

ADENINE NUCLEOTIDE-INDUCED CONTRACTION OF THE INNER MITOCHONDRIAL MEMBRANE

I. General Characterization

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ABSTRACT

The inner membranes of isolated bovine heart mitochondria undergo pronounced contraction upon being exposed to exogenous adenosine diphosphate (ADP), adenosine triphosphate (ATP), and certain other high-energy phosphate compounds. Contraction results in decrease of inner membrane expanse which in turn results in decrease of intracrystal space and increase of mitochondrial optical density (OD). The magnitude of the OD change appears to be proportional to the degree of contraction. Half-maximal contraction can be achieved with ADP or ATP at concentrations as low as about $0.3 \mu\text{M}$. Atractyloside at concentrations as low as about 1.2 nmol/mg mitochondrial protein completely inhibits the contraction. It is concluded from these and other observations that inner membrane contraction occurs as a result of adenine nucleotide binding to the carrier involved in the exchange of adenine nucleotides across the inner mitochondrial membrane.

INTRODUCTION

During the course of metabolic studies on isolated heart mitochondria, sharp, oligomycin-insensitive increases in mitochondrial optical density (OD) were often observed upon exposing the mitochondria to adenosine diphosphate (ADP) or adenosine triphosphate (ATP). This puzzling response became of particular interest when it was learned that the OD change can be prevented or rapidly reversed by adding atractyloside at the same low concentration needed for the inhibition of oxidative phosphorylation. Further examination revealed that less than $1 \mu\text{M}$ adenine nucleotide is required for half-maximal response. This finding, in conjunction with the atractyloside effect, strongly suggested the association of a conformational change with the binding of adenine nucle-

otide to the adenine nucleotide exchange carrier. Support for this interpretation is provided in the present report by electron microscope studies showing that ADP and ATP induce a pronounced contraction of the inner mitochondrial membrane and by specificity studies showing that the nucleotide specificity of the contraction reaction is essentially identical to that described by other workers (1-4) for the adenine nucleotide exchange reaction.

Inner membrane contraction does not require energy derived from respiration or ATP hydrolysis and appears not to be associated with changes in inner compartment volume and matrix density. Consequently, it appears to be unrelated to the mitochondrial contraction which requires energy

from either ATP hydrolysis (5–11) or respiration (9–12) and which is associated with decrease of inner compartment volume and condensation of the matrix (11–14). On the other hand, it is evident from similarities in nucleotide specificity, inhibitor sensitivity, and ultrastructural changes that the inner membrane contraction phenomenon is identical with the adenine nucleotide-induced transformation of heart mitochondria into highly condensed morphological states described by Weber and Blair (15, 16).

A preliminary report of this work has been made (17).

MATERIALS AND METHODS

Bovine heart mitochondria were isolated according to a slight modification of the Nagarse procedure previously described (18, 19). Homogenates of left ventricular myocardium were prepared in 250 mM sucrose-0.1 mM ethylenediaminetetraacetic acid (EDTA)-10 mM Tris-Cl (pH 7.5). Mitochondria were separated by differential centrifugation and were washed twice with 250 mM sucrose-10 mM Tris-Cl (pH 7.8). Washed mitochondria were suspended finally in sucrose-Tris-Cl at concentrations of 70–90 mg protein/ml and were used immediately.

Mitochondrial oxygen consumption, mitochondrial OD, and pH were monitored simultaneously in rapidly stirred suspensions contained in a closed, thermostated, round, precision glass sample chamber (no. 5213, Yellow Springs Instrument Company, Inc., Yellow Springs, Ohio). Oxygen (YSI model 5331) and pH (no. S-30070-10, Sargent-Welch Scientific Co., Skokie, Ill.) electrodes were held in a Lucite plunger similar to the no. 5093 Lucite plunger supplied with the YSI Model 53 Biological Oxygen Monitor. The sample chamber was situated between a light source and a photodetector. The light source consisted of a tungsten lamp and electronic power supply identical to those supplied with the Model 6A Junior Spectrophotometer (Coleman Instruments, Division of Perkin-Elmer Corp., Maywood, Ill.). Light passing through the sample chamber was filtered with a red glass filter (maximum transmission at 800 nm) identical to that supplied with the Spectronic 20 colorimeter (Arthur H. Thomas Co., Philadelphia, Pa.). Light passing through the filter was detected by a 925 vacuum photodiode (peak sensitivity at 800 nm) connected to a Model 11 Laboratory Photometer (Pacific Photometric Instruments, Berkeley, Calif.), modified slightly to provide the proper phototube voltage. The lamp, sample chamber, and phototube were housed in separate compartments constructed from opaque Lucite, the sample chamber having an observation window. The sample chamber and contents were thermostated with

water circulating from an external bath. Narrow, dry light paths to and from the sample chamber were achieved with molded silicone rubber gaskets.

Except where indicated otherwise, sample volume was 8 ml, incubation temperature was 30°C, and mitochondrial protein concentration was 0.25 mg/ml. Additions to the sample chamber, including the mitochondria, were made in small volume (4–30 μ l) to minimize dilution and temperature artifacts. The amounts of materials added are given as final concentrations. The pH given represents the pH of the incubation mixture after all additions were made. Determinations were initiated by adding the mitochondria to the thermo-equilibrated reaction medium. In the apparatus described above, 0.25 mg mitochondrial protein/ml in isotonic sucrose gives an initial OD of approximately 0.6. To achieve suitable accuracy in extracting the OD data from the recorder tracings, the OD scale was routinely expanded threefold immediately after adding the mitochondria.

In preparing mitochondria for electron microscopy, 3-ml samples of buffered mitochondrial suspension (0.5 mg protein/ml) were placed in tubes and rapidly cooled to approximately 0°C by immersion in a salt-water-ice bath for 15–30 s. Chemical fixation was initiated immediately after cooling by rapidly adding 0.02 ml 30% glutaraldehyde. At 1 and 10 min after the glutaraldehyde addition, 0.1 ml of 4% OsO₄ was added and the suspensions were subsequently allowed to stand at 0°C for 30–60 min. Formation of thin, multilayered pellets, dehydration (graded ethanol), embedding (Dow Epoxy Resin in silicone rubber, flat embedding molds), cutting and staining (uranyl acetate and lead citrate) of thin sections, and electron microscopy were carried out as previously described (20).

Sucrose was obtained from Mallinckrodt Chemical Works, St. Louis, Mo., methylene diphosphonate analogs of ADP and ATP from Miles Laboratories Inc., Research Products Division, Kankakee, Ill., and hexokinase (Type F-300), substrate acids, buffers, chelating agents, and nucleotides other than the methylene diphosphonate analogs from Sigma Chemical Co., St. Louis, Mo. Atractyloside was purchased from Calbiochem, Los Angeles, Calif., and was obtained as gifts from A. Bruni and G. P. Brierley. S-13 (5-Cl, 3-*t*-butyl, 2'-Cl, 4'-NO₂-salicylanilide) was a gift from P. Hamm of Monsanto Chemical Co., St. Louis, Mo.

RESULTS

General Characteristics

Fig. 1 presents recorder tracings showing typical OD responses of suspended heart mitochondria to 50 μ M additions of ADP, ATP, and inorganic pyrophosphate (PPi). It can be seen that ADP,

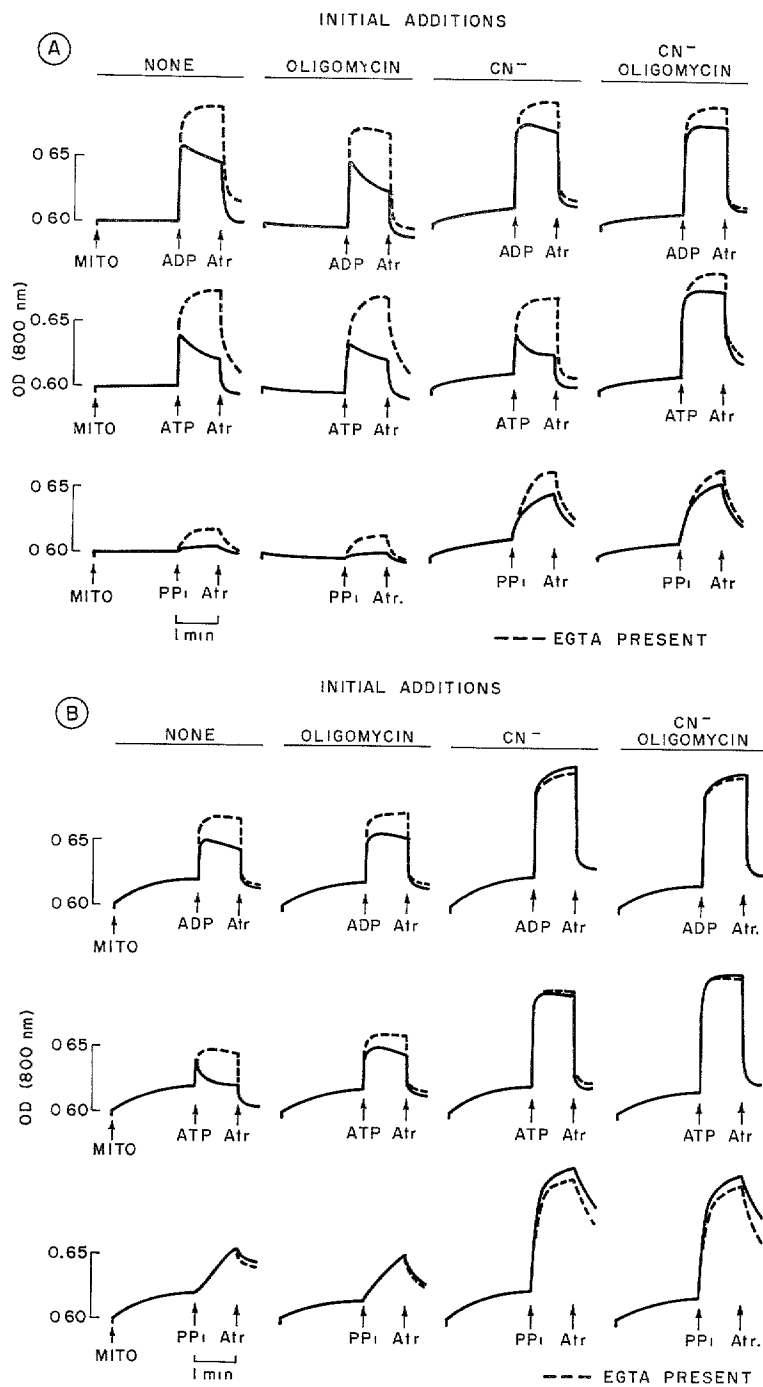


FIGURE 1 A and B Recorder tracings showing the atractyloside-sensitive changes in OD induced by ADP, ATP, and PPi in mitochondria incubated under a variety of conditions. The incubation media of Fig. 1 A contained 250 mM sucrose, 10 mM potassium piperazine-*N,N'*-bis(2-ethanesulfonate) (K-PIPES) (pH 6.8), 2.5 mM malate-pyruvate, and the agents indicated. The incubation media of Fig 1 B differed only in that the sucrose concentration was 200 mM and that 0.1 μ M S-13 was present. The concentrations of materials added were: ADP, 50 μ M; ATP, 50 μ M, PPi, 50 μ M; EGTA, 1 mM, oligomycin, 2.5 nmol/mg protein, CN⁻, 1 mM, atractyloside (Atr), 5 μ M. Respiration rates of mitochondria incubated in the absence of CN⁻ averaged 0.027 (A) and 0.607 (B) μ atom O/min per mg protein. It should be noted that an S-13 concentration approximately 10-fold higher than that used in Fig. 1 B is required for complete uncoupling in rapidly respiring mitochondria.

ATP, and PPI induce sharp, fairly large, atractyloside reversible increases in mitochondrial OD, the magnitudes of which vary considerably with variations in conditions of incubation. Ethylene glycol bis(β -aminoethyl ether) N,N,N',N' -tetraacetic acid (EGTA) markedly increases the magnitude of the OD increase in tightly coupled (Fig. 1 A) and in partially uncoupled (Fig. 1 B) mitochondria energized with oxidizable substrate or ATP. It is evident from the individual and combined effects of cyanide, oligomycin, and S-13 on the magnitude of the OD increases induced in the presence and absence of EGTA that the enhancing effect of the chelating agent decreases as the degree of energization decreases. Results to be presented elsewhere show that the enhancing effect of EGTA is associated with an increase in permeability of the inner mitochondrial membrane and that enhancement is brought about also by a variety of other agents that increase inner membrane permeability (e.g., EDTA, Ca^{++} , organic mercurials).

Mitochondrial energy status also affects the relative magnitudes of the OD changes induced by ADP, ATP, and PPI. Thus, when the mitochondria are in the energized state (Fig. 1 A), ADP induces a larger OD change than either ATP or PPI, whereas when the mitochondria are maintained in the de-energized state due to the presence of cyanide, oligomycin, and S-13 (Fig. 1 B), the magnitudes of the OD changes induced by ATP and PPI exceed that induced by ADP.

Electron micrographs of de-energized mitochondria incubated in the presence and absence of atractyloside, ADP, ADP + atractyloside, ATP, and PPI (Fig. 2) show that the atractyloside-sensitive increase in OD is associated with a striking change in the configuration of the inner mitochondrial compartment (the space bounded by the inner membrane and occupied by the matrix). Mitochondria incubated with either atractyloside (Fig. 2 B) or atractyloside + ADP (Fig. 2 D) are indistinguishable and are characterized by their large, irregular intracristal spaces and generally relaxed appearance, characteristics indistinguishable from those of de-energized mitochondria incubated in the absence of ADP, ATP, PPI, or atractyloside (Fig. 2 A). In marked contrast, mitochondria incubated with ADP, ATP, and PPI in the absence of atractyloside (Figs. 2 C, 2 E, and 2 F, respectively) have relatively small, rounded intracristal spaces and a generally strained appearance. It is

evident that this condition was achieved by contraction of the inner membrane, resulting in a considerable decrease in inner membrane expanse. Consistent with this, the transformation coincides with the formation of a more compact arrangement of the electron-opaque constituents of the inner membrane (Fig. 3) and appears not to be associated with changes in inner compartment volume and matrix density (Figs. 2 and 3). It is clearly evident, however, that the transformation results in an increase in the overall density of and a decrease in the overall volume occupied by the matrix + inner membrane + intracristal space (Fig. 2), these changes appear to occur primarily as a result of the decrease in intracristal space.

Results of electron microscope studies on mitochondria treated with ADP and ATP under the various conditions used in obtaining the data of Fig. 1 suggested that the magnitude of the adenine nucleotide-induced OD change is proportional to the degree of inner membrane contraction. Therefore, procedures were set up to quantitate the degree of contraction by measuring the magnitude of the OD change. Since mitochondrial aggregation and changes in mitochondrial matrix volume due to the movement of solutes into and out of the inner compartment also result in mitochondrial OD changes, it was necessary to select conditions which minimize interference from these sources. As is shown in Fig. 1, the degree of interference can often be estimated simply by determining the degree to which atractyloside reverses the adenine nucleotide-induced OD change.

Most of the inner membrane contraction data presented below were extracted from recorder tracings similar to those shown in Fig. 1 and are presented as percent increase in OD. Except where indicated otherwise, percent increase in OD was determined from the difference between the mitochondrial OD level immediately before adenine nucleotide addition and the highest OD level achieved within 30 s after the addition.

Effect of Adenine Nucleotide Concentration

If it is assumed (*a*) that inner membrane contraction results from the binding of adenine nucleotide (AdN) to a single kind of site in the inner membrane (*b*) that the site is in such a position that bound adenine nucleotide and unbound, exogenous adenine nucleotide are in rapid equilibrium, (*c*) that the equilibrium degree of con-

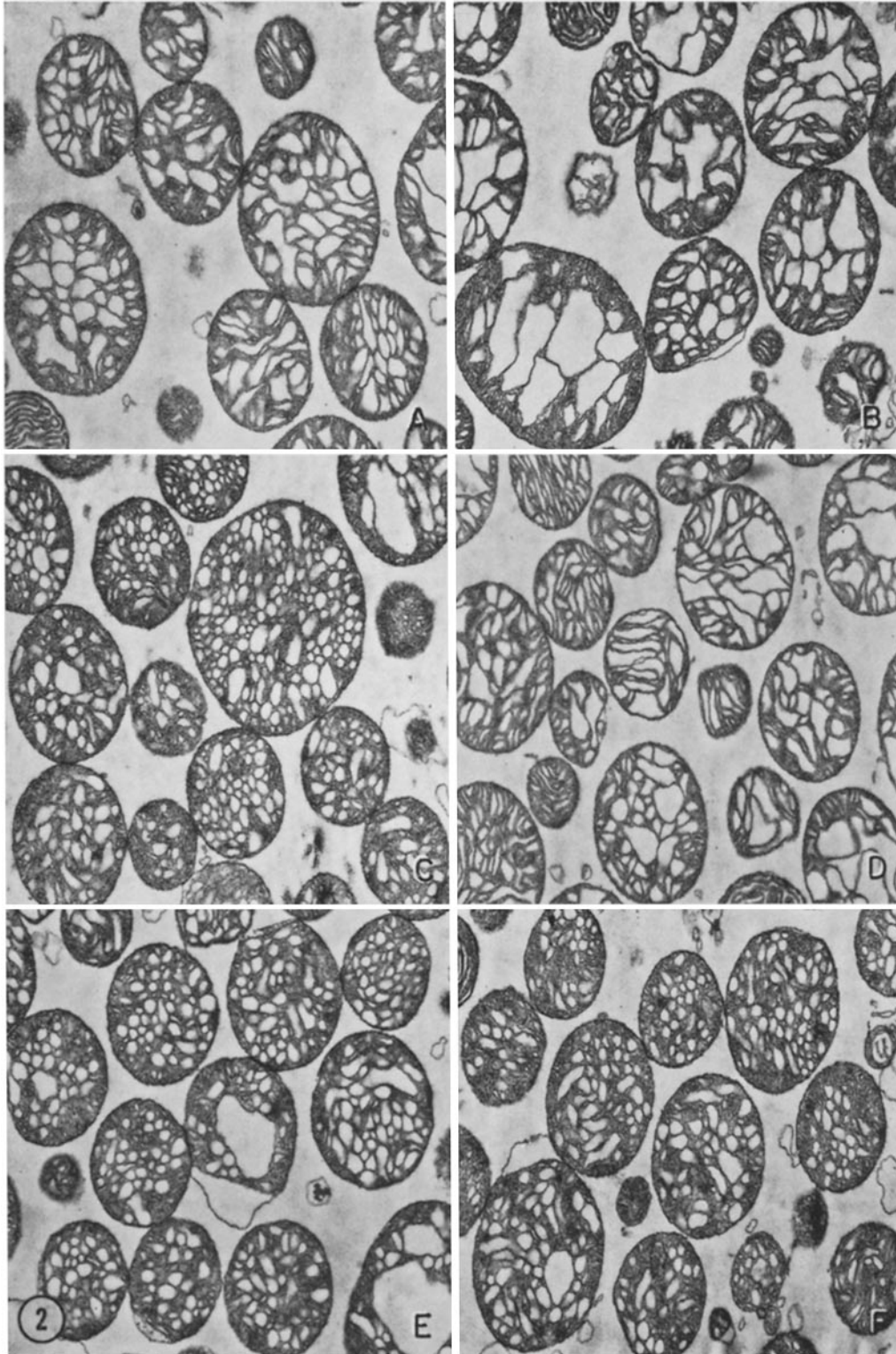


FIGURE 2 A-F Effects of ADP, ATP, PPI, and atractyloside on the ultrastructure of de-energized mitochondria. Mitochondria were incubated at 30°C in media containing 200 mM sucrose, 10 mM K-PIPES (pH 6.5), 2.5 mM malate-pyruvate, 1 mM CN^- , 5 nmol oligomycin/mg protein, 0.2 μM S-13, and where indicated, 10 μM atractyloside. ADP, ATP, and PPI were added to final concentrations of 0.1 mM after 2 min of preincubation. Fixation was initiated after 3 min total incubation. Differential conditions were: (A) none; (B) atractyloside; (C) ADP; (D) ADP + atractyloside; (E) ATP; (F) PPI. $\times 20,000$.

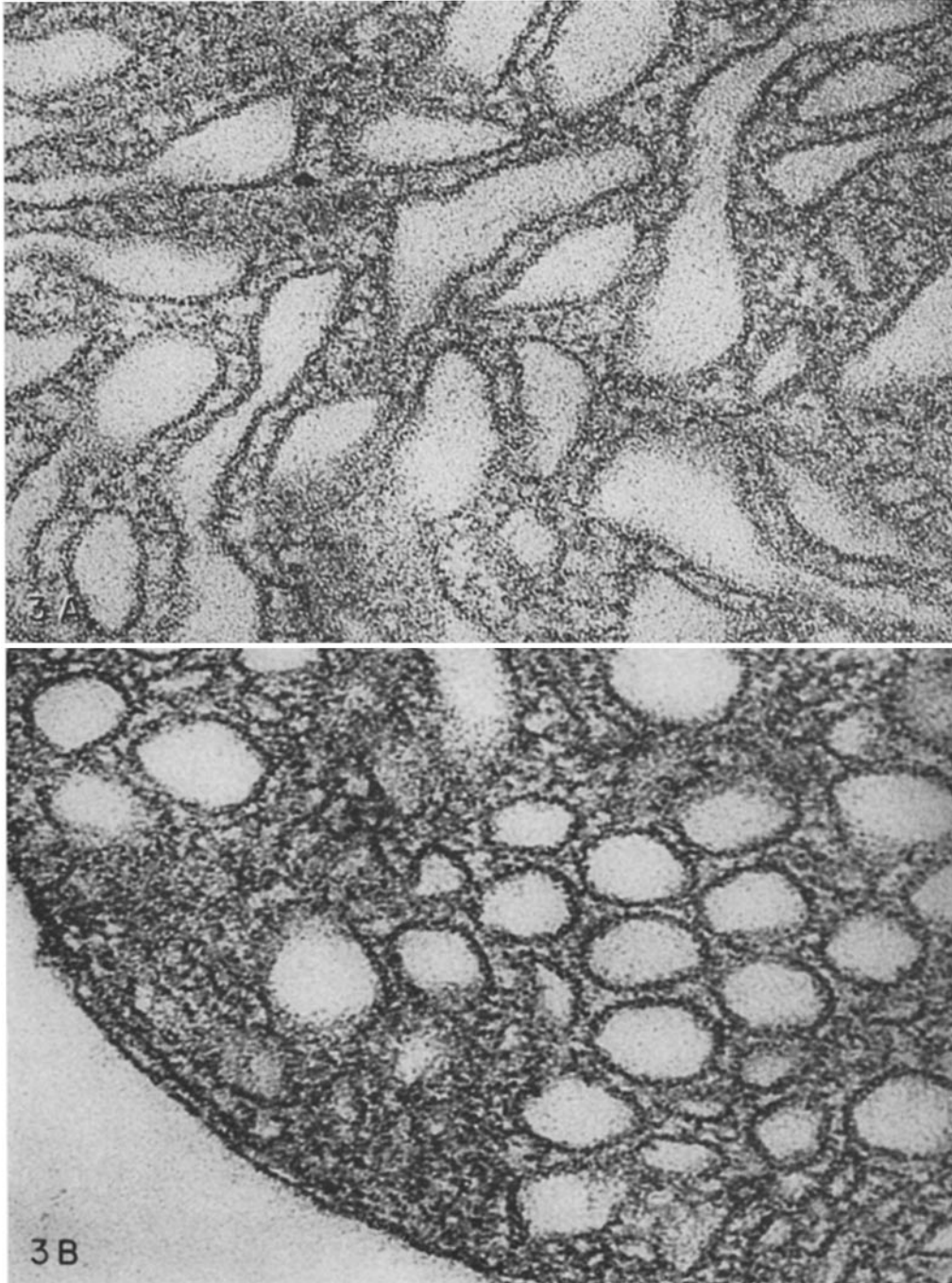


FIGURE 3 A and B High magnification electron micrographs of hypotonically suspended, de-energized mitochondria with inner membranes in relaxed (A) and highly contracted (B) states. Mitochondria were incubated for 5 min at 30°C in media containing 75 mM sucrose, 10 mM K-PIPES (pH 6.8), 0.1 μ M S-19, 2 nmol rotenone/mg protein, and either 10 μ M atractyloside (A) or 0.1 mM PPi (B). \times 140,000.

traction, C , as indicated by the percent increase in OD, is directly proportional to the number of binding sites occupied by adenine nucleotide, and (d) that the binding of adenine nucleotide to one site does not interfere with the binding of adenine nucleotide to adjacent sites, it can be shown (21) that a plot of $1/[\text{AdN}]$ vs $1/C$ should yield a straight line with slope equal to K_D/C_{max} , where K_D is the apparent dissociation constant and C_{max} the degree of contraction when all of the available binding sites are occupied. That these assumptions appear to be valid is shown in Fig. 4,

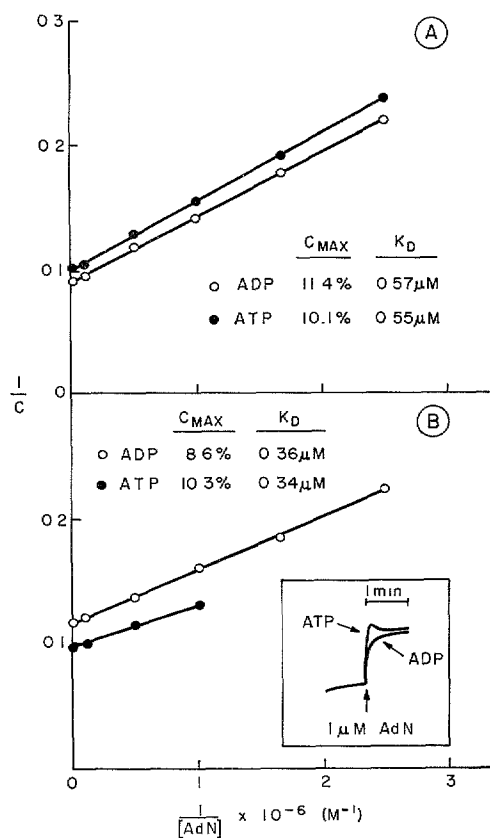


FIGURE 4 A and B Double reciprocal plots of adenine nucleotide (AdN) concentration vs. inner membrane contraction in energized (A) and de-energized (B) mitochondria. Energized mitochondria were preincubated for 2 min in media containing 250 mM sucrose, 10 mM K-PIPES (pH 6.5), 2.5 mM malate-pyruvate, and 1 mM EDTA. De-energized mitochondria were preincubated for 2 min in media containing 200 mM sucrose, 10 mM K-PIPES (pH 6.5), 2.5 mM malate-pyruvate, 0.1 μM S-13, 1 mM CN^- , and 2.5 nmol oligomycin/mg protein. Symbols are defined in the text

where it can be seen that plots of $1/[\text{ADP}]$ and $1/[\text{ATP}]$ vs $1/C$ yield straight lines.

An additional assumption, not mentioned above, is that the equilibrium degree of contraction is adequately represented by the difference between the OD immediately preceding the addition of adenine nucleotide and the highest OD level achieved within 30 s after adding adenine nucleotide. The results of Fig. 1 show that this assumption is reasonably valid for the type of contractile (OD) response obtained under the conditions employed in obtaining the data of Fig. 4. A difficulty not apparent in Fig. 1, however, is that when ATP is added to de-energized mitochondria at a final concentration of less than about 5 μM the contractile response is complicated by the presence of oligomycin-insensitive ATPase activity, resulting in the type of OD response illustrated in the inset of Fig. 4 B. Deviations from linearity in the double reciprocal plots due to this activity were readily detectable only when ATP concentration was lowered beyond 1 μM . Therefore, although it was obvious from the OD response to 1 μM ATP (inset, Fig. 4 B) that the ATPase activity increased the apparent dissociation constant to some extent, results obtained with ATP concentrations as low as 1 μM were used in estimating C_{max} and K_D . Attempts to remove the interference by increasing the oligomycin concentration were not successful.

Nucleotide Specificity

The 2'-deoxy and methylene diphosphonic acid analogs of ADP and ATP were found to be capable of inducing inner membrane contraction at rates comparable to those observed with ADP and ATP. Comparative studies with regard to C_{max} and K_D determinations show (Table I) that the ADP analogs are less effective than ADP in producing inner membrane contraction, the relationships being about the same in energized as in de-energized mitochondria. In marked contrast, the contractile responses to the analogs of ATP differ greatly depending on the energy status of the mitochondria. Thus, the analogs are considerably less effective than ATP in producing contraction in energized mitochondria, whereas they are equally as effective as or more effective than ATP in producing contraction in de-energized mitochondria. Table II shows that the decreased effectiveness of ATP and ATP analogs in producing contraction in energized mito-

TABLE I
Comparison of C_{max} and K_D Values Obtained with ADP, ATP, and 2'-Deoxy and Methylene Diphosphonate Analogs of ADP and ATP

Exp. no	Energy status	AdN	C_{max} K_D	
			%	μM
1	Energized	ADP	11.7	0.48
		d-ADP	10.1	1.26
		AOPCP	9.4	3.88
	De-energized	ADP	8.8	0.44
		d-ADP	8.7	1.12
		AOPCP	7.9	3.48
2	Energized	ATP	10.8	0.45
		d-ATP	8.8	1.40
		AOPCPOP	8.1	2.97
		AOPOPCP	4.8	5.68
	De-energized	ATP	13.4	0.44
		d-ATP	13.2	0.36
		AOPCPOP	13.8	0.21
		AOPOPCP	12.8	0.45

The conditions of incubation were the same as described in the legend of Fig. 4.

chondria is associated with an increased time requirement for the induction of half-maximal contraction. The findings suggest that the rates at which the contractile sites are occupied by ATP and ATP analogs are considerably decreased in energized mitochondria relative to de-energized mitochondria.

Adenosine monophosphate (AMP), inorganic triphosphosphate (PPPi), methylene diphosphonate (PCP), and the 5'-diphosphates and 5'-triphosphates of inosine, uridine, cytidine, and guanosine were also tested for their ability to induce inner membrane contraction in energized and de-energized mitochondria, and, with the exceptions of PPPi and uridine triphosphate (UTP), were found to be quite ineffective (Table III).

Tonicity Dependence

Early in the course of this study it was noted that the tonicity of the suspending medium is an important factor determining the magnitude of the adenine nucleotide-induced increase in OD observed and that the tonicity optimum for the increase varies considerably with variations in

TABLE II
Effects of Temperature and Mitochondrial Energy Status on the Periods of Time Required for Saturating Levels of Adenine Nucleotides to Induce Half-Maximal Degrees of Inner Membrane Contraction

Incubation temperature	AdN	$t_{1/2}^*$	
		Energized	De-energized
$^{\circ}C$		s	s
10	ADP	1.1	1.3
	ATP	3.2	1.3
	AOPCP	1.4	1.9
30	ADP	0.5	0.5
	ATP	0.8	0.5
	AOPCPOP	3.8	0.8
	AOPOPCP	3.1	0.8

Mitochondria were preincubated for 2 min under conditions similar to those described in Fig. 4. Contraction was initiated by rapidly adding 0.1 mM adenine nucleotide.

* The amount of time which elapsed between the point at which an adenine nucleotide-induced increase in mitochondrial OD was first indicated by the recorder and the point at which the equilibrium extent of the increase was half maximal. Values smaller than 0.5 s could not be detected by the methods employed.

other conditions of incubation. These findings are illustrated in Fig. 5, which compares mitochondria incubated in the presence and absence of various combinations of cyanide, S-13, gramicidin, and EDTA. It can be seen that in nonrespiring mitochondria S-13 shifts the tonicity optimum to lower levels and that the shift is increased by gramicidin and EDTA. The common effect of these agents appears to become manifest through a common ability to bring about or accelerate the loss of intramitochondrial solutes. Loss of intramitochondrial solutes results in shrinkage of the inner compartment, condensation of the matrix, and increase of mitochondrial OD. Fig. 1 shows that interference from this source in estimating the degree of inner membrane contraction is small after the 2 min preincubation period used in all of the present studies in which the degree of contraction was estimated from the change in mitochondrial OD.

Electron microscope investigations on the effects of tonicity changes on inner membrane contraction revealed (a) that the large decrease in magnitude of adenine nucleotide-induced increase of mitochondrial OD with decrease of

TABLE III
Nucleotide Specificity of Inner Membrane Contraction

Additions	% increase in OD			
	Energized		De-energized	
	0.5 min	1 min	0.5 min	1 min
AMP	1.0	1.1	0.9	0.9
ADP	12.3	12.2	10.6	10.5
IDP	1.5	1.3	0.2	-0.1
UDP	0.9	1.4	0.8	1.4
CDP	-0.1	-0.1	-0.1	-0.2
GDP	0.1	0.1	-0.1	-0.2
ATP	10.7	10.5	12.4	11.9
ITP	-0.3	-0.3	-0.7	-0.8
UTP	0.3	0.5	7.8	8.5
CDP	-0.3	-0.5	-0.1	-0.1
GTP	-0.3	-0.5	-1.0	-1.3
PPi	2.8	3.3	10.8	11.2
PCP	-0.1	0	0.1	0.1
PPPi	1.6	2.2	11.9	12.0

The conditions of incubation were the same as described in the legend of Fig. 4. The OD changes given represent the difference between the OD immediately before adding 50 μ M of the agent listed and the OD 0.5 and 1 min after the addition. The values are corrected for a small OD change which occurred in the absence of additions.

tonicity from the OD-indicated tonicity optimum for contraction (Fig 5) does not correspond to an actual decrease in inner membrane contraction and (b) that the decrease in magnitude of the OD change with decrease of tonicity closely parallels the decrease in intracristal space which occurs as a result of osmotically induced expansion of the inner mitochondrial compartment (20). These observations suggest that the OD change associated with inner membrane contraction in mitochondria suspended at the OD-indicated tonicity optimum for contraction is due in large part to the increase in average density of the inner membrane + matrix + intracristal space which occurs with inner membrane contraction and which depends on the ability of the mitochondria to undergo a decrease in intracristal space in response to inner membrane contraction (Fig 2).

In contrast, the decrease in magnitude of the adenine nucleotide-induced increase in mitochondrial OD associated with increase of tonicity from the OD-indicated contraction optimum

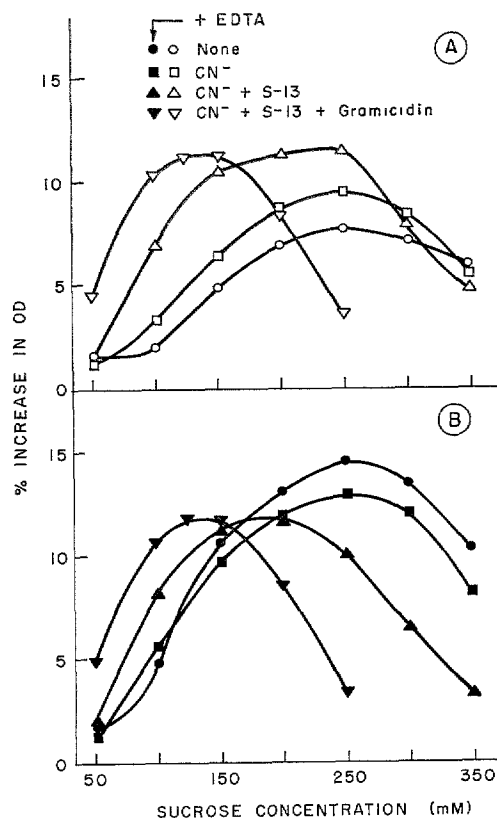


FIGURE 5 A and B Tonicity dependence of ADP-induced inner membrane contraction in mitochondria incubated under a variety of conditions. Mitochondria were preincubated for 2 min in media containing sucrose as indicated, 10 mM K-PIPES (pH 6.4), 2.5 mM malate-pyruvate, and the agents indicated. Contraction was initiated with 50 μ M ADP. Other conditions were: EDTA, 0.1 mM; CN^- , 1 mM; S-13, 0.1 μ M; gramicidin, 0.1 μ g/ml.

(Fig. 5) is closely paralleled by an actual decrease in inner membrane contraction. A likely explanation for this decrease is that the matrix becomes so rigid due to dehydration that it strongly resists the extensive rearrangement required for inner membrane contraction and decrease of intracristal space.

Effect of pH

Fig. 6 presents typical results obtained in determinations on the effect of pH on inner membrane contraction induced with ADP, ATP, and PPI in energized and de-energized mitochondria.

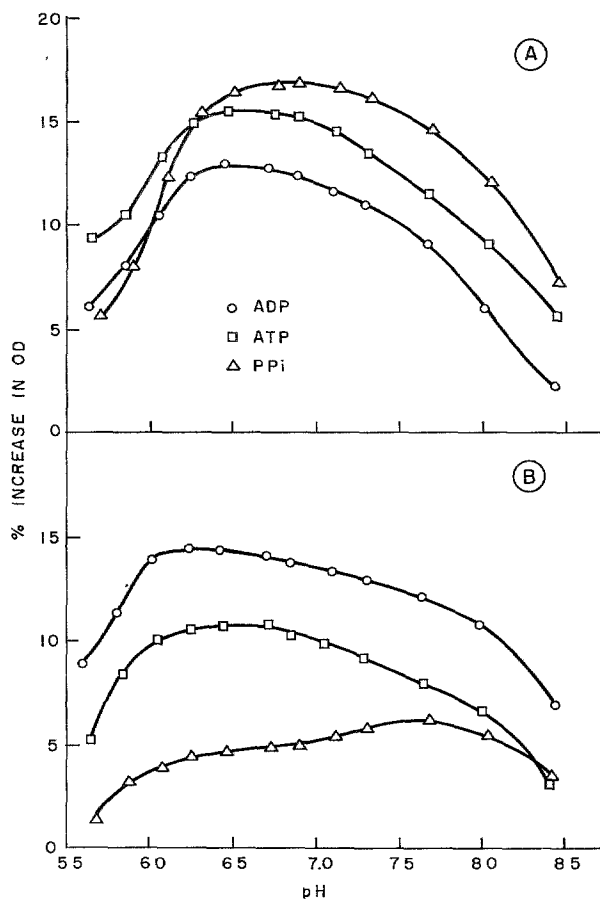


FIGURE 6 A and B pH dependence of inner membrane contraction in de-energized (A) and energized (B) mitochondria. De-energized mitochondria were preincubated for 2 min at the pH indicated in media containing 200 mM sucrose, 10 mM K-PIPES, 2.5 mM malate-pyruvate, 1 mM EGTA, 0.1 μ M S-13, 2 nmol oligomycin/mg protein, and 2 nmol rotenone/mg protein. Energized mitochondria were preincubated for 2 min in media containing 250 mM sucrose, 10 mM K-PIPES, 2.5 mM malate-pyruvate, and 1 mM EGTA. Contraction was initiated by adding 50 μ M of the agents indicated. Different mitochondrial preparations were used in Figs. 6 A and 6 B.

It shows contractile activity to be high over a wide pH range, the optimum for ADP- and ATP-induced contraction in both energized and de-energized mitochondria occurring at approximately 6.5. The higher optimum observed with PPI appeared to result from a more rapid approach to contractile equilibrium at high pH.

Determinations of the effect of pH on K_D with respect to ADP-induced contraction in energized mitochondria revealed that K_D increases as pH is raised or lowered from the 6.5 optimum. However, considerable variation in the extent of the increase was observed among mitochondrial preparations, suggesting that pH

influences K_D via some highly variable characteristic of mitochondria.

Concentration Dependence of Atractyloside Inhibition

It was shown in Fig. 1 that atractyloside strongly inhibits inner membrane contraction. Since atractyloside also strongly inhibits the oxidative phosphorylation of exogenous ADP (22), it was of interest to determine the relative amounts of atractyloside required to inhibit the two reactions. This was done by determining simultaneously the increment of respiratory activity

and the degree of inner membrane contraction induced by ADP in mitochondria preincubated in the presence of oxidizable substrate, inorganic phosphate (Pi), phosphate trap, and different amounts of atractyloside. The results (Fig. 7) show that, although the amounts of atractyloside required to produce complete inhibition of ADP-induced respiration and contraction are about the same, significant differences are observed in the extent of inhibition of the two reactions by atractyloside at concentrations less than those required to produce complete inhibition. Thus, the amount of atractyloside required to produce half-maximal inhibition of contraction is only about half that required to produce half-maximal inhibition of ADP-stimulated respiration. It will be demonstrated elsewhere that similar relationships are observed under conditions where ADP is clearly limiting.

The atractyloside concentration dependence for inhibition of inner membrane contraction was studied under a wide variety of conditions, comparing ADP, ATP, and adenosine 5'-methylene diphosphonate (AOPCP) as inducing agents. In

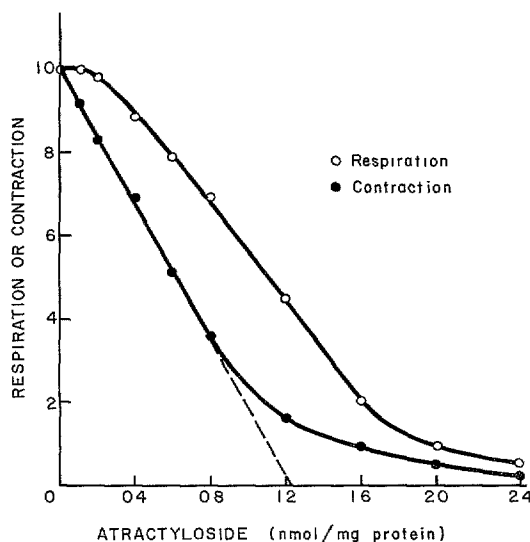


FIGURE 7 Atractyloside inhibition of ADP-induced inner membrane contraction and phosphorylating respiration. Mitochondria were preincubated for 2 min in media containing 250 mM sucrose, 10 mM K-PIPES (pH 6.5), 2.5 mM malate-pyruvate, 1 mM EGTA, 1 mM Pi, 5 mM glucose, 1 mM MgCl₂, 4 Darrow-Colowick U hexokinase/ml, and atractyloside as indicated. Contraction and phosphorylating respiration were initiated by adding 50 μ M ADP.

general, it was found that linear inhibition curves, which extrapolate to about 1.2 nmol atractyloside/mg mitochondrial protein, can be obtained with each of the inducing agents tested. However, in the case of ADP and ATP this result ordinarily requires conditions under which the adenine nucleotide affinity of the contractile site is considerably decreased. It will become clear from results to be presented elsewhere that a linear inhibition curve extrapolating to about 1.2 nmol atractyloside/mg protein is obtained under the conditions of Fig. 7 only because intramitochondrial Pi strongly decreases the affinity of the contractile site, under the conditions employed (1 mM exogenous Pi), the affinity for ADP is decreased to the extent that a K_D of about 5 μ M is observed. It will be shown that this value corresponds closely with the concentration of ADP required to elicit a half-maximal state 3 rate of respiration and that state 3 rate of respiration and equilibrium degree of inner membrane contraction are directly proportional over a wide range of ADP concentrations.

DISCUSSION

The results of this study demonstrate that exogenous ADP, ATP, and certain other high-energy phosphate compounds induce the inner membranes of heart mitochondria to undergo pronounced contraction. Concentration dependence studies suggest that contraction occurs as a result of adenine nucleotides binding to inner membrane sites which are directly accessible to and have a uniformly high affinity for exogenous adenine nucleotides (Fig. 4). The absence of an atractyloside-reversible contracted state of the inner membranes in mitochondria incubated in the absence of exogenous adenine nucleotides suggests that endogenous adenine nucleotides do not have direct access to the sites.

The observation that similar amounts of atractyloside are required to produce complete inhibition of ADP-induced inner membrane contraction and phosphorylating respiration (Fig. 7) suggests that the contractile site is identical with the carrier involved in the exchange of adenine nucleotides across the inner mitochondrial membrane. This is suggested also by the essentially identical nucleotide specificity of the contraction (Tables I and III) and exchange (1-4) reactions and by the similar adenine nucleotide affinity of the binding sites involved in the contraction (Fig. 4) and exchange (23) reactions. An apparent difference in nucleotide specificity conse-

quent to the ability of UTP to induce contraction (Table III) and the inability of UTP to participate in the exchange reaction (4) can be discounted by taking into account the facts that the nucleotide specificity studies on the exchange reaction have been carried out exclusively with energized mitochondria and that UTP induces a significant degree of inner membrane contraction only in de-energized mitochondria (Table III).

Important similarities between the contraction and exchange reactions are observed also with regard to the effects of mitochondrial energy status. In both cases there seems to be energy-dependent discrimination against the reaction of exogenous ATP relative to exogenous ADP. In the case of the exchange reaction, considerable evidence for this discrimination has been provided by Klingenberg and coworkers (4, 24, 25). For example, these workers have shown (4) that under competitive conditions in coupled mitochondria exogenous ADP exchanges with endogenous adenine nucleotides 10–15 times faster than exogenous ATP, whereas in uncoupled mitochondria the exchange of exogenous ATP equals or exceeds that of ADP. Discrimination between exogenous ADP and ATP is considered to be superimposed by an energized state of the membrane upon the basically equal specificity of the carrier for ADP and ATP (4, 25).

Pfaff and Klingenberg (4) have suggested an explanation for the discrimination based on a difference in negative charge on the ADP-loaded and ATP-loaded carrier in conjunction with an energy-dependent membrane potential such as that postulated by Mitchell's chemiosmotic hypothesis (26). The ATP-loaded carrier is considered to have one net negative charge and the ADP-loaded carrier is considered to be neutral. With the energized membrane positively charged outside and negatively charged inside in accordance with the chemiosmotic hypothesis, the inward movement of the negatively charged, ATP-loaded carrier would be resisted by the potential gradient, whereas the movement of the neutral, ADP-loaded carrier would not be resisted and therefore could diffuse freely to the inner side of the membrane to effect the exchange with endogenous ADP or ATP. If the exchange happens to be with endogenous ATP, the carrier complex assumes a net negative charge and, as a consequence, is induced to move down the potential gradient to the outer side of the membrane.

In the case of inner membrane contraction,

energy-dependent discrimination against exogenous ATP relative to exogenous ADP is indicated by the observations that in energized mitochondria inner membrane contraction induced by ATP is less rapid and less extensive than contraction induced by ADP, whereas in de-energized mitochondria contraction induced by ATP is equally as rapid as and more extensive than contraction induced by ADP (Figs. 1, 4, and 6; Tables I–III). Energy-dependent discrimination against contraction induced by analogs of ATP is somewhat greater than is observed with ATP and is detectable also as a large increase in the apparent dissociation constant of the adenine nucleotide-contractile site complex (Table I). Discrimination against contraction induced by UTP, PPI, and PPPI is particularly strong and, in the case of UTP, is so severe as to inhibit contraction almost completely (Table III). The fact that all of the agents that appear to be subject to energy-dependent discrimination have one or more acidic phosphoryl hydroxyl groups than ADP suggests that discrimination may occur by the mechanism based on charge differences suggested by Pfaff and Klingenberg (4). This possibility is further suggested by the observation that PPPI induces a more rapid contraction than PPI in de-energized mitochondria, whereas it produces a less rapid contraction than PPI in energized mitochondria (Table III).

In view of the above indications of the site involved in inner membrane contraction being identical with the adenine nucleotide exchange carrier, it seems reasonable to assume that any mechanism that is invoked to account for the contraction reaction must be consistent with the exchange reaction, and vice versa. An important feature of the exchange reaction that must be accounted for is the strict coupling of the exchange of exogenous adenine nucleotides with endogenous adenine nucleotides; that is, for every adenine nucleotide molecule transported into the mitochondrion via the exchange carrier one is transported out, resulting in the maintenance of a fairly constant level of endogenous adenine nucleotides despite large changes in concentration of exogenous adenine nucleotides (4, 24). To account for this and other characteristics of the exchange reaction, Weidemann et al. (23) have suggested a simple carrier model which seems to be consistent with the observations reported here. The model consists of carrier in which the adenine nucleotide binding site can become oriented to

either side of the membrane in a statistical manner. Exchange coupling is achieved by postulating that only the loaded form of the carrier can move through the membrane. With this restriction the removal of exogenous adenine nucleotides through repeated washings, as is carried out in the isolation of mitochondria, would result in all of the sites becoming trapped in the free form at the outer surface of the membrane.

In addition to providing a satisfactory explanation for the apparent exclusive accessibility of the contractile sites to exogenous adenine nucleotides, this model has the advantage that the manner in which the carrier might operate in bringing about coupled adenine nucleotide exchange and inner membrane contraction is not difficult to visualize. Thus, the carrier might consist of a highly hydrophobic portion which anchors it in the membrane and a positively charged, hydrophilic portion to which the dissociated phosphoryl hydroxyl groups of adenine nucleotides bind. Adenine nucleotide transfer through the membrane could occur as a result of the overall adenine nucleotide-carrier complex being sufficiently hydrophobic to enter and diffuse through the highly hydrophobic phase of the membrane. Exchange coupling could occur as a result of the inability of the charged, hydrophilic portion of the unloaded carrier to move through the membrane. Inner membrane contraction could be explained by assuming that the positively charged site of the unloaded carrier remains embedded in the membrane and that neutralization of the site upon binding adenine nucleotide results in considerable dehydration of the site and decrease of electrostatic repulsion between the site and other positively charged constituents of the membrane.

Contraction by a mechanism of this sort seems consistent with the specificity studies suggesting that the phosphate moiety of adenine nucleotide is the critical requirement for contraction (Table III) and with the ultrastructural studies suggesting that the extent of contraction is large (Figs. 2 and 3). If inner membrane contraction in fact occurs by this mechanism, it is clear from the inability of atractyloside to induce contraction that different receptor sites are involved in the binding of atractyloside and adenine nucleotides.

An interesting feature of the contraction reaction is that ATP and the other inducing agents that appear to be subject to energy-dependent discrimination are capable of inducing a higher degree of contraction in de-energized mitochondria

than are ADP and analogs of ADP (Tables I and III). If it is assumed that contraction results from the negatively charged groups of the inducing agents neutralizing positively charged groups in the inner membrane, as is suggested above, this difference can be accounted for simply on the basis of the fact that the inducing agents that are subject to discrimination possess more acidic phosphoryl hydroxyl groups that may dissociate and neutralize positive charges. However, to be consistent with the mechanism of discrimination suggested by Pfaff and Klingenberg (4), it seems necessary to assume that positive charges in excess of those neutralized by ADP are available for neutralization in de-energized but not in energized mitochondria. Such a condition seems plausible in view of the observations of Azzi (27) suggesting that energization of the inner membrane results in considerable changes in the distribution of charges inside the membrane, positive charges becoming oriented toward the inside and negative charges toward the outside, as would be expected if a membrane potential were formed in accordance with the chemiosmotic hypothesis (26).

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