# ADENINE NUCLEOTIDE-INDUCED CONTRACTION

# OF THE INNER MITOCHONDRIAL MEMBRANE

## II. Effect of Bongkrekic Acid

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## ABSTRACT

In bovine heart mitochondria bongkrekic acid at concentrations as low as about 4 nmol/mg protein (a) completely inhibits phosphorylation of exogenous adenosine diphosphate (ADP) and dephosphorylation of exogenous adenosine triphosphate (ATP), (b) completely reverses atractyloside inhibition of inner membrane contraction induced by exogenous adenine nucleotides, and (c) decreases the amount of adenine nucleotide required to elicit maximal exogenous adenine nucleotide-induced inner membrane contraction to a level which appears to correspond closely with the concentration of contractile, exogenous adenine nucleotide binding sites Bongkrekic acid at concentrations greater than 4 nmol/mg protein induces inner membrane contraction which seems to depend on the presence of endogenous ADP and/or ATP. The findings appear to be consistent with the interpretations (a) that the inner mitochondrial membrane contains two types of contractile, adenine nucleotide binding sites, (b) that the two sites differ markedly with regard to adenine nucleotide affinity, (c) that the high affinity site is identical with the adenine nucleotide exchange carrier, (d) that the low affinity site is accessible exclusively to endogenous adenine nucleotides and is largely unoccupied in the absence of bongkrekic acid, and (e) that bongkrekic acid increases the affinity of both sites in proportion to the amount of the antibiotic bound to the inner membrane.

## INTRODUCTION

Although it has been known for more than a decade that bongkrekic acid is a potent inhibitor of oxidative phosphorylation in mitochondria (1), it has only quite recently been established that this antibiotic, like atractyloside, interferes with phosphorylation by inhibiting the exchange of adenine nucleotides across the inner mitochondrial membrane (2–7). Unlike atractyloside, however, bongkrekic acid inhibits the exchange by markedly increasing the affinity of the adenine nucleotide exchange carrier (4, 5).

It was shown in the preceding communication (8) that exogenous adenosine diphosphate (ADP), adenosine triphosphate (ATP), and certain other high-energy phosphate compounds induce contraction of the inner membranes in heart mitochondria The nucleotide specificity, atractyloside sensitivity, and other characteristics of the reaction suggested that contraction is associated with the binding of the phosphate compounds to the adenine nucleotide exchange carrier The present communication provides further support for this interpretation,

showing that bongkrekic acid produces changes suggestive of increased adenine nucleotide affinity of the contractile site and that the concentration of bongkrekic acid required to bring about the changes is similar to those required to inhibit phosphorylation of exogenous ADP and dephosphorylation of exogenous ATP. In addition, evidence is provided for the existence in the inner mitochondrial membrane of a type of contractile, adenine nucleotide binding site which differs from the exchange carrier with regard to adenine nucleotide affinity and accessibility.

## MATERIALS AND METHODS

Isolation of bovine heart mitochondria and determinations of mitochondrial ultrastructural changes, optical density (OD), and respiratory activity were carried out as previously described (8). ATPase activity was estimated in mitochondria suspended at a concentration of 0.5 mg protein/ml and incubated at 30°C. The reaction was initiated by adding 1 mM ATP to the suspension under rapid stirring. 1 min later a 2 ml aliquot of the incubation mixture was rapidly mixed with 1 ml of cold (0°C) 15% trichloroacetic acid (TCA). Appropriate controls were obtained by mixing the suspension with TCA before adding ATP. The TCA extracts were cleared by centrifugation and analyzed for inorganic phosphate (Pi) according to the method of Fiske and Subbarow (9).

Bongkrekic acid was generously donated by W. Berends; a small amount, originally from W. Berends, was obtained through the courtesy of M. Klingenberg. Other materials were obtained as described in the preceding report (8).

## RESULTS AND DISCUSSION

Preliminary studies suggested that bongkrekic acid produces a similar type of change in two classes of inner membrane contractile sites, the change being detectable in one at a much lower concentration of bongkrekic acid than in the other. Therefore, in conducting dose-response studies, bongkrekic acid concentration was varied over a wide range to include the responses of both classes of sites.

Fig. 1 shows that, as bongkrekic acid concentration is increased from extremely low levels up to approximately 4 nmol/mg mitochondrial protein, the magnitudes of the contractile responses to 50 $\mu$M additions of ADP and ATP are decreased slightly and then increased slightly; as bongkrekic acid concentration approaches the 4 nmol/mg protein level, the contractile responses to ADP and ATP become essentially identical in both
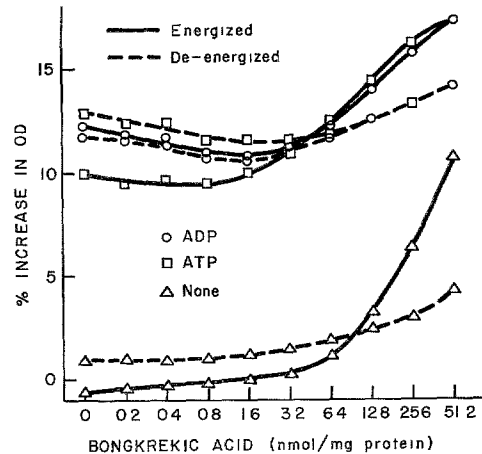


FIGURE 1 Effect of bongkrekic acid on adenine nucleotide-induced inner membrane contraction in bovine heart mitochondria. Energized mitochondria were preincubated in media containing 250 mM sucrose, 10 mM potassium piperazine-$N$,$N'$-bis(2-ethanesulphonate) (K-PIPES) (pH 6.4), 2.5 mM malate-pyruvate, and 1 mM Ethylene glycol bis($\beta$-aminoethyl ether)$N$-$N$,$N'$, $N'$ tetraacetic acid (EGTA) De-energized mitochondria were preincubated in media containing 200 mM sucrose, 10 mM K-PIPES (pH 6.4), 2.5 mM malate-pyruvate, 0 1 $\mu$M S-13, 1 mM CN$^-$, and 2.5 nmol oligomycin/mg protein. Bongkrekic acid (+1 mM NH$_4$OH) was added at the concentrations indicated after 1 min of preincubation. The adenine nucleotides were added 2 min after the bongkrekic acid. The OD changes given represent the maximum change in mitochondrial OD which occurred within 2.5 min after adding bongkrekic acid. Energized and de-energized mitochondria were from different preparations. Note the use of a logarithmic scale on the abscissa.

energized and de-energized mitochondria. Fig 2 shows that 4 nmol/mg protein corresponds to the lowest concentration of bongkrekic acid that produces essentially (a) complete reversal of atractyloside inhibition of ADP-induced inner membrane contraction in energized mitochondria, (b) complete reversal of atractyloside inhibition of both ADP- and ATP-induced contraction in de-energized mitochondria, (c) complete inhibition of phosphorylating respiration in energized mitochondria, and (d) complete inhibition of ATPase activity in de-energized mitochondria.

Increasing the concentration of bongkrekic acid from 4 nmol/mg mitochondrial protein to very high levels results in a considerable increase in the OD of both mitochondria incubated in the presence of added adenine nucleotides and
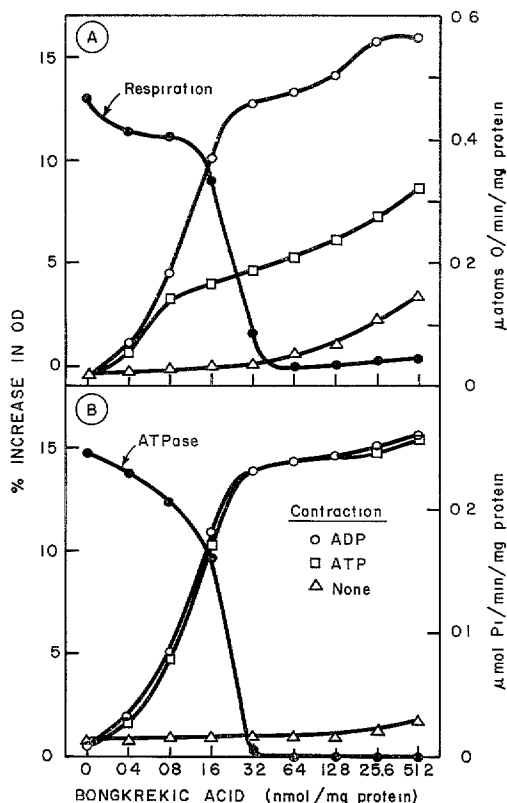
Figure 2 A and B Effects of bongkrekic acid on inner membrane contraction in energized (A) and de-energized (B) mitochondria preincubated with atractyloside, on state 3 respiratory activity (A), and on ATPase activity in de-energized mitochondria (B). In the study on inner membrane contraction, energized mitochondria were preincubated in media containing 250 mM sucrose, 10 mM K-PIPES (pH 6.6), 2.5 mM malate-pyruvate, 1 mM EGTA, and 10 μM atractyloside; de-energized mitochondria were preincubated in media containing 200 mM sucrose, 10 mM K-PIPES (pH 6.4), 2.5 mM malate-pyruvate, 0.1 μM S-13, 1 mM CN⁻, and 10 μM atractyloside. Adenine nucleotides (50 μM) were added after 1 min and bongkrekic acid (+ 1 mM NH₄OH) after 2 min of preincubation. The OD changes given represent the maximum change which occurred within 2 min after adding the bongkrekic acid. In the study on state 3 respiratory activity, mitochondria were preincubated for 2 min in 250 mM sucrose, 10 mM K-PIPES, 2.5 mM malate-pyruvate, 5 mM Pi, 1 mM NH₄⁺, and bongkrekic acid at the concentrations indicated (pH 6 7). State 3 respiration was initiated by adding 0.2 mM ADP. In the study on ATPase activity, mitochondria were preincubated for 2 min under the same conditions described above for de-energized mitochondria except that atractyloside was absent and bongkrekic acid was present. Different mitochondrial

mitochondria incubated in the absence of added adenine nucleotides (Figs. 1 and 2). Electron micrographs of mitochondria incubated in the presence and absence of ADP and low and high concentrations of bongkrekic acid (Fig. 3) demonstrate that the increase in mitochondrial OD induced by high levels of bongkrekic acid in the absence of added adenine nucleotides is associated with changes in mitochondrial ultrastructure indistinguishable from those induced by exogenous ADP in the absence of bongkrekic acid High magnification electron micrographs show that ADP and bongkrekic acid induce similar changes also with regard to the arrangement of the electron-opaque constituents of the inner membrane. It is evident, therefore, that the OD change induced by bongkrekic acid alone is due to inner membrane contraction (8).

The degree of inner membrane contraction induced by bongkrekic acid in the absence of added adenine nucleotides is strongly influenced by preincubation of the mitochondria before addition of bongkrekic acid and by the energy status of the mitochondria during the preincubation period. This can be seen in Fig. 4, which presents recorder tracings showing the effects of brief periods of preincubation in the absence of bongkrekic acid on the magnitude of bongkrekic acid-induced contraction in energized and de-energized mitochondria. The magnitude of bongkrekic acid-induced contraction decreases rapidly with increase of preincubation period, the rate of the decrease being greater in de-energized than in energized mitochondria. Addition of ADP after bongkrekic acid results in a further increase in inner membrane contraction, the magnitude of which is larger as the magnitude of bongkrekic acid-induced contraction is smaller. As the degree of bongkrekic acid-induced contraction becomes small with increase of preincubation period, the degree of ADP-induced contraction approaches that induced by ADP in the absence of bongkrekic acid pretreatment.

Virtually all of the effects of bongkrekic acid at concentrations up to about 4 nmol/mg protein described above can be readily understood in terms of bongkrekic acid increasing the affinity of the atractyloside-sensitive contractile site. The results appear to be consistent with the interpretations (a) that the atractyloside-sensitive site is

preparations were used in Figs. 2 A and 2 B. Note the use of a logarithmic scale on the abscissa.
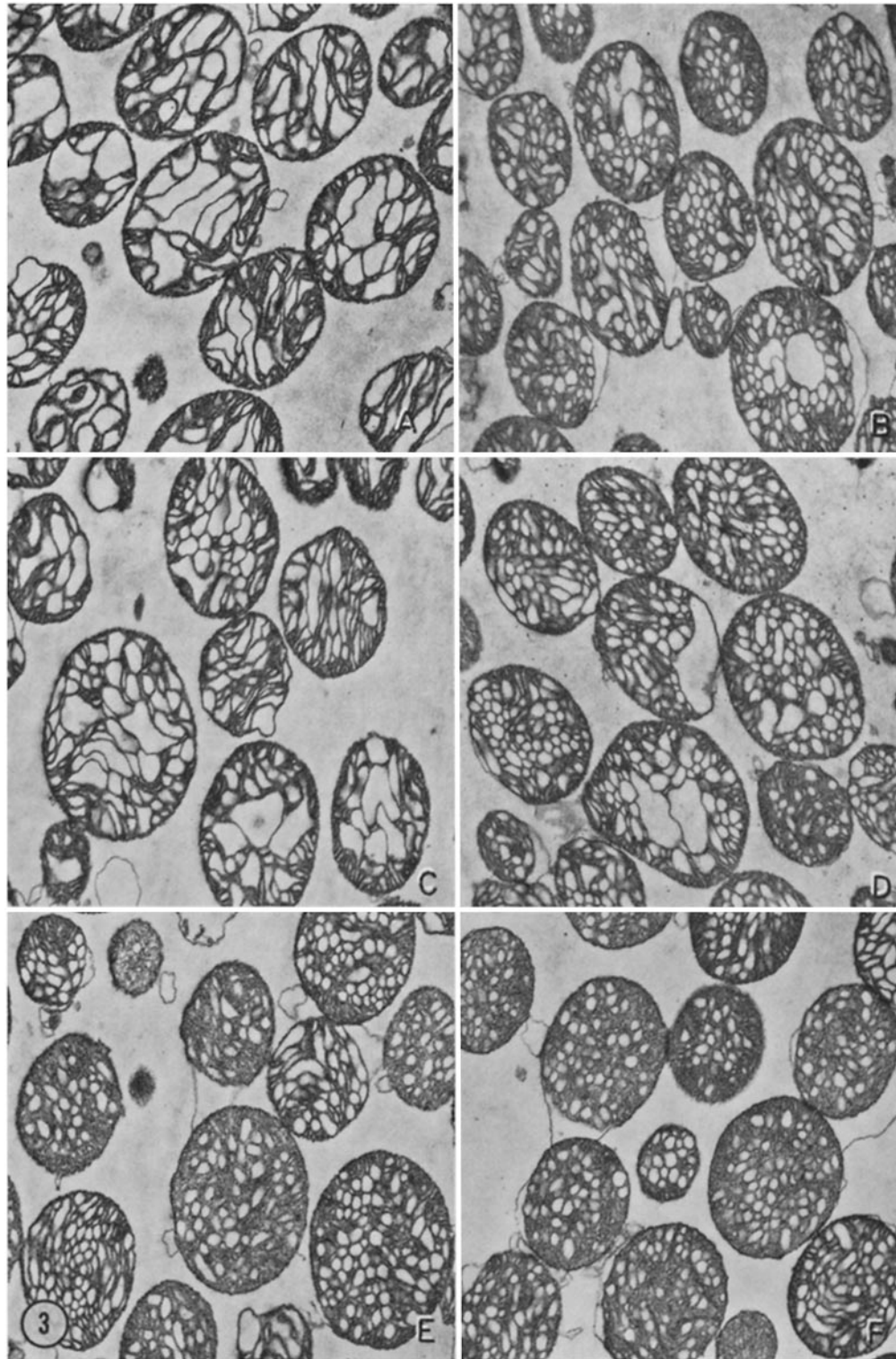
FIGURE 3 A–F  Effects of bongkrekic acid and ADP on the ultrastructure of energized mitochondria. Mitochondria were incubated at 30°C in media containing 250 mM sucrose, 10 mM K-PIPES (pH 6 5), 2.5 mM malate-pyruvate, 3 mM EGTA, 1 mM $NH_4^+$, and bongkrekic acid at the concentrations indicated below. Where used, ADP (0.1 mM) was added after 2 min of preincubation. Fixation was initiated after 4 min total incubation time  Differential conditions were· (A) none; (B) ADP; (C) 4 nmol bongkrekic acid/mg protein; (D) ADP + 4 nmol bongkrekic acid/mg protein; (E) 50 nmol bongkrekic acid/mg protein; (F) ADP + 50 nmol bongkrekic acid/mg protein. × 20,000.
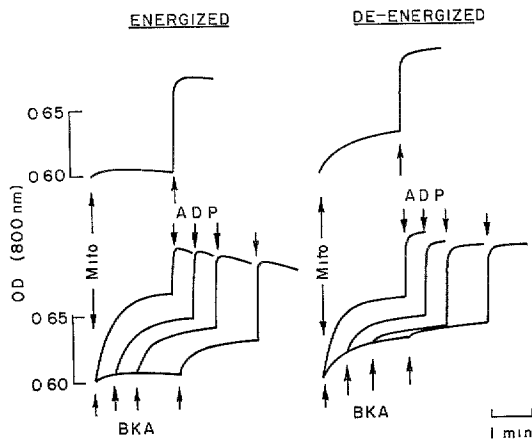
ENERGIZED     DE-ENERGIZED

FIGURE 4   Recorder tracings showing the effects of preincubation period and mitochondrial energy status on inner membrane contraction induced by ADP and bongkrekic acid (BKA) Energized mitochondria were suspended in media containing 250 mM sucrose, 10 mM K-PIPES (pH 6.4), 2.5 mM malate-pyruvate, and 1 mM ethylenediaminetetraacetic acid (EDTA). De-energized mitochondria were incubated in media containing 200 mM sucrose, 10 mM K-PIPES (pH 6 4), 2.5 mM malate-pyruvate, 1 mM CN$^-$, and 0 1 μM S-13. The concentrations of materials added were bongkrekic acid, 20 nmol/mg protein; ADP, 50 μM.

identical with the adenine nucleotide exchange carrier and (b) that bongkrekic acid at concentrations as low as 4 nmol/mg protein increases the affinity of the site to the extent that adenine nucleotide dissociation is largely prevented These interpretations are identical with those made by Klingenberg and co-workers (10) on the basis of direct binding studies (5) and of studies suggesting that bongkrekic acid prevents atractyloside reversal of adenine nucleotide-induced inner membrane contraction (10).

The additional contraction induced by bongkrekic acid at concentrations greater than 4 nmol/mg protein can be explained by assuming (a) that bongkrekic acid increases the affinity of a low affinity, inner membrane, contractile, adenine nucleotide binding site which is accessible exclusively to endogenous adenine nucleotides, is specific for ADP and/or ATP, and is largely unoccupied in the absence of bongkrekic acid and (b) that preincubation of heart mitochondria under the conditions employed in Figs 1, 2, and 4 results in dephosphorylation of endogenous adenine nucleotides. The validity of these as-

sumptions is supported by studies showing that the decline in bongkrekic acid-induced contraction with increase of preincubation period is less rapid and less extensive in mitochondria energized with α-ketoglutarate than in mitochondria energized with succinate (Fig 5), it is established (11, 12) that α-ketoglutarate is particularly effective in maintaining endogenous adenine nucleotides in highly phosphorylated states The validity of the assumptions is supported also by the observation that Pi, despite having a marked inhibitory effect on bongkrekic acid-induced contraction, reverses the inhibitory effect of preincubation on the contraction in energized mitochondria (Fig 5) Results to be presented elsewhere suggest that only intramitochondrial Pi is effective in suppressing bongkrekic acid-induced contraction and that reversal of the contraction by Pi is associated with release of bound adenine nucleotides into the matrix space.

In Figs. 1 and 4 it can be seen that the contraction induced by bongkrekic acid is not entirely additive with that induced by exogenous adenine nucleotide. This could be due to a limited ability of the inner membrane to contract in response to adenine nucleotide binding or to deviation from proportionality in the relationship between inner membrane contraction and mitochondrial OD. Another possibility is that binding of adenine nucleotide to the high affinity, outer sites decreases the affinity of the low affinity, inner sites; an interaction of this sort could explain the observation that addition of exogenous adenine nucleotide to mitochondria in extremely contracted states due to previous incubation in the presence of a high level of bongkrekic acid results in a sharp increase in the level of contraction which is followed immediately by a rapid decrease to a level only slightly greater than that existing before adenine nucleotide addition

In view of the large bongkrekic acid requirement for the induction of inner membrane contraction involving the inner, low affinity sites, it does not seem likely that, if the contraction is in fact due to an increase in affinity of the inner sites, the sites undergo an increase in affinity as a result of bongkrekic acid binding stoichiometrically to the sites or to inner membrane components on which the sites exist The data seem best explained in terms of a mechanism whereby a progressive increase in bongkrekic acid binding to the inner membrane produces a progressive increase in affinity of both inner and outer sites.
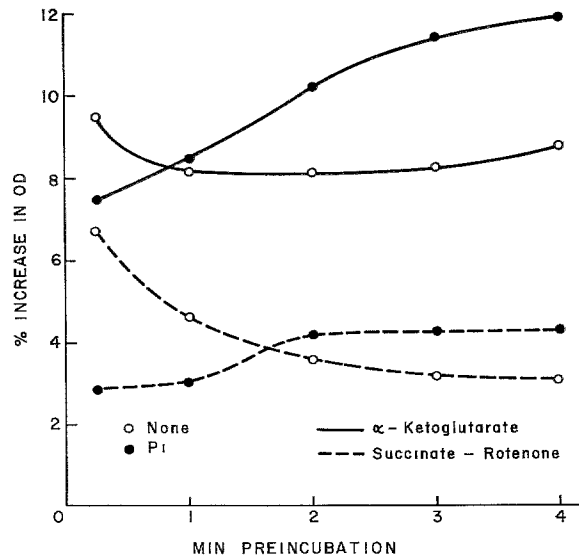
FIGURE 5 Effects of Pi, type of substrate, and length of preincubation period on the magnitude of bong-krekic acid-induced inner membrane contraction in energized mitochondria. Mitochondria were preincubated in media containing 250 mM sucrose, 10 mM K-PIPES (pH 6.4), 1 mM EDTA, 0.1 mM Pi as indicated, and either 5 mM α-ketoglutarate or 5 mM succinate + 2 nmol rotenone/mg protein, as indicated. Bongkrekic acid (25 nmol/mg protein) + 1 mM NH₄OH was added after the periods of preincubation indicated. The OD changes given represent the change in mitochondrial OD which occurred over a period of 2 min after adding the bongkrekic acid. The relatively low initial level of contraction observed in mitochondria preincubated in the presence of Pi is due to a marked inhibitory effect of Pi on bongkrekic acid-induced contraction.

In view of the lipophilic nature of bongkrekic acid (13), it is conceivable that the antibiotic increases the affinity of the sites simply by entering the highly hydrophobic phase of the inner membrane, altering it in such a way as to increase its affinity for the adenine nucleotide-contractile site complexes. According to this mechanism the much lower bongkrekic acid requirement for the production of a detectable increase in the affinity of the outer site would be explained by the much higher intrinsic affinity of the outer site

The fact that bongkrekic acid is required to demonstrate a detectable degree of contraction involving the inner sites suggests that under normal conditions the inner sites are largely unoccupied. Consequently, it seems unlikely that these sites are involved in the exchange of adenine nucleotides across the inner membrane or are related to the inner localized adenine nucleotide exchange carrier sites suggested by Weidemann et al (5, 14). On the other hand, they could be involved in the relatively slow, specific efflux of adenine nucleotides from mitochondria described by

Meisner and Klingenberg (15). Recent studies by Klingenberg et al. (10) and Out et al. (16) have shown that the efflux is sensitive to bongkrekic acid.

In accordance with the above interpretations concerning the outer contractile site, pretreatment of mitochondria with bongkrekic acid makes it possible to estimate the concentration of outer sites simply by titrating the mitochondria with adenine nucleotides. This is demonstrated in Fig. 6, which shows that titration of energized mitochondria pretreated with 4 nmol bongkrekic acid/mg protein with ADP and ATP results in linear increases in contraction. The curves suggest a contractile site concentration of about 1 nmol/mg mitochondrial protein, a value which closely approximates the concentration of atractyloside binding sites estimated with the use of adenosine 5'-methylene diphosphonate (AOPCP) in the same mitochondrial preparation (Fig 6). The results are in fairly good agreement with concentration estimates of high affinity
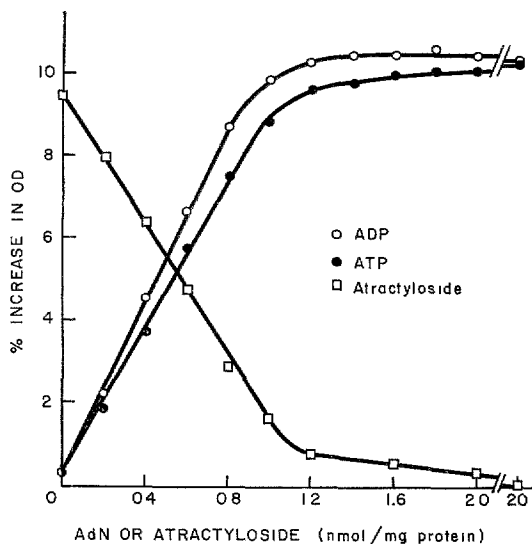
FIGURE 6 Estimation of the concentration of sites involved in inner membrane contraction induced by exogenous adenine nucleotide ($AdN$). Mitochondria of the study in which adenine nucleotide concentration was varied were suspended in media containing initially 250 mM sucrose, 10 mM K-PIPES (pH 6.4), 2.5 mM malate-pyruvate, and 1 mM EDTA Bongkrekic acid (4 nmol/mg protein) was added after 1 min and adenine nucleotide after 2 min of preincubation. The OD changes given represent the maximum change in mitochondrial OD which occurred within 2 min after adding the bongkrekic acid. Mitochondria of the study in which atractyloside concentration was varied were suspended initially as described above except that atractyloside was present as indicated. The mitochondria were preincubated for 2 min and contraction was induced by adding 50 $\mu$M AOPCP.

adenine nucleotide and atractyloside binding sites based on direct binding studies (14, 17)

An additional finding of the study reported in Fig. 6 and of similar studies with de-energized mitochondria is that when low concentrations of adenine nucleotides are used for the induction of inner membrane contraction, bongkrekic acid does not produce equalization of the contractile responses to ADP and ATP This suggests that the equality of 50-$\mu$M concentrations of ADP and ATP in producing contraction in bongkrekic acid-treated mitochondria, as shown in Figs 1 and 2 B, may be due to the presence of adenylate kinase activity and/or small amounts of ADP and ATP impurities in the ATP and ADP solutions used.

Fig. 2 A shows that, in energized mitochondria incubated under conditions where ADP and ATP must overcome the competitive inhibitory effect of atractyloside to produce inner membrane contraction, bongkrekic acid does not bring about equalization of the contractile responses to 50-$\mu$M concentrations of ADP and ATP. Thus, within the limited period allowed for the bongkrekic acid-induced reversal of atractyloside inhibition in the experiment of Fig. 2 A, the magnitude of contraction achieved with ATP was less than half that achieved with ADP. The recorder tracings from which the data were taken suggest that the marked differences observed were due primarily to differences in rate of reversal. The tracings show that, whereas the 2 min period allowed for the reversal by bongkrekic acid at concentrations higher than about 4 nmol/mg protein is more than adequate for the achievement of contractile equilibrium in mitochondria incubated in the presence of ADP, it is far from adequate for the achievement of contractile equilibrium in the presence of ATP. The reversal proceeds very slowly in the presence of ATP, and equilibrium levels of contraction comparable to those achieved with ADP are approached only after several minutes of incubation. In de-energized mitochondria ADP and ATP are equally as effective in promoting the reversal of atractyloside inhibition, and the rates closely approximate that observed in the case of energized mitochondria incubated with ADP. In view of these relationships it seems likely that the relatively slow reversal observed in the case of energized mitochondria incubated with ATP is a manifestation of the energy-dependent discrimination against the interaction of ATP with the outer contractile site discussed in the preceding report (8).

Previous studies have shown that the extent to which bongkrekic acid inhibits the adenine nucleotide exchange reaction and reactions governed by the exchange reaction depends on the ratio of bongkrekic acid to mitochondrial protein (1, 3, 4, 7), the period of time for which the mitochondria are exposed to the inhibitor (2-4, 7), the temperature (3, 4), the pH (6, 7), and the presence or absence of a number of agents, including adenine nucleotides (6, 7), Pi (7), and coenzyme A (18, 19). In general, the findings suggest that bongkrekic acid binds tightly to its site of action and that the rate of binding is influenced by a number of factors.

In the present study, mitochondria were in

most cases preincubated with bongkrekic acid for 2 min at 30°C in media maintained at or near pH 6.5 before evaluating the response. Since there were a number of indications suggesting that 2
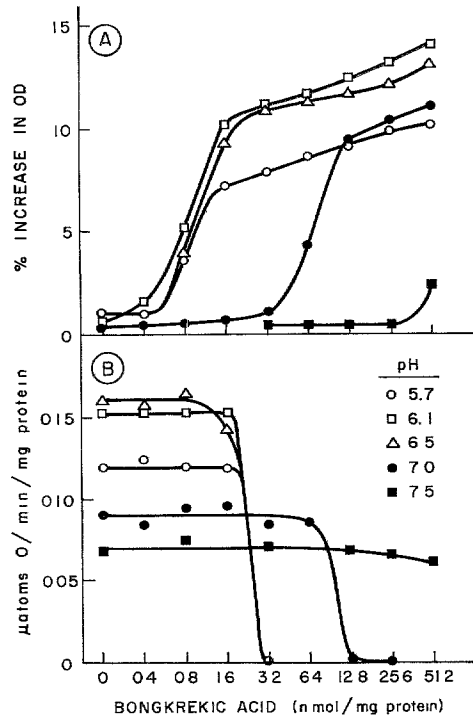


FIGURE 7 A and B Effects of pH on bongkrekic acid-induced inner membrane contraction in de-energized mitochondria preincubated in the presence of ADP and atractyloside (A) and on bongkrekic acid inhibition of ADP-stimulated respiration in uncoupled mitochondria preincubated in the presence of α-ketoglutarate, Pi, and oligomycin (B). Mitochondria of Fig. 7 A were preincubated in media containing 200 mM sucrose, 10 mM K-PIPES, 5 mM α-ketoglutarate, 5 mM Pi, 0.1 µM S-13, 5 nmol oligomycin/mg protein, 1 mM CN⁻, 10 µM atractyloside, and 50 µM ADP (added after 1 min of preincubation). Bongkrekic acid (+1 mM NH₄OH) was added after 2 min of preincubation. The OD changes given represent the maximum change which occurred within 2 min after adding the bongkrekic acid. The mitochondria of Fig. 7 B were preincubated for 3 min under the same conditions described above except that ADP, atractyloside, and CN⁻ were absent and bongkrekic acid (+1 mM NH₄OH) was added after 1 min of preincubation. The respiration rates given represent the increase due to addition of 0.4 mM ADP. Different mitochondrial preparations were used in Figs. 7 A and 7 B. Note the use of a logarithmic scale on the abscissa.

min of preincubation under the conditions employed was not sufficiently long for maximum binding of bongkrekic acid at its site of action, it should be noted that the minimum concentrations of bongkrekic acid required to produce the changes shown in Figs. 1 and 2 likely would have been lower if the mitochondria had been preincubated in the presence of the antibiotic for longer periods before evaluating the responses The observed similarity in concentration dependence of the various bongkrekic acid-induced changes involving the outer contractile site (Figs. 1 and 2) suggests that the conditions of incubation that differed among experiments (e.g , presence vs. absence of atractyloside and Pi, energized state vs. de-energized state) had little influence on the rate of bongkrekic acid binding.

During the course of the present study it was noted that, in accordance with the findings of Kemp et al. (6, 7) with rat liver mitochondria, pH is a particularly important factor affecting the interaction of bongkrekic acid with heart mitochondria This is supported by studies showing that as pH is increased from about 6.5 the amount of bongkrekic acid required to reverse atractyloside inhibition of inner membrane contraction (Fig 7 A) and to inhibit ADP-stimulated respiration (Fig. 7 B) within 2 min increases sharply. Kemp et al (6, 7) noted that inhibition of ADP-stimulated respiration by bongkrekic acid at low pH is not reversed upon raising the pH. We have confirmed this observation with heart mitochondria and have found, in addition, that lowering the pH of mitochondrial incubation mixtures containing 4 nmol bongkrekic acid/mg protein from 7.5 to 6 5 results in immediate inhibition. These findings suggest that high pH decreases the rate of bongkrekic acid penetration to its site of action As was pointed out by Kemp et al. (7), the decrease can be readily explained by assuming that penetration of bongkrekic acid requires that the carboxyl groups of the antibiotic be in the undissociated state. This requirement would be expected if, as suggested above, bongkrekic acid exerts its effect through modification of a highly hydrophobic phase of the inner membrane.

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