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CHARACTERISTICS OF ADENINE NUCLEOTIDE FLUXES AND TRANSPORT IN HUMAN TUMOR MITOCHONDRIA *

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The efflux of adenine nucleotides from three human tumor mitochondria has been investigated with mitochondria prelabeled with radioactive ATP. Uncouplers induce a large efflux of adenine nucleotides from mitochondria from human hepatoma and oat cell carcinoma while efflux from astrocytoma mitochondria is less. This efflux does not require exchangeable anions, i.e., adenine nucleotides or pyrophosphate, in the extramitochondrial medium, and is not sensitive to atractyloside. The efflux is more extensive with dinitrophenol and CCCP than with valinomycin-K^+, and may account for the differential effects of the two types of uncouplers on uncoupler-stimulated ATPase of tumor mitochondria previously reported by us. Dinitrophenol and CCCP do not elicit any efflux of adenine nucleotides from normal liver mitochondria. Efflux of orthophosphate from tumor mitochondria is also greater with dinitrophenol and CCCP; however, the more interesting finding is that the concentration of orthophosphate in these mitochondria is unusually high, i.e., 10–40-times greater than the intramitochondrial phosphate concentration of liver mitochondria. Atractyloside sensitive transport of ATP and ADP in human tumor mitochondria has also been determined. V_{max} values for both ADP and ATP transport are lower than those obtained with liver mitochondria, especially with ADP transport. ATP transport in tumor mitochondria is not affected by CCCP in contrast to the 4–5-fold stimulation observed in liver mitochondria.

Introduction

The most consistently observed abnormality of tumor mitochondria is a lack of stimulation of the mitochondrial ATPase by uncouplers [1–7]. Among these reports, the study conducted by Pedersen and Morris [6] employed the largest number of tumors, including six Morris hepatomas. However, their results were later contradicted by those obtained by Kaschnitz et al. [8] who showed that ATPase in the mitochondria from the same Morris hepatomas could be stimulated by uncouplers. The discrepancy between the results of the two studies was eventually determined to be caused by two important differences in experimental conditions employed by these different investigators. The first difference was in the preparation of mitochondria, where Kaschnitz et al. [8] used much larger amounts of defatted bovine serum albumin in the isolation buffer. The second difference was in the ATPase assay with respect to the order of addition of ATP and uncoupler to the tumor mitochondria.

Barbour and Chan [9] showed that the effect of albumin was two fold. It removed fatty acids and

* Dedicated to Professor Efraim Racker on the occasion of his 70th birthday.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazine; PEI-cellulose, polyethyleneimine cellulose.

it prevented loss of Mg^{2+} from the tumor mitochondria. Hayashi et al. [10] reported that Mg^{2+} loss from tumor mitochondria was further aggravated when these mitochondria were incubated with uncouplers alone. Mg^{2+} loss did not occur if ATP was added to the mitochondria prior to uncouplers, explaining the profound effect of the order of addition of uncoupler and ATP on the uncoupler-stimulated ATPase.

We previously reported that the uncoupler-stimulated ATPase of isolated human tumor mitochondria showed differential responses to protonphoric uncouplers and valinomycin-K^+ [11]. Valinomycin-K^+ always gave rise to a greater stimulation of ATPase activity than dinitrophenol or CCCP on incubation with tumor, fetal liver, and brain mitochondria, but not with adult liver or heart mitochondria. The differential effects of dinitrophenol and valinomycin-K^+ were observed whether Mg^{2+} was present or absent in the reaction mixture, thus this phenomenon could not be attributed entirely to the problem of Mg^{2+} loss which we also observed in the human tumor mitochondria [12]. Preliminary results indicated that there might be significantly greater reduction of the pool size of the adenine nucleotides after tumor mitochondria were incubated with the protonphoric uncouplers than with valinomycin-K^+. As a consequence, subsequent ATP transport rate would be decreased during the ATPase reaction, resulting in a lower ATPase activity.

In this report, we describe experiments carried out with mitochondria preequilibrated with radioactive ATP to address the question of adenine nucleotide fluxes of tumor mitochondria in relation to the uncoupler-stimulated ATPase and tumor cell metabolism.

Materials and Methods

Human tumor materials. Three human tumors were used in this study: an astrocytoma of the brain (T24), an oat cell carcinoma of the lung (T293), and a hepatoma (Li-7). The growth characteristics of these tumors have been described [11–13]. Li-7 was classified as a moderately differentiated hepatoma by Hirohashi et al. [13] based on ultrastructure and the amount of cytoplasm. To obtain 3–6 g of tumor tissues from each mouse, the growth period required was 3–4 weeks for astrocytoma and hepatoma, and 3–4 months for oat cell carcinoma. For this study the astrocytoma were taken from the 64–96th passage. Oat cell carcinoma were from the 25th–33rd passage. Hepatoma (Li-7) were from the 60th–77th passage.

Isolation of mitochondria. Mitochondria from human tumor tissues and from mouse and rat liver were isolated as described previously [11]. Mitochondria from mouse heart were isolated according to Tyler and Gonze [14]. The isolation buffer contained 0.225 M mannitol, 0.075 M sucrose, 2 mM Hepes (K^+), pH 7.4, 0.1 mM EDTA, 1 mg/ml defatted bovine serum albumin and 0.1 mM phenylmethylsulfonyl fluoride.

ATPase assays. ATPase of freshly prepared mitochondria was determined as previously described [12].

Efflux of adenine nucleotides from mitochondria. Efflux of adenine nucleotides was monitored by the appearance of radioactivity from mitochondria previously loaded with radioactive ATP. For loading mitochondria with radioactive ATP, [2,8-^{3}H]ATP (1 mCi/ml, 26–28 Ci/mmol) was added to 20–40 mg mitochondria protein (in 2–3 ml of isolation buffer) so that 1 ml incubation mixture contained 10 μCi radioactivity and 0.3–0.4 nmol ATP. After 20 min at 0°C, 5 volumes of isolation buffer were added and the mitochondrial suspension was centrifuged at 12000 × g for 10 min. The mitochondrial pellet was washed once with the same volume of buffer and the final mitochondrial pellet was then resuspended at 10–20 mg/ml.

Separation of adenine nucleotides on PEI-cellulose columns. Acid soluble nucleotides were extracted from mitochondria or the supernatant solution in the efflux experiments by perchloric acid. Extraction and neutralization were carried out as described by Asimakis and Aprille [15]. AMP, ADP and ATP in the neutralized extract were separated and eluted from PEI-cellulose columns by LiCl solutions as described by Magnusson et al. [16].

Determination of inorganic phosphate. The amount of inorganic phosphate in mitochondria and in the supernatant solution in the efflux experiments was determined by the method of Lohmann and Jendrassik [17] scaled down to 1.25 ml.

Adenine nucleotide translocation. Adenine nucleotide transport (ADP or ATP) was carried out similar to that described by Barbour and Chan [18]. [2-^{3}H]ADP or [2,8-^{3}H]ATP (in 50 μl volume with specific radioactivity of 3000 cpm/nmol) was added to 0.95 ml reaction mixture so that the final composition of the solution was 50 mM Tris-HCl, pH 7.4, 50 mM sucrose, 75 mM KCl, 1 mM EDTA, 1 mg/ml defatted bovine serum albumin and 0.5–1 mg mitochondrial protein. The reaction was carried out for 5–10 s at 0–4°C and was stopped by the addition of 50 nmol atractyloside (in 20 μl volume). The mitochondrial suspension was centrifuged for 5 min at 8000 × *g* in a Beckman 12 microfuge. The pellet was resuspended in 1 ml of reaction mixture containing 50 μM atractyloside and then centrifuged again. The pellet was dissolved in 0.4 ml 2% SDS overnight and then counted in 10 ml ACS counting solution.

Materials. [2,8-^{3}H]ATP (28 Ci/mmol) was purchased from ICN. [2-^{3}H]ADP (16 Ci/mmol) and ACS counting solution were purchased from Amersham International. Atractyloside and PEI-cellulose were from Sigma Co.

Results

Differential effects of 2,4-dinitrophenol and valinomycin-K$^+$ on uncoupler-stimulated ATPase of tumor mitochondria was observed when preincubation was at 22°C but not at 0°C

Fig. 1 shows that when human hepatoma mitochondria were incubated with dinitrophenol at 22°C before the addition of ATP, the ATPase activity decreased with the length of incubation time (lower curves in Fig. 1a and 1b). This occurred regardless of the absence (lower curve in Fig. 1a) or the presence of Mg^{2+} (lower curve in Fig. 1b). When incubation was carried out at 0°C, there was only a slight decrease of activity (upper curves of Fig. 1a and 1b).

Table I shows that when mitochondria from oat cell carcinoma and hepatoma were incubated with uncouplers at 22°C, the dinitrophenol-stimulated ATPase was only a fraction of the (valinomycin-K$^+$)-stimulated activity. If the incubation with uncouplers was carried out at 0°C, then the dinitrophenol-stimulated activity approached the (valinomycin-K$^+$)-stimulated activity.

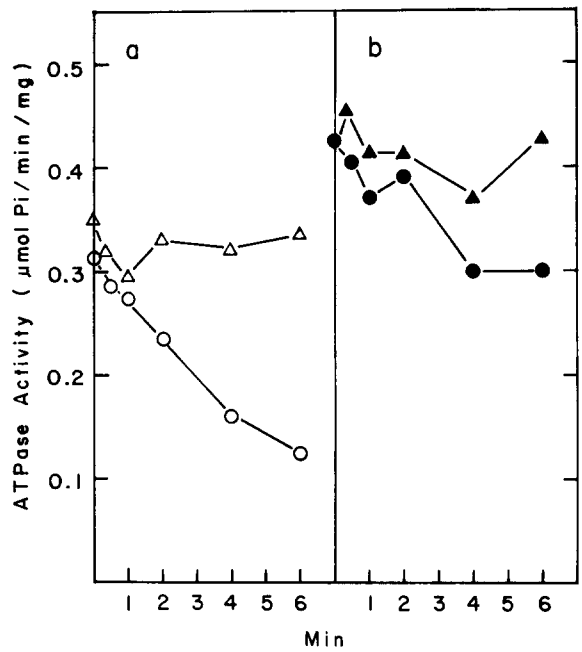

Fig. 1. Effect of incubation of hepatoma mitochondria with dinitrophenol at different temperatures on the uncoupler-stimulated ATPase. Mitochondria (300 μg) from human hepatoma (Li-7) were incubated with 30 μM dinitrophenol in 1 ml reaction mixture containing 50 mM Tris-HCl, pH 7.4, 50 mM sucrose, 75 mM KCl, 1 mM EDTA, and 1 mg/ml defatted bovine serum albumin with (Fig. 1b) or without (Fig. 1a) 5 mM $MgCl_2$ at either 22°C (○, ●) or at 0°C (△, ▲) for the indicated length of time. ATPase reaction was inititated by the addition of 5 μmol ATP and the reaction was carried out for 5 min at 37°C.

Similar ATPase activities were obtained with astrocytoma mitochondria with dinitrophenol and valinomycin-K$^+$ whether incubation with the uncouplers was at 22°C or 0°C. However, this is also the tumor system where the differential effects of dinitrophenol and valinomycin-K$^+$ are the smallest.

Efflux of adenine nucleotide from tumor mitochondria

If the differential effects of protonphoric uncouplers and valinomycin-K$^+$ on the uncoupler-stimulated ATPase were due to different extent of efflux of exchangeable adenine nucleotides from the mitochondria, one should be able to demonstrate this by monitoring the efflux of radioactivity from tumor mitochondria prelabeled with radioac-

TABLE I

ACTIVITY OF UNCOUPLER-STIMULATED ATPase WHEN TUMOR MITOCHONDRIA WERE INCUBATED WITH UNCPOUPLERS AT 22°C AND 0°C.

Mitochondria (0.3–0.7 mg protein) were incubated for 6 min in 1 ml reaction mixture in the absence or presence of 5 mM $MgCl_2$. When dinitrophenol or valinomycin were present, at least three concentrations of dinitrophenol (15–100 μM) and valinomycin (2–20 ng) were used. This was necessary because the concentration of uncoupler which elicited maximal ATPase activity varied with the type of tumor mitochondria used and the experimental conditions (see Refs. 11 and 12). After incubation at 22°C or 0°C, ATP (5 μmol) was added and the ATPase reaction was carried out at 37°C for 5 min. The maximal ATPase activities elicited by dinitrophenol and valinomycin, and the control ATPase activity (obtained in the absence of any uncouplers) are presented.

Mitochondria from	Mg^{2+}	ATPase activity (μmol Pi/min per mg) when preincubation was at					
		22°C			0°C		
		No addition	Dinitrophenol	Valinomycin	No addition	Dinitrophenol	Valinomycin
Oat cell carcinoma (T293)	–	51	131	419	73	663	619
	+	34	284	916	63	727	904
Hepatoma (Li-7)	–	30	41	225	52	230	358
	+	50	143	383	47	265	383
Astrocytoma (T24)	–	23	112	148	27	118	136
	+	87	215	244	73	179	215

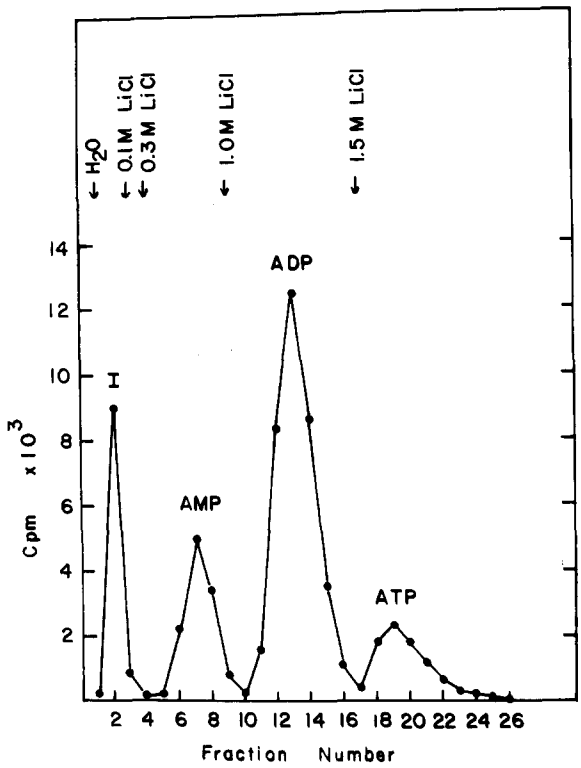

Fig. 2. Distribution of tritium label in the adenine derivatives of hepatoma mitochondria after uptake of [2,8-^{3}H]ATP. Human hepatoma mitochondria (8.8 mg) in 0.5 ml isolation buffer were incubated with 6 μCi of [2,8-^{3}H]ATP (0.21 nmol). After 20 min at 0°C, the loaded mitochondria were centrifuged and washed as described in Materials and Methods. After resuspension, an aliquot of the mitochondria (1.2 mg) was extracted with $HClO_4$. An aliquot of the neutralized extract was applied to a column of PEI-cellulose (2 cm height) packed in a Pasteur pipette. The column was washed with solutions as indicated in the figure. One ml fractions were collected and the radioactivity in each fraction was determined by liquid scintillation counting after mixing the entire fractions with 10 ml ACS counting solution.

tive adenine nucleotides. Furthermore, if the exchangeable adenine nucleotides pool size is crucial in determining the magnitude of the uncoupler-stimulated ATPase, one would also predict a greater efflux at 22°C than at 0°C.

Fig. 2 shows that under the specified labelling conditions, the label from [^{3}H]ATP taken up by hepatoma mitochondria was distributed into AMP, ADP, ATP and substances not absorbed by PEI-cellulose (peak I), which is probably adenosine. The relative magnitude of the four peaks varied with the type of mitochondria used, probably reflecting the ATPase, the adenylate kinase, and other adenine nucleotide metabolizing enzyme activities of that particular type of tumor mitochondria.

TABLE II

EFFLUX OF TRITIATED ADENINE DERIVATIVES FROM MITOCHONDRIA PRELABELED WITH [^{3}H]ATP

Mitochondria (10–20 mg/ml) were incubated at 0°C with [2,8-^{3}H]ATP (10 μCi/ml, 0.36 μM) for 20 min. The mitochondrial suspension was then centrifuged and washed as described under Materials and Methods. For efflux experiments, 0.3–0.6 mg labeled mitochondria were added to 1 ml reaction mixture, after 6 min at 0°C or 22°C, the tubes were centrifuged at 0°C at 12000 × g for 3 min. Aliquots (0.2 ml) of the supernatant were counted. An aliquot of the mitochondrial suspension (20 μl) was also counted. Data were expressed as the percentage of the counts originally present in the labeled mitochondria.

Temperature	Addition during incubation	Per cent of total radioactivity			
		Liver	Oat cell carcinoma (T-293)	Hepatoma (Li-7)	Astrocytoma (T-24)
22°C	None	9.2	11.4	25.4	15.3
	Dinitrophenol	11.7	56.4	40.7	23.2
	CCCP	13.5	66.8	50.2	31.5
	Valinomycin	20.3	33.6	35.7	21.4
0°C	None	8.5	8.8	16.8	13.9
	DNP	11.3	9.3	19.0	16.0
	CCCP	13.3	12.2	25.8	18.4
	Valinomycin	8.0	14.2	20.7	14.2

Efflux studies under various conditions were carried out using these prelabeled mitochondria and the results are summarized in Table II. It can be seen that there was little efflux of radioactivity from liver mitochondria at either 0°C or 22°C. At 22°C, there was usually a significant increase in efflux with valinomycin in the presence of K^+ while CCCP and dinitrophenol had little effect. There are three points worth noting in the results obtained with the tumor mitochondria: (1) astrocytoma mitochondria showed the least basal efflux whereas the hepatoma mitochondria always showed much greater basal efflux at 22°C; (2) efflux at 22°C was greater than at 0°C; (3) at 22°C, dinitrophenol and CCCP caused a much greater efflux than valinomycin. The stimulation of efflux by protonphoric uncouplers was concentration dependent (Fig. 3). Maximal efflux was attained in a very short time (Fig. 4a). The uncoupler induced efflux at 22°C was not prevented by the addition of Mg^{2+}, and was not inhibited by atractyloside, *N*-ethylmaleiimide or dibucaine, a phospholipase inhibitor (data not shown).

In addition to the adenine compounds, we also found inorganic phosphate in the supernatant in the efflux experiment. In contrast to the rapid efflux of radioactivity, the continuous release of phosphate could be demonstrated (Fig. 4b). Phosphate release was always greater in the presence of protonphoric uncouplers (Table III), and was inhibited by *N*-ethylmaleiimide (data not shown). A very interesting point revealed by the data in

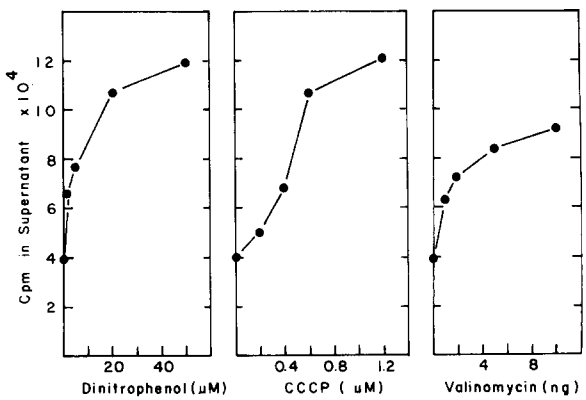

Fig. 3. Uncoupler concentration dependent efflux of tritium label from mitochondria preloaded with [2,8-^{3}H]ATP. Mitochondria (0.44 mg containing 169000 cpm) from human oat cell carcinoma preloaded with [2,8-^{3}H]ATP were added to 2 ml reaction mixture containing various amounts of dinitrophenol, CCCP and valinomycin. After 6 min at 22°C the suspension was centrifuged at 0°C for 3 min at 12000 × g. An aliquot (0.2 ml) of the supernatant was taken for counting.

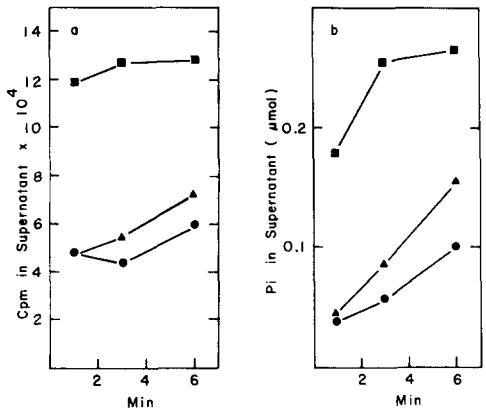

Fig. 4. Efflux of tritium label and inorganic phosphate from mitochondria preloaded with [2,8-^{3}H]ATP with time. Mitochondria (1.03 mg containing 192000 cpm) from oat cell carcinoma preloaded with [2,8-^{3}H]ATP were added to 2 ml reaction mixture without (●) or with 60 μM dinitrophenol (■) or 10 ng/ml valinomycin (▲). After the indicated time, the mixture was centrifuged at 0°C for 3 min at 12000× *g*. One aliquot (0.2 ml) of the supernatant was taken for counting (Fig. 4a). To another aliquot (1 ml) of the supernatant was added 0.1 ml of cold 50% trichloroacetic acid. After centrifugation, P_i determination was carried out on the clarified supernatant (Fig. 4b).

Table III was that the phosphate concentration in all three tumor mitochondria were at least 10-times higher than those in liver or heart mitochondria.

Analysis of the adenine compounds leaving and remaining in the tumor mitochondria was conducted. Fig. 5 shows the results of an efflux experiment obtained with mitochondria from oat cell carcinoma. When tumor mitochondria were incubated alone (Fig. 5a), relatively small amounts of radioactivity were found in the supernatant (dashed line), the composition of which reflected the distribution pattern inside the mitochondria (solid line). When these mitochondria were incubated with CCCP (Fig. 5b), more than half of the total radioactivity was found in the supernatant solution which contained peak I material, AMP and ADP (dashed line). Interestingly, although there was still ATP remaining inside the mitochondria (solid line), very little of it was found in the supernatant solution.

Transport of adenine nucleotides

In addition to the unusual efflux patterns of adenine nucleotides and their metabolites seen with tumor mitochondria, adenine nucleotide translocation in tumor mitochondria also shows different characteristics from that of liver mitochondria. These studies were carried out at 3–100 μM substrate concentrations. A representative Lineweaver-Burk plot derived from data obtained with mitochondria from oat cell carcinoma

TABLE III

INORGANIC PHOSPHATE CONTENT OF TUMOR MITOCHONDRIA AFTER INCUBATION WITH UNCOUPLERS

Tumor mitochondria (0.2–0.5 mg/ml) were incubated without or with 60 μM dinitrophenol, 1.2μM CCCP or 10 ng/ml valinomycin for 6 min at 22°C or 0°C. After centrifugation, the supernatant was removed and 0.5 ml cold 5% trichloroacetic acid was added to the mitochondrial pellet. The pellet was sonicated briefly in a bath type sonicator and the suspension was centrifuged to precipitate denatured mitochondria. An aliquot (0.4 ml) of the clarified supernatant was used for determination of inorganic phosphate. n.d., not determined.

Temperature	Addition during incubation	μmol P_i/mg protein			
		Liver	Oat cell carcinoma (T-293)	Hepatoma (Li-7)	Astrocytoma (T-24)
22°C	None	n.d.	0.35	0.08	0.129
	Dinitrophenol	n.d.	0.06	0.015	0.053
	CCCP	n.d.	0.08	0.003	0.003
	Valinomycin	n.d.	0.29	0.048	0.070
0°C	None	0.009	0.37	0.103	0.146
	DNP	n.d	0.34	0.093	0.127
	CCCP	n.d.	0.35	0.083	0.125
	Valinomycin	n.d.	0.40	0.101	0.141

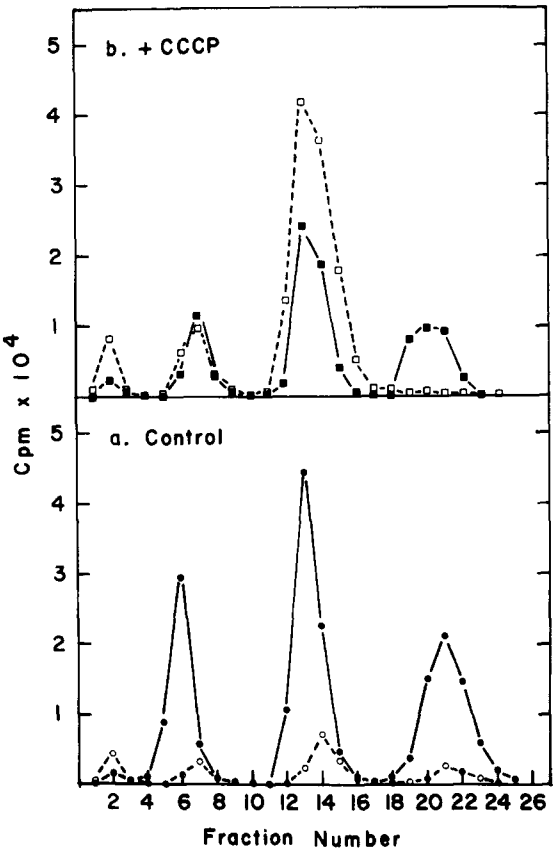

Fig. 5. Distribution of tritium label in the adenine derivatives in the supernatant and the mitochondria in an efflux experiment. Mitochondria (0.45 mg containg 169000 cpm) from oat cell carcinoma preloaded with [2,8-^{3}H]ATP were added to 2 ml reaction mixture without (○, ●) and with 1.2 μM CCCP (□, ■). The mixture was centrifuged after 7 min at 22°C. To 1 ml of the supernatant was added 0.15 ml of 40% $HClO_4$. To the mitochondrial pellet was added 1 ml of 5% $HClO_4$. The perchloric acid extract was neutralized and an aliquot (0.2–0.4 ml) was used for chromatography on PEI-cellulose. Data presented were counts in the entire samples. ------, counts in the supernatant; ———, counts in the mitochondrial pellet.

is shown in Fig. 6. Table IV summarizes the kinetic parameters for adenine nucleotide translocation in normal and tumor mitochondria. It can be seen that the general trend was that both K_m and V_{max} values were decreased with respect to both ADP and ATP transport in tumor mitochondria. In particular, V_{max} values for ADP transport of tumor mitochondria was only half or even less of the V_{max} values obtained with liver and heart mitochondria.

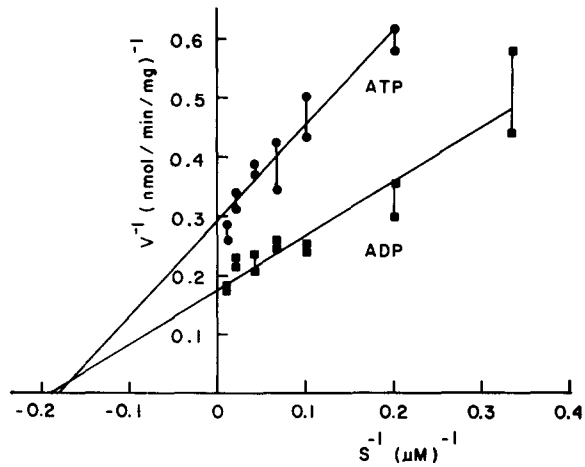

Fig. 6. Lineweaver-Burk plots of ADP and ATP transport in the mitochondria of human oat cell carcinoma. Mitochondria (0.81 mg) were used in each assay. ATP and ADP concentrations varied between 3 and 100 μM.

Another characteristics of the adenine nucleotide transport in tumor mitochondria was that in contrast to the 4–5-fold activation seen in normal liver mitochondria, ATP transport in tumor mitochondria was not or only very slightly stimulated by CCCP (Fig. 7). Interestingly, ATP transport rate in heart mitochondria was very high in the absence of any uncouplers. Addition of CCCP resulted in a two-fold stimulation.

TABLE IV

KINETIC PARAMETERS FOR ADP AND ATP TRANSPORT IN LIVER, HEART AND TUMOR MITOCHONDRIA

ADP and ATP transport were determined as described under Materials and Methods. Values of K_m and V_{max} were obtained from linear regression calculation. K_m values are expressed in μM. V_{max} values are expressed in nmol ADP or ATP per min per mg protein.

Mitochondria from	ADP transport		ATP transport	
	K_m	V_{max}	K_m	V_{max}
Liver (mouse)	6.0	11.9	45.2	6.5
Liver (rat)	10.9	14.1	22.7	5.4
Heart (mouse)	2.5	20.0		
Astrocytoma (T24)	14.4	2.3	25.2	3.1
Oat cell carcinoma (T293)	5.2	5.6	5.5	3.4
Hepatoma (Li-7)	1.7	3.0	6.0	2.6
Melanoma (T355)	2.2	3.4	3.2	2.8

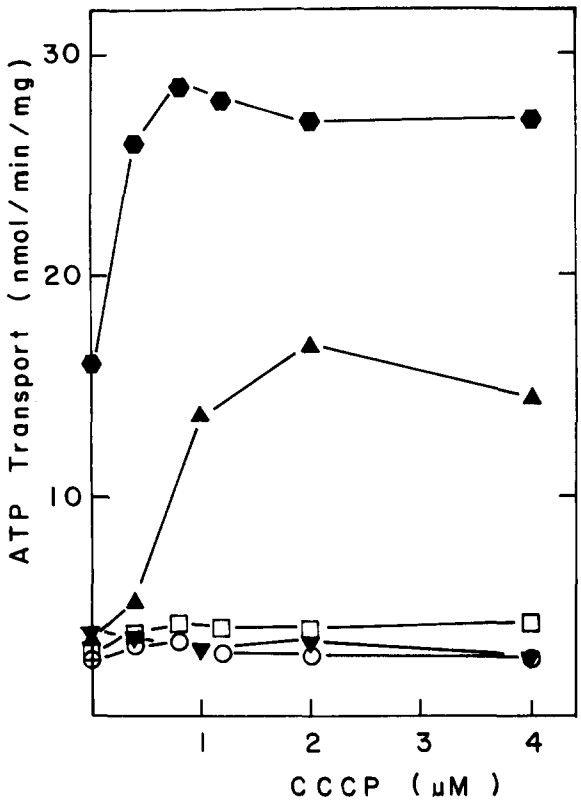

Fig. 7. Effect of CCCP on ATP transport of various mitochondria. ATP transport was carried out at 0°C in 1 ml reaction mixture containing 0.4–1.0 mg mitochondrial protein and varying concentrations of CCCP. Transport reaction was initiated by the addition of 50 nmol [2,8-^{3}H]ATP and stopped 5 s (for heart mitochondria) or 10 s later by the addition of atractyloside. Mitochondria were from mouse heart (●), mouse liver (▲), human astrocytoma (○), human oat cell carcinoma (□) and human hepatoma (▼).

Discussion

Several laboratories have reported that the uncoupler-stimulated ATPase of mitochondria obtained from animal and human tumors is different from that of normal tissues. When these mitochondria were incubated with dinitrophenol or CCCP at room temperature before the addition of ATP, the ATPase activity obtained was invariably lower than when the addition of ATP preceded or coincided with the addition of these uncouplers [8,10,11,19]. We extended this finding to show that this particular response was only observed with protonphoric uncouplers, but not with valinomycin-K^+ [11]. The valinomycin-K^+ system, then, was used as an internal control in identifying the factors responsible for deterioration of ATPase activity when tumor mitochondria were incubated with dinitrophenol or CCCP. Several lines of investigation suggested that the protonphoric uncouplers induced efflux of exchangeable adenine nucleotides. Direct measurement of individual adenine nucleotides was, however, inconclusive [11].

In this paper, we have reinvestigated this aspect of the problem using tumor mitochondria labeled with radioactive ATP. Chromatographic analyses of adenosine phosphate esters revealed that adenine-labeled ATP taken up by the mitochondria quickly equilibrated with ADP and AMP even at 0°C. The relative distribution of radioactivity in the three adenine nucleotides was similar to that obtained by quantitative determination [11]. Thus, this system was suitable for monitoring the efflux of adenine nucleotides.

In the tumor systems where the differential effects of dinitrophenol and valinomycin-K^+ on the ATPase were very large, e.g. hepatoma and oat cell carcinoma, dinitrophenol and CCCP clearly induced a large efflux of labeled adenine nucleotides, the extent of which was always greater than that induced by valinomycin-K^+. Under the same conditions, there was little loss of labeled adenine nucleotides from liver mitochondria. The uncoupler induced efflux of adenine nucleotides from tumor mitochondria differed from that observed by D'Souza and Wilson [20] in that PP_i was not required in the extramitochondrial solution and the process was not inhibited by atractyloside. The efflux from tumor mitochondria was not due to a general membrane leakage since EDTA was present in the reaction mixture to suppress phospholipase action which could disrupt membrane structure. On the other hand, EDTA may chelate divalent ions which stabilize the mitochondrial membranes. However, neither addition of Mg^{2+} nor omission of EDTA affected the uncoupler-induced efflux of adenine nucleotides (data not shown). Furthermore, two other observations argue against a non-specific membrane leakage as the basis for the adenine nucleotide efflux. The first observation was that although AMP and ADP were released from tumor mitochondria in the

presence of CCCP, ATP remained inside the mitochondria (Fig. 5b). The second observation was that inorganic phosphate was also released from tumor mitochondria in the presence of CCCP, however it followed a different time course than the efflux of adenine nucleotides (Fig. 4).

The uncoupler stimulated efflux of inorganic phosphate has been previously documented [21]. In our experiment, this process was completely inhibited by *N*-ethylmaleiimide, inhibitor of the mitochondrial phosphate transporter (data not shown). Considerably less phosphate was released from the mitochondria after incubation with valinomycin-K^+ (Fig. 4b and Table III). It is not known if the intramitochondrial concentration of P_i has a direct effect on the ATPase although Mitchell and Moyle [22] have shown that the ATPase activity was stimulated by P_i.

An unexpected finding was that the phosphate content of tumor mitochondria was 10–40-times higher than that found in liver or heart mitochondria. This could be the results of (i) high cytosolic phosphate concentration in these tumor tissues, (ii) a more active phosphate transport system in the tumor mitochondria, or (iii) co-transport with Ca^{2+}, which is accumulated and retained tenaciously in some tumor mitochondria [23,24]. The mechanism of phosphate accumulation as well as the uncoupler-induced adenine nucleotide efflux in tumor mitochondria are currently under investigation.

In this study, we have also examined the forward exchange of ADP and ATP. The only systematic and thorough study on this subject was conducted by Barbour and Chan [25], whose report on adenine nucleotide translocation in mitochondria of Morris rat hepatomas appeared while this manuscript was in preparation. Our results, obtained with human tumors, agreed with theirs in that V_{max} for both ADP and ATP transport in tumor mitochondria was lower than in liver mitochondria, especially that of ADP. Barbour and Chan attributed the low transport rate to a reduced content of endogenous adenine nucleotides which appeared to be the result of ischemia in tumors of large size. We do not believe this is the sole cause for the lower rates since the intramitochondrial adenine nucleotide content of astrocytoma and oat cell carcinoma was reasonably high (Table V of Ref. 11). It is possible that the lower transport activity is an inherent property of these tumor mitochondria.

The lack of response of ATP transport to CCCP is another distinctive feature of tumor mitochondria. Barbour and Chan [25] presented similar data showing that V_{max} values for ATP transport in Morris hepatoma mitochondria was not affected by the addition of CCCP. However, the results of this study and that of Barbour and Chan differ significantly with respect to the effect of CCCP on ATP transport of normal liver mitochondria. We routinely observe 3–5-fold activation of ATP transport in either mouse or rat liver mitochondria by CCCP (Fig. 7), which agree with the original results of Pfaff and Klingenberg [26]. However, Barbour and Chan [18,25] only found minimal activation of ATP transport in liver mitochondria by CCCP.

The effect of CCCP on ATP transport has been explained by the ability of CCCP to abolish the membrane potential which opposes the electrogenic exchange of ATP^{4-} (out) and ADP^{3-} (in) [27]. However, it has also been proposed that there is an electroneutral element in this exchange, i.e. ATP^{4-} (out) $+H^+ \leftrightarrow$ ADP (in) [26]. The lack of response of ATP transport to CCCP in tumor mitochondria could be due to: (i) extramitochondrial ATP exchanges only for intramitochondrial ATP, or (ii) tumor mitochondria are already in an uncoupled state. The first possibility is amenable to experimentation. The second possibility is not very likely since we have shown that these mitochondria exhibited respiratory control [12]. Nevertheless, the contribution of the pH gradient and membrane potential to the protonmotive force in these tumor mitochondria has yet to be determined.

Related to the effect of CCCP on ATP transport, it is interesting to note that the rate of ATP transport in heart mitochondria obtained in the absence of any uncouplers is equivalent to the maximal uncoupler activated rate in liver mitochondria. The question remains why ATP transport in heart mitochondria is not subjected as severely to the constraints of membrane potential.

Regardless of the mechanism responsible for the uncoupler stimulated efflux of adenine nucleotides and the low rates of transport in tumor

mitochondria, there are interesting consequences for the physiology of the tumor cells. Many tumors have a reduced content of mitochondria [19]. The yield of mitochondria from the human tumors we used were usually 1–2% of total cell protein compared to 10–20% as obtained with liver or heart tissues. Since mitochondria are usually the major users of cellular ADP, a combination of much reduced mitochondrial content and lower ADP transport rate will certainly increase the cellular ADP and P_i concentrations which have been shown to be important factors in the regulation of aerobic glycolysis [28,29]. In addition, efflux of adenine nucleotides, which reduce the exchangeable adenine nucleotide pool size, and a decreased ATP transport due to a lack of uncoupler stimulation could well explain the low activity of the uncoupler-stimulated ATPase in tumor mitochondria as a result of a decrease in ATP uptake.

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References

1 Emmelot, P., Bos, C.J., Brombacher, P.J. and Hampe. J.F. (1959) Br. J. Cancer 13, 343–379
2 Devlin, J.M. and Pruss, M.P. (1962) Proc. Am. Assoc. Cancer Res. 3, 315
3 Kolárov, J., Kužela, S., Krempaský, V. and Ujházy, V. (1973) Biochem. Biophys. Res. Commun. 55, 1173–1179
4 Thorne, R.F.W. and Bygrave, F.L. (1973) Cancer Res. 33, 2562–2567
5 LaNoue, K.F., Hemington, J.G., Ohnishi, T., Morris, H.P. and Williamson, J.R. (1973) in Hormones and Cancer (McKerns, K.W., ed.), pp. 131–167, Academic Press, New York
6 Pedersen, P.L. and Morris, H.P. (1974) J. Biol. Chem. 249, 3327–3334
7 Senior, A.E., McGowan, S.E. and Hilf, R. (1975) Cancer Res. 35, 2061–2067
8 Kaschnitz, R.M., Hatefi, Y. and Morris, H.P. (1976) Biochim. Biophys. Acta 449, 224–235
9 Barbour, R.L. and Chan, S.H.P. (1978) J. Biol. Chem. 253, 367–376
10 Hayashi, J.-I., Gotoh, O. and Tagashira, Y. (1980) Arch. Biochem. Biophys. 205, 27–35
11 Knowles, A.F. (1982) Biochim. Biophys. Acta 681, 62–71
12 Knowles, A.F. and Kaplan, N.O. (1980) Biochim. Biophys. Acta 590, 170–181
13 Hirohashi, S., Shimosato, Y., Kameya, T., Koide, T., Mukojima, T., Taguchi, Y. and Kageyama, K. (1979) Cancer Res. 39, 1819–1828
14 Tyler, D.D. and Gonze, J. (1967) in Methods in Enzymology (Estabrook, R.W. and Pullman, M.E., eds.), Vol. 10, pp. 75–77, Academic Press, New York
15 Asimakis, G.K. and Aprille, J.R. (1980) Arch. Biochem. Biophys. 203, 307–316
16 Magnusson, R.T., Portis, A.R., Jr. and McCarty, R.E. (1976) Anal. Biochem. 72, 653–657
17 Lohmann, K. and Jendrassik, L. (1926) Biochem. Z. 178, 419–426
18 Barbour, R.L. and Chan, S.H.P. (1981) J. Biol. Chem. 256, 1940–1948
19 Pedersen, P.L. (1978) in Progress in Experimental Tumor Research (Wallach, D.F.H., ed.), Vol. 22, pp. 190–274, Karger, Basel
20 D'Souza, M.M. and Wilson, D.F. (1982) Biochim. Biophys. Acta 680, 28–32
21 Papa, S., Zanghi, M.A., Paradies, G. and Quagliariello, E. (1970) FEBS Lett. 6, 1–4
22 Mitchell, P. and Moyle, J. (1973) Bioenergetics 2, 1–11
23 Thorne, R.F.W. and Bygrave, F.L. (1974) Biochem. J. 144, 551–558
24 Villalobo, A. and Lehninger, A. (1980) J. Biol. Chem. 255, 2457–2464
25 Barbour, R.L. and Chan, S.H.P. (1983) Cancer Res. 43, 1511–1517
26 Pfaff, E. and Klingenberg, M. (1968) Eur. J. Biochem. 6, 66–79
27 Klingenberg, M. and Rottenberg, H. (1977) Eur. J. Biochem. 73, 125–130
28 Wu, R. and Racker, E. (1959) J. Biol. Chem. 234, 1029–1035
29 Racker, E., Johnson, J.H. and Blackwell, M.Y. (1983) J. Biol. Chem. 258, 3702–3705