ON THE PARTICIPATION OF FLAVIN IN MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE REACTIONS

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SUMMARY

The effect of atebrin, a flavoenzyme inhibitor, was studied on the ATPase reactions of rat-liver mitochondria. The ATPase induced by 2,4-dinitrophenol is stimulated at low (0.75 m*M*) and inhibited by high (3 m*M*) concentrations of atebrin. Amytal, in concentrations which in themselves do not affect the dinitrophenol-induced ATPase, greatly promotes the inhibition by atebrin. Addition of FMN or FAD (7 m*M*) counteracts the effects of atebrin both in the presence and absence of amytal.

The Mg^{++}-activated ATPase of mitochondria treated with 0.1 % deoxycholate is inhibited by atebrin even at concentrations that stimulate the dinitrophenol-induced ATPase of the intact mitochondria. This inhibition is not relieved by added flavin nucleotides; on the contrary, the latters inhibit the Mg^{++}-activated ATPase and their inhibitory effect is additive to that of atebrin.

Agents which reduce the mitochondrial respiratory catalysts, such as succinate or dithionite, enhance the Mg^{++}-activated ATPase reaction of mitochondria treated with 0.1 % deoxycholate. Dithionite, but not succinate, removes the inhibition of the Mg^{++}-activated ATPase by atebrin.

The implications of the experimental data for the mechanism of mitochondrial ATPase reactions are discussed.

INTRODUCTION

It was concluded in a previous paper[1] that the P_i–ATP exchange reaction, which takes place in intact mitochondria, is connected to a great extent with the first of the three phosphorylations accompanying the aerobic oxidation of DPNH. It was postulated that the exchange reaction involves two reversible steps of electron transfer along the respiratory chain, one between DPNH and the diaphorase flavin, and a second between the diaphorase flavin and the subsequent carrier. FADH ~ P was suggested to constitute an intermediate in this reaction sequence, the phosphate group being released and transferred to ADP when FADH ~ P is oxidized by the

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; FAD and $FADH_2$, oxidized and reduced, flavin-adenine-dinucleotide; FADH ~ P, flavin-adenine-dinucleotide, reduced phosphorylated form; FMN, flavin mononucleotide; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)-aminomethane.

References p. 10.

subsequent carrier. These conclusions were arrived at in cooperation with GRABE[2], who has obtained theoretical support for this reaction sequence and pointed out that the phosphate group in the FADH ~ P is probably attached to the C-2 atom of the isoalloxazine ring of the reduced FAD.

Previous experiments[3] had revealed that the DNP-activated ATPase of intact mitochondria and the Mg^{++}-activated ATPase of structurally disorganized mitochondria are closely interrelated, the latter involving a part of the reaction sequence accounting for the former. It was considered that the DNP-induced ATPase reaction is mainly related to the same phosphorylation step as the P_i–ATP-exchange reaction, and that it involves, like the exchange, a reversible two-step transfer of electrons in the diaphorase region.

In the present paper the effects of the flavin antagonist, atebrin*, on mitochondrial oxidative phosphorylation and related reactions will be described. Atebrin is known from previous studies to inhibit the DPNH-cytochrome *c*-reductase and D-amino acid oxidase reactions[4–6] and to uncouple oxidative phosphorylation[7]. Interest was focused primarily on the effects of atebrin on the mitochondrial ATPase reactions. The present data provide further support for the previous conclusion concerning the involvement of flavin in the DNP-induced ATPase; they suggest, furthermore, that the Mg^{++}-activated ATPase reaction of structurally disorganized liver mitochondria is, like the DNP-induced ATPase reaction of the intact mitochondria, a flavin-dependent reaction.

EXPERIMENTAL

Rat-liver mitochondria, twice washed, were prepared as described previously[8].

For tests of oxidative phosphorylation and P_i–ATP exchange, mitochondria, corresponding to 200 mg (wet weight) liver, in 1-ml aliquots of a sucrose suspension were added to the reaction vessels. The final volume of the reaction mixture was 2 ml. The tests were performed as described previously[1].

For ATPase tests, the mitochondrial pellets were suspended in sucrose to give 1 ml of a 0.25 *M* sucrose suspension containing mitochondria from 50 mg liver. In the case of deoxycholate treatment the suspension was diluted with a deoxycholate–sucrose mixture (pH 7.5) to give a 0.25 *M* sucrose suspension containing the deoxycholate concentrations indicated in the different experiments. The deoxycholate was allowed to act for 2 min at room temperature. 1 ml-aliquots of the sucrose suspension were added to the incubation tubes containing 10–50 μmoles Tris buffer (pH 7.5), 10 μmoles ATP (pH 7.5), 150 μmoles sucrose and, when used, 8 μmoles Mg^{++} and 0.2 μmole DNP. The final volume was 2.0 ml, and the final concentration of deoxycholate in the ATPase test was half of that used in preincubation. The tubes were incubated for 20 min at 30° and the reaction was stopped with 1.0 ml 1.5 *M* $HClO_4$. Inorganic phosphate was determined according to ERNSTER *et al.*[9].

RESULTS

Table I illustrates the effects of atebrin on oxidative phosphorylation and P_i–ATP exchange. Atebrin caused a depression of the phosphorylation as well as the respira-

* Synonyms: quinacrine, atabrine or 3-chloro-7-methoxy-9-(1-methyl-4-diethylamino-butylamino) acridine.

References p. 10.

tion, the latter effect being more pronounced with glutamate than with succinate as substrate. The concentration of atebrin required for an almost complete suppression of the phosphate uptake was about 4 mM, in agreement with previous data by HUNTER[7]. Atebrin also inhibited the P_i–ATP exchange; complete inhibition ensued at a concentration of 8 mM.

TABLE I

EFFECTS OF ATEBRIN ON OXIDATIVE PHOSPHORYLATION AND P_i–ATP EXCHANGE

Conditions for oxidative phosphorylation: Each Warburg vessel contained mitochondria from 200 mg (wet weight) rat liver, 20 μmoles substrate, 30 μmoles $^{32}P_i$, 8 μmoles Mg^{++}, 3 μmoles ATP, hexokinase in excess (together with 60 μmoles glucose), 50 μmoles Tris buffer (pH 7.5) and 125 μmoles sucrose. The final vol. was 2.0 ml, gas phase air, temperature 30° and time of incubation 20 min. Conditions for P_i–ATP exchange: mitochondria from 200 mg liver, 30 μmoles ATP, 20 μmoles $^{32}P_i$, 8 μmoles Mg^{++}, 100 μmoles Tris buffer (pH 7.5) and 125 μmoles sucrose. Final vol. was 2.0 ml. Temperature 22°. The % value was obtained by substracting the % phosphate exchanged after 3-min incubation from the value obtained after 13-min incubation.

Atebrin mM	*Succinate*			*Glutamate*			*P_i-ATP exchange %*
	μatoms ΔO	*μmoles ΔP*	*P/O*	*μatoms ΔO*	*μmoles ΔP*	*P/O*	
0	8.9	17.8	2.0	8.1	22.7	2.8	14.5
1	7.9	8.5	1.1	7.8	16.5	2.1	11.8
2	5.0	2.1	0.4	5.4	6.6	1.2	8.5
4	4.9	0.6	0.1	0.0	1.0	—	3.9
8	3.2	0.4	0.1	0.0	0.7	—	0.4

Fig. 1 shows the effect of varying concentrations of DNP on the ATPase activity of intact mitochondria. Maximum activity was obtained at about 0.1 mM DNP which is in agreement with earlier observations[10].

In Fig. 2 the effect of varying concentrations of atebrin on the rate of the DNP-induced ATPase reaction is shown. In low concentrations atebrin stimulated the

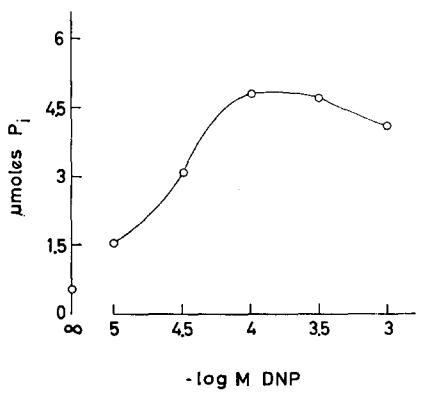

Fig. 1. Influence of DNP concentration on the ATPase activity of intact liver mitochondria. Each tube contained mitochondria from 50 mg wet weight liver, 10 μmoles ATP, 10 μmoles Tris (pH 7.5) and DNP in 2.0 ml 0.25 M sucrose, and was incubated for 20 min at 30°.

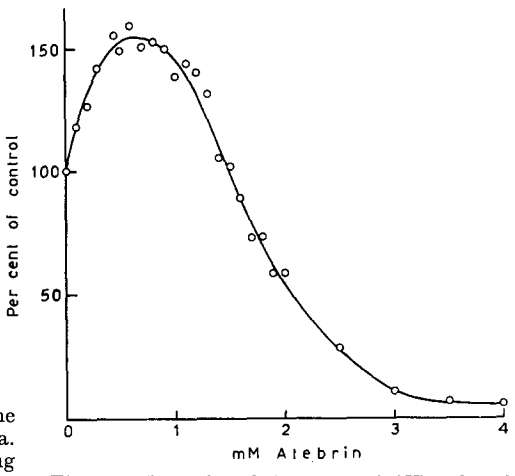

Fig. 2. Effect of atebrin on the DNP-induced ATPase. Experimental conditions as in Fig. 1. The DNP concentration was 0.1 mM.

References p. 10.

reaction rate, giving a maximum stimulation by about 50 % at a concentration of about 1.5 m*M*. The extent of the maximum stimulation varied somewhat from one experiment to another, but it never exceeded 60 %. In the higher concentration range the atebrin effect changed to an inhibition, which was almost complete at 4 m*M*, *i.e.* at the concentration which was previously required for the complete inhibition of phosphate uptake.

Also with acriflavin, another compound known to interfere with flavins[6], it has been possible to demonstrate the same effects on the DNP-induced ATPase (Fig. 3).

As shown in Fig. 4, the stimulating effect of low concentrations of atebrin was present even if DNP was added in sub-optimal concentrations. With no DNP, atebrin in itself induced a slight ATPase activity (*cf.* also Fig. 5).

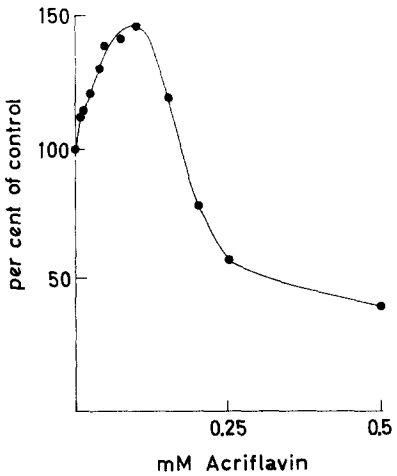

Fig. 3. Effect of acriflavin on the DNP-induced ATPase. Experimental conditions as in Fig. 2.

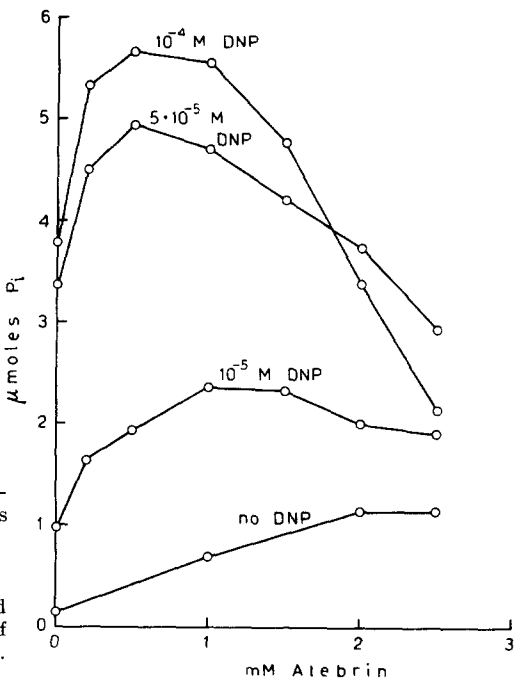

Fig. 4. Effect of atebrin on ATPase induced by addition of suboptimal concentrations of DNP. Experimental conditions as in Fig. 2.

Fig. 5 compares the effects of atebrin on the DNP-induced ATPase of intact mitochondria and the Mg^{++}-activated ATPase of mitochondria treated with 0.1 % deoxycholate. As has been shown previously[3] this treatment elicits a maximum activity of the Mg^{++}-activated ATPase. As can be seen in Fig. 5, this reaction is strongly inhibited by atebrin; the inhibition is present also at low concentrations of atebrin, concentrations which were stimulatory in the case of the DNP-induced ATPase. Fig. 5 also shows that the slight ATPase activity elicited by atebrin alone in fresh mitochondria can be depressed by about 50 % by the addition of 4 m*M* Mg^{++}.

It has been found previously that both the disappearance of the DNP-activated ATPase, and the appearance of the Mg^{++}-activated ATPase upon treatment of mitochondria with increasing concentrations of deoxycholate, take place according to a biphasic curve, showing an intermediate maximum in the medium range of deoxycholate concentration. When testing the effects of atebrin on the two types of

References p. 10.

ATPase at varying concentrations of deoxycholate it was found that the DNP-activated ATPase was stimulated by low, and inhibited by high, concentrations of atebrin within the entire range of deoxycholate concentrations where there was a measurable DNP-activated ATPase activity. The Mg^{++}-activated ATPase, on the other hand, revealed a gradual shift in its behavior against atebrin along the deoxycholate concentration scale. Thus, at low deoxycholate concentrations, and up to the first maximum of activity, the Mg^{++}-activated ATPase was stimulated by low, and inhibited by high concentrations of atebrin; whereas the final Mg^{++}-activated ATPase, that present at 0.1 % deoxycholate, was strongly inhibited even by low concentrations of atebrin. A typical experiment is shown in Fig. 6. This finding conforms to the earlier conclusion[3] that the ATPase activities appearing at low and medium concentrations of deoxycholate are more similar in their properties to the DNP-activated ATPase of the intact mitochondria than to the Mg^{++}-activated ATPase of the mitochondria treated with 0.1 % deoxycholate.

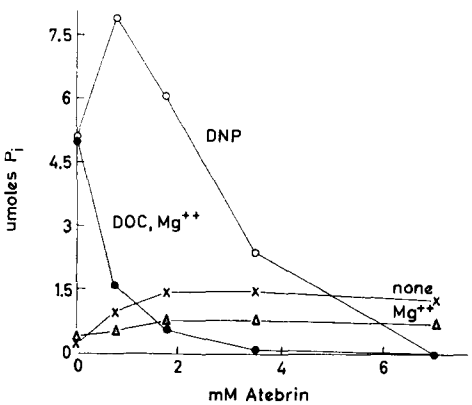

Fig. 5. Effect of atebrin on mitochondrial ATPases under various conditions. Experimental conditions as in Fig. 1. Where indicated 0.1 m*M* DNP and 4 m*M* Mg^{++} were present. For deoxycholate (DOC) additions see the experimental section.

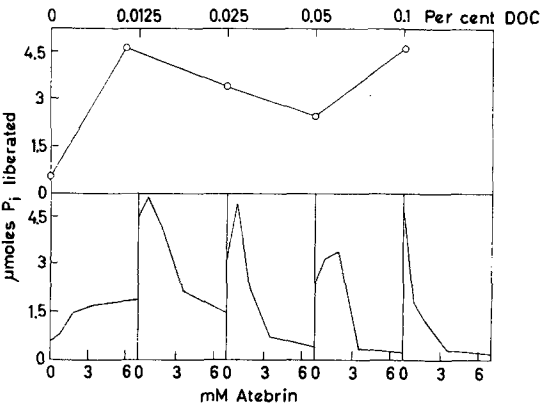

Fig. 6. Effect of atebrin on the Mg^{++}-activated ATPase at various concentrations of deoxycholate (DOC). For experimental conditions see the experimental section.

Since the dualistic effect of atebrin on the DNP-activated ATPase was thought to be due to a shifting of the rate-limiting step within this reaction sequence, it seemed to be of interest to investigate how atebrin influences the susceptibility of the DNP-activated ATPase to respiratory inhibitors. It has been found previously[3] that amytal exerted a certain inhibition on the DNP-activated ATPase, while antimycin A and cyanide had no significant effect. As shown in Fig. 7 this situation is dramatically altered when the effects of these respiratory inhibitors are studied in the presence of atebrin. Cyanide and, to a lesser extent, antimycin A caused a marked inhibition of the DNP-activated ATPase, especially on the descending side of the atebrin curve. This effect was still more marked in the case of amytal, which caused an almost complete depression of DNP-activated ATPase even in the ascending phase of the atebrin curve. In all three cases, succinate accentuated the inhibitory effects of cyanide, antimycin A and amytal.

In the case of the Mg^{++}-activated ATPase of mitochondria treated with 0.1 %

References p. 10.

deoxycholate, the activity of which was previously[3] found not to be influenced by amytal or antimycin A, the addition of these agents did not potentiate the inhibition caused by atebrin.

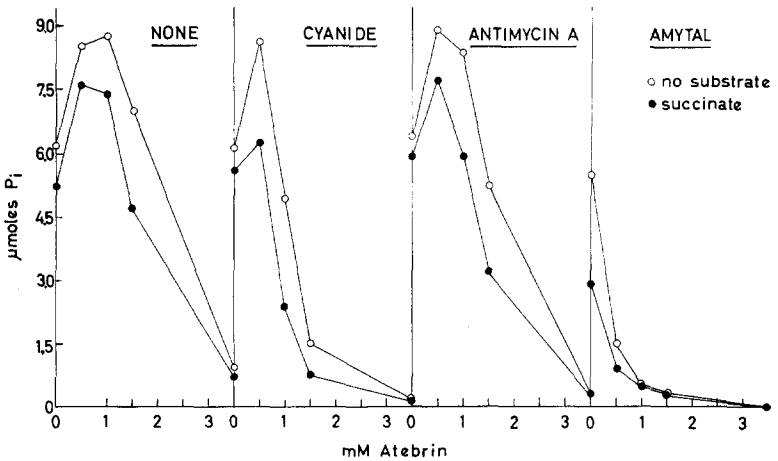

Fig. 7. Effect of atebrin on DNP-induced ATPase in the presence of certain respiratory inhibitors. Experimental conditions as in Fig. 2. Additions: Amytal, 2 mM; cyanide, 1 mM; antimycin A, 1 μg/ml.

It was found, on the other hand, that cyanide or succinate, alone or in combination, caused a slight but significant stimulation of this ATPase activity. Some typical data are shown in Table II. A similar effect could also be obtained, and even to a larger extent, by the addition of dithionite, if the addition was made to a system containing cyanide. It is noteworthy that this effect was not present if antimycin A was substituted for cyanide. From Table III, in which an experiment of this type is shown, it can be seen that the added amount of dithionite had to be kept below 5 μmoles per sample in order to give maximum stimulation. This relatively small amount of dithionite was rapidly oxidized by way of the respiratory chain (at a much higher rate that the auto-oxidation of dithionite), and after a few minutes of incubation no dithionite was left in the system. The role of added cyanide may thus be explained

TABLE II

EFFECTS OF CYANIDE AND SUCCINATE ON THE Mg^{++}-ACTIVATED ATPASE

For the general conditions for measuring the Mg^{++}-activated ATPase see experimental section. Where indicated, the concentration of cyanide was 1 mM and that of succinate 10 mM. Time of incubation 20 min, temperature, 30°.

Expt.	*Atebrin mM*	*ATPase (µmoles P_i liberated)*			
		None	*Cyanide*	*Succ.*	*Cyanide + succ.*
1	0	5.1	6.1	6.6	7.2
2	0	5.0	6.3	7.0	7.1
	0.75	1.5	1.5	1.6	2.0
	1.75	0.6	0.6	0.6	0.5
	3.5	0	0	0	0

References p. 10.

in terms of a preservation of the respiratory catalysts in the reduced state after all dithionite had been consumed. The significance of these findings will be discussed below.

TABLE III

EFFECT OF DITHIONITE ON Mg^{++}-ACTIVATED ATPASE IN THE PRESENCE OF CYANIDE AND ANTIMYCIN A

For the experimental conditions see experimental section. Where indicated the concentration of cyanide was 1 mM, and that of antimycin A was 2 μg/tube. Time of incubation, 20 min. Temperature, 30°.

Dithionite µmoles/tube	*ATPase (µmoles P_i liberated)*		
	None	*Cyanide*	*Antimycin A*
0	4.3	5.4	4.5
1	4.9	9.2	4.9
2	4.7	9.0	4.9
3	4.1	8.3	4.0
5	3.1	4.6	3.0

As shown in Table IV, dithionite was also able to prevent the inhibition of the Mg^{++}-activated ATPase by atebrin. In the presence of atebrin, however, dithionite + cyanide gave no higher activity than did dithionite alone.

In Fig. 8, the effects of added FAD and FMN on the DNP-induced ATPase are demonstrated. These compounds were able to counteract both effects of atebrin, the stimulation as well as the inhibition. FMN or FAD were able to prevent the atebrin effect even if the latter was potentiated by amytal. As is shown in Fig. 8, at low concentrations of FMN or FAD there can still be seen a stimulation by low, and an inhibition by high concentrations of atebrin. At higher concentrations of FMN or FAD, the DNP-activated ATPase appears more or less unaffected by atebrin. These effects of added flavins were not duplicated by DPN, DPNH, TPN or TPNH.

The addition of FMN or FAD to the Mg^{++}-activated ATPase of mitochondria treated with 0.1 % deoxycholate did not relieve the atebrin inhibition, as demonstrated in Table V, but resulted itself in an inhibition, similar and additive to that caused by atebrin.

TABLE IV

EFFECT OF DITHIONITE ON THE Mg^{++}-ACTIVATED ATPASE IN THE PRESENCE OF ATEBRIN

For experimental conditions see experimental section. Where indicated the concentration of cyanide was 1 mM and the added amount of dithionite was 1 μmole/tube. Time of incubation, 20 min. Temperature, 30°.

Additions	*Expt. 1*		*Expt. 2*	
	ATPase (µmoles P_i liberated)			
	without atebrin	*with atebrin*	*without atebrin*	*with atebrin*
None	6.2	1.7	5.7	2.2
Dithionite	7.5	6.3	6.8	6.7
Cyanide	6.5	1.5	6.2	2.7
Dithionite + cyanide	8.6	6.0	7.5	6.5

References p. 10.

The selective inhibitory effect of 2,6-dichlorophenolindophenol on the DNP-induced ATPase of intact mitochondria, reported previously[3], was not influenced by atebrin. The ATPase activity elicited by the dye in itself was stimulated by low, and inhibited by high concentrations of atebrin, in the same way as the DNP-induced ATPase.

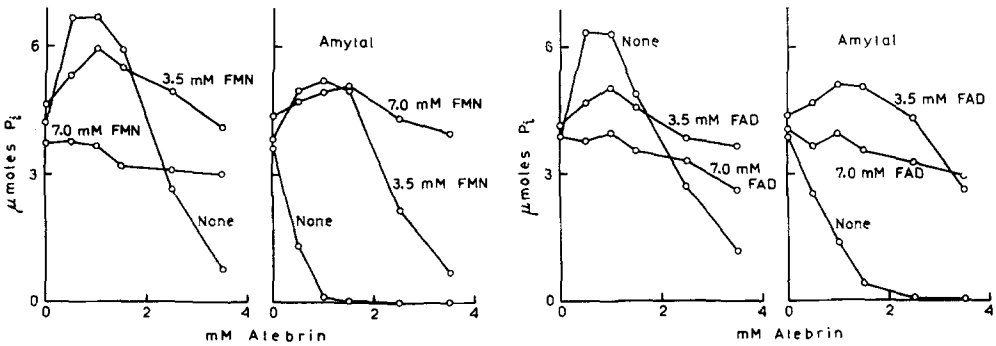

Fig. 8. Effects of FMN and FAD on the DNP-induced ATPase in the presence of atebrin. Where indicated 2 m*M* amytal was present. Experimental conditions as in Fig. 2.

TABLE V

EFFECT OF ATEBRIN ON THE Mg^{++}-ACTIVATED ATPASE IN THE PRESENCE OF ADDED FMN AND FAD

For experimental conditions see experimental section. Time of incubation, 20 min. Temperature, 30°

Expt.	*Flavin (mM)*	*ATPase (μmole P_i liberated)*		
		Atebrin		
		None	*1.5 mM*	*3.5 mM*
1	None	4.4	2.5	1.4
	FMN (0.75)	3.5	2.1	1.6
	FMN (2.25)	3.4	1.7	1.6
	FMN (3.5)	2.6	1.4	0.9
	FMN (7.0)	1.9	1.4	0.9
2	None	3.5	2.7	1.4
	FAD (0.35)	3.5	2.7	1.6
	FAD (3.5)	2.5	1.4	1.1
	FAD (7.0)	1.8	1.3	0.7

DISCUSSION

The present data appear to support the conclusion that flavin (FMN or FAD) is involved in the mitochondrial ATPase reactions. Before discussing the possible implications of this conclusion for the mechanism of phosphorylation coupled to electron transport it seems important to summarize some points, which are believed to justify the view that the effects of atebrin, reported in the present paper, are really due to a competition with flavin, rather than to some unspecific type of action. These points are as follows:

References p. 10.

1. Atebrin is a known inhibitor of flavoenzymes, such as DPNH-cytochrome *c* reductase and D-amino acid oxidase[5-7,11]*.

2. The effects described in the present paper could be duplicated with other flavin antagonists, such as acriflavin[6,11], and also with promazine and certain promazine derivatives[13], as will be demonstrated in a forthcoming paper[14].

3. The characteristic dual effect of flavin antagonists on the DNP-induced ATPase (consisting of a stimulation at low, and an inhibition at high concentrations) was not duplicated by any inhibitor of this reaction of the non-flavin-antagonist type, such as amytal[3], azide[3,15,16], thyroxine** or *p*-chloromercuribenzoate[3,10].

4. Respiratory inhibitors were able to aggravate the inhibitory effect of atebrin on the DNP-induced ATPase, especially in the simultaneous presence of a hydrogen source such as succinate. This indicates that atebrin renders the DNP-induced ATPase more dependent on an appropriate oxidation-reduction state of the respiratory chain. The aggravation was greatest with amytal, which is a specific inhibitor of the flavoprotein site of the mitochondrial DPNH-cytochrome *c*-reductase system[17].

5. Addition of FMN or FAD was able to reverse the effect of atebrin on the DNP-induced ATPase. The reversal could not be due to a chemical binding of atebrin by the added flavins, since no similar effect could be obtained in the case of the Mg^{++}-activated ATPase. The effects of added FMN or FAD could not be duplicated with any non-flavin type of compound tested, *e.g.* with pyridine nucleotides.

The present finding that the DNP-induced ATPase involves flavin is in agreement with conclusions drawn from earlier studies[1] on the effects of amytal on this reaction and on the P_i–ATP exchange. These studies[1] have previously led to postulation of a reaction scheme for the DNP-induced ATPase, which in a simplified form, can be written as follows:

$$ATP + FADH_2 \rightleftarrows ADP + FADH \sim P \quad (1)$$

$$FADH \sim P + DPN^+ + OH^- \longrightarrow FAD + DPNH + P_i \quad (2)$$

$$FAD + DPNH + H^+ \longrightarrow FADH_2 + DPN^+ \quad (3)$$

$$\text{Net: } ATP + H_2O \longrightarrow ADP + P_i$$

Furthermore it has been concluded previously[3] that the DNP- and Mg^{++}-activated ATPases involve at least one common step and that the Mg^{++}-activated ATPase probably does not involve an active electron transport. In view of these conclusions it might be envisaged that the Mg^{++}-activated ATPase reaction consists of reaction (1) followed by a hydrolysis of the $FADH \sim P$ into $FADH_2$ and P_i.

The finding that low concentrations of the flavin antagonists stimulate the DNP-induced ATPase is not yet fully understood. If the scheme given in reactions (1)–(3) is correct, one must assume that all three of these reactions are more or less sensitive to flavin antagonists since all of them involve flavin. The stimulation caused by low concentrations of these agents may then be due to the fact that the reaction which is the most sensitive to the flavin antagonists is not rate-limiting in the overall process,

* Early data[5] concerning the inhibitory effect of atebrin on glucose-6-phosphate dehydrogenase originate from the time when it was believed that reduced pyridine nucleotides reduce 2,6-dichlorophenolindophenol non-enzymically[12] and when the activity of the glucose-6-phosphate dehydrogenase was measured using this dyestuff as a hydrogen acceptor. Very probably, the enzyme preparation used in these studies was contaminated with diaphorase. Using the reduction of TPN (recorded spectrophotometrically at 340 mμ) as a measure of the glucose-6-phosphate dehydrogenase activity, we found no inhibition with atebrin (unpublished data).

** Unpublished data.

References p. 10.

and that a partial inhibition of this reaction promotes the reaction that is rate-limiting. The potentiating effect of amytal on the atebrin effect may serve as a clue in a further elucidation of this phenomenon.

The failure of added flavins to relieve the inhibition by atebrin of Mg^{++}-activated ATPase might be explained by the fact that this reaction involves only the reduced form of flavin. The inhibitory effect of added (oxidized) flavins in themselves may then be explained in the same way. The stimulation of the Mg^{++}-activated ATPase reaction by dithionite (recently observed also by MYERS AND SLATER[18]) or by succinate, and the preventive effect of the former against inhibition by atebrin, fit into this explanation if one assumes that an insufficient reduction of the flavin constitutes the limiting factor in the Mg^{++}-activated ATPase of the deoxycholate-treated mitochondria.

In the present discussion only flavin has been considered as a participant in the Mg^{++}-activated ATPase. As there exist two further sites of phosphorylation along the electron-transport chain there might well exist two more Mg^{++}-activated ATPases, corresponding in their mechanism to the one in the flavin region, but involving the reduced forms of two further respiratory chain intermediates, *e.g.* two quinones. There exists then the possibility that added reducing agents stimulate the Mg^{++}-activated ATPase by reducing these two carriers as well. The finding that antimycin A abolishes the stimulatory effect of dithionite on the Mg^{++}-activated ATPase may support this concept.

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