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OXIDATIVE PHOSPHORYLATION PROPERTIES OF MITOCHONDRIA ISOLATED FROM TRANSPLANTED HEPATOMA

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SUMMARY

Mitochondria were isolated from Morris hepatomas with rapid (types 3683, 7777, and 3924A) and intermediate (types 5123D and 7800) growth rates, using proteolytic digestion of minced tumor tissue to release the particles. Mitochondria isolated by the same procedure from rat liver were employed as controls. All the hepatoma mitochondria were capable of coupled respiration with normal phosphorylation yields (ADP/O) and respiratory control ratios ranging from 2 to considerably more than 10. Particles from hepatomas 7777 and 7800 exhibited properties closest to liver mitochondria, while those from hepatomas 3683 and 3924A showed the greatest difference. All the hepatoma mitochondria were capable of oxidizing succinate, 3-hydroxybutyrate and monoamines. However, the oxidation rates of the latter two substrates by mitochondria from hepatomas 3683 and 3924A were only a fraction of the control rates. These differences appeared to be due, at least in part, to the structural instability of the isolated hepatoma mitochondria. In contrast to the reports of others, all hepatoma mitochondria exhibited considerable stimulation of ATPase activity by uncouplers. Maximal stimulation of ATPase activity by representatives of three classes of uncouplers was in all instances comparable to the values obtained for rat liver mitochondria.

INTRODUCTION

The importance of energy metabolism in malignant physiology and the early observations of Warburg [1] that in many cancer cells glycolysis is a stronger feature than aerobic respiration have prompted extensive studies of mitochondrial abundance (i.e., concentration and activity of a marker enzyme), structure, and function in malignant cells and tissues [2–8]. Cell lines of choice have been the transplantable

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Morris hepatomas because of the considerable stability of each tumor cell line, the availability of cell lines with rapid, slow, and intermediate growth rates, and the possibility of using normal and host liver mitochondria as reasonable controls [9].

It has been reported that mitochondria isolated from such transplantable hepatomas differ in a number of important respects from normal and host liver mitochondria. A common observation has been that hepatoma mitochondria have poor respiratory characteristics, such as low respiratory rates and low or lack of respiratory control [4, 6, 8]. Mitochondria isolated from Morris hepatoma 3924A were found to be unable to oxidize 3-hydroxybutyrate and deficient in the outer membrane enzyme monoamine oxidase [4]. Ohnishi et al. [6] reported that in mitochondria isolated from the slow-growing Morris hepatoma 16 electron paramagnetic resonance (EPR) signals associated with NADH dehydrogenation were significantly diminished, and that in mitochondria from the fast-growing Morris hepatoma 7777 all EPR signals exhibited considerably lowered intensity. They concluded that the diminished EPR signals correlated well with the lowered respiratory activities of their preparations of hepatoma mitochondria, as compared to host or normal liver mitochondria. Furthermore, a view expressed by several laboratories is that mitochondria isolated from transplantable hepatomas (mainly Morris hepatomas) exhibit little or no uncoupler-stimulated ATPase activity [2, 5, 7, 8]. Pedersen et al. [5] showed that in mitochondria from Morris hepatoma 7800, the lack of uncoupler-stimulated ATPase activity could not be correlated, however, with an absence or a deficiency of the membrane-bound ATPase. Hence, Pedersen and Morris [8] speculated that such mitochondria might contain an additional site of uncoupler action to account for uncoupling of energy-linked functions without stimulation of ATP hydrolysis*.

The present paper is concerned with the respiratory properties of mitochondria isolated from several Morris hepatomas with fast and intermediate growth rates. Parameters studied were oxidative phosphorylation and respiratory control with succinate and 3-hydroxybutyrate as substrates, monoamine oxidase and 3-hydroxybutyrate dehydrogenase activities, and ATPase activities in the presence and absence of the uncouplers DNP**, CCCP, and S-13. Results have indicated that the differences between hepatoma and host or normal liver mitochondria are only quantitative. Essentially, all tumor mitochondria had measurable to normal levels of 3-hydroxybutyrate and monoamine oxidase activities, normal uncoupler-stimulated ATP activities, and were capable of oxidative phosphorylation with respiratory control and normal P/O values. A preliminary report of these studies has been presented [12].

METHODS AND MATERIALS

Isolation of mitochondria. The following procedure is based on the methods described by Hatefi et al., Bullock et al., Schnaitman and Greenawalt, and by Loewenstein et al. [13–16]. Rats were anaesthesized with ether, and the tumors were removed

* This hypothesis pre-dated the work of this laboratory, which showed that in beef heart mitochondria there exists a specific uncoupler-binding site located in the inner membrane and distinct from the F_1(ATPase) [10, 11].

** Abbreviations: DNP, 2,4-dinitrophenol; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4 -nitrosalicylanilide; HEPES, *N*-2-hydroxyethylpiperizane-*N* -ethanesulfonic acid.

and freed of necrotic areas and connective tissue. All subsequent operations were carried out at 0–4 °C. The tumors were washed with an ice-cold solution containing 220 mM mannitol, 70 mM sucrose and 2 mM HEPES (pH 7.4 at 25 °C). This solution will be referred to as the isolation medium. The tissue was then minced thoroughly, pulped and suspended in the isolation medium (2 ml per g tissue). 1 mg Nagarse protease (Nagase Co., Ltd., Osaka, Japan) was added per g tissue, and the mixture was stirred vigorously for 20 min. After that time, 2 ml isolation medium (containing 2 % (w/v) of bovine serum albumin) were added per g tissue. The homogenate was centrifuged at $3200 \times g$ (average) for 1 min and the sediment was discarded. All centrifugation times are exclusive of acceleration and deceleration times. The supernatant was further centrifuged at $17\,000 \times g$ (average) for 2 min. The resulting pellets were rinsed thoroughly with the isolation medium (containing 1 % bovine serum albumin) and resuspended gently by hand in a glass homogenizer in 2 ml of the same solution per g tissue. After recentrifugation, this washing procedure was repeated once. Finally the mitochondria were suspended in the isolation medium containing 1 % bovine serum albumin. Protein concentration was measured by the Biuret method [17]. The mitochondrial protein concentration in the final suspensions was adjusted to 20 mg/ml. Due to the presence of albumin in the mitochondrial suspensions, a ratio of 2 : 1 (w/w) between mitochondrial protein and serum albumin was maintained in all enzyme assays.

Determination of enzymatic activities. All assays were carried out at 30 °C. Measurements of respiratory control and ADP/O ratios were carried out polarographically, using a Clark type oxygen electrode. The assay medium (1.7 ml) contained 250 mM mannitol, 20 mM KCl, 10 mM potassium phosphate and 5 mM $MgCl_2$ at pH 7.4. Respiratory substrates were succinate (5 mM, plus 3 μM rotenone) and 3-hydroxybutyrate (10 mM). The mitochondrial protein concentrations were 0.6 and 1.2 mg/ml, in conjunction with succinate and 3-hydroxybutyrate as substrates, respectively. ATPase activity was assayed by following the pH change associated with the hydrolysis of ATP [18, 19]. The assay volume was 3.4 ml of 150 mM KCl, 5 mM $MgCl_2$ and 3.3 mM glycylglycine at pH 7.400 ± 0.050 at 30 °C. The pH changes were measured by a glass electrode connected to an Orion 801 pH meter and a Honeywell 193 Laboratory recorder (sensitivity 2 mV). The substrate was 1 mM ATP plus 1 mM $MgCl_2$, adjusted to pH 7.40. Under these conditions the "acidification factor" ($\Delta H^+/\Delta P_i$) was 0.83 ± 0.06 (S.D., $n = 4$). The mitochondrial protein concentration was 0.3 mg/ml. In some experiments (see Table VI) and for the determination of the $\Delta H^+/\Delta P_i$ quotient, the ATPase activity was determined also by direct measurement of the amount of phosphate released [20] in 1-ml aliquots of the reaction mixtures. Acid phosphatase was assayed spectrophotometrically [21], using 0.3 mM carboxyphenylphosphate as the substrate in 150 mM sodium acetate buffer (pH 5.0 at 25 °C) containing 0.05 % (v/v) Triton X-100. The concentration of mitochondrial protein was 0.5 mg/ml. Monoamine oxidase was determined as described by Schnaitman et al. [22] in the presence of 0.05 % (v/v) Triton X-100 and at a mitochondrial protein concentration of 0.5 mg/ml. Control experiments with rat liver mitochondria showed that the enzyme was not inhibited by Triton up to at least 0.2 %. 3-Hydroxybutyrate dehydrogenase was assayed in the mitochondrial preparations after a freeze-thaw treatment (24 h at −20 °C) by following the reduction of NAD spectrophotometrically as described by Nielsen and Fleischer [23]. The mitochondrial protein

TABLE I

PREPARATIONS OF MORRIS HEPATOMA MITOCHONDRIA

Hepatoma type	Tumor generation numbers	Tumor growth time (days)	Yield of tissue (g/animal)	Yield of mitochondrial protein (mg/g tumor tissue)
3683	530	10	2.0	0.4
7777	108, 109, 115	10–12	0.5–0.8	1.1–1.9
3924A	322, 325	12–15	1.5–8.0	0.8–1.8
5123D	115	21	0.7	2.5
7800	84, 87	20–29	3.0–3.9	1.0–2.2

concentrations in the assays ranged from 0.1 to 0.4 mg/ml. All assays for measurement of 3-hydroxybutyrate dehydrogenase activity were performed in the presence of 5 μM rotenone.

Hepatoma-bearing animals were supplied by Dr. H. P. Morris. Female ACI rats were hosts for hepatomas 3683 and 3924A; female Buffalo rats were hosts for the other hepatomas and were used for normal liver controls. All hepatomas were grown subcutaneously. Table I presents data concerning hepatoma types, tumor generation numbers, growth times, and the yields of the preparations. Bovine serum albumin (Cohn fraction V) and *o*-carboxyphenylphosphate were obtained from Sigma Chemical Co., and CCCP from Calbiochem. Rutamycin and S-13 were gifts from Eli Lilly and Monsanto Commercial Products Co., respectively. All other chemicals were reagent grade.

RESULTS

Isolation and general properties of hepatoma mitochondria. Table I summarizes data obtained from 13 mitochondrial preparations from hepatomas of rapid (types 3683, 7777, and 3924A) and intermediate (types 5123D and 7800) growth rates. For each preparation the tumor tissue from 15–30 rats was pooled. The tumor generation numbers are given for comparison with material used in the earlier studies of others (e.g., ref. 4). The tumor growth time represents the number of days between inoculation and harvest of the hepatomas. Up to the harvest times shown, necrosis was found to be minimal. The yield of hepatoma tissue per animal was found to vary widely among the various cell lines. The last column of Table I gives the yield of mitochondrial protein obtained from each type of hepatoma. In all instances the yield was much lower than that of rat liver. For liver, the amount of mitochondrial protein isolated by our procedure was about 10 mg protein per g tissue.

Table II shows comparative values for the activities of the lysosomal marker enzyme, acid phosphatase, and of two enzymes characteristic of the outer and inner mitochondrial membranes. The amount of lysosomal material present in the mitochondrial preparations, as judged from acid phosphatase activity [16], was found to be in the same range for all preparations. The only exception was mitochondria from hepatoma 7800 which in some preparations contained a somewhat higher acid phosphatase activity. Monoamine oxidase activity in mitochondria from hepatomas

TABLE II

ACTIVITIES OF ACID PHOSPHATASE, MONOAMINE OXIDASE AND 3-HYDROXYBUTYRATE DEHYDROGENASE IN PREPARATIONS OF MORRIS HEPATOMA AND RAT LIVER MITOCHONDRIA

Hepatoma type	Acid phosphatase (nmol salicylic acid · min^{-1} · mg^{-1} protein)	Monoamine oxidase (nmol benzaldehyde · min^{-1} · mg^{-1} protein)	3-Hydroxybutyrate dehydrogenase (nmol NADH · min^{-1} · mg^{-1} protein
3683	62	0.7	16
7777	34	0.3–3.7	48–106
3924A	42–53	0.3–1.1	2.6–4.3
5123D	73	6.2	21
7800	52–116	6.8–8.5	150–234
Liver	29–66	7.6–10.5	161–418

7800 and 5123D was found to be only slightly lower than in rat liver mitochondria. About one third of the normal activity was present in mitochondria from hepatoma 7777, and only about 10 % or less of the normal value could be measured in mitochondria from hepatomas 3683 and 3924A. Mitochondria from tumor 3924A were previously reported by others to contain no monoamine oxidase at all. [4]. 3-Hydroxybutyrate dehydrogenase activity of hepatoma 7800 was similar to that of liver mitochondria. The other hepatomas exhibited clearly lower values. Hepatoma 3924A contained only about 1 % of the normal value.

Respiratory characteristics of hepatoma mitochondria. Table III summarizes the values of respiratory rates, respiratory control, and ADP/O ratios determined for the preparation of hepatoma and liver mitochondria. It is evident from the respiratory control and P/O ratios listed in the table that all hepatomas yielded well-coupled mitochondria. Indeed, hepatomas 7800 and 7777 yielded very tightly coupled mito-

TABLE III

OXIDATIVE PHOSPHORYLATION PROPERTIES OF MORRIS HEPATOMA AND RAT LIVER MITOCHONDRIA

Respiration rates are expressed in ng atoms oxygen · min^{-1} · mg^{-1} protein. RCR, respiration control ratio.

Hepatoma type	Succinate			3-Hydroxybutyrate		
	Respiration rate	RCR	ADP/O	Respiration rate	RCR	ADP/O
3683	180–230	2.2– 2.7	1.0–1.1	54*	–	–
7777	112–260	2.5–> 10	1.1–1.4	34– 55	3.7–> 10	1.7–2.3
3924A	158–357	2.1– 6.5	1.0–1.4	24– 62	1.5– 5.7	1.2–2.3
5123D	190–207	4.4– 5.3	1.2–1.3	39– 54	2.1– 2.5	1.7–2.0
7800	213–289	3.5–> 10	1.1–1.4	55–158	3.1–> 10	1.5–2.6
Liver	178–309	2.8–> 10	1.1–1.5	44–120	3.8–> 10	1.9–2.4

* In the presence of 1.2 μM S-13.

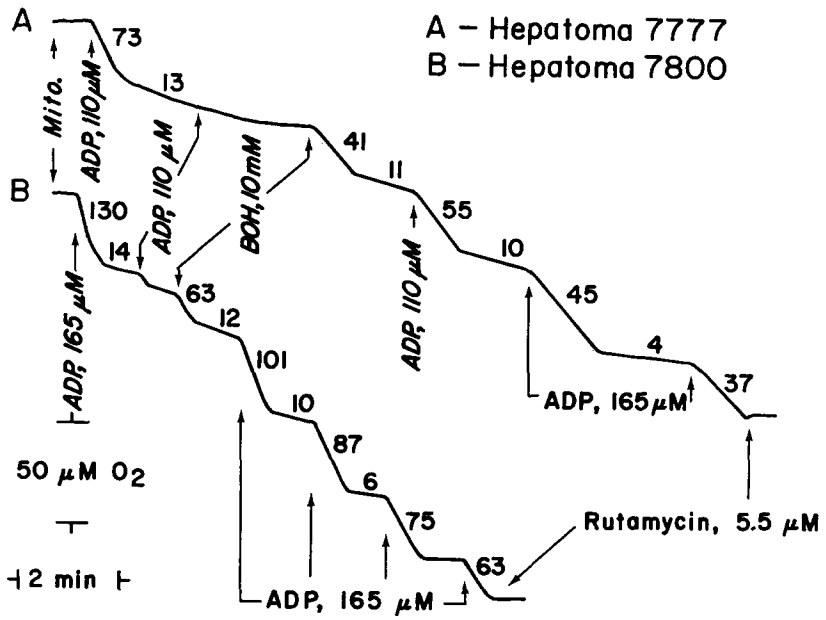

Fig. 1. Oxidative phosphorylation and coupling characteristics of mitochondria from hepatomas 7777 (A) and 7800 (B) respiring on 3-hydroxybutyrate (BOH). Assay conditions were as described in Methods and Materials. In this and in Fig. 2, the numbers shown next to various segments of the oxygraph traces indicate respiration rates in ng atoms oxygen · min^{-1} · mg^{-1} · protein.

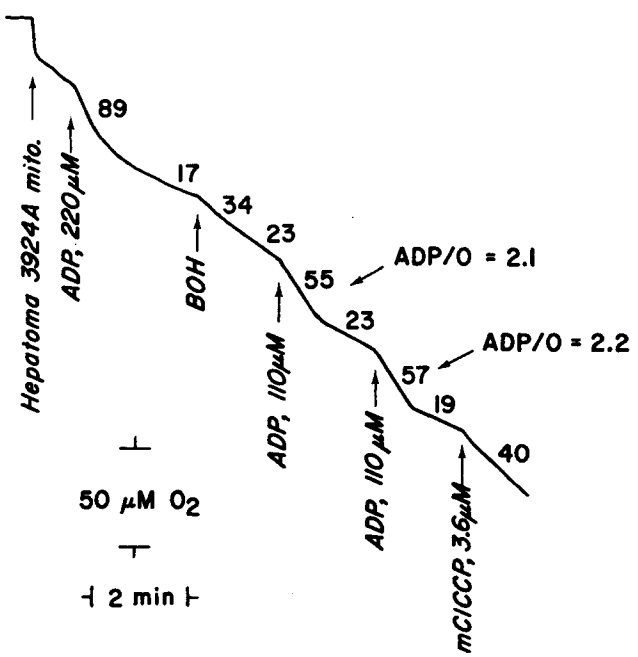

Fig. 2. Oxidative phosphorylation properties of mitochondria from hepatoma 3924A respiring on 3-hydroxybutyrate (BOH). The respiration rates are shown as in Fig. 1.

chondria (respiratory control ratio > 10) (Fig. 1) comparable to those isolated from normal or host livers. Others [8] have reported previously that mitochondria from hepatoma 7777 lack respiratory control.

The mitochondria from all hepatomas used both succinate and 3-hydroxybutyrate as respiratory substrates. In the case of 3-hydroxybutyrate, it was necessary to remove endogenous substrates by preincubation of mitochondria with ADP before the addition of 3-hydroxybutyrate (see Fig. 2). Thus, it was possible to distinguish the relatively slow rate of 3-hydroxybutyrate oxidation from the faster oxidation rate of endogenous substrates. Fig. 2 also shows that, contrary to the reports of others [4], mitochondria from hepatoma 3924A can oxidize 3-hydroxybutyrate when pretreated in the manner described. With mitochondria from tumor 3683, however, it was necessary to add uncoupler to demonstrate the oxidation of 3-hydroxybutyrate. In general, the coupled oxidation rates obtained with 3-hydroxybutyrate were lower in most tumor mitochondria, especially in preparations from hepatomas 5123D, 3924A and 7777, whereas the rates of coupled respiration measured with succinate were about equal for liver and hepatoma mitochondria. The ADP/O ratios, on the other hand, were found to be in the same range for both normal and tumor mitochondria with either substrate.

Uncoupler-stimulated ATPase. The ability of the uncoupler, DNP, to stimulate ATPase activity in all preparations of hepatoma and liver mitochondria is demonstrated by the data summarized in Table IV. The stimulation of ATPase activity by DNP ranged from about 2-fold (hepatoma 3924A) to more than 10-fold (hepatomas 7800 and 5123D). In all cases, the DNP-stimulated ATPase activity could be inhibited completely by rutamycin or atractyloside. Table IV also lists the activity levels of "latent" ATPase in the mitochondrial preparations. It is seen that mitochondria from the fast growing tumors exhibited relatively higher latent ATPase activities. Nevertheless, the low levels of this activity are indicative (as are the respiratory control ratios in Table III) of the degree of intactness of mitochondria in the respective preparations. The last column of Table IV shows the ATPase activities of the mitochondrial preparations after a freeze-thaw treatment. These activities were comparable to the activities elicited by DNP.

TABLE IV

ATPase ACTIVITIES OF MORRIS HEPATOMA AND RAT LIVER MITOCHONDRIA

Hepatoma type	ATPase activity (nmol ATP · min^{-1} · mg^{-1} protein)		
	Latent	Plus 49 μM DNP[a]	After freeze-thaw[b]
3683	110–146	338–415	251
7777	57– 90	234–290	327–376
3924A	77– 94	170–286	253–381
5123D	12– 19	309–321	407–420
7800	15– 72	186–429	143–262
Liver	27– 50	184–298	298–377

[a] DNP was added after ATP. As shown in Fig. 3, 49 μM DNP resulted in maximal stimulation.
[b] Mitochondria were frozen 16 h at −20 °C, then thawed and assayed.

TABLE V

UNCOUPLER-STIMULATED ATPase IN MORRIS HEPATOMA AND RAT LIVER MITOCHONDRIA: MAXIMAL ACTIVITIES AND CORRESPONDING UNCOUPLER CONCENTRATIONS

Uncoupler concentrations given are approximately those required for maximal activation. All uncouplers were added as ethanolic solutions before ATP. The final concentration of ethanol was $\leqq 3\,\%$ (v/v). This concentration of ethanol did not inhibit the DNP-stimulated ATPase of liver mitochondria. Activity is expressed in nmol ATP $\cdot$ min^{-1} $\cdot$ mg^{-1} protein, rounded to the nearest decade.

Hepatoma type	DNP		S-13		CCCP	
	Concentration (μM)	Activity	Concentration (μM)	Activity	Concentration (μM)	Activity
3683*	2	230	0.002	260	0.03	270
7777	> 20	200	> 0.6	230	0.06*	80
3924A	> 10	200	0.003	240	0.13	150
5123D	> 24	240	> 0.02	200	0.3	200
7800	> 30	300	> 0.02	200	> 0.2	200
Liver	> 50	300	> 0.2	250	> 0.03	150

* Because of the scarcity of material, the uncoupler concentrations required for maximal stimulation were not fully investigated in these instances.

The mitochondrial preparations were also tested for the response of their ATPase to various concentrations of DNP, S-13 and CCCP. Results are presented in Table V and in Figs. 3A to 3C. Since a very wide range of uncoupler concentrations (six orders of magnitude) was examined, it was necessary to present the figure data in semilogarithmic plots. Also, it should be kept in mind that bovine serum albumin was present in all the assays (see Methods and Materials).

Figs. 3A to 3C depict the effects of the above uncouplers on ATPase activities in three characteristic cases: mitochondria from rat liver and hepatomas of rapid (3924A) and intermediate (7800) growth rates. The curves show in most instances broad peaks of activity with little inhibition at high concentrations of uncoupler. The locations of these peaks relative to uncoupler concentration is characteristic for each uncoupler (see also Table V). In the case of DNP, maximal ATPase activities were found at 10–15 μM DNP for both normal and tumor mitochondria. As expected, the more potent uncouplers, S-13 and CCCP, elicited their maximal effects at far lower concentrations. As seen in Table V and Figs. 3A to 3C, the maximal activities of the uncoupler-stimulated ATPase were roughly comparable to the ATPase activities exposed by disruption of mitochondria by freeze-thaw treatment (see Table IV). Only in the case of CCCP was the stimulated ATPase activity in some instances lower than the activity exposed by freezing-thawing.

Since the above ATPase activities were measured potentiometrically with a pH-meter, the uncoupler-stimulated ATPase activity of mitochondria from tumor 7800 was also studied by direct measurement of the inorganic phosphate released. This hepatoma was selected because its ATPase has been studied in detail by others [5, 8]. Table VI summarizes the results obtained with the three uncouplers added to the assay medium both before and after ATP. In all cases, the estimated rates of

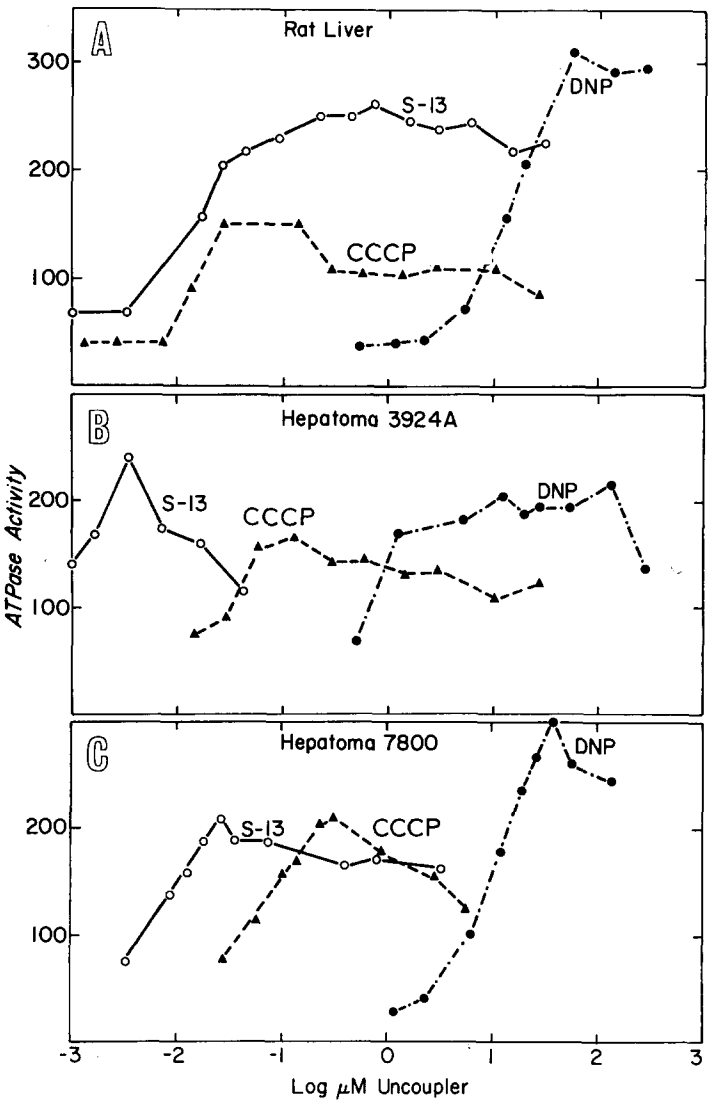

Fig. 3. Concentration effects of DNP, S-13, and CCCP on stimulation of ATPase activity of mitochondria isolated from (A) rat liver, (B) hepatoma 3924A, and (C) hepatoma 7800. ATPase activity is in nmol ATP hydrolyzed · min^{-1} · mg^{-1} protein.

inorganic phosphate release were found to be in good agreement with the rates calculated from pH changes. Also in both assays a higher activity was measured when DNP and S-13 were added to the assay medium after ATP than before.

DISCUSSION

The results presented in this paper show that mitochondria isolated from five Morris hepatomas with rapid and intermediate growth rates possess properties com-

TABLE VI

UNCOUPLER-STIMULATED ATPase IN MITOCHONDRIA FROM MORRIS HEPATOMA 7800: COMPARATIVE ASSAYS OF H^+ APPEARANCE AND PHOSPHATE LIBERATION

ATP hydrolysis was followed by measuring the pH changes as described in Methods and Materials. After 2–3 min, aliquots were withdrawn for measurement of inorganic phosphate (P_i) formation. The pH changes occurred at constant rate during this time, except in the two instances marked with asterisk. In these cases the rates slowed down with time. Thus the values listed were calculated by averaging the initial rate and the rate at the time of sampling (about 6 min after the start of the reaction). The following concentrations of uncouplers were used: DNP, 50 μM; S-13: 0.03 μM and CCCP, 0.29 μM. The reaction rates are expressed as nmol $P_i \cdot min^{-1} \cdot mg^{-1}$ protein for P_i liberation, and as nmol ATP $\cdot min^{-1} \cdot mg^{-1}$ protein for H^+ appearance.

	Reaction rates		
	DNP	S-13	CCCP
Addition sequence (a) (mitochondria-ATP-uncoupler)			
H^+ appearance	381	321	265
P_i liberation	399	294	232
Addition sequence (b) (mitochondria-uncoupler-ATP)			
H^+ appearance	261	115*	226
P_i liberation	226	150*	239

parable to those of normal rat liver mitochondria. The parameters compared were rates of respiration and phosphorylation, ADP/O values, respiratory control ratios, and the stimulatory effects of uncouplers on mitochondrial ATPase. Among the hepatoma mitochondria tested, those from tumor types 7777 (rapid growth rate) and 7800 (intermediate growth rate) exhibited properties closest to liver mitochondria, while particles from the fast-growing tumors 3683 and 3924A showed the greatest difference. Thus, as compared to liver mitochondria, the latter two preparations had low monoamine oxidase, low 3-hydroxybutyrate dehydrogenase (Table II), and high latent ATPase activities (Table IV). As regards 3-hydroxybutyrate dehydrogenase, it is interesting that this activity was considerably higher when measured under phosphorylating conditions in fresh preparations of mitochondria from 3683 and 3924A tumors (Table III) than in mitochondria frozen for 24 h at −20 °C, then thawed and assayed the next day (Table II). Since the relatively high latent ATPase activity of 3683 and 3924A mitochondria suggests structural damage and partial exposure of the ATPase enzyme, it is possible that the low 3-hydroxybutyrate dehydrogenase and monoamine oxidase activities are also consequences of the fragile nature of these mitochondria. This interpretation explains the discrepancy between the 3-hydroxybutyrate dehydrogenase activities of fresh and 24-h aged, frozen-thawed mitochondria from 3683 and 3924A tumors. Anyhow, it is important to note that, in contrast to the report of others [4], all the tumor mitochondria tested showed measurable to normal monoamine oxidase and 3-hydroxybutyrate dehydrogenase activities. Hence, the differences with normal rat-liver mitochondria are not qualitative, but only quantitative. Furthermore, it seems possible that these quantitative differences are related more to the structural stability of various particle preparations

than to the amount and activity of each enzyme present in the intact mitochondria.

A point of considerable interest, which is also in disagreement with the previous reports of others [2, 5, 7, 8], is that all the tumor mitochondria tested in our studies showed considerable ATPase activation by uncouplers. The compounds tested represented three major classes of uncouplers, namely, substituted phenols, phenylhydrazones, and salicylanilides. In all cases, maximal ATPase activation was obtained in the expected concentration range for each uncoupler. In addition, the maximal activities of uncoupler-stimulated ATPase were comparable to the maximal activities measured (a) for rat-liver mitochondria (Table V), and (b) after disruption of mitochondria by freeze-thawing (Table IV).

The reason for the discrepancy between our data and those of others is rather difficult to understand. Our results on the coupling properties of tumor mitochondria clearly indicate that the isolated mitochondria are in relatively good physical condition. Hence, it might be inferred that our isolation procedure is probably milder and less damaging to the particles than the procedures used by others. While, as discussed above, this may account for our finding of monoamine oxidase and 3-hydroxybutyrate dehydrogenase activities in all preparations of tumor mitochondria tested, it is difficult to consider partial damage to mitochondria as basis for the absence of uncoupler-stimulated ATPase activity reported by others. As shown in Table IV, deliberate membrane disruption by freeze-thawing of the tumor mitochondria resulted in the exposure of ATPase activities of 250–400 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} protein. Pedersen and Morris [8] obtained DNP-stimulated ATPase activities in this range for liver mitochondria, but their hepatoma mitochondria did not exhibit ATPase activities greater than 100 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} protein before or after addition of uncouplers. Thus, the problem of lack of uncoupler stimulation could not be attributed to a high starting (latent) ATPase activity of their preparations. As compared to our results, their latent ATPase activities in the absence of uncouplers should have had the capacity for severalfold stimulation upon addition of uncouplers or membrane disruption by freeze-thawing. Yet little or no activation, and in some cases a slight inhibition, was observed in the presence of uncouplers. One obvious difference between our preparations of tumor mitochondria and those of others is that we used Nagarse protease, instead of mechanical devices, to release the mitochondria of tumor cells. It is faintly possible, therefore, that protease treatment might have evoked uncoupler-stimulated ATPase activity in our preparations of tumor mitochondria. If so, we would then have to conclude further that such treatment confers additional "normal" properties to these mitochondria (such as 3-hydroxybutyrate dehydrogenase and monoamine oxidase activities, good respiratory control, and phosphorylation yield), which is not very likely.

Whatever the reason for the differences between our results and those of others, it is clear from the data presented here that when isolated and assayed by the procedures described above, hepatoma mitochondria do not appear to be qualitatively different from normal liver mitochondria in their coupling properties and ATPase activities. Therefore, it seems that possible effects of cellular transformation on mitochondrial function might have to be sought at a more subtle level than hitherto explored.

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