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DIFFERENTIAL EFFECTS OF 2,4-DINITROPHENOL AND VALINOMYCIN ($+K^+$) ON UNCOUPLER-STIMULATED ATPase OF HUMAN TUMOR MITOCHONDRIA

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The uncoupler-stimulated mitochondrial ATPase of four human tumors, mouse kidney, brain and fetal liver exhibited a characteristic behavior when preincubated with the H^+-conducting uncouplers, dinitrophenol, CCCP, S-13 and gramicidin. The ATPase activity was considerably lower with preincubation than without. Preincubation with valinomycin ($+K^+$), on the other hand, did not result in a significant decrease of the ATPase activity. These results may be contrasted with those obtained with liver or heart mitochondria, the ATPase activity of which did not suffer any loss when preincubated with dinitrophenol. The effect of preincubation with dinitrophenol on the tumor mitochondria could not be accounted for by dinitrophenol-induced Mg^{2+} efflux, since the differential effects of dinitrophenol and valinomycin ($+K^+$) remained even when ATPase activity was determined in presence of Mg^{2+}. Small amounts of ATP and ADP in the preincubation mixture containing dinitrophenol protected against the decay of the ATPase activity, implicating the exchangeable adenine nucleotides in the tumor mitochondria. In a model system where liver mitochondria were depleted of their adenine nucleotides, a lower ATPase activity was indeed obtained. However, direct determination of the concentations of adenine nucleotides in dinitrophenol- and valinomycin-treated tumor mitochondria revealed only slight differences.

Introduction

The uncoupler-stimulated ATPase of tumor mitochondria has been the subject of many studies [1–9]. In most cases, respiratory control could be demonstrated in mitochondria from tumors of various types [2,5,9,10]. The behavior of uncoupler-stimulated ATPase in the same tumor mitochondria, on the other hand, was at variance with that of some normal mitochondria. Briefly, tumor mitochondrial ATPase activity elicited by uncouplers such as dinitrophenol or CCCP was low and sometimes absent when compared with liver mitochondria [1–4]. Later studies have demonstrated that this anomalous behavior was due, in part, to leakage of Mg^{2+} from tumor mitochondria [6,8,9]. In addition, higher ATPase activity could be obtained if ATP was added before dinitrophenol or CCCP during the ATPase assays [5,7,9].

We have previously reported [8] that mitochondria from three transplantable human tumors, an astrocytoma, an oat cell carcinoma and a melanoma, were capable of oxidative phosphorylation, although respiratory control was impaired. Each mitochondrial preparation appeared to have large amounts of endogenous uncouplers and one

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; 1799, 2,6-dihydroxy-1,1,1,7,7,7-hexafluoro-2,6-bistrifluoromethylheptan-4-one; S-13, 2,5-dichloro-3-(*tert*-butyl)-4-nitrosalicylanilide.

of them readily lost Mg^{2+}. When these unfavorable conditions were prevented by inclusion of defatted albumin and Mg^{2+} in the assay media, significant stimulation of the tumor mitochondrial ATPase by dinitrophenol and CCCP could be observed. In this paper, we report that the uncoupler-stimulated ATPase of some of the human tumor mitochondria showed a much different behavior toward valinomycin ($+K^+$) than toward dinitrophenol or CCCP. Possible causes for this discrepancy are considered.

Materials and Methods

Human tumor materials. The four tumors used in this study were an astrocytoma (T24), an oat cell carcinoma (T293), a melanoma (T355) and a hepatoma (Li-7). The first two tumors were used in our previous study [8]. The astrocytoma were taken from the 43rd–63rd passage. Oat cell carcinoma were taken from the 17th–24th passage. Melanoma (T355) were taken from the 8th–13th passage. Hepatoma (Li-7) were taken from the 52nd–59th passage.

Isolation of mitochondria. The isolation buffer consisted of 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, pH 7.4, 0.1 mM EDTA, and defatted bovine serum albumin (1 mg/ml). Tumor mitochondria were isolated from homogenates as described previously [8]. The yield of tumor mitochondria (1–2 mg/g tissue) was very much less than that obtained with normal tissue (5–20 mg/g tissue). Mitochondria from liver, heart and kidney were isolated from 10% homogenates according to the method of Schnaitman and Greenawalt [11]. Mitochondria from brain were isolated as follows. The grey matter of the mouse brain was homogenized in 7 vol. of isolation buffer. The homogenate was centrifuged at $3000 \times g$ for 30 s. The supernatant was then centrifuged at $12000 \times g$ for 5 min. The pellet was washed twice with the isolation buffer and care was taken to decant off as much as the white fluffy layer as possible.

ATPase assays. ATPase of freshly prepared mitochondria was determined as previously described [8]. Due to slight contamination of non-mitochondrial ATPase in the tumor mitochondria fraction, ATPase assays were also carried out with oligomycin (2 μg). The oligomycin-insensitive ATPase activity was then used for correction. All data shown in this paper represent oligomycin-sensitive mitochondrial ATPase activity.

Depletion of mitochondrial adenine nucleotides. The content of adenine nucleotides of mouse liver mitochondria was decreased by incubating the mitochondria with pyrophosphate as described by Asimakis and Aprille [12].

Determination of adenine nucleotide concentration. To 1–1.5 ml of mitochondria suspension (approx. 10 mg/ml) was added 0.15 vol. of ice-cold 40% $HClO_4$. The denatured protein was removed by centrifugation and the supernatant was neutralized by addition of a solution of 1.65 M K_2CO_3 in 0.43 M triethanolamine as described by Asimakis and Aprille [12]. The neutralized extract was assayed for concentration of ADP and AMP by the method of Adam [13]. ATP was determined by bioluminescence in the firefly luciferase-luciferin system as described by Seliger and McElroy [14].

Materials. ATP was purchased from Boehringer-Mannheim. All other chemicals and enzymes were obtained from Sigma Chemical Co. S-13 and 1799 were generous gifts from Dr. E. Racker of Cornell University.

Results

Differential effects of valinomycin ($+K^+$) and dinitrophenol on the uncoupler-stimulated ATPase of human tumor mitochondria

When valinomcyin was used (in the presence of K^+) as an uncoupler for stimulation of mitochondrial ATPase, the resultant ATPase activity was found to be much higher than that stimulated by dinitrophenol. This effect is illustrated in Fig. 1 for mitochondria isolated from oat cell carcinoma. In this experiment, mitochondria were preincubated with the uncouplers for 6 min at room temperature before reaction was initiated by addition of ATP. Since Mg^{2+} leakage did occur in some tumor mitochondria, all assays were conducted with and without 5 mM $MgCl_2$. It can be seen from Fig. 1 that ATPase activity stimulated by valinomycin ($+K^+$) was about 3-fold greater than that observed with dinitrophenol in the absence of Mg^{2+}. Even with the addition of Mg^{2+}, ATPase activity stimulated by valinomycin ($+K^+$)

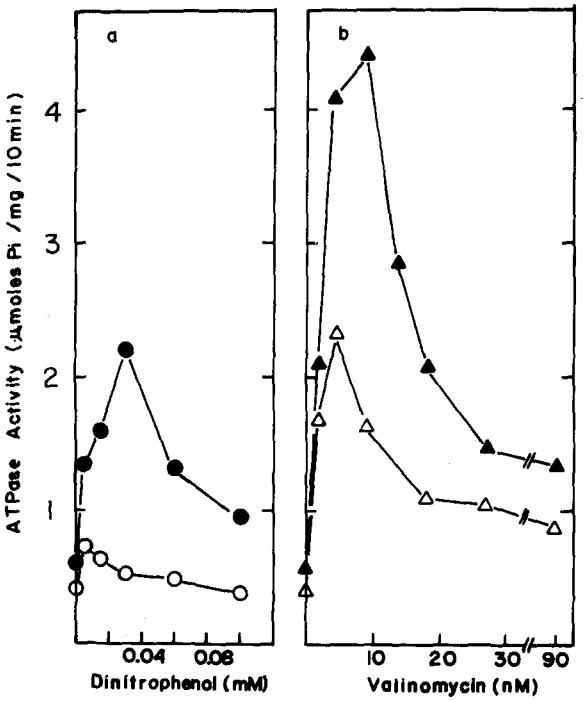

Fig. 1. Stimulation of mitochondrial ATPase from human oat cell carcinoma by dinitrophenol and valinomycin ($+K^+$). Mitochondria (490 μg) from oat cell carcinoma were incubated in a reaction mixture containing 50 mM Tris-HCl, pH 7.4, 50 mM sucrose, 75 mM KCl, 1 mM EDTA and defatted albumin (1 mg/ml) in the absence (○,△) and presence (●,▲) of 5 mM $MgCl_2$ with various concentrations of dinitrophenol (a) and valinomycin (b). After 6 min at room temperature, reaction was initiated by the addition of 5 μmol ATP and the reaction was carried out for 10 min at 37°C.

was still 2-fold higher than the dinitrophenol-stimulated activity. The differential effects of valinomycin ($+K^+$) and dinitrophenol on uncoupler-stimulated ATPase were not observed with mitochondria from liver or heart (data not shown).

The discrepancy between the dinitrophenol-stimulated and valinomcyin ($+K^+$)-stimulated ATPase was most pronounced in the mitochondria of oat cell carcinoma under the specified assay condition. Similar discrepancies were observed with mitochondria from hepatoma (Li-7), melanoma (T355) and astrocytoma (T24). These results are summarized in Table I. The differential effects observed with mitochondria from the astrocytoma and melanoma were less noticeable when

TABLE I

DIFFERENTIAL EFFECTS OF VALINOMYCIN ($+K^+$) AND DINITROPHENOL IN THE STIMULATION OF TUMOR MITOCHONDRIAL ATPase IN THE PRESENCE AND ABSENCE OF Mg^{2+}

ATPase assays with mitochondria from individual tumors were carried out as in Fig. 1. For each condition (dinitrophenol, valinomycin, Mg^{2+}), a titration curve with at least five different concentrations of the uncouplers was obtained. The maximal activity (after correcting for the oligomycin-insensitive portion) is presented in this table. Values are expressed as μmol P_i/mg per 10 min.

Mitochondria from	Dinitrophenol-stimulated ATPase		Valinomycin ($+K^+$)-stimulated ATPase	
	$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
Oat cell carcinoma (T293)	0.98	1.74	1.85	3.71
Hepatoma (Li-7)	0.64	1.48	1.30	2.38
Melanoma (T355)	1.24	2.02	2.39	3.06
Astrocytoma (T24)	1.02	1.35	1.79	2.06

ATPase activity was measured in the presence of Mg^{2+}. Mitochondria from these two tumors lost less Mg^{2+} than mitochondria from hepatoma and oat cell carcinoma.

Effect of K^+ on the uncoupler-stimulated ATPase

The uncoupling effect of valinomycin was absolutely dependent on the presence of K^+ [15]. It has also been reported that K^+ was required for dinitrophenol-stimulated ATPase activity, although the effect of K^+ was apparent only if the mitochondria were depleted of K^+ [16]. In order to determine whether the lower ATPase activity stimulated by dinitrophenol was due to a deficiency in intramitochondrial K^+, ATPase activity was determined at a constant uncoupler concentration and varying K^+ concentrations, the results being shown in Fig. 2. It can be seen that the valinomycin-stimulated ATPase activity increased with increasing concentration of K^+, reaching maximal activity at 75 mM whereas the dinitrophenol-stimulated ATPase activity was insensitive to K^+. We conclude that the lower ATPase activity stimulated by dinitrophenol was

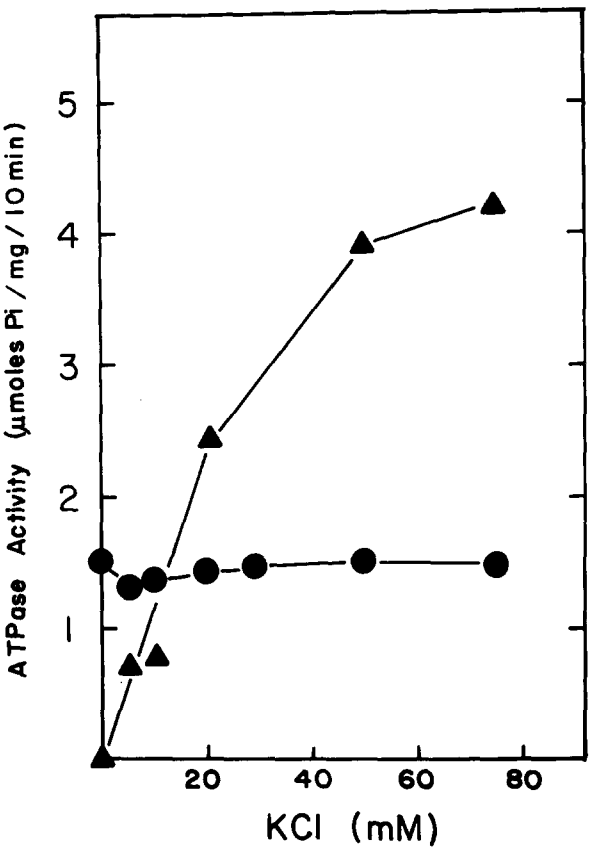

Fig. 2. Effect of KCl concentration on the uncoupler-stimulated mitochondrial ATPase from oat cell carcinoma. In this experiment, the reaction mixture contained 50 mM Tris-HCl, pH 7.4, 50 mM sucrose, 1 mM EDTA, defatted albumin (1 mg/ml) and various concentrations of KCl. Mitochondria (410 μg) from oat cell carcinoma were incubated in the reaction mixture with either 30 μM dinitrophenol (●) or 9 nM valinomycin (▲) for 6 min at room temperature before starting the reaction by the addition of ATP.

not due to a deficiency in K^+ in the dinitrophenol-treated mitochondria.

Effect of other uncouplers on the tumor mitochondrial ATPase

CCCP, 1799, S-13 and gramicidin were tested for stimulation of ATPase activity. The results obtained with mitochondria from oat cell carcinoma are summarized in Table II. CCCP, 1799 and S-13, which are H^+ ionophores, brought about lower activity than valinomycin ($+K^+$). Gramicidin, an ionophore with little selectivity toward monovalent cations [17], had the same effect as the H^+ ionophores.

TABLE II

EFFECT OF VARIOUS UNCOUPLERS ON THE UNCOUPLER-STIMULATED MITOCHONDRIAL ATPase OF OAT CELL CARCINOMA

Assay conditions were similar to those described in the legend of Fig. 1. $MgCl_2$ (5 mM) was present in the reaction mixture. For each uncoupler, at least five different concentrations were used. Maximal ATPase activity elicited by a particular uncoupler concentration is presented in the table.

Expt. No.	Uncoupler	Uncoupler concentration (μM)	Uncoupler-stimulated ATPase (μmol P_i/mg per 10 min)
1	Dinitrophenol	30	1.63
	CCCP	2	1.12
	Valinomycin	$9 \cdot 10^{-3}$	3.86
2	Dinitrophenol	30	1.12
	S-13	$6 \cdot 10^{-2}$	0.62
	1799	20	1.25
	Valinomycin	$9 \cdot 10^{-3}$	3.00
3	Dinitrophenol	15	1.98
	Gramicidin	20 ng	1.72
	Valinomycin	$9 \cdot 10^{-3}$	5.47

Effect of the order of addition of uncouplers and ATP on the uncoupler-stimulated ATPase of tumor mitochondria

Several investigators have observed that prolonged incubation of tumor mitochondria [7,18,19] and mouse brain and fetal liver mitochondria [19] with dinitrophenol resulted in much lower ATPase activity than when ATP addition preceded dinitrophenol. We have carried out similar experiments with dinitrophenol and valinomycin, the results obtained with mitochondria from oat cell carcinoma being illustrated in Fig. 3. Preincubation of mitochondria with dinitrophenol for 6 min at room temperature resulted in very low ATPase activity, especially in the absence of Mg^{2+} (Fig. 3a). When mitochondria were introduced into a mixture containing ATP and dinitrophenol, the resultant ATPase activity was much higher (Fig. 3a and b). With valinomycin ($+K^+$), the ATPase activity from mitochondria with or without 6 min preincubation with the uncoupler was similar (data not shown). It can be seen that if mitochondria

TABLE III

EFFECT OF PREINCUBATION OF MITOCHONDRIA WITH DINITROPHENOL AND VALINOMYCIN ON THE UNCOUPLER-STIMULATED ATPase

Experimental conditions were the same as those described in the legend to Table II. When mitochondria were not preincubated with uncouplers, the reaction was initiated by the addition of mitochondria to reaction mixture containing uncouplers and ATP. Values are expressed as μmol P_i/mg per 10 min.

Mitochondria from	6 min preincubation with uncouplers	Dinitrophenol-stimulated ATPase		Valinomycin ($+K^+$)-stimulated ATPase	
		$-Mg^{2+}$	$+Mg^{2+}$	$-Mg^{2+}$	$+Mg^{2+}$
Oat cell carcinoma (T293)	+	0.54	2.10	1.49	3.55
	−	1.57	2.78	1.82	3.30
Hepatoma (Li-7)	+	0.56	1.57	1.47	2.56
	−	1.73	2.88	1.87	3.00
Melanoma (T355)	+	1.24	2.02	2.39	3.06
	−	2.55	2.74	2.44	2.63
Astrocytoma (T24)	+	0.50	0.97	1.28	1.59
	−	0.86	1.16	1.24	1.39
Mouse brain	+	1.60	1.72	2.43	2.88
	−	5.23	3.12	3.89	2.73
Mouse fetal liver	+	0.70		0.90	
	−	3.00		1.90	
Mouse kidney	+	2.74	2.86	5.29	6.28
	−	5.85	5.32	5.80	5.59
Mouse liver	+	5.96		2.58	
	−	6.25		2.83	
Mouse heart	+	12.23		7.23	
	−	12.39		5.84	

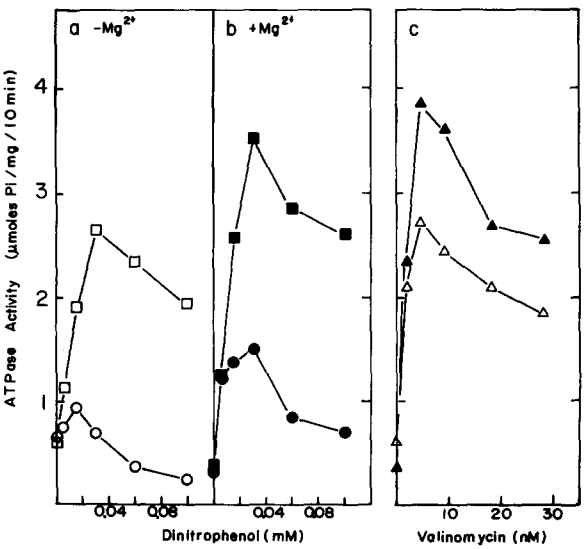

Fig. 3. Effect of preincubation with dinitrophenol on the uncoupler-stimulated mitochondrial ATPase of oat cell carcinoma. Mitochondrial ATPase was assayed in the absence (□,○,△) and presence (■,●,▲) of 5 mM $MgCl_2$. In one set of assays, mitochondria were preincubated with dinitrophenol for 6 min at room temperature before the addition of ATP (○,●). In a second set of assays, mitochondria were added to reaction mixtures containing dinitrophenol and ATP (□,■). In the experiment with valinomycin (c), mitochondria were added to reaction mixture containing valinomycin and ATP.

were not preincubated with dinitrophenol, the maximal dinitrophenol-stimulated ATPase activity approached that elicited by valinomycin ($+K^+$) (Fig. 3c).

Table III summarizes the results obtained with mitochondria from the other three transplantable human tumors and mitochondria from mouse liver, heart, kidney, brain and fetal liver. Except for liver and heart, mitochondria from all tissues suffered a loss of ATPase activity after preincubation with dinitrophenol, but not with valinomycin ($+K^+$).

Reduction of ATPase activity was dependent on time of preincubation with dinitrophenol and was prevented by ATP and ADP

Fig. 4 shows that the deccrease in ATPase activity depended on the length of the preincubation time, with or without Mg^{2+}. Fig. 4a shows the results obtained with mitochondria from hepatoma (Li-7) where deterioration of activity was considerable regardless of the presence of Mg^{2+}. Mitochondria from oat cell carcinoma showed similar behavior. Fig. 4b shows the results obtained with mitochondria from melanoma (T355) in which deterioration of ATPase activity was much less in the presence of Mg^{2+}. Mitochondria from astrocytoma behaved in a similar way.

The presence of ATP in the preincubation mixture prevented the decrease in uncoupler-stimulated ATPase activity. This is shown in Fig. 5. In these experiments, mitochondria were added to reaction mixture containing a constant amount of dinitrophenol and various concentrations of either

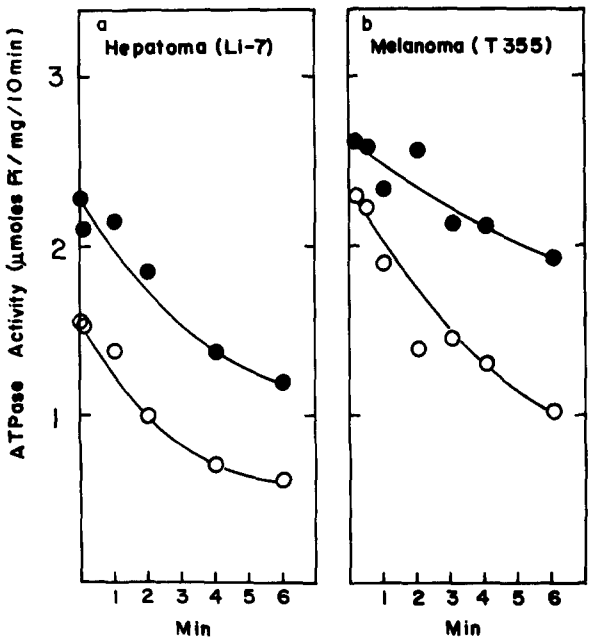

Fig. 4. Effect of the length of the preincubation period with dinitrophenol on the subsequent uncoupler-stimulated ATPase. (a) Mitochondria from hepatoma (Li-7) (530 μg) were incubated with 60 μM dinitrophenol with (●) and without (○) 5 mM $MgCl_2$ for various lengths of time at room temperature before ATPase reaction was initiated by the addition of ATP. (b) Mitochondria from melanoma (T355) (500 μg) were used.

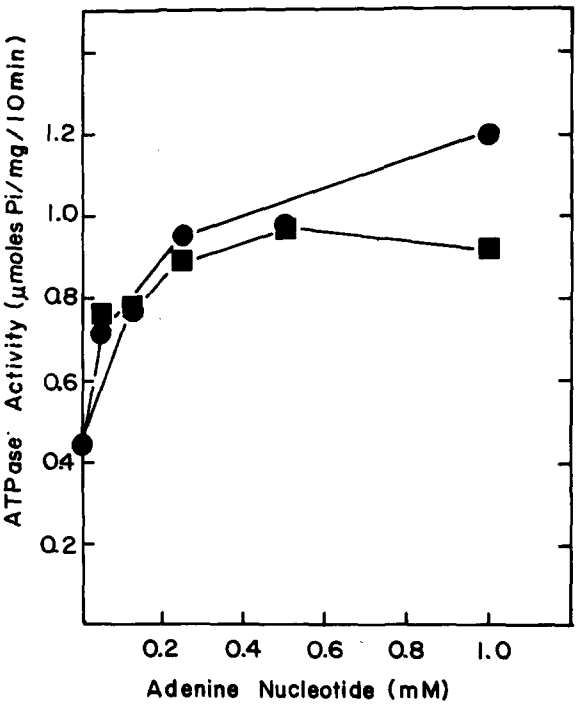

Fig. 5. Protective effect of ATP and ADP against deterioration of tumor mitochondria preincubated with dinitrophenol. Mitochondria from oat cell carcinoma (480 μg) were incubated with 30 μM dinitrophenol and various amounts of ATP (●) and ADP (■) in 1 ml reaction mixture without Mg^{2+}. Reaction was initiated by the addition of 5 μmol ATP and was carried out at 37°C.

ATP or ADP and incubated at room temperature for 6 min. Reaction was then initiated by the addition of 5 μmol ATP. Data presented in Fig. 5 were corrected for a small amount of ATP hydrolysis which occurred during the preincubation period. It can be seen that when ATP was present in the preincubation mixture, subsequent ATPase activity was considerably enhanced. ADP had a similar protective effert, especially in the absence of Mg^{2+}, but GTP and CTP were without effect (data not shown).

Effect of depletion of adenine nucleotides on the uncoupler-stimulated ATPase of liver mitochondria

The protective effect of ATP and ADP in preventing the tumor mitochondrial ATPase activity from deteriorating during incubation with dinitrophenol could depend on maintenance of intramitochondrial adenine nucleotide pool size. If dinitrophenol (but not valinomcyin ($+K^+$))

TABLE IV

ADENINE NUCLEOTIDE CONCENTRATIONS OF CONTROL MITOCHONDRIA AND MITOCHONDRIA PREINCUBATED WITH DINITROPHENOL OR VALINOMYCIN ($+K^+$)

Mitochondria (10–20 mg) were added to solutions containing 50 mM Tris-HCl, pH 7.4, 50 mM sucrose, 75 mM KCl, 1 mM EDTA and defatted bovine serum albumin (1 mg/ml) with or without the indicated concentrations of uncouplers so that the final protein concentration was similar to that used in the ATPase assays (400–600 μg protein/ml). The incubation with the uncouplers were carried out for 6 min at room temperature and then the mitochondrial suspensions were centrifuged for 10 min at 8000$\times g$ at 0°C. The mitochondrial pellets were gently rinsed with 2 ml of isolation buffer and then resuspended in 1–2 ml of isolation buffer. Adenine nucleotides were extracted from these mitochondria by perchloric acid and their concentrations determined as described in Materials and Methods.

Mitochondria from	Uncoupler	Uncoupler concentrations (μM)	Adenine nucleotides (nmol/mg protein)			
			ATP	ADP	AMP	Total
Liver	None	0	3.30	9.33	4.76	17.39
	Dinitrophenol	300	0.71	4.68	8.02	13.41
	Valinomycin	$5.4 \cdot 10^{-3}$	1.10	4.66	3.71	9.47
Oat cell carcinoma (T293)	None	0	2.55	3.47	1.11	7.13
	Dinitrophenol	30	0.84	1.55	1.67	4.06
	Valinomycin	$0.9 \cdot 10^{-3}$	1.2	3.76	1.98	6.94
Astrocytoma (T24)	None	0	8.65	3.78	2.39	14.82
	Dinitrophenol	30	1.62	3.4	0.96	5.98
	Valinomycin	$9 \cdot 10^{-3}$	2.13	3.47	1.35	6.95
Melanoma (T355)	None	0	0.74	1.96	0.86	3.56
	Dinitrophenol	100	0.32	1.21	0.75	2.28
	Valinomycin	$9 \cdot 10^{-3}$	0.58	1.45	0.69	2.72
Hepatoma (Li-7)	None	0	3.18	2.87	0.49	6.54
	Dinitrophenol	15	1.32	1.6	0.55	3.47
	Valinomycin	$1.8 \cdot 10^{-3}$	1.19	1.78	0.37	3.34

induced an efflux of adenine nucleotides, the concentration of adenine nucleotides inside the mitochondria could decrease. Furthermore, if adenine nucleotide transport was strictly an exchange process, ATP transport would be less in the dinitrophenol-treated mitochondria, resulting in lower ATPase activity. Such a phenomenon could be demonstrated in a model system, i.e., liver mitochondria whose intramitochondrial adenine nucleotide concentrations were reduced by incubating the mitochondria with pyrophosphate. By using the conditions employed by Asikamis and Aprille [12], adenine nucleotide concentrations in liver mitochondria could be reduced more than 70%. Fig. 6 shows that the uncoupler-stimulated ATPase was substantially diminished in the pyrophosphate-treated liver mitochondria, simulating the behavior of tumor mitochondria pretreated with dinitrophenol.

Lack of difference in adenine nucleotide concentrations in tumor mitochondria treated with dinitrophenol and valinomycin ($+K^+$)

The concentrations of adenine nucleotides in control tumor mitochondria and tumor mitochondria treated with dinitrophenol and valinomycin ($+K^+$) were determined. One concentration of uncoupler was used, that being the concentration which elicited maximal activity, since large amounts of mitochondria were required. From the data presented in Table IV, several conclusions can be drawn. (1) Preincubation of mitochondria with uncouplers resulted in a decrease in intramitochondrial ATP. (2) In general, there was a reduction of total adenine nucleotides after incubation of mitochondria with either dinitrophenol or valinomycin. (3) With the exception of mitochondria obtained from oat cell carcinoma, there was little difference between the adenine

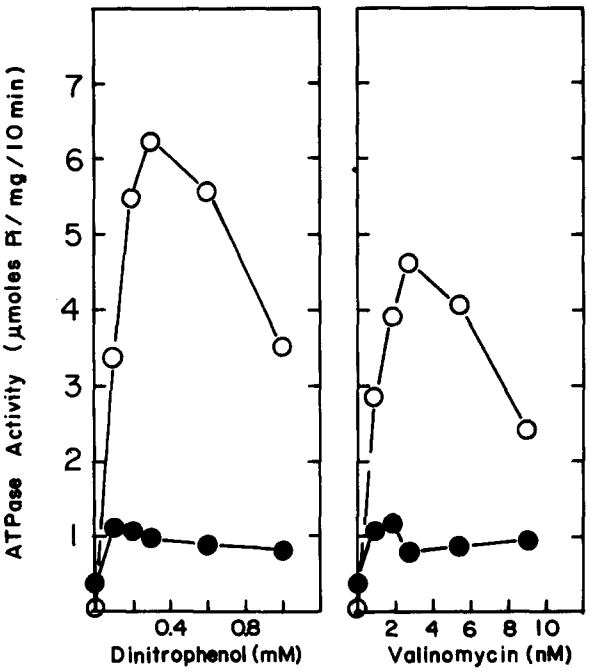

Fig. 6. Effect of depletion of adenine nucleotides from liver mitochondria on uncoupler-stimulated ATPase. To deplete adenine nucleotides from mitochondria, the following incubation was carried out: To 30-ml solutions containing 0.225 M sucrose, 75 mM KCl and 5 mM Tris-HCl, pH 7.4, with (●) and without (○) 5 mM sodium pyrophosphate was added 1 ml suspension of mouse liver mitochondria (30 mg protein). After 5 min incubation at 30°C, the mitochondria suspension was centrifugated at 9000×g for 10 min at 0°C. The pellet was resuspended in the isolation buffer. A portion of it was used for determining uncoupler-stimulated ATPase. A second portion was used for determination of adenine nucleotide concentrations. The concentrations of the individual adenine nucleotides were as follows: In the control mitochondria there were 3.66 nmol ATP, 8.21 nmol ADP and 4.48 nmol AMP per mg protein. In the pyrophosphate-treated mitochondria there were 0.55 nmol ATP, 1.69 nmol ADP and 1.51 nmol AMP per mg protein.

nucleotide concentrations of the dinitrophenol- or valinomycin ($+K^+$)-treated mitochondria. Preincubation of mitochondria from oat cell carcinoma with dinitrophenol consistently released a greater quantity of adenine nucleotides than did valinomycin ($+K^+$).

Discussion

A unique characteristic of many tumor mitochondria is the response of ATPase toward dinitrophenol. Under the assay conditions employed by several groups [1–4], the tumor mitochondria showed a weak response to dinitrophenol in contrast to mitochondria from control tissues. It was found later that ATPase activity approaching that of the control tissue mitochondria could be elicited in tumor mitochondria if ATP was added to the tumor mitochondria prior to the addition of dinitrophenol [5,7,18,19]. From the results of their recent investigation, Hayashi et al. [9] concluded that one of the reasons for the low ATPase activity of tumor mitochondria was leakage of endogenous Mg^{2+} upon incubation of the tumor mitochondria with uncoupler, i.e., dinitrophenol.

In this report, we have shown that mitochondria from several human tumors also exhibited this behavior toward dinitrophenol, i.e., the uncoupler-stimulated activity was greatly reduced if the tumor mitochondria were preincubated with dinitrophenol for a short period of time before the addition of ATP. Furthermore, we showed that this was a unique response of tumor mitochondria toward all the H^+-conducting uncouplers. When mitochondria were preincubated with valinomycin ($+K^+$), similar decay in ATPase activity was not observed. We also observed that the valinomycin ($+K^+$)-stimulated activity in freshly prepared tumor mitochondria approached that of the freeze-thawed mitochondria when the ATPase activity was assayed with an ATP-regenerating system (data not shown). These results would imply that with valinomycin ($+K^+$), the tumor mitochondrial ATPase was operating at the same capacity as in freeze-thawed mitochondria where all transport barriers were destroyed. Therefore, instead of asking why tumor mitochondrial ATPase (and also mitochondrial ATPase of mouse brain, fetal liver and kidney) exhibited a characteristic behavior toward H^+-conducting uncouplers, the question now concerns the difference in the intramitochondrial environment generated by preincubation with dinitrophenol (or other H^+ ionophores) and valinomycin ($+K^+$) so that the ATPase is operating at a diminished rate in the former situation.

It is known that aside from its uncoupling action, dinitrophenol has other effects on mitochondria. (1) Addition of dinitrophenol to mitochondria caused K^+ efflux if the ex-

tramitochondria K^+ concentration was low [20]. (2) It has been reported that dinitrophenol induced Mg^{2+} leakage from tumor mitochondria [9]. (3) When mitochondria were incubated with dinitrophenol in hypotonic solution, phospholipase activity was activated [21]. It is easy to see that the consequence of activated phospholipase on the mitochondrial membranes can be far-reaching, thus affecting various functions of the mitochondria. In our experiments, we have ruled out the above three occurrences as the bases for the differential effects of dinitrophenol and valinomycin ($+K^+$) on tumor mitochondria. Since the ATPase assay was carried out in media containing 75 mM KCl, it was unlikely that there could be a K^+ deficiency in the dinitrophenol-treated mitochondria. Furthermore, the dinitrophenol-stimulated ATPase activity was independent of K^+ concentration in the reaction mixture (Fig. 2), which would imply that even when K^+ was absent from the reaction mixture, there was sufficient K^+ inside the tumor mitochondria to support the ATPase reaction. We have also shown that although the differential effects of the two uncouplers were less marked in the presence of exogenous Mg^{2+}, the effects were not abolished (Figs. 1, 3 and 4, and Tables I and III), therefore a factor aside from Mg^{2+} leakage must be responsible for the differences. Finally, we have observed that dibucaine (a phospholipase inhibitor) did not prevent the decrease in ATPase activity in dinitrophenol-treated mitochondria (data not shown).

Of various other possible causes, we had thought the most likely explanation for the differential effects of dinitrophenol valinomycin ($+K^+$) was a reduced pool of exchangeable adenine nucleotides in the dinitrophenol-treated tumor mitochondria. This is based on the following three lines of reasoning: (1) The effect of dinitrophenol could be partially prevented by ATP and ADP (Fig. 5). (2) Liver mitochondria in which adenine nucleotides had been reduced to a low level (25% of that of the control) showed little uncoupler-stimulated ATPase (Fig. 6). (3) There was a close relationship between the adenine nucleotide pool and uncoupler-stimulated ATPase activity in two other systems: (a) higher uncoupler-stimulated ATPase activity of liver mitochondria from glucagon-treated rats was correlated to a larger pool of exchangeable adenine nucleotides [22], and (b) increase in intramitochondrial adenine nucleotides in neonatal liver mitochondria [23] accompanied the transition of the ATPase from its more tumor-like behavior toward dinitrophenol to a normal behavior seen with adult liver mitochondria [19]. However, when we determined the concentrations of adenine nucleotides of dinitrophenol- and valinomycin ($+K^+$)-treated mitochondria, only small differences were observed except in the mitochondria of oat cell carcinoma where we consistently observed a lower adenine nucleotide concentration in dinitrophenol-treated mitochondria.

We believe there should be a common cause underlying the characteristic behavior of the ATPase of tumor mitochondria and mitochondria from some normal tissues which we described in this report. It is obvious that other aspects of the tumor mitochondria need to be examined in detail with regard to their relationship to the uncoupler-stimulated ATPase. Such studies would also result in a better understanding of the mechanism of uncouplers in general.

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