Phosphorylation and ATPase Activity of Liver Mitochondria from Swiss Mice Bearing Krebs-2 Carcinoma.* (29462)

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The massive depletion of carcass fat during the growth of Walker carcinosarcoma-256 in the rat has been reported by Mider *et al* (1) and Haven *et al*(2). The latter authors have related this phenomenon to the tumor mass. Since the caloric expenditure of such tumor-bearing animals was greater than that of controls, one possible explanation was that fat was mobilized to meet the caloric requirements of tumor protoplasm synthesis(3,4).

Recently(5) it has been observed that Swiss mice bearing Krebs-2 carcinoma undergo loss of approximately 50% of their body fat by the end of the first week following subcutaneous inoculation of the tumor. During the same time span, however, no appreciable tumor mass had yet developed. Thus, the "mobilization for energy" concept (3) does not appear adequate to explain the lipolysis prior to rapid tumor growth, and a different mechanism for this case should be sought. In studies of pair-fed animals, anorexia was ruled out as a possible cause of fat depletion(5). No change in body weight was observed during this period; accumulation of water apparently offsets the loss of weight expected in view of the lipolysis that occurs(5).

An attractive and obvious hypothesis is that the inoculated tumor cells might in some way alter the energy-conserving systems of the host, *i.e.*, uncouple oxidative phosphorylation, thus causing an increased utilization of oxidizable substrates, such as lipid. The results reported here demonstrate, however, that oxidative phosphorylation is not impaired in isolated liver mitochondria from mice in which Krebs-2 has grown for 5 days.

Materials and methods. Adult, non-growing Swiss Ha/ICR male mice, averaging 38 g in weight, were inoculated subcutaneously on the back with 0.1 ml of a bacteriologically sterile suspension of Krebs-2 carcinoma cells in undiluted ascitic fluid(5). On day three, 4 groups were formed by random allocation; Groups I and Ia were normal mice, and II and IIa were tumor-bearing mice. Groups I and II, shipped by air express from Roswell Park Memorial Inst. and received the same day at the Merck Sharp & Dohme Research Laboratories, were used for isolation of liver mitochondria. With Groups Ia and IIa, total fat was determined as described previously (5).

For isolation of mitochondria from livers of both control and tumor-bearing animals, the mice were stunned by cervical fracture and bled by decapitation. The livers were removed and placed in ice-cold 0.25 M sucrose immediately. Mitochondria were isolated in 0.25 M sucrose by the method of Hogeboom *et al*(6); a 20% homogenate of liver was prepared in a Potter-Elvehjem tissue homogenizer. To obtain sufficient mitochondria for simultaneous measurements of oxidative phosphorylation and ATPase, livers of 4 to 5 animals from each group were combined. The mitochondria, after 2 washes, were suspended in the isolation medium to a final volume of 0.5 to 0.7 g wet weight of liver per ml. The entire preparation was carried out at 0-5°C and the mitochondria were used immediately. The nitrogen content of the mitochondrial suspension, determined by a modified Biuret method, ranged from 1.6 to 2.2 mg per ml. In most instances preparations from the control and treated animals were assayed the same day.

The standard incubation medium (3.0 ml) for measurements of respiration and phosphorylation contained the following: 30 μmoles phosphate buffer (pH 7.4), 20 μmoles

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TABLE I. Effect of Krebs-2 Carcinoma on Fat Content of Swiss Mice.

Exp	No. of animals	Animals	Total fat, g/animal	% decrease
1	6	Normal	3.8 ± 1.0	—
	6	Tumor bearing	2.7 ± .8*	29
2	6	Normal	3.8 ± 1.0	—
	6	Tumor bearing	2.6 ± .8*	33

* $P < .05$

$MgCl_2$, 150 μmoles KCl, 50 μmoles glucose, 150 KM units hexokinase (1.0 mg Sigma Type III), 3.0 μmoles ADP, and mitochondria (0.8 to 1.1 mg N). Where indicated 60 μmoles of succinate, α-ketoglutarate, D,L-glutamate, or β-hydroxybutyrate were added after an 8 minute preincubation. Oxygen uptake was measured by conventional manometric techniques at 30°C and inorganic orthophosphate by the method of Lowry and Lopez(7). Phosphate uptake was measured as the difference between the content of inorganic phosphate in zero time controls and the amount remaining in the reaction medium after incubation for 20 to 30 minutes. The reactions were terminated by addition of 0.2 ml of 4.8 M trichloroacetic acid.

The ATPase activity of the mitochondrial preparations was assayed in the following standard medium (4.0 ml); 40 μmoles tris-(hydroxymethyl) aminomethane (pH 7.4), 300 μmoles KCl, 30 μmoles ATP, and mitochondria (0.28 to 0.48 mg N). Incubation was carried out at 25°C, with 1.0 ml samples removed at 0, 10 and 20 minutes, for determination of inorganic phosphate release after termination of the reaction with trichloroacetic acid.

Hexokinase, Type III from yeast, ATP, DPN and cytochrome c from Sigma; ADP and D,L-β-hydroxybutyrate from Nutritional Biochemicals; α-ketoglutarate from Calbiochem; succinate and D,L-glutamate from Merck & Co., Inc.; and crystallized bovine plasma albumin from Armour Pharmaceutical Co. were used without further purification. Other chemicals were from commercial sources.

Results. The decrease in total fat of mice from 2 representative experiments, 5 days after injection of 0.1 ml of Krebs-2 carcinoma cells, is presented in Table I. Five days post-inoculation was selected for this study, because at this time the tumor was not detectable by palpation, although a significant decrease in body fat had occurred. Thus the lipolysis that occurred in the first week cannot be explained by a requirement for oxidizable substrates for rapid tumor growth. In recent experiments(8), no alterations in body temperature of Swiss mice were observed during the first week after tumor implantation. This fact, and the ability of bacteriologically sterile, non-growing tumor preparations, obtained by repeated freezing and thawing, to reproduce fat loss(5) would suggest that a transmitted bacterial infection was not the explanation for the early lipolysis. At the present time it cannot be excluded that the observed lipolysis is due to an undetected viral infection.

The results of the determination of respiratory activity and oxidative phosphorylation of mitochondria from livers of the 2 groups of animals are presented in Table II. No significant difference was observed in the rates of oxygen uptake or in P/O ratios

TABLE II. Respiration and Oxidative Phosphorylation of Mouse Liver Mitochondria.

		Substrate					
		Succinate		α-Ketoglutarate		Glutamate	
Liver source	Additions to assay system	O_2 uptake*	P/O	O_2 uptake*	P/O	O_2 uptake*	P/O
Normal animals	None	60	1.9	25	3.1	26	2.8
	10 mg albumin	56	2.1	30	2.8	—	—
	3 μmoles DPN	—	—	26	3.0	27	2.7
Tumor-bearing animals	None	53	1.9	27	3.0	22	2.6
	10 mg albumin	56	2.0	31	2.9	—	—
	3 μmoles DPN	—	—	28	3.0	24	2.5

* O_2 uptake—values are μatoms O_2/hr/mg mitochondrial N.

TABLE III. ATPase Activity of Mouse Liver Mitochondria.

Liver source	ATPase activity*		
	Latent	+Mg^{++}	+2,4-Dinitrophenol
Normal animals	5.1	8.2	29.5
Tumor-bearing animals	4.2	5.6	25.4

* 6.6 mM $MgCl_2$ and 0.1 mM 2,4-dinitrophenol added where indicated. Values are μmoles phosphate released/10 min/mg N and are the average of 2 separate experiments.

between the control and treated animals with succinate, α-ketoglutarate or glutamate as substrate. Studies by various investigators (9-11) have indicated that if oxidative phosphorylation is impaired, the presence of serum albumin in the assay system often stimulates phosphate uptake, particularly if uncoupling could be due to fatty acids(12, 13). As reported in Table II, serum albumin had no significant effect on phosphorylation of either mitochondrial preparation.

With α-ketoglutarate and glutamate, added DPN did not stimulate respiration (Table II), in contrast to the observation by others (14,15) of a stimulation by DPN of the oxidation of DPN-linked substrates by liver mitochondria from tumor-bearing animals. Addition of cytochrome c, in the presence and absence of DPN, had no effect.

Results with β-hydroxybutyrate as substrate were variable: rate of oxygen uptake was lower than the controls with some preparations from treated animals, while with others there were no differences. With both the treated and controls, however, the P/O ratios were the same. DPN and cytochrome c stimulated respiration of both treated and controls.

No significant difference between the preparations was observed in the values for respiratory control (ratio of Δ O_2 uptake in presence of ADP/Δ O_2 uptake when ADP was exhausted), as measured polarographically with a micro-platinum electrode(16). Values of respiratory control for mitochondria from the treated and controls were from 3 to 4 with succinate or glutamate as substrate.

The ATPase activities of the 2 mitochondrial preparations under study were determined, and representative results are shown in Table III. There was no significant difference in the latent ATPase or in the ATPase activity stimulated by Mg^{++} or 2,4-dinitrophenol. The levels of latent and Mg^{++}-ATPase presented in Table III for both preparations were higher than usually observed; mitochondrial preparations from both groups of animals have yielded values approaching zero. The results indicate that the mitochondria are essentially intact, since aged or disrupted mitochondria demonstrate high latent and Mg^{++}-stimulated ATPase (17). Similarly, altered mitochondria manifest a reduced stimulation of ATPase by dinitrophenol, which was not observed in this study(17).

Liver mitochondria isolated from treated animals 6 and 7 days after injection yielded the same results for oxidative phosphorylation and ATPase as those from animals 5 days post-injection.

Discussion. As demonstrated, liver mitochondria from animals 5, 6 and 7 days after injection of Krebs-2 carcinoma had activities for respiration, oxidative phosphorylation, respiratory control and ATPase similar to control animals, indicating that the lipolysis which occurs prior to rapid growth of the tumor is not caused by a change in the energy conserving reactions of liver mitochondria. These results are similar to those reported by Greene(14), who observed no change in oxidative phosphorylation of liver mitochondria from rats bearing the Walker-256. The results reported here and by Greene (14) are in contrast to the report of Nanni (18) who has observed a decrease in phosphorylating efficiency of mitochondria isolated from liver and kidneys of rats bearing a sarcoma. In the studies of both Greene(14) and Nanni(18), however, the tumors were very large (15 to 35% of body weight), whereas in this report no appreciable tumor mass had developed at time of measurement.

Greene(14) observed a decreased oxidation of β-hydroxybutyrate by mitochondria from the tumor-bearing animals which was stimulated by DPN; Aboobaker and Sahasrabudhe (15) have also reported a similar effect with

both β-hydroxybutyrate and α-ketoglutarate as substrate. In both reports it was suggested that the stimulation by DPN reflected a deficiency in mitochondrial pyridine nucleotide. The lack of a requirement for DPN with α-ketoglutarate and glutamate reported here suggests that there is no deficiency of mitochondrial DPN. The variation observed in this study in the oxidation of β-hydroxybutyrate may be a manifestation of some subtle change of the mitochondria, not yet understood, which alters the activity of β-hydroxybutyrate dehydrogenase. A complete explanation for the decreased activity with this substrate must await a better understanding of the properties of the enzyme.

Although the isolated liver mitochondria were not uncoupled in assays *in vitro,* one cannot rule out the possibility of some alteration in the coupling mechanism *in vivo.* It is conceivable that an uncoupling agent could be removed during the isolation procedure. The results do suggest, however, that no major morphological changes of the mitochondria have occurred, such as might be caused by fatty acids. Measurements of the integrity of oxidative phosphorylation *in vivo* will require development of new methodology.

Summary. Subcutaneous injection of Krebs-2 carcinoma into male Swiss mice caused approximately a 30% depletion of body fat after 5 days. At this time the tumor was not detectable by palpation. There was no significant difference in respiration and oxidative phosphorylation between isolated liver mitochondria from control mice and treated mice 5 days post-inoculation. No change in the latent ATPase or the Mg^{++}-, and 2,4-dinitrophenol stimulated ATPase activities of the mitochondrial preparation from treated animals was observed. The results suggest that the lipolysis that occurs in the tumor-bearing animals cannot be explained on the basis of a change in the energy coupling reactions of the host liver.

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