Demonstration of the Relationship between the Adenine Nucleotide Carrier and the Structural Changes of Mitochondria as Induced by Adenosine 5′-Diphosphate†

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ABSTRACT: The specific interaction of various ligands with the mitochondrial ADP carrier was explored on the basis of structural changes of mitochondria (as measured by absorbance changes) and the binding of [^{14}C]ADP. A detailed insight into the function of a membrane carrier was obtained supporting strongly the mobile (reorienting) carrier model on a molecular basis. The linkage of absorbance changes at 546 nm to the binding of ADP at the carrier is demonstrated by quantitative agreement between absorbance change and binding extent. The absorbance increase (*i.e.* contraction of mitochondrial matrix) has a low K_m for ADP (4 μM), similar to that for ADP binding to the carrier. The contraction is also highly specific for ADP and ATP. From the absorbance decrease (expansion) induced by atractylate an apparent $K_d = 3 \times 10^{-8}$ M is obtained, in agreement with [^{35}S]atractylate-binding studies. In contrast to atractylate, bongkrekate further increases the absorbance, in agreement with binding studies. In general, depending on time and on bongkrekate concentration the increases of absorbance and of binding occur in parallel. The rate of the bongkrekate-induced absorbance increase is relatively slow and increases strongly with H^+ concentration, indicating a requirement for diffusion of undissociated bongkrekate through the membrane. The rate of the bongkrekate effect has a high temperature dependence (E_A = 23 kcal). The bongkrekate-induced absorbance increase is competitively inhibited by atractylate and completely inhibited by carboxyatractylate. The results are best explained by postulating that absorbance changes reflect carrier localization on the inner surface or the outer surface. The expanded state is associated with the accumulation of carrier on the outer surface and the contracted state with its accumulation on the inner surface. Atractylate as an impermeable ligand traps the carrier on the outside whereas bongkrekate as a permeable ligand fixes and traps the carrier on the inside. In both cases the carrier is immobilized. On binding ADP, the carrier becomes mobile and distributes both on the inside and outside according to the ADP concentration gradient.

In the last few years the adenine nucleotide carrier of the inner mitochondrial membrane has been investigated by measuring the binding of ADP to mitochondria (Weidemann *et al.*, 1970). A definition of the carrier sites was possible by combining the binding of ADP and ATP with other ligands such as inhibitors of the adenine nucleotide transport, atractylate and bongkrekate. The mutual interaction of these ligands gave a dynamic picture of the carrier as correlated to the translocation mechanism (Weidemann *et al.*, 1970; Klingenberg *et al.*, 1973; Klingenberg and Buchholz, 1973). However, the kinetic resolution in these binding studies was limited.

In this paper, another indirect method for studying binding and dynamic behavior of the carrier will be reported, which has certain advantages over direct binding studies, in particular permitting higher time resolution as required for the kinetic analysis of carrier dynamics. This method is based on small structural changes of mitochondria induced by the addition of the ligands to the adenine nucleotide carrier, which is independent of osmotic changes and of correlated energy transfer. These changes are paralleled by a slight swelling or shrinkage of the mitochondria and are reflected in turbidity changes of a mitochondrial suspension.

"Swelling–shrinkage" phenomena of mitochondria were first reported (Bücher and Klingenberg, 1958; Packer, 1960) in conjunction with the transition from the "controlled" to the "active" respiratory state on addition of ADP and are in the literature generally called "small amplitude shrinkage." Among further reports (Harris *et al.*, 1968), the studies of Hackenbrock (1966, 1968) defined "condensed" conformation of the mitochondria as induced by ADP in contrast to the "orthodox" conformation in an energized state. However, Stoner and Sirak (1970) and Weber and Blair (1970) stated that the ADP- and ATP-induced shrinkage is independent of energy transfer to and from these nucleotides. Stoner and Sirak (1970) showed that only a very low concentration of ADP and ATP is required for the shrinkage and that this is sensitive to atractylate.

In view of our detailed studies on the binding of ADP and the inhibitors (Weidemann *et al.*, 1970; Klingenberg and Buchholz, 1973), these structural changes were examined to determine if they were correlated to the binding at the ADP carrier and not to the transport of ADP and ATP into the matrix. A critical test is furnished by bongkrekate, which has been shown to induce an apparent increase of the binding of ADP to the membranes in contrast to atractylate, although both substances are inhibitors of the transport. In the first report (Klingenberg *et al.*, 1971b) we demonstrated that both these ligands have opposite effects on the turbidity and that therefore these phenomena are related to the binding of ADP at the carrier rather than to the transport of ADP. It was therefore possible to follow the binding to the carrier by recording the turbidity of the mitochondria as their absorbance, although the exact nature of the changes remains unknown. The advantages of this method are demonstrated in the present detailed account, which leads to a unique insight into the carrier function in a biomembrane.

† From the Institute of Physical Biochemistry, the University of Munich, Munich, Germany. *Received August 7, 1973.* This investigation was supported by the Sonderforschungsbereich 51.

‡ Research fellow of the Deutsche Forschungsgemeinschaft.

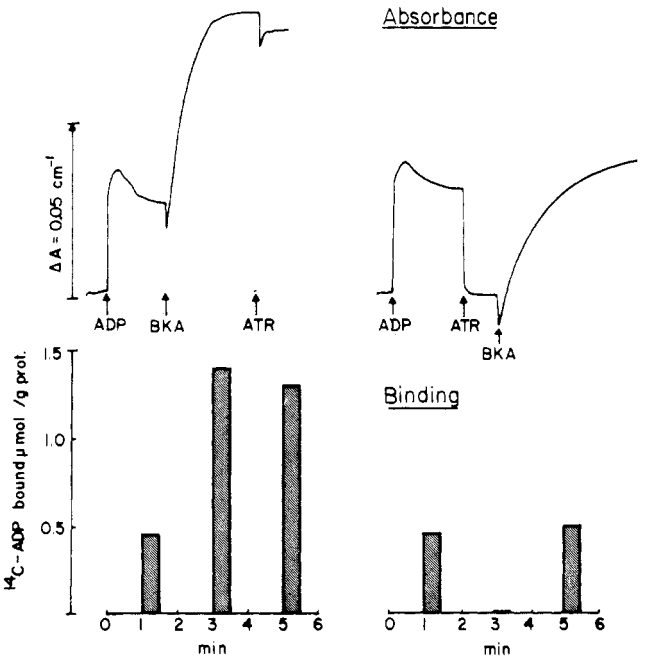

FIGURE 1: Comparison of [^{14}C]ADP binding with the absorbance changes of mitochondrial suspension as induced by addition of ligands to the ADP carrier. Photometric recording at 546 nm of a suspension of beef heart mitochondria (0.4 mg of protein/ml). In parallel experiments under exactly the same incubation conditions, the binding of [^{14}C]ADP was measured in samples successively withdrawn from the incubation at the times indicated for determination of binding (see Methods). Incubation conditions: 0.25 M sucrose, 20 mM morpholinopropanesulfonic acid, 1 mM EDTA, pH 6.5, 20°; ADP, 5 μM; atractylate, 1 μM; bongkrekate, 5 μM.

Recently Stoner and Sirak (1973a,b) published a detailed account of their work, which in part overlaps with the results of the present work. The interpretation of their results follows our concept of the ADP carrier involvement (Klingenberg *et al.*, 1971b). However, their interpretation of the bongkrekate effects comes to entirely different conclusions.

Methods

Beef heart mitochondria were prepared according to the following procedure: fresh beef hearts were carefully stripped of connective tissue and any fat tissues. The muscle pieces were minced in a meat grinder and then mixed with 0.25 M sucrose–20 mM Tris in equal portions. To this concentrated meat suspension 0.5 mg of bacterial proteinase/g of muscle was added as a powder. This suspension was stirred for 15 min at 2° and kept at pH 7.0 by the addition of 1 M Tris solution. After dilution with a medium of 0.25 M sucrose–20 mM Tris-HCl (pH 7.2), this suspension was homogenized in a high-speed blender for 15 sec. The homogenate was centrifuged at 600*g* and refrigerated for 20 min. The supernatant was filtered through cheesecloth and again centrifuged at 5000*g* for 20 min. After careful removal of accumulated fat in the upper layer, the supernatant was decanted and the sediment rehomogenized with incubation medium and centrifuged a second time for 20 min at 5000*g*. This sediment was resuspended for the stock solution containing about 50 mg of protein/ml. The yield was about 5 mg of mitochondrial protein/g of fresh weight muscle.

For measuring the swelling–shrinkage of the mitochondria, the absorption changes in the mitochondrial suspension were recorded at 546 nm. The emerging light has to be screened in order that the scattered light will be discarded. The Eppendorf photometer was favorable for this purpose. The absorbance changes were continuously recorded on an absorbance scale extended to 0.1 cm^{-1} with a light path in the cuvet of 5 mm. The suspension of mitochondria which yielded the largest absorbance changes had about 0.5 mg of protein/ml. The total absorbance of this suspension ranged from $A = 0.9$ to $A = 1.2\ cm^{-1}$.

For comparison of [^{14}C]ADP binding two procedures were employed: aliquots of 0.5 ml were removed from the cuvet under simultaneous recording and immediately after removal the samples were centrifuged and the [^{14}C]ADP bound was determined in the sediments as described before (Weidemann *et al.*, 1970). In kinetic experiments (Figure 6) mitochondria were exposed to ADP and the inhibitors placed in parallel incubations under identical conditions and then rapidly separated by pressure filtration. For this procedure the mitochondrial suspension was filtered under 5 atm of pressure through 0.65- and 0.45-μ Millipore filter combinations. A specially designed apparatus was used with a rapid mixing and preprogrammed timing between mixing, filtration, and pressure exposure. The filters with the mitochondria were added to the counting vials where they were dissolved in the scintillation fluid by sonication.

The decrease of specific activity of [^{14}C]ADP used in the binding studies was accounted for by assuming equilibration of the added [^{14}C]ADP with endogenous ADP. This was assumed in the experiment of Figure 7 as about 3.5 μmol of ADP/g of protein.

Results

Correlation between Mitochondrial Shrinkage and Swelling to the ADP Binding. A quantitative correlation between the structural changes of mitochondria and the binding of ADP seems to be of great importance in order to substantiate the assumption that the changes reflect binding of ADP to the carrier. An example of this correlation in a basic experiment is shown in Figure 1, where parallel to the absorbance recording the binding of [^{14}C]ADP is measured in the various structural states. Addition of ADP produces a small contraction which is subsequently largely abolished by atractylate. A low amount of atractylate is chosen so that subsequent addition of bongkrekate is still able to induce a contraction despite the competitive effect of atractylate.

The specific binding as plotted in Figure 1 is defined as a binding of ADP above the binding of 4.3 μmol of ADP/g of protein. This specific binding is removable by atractylate (Weidemann *et al.*, 1970) and amounts here to 0.45 μmol/g of protein. After addition of bongkrekate a considerable increase of absorbance is observed, which is then decreased only to a small extent by atractylate. The corresponding binding increases about threefold to 1.4 μmol/g of protein in agreement with the absorbance changes. In the second experiment reported in Figure 1, the order of addition is ADP, atractylate, and then bongkrekate. The absorbance increase by bongkrekate reaches approximately the same level as ADP alone. The same holds for the [^{14}C]ADP binding.

These and further results presented below show that the absorbance increase, *i.e.* contraction, under the present conditions reflects quantitatively the "specific" binding of ADP, which has been regarded as the binding of ADP to the ADP carrier. However, there are instances where this relation does not hold.

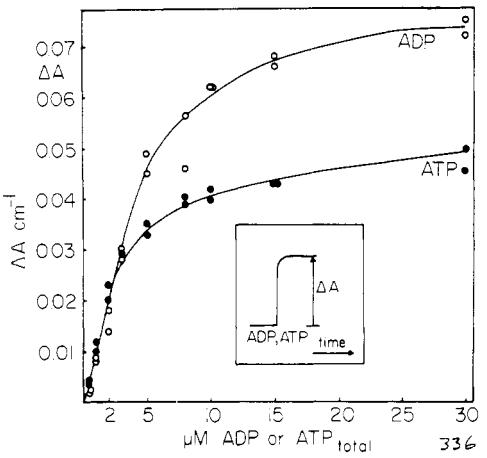

FIGURE 2: ADP- or ATP-induced contraction as a function of the concentration of ADP and ATP. The maximal absorbance increase immediately after addition of ADP and ATP was measured and plotted as a function of the total concentration of ADP and ATP.

Dependence of the Absorbance Change on the Concentration of ADP, ATP, and Atractylate. The absorbance change induced by increasing concentrations of ADP or ATP is given in Figure 2. The maximum absorbance change obtained with ATP is considerably smaller than with ADP. The free concentrations of ADP and ATP have to be calculated by an iterative procedure in order to obtain a plot of $1/\Delta A$ *vs.* $1/[ADP]_{free}$ (not shown). From these plots an apparent K_d = 4 μM ADP and K_d = 3 μM for ATP are obtained. It is remarkable that these K_d values agree approximately with the K_d determined earlier for the specific portion of ADP binding to mitochondrial membranes (Weidemann *et al.*, 1970). Studies on the specificity of the ligand for this effect showed complete agreement with the high specificity for the specific binding (Weidemann *et al.*, 1970): d-ADP, AMP-PNP. AMP-PCP caused contraction with decreasing effectiveness. No contraction is obtained with AMP and nucleotides with other bases. The specificity of binding will be treated separately together with further material on the energy dependence of binding.

The absorbance decrease, *i.e.* decontraction, which is induced by atractylate after previous addition of ADP, has been titrated with atractylate as shown in Figure 3. At about 1 μmol of atractylate/g of protein the curves approach a maximum indicating the high affinity of atractylate to the membranes. A reciprocal plot similar to that for the ADP effect is not possible in view of the very small amount of free atractylate to be expected and the resulting uncertainty in the calculation of the free atractylate. Therefore, a "ligand conservation plot" is shown, where the degree of absorbance decrease (α) is related to the total amount of atractylate added according to the following relation (Webb, 1963)

$$N_0/\alpha = [K_d/(1 - \alpha)] + C_0$$

where N_0 = total amount of atractylate added, C_0 = number of carrier sites/gram of protein, and K_d = the dissociation constant of the carrier–atractylate complex.

As shown in the insert of Figure 3, a clustering of points is obtained at the lower end and, therefore, only an approximate line can be drawn for the evaluation of K_d and extrapolation of the number of binding sites. The tendency for clustering is a disadvantage of this relation. The evaluated $K_d = 3 \times 10^{-8}$ M for atractylate is somewhat smaller than that measured by binding studies with [^{35}S]atractylate ($K_d = 1 \times 10^{-7}$ M (Klingenberg *et al.*, 1973)). The number of binding sites as evaluated from the plot (C_0 = 1.3 μmol/g of protein) corresponds approximately to the number obtained by the direct [^{35}S]atractylate binding studies (Klingenberg *et al.*, 1971a, 1973). Thus, the absorbance decrease induced by atractylate appears to correlate to the binding of this ligand at the membrane.

The Dependence on ADP of the Bongkrekate Effect. The absorbance increase induced by bongkrekate addition after ADP is followed at increasing concentrations of bongkrekate

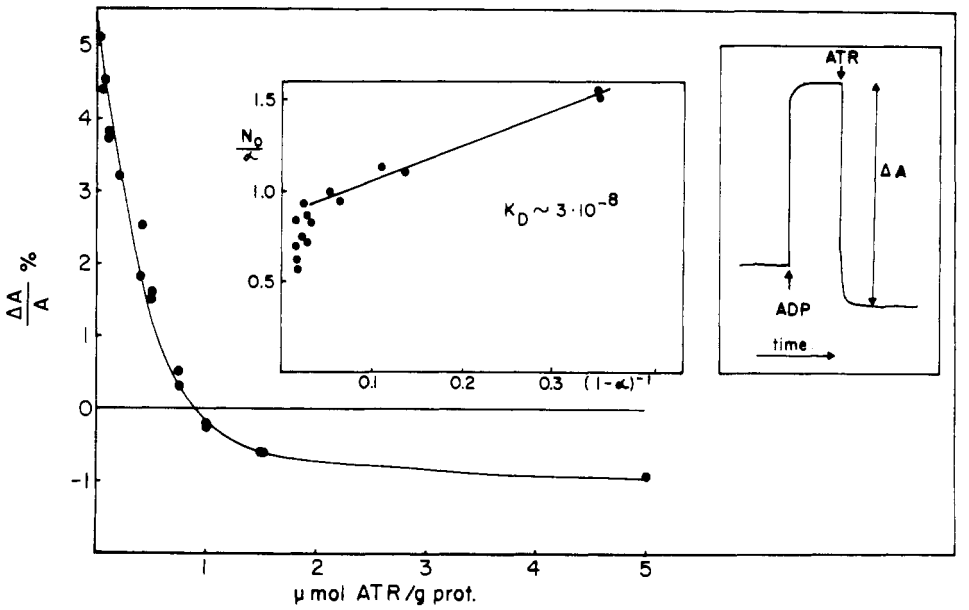

FIGURE 3: The reversal by atractylate of ADP-induced contraction as a function of the atractylate concentration. Absorbance increase was induced by 10 μM ADP and subsequently reversed by addition of atractylate (see right insert). The data are replotted according to the "ligand conservation" plot (see text) as shown in the left insert: 0.25 M sucrose, 20 mM morpholinopropanesulfonic acid, and 1 mM EDTA, final volume 1 ml.

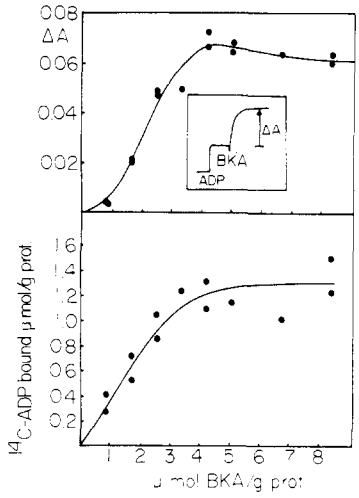

FIGURE 4: Increase of contraction by bongkrekate; comparison of absorbance increase and [^{14}C]ADP binding as a function of bongkrekate concentration. First 5 μM [^{14}C]ADP is added and subsequently bongkrekate in increasing concentrations. The extent of additional absorbance change is measured about 2 min after bongkrekate addition. At this time also samples of 0.5 ml were removed from the cuvet and were immediately centrifuged for determination of [^{14}C]ADP binding: 0.69 mg of beef heart mitochondria in 0.25 M sucrose, 20 mM morpholinopropanesulfonic acid, and 1 mM EDTA (1 ml), pH 6.5, 20°.

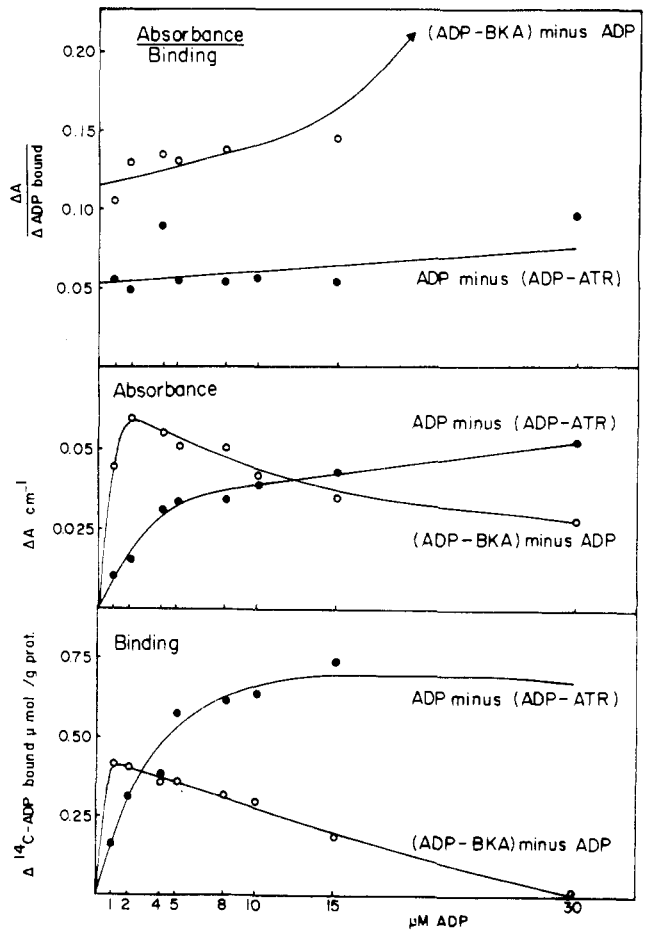

FIGURE 5: Correlation of shrinkage to [^{14}C]ADP binding under the influence of bongkrekate. In parallel samples the binding of [^{14}C]-ADP was measured as well as the absorbance increase, both after addition of [^{14}C]ADP and after further addition of bongkrekate: atractylate, 10 μM; bongkrekate, 5 μM; other conditions as described in Figure 4.

as shown in Figure 4, and is compared with the increase of [^{14}C]ADP binding as induced by bongkrekate in the same samples. There is an approximate agreement between both curves in that saturation is reached at about 4 μmol of bongkrekate/g of protein. This demonstrates that the bongkrekate-induced absorbance increase corresponds also to an increase of ADP binding similar to the one for ADP alone. Attempts to determine the K_d for bongkrekate in a "ligand conservation plot" were unsuccessful because of too much scatter. The bongkrekate-induced absorbance increase is dependent on ADP as was shown before (Klingenberg *et al.*, 1971b). This was in agreement with the mutual dependence on ADP of the bongkrekate effect at the carrier as elucidated previously (Klingenberg and Buchholz, 1973). The residual small and slow absorbance increase induced by bongkrekate alone can be attributed to the slow leakage of small amounts of endogenous ADP and ATP. The dependence on ADP of the bongkrekate-induced shrinkage indicated first the relation of this bongkrekate effect to the ADP carrier.

The correlation between the bongkrekate-induced absorbance change and the ADP binding is also followed by varying ADP concentration (Figure 5). Here in particular the comparison of the binding of ADP alone and the additional binding induced by bongkrekate is of interest. For the "specific" binding, the difference of total binding of ADP minus binding after addition of atractylate (case: ADP minus ADP–atractylate) is measured. In this case ΔA increases continuously throughout the whole range coming close to saturation around 5 μM ADP. In contrast ΔA in the case ADP–bongkrekate minus ADP reaches saturation at only 2 μM ADP and then slowly decreases. This is due to the fact that the sum of absorbance changes reaches its maximum at about 2 μM ADP and therefore the additional increase by bongkrekate becomes smaller because of the increasing share of ΔA occurring initially on addition of ADP.

The curves for the ADP binding (corrected for dilution by endogenous adenine nucleotides; see Methods) correlate only qualitatively with the corresponding curves of ΔA. Two discrepancies appear at first sight: at 1 μM ADP the bongkrekate-induced binding has reached saturation, whereas ΔA is not yet maximal. At high ADP concentration additional binding approaches zero, whereas the ΔA does not decrease below 50% of the maximum ΔA. For facilitating this comparison, the ratio of $\Delta A/\Delta$[ADP] is plotted. The absorbance change per binding change is nearly two times larger for the bongkrekate-induced binding than for the binding of ADP alone. This difference becomes larger with the increasing concentration of bongkrekate. Obviously there is no quantitative relation between ΔA and ADP binding. The bongkrekate-induced ΔA is relatively large as compared to the binding and becomes nearly independent of additional binding at high ADP concentrations. An explanation for this discrepancy will be offered in the Discussion.

Also the kinetics of the absorbance changes can be correlated to corresponding binding changes of ADP and, therefore, used as a convenient means of following kinetics of ADP binding. This correlation is shown in Figure 6, where the relatively slow absorbance increase induced by bongkrekate is kinetically resolved. In a parallel manner under the same incubation conditions the kinetics of ADP binding are measured by rapid pressure filtration (see Methods). Both results are superimposed (in Figure 6) demonstrating the relationship between the absorbance change and ADP binding during the

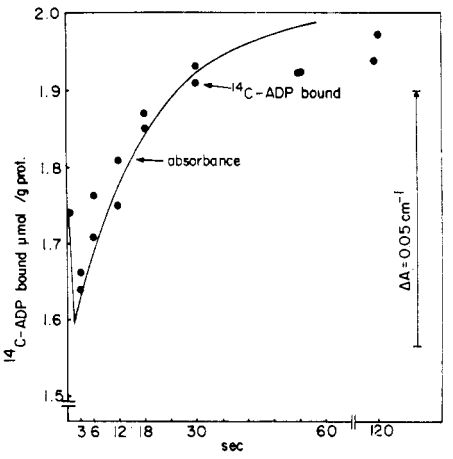

FIGURE 6: Correlation between the kinetics of the bongkrekate-induced shrinkage and ^{14}C binding. Parallel data show bongkrekate-induced absorbance changes together with measurements of ^{14}C binding in separate samples. First ADP and then bongkrekate are added. Thereafter, at the times indicated, the mitochondria are separated by a rapid pressure filtration technique (see Methods). In order to examine the fitting of the kinetic data, the maximum range of binding increase has been set equal to the absorbance increase for superimposing the binding data with the absorbance curve. The binding is calculated so as to take into account a lowering of specific activity by endogenous ADP–ATP assuming 5 µmole of ADP or ATP/g of protein: 0.8 mg of protein in 0.25 M sucrose, 20 mM Tris-maleate, and 1 mM EDTA, pH 6.5, 20°.

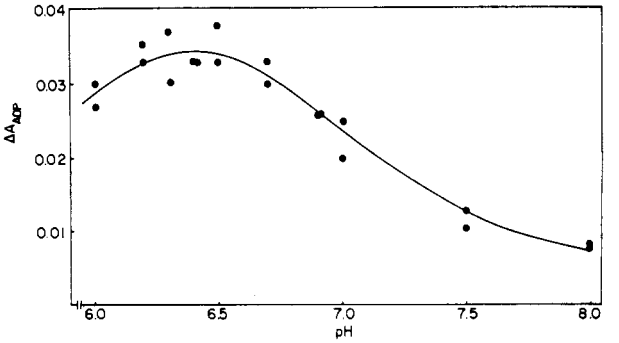

FIGURE 7: pH dependence of the contraction induced by ADP. The extent of absorbance increase is measured 30 sec after addition of ADP: 0.6 mg of beef heart mitochondria in 0.25 M sucrose, 20 mM Tris-maleate (at the pH indicated), and 1 mM EDTA.

time course. Even an initial absorbance decrease after addition of bongkrekate is reflected in a decrease of ADP binding (at 3 sec). This "anomaly" of the bongkrekate effect will be discussed below too.

Influence of pH on the Absorbance Change. The pH has been found to have marked effects on the specific ADP binding and in particular on the additional bongkrekate-dependent increase of ADP binding (Weidemann *et al.*, 1970; Klingenberg and Buchholz, 1973). In the absorbance changes corresponding effects can be observed. The absorbance increase induced by the addition of ADP has a pronounced pH dependence between pH 6 and 8 (Figure 7). It has a maximum at pH 6.4 and decreases at pH 8 to one-third, with an apparent pK = 7.1.

The pH dependence of the bongkrekate-induced ΔA is more difficult to evaluate because of the slowness of the absorbance change above pH 6.8 as illustrated in the insert of Figure 8. On the other hand, this offers the possibility of following the kinetics. The sigmoidal absorbance change after bongkrekate addition at pH 7 is caused by the counteraction of the initial absorbance decrease after the addition of bongkrekate and will be discussed subsequently. Obviously the absorbance follows a complicated course at higher pH which does not permit one to evaluate an actual initial rate. Most reproducible results could be obtained by evaluating the rate in the range where the absorbance curve rises above the level reached before addition of bongkrekate. The slope is here nearly maximal, *i.e.* close to the inflection point. From pH 6.0 to 7.0 the rate decreases about 100-fold. For better understanding of the pH influence the log of the rate of absorbance change for bongkrekate (log v_b) was plotted against the pH. An approximate straight line is obtained between pH 7.2 and 6.5 with a slope of 2.4 and in the range below pH 6.5 with a slope of 0.5. The extremely strong pH dependence of the bongkrekate-induced absorbance change can be described by the relation $v^* = kH^{2.4}$. This unusual pH dependence is an important clue for understanding why the bongkrekate effect on mitochondria as expressed in the absorbance change, in the increase of ADP binding and the simultaneous inhibition of ADP transport, takes an unusually long time in becoming effective at neutral pH (Klingenberg and Buchholz, 1973). Other ligands to the adenine nucleotide carrier, such as atractylate and also ADP, do not exhibit this time delay. Only at low pH binding of bongkrekate is as fast as expected for "normal" binding and then difficult to resolve kinetically.

The proportion factor 2.4 indicates that three H^+-dissociating groups are involved. These can easily be found in the three carboxylic groups of bongkrekate (Lijmbach *et al.*, 1971),

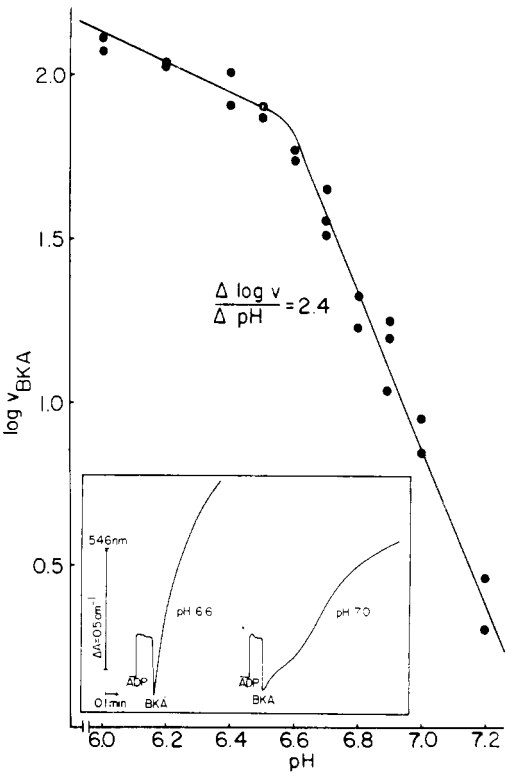

FIGURE 8: Dependence of the rate of the bongkrekate-induced contraction on pH. At first, 4 µM ADP is added, followed by 5 µM bongkrekate 2 min later. (The contraction rate was measured at the time when the graph reached the original ADP level following the initial absorbance decrease.) 0.5 mg of protein in 0.25 M sucrose, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, and 1 mM EDTA (1 ml), 22°. The insert shows an example of a photometric absorbance recording.

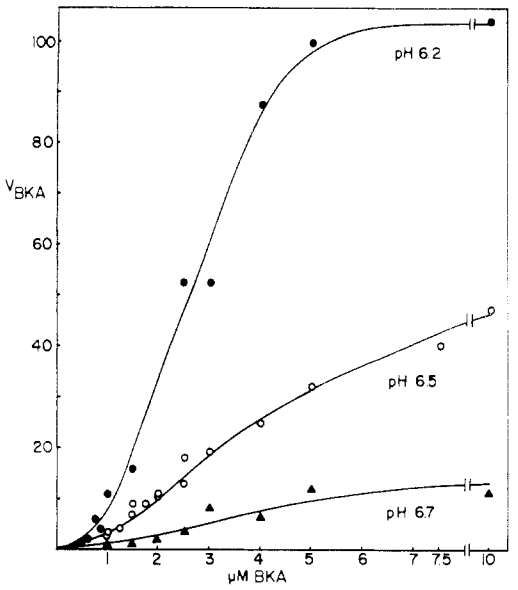

FIGURE 9: Mitochondrial contraction as a function of bongkrekate concentration at different pH values. Incubation of 0.5 mg of protein/ml, 20 mM Tris-maleate, and 10 μM ADP. For other conditions see Figure 8.

whereas their occurrence in the carrier protein would be uncommon. The three pK values of bongkrekate can be expected to be below pH 7 so that for the concentration of undissociated bongkrekate [$BKAH_3$] eq 1 follows. Assuming K_1,

$$[BKAH_3] = \frac{[BKA^{3-}][H^+]^3}{K_1K_2K_3} \quad (1)$$

K_2, and $K_3 \simeq 10^{-5}$ M it follows at pH 7 that $[BKAH_3] = 10^{-6} \times [BKA^{3-}] \simeq 10^{-6} \times [BKA]_t$. With $[BKA]_t = 10^{-5}$ M it follows that $[BKAH_3] = 10^{-11}$ M.

Obviously the undissociated form [$BKAH_3$] is rate limiting in the bongkrekate-induced absorbance increase. The important consequences of this result on the mechanism of the bongkrekate effect in particular and the relation between carrier mechanism and absorbance changes will be discussed below.

The interrelation of the rate of bongkrekate-induced absorbance change to pH and to bongkrekate concentration is studied in Figure 9. At pH 6.2 the rate appears to be saturated at a lower concentration of bongkrekate than at pH 6.5. It is also to be noted that the sigmoidal dependence on the bongkrekate concentration appears to increase with the pH. This is analogous to the pH influence on the sigmoidicity reported for the bongkrekate-induced ADP binding (Klingenberg and Buchholz, 1973). Furthermore there is a correlated disappearance of the sigmoidicity in the time course at lower pH as shown in the insert of Figure 8.

Competition between Atractylate and Bongkrekate. As shown already in Figure 1, bongkrekate is able to overcome the suppression by atractylate to some extent and to increase simultaneously the binding of ADP. Also from other binding experiments it is known that bongkrekate is a strong competitor of atractylate with respect to its opposite effect on the specific ADP binding (Weidemann *et al.*, 1970; Klingenberg and Buchholz, 1973).

The competition between bongkrekate and atractylate can be quantitatively analyzed in terms of the kinetics of the absorbance increase induced by bongkrekate (BKA) after the addition of varying amounts of atractylate (ATR). This competition can be described by the following equation, assuming a simple linear binding competition for the same site.

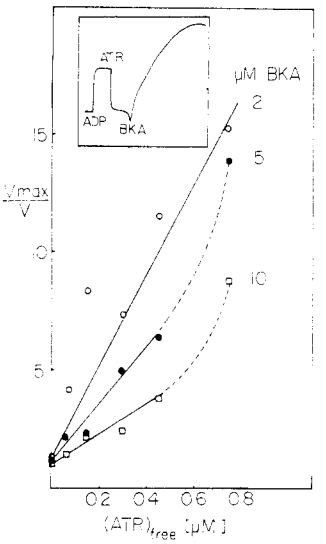

FIGURE 10: The competitive action of atractylate (ATR) on the bongkrekate (BKA)-induced rate of contraction. Plot according to eq 2. For the sequence of addition see insert. 0.4 mg of protein in 0.25 M sucrose, 20 mM Tris-maleate, 1 mM EDTA, and 10 μM ADP, pH 6.5, 20°. For the calculation of free atractylate see text.

μM BKA	S	$S \times [BKA] = \frac{K_D^{BKA}}{K_D^{ATR}}$
2	2	4
5	1.2	6
10	0.6	6

$$\frac{V_{max}}{V} = 1 + \frac{K_d^{BKA}}{[BKA]}\left(1 + \frac{[ATR]}{K_d^{ATR}}\right) \quad (2)$$

In a plot of V_{max}/V against [atractylate], the slope is $S = (1/[BKA])(K_d^{BKA}/K_d^{ATR})$.

A plot of V_{max}/V *vs.* [atractylate] should give a straight line with slopes decreasing with higher bongkrekate concentrations. The results of such an experiment are given in Figure 10 where the free concentration of atractylate has been calculated from the total concentration assuming for atractylate a $K_d = 2 \times 10^{-7}$ M and a number of binding sites (C) = 0.8 μmol/g of protein, according to the following relation.

$$[ATR]_{free} = \frac{[ATR]_t K_d}{K_d + C} \quad (3)$$

The experimental points can be approximated by straight lines which intersect the ordinate at nearly the same point on varying concentration of bongkrekate. The slopes are evaluated in the legend at the three bongkrekate concentrations for the ratio of the K_d of atractylate to bongkrekate. An approximate constant ratio is obtained for the three bongkrekate concentrations amounting to about sixfold higher K_d for atractylate than for bongkrekate.

It was of interest to study also the effect of carboxyatractylate on the mitochondrial absorbance changes. As was shown (Klingenberg *et al.*, 1973) carboxyatractylate causes an absorbance decrease similar to atractylate. However, the subsequent addition of bongkrekate is unable to reverse the effect of carboxyatractylate, indicating a much higher affinity of carboxyatractylate which cannot be overcome by bongkre-

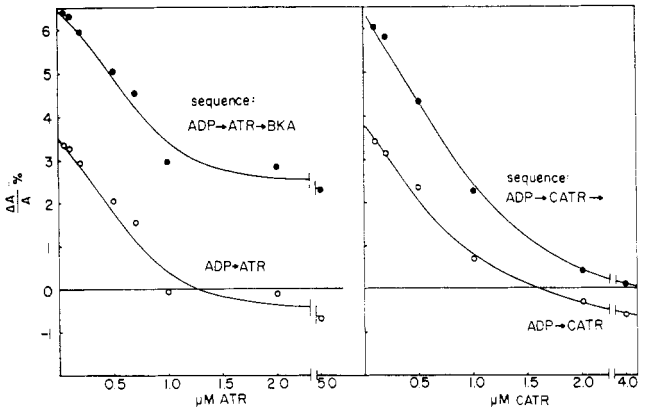

FIGURE 11: The competitive influence of actractylate and carboxyatractylate on the bongkrekate-induced absorbance increase. For conditions of incubation see Figure 10. The sequence of additions is indicated in the figure. ADP, 2 min; atractylate, 1 min; bongkrekate, 4 min.

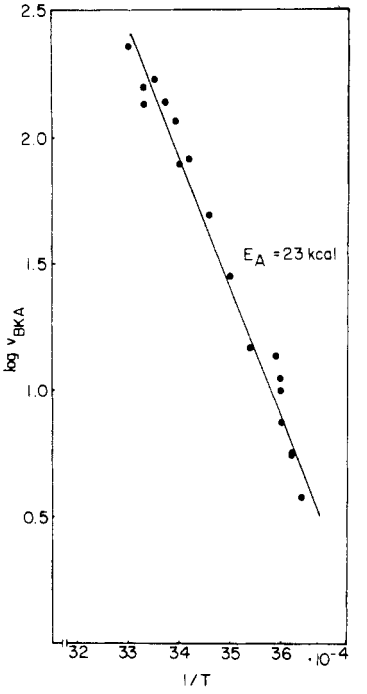

FIGURE 12: Influence of temperature on bongkrekate-induced contraction. Arrhenius plot. The rate of bongkrekate (10 µM) induced contraction after addition of ADP (10 µM) was measured over a range from 6 to 31°: 0.65 mg of protein/ml in 0.25 M sucrose, 10 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid), pH 6.8.

kate. This previously reported finding (Klingenberg *et al.*, 1971b) was later substantiated by measuring directly the binding of [^{35}S]carboxyatractylate to the membranes (Klingenberg *et al.*, 1973).

The difference in the contraction effects between atractylate and carboxyatractylate is demonstrated by comparing the competition between these ligands and bongkrekate on a more quantitative basis as shown in Figure 11. The absorbance decreases induced by the addition of atractylate and carboxyatractylate are both followed as a function of the concentration of these ligands and are shown to follow very similar curves. Both for atractylate and carboxyatractylate an apparently equal K_M (for atractylate 0.65 and for carboxyatractylate 0.70 µM) is found. The subsequent absorbance increase, however, differs in its concentration dependence on atractylate and carboxyatractylate. The net absorbance increase induced by bongkrekate remains approximately constant in the presence of atractylate over the whole range of atractylate concentrations; however, it decreases strongly with higher carboxyatractylate concentration. Thus the difference between atractylate and carboxyatractylate is not obvious on titrating these ligands alone but becomes apparent only by following the competition with bongkrekate. This has also been shown by comparing the influence of bongkrekate on the binding of [^{35}S]atractylate and carboxyatractylate (Klingenberg *et al.*, 1973).

Temperature Dependence of the Bongkrekate-Induced Rate of the Absorbance Increase. The temperature dependence of the rate of bongkrekate-induced absorbance increase was studied over a wide temperature range from 6 to 31°; see Figure 12. The Arrhenius plot gives over the whole range a straight line from which an activation energy E_A = 23 kcal can be derived. This corresponds to an unusually high temperature dependence with Q_{10} = 4. The high-temperature dependency would be in accordance with the assumption that the bongkrekate-induced rate of absorbance change reflects the diffusion rate of bongkrekate through the membrane. This rate should then be determined also by the viscosity of the membrane which can be expected to have much higher temperature dependency than an enzymic reaction. It should be mentioned that unusually high activation energies have been found for the overall ADP translocation in rat liver mitochondria (Pfaff *et al.*, 1969). In this case the translocation step of the carrier appears to be the rate-limiting step. However, also here in principle the viscosity of the lipid phase might be determining the translocation.

The slowness of the bongkrekate-induced absorbance change raises the question whether the preincubation with bongkrekate alone may produce a rapid absorbance change on subsequent addition of ADP. The absorbance increase could, therefore, be expected to be biphasic, with first a rapid and a remaining slower phase, where the rapid phase should increase with the time between the addition of bongkrekate and ADP. The absorbance change at 6 sec after ADP addition represents an approximate measure of the rapid phase (Figure 13). The results show that the rapid phase indeed increases with the preincubation time. The time required for half-maximum acceleration increases considerably at higher pH, as can be expected from the decreased rate of bongkrekate-induced absorbance in the case ADP–bongkrekate (see insert in Figure 8). At pH 6.9 even after 1.5 min only about 50 or 60% of the ΔA are converted to the rapid phase. This indicates that the cooperative effect between ADP and bongkrekate which has been noted earlier in binding studies (Erdelt *et al.*, 1972) particularly at pH 7.0 becomes important only at higher pH.

Discussion

The binding of adenine nucleotides to mitochondrial membranes had been extensively investigated in this laboratory for the elucidtaion of a specific binding to the ADP–ATP carrier (Weidemann *et al.*, 1970). The portion of the ADP binding linked to the carrier was measured as the atractylate-removable portion. This binding is distinguished by its high affinity for ADP and its high specificity for the ligands. It suggested to us that the absorbance changes, *i.e.* structural changes of mitochondria, induced by low amounts of ADP as originally reported by Stoner and Sirak (1970) are caused by the binding of ADP to the carrier (Klingenberg *et al.*, 1971b).

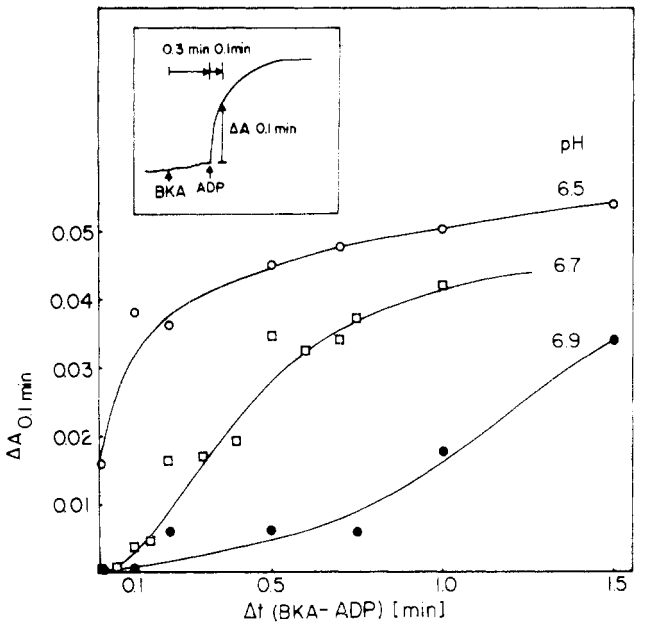

FIGURE 13: Influence of preincubation of mitochondria with bongkrekate on the absorbance increase after addition of ADP. Mitochondria (0.5 mg of protein in 0.25 M sucrose, 20 mM Tris-maleate, and 1 mM EDTA, 20°) were incubated for the time indicated with 4 μM bongkrekate. After preincubation, 5 μM ADP was added and the absorbance reached within 0.1 min was measured.

The close correlation shown in this publication between certain structural changes and the binding of ADP or ATP to the mitochondria demonstrates that the binding of ADP or ATP is a primary, although possibly indirect cause for the structural change. Alternative explanations as based on binding of ADP to other sites can be ruled out mainly because of the sensitivity of ADP binding to atractylate and the insensitivity to oligomycin (see also Weber and Blair 1970). This excludes the possibility that the interconversion of ADP or ATP associated with energy transfer causes these structural changes. The most striking analogy is the opposite effect of atractylate and bongkrekate on the ADP-induced structural change, which has been previously reported by us (Klingenberg *et al.*, 1971b). The complicated response of binding to these highly specific inhibitors (Weidemann *et al.*, 1970; Klingenberg and Buchholz, 1973) is correlated here to the structural changes.

The results also illustrate the agreement of both effects with respect to the specificity, as shown by the K_M and the high sensitivity to changes in the nucleotide structure of the base, ribose, or phosphate region. Even when it is possible to resolve the kinetics, as in the case of the bongkrekate-induced increase of ADP binding, agreement with the time course of absorbance increase was found. A number of additional parameters, such as the strong influence of pH and of temperature on the bongkrekate-induced effects and the difference between the affinity of atractylate and carboxyatractylate, further support the correlation of the structural changes to the ADP carrier.

With these provisions the absorbance changes induced by the structural changes are a valuable tool for gaining information on the function of the carrier, its interaction with the ligands, and possible transport mechanism, although the nature of these structural changes is not explained. The more detailed analysis of the data will show that the structural change actually reflects carrier distribution across the membrane and, therefore, it is correlated to the ADP binding insofar as it influences this carrier distribution.

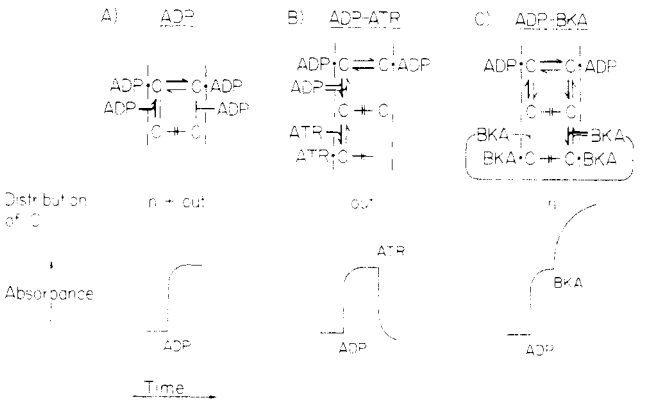

FIGURE 14: Interpretation of the ligand-induced absorbance changes in terms of the reorientation of the mobile carrier to the two sides of the membrane.

Structural Changes and Mobility of the Adenine Nucleotide Carrier. The most fruitful model for elucidating the structural changes linked to the ADP carrier is based on the mobile or reorientating carrier concept. The present results provide evidence only for the mobility of the substrate binding site of the carrier. This does not necessarily imply diffusion of the carrier through the membrane. The essential features of this "mobile" carrier are the following. (a) The carrier is mobile only when loaded with the ligand ADP or ATP and immobilized when it is unloaded. (b) The carrier can be distributed statistically toward the inside or outside of the membrane, both in the loaded or unloaded state. The carrier distribution across the membrane can be described in simple terms as a function of the ADP concentration, using the reaction sequence

$$ADP_e + C_e \overset{K_d}{\rightleftarrows} C\cdot ADP_e \overset{K}{\rightleftarrows} C\cdot ADP_i \overset{K_d}{\rightleftarrows} C_i + ADP_i \quad (4)$$

where e = external, i = internal, C = carrier, and K = distribution constant across the membrane and eq 5, where C_t = total amount of carrier.

$$\frac{C_t}{[C_i] + [C\cdot ADP_i]} = 1 + \frac{1}{K_d}\frac{1 + K_d/[ADP_e]}{1 + K_d/[ADP_i]} \quad (5)$$

Assuming saturation by ADP inside, it follows that

$$([C_i] + [C\cdot ADP_i])/C_t = \frac{[ADP_e]K}{[ADP_e](1 + K) + K_d} \quad (6)$$

(c) The carrier is immobilized on either side of the membrane when loaded with an inhibitor.

With these principles, the structural changes can be correlated to the mobile carrier with the following postulates. (1) The structural change reflects distribution of carrier across the membrane, independent of whether or which ligand is bound to the carrier. (2) The absorbance increase reflects accumulation of the carrier inside and *vice versa*. (3) "Mobilizing" ligands such as ADP or ATP cause distribution of the carrier to both sides of the membranes; immobilizing ligands produce one-sided distribution, either to the outside (atractylate, carboxyatractylate) or to the inside (bongkrekate) (Figure 14).

With these postulates most of the experimental results, in particular the effect of the inhibitors, can be satisfactorily ex-

plained. Before addition of any ligand, the mitochondria are in a relatively low absorbance state corresponding to the structural "relaxed" state. This fits the postulated model since it can be expected that in the absence of external ADP but sufficient endogenous ADP, the carrier is accumulated on the outer surface in the unloaded form. Carriers on the inside binding endogenous ADP become mobile, migrate to the outer surface, and after losing ADP are trapped as immobile "empty" carrier. On the addition of ADP these carriers become mobile again and migrate to the inner surface (Figure 14A). This is reflected in the absorbance increase with increasing concentration of ADP or ATP (Figure 2). The carrier should distribute to the inside proportional to the binding increase following eq 6. This is fulfilled by the close relationship of the absorbance increase to the binding (Figures 2 and 5). On saturation with ADP the carrier should be distributed partially to the in- or outside. As a result the absorbance increase on saturation with ADP does not reach the same extent as the increase induced by bongkrekate.

The impermeability of the mitochondrial membrane to atractylate and several other effects suggests that these inhibitors affect the carrier only from the outside (Weidemann *et al.*, 1970; Klingenberg *et al.*, 1971a,b, 1973). According to the reorienting model therefore with atractylate the carrier should accumulate on the outer surface in the form of the atractylate–carrier complex providing that the carrier is rendered mobile by sufficient ADP inside (Figure 14B). In accordance atractylate abolishes the ADP-induced absorbance increase when added before or after ADP. The concentration dependence of the absorbance decrease by atractylate (Figure 3) is closely related to the corresponding dependence of the binding of atractylate (unpublished). Also the absorbance decrease by atractylate below the basic absorbance level is explained. In the absence of ADP (before addition of atractylate) a small amount of exogenous ADP originating from endogenous ADP may cause the orientation of a small carrier portion to the inside. The addition of atractylate brings about the outward localization also of this portion. Thus with atractylate and carboxyatractylate the complete accumulation of carrier on the outer surface is reached.

Mechanism of the Bongkrekate-Induced Shrinkage. The use of bongkrekate has been of great value in revealing the nature of the absorbance changes and also the mechanism of the carrier. The surprising increase of ADP binding to the membranes by bongkrekate opposite to the effects of atractylate provides strong support for relating absorbance changes to carrier reorientation. Furthermore, here the carrier distribution model gives valuable assistance in explaining the variety of rather complicated effects of bongkrekate, in particular when comparing binding and structural change (Figure 14c). The strong absorbance increase by bongkrekate would be explained by postulating that bongkrekate binds to the carrier preferentially from the inside resulting in an inside accumulation of the carrier by forming the tight bongkrekate–carrier complex. For this reaction the carrier must be mobilized on the outer surface by external ADP, in accordance with the requirement of ADP for the bongkrekate-induced absorbance increase.

The model also explains why the absorbance increases more than the binding of ADP when bongkrekate is introduced (Figures 4 and 5). When the membranes are saturated with ADP and the distribution inside to outside according to eq 6 is reached, addition of bongkrekate can be expected to pull the residual carriers from the outside to the inside by the trapping effect. Under this condition no further increase of the ADP binding occurs. At nonsaturating concentration of ADP the parallel increase of ADP binding and absorbance by bongkrekate is explained by the reaction sequence introduced earlier during the binding studies. All free carrier sites become occupied with ADP forming mobile ADP–carrier complexes and are then trapped on the inside with release of ADP (Figure 14). Thus the additional ADP binding corresponds to the free carrier which eventually becomes occupied by ADP, moves across the membrane, and releases ADP to the inside.

This analysis shows that it is the shift of the carrier to the inside which induces the structural change independent of ADP binding. ADP induces the absorption increase insofar as it promotes carrier distribution to the inside, either alone or together with bongkrekate.

It is understandable that bongkrekate can bind to the carrier from the inside since as a lipophilic substance it should penetrate the membrane, in contrast to atractylate. It can be expected that only the undissociated form of bongkrekate is able to penetrate the membrane. This should result in a strong pH dependence of the distribution of bongkrekate across the membrane according to the following relation (see above).

$$[BKA_i]/[BKA_e] = [H_e^+]^3/[H_i^+]^3$$

Thus bongkrekate should accumulate inside up to 1000-fold per $\Delta pH = 1$ across the membrane. This may furnish a simple explanation for the preference of bongkrekate binding to the inside as deduced from the model.

A clear dependence on the pH cannot be observed for the *extent* of the bongkrekate-induced shrinkage since it is difficult to obtain final values at low ΔA because of the very slow absorbance increase. However, the *rate* of bongkrekate-induced shrinkage gives the possibility to measure the pH influence since the rate limiting step for the bongkrekate-induced contraction is probably the permeation of bongkrekate. As was discussed above at pH 7, assuming $pK = 5$ for the three carboxylic groups of bongkrekate, only about a portion of 10^{-6} of the total bongkrekate (BKA_t) is in the $BKAH_3$ form. The rate of permeation (v) and the corresponding rate of shrinkage should therefore decrease threefold with increasing pH.

$$v = k[BKAH_3] = k'[BKA_t][H^+]^3$$

$$\text{since } [BKA^{3-}] \simeq [BKA_t]$$

$$\log v = \log [BKA_t] - 3pH + k''$$

The extremely strong pH dependence of the shrinkage rate (Figure 8) can thus be satisfactorily explained. The observed slope, $\Delta \log v/\Delta pH = 2.4$, approaches the theoretical value of 3. At pH 6.5 the rate becomes so high that other steps become rather limiting, *e.g.*, the rate of binding of bongkrekate to the carrier.

A transitional binding of bongkrekate also to the outside should be possible and would explain why at higher pH bongkrekate leads initially to a rapid absorbance decrease, similar to atractylate (see insert of Figure pH 7).

The temperature dependence of the ra of the bongkrekate-induced absorbance increase is unusua high ($Q_{10} = 4$). In view of the assumption that this rate may reflect the diffusion of bongkrekate through the membrane, it may be due to the change of viscosity with the temperature. It is remarkable that the change with temperature is continuous, since no phase

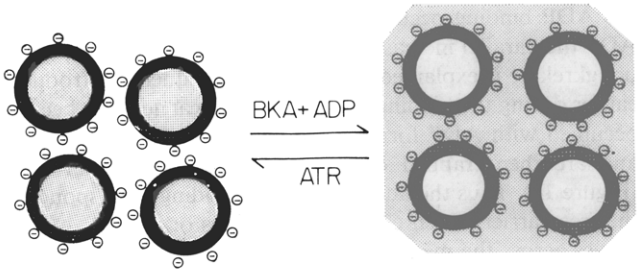

FIGURE 15: Interpretation of the transition from the vesicular (= decontracted) to the vacuolar (= contracted) state of mitochondrial matrix. The change in magnitude of one surface area as compared to the other leads to a reversion of the membrane configuration caused by charge excess on the membrane surfaces.

transition is apparent. Similar high temperature dependence was found for the rate of the ADP–ATP exchange in rat liver mitochondria (Pfaff *et al.*, 1969). This exchange is probably limited by the movement of the carrier through the membrane and may, therefore, likewise reflect a viscosity change in the membrane phase.

The interpretation offered by Stoner and Sirak (1973a) does not include a distribution or reorientation of the carrier on both membrane sides. They assume that ADP, by neutralizing the positive charges of the carrier, allows the carrier to move deeper into the membrane causing the contraction. They are unable to interpret the effect of bongkrekate as being caused by specific binding to the carrier, but rather they assume that bongkrekate affects the lipid phase and thus only indirectly influences the carrier. However, there are numerous arguments for the specific binding of bongkrekate to the carrier to the same binding site as atractylate or ADP (Klingenberg *et al.*, 1973).

On the Nature of the Morphological Changes. The nature of the observed morphological changes is of great importance to the eventual understanding of the mechanism of the ADP carrier. It is difficult to comprehend that at a density on the membrane surface of about 1 per 250 nm^2, as calculated from [^{35}S]atractylate binding (Klingenberg *et al.*, 1973), the movement of these carrier molecules can change the overall configuration of the membrane. With the assumption of mol wt ~300,000 for the carrier, only about 5% of the membrane protein could be accounted for by the ADP carrier. It is improbable that only the redistribution of such a small portion of protein could lead to the observed conformational changes of the membrane.

Some clues may be derived from a closer examination of the morphology of these changes. As shown before (Klingenberg *et al.*, 1973), these changes depend on the network of the cristae system, which has a tendency toward a more vesicular form in the presence of atractylate, and toward a more vacuolar configuration in the presence of bongkrekate. There is no evidence of configurational changes of the membrane itself. These morphological transitions may be simply caused by change of one membrane surface area relative to the opposite surface area. Increase of the outer surface area relative to the inner surface area leads to the vesicular form and *vice versa* to the vacuolar form. One possible explanation for such a fundamental change, induced by such specific interaction, would be the accumulation of either positive or negative surface charges (Figure 15). It can be visualized that these changes are produced by various specific or unspecific effectors. This is suggested by experiments using the lipophilic cation octylguanidine, which was observed to induce an absorbance increase similar to that caused by ADP (Schäfer *et al.*, 1972). This cation probably orients itself in the membrane to one side forming a layer of excess positive charges (Schäfer and Bojanowski, 1972).

In the present case it can be visualized that the surface charge accumulation is produced by the preferential orientation of the carrier to one membrane side by a mechanism not yet known. Possibly a reorientation of carrier-associated anionic phospholipids is involved.

Acknowledgments

We thank Miss B. Schmiedt for her skillful technical assistance. Bongkrekate was a kind gift of W. Berends, Delft.

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