incubated at 19 °C with bongkrekic acid for 1.5 min before the addition of ATP ($1 \cdot 10^{-4}$ M), no inhibition was observed with $1.9 \cdot 10^{-6}$ M bongkrekic acid and 50% with $9.5 \cdot 10^{-6}$ M bongkrekic acid.

*Addition of ADP*

When various amounts of ADP (final concentration between $3 \cdot 10^{-5}$ and $6 \cdot 10^{-4}$ M) were added to mitochondria in the presence or absence of oligomycin, either no change or only a small increase in fluorescent intensity was observed. Although the addition of ADP alone to mitochondria had negligible effects on the fluorescent intensity, the addition of ADP did influence the changes seen with ATP. In the presence of oligomycin (3.3 μg/ml), the addition of ADP to mitochondria prior to the addition of ATP reduced the ATP dependent-oligomycin insensitive decrease in fluorescent intensity as seen in Fig. 4. A 50% reduction was seen with $2.3 \cdot 10^{-4}$ M ADP when $1 \cdot 10^{-4}$ M ATP was used. With $1.3 \cdot 10^{-5}$ M ATP 50% reduction occurred with $4.3 \cdot 10^{-5}$ M ADP.

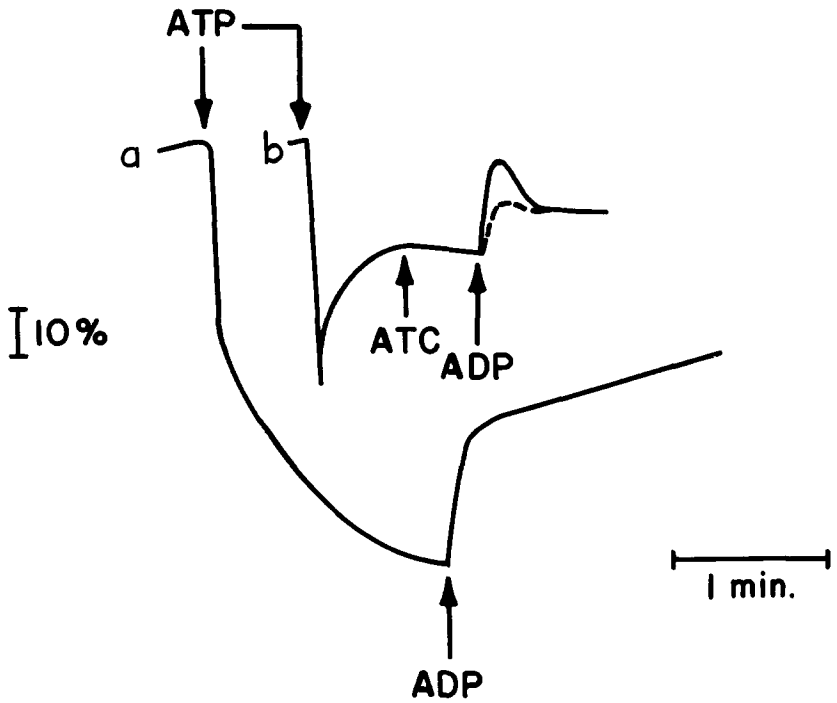


Fig. 5. Relative change in fluorescent intensity of 3,3-dipropylthiocarbocyanine iodide in a suspension of rat liver mitochondria after the addition of ATP (final concentration equals $1 \cdot 10^{-4}$ M) in the absence (a) or presence (b) of 3.3 μg/ml oligomycin. ADP (final concentration, $6.5 \cdot 10^{-4}$ M) added where indicated. In (b), solid line represents trace in the absence of atractyloside while the broken line (– – –) indicates the change in the presence of atractyloside (final concentration, $1.7 \cdot 10^{-6}$ M) added at the point marked ATC.

When ADP was added after ATP (after the fluorescent intensity had returned to a constant level) in the presence of 3.3 μg/ml oligomycin, a rapid increase in fluorescent intensity was recorded (Fig. 5b). This increase, which was inhibited by atractyloside (Fig. 5b) or bongkrekic acid (not shown), was transitory with the fluorescent intensity returning towards the original level within 20 s.

If ADP (final concentration equals $6.5 \cdot 10^{-4}$ M) was added to mitochondria which had been incubated with ATP in the absence of oligomycin (Fig. 5a) or in the presence of low concentrations of oligomycin, there was an increase in fluorescent intensity which occurred in two phases: an initial rapid increase comparable to that seen in the presence of 3.3 μg/ml oligomycin, followed by a slow rise in fluorescent intensity.

*Addition of AMP*

A small rise (up to 5 %) in fluorescent intensity was recorded when concentrations of AMP up to 2 mM were added to mitochondria in the presence of oligomycin. In this range of AMP concentrations the fluorescent intensity then returned to within 2 % of the original value in 30 s. With the addition of higher concentrations of AMP, however, the fluorescence level remained somewhat higher than the level

seen prior to the addition of AMP. The increase observed upon the addition of AMP was not inhibited by the presence of atractyloside. The presence of AMP reduced the decrease in fluorescent intensity observed when ATP was added. The relationship between inhibition of the ATP dependent-oligomycin insensitive change and the logarithm of AMP concentration is presented in Fig. 4. The concentrations of AMP (2 mM or below) presented in Fig. 4 are limited to those which had only a small transient influence on fluorescent intensity.

*Addition of other agents*

The addition of 2,4-dinitrophenol (final concentration $2 \cdot 10^{-5}$ M) led to an increase (approx. 8 %) in fluorescent intensity which returned to the original level in 2–3 min. When ATP ($1 \cdot 10^{-4}$ M) was added in the presence of 2,4-dinitrophenol, only a very small decrease, if any (2 % or less) was observed.

The addition of either EDTA (to 1 mM) or $MgCl_2$ (to 1 mM) led to a prolonged increase in fluorescent intensity and hence the effects of these components were not further examined.

## DISCUSSION

Evidence that the fluorescent intensity of the dye 3,3′-dipropylthiocarbocyanine iodide is a function of membrane potential in mitochondria has been presented previously [5]. In the range of fluorescent intensities seen in the present study, the fluorescent intensity is directly proportional to external $K^+$ in the presence of valinomycin (unpublished observations) and hence it is likely that the fluorescence intensity is directly proportional to membrane potential. The addition of ATP in the presence or absence of oligomycin led to a decrease in fluorescent intensity indicative of a hyperpolarization (internal potential becomes more negative). Since the slow phase of the response to ATP was inhibited by oligomycin, this phase appears to be the result of the activity of an ATPase which drives protons across the membrane. The rapid phase of the response to ATP which is insensitive to oligomycin could result from the electrogenic exchange of $ATP_{external}^{4-}$ for $ADP_{internal}^{3-}$ as proposed by Klingenberg [1, 2]. A number of observations indicate that the changes in fluorescence and hence potential in the presence of oligomycin result from the exchange of $ATP^{4-}$ and $ADP^{3-}$ through an adenine nucleotide exchange mechanism. First, the percentage decrease, assuming equivalence to change in membrane potential, upon the addition of ATP shows saturation with a half maximal activity of $2 \cdot 10^{-5}$ M ATP. This estimate compares well with a $K_m$ of $1.2 \cdot 10^{-5}$ M ATP reported for the ATP/ADP exchange system [2]. Second, the order for inhibition of the effect of ATP on fluorescent intensity, viz: atractyloside, bongkrekic acid > ADP > AMP, is identical to that of the inhibition of ATP/ADP exchange [2]. The effectiveness of bongkrekic acid in our experiments exhibited the same marked dependence on pH and temperature reported in studies in which ADP binding or exchange was measured [11, 12]. Attempts were made to determine whether or not there was quantitative agreement between the above results with atractyloside and ADP and those of Klingenberg and his associates. Calculations were made for the concentrations of these two inhibitors required to give 50 % inhibition of ATP translocation using the constants (Table I) given by Klingenberg [2] and assuming competitive inhibition (Type 1a, fully competitive, see

TABLE I

Calculated concentrations of atractyloside and ADP giving 50 % inhibition of the entrance of ATP into rat liver mitochondria via adenine nucleotide exchange system compared to concentrations observed to give 50 % inhibition of the change in fluorescent intensity with ATP. In the calculation, competitive inhibition and the following constants [2] are assumed: $K_m$(ATP) = $K_i$(ADP) = $1.2 \cdot 10^{-5}$ M and $K_i$(atractyloside) = $2 \cdot 10^{-8}$ to $1 \cdot 10^{-7}$ M.

| ATP (M) | ADP (M) | | Atractyloside (M) | |
|---|---|---|---|---|
| | Calculated | Observed | Calculated | Observed |
| $1 \cdot 10^{-4}$ | $1.1 \cdot 10^{-4}$ | $2.3 \cdot 10^{-4}$ | $1.9 \cdot 10^{-7}$–$1 \cdot 10^{-6}$ | $1.5 \cdot 10^{-6}$ |
| $3.3 \cdot 10^{-5}$ | – | – | $7.4 \cdot 10^{-8}$–$3.7 \cdot 10^{-7}$ | $5 \cdot 10^{-8}$ |
| $1.3 \cdot 10^{-5}$ | $2.5 \cdot 10^{-5}$ | $4.3 \cdot 10^{-5}$ | – | – |

ref. 13). The value of $K_m$ ATP was taken to be equal to $1.2 \cdot 10^{-5}$ M, for according to Klingenberg $K_m$ ATP will be dependent on potential and will be equal to $1.2 \cdot 10^{-5}$ M when the potential is near zero. Under the experimental conditions of no added substrate plus inhibition of endogenous metabolism with rotenone, the membrane potential is small (−30 mV) (Laris, unpublished observations). Calculations are given in Table I for 50 % inhibition of transport together with observed values of 50 % inhibition of the influence of ATP on fluorescence. There is good agreement between values reported above and those predicted from Klingenberg's data. Complete inhibition with bongkrekic acid was seen in the range $5 \cdot 10^{-7}$ M to $1 \cdot 10^{-6}$ M in these studies and those of Klingenberg [11]. Calculations of the concentration of bongkrekic acid which would be expected to give 50 % inhibition were made assuming non-competitive inhibition and a $K_i$ of $2 \cdot 10^{-8}$ M [2] employing various approaches described by Dixon and Webb [13]. In all cases where the calculation was possible, the calculated bongkrekic acid concentration for 50 % inhibition was approx. 10 % of that observed in our experiments. Two possible reasons for the discrepancy are: (1) the types of non-competitive inhibition tested do not apply or (2) since the effectiveness of bongkrekic acid is so sensitive to temperature and pH, the value of $K_i$ employed is not the correct one for our system. The inhibition seen with AMP appears to be somewhat greater than that predicted by Klingenberg et al. [1]. There is the possibility, however, that the inhibition results from the production of ADP from AMP and ATP via the reaction catalyzed by adenylate kinase. The small increase in fluorescent intensity seen with AMP alone is apparently not related to the adenylate nucleotide translocator for it is insensitive to atractyloside. The addition of ADP after ATP led to an increase in fluorescence (to a more positive potential) which occurred in two phases, a rapid phase which occurred in the presence of oligomycin and a slow phase which was not seen in the presence of oligomycin. The slow phase, therefore, appears to be the result of the inhibition of the mitochondrial ATPase activity while the rapid phase which was inhibited by atractyloside and bongkrekic acid is the result of an exchange of $ADP_{external}^{3-}$ for $ATP_{internal}^{4-}$. Finally, the decrease in fluorescent intensity with ATP is not seen or is reduced in the presence of 2,4-dinitrophenol. In the presence of this uncoupler, protons would presumably rapidly traverse the membrane balancing the charge due to the ATP/ADP exchange.

Studies on the dinitrophenol stimulated ATPase of mitochondria treated with 3.3 μg/ml indicated that this enzymatic activity was not completely inhibited. Are the changes in fluorescent intensity reported here as the ATP-dependent oligomycin sensitive response the result of alterations of this residual ATPase activity or are they related to an electrogenic ATP/ADP exchange mechanism? The transient results seen with ADP added after ATP in the presence of oligomycin are important in distinguishing between these two possibilities. It is difficult to understand why the influence of ADP should be only transient if we are measuring only some aspect of residual ATPase activity. Furthermore, the dinitrophenol-stimulated ATPase activity recorded in the presence of 3.3 μg/ml oligomycin is linear for a 2-min period while the fluorescent intensity rapidly decreases and then increases after the addition of ATP.

An estimation was made of the magnitude of the drop in the potential with $1 \cdot 10^{-4}$ M ATP in the presence of oligomycin. This estimate was made using the "null point" method described previously [5]. In essence, the levels of fluorescent intensity before and after ATP addition are matched to the same levels in the presence of valinomycin and particular concentrations of $K^+$. The potentials are calculated assuming that the membrane potential is equal to the $K^+$ equilibrium potential in the presence of valinomycin. According to this method, the potential fell from approximately $-30$ mV to $-50$ mV with $1 \cdot 10^{-4}$ M ATP.

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