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Mitochondrial ATPase Activities of Hepatoma BW7756 and Ascites Tumor Cells

INFLUENCE OF MG^{2+} IONS, FREE FATTY ACIDS, AND UNCOUPLERS*

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ATPase activity of mitochondria isolated from Ehrlich ascites carcinoma and hepatoma BW7756 declines over time when assayed in the absence of added magnesium ions. In contrast, rat liver mitochondria when assayed in the presence or absence of added magnesium ions or the tumor mitochondria when assayed in the presence of added magnesium ions, maintain a constant level of ATPase activity when incubated in 0.25 M sucrose at 0°. Studies monitoring loss of endogenous magnesium from normal and tumor mitochondria reveal that the tumor mitochondria are much more leaky to magnesium, and that this loss of endogenous magnesium can be effectively blocked by addition of bovine serum albumin (albumin). Tumor mitochondria when isolated in the absence of albumin contain more than twice the levels of free fatty acids than rat liver mitochondria, and exhibit only a slight to moderate stimulation of ATPase activity in response to 2,4-dinitrophenol. In contrast, when tumor mitochondria are isolated in the presence of albumin, the resultant mitochondria contain lower levels of free fatty acids, and latent ATPase activity can be greatly stimulated by addition of 2,4-dinitrophenol. Adenine nucleotide translocase activity of tumor mitochondria is enhanced more than 5-fold in mitochondria isolated in the presence of albumin compared to tumor mitochondria isolated in the absence of albumin. In all assays, the maximum specific activity for tumor mitochondrial nucleotide translocation was less than 50% of that exhibited by rat liver mitochondria.

The results in this paper strongly indicate (*a*) the low uncoupler-stimulated ATPase activity in mitochondria isolated from Ehrlich ascites carcinoma and hepatoma BW7756 cells is due to high levels of endogenous free fatty acids that are inhibiting adenine nucleotide translocation, while simultaneously inducing the loss of endogenous magnesium; and (*b*) that when tumor mitochondria are isolated and incubated in the presence of albumin the high levels of uncoupler stimulation observed are due to the prevention of mitochondrial magnesium loss and activation of the adenine nucleotide translocation.

A working hypothesis concerning the transformation from the normal to the neoplastic state is that during this transition, the molecular mechanisms responsible for controlling cell growth are lacking or altered. Implicit in this theme, is that the ATP-generating reactions must somehow be responsive to this transition, and thus provide the neoplastic cell with sufficient quantities of ATP necessary for the accelerated biosynthetic reactions.

In most normal tissues, the mitochondria are primarily responsible for the production of ATP, whereas in many neoplastic tissues, high aerobic glycolytic activity, occurring in the cytoplasm, is largely responsible for ATP production. The phenomenon of high aerobic glycolysis had been vigorously studied and mitochondria isolated from a wide variety of neoplastic tissues were shown to possess low efficiency for ATP production and reduced responsiveness to uncoupler-stimulated ATPase activity (1-4). Pedersen and Morris (5) reported that ATPase activity of mitochondria isolated from various Morris hepatoma cell lines was not stimulated by uncouplers in contrast to the uncoupler-sensitive oxidative phosphorylation in these mitochondria, and proposed that a second uncoupler binding site is expressed during the transition from the normal to the neoplastic state. Recently, Kaschnitz *et al.* (6) reported essentially contradictory results to those of Pedersen and Morris when the same tumor mitochondria were isolated in the presence of 1% bovine serum albumin. However, Kaschnitz *et al.* did not comment on the necessity of using albumin[1] during mitochondria isolation. In fact, the precise mode of action of albumin on tumor mitochondria has not been clearly defined in the literature.

In the present paper, we have investigated the action of albumin on uncoupler-stimulated ATPase activity in tumor mitochondria and present evidence suggesting that albumin is able to affect the magnitude of uncoupler-stimulation by increasing adenine nucleotide translocase activity and preventing the simultaneous loss of endogenous magnesium ions in the tumor mitochondria. Moreover, the data presented

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[1] The abbreviation used is: albumin, bovine serum albumin.

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suggest that the action of albumin on mitochondrial activity is mediated through its ability to influence the endogenous free fatty acid levels. A preliminary report of these results has been presented (7).

EXPERIMENTAL PROCEDURES

Materials

ATP, phosphoenolpyruvate, pyruvate kinase, *m*-chlorocarbonylcyanide phenylhydrazone, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide, oligomycin, atractyloside, and sodium dodecyl sulfate were obtained from Sigma Chemical Co., Saint Louis, Mo. Bovine serum albumin, Pentex Fraction 5, was obtained from Miles Laboratories Inc. Research Products [^{14}C]ATP, 42 μOi/μmol, was purchased from New England Nuclear. Special enzyme grade sucrose was obtained from Schwarz/Mann Research Laboratories. All spectrophotometric determinations were performed with a Perkin Elmer model 124 dual beam spectrophotometer. Magnesium analysis was performed using a Perkin Elmer model 303 absorption spectrophotometer. Radioactivity was measured using liquid scintillation counting with a Beckman model LS 255 scintillation counter, with an NIH mixture: toluene, Triton X-100, and H_2O, 6:3:1 (v/v), respectively, with a final concentration of 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP)/liter of mixture. All samples were counted to less than 3% error. Male Sprague-Dawley rats and female CD-1 strain mice were obtained from Taconic Farms, Germantown, N. Y. C57LJ female mice were obtained from Jackson Laboratories, Bar Harbor, Maine, as was the hepatoma BW7756 cell line. Ehrlich Lettré cells were grown in female Ha/ICR or CD-1 mice (or both) and maintained by 9- to 12-day transfers. The line was a gift of Dr. T. Hauschka, Roswell Park Memorial Institute, Buffalo, N. Y.

Methods

ATPase Assays – Mitochondrial ATPase activity was determined using the procedures of Pedersen and Morris (5) and Chefurka (8). Following the former procedure (ATPase assay 1), approximately 1 mg of mitochondria was suspended in a medium containing 55 mM sucrose and 11 mM imidazole, pH 6.9, in a final volume of 0.97 ml for 5 min at room temperature. Samples were subsequently incubated at 30° for 5 min followed by the addition of 6 μmol of ATP in a volume of 30 μl into each tube. The reaction was allowed to continue for 10 min at 30° and stopped by the addition of 1.0 ml of 5% trichloroacetic acid. A zero time control was run for each set of determinations in which trichloroacetic acid was added before addition of substrate. Phosphate analysis was performed using the procedure of Lohman and Jendrassik (9). Determination of ATPase activity as described by Chefurka (ATPase assay 2) was carried out as follows. In a reaction medium containing 75 mM KCl, 1 mM sodium EDTA, pH 7.2, 50 mM sucrose, and 50 mM Tris/acetate, pH 7.2, approximately 1 mg of mitochondria was incubated for 5 min at room temperature in a final volume of 0.985 ml. The mixture was then transferred to a 30° water bath and incubated for 5 min followed by the addition of 3 μmol of ATP in a volume of 15 μl. After 10 min, the reaction was stopped as above and phosphate analysis performed as before (9). Other additions were made as indicated in the legends of the figures.

Adenine Nucleotide Translocase Assay – Mitochondrial uptake of [^{14}C]ATP at 0° was monitored essentially as described by Vignais *et al.* (10) except that ATP was substituted for ADP. In a final volume of 1.0 ml, 0.5 to 1.0 mg of mitochondria was suspended in a medium containing 110 mM KCl, 20 mM Tris/HCl buffer, pH 7.4, and 0.2 mM sodium/EDTA (KCl/Tris/EDTA medium), and incubated at 0° for 10 min. The reaction was started by the addition of 70 nmol of [^{14}C]ATP (5,000 to 7,000 cpm/nmol) and stopped at appropriate times by addition of 50 nmol of atractyloside followed immediately by centrifugation at 12,000 × *g* for 3 min. The pellet was dissolved in 100 μl of 0.5% sodium dodecyl sulfate, added to a vial containing 5.0 ml of NIH mixture, and counted by liquid scintillation.

Determination of Mitochondrial Magnesium Flux – Approximately 1 mg of mitochondria was incubated in the presence of 3.0 ml of 0.25 M sucrose and 5 mM Tris/Cl, pH 7.4, at room temperature for 30 min and separated by centrifuging for 5 min at 15,000 × *g*. The supernatant was saved and the pellet dissolved in 3.0 ml of 0.5% sodium dodecyl sulfate. Magnesium contents in the supernatant and pellet were determined by atomic absorption spectroscopy. Standard curves of 0 to 10.0 ppm of magnesium were run for each set of determinations in the presence of 0.25 M sucrose and 0.5% sodium dodecyl sulfate for internal corrections.

Free Fatty Acid Determination – The endogenous free fatty acid content in mitochondria was determined by the procedure of Falholt *et al.* (11) as modified in our laboratory[2] as follows. Approximately 1 mg of mitochondria, suspended in 3.0 ml of 0.25 M sucrose 5 mM Tris/Cl, pH 7.4, was added to a medium containing 2.5 ml of 50 mM NaH_2PO_4, titrated to pH 3.0 with 50 mM H_3PO_4, followed by a 30-min incubation at 0°. To this solution was added 7.5 ml of chloroform, heptane, and methanol (4:3:2, v/v) and the mixture was mixed on a Vortex mixer at room temperature for 3 min. All further manipulations were carried out at room temperature. The two phases were separated by centrifuging at 2500 rpm in a clinical centrifuge for 5 min, followed by removal of the top aqueous phase by aspiration. Absolute methanol (0.5 ml) was added to the remaining organic phase, to disrupt an occasional gel-like formation due to denatured protein, and the mixture was mixed on a Vortex mixer for 15 s. The methanol was removed by adding 5.0 ml of 0.01 N H_2SO_4, mixing on a Vortex mixer for 1 min, and separating the phases by centrifugation as described above; the remaining denatured protein and the aqueous phase were removed by aspiration. Then 4.0 ml of the organic phase was subsequently transferred to a clean screw cap test tube to which was added 2.0 ml of freshly prepared copper nitrate mixture containing 10 ml of 0.5 M $CuNO_3$, 10 ml of 1.0 M triethanolamine, and 6.0 ml of 1.0 N NaOH in a final volume of 100 ml to which was added 33 g of NaCl. The two phases were mixed by vortexing for 3 min and separated as described above. Subsequently, 3.0 ml of the top organic phase was transferred to another screw cap test tube, and 0.5 ml of 0.4% diphenylcarbazide in absolute ethanol was added. The pink color was allowed to develop for 15 min and the absorbance was measured at 553 nm. A standard curve was run for each set of determinations using 0 to 120 nmol of palmitic acid.

Isolation of Mitochondria – Rat liver mitochondria were isolated from male Sprague-Dawley rats weighing 250 to 350 g by the method of Johnson and Lardy (12) in a 0.25 M sucrose medium with or without 2 mg/ml of albumin. When mitochondria were isolated in the presence of albumin, the final pellet was also resuspended in the albumin/sucrose medium.

Isolation of Ehrlich ascites carcinoma mitochondria was performed using the procedure of Wu and Sauer (13). Female Ha/ICR (or CD-1) strain mice were injected with 0.1 ml of peritoneal ascites fluid and cells from five to seven mice were harvested 9 to 12 days later. Mice were killed by cervical dislocation and mitochondria were isolated as follows. Cells were collected by centrifuging at 900 × *g* for 5 min and washed with 1:4 diluted Krebs balanced salt solution. The erythrocyte-free tumor cells were subsequently washed twice with a medium containing 0.25 M sucrose, 1 mM EDTA, pH 4.5 (sucrose/EDTA medium). The washed cells were resuspended in 40 ml of the sucrose/EDTA medium containing 0.25 mM citric acid, and disrupted using a Dounce glass homogenizer followed by homogenization and centrifugation as before. The supernatants were combined and centrifuged for 10 min at 10,000 × *g*. The pellet was resuspended in 30 ml of 0.25 M sucrose and recentrifuged for 5 min at 900 × *g*. Mitochondria were separated from the supernatant by a 10,000 × *g* spin for 10 min, with the final pellet resuspended in a minimal volume of 0.25 M sucrose. Since it was desirable to obtain more tightly coupled mitochondria for comparison when albumin was used, albumin was added from the moment of cell breakage through the final resuspension of the mitochondrial pellet. Hepatoma BW7756 was transplanted and grown subcutaneously in C57LJ female mice and harvested 21 to 25 days later. Hepatoma-bearing mice were killed by cervical dislocation and mitochondria were isolated using the same procedure as for rat liver, in the presence or absence of albumin, with albumin present in the final resuspension of the mitochondrial pellet. Typical yields from three to four tumor-bearing mice were 50 to 60 mg of mitochondrial protein.

RESULTS

Stability of Mitochondrial ATPase to in Vitro Aging – Fig. 1 summarizes the results of experiments measuring the stability of ATPase activity in mitochondria isolated from Ehrlich ascites, hepatoma BW7756, and rat liver cells during incubation at 0°. As seen in Fig. 1*A*, ATPase activities in hepatoma BW7756 and Ehrlich ascites mitochondria declined by 50 and

[2] M. A. Nixon and S. H. P. Chan, manuscript to be submitted.

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78%, respectively, when assayed at 21 h in the absence of added magnesium ions, whereas ATPase activity in rat liver mitochondria increased slightly. However, as seen in Fig. 1*B*, when 5.0 mM $MgCl_2$ was included in thè assay medium, mitochondrial ATPase activity from all three tissues was constant during the same time period. In view of the fact that mitochondrial ATPase requires magnesium for its activity, we interpret these results to indicate that possibly magnesium was leaking out of the mitochondria during the incubation, resulting in the lowered ATPase activity.

Magnesium Loss in Normal and Tumor Mitochondria – To examine the hypothesis that the decline of ATPase activity in tumor mitochondria might be due to the loss of endogenous magnesium during incubation at 0°, the following sets of experiments were conducted. Mitochondria from rat liver, Ehrlich ascites, and hepatoma BW7756 were incubated in the presence of a sucrose/Tris buffer medium, as described under "Experimental Procedures," at room temperature for 30 min. After this period of incubation, the mitochondria were separated from the medium by centrifugation and magnesium determinations were performed on the supernatant and pellet. By simply comparing the amount of magnesium found in the supernatant to the total amount originally present prior to the incubation procedure, the extent to which mitochondria lost endogenous magnesium was calculated. Data presented in the histogram of Fig. 2 are the results of such experiments which clearly illustrate that both Ehrlich ascites and hepatoma BW7756 mitochondria are much more susceptible to the loss of endogenous magnesium than are rat liver mitochondria. For instance, whereas most of the endogenous magne-

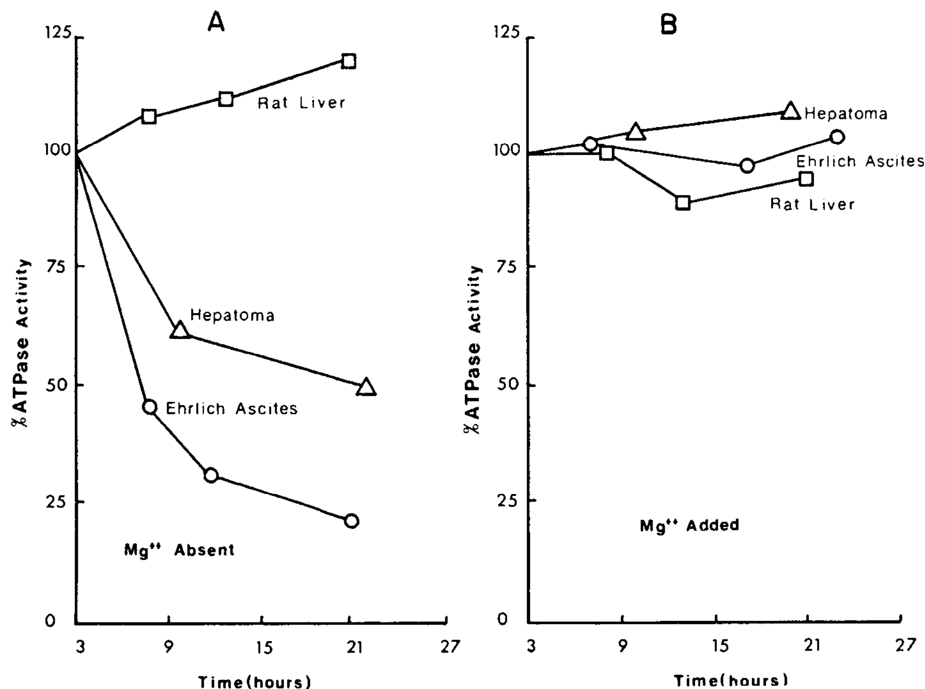

FIG. 1. Stability of ATPase activity of mitochondria from rat liver, Ehrlich ascites cells, and hepatoma BW7756. *A*, ATPase activity in mitochondria isolated from rat liver, Ehrlich ascites, and hepatoma BW7756 cells during incubation at 0°. Time indicated on the *abscissa* is the number of hours after killing of animals when ATPase assay was performed. ATPase assay 1 procedure was used as described under "Experimental Procedures." Initial enzyme specific activities at 3 h were 12, 24, and 41 μmol of ATP hydrolyzed/mg of protein/min for hepatoma BW7756, rat liver, and Ehrlich ascites mitochondria, respectively. *B*, assay procedure was identical as in *A*, except that 5.0 mM $MgCl_2$ was present in the assay medium. Initial enzyme specific activities at 3 h were 33, 20, 82 nmol/mg/min for hepatoma BW7756, rat liver, and Ehrlich ascites mitochondria, respectively. Substituting $MgSO_4$ for $MgCl_2$ in the assay medium gave identical results.

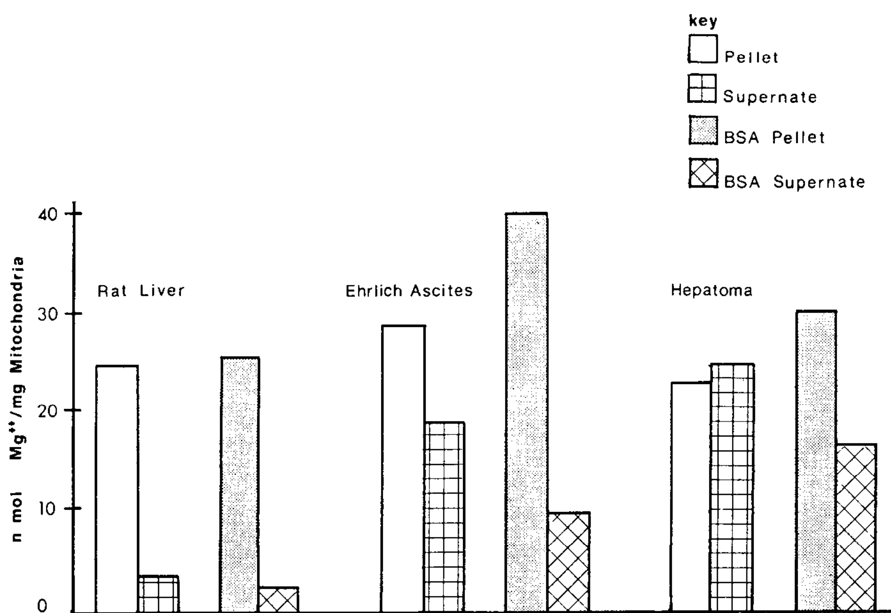

FIG. 2. Contents of magnesium in mitochondrial preparations. Approximately 1 mg of rat liver, Ehrlich ascites, and hepatoma BW7756 mitochondria were incubated in the presence of 3 ml of 0.25 M sucrose, 5 mM Tris/Cl, pH 7.4, with and without 1 mg of bovine serum albumin (*BSA*) as indicated for 30 min at room temperature. Mitochondria were separated from supernatant by centrifugation, and magnesium determinations were performed on the supernatant and pellet as described under "Experimental Procedures."

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sium is retained in the mitochondrial pellet from rat liver, with only a small amount of magnesium appearing in the supernatant, 20 and 25 nmol of magnesium/mg of mitochondria were found in the supernatants of Ehrlich ascites and hepatoma BW7756 mitochondria incubated in the medium as described above. When 1 mg of albumin was added during the incubation, the loss of endogenous magnesium was greatly reduced. This protective effect of albumin on the mitochondrial magnesium loss was particularly prominent with the tumor mitochondria. Specifically, as shown in the figure, the loss of endogenous magnesium in Ehrlich ascites and hepatoma BW7756 mitochondria was reduced to 10 and 18 nmol/mg, respectively. Albumin added to rat liver mitochondria also resulted in a slight, but reproducible, reduction in the loss of endogenous magnesium.

Since the above experiments only demonstrate that tumor mitochondria lose significant amounts of magnesium ion at room temperature, it was of interest to investigate to what extent this same phenomenon occurred at 0°. When the mitochondria from the three different tissues were incubated at 0°, an increased amount of magnesium was found leaking out of the mitochondria. After 24 h of incubation, 64 and 57% of the total magnesium contents had leaked out of Ehrlich ascites and hepatoma BW7756 mitochondria, respectively, compared to 19% for rat liver mitochondria. In addition, the total magnesium contents in mitochondria freshly isolated from rat liver, Ehrlich ascites cells, and hepatoma BW7756 were, respectively, 31.7, 45.0, and 49.1 nmol/mg of mitochondrial protein.

When 2 mg/ml of albumin was included in the isolation medium, the magnesium contents of mitochondria from the three tissues were, respectively 35.5, 59.2, and 60.7 nmol/mg of mitochondrial protein. In all cases, there is a higher magnesium content in mitochondria isolated in the presence of albumin than in sucrose, suggesting that a partial depletion of endogenous magnesium occurs during isolation of mitochondria in the presence of sucrose alone. In addition, experimental results reveal that mitochondria isolated in the presence of albumin were found to be much less leaky to magnesium when incubated under conditions described in Fig. 2. Finally, the results also indicate that both Ehrlich ascites and hepatoma BW7756 mitochondria have a higher total magnesium content than rat liver mitochondria/mg of protein basis.

Effects of Oleic Acid and Albumin on Mitochondrial Magnesium Loss – Since mitochondria were shown to contain free fatty acids (14), we suspected that the inhibition of mitochondrial magnesium loss by albumin may be related to its fatty acid binding capacity. In a similarly designed experiment as that described in Fig. 2, we could show the loss of endogenous magnesium from rat liver mitochondria during a 30-min incubation at room temperature in the presence of the added oleic acid (Fig. 3). As seen in Fig. 3*A*, when mitochondria are incubated in the presence of increasing concentrations of oleic acid, there is a corresponding increased loss of magnesium, with a maximal loss of 18 nmol/mg of mitochondria at 100 μM concentration of the fatty acid. However, as shown in Fig. 3*B*, if rat liver mitochondria are first preincubated with the indicated amounts of albumin, followed by the addition of 100 μM oleic acid, the subsequent loss of endogenous magnesium induced by the fatty acid is largely prevented. Thus, the results in Fig. 3 demonstrate that fatty acids themselves are capable of inducing the loss of endogenous magnesium, and that albumin is largely able to prevent this effect.

To further investigate the action of albumin on the inhibition of mitochondrial magnesium loss, the following series of experiments was performed. Freshly isolated rat liver mitochondria were rapidly freeze-thawed and aliquots of mitochondria were immediately incubated under conditions described in Fig. 2. After a freeze-thaw treatment, rat liver mitochondria become very leaky to magnesium, losing more than 15 nmol/mg of mitochondria after 30 min at room temperature. However, if the freeze-thawed mitochondria are incubated in a medium containing albumin, the loss of endogenous magnesium is, again, largely prevented. Since it is expected that a freeze-thaw treatment results in the rupture of the outer mitochondrial membrane, these results suggest that albumin may unexpectedly exert an effect on the damaged mitochondrial membrane to reduce its magnesium loss.

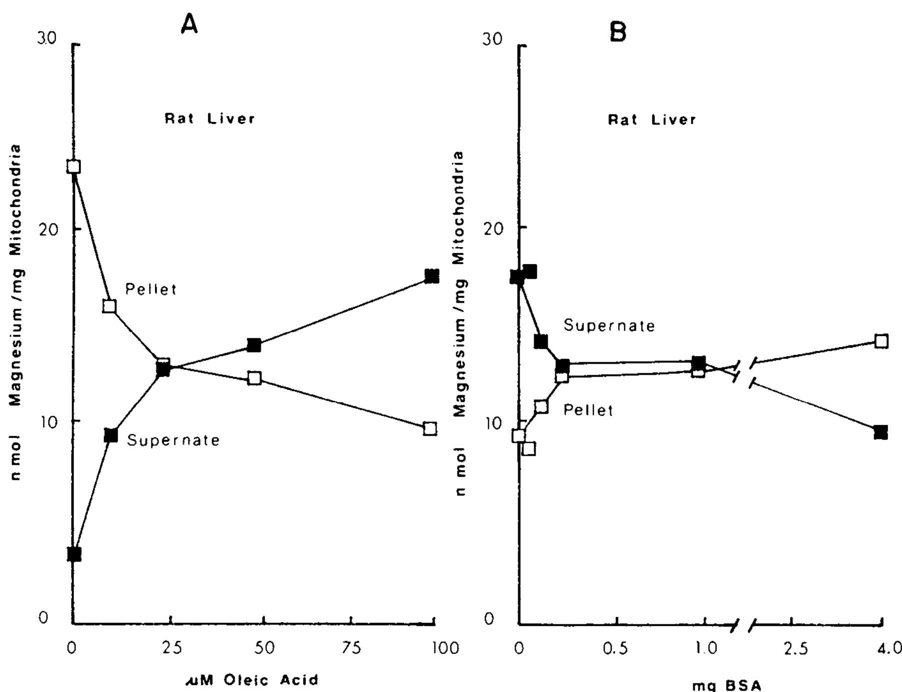

FIG. 3. Effects of oleic acid and albumin on contents of magnesium in rat liver mitochondria. *A*, freshly isolated rat liver mitochondria were incubated under the same conditions as described in Fig. 2, in the presence of the indicated concentrations of oleic acid. Magnesium content in the supernatant and pellet were determined as described under "Experimental Procedures." *B*, rat liver mitochondria were incubated under the same conditions as described in Fig. 2 with the following modifications. The freshly isolated mitochondria were first preincubated in the presence of the indicated amounts of bovine serum albumin for 5 min followed by the addition of 100 μM oleic acid. After an additional 25 min of incubation, the mitochondria were centrifuged and magnesium contents of the supernatant and pellet were determined. *BSA*, bovine serum albumin.

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The concept that free fatty acids can induce the loss of endogenous magnesium from mitochondria leads to the prediction that tumor mitochondria are more leaky to magnesium than their normal counterpart because they contain a higher level of endogenous free fatty acids. Experimental data confirm this prediction that mitochondria from Ehrlich ascites cells and hepatoma BW7756 contain, respectively, 88.4 and 80.5 nmol of free fatty acids/mg of mitochondrial protein while rat liver mitochondria contain only 38.7 nmol/mg of protein. These results demonstrate that tumor mitochondria freshly isolated in sucrose contains more than double the amount of endogenous free fatty acid present in rat liver mitochondria. Further evidence supporting this concept is that tumor mitochondria isolated in the presence of albumin, in addition to being less leaky to magnesium, contain a lower concentration of free fatty acids (82.7 and 71.6 nmol/mg of mitochondrial protein for Ehrlich ascites and hepatoma cells, respectively); while rat liver mitochondria isolated in the presence of albumin contain essentially the same amount of free fatty acids (37.6 nmol/mg of protein).

Since assays monitoring the inhibition of mitochondrial magnesium loss by albumin were performed at room temperature, it was of interest to also examine the effects of albumin on the removal of mitochondrial free fatty acids at the same temperature. Fig. 4 shows that when freshly isolated mitochondria were incubated for 30 min with the indicated amounts of albumin, a maximum of 22 and 30.5% of the total free fatty acid content was removed in hepatoma BW7756 and Ehrlich ascites mitochondria, respectively, compared to less than 4% with rat liver mitochondria, thereby indicating that either a higher percentage of fatty acids are more easily removed or are more accessible to albumin in tumor *versus* normal mitochondria. Although the amounts of fatty acids depleted from the tumor mitochondria are low, data presented above would suggest that removal of fatty acids may account, at least in part, for the action of albumin on the prevention of mitochondrial magnesium loss. Finally, when mitochondria were incubated at 0°, there was a gradual increase in free fatty acid content (probably due to phospholipase activity) paralleled, as mentioned previously, by an increased loss of endogenous magnesium. Specifically, it was found that after 24 h, the free fatty acid content in tumor mitochondria had increased to over 100 nmol/mg of mitochondria.

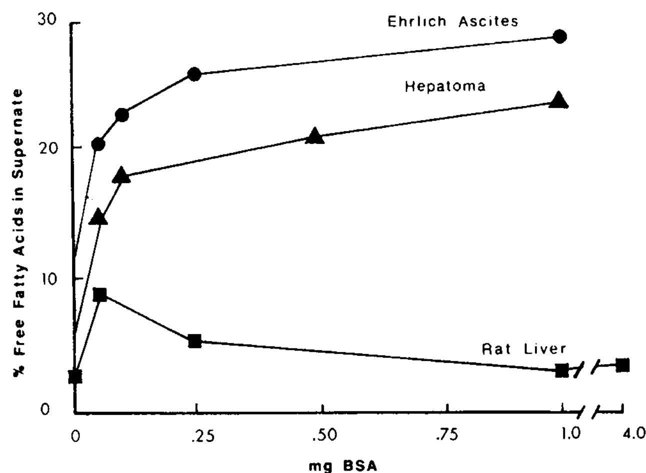

FIG. 4. Effects of albumin on contents of free fatty acids in mitochondrial preparations. Freshly isolated rat liver, Ehrlich ascites, and hepatoma BW7756 mitochondria were incubated under conditions as described in Fig. 2 with the indicated amounts of albumin. Fatty acid determinations were performed as described under "Experimental Procedures." Initial fatty acid levels were 38, 88, and 69 nmol/mg for rat liver, Ehrlich ascites, and hepatoma BW7756 mitochondria, respectively. *BSA*, bovine serum albumin.

Mitochondrial ATPase Activity in Presence of 2,4-Dinitrophenol — Having determined that albumin was able to maintain the magnesium and lower the fatty acid levels in isolated tumor mitochondria, we performed the following series of experiments to investigate these effects on the activity of adenine nucleotide translocase and mitochondrial ATPase. Fig. 5 describes the response of mitochondrial ATPase activity to the uncoupler 2,4-dinitrophenol in freshly isolated Ehrlich ascites mitochondria. It is clearly seen that in the absence of added magnesium, the addition of 200 μM 2,4-dinitrophenol increases ATPase activity by approximately 2.5-fold. However, if the mitochondria are subsequently incubated at 0° and assayed at the indicated times, the uncoupler-stimulated ATPase activity rapidly declines. If magnesium is included in the assay medium, however, ATPase activity is constant during the same time period.

Since free fatty acids are known uncouplers of oxidative phosphorylation, any increase in their endogenous level should lead to an increase in ATPase activity. Thus, the observed loss of 2,4-dinitrophenol-activated ATPase activity incurred during incubation at 0° is, initially, somewhat dis-

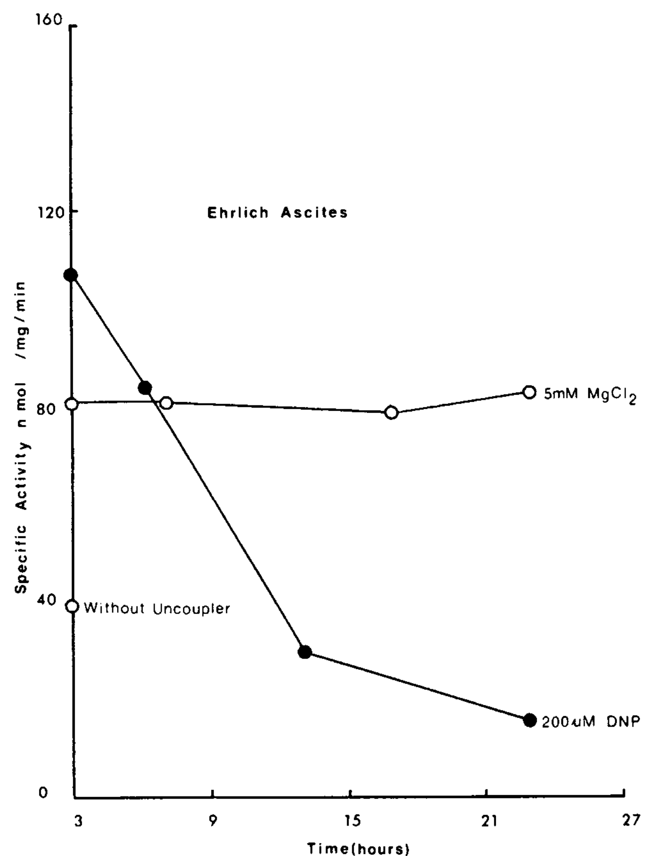

FIG. 5. Effects of 2,4-dinitrophenol and magnesium on ATPase activity of Ehrlich ascites mitochondria. Ehrlich ascites mitochondria were incubated at 0° and assayed at the indicated times according to the ATPase assay 1 procedure described under "Experimental Procedures" with 200 μM 2,4-dinitrophenol and with 5 mM $MgCl_2$. Substituting 2,4-dinitrophenol with 10 μM *m*-chlorocarbonylcyanide phenylhydrazone or 0.15 μM 5-chloro-3-*t*-butyl-2'-chloro-4-nitrosalicylanilide gave similar results. *DNP*, 2,4-dinitrophenol.

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turbing particularly since fatty acid levels are known to increase as a consequence of aging. However, the results in Fig. 5 are justified when it is recalled that during the same time period most of the endogenous magnesium has leaked out of the mitochondria. In addition, Fig. 6 shows that fatty acids will only increase ATPase activity when assayed in the presence of magnesium. Specifically, in the presence of 2.5 mM $MgSO_4$, oleic acid will increase ATPase activity in Ehrlich ascites mitochondria by approximately 3-fold, with maximum activation achieved whether or not 2,4-dinitrophenol is present. Also in the absence of magnesium, oleic acid will inhibit both 2,4-dinitrophenol-stimulated ATPase activity, and that of the untreated mitochondria.

It is known that the concentrations of 2,4-dinitrophenol used in Fig. 5 can activate ATPase activity in rat liver mitochondria by 5- to 10-fold, and that this response is dependent on the presence of potassium ions (15, 16). Since the uncoupler assay described in Fig. 5 was performed in the absence of added potassium, we decided to examine whether or not the only moderate activation of ATPase activity was due to the absence of this ion in the assay medium. Fig. 7 compares the effects of increasing concentrations of 2,4-dinitrophenol on mitochondria freshly isolated in the presence of sucrose, and assayed in a medium containing 75 mM KCl. It can be clearly seen that, in marked contrast to rat liver mitochondria, which exhibit nearly a 5-fold increase in ATPase activity, hepatoma BW7756 mitochondria are almost completely insensitive to uncoupler stimulation, whereas Ehrlich ascites mitochondrial ATPase activity is only moderately activated. Thus, these results are somewhat in agreement with those previously reported (5) showing that uncoupler-stimulated ATPase activity from various Morris hepatoma cell lines was almost completely absent, and that uncoupler activation could not be restored upon addition of potassium ions.

The fact that fatty acids have been demonstrated to be inhibitors of 2,4-dinitrophenol stimulation (Fig. 6), which is probably partly due to the induced loss of endogenous magnesium, and that tumor mitochondria contain high levels of fatty acids raises the following question: What would be the effect on uncoupler-stimulated ATPase activity, when tumor mitochondria are isolated under conditions in which they contain a lower concentration of free fatty acids, and are less leaky to magnesium? The answer to this question is shown in Fig. 8.

It has been previously determined that if tumor mitochondria are freshly isolated in the presence of albumin, the resultant mitochondria are less leaky to magnesium and contain a lower content of free fatty acids. Under these conditions, tumor mitochondrial ATPase activity can be activated up to the level exhibited by normal mitochondria. Specifically, a titration with 2,4-dinitrophenol of mitochondria isolated in the presence of albumin resulted in a maximum stimulation of more than 4.5-fold for both hepatoma BW7756 and Ehrlich ascites mitochondria, compared to a similar

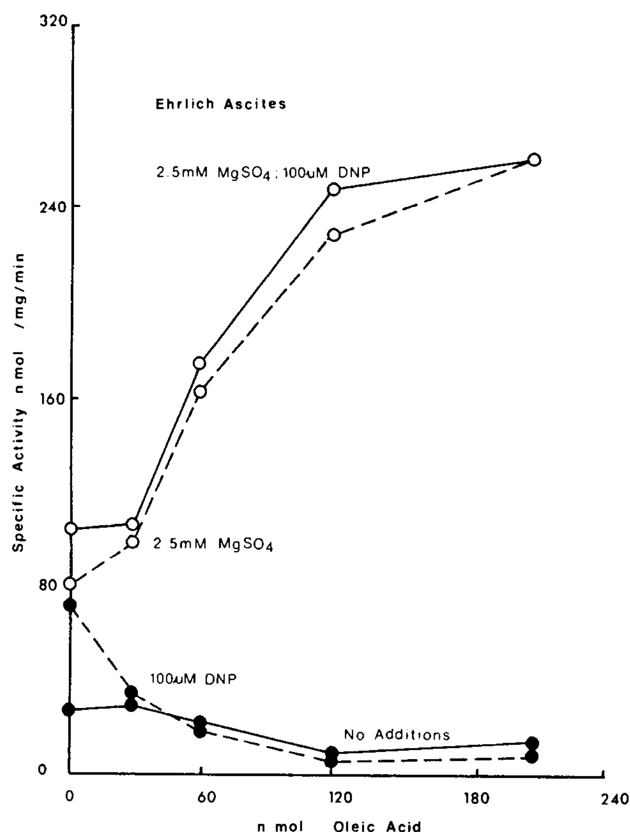

FIG. 6. Effects of oleic acid, 2,4-dinitrophenol, and magnesium on ATPase activity of Ehrlich ascites mitochondria. Ehrlich ascites mitochondria were assayed using the ATPase assay 2 procedure containing 75 mM KCl as described under "Experimental Procedures." Additions of 2.5 mM $MgSO_4$ and 100 μM 2,4-dinitrophenol are indicated. *DNP,* 2,4-dinitrophenol.

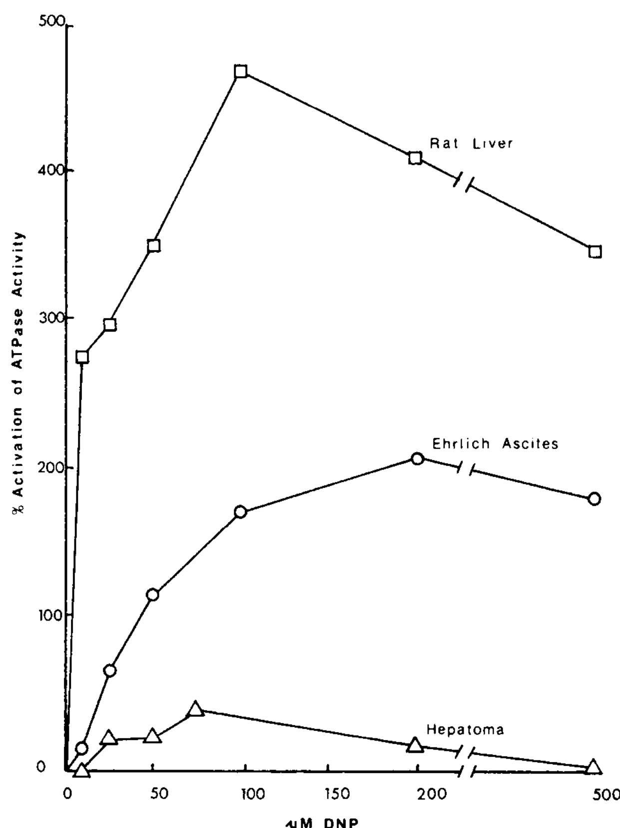

FIG. 7. Effects of 2,4-dinitrophenol on ATPase activities of mitochondrial preparations. Rat liver, Ehrlich ascites, and hepatoma BW7756 mitochondria were freshly isolated in the presence of sucrose and assayed according to the ATPase assay 2 procedure described under "Experimental Procedures." Initial enzyme specific activities were 6, 16, and 40 nmol/mg/min for hepatoma BW7756, Ehrlich ascites, and rat liver mitochondria, respectively. *DNP,* 2,4-dinitrophenol.

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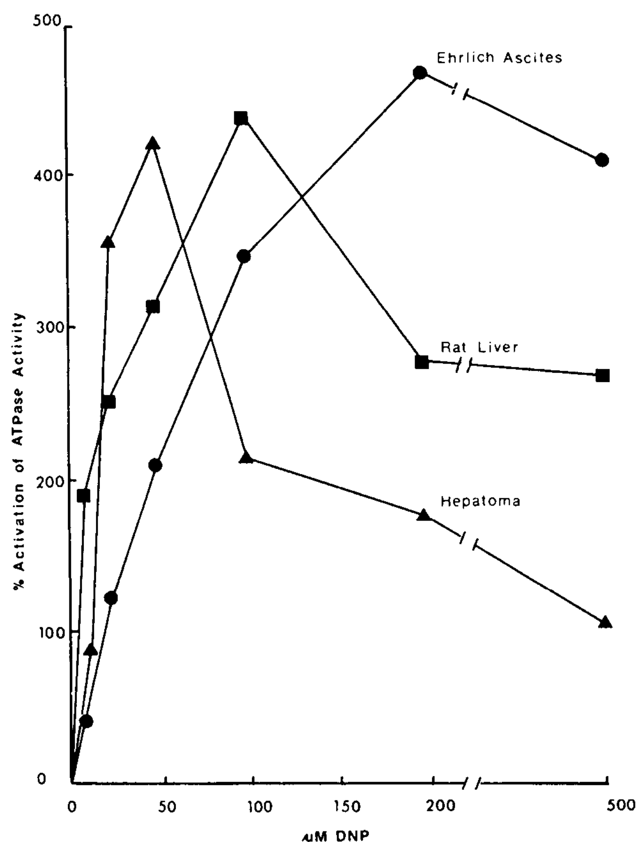

FIG. 8. Effects of 2,4-dinitrophenol on ATPase activities of mitochondria isolated in the presence of albumin. Rat liver, Ehrlich ascites, and hepatoma BW7756 mitochondria were isolated in the presence of 0.25 M sucrose supplemented with 2 mg/ml of albumin and immediately assayed using the ATPase assay 2 procedure as described under "Experimental Procedures." Initial specific enzyme activities were 8, 29, and 57 nmol/mg/min for hepatoma BW7756, Ehrlich ascites, and rat liver mitochondria, respectively. *DNP*, 2,4-dinitrophenol.

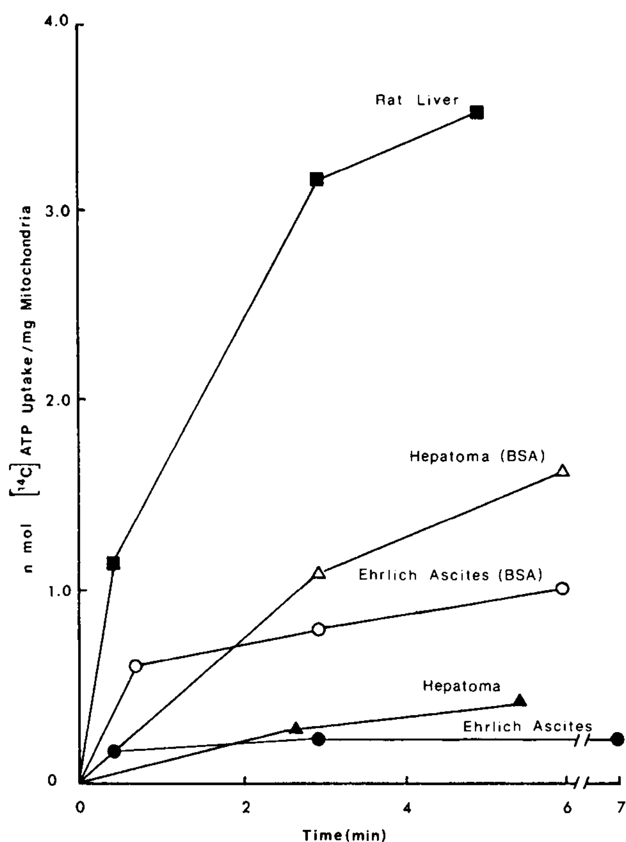

FIG. 9. Adenine nucleotide translocase activities of mitochondrial preparations. Rat liver, Ehrlich ascites, and hepatoma BW7756 mitochondria were isolated in the presence and absence of 2 mg/ml of albumin indicated and incubated at 0° in a KCl/Tris/EDTA medium as described under "Experimental Procedures" for 10 min followed by the addition of 70 nmol of [^{14}C]ATP in the presence of 1.3 units of pyruvate kinase, 26 μM phosphoenolpyruvate, and 26 μM $MgCl_2$. The incubations were stopped at the indicated times with 50 μM atractyloside. The mitochondrial pellets were centrifuged down and counted for radioactivity as described in the text. *BSA*, bovine serum albumin.

stimulation in rat liver mitochondria. It is also clear that, whereas rat liver mitochondria have a maximum activation of ATPase activity at 100 μM 2,4-dinitrophenol, hepatoma BW7756 and Ehrlich ascites mitochondria exhibit maximum activation at 50 and 200 μM 2,4-dinitrophenol, respectively.

Mitochondrial Adenine Nucleotide Translocase Activity— Data presented thus far have emphasized the fact that the fatty acid-induced magnesium loss in tumor mitochondria may be responsible for the low levels of uncoupler-stimulated ATPase activity. However, in intact mitochondria, ATPase activity is also dependent on the availability of ATP and thus it was of interest to determine whether the elevated fatty acid levels had an effect on ATP translocation. Data in Figs. 9 and 10 are the results of experiments measuring the rate of uptake of [^{14}C]ATP by mitochondria when assayed at 0° according to the procedure of Vignais *et al.* (10). Fig. 9 shows that tumor mitochondria isolated in the presence of albumin, which exhibit high levels of uncoupler stimulation, also have a greater than 5-fold increased adenine nucleotide translocase activity compared to the sucrose-isolated tumor mitochondria. For instance, after a 7-min incubation with the labeled nucleotide, Ehrlich ascites mitochondria freshly isolated in sucrose accumulated only 0.26 nmol of ATP/mg compared to 1.36 nmol of ATP/mg with mitochondria isolated in the presence of albumin. Slightly higher values of ATP uptake were observed using freshly isolated hepatoma BW7756 mitochondria. After only a 6-min incubation, 0.31 and 1.67 nmol of ATP/mg were accumulated in hepatoma mitochondria isolated without and with albumin, respectively. Finally, in all cases, the maximum translocase activity measured in tumor mitochondria was less than 45% of that of rat liver mitochondria.

Thus, the data clearly imply that the elevated free fatty acid levels in tumor mitochondria result in an inhibition of nucleotide translocation and that albumin is able largely to reverse this effect. Further evidence supporting this view is presented in Fig. 10, in which the effects of exogenously added fatty acids were monitored on nucleotide translocation. It can be clearly seen that when rat liver, Ehrlich ascites, or hepatoma BW7756 mitochondria are incubated in the presence of additional 100 μM oleic acid, ATP uptake is almost completely abolished, thereby directly demonstrating that fatty acids themselves are also inhibitors of nucleotide translocation although much less potent than fatty acyl coenzyme A (17). And finally it was observed that if 1 mg of albumin was added to rat liver mitochondria that had been preincubated with 100 μM oleic acid, the albumin was able effectively to reverse the inhibitory action of the fatty acid on nucleotide translocation.

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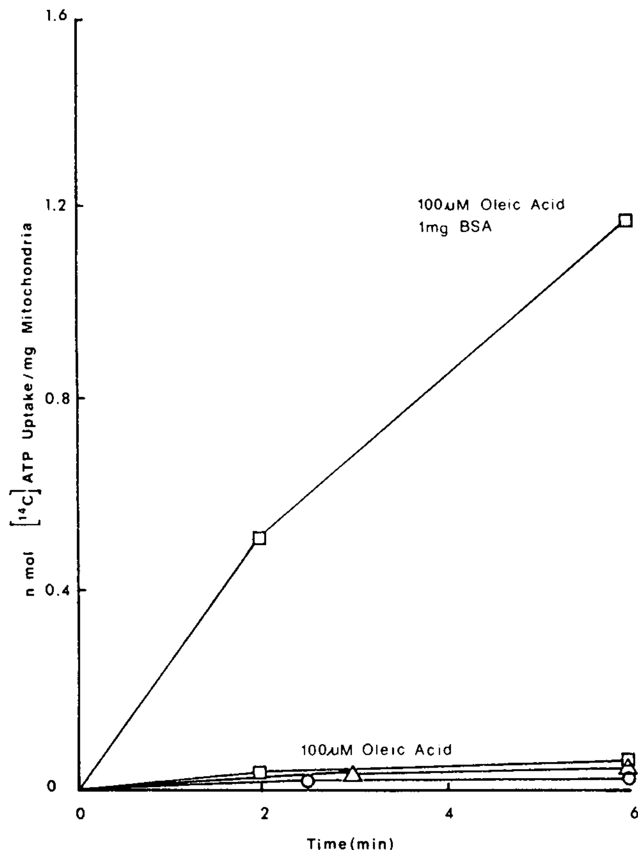

FIG. 10. Effects of oleic acid and albumin on adenine nucleotide translocase activities of mitochondrial preparations. Rat liver (□——□), Ehrlich ascites (○——○), and hepatoma BW7756 (△——△) mitochondria were isolated at 0.25 M sucrose and incubated in a medium at 0° containing 100 μM oleic acid for 10 min before addition of substrate for adenine nucleotide translocase activity as described in the text. Rat liver mitochondria incubated with 100 μM oleic acid for 10 min followed by the addition of 1 mg of albumin. After an additional 5-min incubation, assays were started by the addition of 70 nmol of [^{14}C]ATP and stopped at the indicated times with 50 μM atractyloside as described for Fig. 9. *BSA*, bovine serum albumin.

DISCUSSION

Properties of ATPase Activity of Tumor Mitochondria—The reduced responsiveness of mitochondrial ATPase from neoplastic tissues to uncoupling agents appear to be a frequently observed phenomenon (1-5). Pedersen *et al.* (5, 18) observed that mitochondria isolated from the Morris hepatoma cell lines as well as two ascites cells were almost completely resistant to uncoupler stimulation in sharp contrast to normal liver mitochondria. On the other hand, since neither the forward and reverse energy-dependent reactions in one of the hepatoma mitochondria are different from normal tissue mitochondria; namely they are all sensitive to uncouplers, Pedersen proposed that perhaps the hepatoma mitochondria in particular and tumor mitochondria in general, contain two principal sites of action for uncoupling agents; one of which becomes expressed during the normal to neoplastic transition, and the other is common to both normal and neoplastic tissues. However, recently Kaschnitz *et al.* (6) re-examined the observations of Pedersen, and found that when the same Morris hepatoma mitochondria were isolated in the presence of higher albumin concentration, high levels of uncoupler-stimulated ATPase activity could be achieved. Kaschnitz *et al.* could not explain the apparent discrepancy. Assays monitoring uncoupler stimulation are traditionally performed in the absence of added magnesium, in order to avoid confusing magnesium *versus* uncoupler stimulation. Unfortunately since the assays determining uncoupler stimulation by Kaschnitz *et al.* were performed in the presence of added magnesium, it is difficult to determine what proportion of the observed ATPase activity is due to magnesium activation and what proportion to uncoupler stimulation.

Results reported in this study explain the discrepancy between the studies of Pedersen *et al.* (5, 18) and Kaschnitz *et al.* (6). The stability of ATPase activity in mitochondria isolated from both normal and the tumor tissues was found to be dependent on endogenous magnesium content of the mitochondrial preparation. It is clear that the prerequisite condition for uncoupler to stimulate tumor mitochondrial ATPase activity is that the tumor mitochondria must contain certain endogenous concentrations of magnesium ions. If and when some magnesium ions are leaked out of the tumor mitochondria, added uncoupler will have no effect. Only when magnesium ions are retained in the mitochondria as in the case of using 2% albumin in the isolation medium, uncouplers will greatly stimulate ATPase activity as observed by Kaschnitz *et al.* Since these observations were obtained with both a solid tumor of intermediate growth rate and an ascites cell line of relatively fast growth rate,[3] we believe the leakiness of magnesium ions in tumor mitochondria is a widespread characteristic of a variety of tumors of different growth rates and degrees of differentiation. Thus, it seems that it is not necessary to postulate an additional site of action for uncoupling agents during the normal to neoplastic transition. The apparent lack of uncoupler-stimulated ATPase in tumor mitochondria is but a direct consequence due to the leakiness of magnesium in these tumor mitochondria. In view of the results reported here, we agree with a previous report (19) that the degree of respiratory control is not the only critical parameter for measuring "intactness" of a mitochondrial preparation, the effect of added Mg^{2+} on the respiratory control ratio (19, 20) and on ATPase activity is also a useful parameter to determine the "intactness" of a preparation.

Free Fatty Acid Levels in Tumor Mitochondria—The free fatty acid levels in both Ehrlich ascites and the hepatoma mitochondria are more than double those of rat liver mitochondria. It was also observed that the already high levels of free fatty acids in the tumor mitochondria would be further increased by incubation *in vitro*. High mitochondrial free fatty acids content is not restricted to diseased tissues of origin, but high levels were also found in liver mitochondria from experimentally alloxan-induced diabetic rats (21) resulting in uncoupling of oxidative phosphorylation. *In vitro* aging on mouse liver mitochondria (8, 22) and rat liver mitochondria (14) resulted also in an increase in endogenous free fatty acid levels probably due to the phospholipase activity (23) with a paralleled uncoupling of oxidative phosphorylation. This study extended these observations in tumor mitochondria and the results indicate that the modes of action of the elevated free fatty acids are (*a*) inducing the loss of magnesium ions in these mitochondria and (*b*) inhibiting the adenine nucleotide translocase activity. Since free fatty acids are known to bind divalent cations (24, 25), the elevated free fatty acids become inhibitors of uncoupler-stimulated ATPase activity either di-

[3] Mitochondria isolated from L1210 ascites tumor cells in our laboratory were also found to be similarly leaky to magnesium ions and contain higher levels of free fatty acids.

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rectly by depleting available magnesium ions or by inhibiting the entry of ATP into the tumor mitochondria or both. In any case, it is clear that in the presence of added magnesium ions, fatty acids greatly stimulate tumor mitochondrial ATPase activity regardless of the presence or absence of added uncouplers. It is also known that uncoupling agents can activate phospholipase activity (26) thereby increasing the free fatty acid content, and that when specific inhibitors of the phospholipase are incubated with isolated mitochondria energy-linked functions are maintained for long periods of time (23, 27). It is interesting to note in the present study that in Ehrlich ascites mitochondria, complete loss of uncoupler-stimulated ATPase activity and respiratory control was accompanied by an increase in free fatty acid levels of about 17 nmol/mg, in agreement with two previous reports (22, 27), suggesting that the hydrolyzed phospholipids must have occupied key sites in the maintenance of energy coupling.

Mechanism of Action of Albumin—Serum albumin has been routinely used in the isolation and assaying mitochondrial preparations for its high affinity of depleting free fatty acids (28, 29); the latter compounds have been extensively documented to be inhibitors of oxidative phosphorylation (30–35). However, few studies have been reported delineating the precise mechanism of action of serum albumin on mitochondrial function. Results in this report demonstrate that not only tumor mitochondria isolated in the presence of albumin contain less fatty acids but also that the presence of albumin can largely prevent the loss of magnesium ions from tumor mitochondria. This last finding was unexpected and turns out to be the key observation in explaining the apparent discrepancy in the literature concerning the apparent lack of uncoupler-stimulated ATPase activity in tumor mitochondria. It should be emphasized that although substantial alterations in enzyme activity were seen with mitochondria isolated in the presence of albumin, the albumin medium had no observable effect on the apparent mitochondrial ultrastructure and morphology as concluded from examining the thin sections of mitochondrial preparations in the electron microscope, which is in agreement with a previous report (36). It is clearly demonstrated in this report that albumin can effectively block the loss of magnesium ion from both intact tumor and freeze-thawed or fatty acid-treated normal mitochondria. This raises the question as to the location of the albumin binding site in the mitochondrial membrane. Following the techniques of Sternberger (37) and the procedures of Hackenbrock and Hammon (38) we have obtained preliminary results using ferritin-conjugated albumin antibodies which show that the ferritin · antibody complex is located exclusively on the outside of the outer membrane, thus suggesting that the prevention of magnesium loss in intact tumor mitochondria by albumin is mediated through its interaction at the outer membrane. It was of interest to note that only a small amount of albumin was necessary to accomplish this effect.

Factors That Are Responsible for Low Specific ATPase Activity in Tumor Mitochondria—Since it has been demonstrated in this study that free fatty acids themselves are inhibitors of adenine nucleotide translocation, it seems reasonable to anticipate that the low specific ATPase activity may in part be due to reduced translocase activity in the tumor mitochondria. Indeed, in agreement with a previous report (39), the rate of nucleotide translocation of ATP was less than 25% of that of rat liver mitochondria. Furthermore, it should be emphasized that the low specific activity of ATPase in tumor mitochondria could be a function of simply fewer F_1 molecules present in tumor mitochondria. Many mitochondrial components in tumor tissues, such as cytochromes (40) and β-hydroxybutyrate dehydrogenase (41) have been shown to be in lower concentration in comparison with normal tissue mitochondria. Indeed it was observed in our laboratory that when sonicated submitochondrial particles were prepared from tumor mitochondria, the maximum specific ATPase activities obtained were 303 and 130 nmol/mg/min for Ehrlich ascites and hepatoma BW7756, respectively, compared to approximately 650 nmol/mg/min for rat liver submitochondrial particles. Finally, it is known that in normal mammalian mitochondria, ATPase activity is suppressed by the presence of an endogenous inhibitor protein (42, 43). Preliminary studies in our laboratory on the isolation of the endogenous inhibitor protein from Ehrlich ascites mitochondria have indicated that a 3- to 4-fold higher quantity of endogenous inhibitor protein can be extracted from the tumor mitochondria. This observation is of particular interest since it has been demonstrated that the inhibitor protein may also interact with the adenine nucleotide translocase enzyme (44), thereby exerts a regulatory function during energy conservation.

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