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Characterization of the Kinetics and Mechanism of the Mitochondrial ADP-ATP Carrier*

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Initial velocity measurements of [^{3}H]ADP and [^{3}H]-ATP uptake in rat liver mitochondria were taken as a function of the mitochondrial energy state over a wide range of external nucleotide concentrations in an effort to understand kinetic anomalies (*i.e.* nonlinearity) often seen in published double reciprocal plots for adenine nucleotide transport. In addition, the mechanism of carrier transport was examined by varying the size of the exchangeable [ADP] plus [ATP] pool. Results obtained showed that plots of v^{-1} *versus* [ADP]$^{-1}$ or [ATP]$^{-1}$ under both high energy and uncoupled conditions were nonlinear and biphasic, suggesting the presence of (*a*) two carriers with different kinetic properties; (*b*) negative cooperativity among adjacent carrier subunits or clusters of carriers in the membrane; or (*c*) endogenous, tight-binding competitive inhibitors (*e.g.* long chain acyl-CoA esters).

Determinations of the apparent kinetic constants at higher substrate concentrations indicated that under energized conditions K_m and V_{max} values for ATP uptake are greater than K_m and V_{max} values for ADP uptake but that under de-energized conditions the opposite was observed. Changes in the K_m/V_{max} ratio, as a function of the mitochondrial energy state, were mainly due to the 10-20-fold increase and decrease in the K_m for ADP and ATP uptake, respectively, upon a high energy to uncoupled state transition.

Variation in the size of the intramitochondrial exchangeable pool was achieved by treating mitochondria with potassium arsentate which lowered the ATP/ADP ratio as well as the total amounts of [ADP] plus [ATP] while increasing the level of [AMP]. The double reciprocal plots at various internal [ADP] plus [ATP] levels also showed biphasic patterns which extrapolated to yield two sets of intersecting lines on the abscissa. This pattern was observed for both ADP and ATP uptake and was independent of the mitochondrial energy state. These results indicate formation of a ternary complex as an integral part of the exchange process. Such an intermediate is inconsistent with the widely accepted ping-pong mechanism for nucleotide transport. A model for carrier transport which is consistent with the formation of a carrier-(nucleotide)$_2$ ternary complex and with previous results from inhibitor-binding studies is presented.

Kinetic studies of mitochondrial adenine nucleotide transport have largely focused on the substrate specificity and factors which influence the rates of nucleotide exchange. These studies have shown that: (*a*) transport occurs by a one for one exchange between intra- and extramitochondrial adenine nucleotides (1, 2); (*b*) uptake (3-6) and release (7) of nucleotides is highly specific for ADP and ATP; (*c*) rates of nucleotide exchange are influenced by the size (8), chemical activity (7-9), and the ATP/ADP ratio (10, 11) of the endogenous exchangeable nucleotide pool; and (*d*) rates are also influenced by the levels of endogenous inhibitors such as long chain acyl-CoA esters (12, 13) and the energy state of mitochondria (8, 11, 14-19).

The latter finding that rates of nucleotide uptake are influenced by energization of mitochondria has received considerable attention since it suggests that ADP-ATP exchange is electrogenic (8, 15, 20, 21). This concept has been investigated by several groups and good evidence has been obtained indicating that the membrane potential generated by substrate oxidation is the driving force for asymmetric nucleotide exchange (21, 22).

The details by which the membrane potential modulates the rates of nucleotide exchange are unknown, although two explanations have been offered. The first proposes that the membrane potential, poised negative inside, retards the movement of carrier-ATP relative to carrier-ADP complexes toward the inside due to one extra negative charge on ATP at physiological pH (4, 23). The second suggests that the membrane potential directly influences the conformation of the carrier and, thus, the apparent kinetic constants for ADP and ATP uptake (14). Discrimination between these two hypotheses is important since both offer an explanation for why the ATP/ADP ratio is greater outside than inside under energized conditions (24). We have re-examined the kinetic data reported in the literature and, in particular, the data which support the above proposals and have observed an inconsistency concerning the presentation of velocity data in double reciprocal plots.

In many instances (for examples see Refs. 3, 14), the data presented describe a nonlinear (concave-down) relationship of v^{-1} *versus* [substrate]$^{-1}$, although the authors would have them fit a straight line. In addition, in those studies in which nonlinearity was recognized (for example, Ref. 7), a discussion as to its significance was usually not presented. Accordingly, we have examined the phenomenon of nonlinearity seen in double reciprocal plots by measuring rates of nucleotide uptake over a much larger substrate concentration range than was used in previous studies. We have also examined the mechanism of nucleotide transport by means of initial velocity measurements under both high and low energy states in an effort to understand the details by which the membrane potential influences rates of nucleotide exchange. A study of this nature seems particularly important since, although the mechanism of nucleotide exchange reported is equivalent to ping-pong (25, 26), this proposal has not been verified by initial velocity measurements. Instead, efforts to describe the

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mechanism of transport have relied exclusively on inhibitor- and nucleotide-binding studies.

The results presented here show that, under both uncoupled and high energy conditions, double reciprocal plots of ADP and ATP uptake are nonlinear and appear distinctively biphasic. Moreover, instead of parallel lines, a pattern of intersecting lines is obtained for plots of v^{-1} *versus* (nucleotide)$^{-1}$ at various fixed internal nucleotide concentrations demonstrating the existence of a ternary complex and thus excluding a ping-pong mechanism for carrier transport.

MATERIALS AND METHODS

ADP-ATP, oligomycin, ruthenium red, NADH, NADP, carboxyatractyloside, *m*-chlorocarbonylcyanide phenylhydrazone, bovine serum albumin, pyruvate kinase, phosphoenolpyruvate, hexokinase, yeast glucose 6-phosphate dehydrogenase, lactate dehydrogenase, myokinase, and A23187 were purchased from Sigma. Dibucaine (Nupercaine) was from K and K Laboratories, Hicksville, New York. [2,8-^{3}H]ADP (26.4 Ci/mmol) and [2-^{3}H]ATP (16 Ci/mmol) were obtained from New England Nuclear and Amersham, respectively. Male Sprague-Dawley rats (250–350 g) were from Taconic Farms, Germantown, New York.

Isolation of Mitochondria—Rat liver mitochondria were isolated from male Sprague-Dawley rats by the method of Johnson and Lardy (27) in 0.25 M sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, pH 7.4, 1 mM EDTA, 1% bovine serum albumin, and 200 μM dibucaine.

Determination of Mitochondrial Adenine Nucleotides—Mitochondrial adenine nucleotide levels were measured by enzymatic analysis of neutralized perchloric acid extracts obtained from 10–15 mg of mitochondria. AMP and ADP levels in the neutralized extracts were analyzed by the method of Jaworet *et al.* (28) while ATP levels were measured by the procedure of Lamprecht and Trauschold (29).

Adenine Nucleotide Transport Assay—Mitochondrial uptake of [^{3}H]ADP and [^{3}H]ATP was measured using the carboxyatractyloside inhibitor stop technique described previously (13). In a final volume of 47.5 ml, 30–50 mg of mitochondria were suspended in a medium containing 116 mM KCl, 21 mM Tris/HCl, pH 7.4, 1.05 mM EDTA (KCl medium), 5.26 mM 2-oxoglutarate, and, unless otherwise stated, 5.26 μM ruthenium red. After a 5-min incubation at room temperature, 100 μg of oligomycin was added to the mitochondrial suspension and chilled to 2°C. The assay was started by adding titrating amounts (50 μl) of radioactive ADP or ATP (using a series of Hamilton syringes) to microfuge tubes containing 0.95-ml aliquots of the suspension under constant mixing in a Vortex mixer. After 12 s, the reaction was stopped by injecting 50 μl of 200 μM carboxyatractyloside (using another series of syringes), and the mitochondria were subsequently pelleted by centrifuging at 12,000 × *g* for 4 min. The supernatant was removed by aspiration and the mitochondria were washed by resuspending the pellet in 1 ml of KCl medium containing 10 nmol of carboxyatractyloside and centrifuged as before. The pellet was dissolved in 0.2 ml of 2% sodium dodecyl sulfate, transferred to a scintillation vial containing 3.0 ml of counting fluid, and counted by liquid scintillation. At this temperature (2°C) and under these conditions (both in the presence and absence of uncouplers), the first order rate constant for nucleotide uptake remained unchanged during the first 15–20 s of the assay. Therefore, the 12-s time point is chosen for the kinetic analysis. Corrections for nonspecific counts trapped by mitochondria in the adherent fluid and sucrose-permeable space were made by subtracting values obtained for controls in which the inhibitor was added before the labeled nucleotide.

The apparent kinetic constants were calculated from linear portions of the curve by the direct linear plot method of Eisenthal and Cornish-Bowden (30). Mitochondrial protein determinations were by the biuret method (31).

RESULTS

Velocity Dependence on External Substrate Concentrations—The double reciprocal plots shown in Fig. 1 summarize results of initial velocity studies characterizing the effects of changes in the mitochondrial energy state on the apparent kinetic constants for ADP (Fig. 1*A*) and ATP (Fig. 1*B*) uptake. As seen in the figure, the plots are biphasic. In addition, this pattern is seen in both energized and uncoupled mitochondria. These results, in general, constrast with previous studies which claimed that the kinetics of nucleotide uptake followed the classical Michaelis-Menten type (10).

It is evident from these data that a wide nucleotide-concentration range must be used in order to detect the biphasic character of the plot. In the present study, we have varied the external ADP and ATP concentrations up to 200- and 350-fold, respectively. Most investigations have employed a narrow range of nucleotide concentration and thus the biphasic nature of the kinetic plots has not been recognized. This has resulted in large differences in reported kinetic constants and the effects of membrane potential on rates of nucleotide exchange. Apparent kinetic constants reported in the literature have been obtained by extrapolating the linear regions of plots corresponding to high (7) and low (8) substrate concentrations and thus have differed by more than 10-fold.

Souverijn *et al.* (14) reported that the K_m and V_{max} values for ADP uptake were insensitive to changes in the mitochondrial energy state. This contrasts with the data shown in Fig. 1*A* and a previous report by Vignais *et al.* (19) which demonstrates that both the K_m and V_{max} values for ADP uptake are greater in uncoupled than in coupled mitochondria. Careful examination of the data in Fig. 1*A* shows that the K_m and V_{max} values for ADP uptake are similar in coupled and uncoupled mitochondria if the kinetic constants are obtained in the substrate concentration range used by these authors (1–15 μM). This, however, would correspond to an extrapolation to the left of the break point in coupled mitochondria and to the right in uncoupled mitochondria. Such comparison is clearly invalid. The shift in the break point for the kinetic plot of ADP uptake upon a change in the membrane potential is evident only if a broad substrate concentration range is used.

Also seen in Fig. 1 is that the biphasic nature of the plot seems to vary with the energy state of mitochondria. Less markedly biphasic plots are seen for ATP uptake in uncoupled than in coupled mitochondria and for ADP uptake in coupled than in uncoupled mitochondria. This effect of the membrane potential may be only apparent if the following two points are considered. First, at low external substrate concentrations, the leakage of unlabeled internal exchangeable adenine nucleotides will result in a higher external substrate concentration and a lower isotope specific activity than is realized. This effect will result in an overestimation of the slope of the plot, particularly at lower substrate concentrations, and thereby an underestimation of its biphasic character. We have attempted to minimize the leakage of internal adenine nucleotides by isolating mitochondria in an isotonic sucrose medium supplemented with 1% albumin and 200 μM dibucaine. Both these agents have been shown previously to protect the integrity of mitochondrial membranes, especially at elevated temperatures (32). Under these conditions, we have estimated that the loss of internal nucleotides is on the order of 5%. This loss corresponds to a maximum leakage of 0.25 nmol/mg of exchangeable nucleotides for assays in which 1 mg of mitochondria containing 5 nmol of internal ADP plus ATP were used and thus introduces up to 125% error in the value of the lowest substrate concentration (0.2 μM). We have not attempted to make corrections for this error since the chemical activity of these nucleotides would likely be less than those added externally and the ability for these nucleotides to interfere with transport assays would depend on their ATP/ADP ratio and the energetic state of mitochondria. For example, the leakage of nucleotides having a high ATP/ADP ratio would hardly affect the uptake of ADP in coupled mitochondria since the K_m for ATP uptake under these conditions is 20–50-fold higher than that of ADP uptake.

The second point deals with the effects of residual adenylate

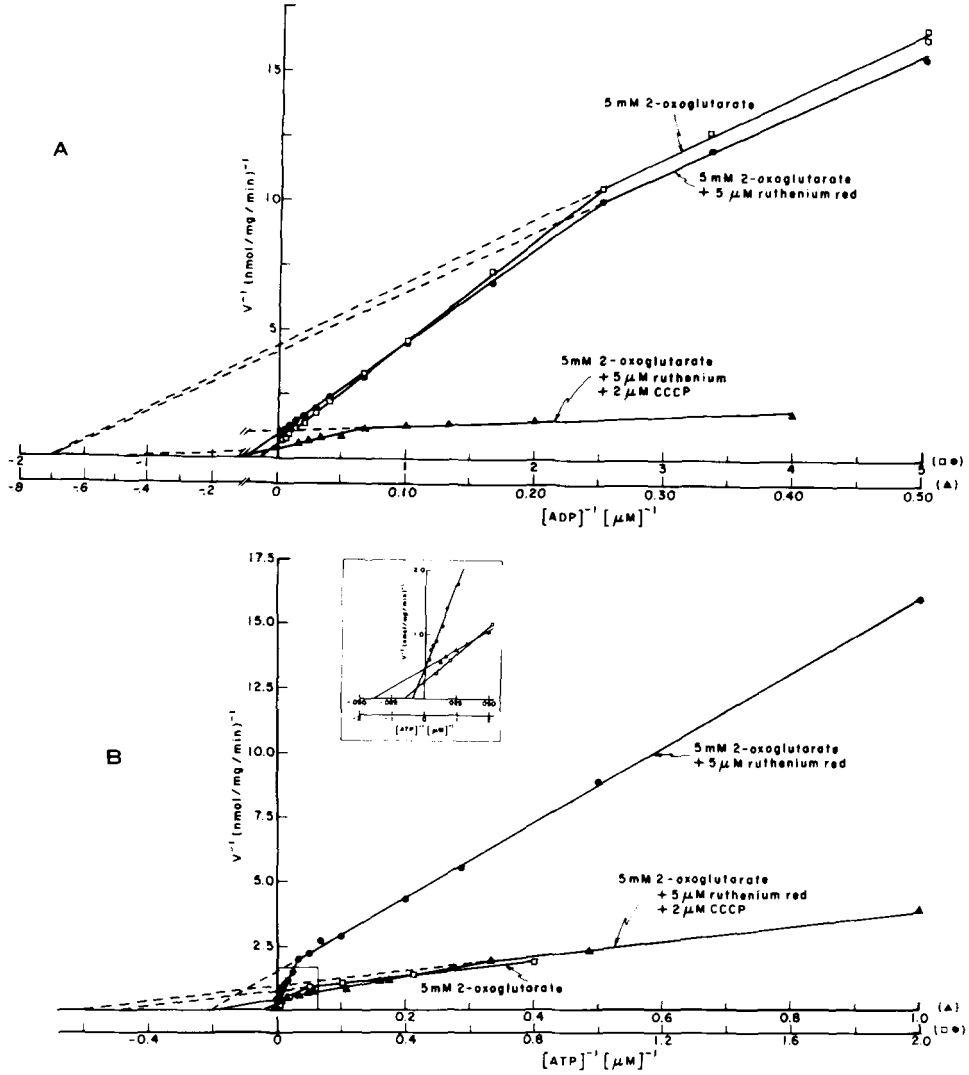

FIG. 1. **Lineweaver-Burk plots of ADP and ATP uptake: effects of ruthenium red and mitochondrial energy states.** Initial velocities of [^{3}H]-ADP (*panel A*) and [^{3}H]ATP (*panel B*) uptake were measured as described under "Materials and Methods" in the presence (●——●) and absence (□——□) of ruthenium red and the presence of 2 μM carbonyl cyanide *m*-chlorophenylhydrazone plus ruthenium red (▲——▲). The reaction was started by adding 50 μl of labeled nucleotide (ADP, 0.2–40 μM final concentration, with specific activity ≥3000 cpm/nmol; and ATP, 1–350 μM final concentration, with specific activity ≥2000/cpm/nmol) and stopped after exactly 12 s by adding 50 μl of 200 μM carboxyatractyloside. Final concentrations of chemical compositions in the assay medium after addition of nucleotides were 110 mM KCl, 20 mM Tris-HCl, pH 7.4, 0.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10 μM dibucaine, 0.5 mg/ml of serum albumin, 2 μg/ml of oligomycin, 1.05 mM EDTA, 5 mM 2-oxoglutarate, 12.5 mM succrose and, where indicated, 5 μM ruthenium red and 2 μM carbonyl cyanide *m*-chlorophenylhydrazone. Note the two different scales on the abscissa. The upper and lower scales on the abscissa refer to assays measured in the presence and absence of the uncoupler, respectively. The *inset* in *panel B* is an expanded scale of data points to the left of the breakpoint. Data shown were averaged values from duplicate sets. Average error after correcting for zero time values was ≤5% ± S.E.

kinase activity. Duée et al. (6) have shown adenylate kinase activity is very low or absent in the presence of 1 mM EDTA. However, even if some activity remains, it can be shown that only the slope of the plot is influenced (overestimated), particularly at low substrate concentrations. This is true since adenylate kinase will convert substrate (ADP or ATP) to "nonsubstrate" (AMP) while not affecting the specific activity of the isotope. In addition, a brief consideration of the Michaelis-Menten equation demonstrates that the per cent conversion of substrate to nonsubstrate decreases with increasing substrate concentrations. Thus, the net effect of these two factors would overestimate the slope of the plot to the right of the break point and therefore again underestimate the biphasic character of the plot.

The significance of biphasic velocity dependence on substrate concentration indicates (*a*) the presence of two carriers having different kinetic properties, (*b*) negative cooperativity between adjacent carrier subunits or clusters of carriers in the membrane, or (*c*) the presence of endogenous, tight-binding competitive inhibitors. A more detailed discussion of these interpretations is given in the next section.

Data in Table I show the effects of the mitochondrial energy state on the apparent kinetic constants for ADP and ATP uptake in mitochondria obtained at high substrate concentrations. The K_m and V_{max} values for ADP and ATP uptake in coupled mitochondria and V_{max} values for ATP uptake in uncoupled mitochondria are similar to those reported by Souverijn *et al.* (14), although the V_{max} value for ADP uptake under our conditions is somewhat lower in coupled and higher in uncoupled mitochondria. In disagreement with these authors (14) are the K_m values for ADP and ATP uptake in uncoupled mitochondria. We have observed that the K_m value for ADP uptake increased from 2–4 to 20–35 μM upon uncoupling whereas the K_m value for ATP uptake was lowered from 100–120 to 6–10 μM. However, Souverijn *et al.* (14) reported that the K_m values for ADP and ATP uptake were both equal to 1–2 μM. As discussed above, the discrepancy in data between the two groups may stem from the fact that these authors used a much narrower substrate range and thus would not have been expected to detect the higher K_m values seen at higher substrate concentrations.

In summary, the K_m and V_{max} values for ATP uptake are greater than the K_m and V_{max} values for ADP uptake in coupled mitochondria whereas, in uncoupled mitochondria, the reverse is true. Furthermore, uncoupling of mitochondria lowers the K_m value for ATP uptake whereas the K_m and V_{max} values for ADP uptake are increased.

Importance of Ruthenium Red in Transport Assay—Siliprandi *et al.* (33) have reported that, when coupled rat liver mitochondria were incubated in a medium containing an oxidizable substrate, but lacking Mg^{2+}, mitochondria lose internal Mg^{2+}. Inhibitors of the respiratory chain, uncoupling agent, and ruthenium red prevented this magnesium loss, suggesting that recycling of the endogenous Ca^{2+} was respon-

sible for the loss of Mg^{2+} (33). We have repeated these experiments under conditions in which nucleotide transport assays are conducted and have obtained similar results. The relevance of this point to the present work is that mitochondria assayed in the absence of added ruthenium red will have lower endogenous Mg^{2+} levels under coupled than uncoupled conditions. Previous studies by Pfaff *et al.* (8), Verdouw and Bertina (9), and Klingenberg (7) have shown that the preferred substrate for nucleotide transport is the free nucleotide rather than the Mg^{2+}·nucleotide complex. Consequently, reduction in the endogenous Mg^{2+} levels under coupled conditions would increase the levels of free endogenous nucleotides and, therefore, transport rates. This concept is consistent with the report by Duszynski *et al.* (34), who showed that addition of the ionophore A23187 to mitochondria increases the V_{max} for ADP uptake. Furthermre, because ruthenium red can inhibit state 4 respiration (33) (which we confirmed in our laboratory) and, therefore, presumably increase the membrane potential, the K_m value for transport will also be affected. The effects of ruthenium red on coupled ADP and ATP transport activity are also shown in Fig. 1, *A* and *B*. The omission of ruthenium red from the assay medium resulted in higher K_m and V_{max} values for ADP uptake at high ADP concentration and a higher V_{max} and lower K_m value for ATP uptake at high ATP concentration. In uncoupled mitochondria, ruthenium red had no effect on ADP transport. These results agree with the presumption that the membrane potential is lower and chemical activity of the exchangeable pool higher in coupled mitochondria assayed in the absence of ruthenium red.

TABLE I

Apparent kinetic constants of adenine nucleotide transport at high substrate concentrations

The apparent kinetic constants shown in this table were calculated by the direct linear plot method of assays measuring ADP and ATP uptake rates under coupled and uncoupled conditions in the presence of ruthenium red. The ranges shown are values obtained from several determinations.

	Uncoupled		Coupled	
	K_m	V_{max}	K_m	V_{max}
	μM	nmol/mg/min	μM	nmol/mg/min
ADP	20–35	3–5	2–4	1–1.5
ATP	6–10	2–3	100–120	2–3

Velocity Dependence on Internal Substrate Concentration—The mechanism of nucleotide transport has been proposed to operate as a mobile carrier having a single ligand-binding site to which both inhibitors and substrates can bind (25, 35, 36) and as a double site gated channel (26). Both these models describe an obligatory one for one exchange in which substrate is alternately bound on either side of the membrane

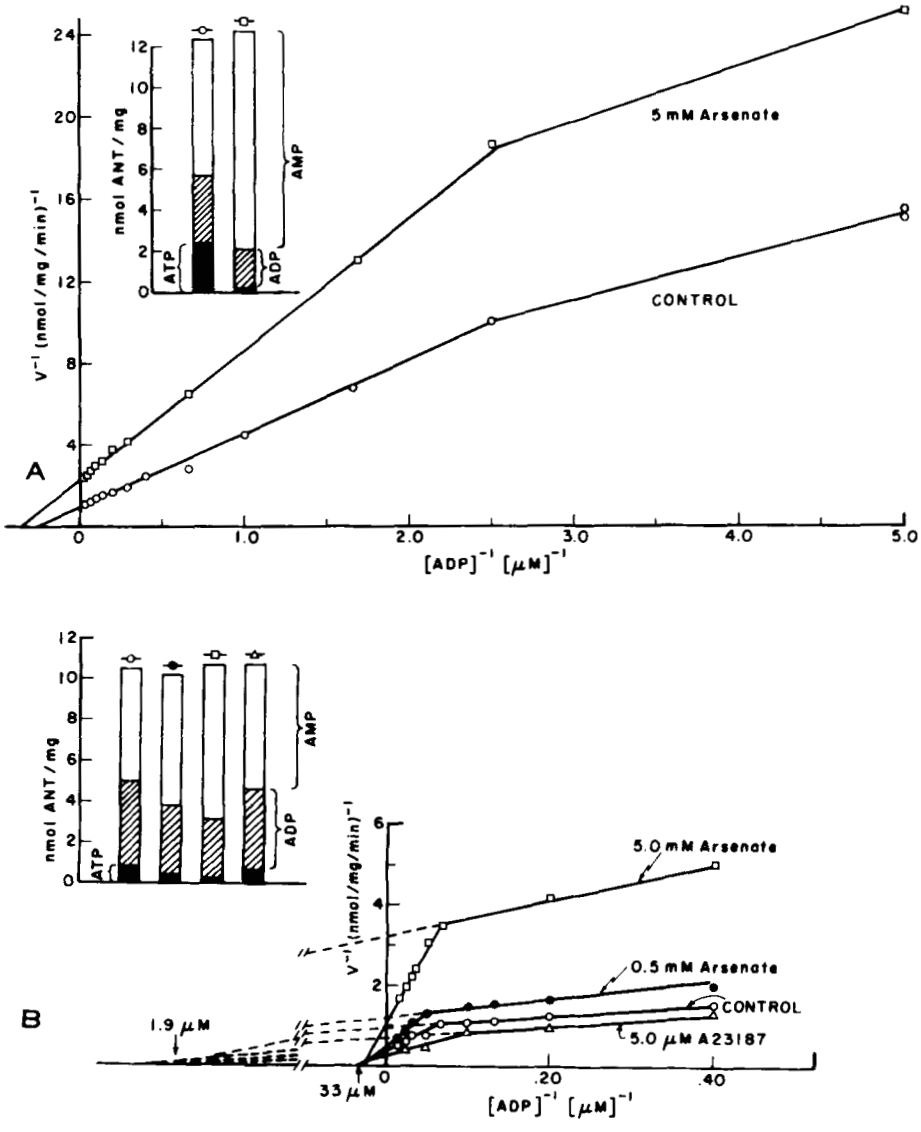

FIG. 2. **Lineweaver-Burk plots of ADP uptake in coupled and uncoupled mitochondria: effects of internal exchangeable nucleotide pool sizes.** Variation in the size of the internal exchangeable nucleotide pool was achieved by adding the indicated amounts of potassium arsenate or ionophore A23187 to the preincubation medium. Assays under coupled conditions (*panel A*) were measured as described in the legend to Fig. 1, in the presence of 5 mM 2-oxoglutarate and 5 μM ruthenium red with (□——□) and without (○——○) 5 mM potassium arsenate. Uncoupled rates of ADP uptake (*panel B*) were measured exactly as in *panel A*, in the absence (○——○) or in the presence of 0.5 mM potassium arsenate (●——●), 5 mM potassium arsenate (□——□), or 5 μM A23187 (△——△) in addition to 2 μM carbonyl cyanide *m*-chlorophenylhydrazone during the preincubation. The distribution of intramitochondrial adenine nucleotides under the above assay conditions are shown in the histograms of *panels A* and *B*. Subsequent to a 5-min preincubation at room temperature, internal adenine nucleotides were extracted by resuspending 10–15 mg of mitochondrial pellet in 1–2 ml of 5% perchloric acid. The mixture was allowed to stand on ice for 30 min followed by addition of KOH for neutralization and desalting. The levels of AMP, ADP, and ATP levels in the neutralized extracts were determined by enzymatic analysis as described under "Materials and Methods."

and thus describes a ping-pong mechanism (37). This mechanism can be easily verified if changes in the internal pool size do not alter the apparent K_m/V_{max} ratio.

To modify the internal pool size, mitochondria were treated with potassium arsenate which lowered the internal ATP/ADP ratio and [ATP] plus [ADP] levels (8). The effects of arsenate on the distribution pattern of internal adenine nucleotides are shown in the histograms of Figs. 2 and 3. The [ADP] plus [ATP] levels for freshly isolated mitochondria incubated under conditions used in these experiments were approximately 5 nmol/mg whereas the total adenine nucleotide level varied from 10.5–13.0 nmol/mg. This range is in agreement with literature values (6). The ATP/ADP ratio in coupled mitochondria was greater than in uncoupled mitochondria and was less than 0.65 in all cases. This value is low because phosphate was not added to the medium.

Double reciprocal plots shown in Figs. 2 and 3 are the results of initial velocity studies examining the effects of reduced internal pool size on ADP and ATP uptake in coupled and uncoupled mitochondria. Contrary to expected results, the apparent K_m/V_{max} ratio was not constant and two sets of lines, which intersect very near or on the abscissa, were obtained.

The fact that the lines intersect on the abscissa indicates that $K_m = K_d$. Furthermore, the change in the K_m value for nucleotide uptake, seen by energization (ATP uptake) or deenergization (ADP uptake) of mitochondria, is reflected by a change in the K_d value because the intersecting lines converge on the abscissa under both conditions. This conclusion was reached earlier by Souverijn *et al.* (14) who reported that the increase in the K_m value for ATP uptake, upon energization of mitochondria, was due to an increase in the K_d value since the V_{max} value was unaffected. However, it should be pointed out that this explanation would only hold true for a reaction involving a single substrate which, subsequent to binding, was immediately converted to product without intervening steps. From the data described in this report, it is clear that the mechanism of transport is far more complex than previously recognized and thus it is only coincidental that similar conclusions were reached. As will be discussed below, other expla-

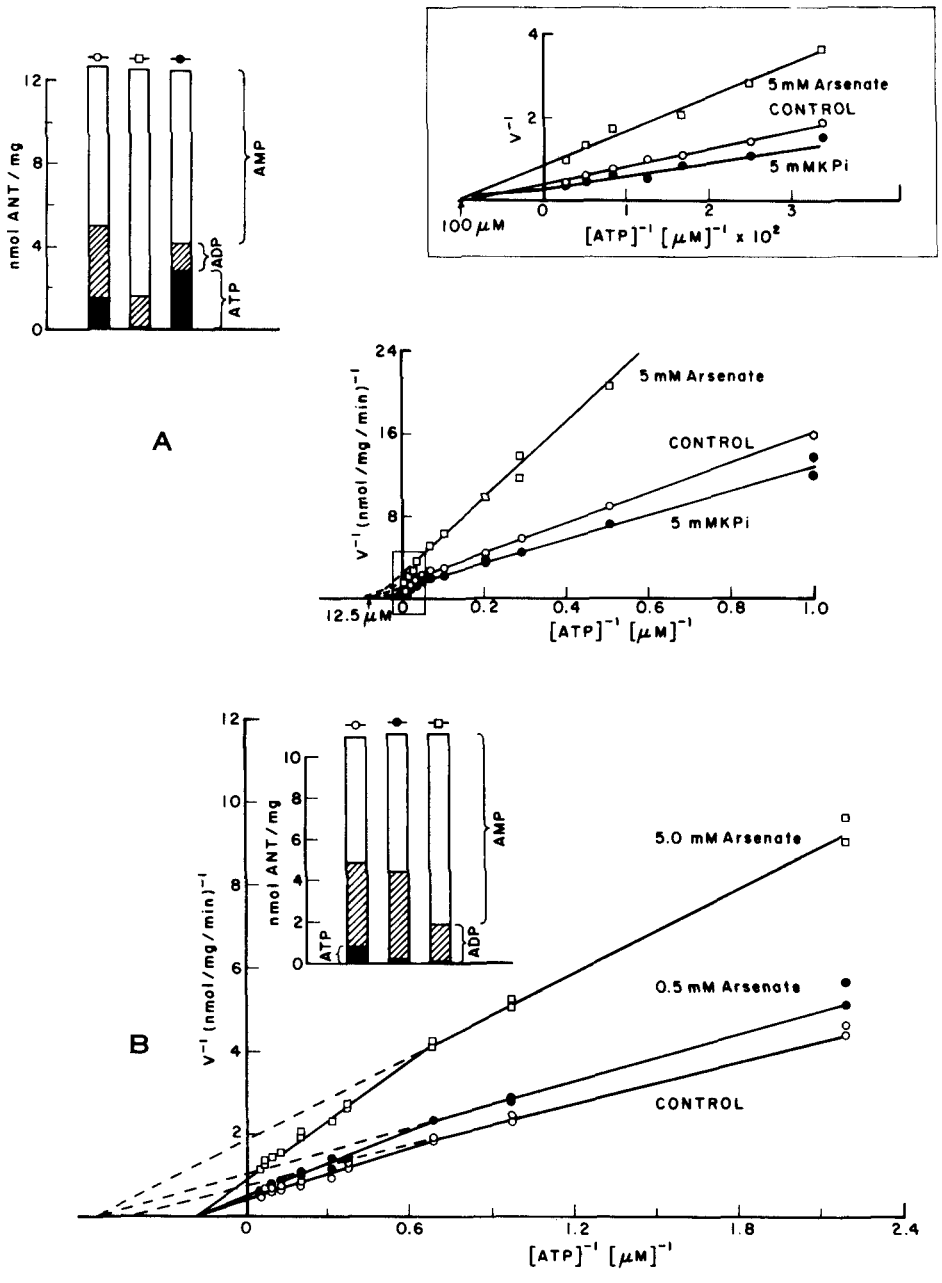

FIG. 3. **Lineweaver-Burk plots of ATP uptake in coupled and uncoupled mitochondria: effects of internal exchangeable nucleotide pool sizes.** Reduction in the size of the exchangeable pool was achieved by adding the indicated amounts of potassium arsenate to the preincubation medium as described in the legend to Fig. 2. ATP uptake rates under coupled conditions (*panel A*) were measured in the same way as described in the legends to Figs 1 and 2, in the presence of 5 mM 2-oxoglutarate and 5 μM ruthenium red with (□——□) and without (○——○) 5 mM potassium arsenate. Assays in the presence of 5 mM potassium phosphate (●——●) were measured as follows. Mitochondria were preincubated at room temperature for 5 min with 5 mM potassium arsenate, pelleted by centrifugation, and resuspended in the same medium except potassium phosphate was substituted for arsenate. The suspension was subsequently incubated at room temperature for an additional 5 min, cooled to 2°C, and assayed as before. The *inset* is an expanded scale of data points to the left of the breakpoint. Rates of ATP uptake in uncoupled mitochondria (*panel B*) were measured exactly as in *panel A* in the presence of 2 μM carbonyl cyanide *m*-chlorophenylhydrazone plus 0 (○——○), 0.5 mM (●——●), and 5 mM (□——□) potassium arsenate in the preincubation mix. The distribution of intramitochondrial AMP, ADP, and ATP levels assayed under coupled and uncoupled conditions are shown in the histograms of *panels A* and *B*, respectively. Extraction and analysis of adenine nucleotides were performed as described in the legend to Fig. 2.

nations can account for a change in K_m value with variations in the mitochondrial energy state without proposing a change in K_d value.

It is important to demonstrate that lower transport rates in the presence of arsenate are due to a reduced internal pool size and not to an irreversible inactivation of the carrier by arsenate. Results in Fig. 3*A* show that potassium phosphate can reverse arsenate inhibition of transport (V_{max} increases from 1.14 to 3.33 nmol/mg/min) and that higher transport rates are accompanied by increased internal [ADP] plus [ATP] levels as well as a higher ATP/ADP ratio. It is interesting to note that the maximum velocity in these mitochondria is even greater than control mitochondria although the exchangeable pool size is somewhat lower. This indicates that a high ATP/ADP ratio inside activates exchange and suggests that $[ATP]_{in}$ is the preferred subsrate in coupled mitochondria. A similar conclusion was reached previously by Klingenberg (15, 18).

Data shown in Fig. 2*B* provides additional evidence that variations in the size of the exchangeable pool influences transport rates. Incubation of mitochondria in the presence of the ionophore A23187 lowers endogenous Mg^{2+} levels from 15.5 to 1.5 nmol/mg and increases transport rates by 34%, suggesting that higher free ADP and ATP levels increase transport activity. These data coincide with the report by Pfaff et al. (8) who showed that reduction in the internal pool size resulted in a lowering of the first order rate constant for nucleotide uptake.

The intersecting line pattern shown in these figures can be interpreted to indicate that (*a*) if two separate carriers do exist, then the mechanism for both carriers is sequential; (*b*) ternary complex formation occurs on both subunits of the dimer which exhibit negative cooperativity or a single ternary complex is formed per dimer which interacts with an adjacent dimer; or (*c*) formation of a second ternary complex is only apparent due to the presence of reversibly tight-binding competitive inhibitors.

The ratio of the K_m values for internal ATP and ADP can be deduced from the data shown in Figs. 2 and 3. If the K_m value for ADP_{in} and ATP_{in} are markedly different from each other, then changes in their ratio would not be expected to result in a common intersection on the abscissa as the total amount of $[ADP]_{in}$ plus $[ATP]_{in}$ was reduced, but rather the intersections would converge to form a line between two points on the abscissa. These points would correspond to the apparent K_m values for external nucleotide uptake in the presence of only ADP and only ATP on the inside. Variations in the internal ATP/ADP ratio (<0.1 to 2.0) did not influence the apparent K_m values for external ADP or ATP under high energy or uncoupled conditions. Therefore, it follows that the K_m values for internal ADP and ATP must be similar and their ratio remains constant, regardless of the mitochondrial energy state. This conclusion is supported by the recent studies of Klingenberg (7) using submitochondrial particles, who observed that the differences between ADP and ATP as substrates were minor and independent of the energy state (*cf.* Ref. 38 in which K_m values of 30 μM and 20 μM for ADP uptake, respectively, were observed in submitochondrial particles).

DISCUSSION

Results presented here demonstrate that nucleotide uptake does not follow classical Michaelis-Menten type kinetics and that the mechanism of carrier transport is sequential. The following discussion evaluates the interpretations offered to account for biphasic kinetics and the significance of biphasic kinetic plots with regards to reported kinetic constants. In addition, the results of inhibitor-binding studies previously reported are re-examined in light of the present findings and a model of carrier transport consistent with these data is offered.

Significance of Biphasic Plots of Velocity Versus Substrate Concentration—We have interpreted the occurrence of biphasic kinetics for nucleotide uptake to indicate: (*a*) the presence of two carriers having different kinetic properties; (*b*) negative cooperativity among carrier molecules in the membrane; or (*c*) the presence of endogenous, tight-binding competitive inhibitors. Evidence that a second carrier may catalyze the exchange of adenine nucleotides includes reports demonstrating substrate and inhibitor cross-reactivity between the citrate and the adenine nucleotide carrier. These studies have shown that phosphoenolpyruvate is a substrate for both carriers (39) and that atractyloside (40), carboxyatractyloside (41), and long chain acyl-CoA esters (41, 42) inhibit citrate transport, although at higher concentrations than needed for inhibition of nucleotide transport. In addition, we have determined that, in contrast to a previous report (40), 1,2,3,-benzenetricarboxylate is not only an inhibitor of citrate transport, but also acts as a competitive inhibitor of nucleotide uptake, having a K_i value of 2.4 mM (results not shown).

Strong evidence against two carriers comes from results demonstrating that a single molecular species is responsible for nucleotide exchange. Saturation of carboxyatractyloside-binding sites is coincident with complete inhibition of transport activity indicating that the inhibitor binds only to the carrier at the concentrations used (35, 43). These data, together with results showing that the isolated carboxyatractylate-binding protein migrates as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (44) and isoelectric focusing gels (45) suggests that if two independent carriers do exist, then their physical properties must be very similar or identical. In view of the large difference in the apparent kinetic constants obtained at low and high substrate concentrations, we consider it less likely that two carriers having these physical properties could account for the kinetic data.

Interpretation of biphasic kinetic plots in terms of negative cooperativity would necessarily predict biphasic mass action plots for substrate binding. Weidemann *et al.* (46) have reported biphasic Scatchard plots for ADP and ATP binding to the carrier but argued that high and low affinity binding represent carrier sites oriented toward the inner and outer surfaces of the membrane, respectively. In addition, only the larger proportion of low affinity sites on the outside reflects the true K_d value since the high affinity sites in contact with the residual endogenous adenine nucleotide pool would be fully saturated at low external substrate concentration giving a low apparent K_d value. This proposal was supported by the finding that the proportion of high affinity binding sites was roughly equivalent to the decrease in [^{14}C]ADP "binding" to carrier sites when bongkrekic acid was added prior to labeled nucleotide (46). These authors concluded, therefore, that only a single class of low affinity sites exists for the binding of external substrates. This conclusion does not support the possibility that negative cooperativity could account for the biphasic kinetic plots.

Atractyloside and carboxyatractyloside are known to bind to the carrier exclusively on the cytoplasmic side of the inner membrane in a 1:1 ratio to the external nucleotide removed, indicating the identity of substrate and inhibitor-binding sites (35). Thus, if nonlinear Scatchard plots for substrate binding were due to cooperative interactions, then similar nonlinear plots might be expected for inhibitor binding. Vignais and coworkers reported that carboxyatractyloside binding to liver

mitochondria exhibited positive cooperativity (43, 47–49) and that these interactions were increased by addition of ADP (43, 47, 48) and abolished by sonication or lipid depletion (47, 49). Klingenberg and co-workers have also observed positive cooperativity for inhibitor binding but suggested that nonlinear mass action plots were caused by retardation of equilibrium due to high affinity binding (35, 50). These observations seem opposite of what would be predicted. However, when it is considered that the structure of these inhibitors differs widely from those of the natural substrates, these results should perhaps only be viewed to indicate that cooperative interactions between binding sites can occur. Indeed, negative cooperativity of atractyloside and carboxyatractyloside binding to sonic beef heart particles has been reported (51).

Studies characterizing the binding of ATP to mutant yeast mitochondria may also provide additional evidence supporting negative cooperativity. The mutations involved resulted in a loss of the high affinity ATP-binding site (52, 53) suggesting that modification of the carrier and/or its lipid environment disrupts cooperative binding interactions. This explanation is not exclusive since these mutations may also alter the distribution of carrier sites in favor of a C-state orientation and thus would not indicate loss of cooperativity for ligand binding. In summary, although it would seem that strong evidence favoring negative cooperativity is still lacking, the possible existence of these interactions should not be dismissed at this time.

The third interpretation of nonlinear kinetic plots is that such behavior is due to the presence of competitive tight-binding inhibitors. Valid application of the Michaelis-Menten equation to reactions occurring in the presence of inhibitors is based on the assumption that the free inhibitor concentration is not affected by substrate-dependent changes in the fraction of enzyme inhibited. As discussed by Henderson (54), this assumption is no longer valid when the ratio of E_t/K_i (where E_t is the total enzyme concentration) is ≥ 0.01, and thus the concentration of enzyme-inhibitor complex must be accounted for in the derived velocity equation. Under normal assay conditions, a ratio of $E_t/K_i \geq 0.01$ would only occur when the inhibitor exhibits very high affinity for the enzyme. For a reaction catalyzed by a sequential mechanism, tight-binding competitive inhibitors will yield a series of increasingly nonlinear, concave-down plots (55). The results shown in this report fit this description well and may provide evidence for the existence of such inhibitors in mitochondria. Morel *et al.* (41) have demonstrated that additions of palmitoyl-CoA to liver mitochondria competitively inhibits ADP uptake and that the kinetic plots in the presence of the inhibitor were increasingly nonlinear (*i.e.* concave down), at higher inhibitor concentrations, suggesting that acyl-CoA esters behave as tight-binding inhibitors. Moreover, it is known that isolated liver mitochondria contain up to 1 nmol/mg of long chain acyl-CoA esters (56) and that removal of these inhibitors by addition of serum albumin or fatty acid-binding protein can increase transport activity (13). Thus, it would seem that long chain acyl-CoA esters present in mitochondrial preparations may well influence the rates of nucleotide exchange as well as the shape of the resulting kinetic plots.

In conclusion, among the above alternative explanations offered to account for the biphasic kinetic plots, little evidence was found in the literature supporting the existence of two different carriers, and, in the absence of further experimental studies, it is not possible to differentiate between negative cooperativity and the possible inhibitory effects of endogenous acyl-CoA esters. Distinction between the latter two alternatives could be accomplished by measuring rates of nucleotide uptake under different energy states in submitochondrial particles prepared in the presence of excess amounts of defatted serum albumin to remove acyl-CoA esters from both the inner and outer surfaces of the inner membrane. Homogenous kinetic plots would favor the effects of acyl-CoA esters while biphasic plots would support negative cooperativity.

An important consideration to be drawn from these studies is that, because the kinetic plots are biphasic, the apparent kinetic constants obtained depend on the substrate concentration range chosen. The values reported in Table I were obtained at high substrate concentrations. We have chosen to report only these values since the significance of kinetic constants obtained at low substrate concentrations would depend on the interpretation of the biphasic kinetic plot (see above). For example, if it is concluded that biphasic kinetics is a result of negative cooperativity or the presence of two separate carriers, then the existence of both high and low affinity sites can be considered real and the "true" value of the apparent kinetic constants can be calculated by subtracting their additive effects on the overall rate of transport. However, the intercepts extrapolated on the abscissa and ordinate at low substrate concentration would not be equal to the concentration of substrate necessary for half-maximum velocity and the maximum velocity, respectively, if biphasic kinetics is a result of endogenous acyl-CoA ester inhibition.

Additional evidence supporting the biphasic character of nucleotide uptake is reported by Verdouw and Bertina (9) who demonstrated the existence of two apparent affinities for ATP in the uncoupler-stimulated ATPase reaction ($K_{m_1} = 6.7$ μM, $K_{m_2} = 63$ μM). Since both K_m values are sensitive to atractyloside, these values likely reflect a biphasic affinity of the nucleotide carrier for ATP uptake. The ratio of low/high affinities of the transporter for ATP in this study is in remarkable agreement with that obtained here (*i.e.* 9:1, see Fig. 1*B*).

The dependency of nucleotide transport rates on the mitochondrial energy state has been investigated by several groups, the results of which have yielded only fair agreement. Klingenberg and Pfaff (3) reported that the K_m (11 μM) for ATP uptake was greater than the K_m (1 μM) for ADP uptake and that uncoupling of mitochondria abolished this difference by lowering the K_m value for ATP uptake. Souverijn *et al.* (14) reported rather similar findings except that the K_m value for ATP uptake was on the order of 150 μM in coupled mitochondria. In retrospect, this discrepancy is understandable since the K_m values reported by Klingenberg and Pfaff (3) and Souverijn *et al.* (14) were obtained at low and high substrate concentrations, respectively.

Data in Table I shows that the K_m and V_{max} values for ADP uptake increased in uncoupled mitochondria. This finding is consistent with the more recent report by Vignais *et al.* (19) and contrasts the earlier reports of Pfaff *et al.* (8) and Souverijn *et al.* (14) who observed no change in the K_m value for ADP uptake upon uncoupling. The previous study by Vignais *et al.* (10) also seems consistent with the present findings since they observed that the K_m and V_{max} values for ADP uptake were greater in a high energy (succinate-present) than low energy (succinate-absent) state. However, these authors concluded that the K_m value for external ADP was dependent on the intramitochondrial ATP/ADP ratio (higher K_m values obtained at low ratios) since this ratio also changed under these conditions. Data in Figs. 2 and 3 contradict this conclusion by showing that the K_m value for ADP and ATP uptake in coupled and uncoupled mitochondria remains unaffected when the internal ATP/ADP ratio is varied over a range of <0.1 to 2.0. Although this variation is less than that of 0.13 to 5.8 as reported by Vignais *et al.* (10), we believe that the sensitivity of the K_m value for ADP uptake to changes in the

membrane potential is explained by factors other than the internal ATP/ADP ratio.

Mechanism of Adenine Nucleotide-carrier Transport—Klingenberg and co-workers (25, 36, 57) have proposed that the mechanism of nucleotide transport operates by an alternating reorientation of a single ligand-binding site to which both inhibitors and substrates can bind. This model is based primarily on the observation of mutually exclusive inhibitor binding *i.e.* binding of bongkrekate to the carrier on the matrix side of the membrane prevents binding of carboxyatractyloside on the cytoplasmic side (35) and vice versa (25, 58)). A key observation which led to this proposal was that bongkrekate binding was dependent on the presence of external ADP (58). This indicated that the binding site of bongkrekate was not available until ADP_o bound to the carrier, underwent translocation, and was released on the matrix side, thus exposing the inhibitor-binding site. The displacement of ADP_i bound to the carrier by bongkrekate, with the ensuing inhibition of transport activity, traps the ADP inside. This effect resulted in the large increase in the apparent affinity of the carrier for ADP and led to the proposal that bongkrekate inhibited transport by forming a carrier·ADP·bongkrekate ternary complex (58–60). The fact that ADP was not actually tightly bound to the carrier was shown conclusively by Klingenberg (61) who demonstrated that nucleotide initially bound to the carrier was able to participate in phosphorylation and dephosphorylation reactions. This result indicated that only a binary carrier-bongkrekate complex was formed.

Lauquin and Vignais (62) have recently detected the formation of a carrier·ADP·bongkrekate ternary complex. They showed that preincubation of mitochondria in the presence of ADP and bongkrekate resulted in uncompetitive inhibition indicating that bongkrekate was bound to a site other than the ADP site. These results would not be contradictory if a mechanism of transport could account for the presence of both binary carrier-bongkrekate and ternary carrier·ADP·bongkrekate complexes. The fact that bongkrekate seemingly displaces bound ADP while still forming a ternary complex suggests the presence of a carrier·$[ADP]_2$ complex during the normal operation of transport. This idea is consistent with data presented in this report showing an intersecting line pattern in the kinetic plots. A model is summarized in Fig. 4 in which we propose that carrier transport follows a reciprocating ordered sequential mechanism. The key features of this model are that it can account for mutually exclusive inhibitor binding, distinct binding sites for atractyloside and bongkrekate, and formation of binary and ternary bongkrekate-carrier complexes and ternary substrate-carrier complexes.

The working of the model is as follows. External nucleotide, $ANP_e^{S_1*}$ (depicted here as radioactive), binds to the carrier and induces a conformational change activating the carrier to a mobile state. In this conformation, the carrier·$ANP_e^{S_1*}$ complex reorients across the membrane (external substrate S_1* becomes internal product P_1*). Once on the inside, the carrier then assumes a conformation to which the second substrate (internal nucleotide, $ANP_i^{S_2}$) binds, prior to releasing $ANP_i^{P_1*}$, and forms a kinetically stable carrier-$[substrate]_2$ complex. Dissociation of P_1* occurs and the carrier·$ANP_i^{S_2}$ complex then assumes the mobile conformational state and translocation back across the membrane occurs (internal substrate S_2 becomes external P_2). The carrier then returns to the nonmobile conformation from which $ANP_e^{P_2}$ dissociates. This model thus describes the reorientation of a single ligand-binding site. When the binding site is oriented on the outside, inhibitors (atractyloside, carboxyatractyloside, and acyl-CoA esters) compete with external nucleotides for the carrier. The observation that bongkrekate binding is dependent on external ADP is also accounted for since bongkrekate does not bind until reorientation to the inside has occurred. In addition, the model predicts separate bongkrekate- and atractyloside-binding sites as recently suggested by Block *et al.* (26).

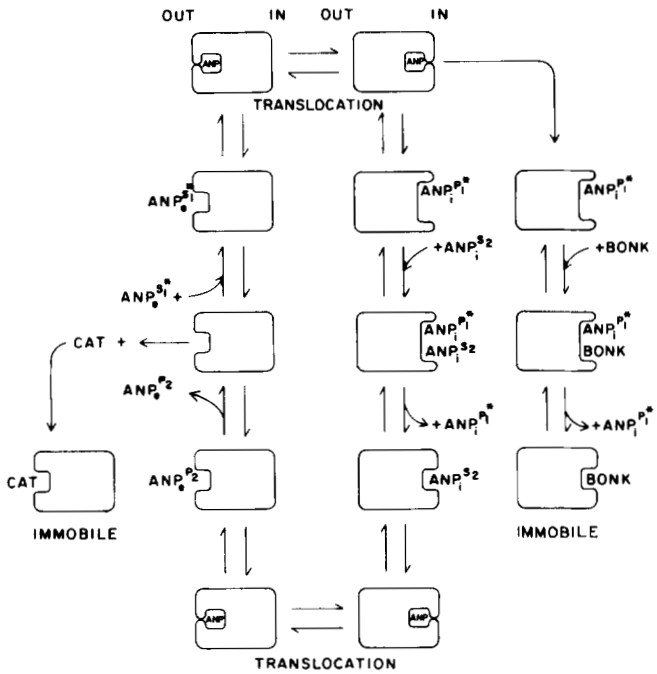

FIG. 4. **A proposed model of mitochondrial adenine nucleotide carrier transport.** This ordered sequential model (see text for detailed description) is consistent with published results on inhibitor-binding studies and data presented in this report although the existence of the carrier·$[nucleotide]_2$ ternary complex in the matrix remains to be experimentally verified. Other models including (*a*) one nucleotide-binding site on each surface of the carrier in the membrane with either sequential or random nucleotide binding and (*b*) two nucleotide-binding sites on the outer surface are also possible but less likely candidates. This model does not commit itself to the question of whether the active carrier species is a monomer, dimer, or clusters of carrier subunits. *BONK*, bongkrekic acid; *CAT*, carboxyatractyloside.

The formation of binary and ternary bongkrekate·carrier complexes is predicted as follows. Bongkrekate binds to the carrier in place of internal substrate and induces the release of the newly translocated nucleotide, $ANP_i^{P_1*}$. In this manner, bongkrekate does not directly compete with $ANP_i^{P_1*}$ as suggested by Klingenberg et al. (59), but rather acts as an internal substrate-inducing displacement of the bound nucleotide. The resulting carrier·bongkrekate complex is not mobile and thus inhibits transport. This formation can thus account for the displacement of radioactive ADP by bongkrekate, which is then available to participate in phosphotransfer reactions. It also accounts for the formation of a ternary carrier·ADP·bongkrekate complex suggested by Lauquin and Vignais (62).

This model of carrier transport is similar to the mechanism recently proposed for the glutamate-aspartate carrier by Murphy *et al.* (63). According to their model, the carrier operates by the alternating reorientation of a single ligand-binding site in which the binding of aspartate is necessary for release of glutamate, thus forming a ternary complex. It is of interest to note that these authors also observed in the kinetic plots a series of intersecting lines on the abscissa when the internal aspartate levels were varied.

REFERENCES

1. Klingenberg, M., and Pfaff, E. (1966) in *Regulation of Metabolic*

Processes in Mitochondria (Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., eds) Vol. 7, p. 180–218, BBA Library, Elsevier, Amsterdam
2. Pfaff, E., Klingenberg, M., and Heldt, H. W. (1965) *Biochem. Biophys. Acta* **104,** 312–315
3. Klingenberg, M., and Pfaff, E. (1968) in *Metabolic Roles of Citrate* (Goodwin, T. W., ed) pp. 105–122, Academic Press, New York
4. Pfaff, E., and Klingenberg, M. (1968) *Eur. J. Biochem.* **6,** 66–79
5. Winkler, H. H., Bygrave, F. L., and Lehninger, A. L. (1968) *J. Biol. Chem.* **243,** 20–28
6. Duée, E. D., and Vignais, P. V. (1969) *J. Biol. Chem.* **244,** 3920–3931
7. Klingenberg, M. (1977) *Eur. J. Biochem.* **76,** 553–565
8. Pfaff, E., Heldt, H. W., and Klingenberg, M. (1969) *Eur. J. Biochem.* **10,** 484–493
9. Verdouw, H., and Bertina, R. M. (1973) *Biochim. Biophys. Acta* **325,** 385–396
10. Vignais, P. V., Vignais, P. M., Lauquin, G., and Morel, F. (1973) *Biochimie (Paris)* **55,** 763–778
11. Out, T. A. (1977) Ph.D. thesis, University of Amsterdam
12. Lerner, E., Shug, A. L., Elson, C., and Shrago, E. (1972) *J. Biol. Chem.* **247,** 1513–1519
13. Chan, S. H. P., and Barbour, R. L. (1979) in *Membrane Bioenergetics* (Lee, C. P., Schatz, G., and Ernster, L., eds) pp. 521–532, Addison-Wesley, Massachusetts
14. Souverijn, J. H. M., Huisman, L. A., Rosing, J., and Kemp, A., Jr. (1973) *Biochim. Biophys. Acta* **305,** 185–198
15. Klingenberg, M. (1975) in *Energy Transformation in Biological Systems* Vol. 31, Ciba Foundation Symposium, pp. 105–124, Associated Scientific Publishers
16. Lauquin, G. J. M., Villiers, C., Michejda, J. W., Hryniewiecka, L. V., and Vignais, P. V. (1977) *Biochim. Biophys. Acta* **460,** 331–345
17. Lauquin, G. J. M., Villiers, C., Michejda, J., Brandolin, G., Boulay, F., Cesarini, R., and Vignais, P. V. (1978) in *The Proton and Calcium Pumps* (Azzone, G. F., Avron, M., Metcalfe, J. C., Quagliariello, E., and Siliprandi, N., eds) pp. 251–262, Elsevier, Amsterdam
18. Klingenberg, M. (1979) in *Cation Flux Across Biomembranes* (Mukohata, Y., and Packer, L., eds) pp. 387–397, Academic Press, New York
19. Vignais, P. V., Lauquin, G. J. M., and Vignais, P. M. (1976) in *Mitochondria Bioenergetics and Membrane Structure* (Packer, L., and Gomez-Puyou, A., eds) pp. 109–125, Academic Press, New York
20. Laris, P. C. (1977) *Biochim. Biophys. Acta* **459,** 110–118
21. LaNoue, K., Mizani, S. M., and Klingenberg, M. (1978) *J. Biol. Chem.* **253,** 191–198
22. Klingenberg, M., and Rottenberg, H. (1977) *Eur. J. Biochem.* **73,** 125–130
23. Klingenberg, M., Wulf, R., Heldt, H. W., and Pfaff, E. (1969) in *Mitochondria: Structure and Function* (Ernster, L., and Drahota, Z., eds) pp. 59–77, Academic Press, New York
24. Heldt, H. W., Klingenberg, M., and Milovancev, M. (1972) *Eur. J. Biochem.* **30,** 434–440
25. Klingenberg, M., and Buchholz, M. (1973) *Eur. J. Biochem.* **38,** 346–358
26. Block, M. R., Lauquin, G. J. M., and Vignais, P. V. (1979) *FEBS Lett.* **104,** 425–430
27. Johnson, D., and Lardy, H. A. (1967) *Methods Enzymol.* **10,** 94–96
28. Jaworet, D., Gruber, W., and Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed) pp. 2127–2131, Academic Press, New York
29. Lamprecht, W., and Trauschold, I. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed) pp. 2101–2109, Academic Press, New York
30. Eisenthal, R., and Cornish-Bowden, A. (1974) *Biochem. J.* **139,** 715–720
31. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) *J. Biol. Chem.* **177,** 751–766
32. Scarpa, A., and Lindsay, J. G. (1972) *Eur. J. Biochem.* **27,** 401–407
33. Siliprandi, D., Toninello, A., Zoccarato, F., and Siliprandi, N. (1977) *Biochem. Biophys. Res. Commun.* **78,** 23–27
34. Duszynski, J., Savina, M. V., and Wojtczak, L. (1978) *FEBS Lett.* **86,** 9–13
35. Klingenberg, M., Grebe, K., and Scherer, B. (1975) *Eur. J. Biochem.* **52,** 351–363
36. Klingenberg, M., Scherer, B., Stengel-Rutkowski, L., Buchholz, M., and Grebe, K. (1973) in *Mechanisms of Bioenergetics* (Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E., and Siliprandi, N., eds) pp. 257–284, Academic Press, New York
37. Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67,** 104–137
38. Klingenberg, M., Riccio, P., Aquila, H., Schmiedt, B., Grebe, K., and Topitsch, P. (1974) in *Membrane Proteins in Transport and Phosphorylation* (Azzone, G. F., Klingenberg, M. E., Quagliariello, E., and Siliprandi, N., eds) pp. 229–243, North Holland, Amsterdam
39. Chudapongse, P., and Haugaard, N. (1973) *Biochim. Biophys. Acta* **307,** 599–606
40. Shug, A. L., and Shrago, E. (1973) *Biochem. Biophys. Res. Commun.* **53,** 659–665
41. Morel, F., Lauquin, G., Lunardi, S., Duszynski, J., and Vignais, P. V. (1974) *FEBS Lett.* **39,** 133–138
42. Halperin, M. L., Robinson, B. H., and Fritz, I. B. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69,** 1003–1007
43. Vignais, P. V., Vignais, P. M., and Defaye, G. (1971) *FEBS Lett.* **17,** 281–288
44. Klingenberg, M., Riccio, P., and Aquila, H. (1978) *Biochim. Biophys. Acta* **503,** 193–210
45. Klingenberg, M., Aquila, H., Riccio, P., Buchanan, B. B., Eiermann, W., and Hackenberg, H. (1975) in *Electron Transfer Chains and Oxidative Phosphorylation* (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C., and Siliprandi, N., eds) pp. 431–438, North Holland, Amsterdam
46. Weidemann, M. J., Erdelt, H., and Klingenberg, M. (1970) *Eur. J. Biochem.* **16,** 313–335
47. Vignais, P. V., Vignais, P. M., and Defaye, G. (1973) *Biochemistry* **12,** 1508–1519
48. Vignais, P. V., Vignais, P. M., Defaye, G., Lauquin, G., Doussiere, J., Chabert, J., and Brandolin, G. (1973) in *Mechanisms in Bioenergetics* (Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E., and Siliprandi, N., eds) pp. 323–335, Academic Press, New York
49. Vignais, P. V., Vignais, P. M., Defaye, G., Chabert, J., Doussiere, J., and Brandolin, G. (1972) in *Biochemistry and Biophysics of Mitochondrial Membranes* (Azzone, G. F., Carafoli, E., Lehninger, A. L., Quagliariello, E., and Siliprandi, N., eds) pp. 447–464, Academic Press, New York
50. Riccio, P., Scherer, B., and Klingenberg, M. (1973) *FEBS Lett.* **31,** 11–14
51. Klingenberg, M. (1974) in *Dynamics of Energy Transducing Membranes* (Ernster, L., Estabrook, R. W., and Slater, E. C., eds) pp. 511–528, Elsevier, Amsterdam
52. Perkins, M. E., Haslam, J. M., Klyce, H. R., and Linnane, A. W. (1973) *FEBS Lett.* **36,** 137–142
53. Haslam, J. M., Astin, A. M., and Nichols, W. W. (1977) *Biochem. J.* **166,** 559–563
54. Henderson, P. J. F. (1972) *Biochem. J.* **127,** 321–333
55. Morrison, J. F. (1969) *Biochim. Biophys. Acta* **185,** 269–286
56. Halperin, M. L., Cheeman-Dhadli, S., Taylor, W. M., and Fritz, I. B. (1975) in *Advances in Enzyme Regulation* (Weber, G., ed) Vol. 13, pp. 435–445, Pergamon Press
57. Klingenberg, M., Riccio, P., Aquila, H., Buchanan, B. B., and Grebe, K. (1976) in *The Structural Basis of Membrane Function* (Hatefi, Y., and Djaradi-Ohanianc, L., eds) pp. 293–311, Academic Press, New York
58. Erdelt, H., Weidemann, M. J., Buchholz, M., and Klingenberg, M. (1972) *Eur. J. Biochem.* **30,** 107–122
59. Klingenberg, M., Buchholz, M., Erdelt, H., Falkner, G., Grebe, K., Kadner, H., Scherer, B., Stengel-Rutkowski, L., and Weidemann, M. J. (1972) in *Biochemistry and Biophysics of Mitochondrial Membranes* (Azzone, G. F., Carafoli, E., Lehninger, A. L., Qaugliariello, E., and Siliprandi, N., eds) pp. 465–486, Academic Press, New York
60. Weidemann, M. J., Erdelt, H., and Klingenberg, M. (1970) *Biochem. Biophys. Res. Commun.* **39,** 363–370
61. Klingenberg, M. (1976) *Eur. J. Biochem.* **65,** 601–605
62. Lauquin, G. J. M., and Vignais, P. V. (1976) *Biochemistry* **15,** 2316–2322
63. Murphy, E., Coll, K. E., Viale, R. O., Tischler, M. E., and Williamson, J. R. (1979) *J. Biol. Chem.* **254,** 8369–8376