The Isolation from Thyrotoxic and Diabetic Rats of Skeletal Muscle Mitochondria Showing Tight Coupling, High Respiratory Indices, and Normal Adenosine Triphosphatase Activities*

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ABSTRACT: When skeletal muscle mitochondria are isolated and subsequently tested under carefully controlled conditions, neither thyrotoxicosis nor alloxan diabetes causes any aberration in oxidative activity, respiratory control, or adenosine triphosphatase (ATPase) activities. As with mitochondria from untreated rats, such mitochondria, when isolated in the presence of heparin and incubated in the presence of albumin and excess EDTA, show very high oxidative activity and very tight coupling and give theoretical adenosine diphosphate:oxygen ratios for all substrates tested.

Again as with mitochondria from untreated rats, the findings establish the nonidentity of the two ATPases found in such mitochondria but they indicate no aberration in ATPase activities. Thyroxine *in vitro* failed to affect the Mg^{2+}-ATPase but it inhibited the respiratory-chain coupling site. The observance of an effect of thyroxine *in vitro* without any effect of the hormone being shown by mitochondria isolated from the thyrotoxic rat would appear to indicate that thyroxine acts by binding to the outer membrane of the mitochondrion. *In vitro* addition of pork amorphous insulin failed to affect ATPase activities or respiratory coupling in mitochondria from untreated or alloxan diabetic rats.

The administration of large doses of thyroxine or the direct addition of thyroxine *in vitro* has been shown to cause uncoupling of oxidation from phosphorylation in liver mitochondria (Lardy and Feldott, 1951; Crane *et al.*, 1951). Skeletal muscle mitochondria, of patients exhibiting elevated metabolic rates of nonthyroid origin, showed lowered respiratory control and relatively high endogenous ATPase[1] activity (Azzone *et al.*, 1961). Thyroxine *in vitro* has been shown to stimulate ATPase activity in the presence of $MgCl_2$ in rat liver mitochondria (Chance and Hollunger, 1963; Bronk, 1965). The results of these workers support the concept that a deficient control of respiration by available phosphate acceptor, paralleled by an increased endogenous ATPase activity, constitutes the primary manifestation of the hypermetabolic state at the mitochondrial level. On the other hand, Tata *et al.* (1963) failed to show an alteration of P:O ratio or of respiratory index for either liver or muscle mitochondria when a change in basal metabolic rate was effected by doses of thyroxine small enough to have no effect on growth rate. However, they did show an enhancement of oxidative and phosphorylative activity following such thyroxine treatment.

Randle and Smith (1957, 1958) proposed that insulin acts to uncouple some form of energy transfer which is necessary to maintain the muscle barrier to glucose. Krahl (1957) suggested that insulin acts on the structure of the mitochondria so as to change the efficiency of substrate oxidation and energy trapping. Several groups of workers have found an impairment of both oxygen consumption and oxidative phosphorylation in liver mitochondria and heart homogenates of diabetic rats and depancreatized cats (Polis *et al.*, 1949; Goranson and Erulkar, 1949; Vester and Stadie, 1957; Hall *et al.*, 1960; Haugaard and Haugaard, 1964). However, Parks *et al.* (1955) could detect no change in the P:O ratios of rat liver mitochondria after the induction of alloxan diabetes and no effect of insulin *in vitro*.

The results of the study to be presented here show that when skeletal muscle mitochondria are isolated and subsequently tested under very carefully controlled conditions, neither thyrotoxicosis nor alloxan diabetes causes any aberration in oxidation, oxidative phosphorylation, or ATPase activities.

Experimental Section

Treatment of Animals. Young male, Hooded rats were made thyrotoxic by the inclusion of 0.01% sodium L-thyroxine admixed in a diet of powdered Purina rat

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[1] Abbreviations used: ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; albumin, bovine serum albumin; ADP:O, ratio of ADP utilization in micromoles to O uptake in microgram-atoms; P:O, ratio of P_i uptake in micromoles to O uptake in microgram-atoms; PMB, *p*-mercuribenzoate; NEM, *N*-ethylmaleimide; DPN, diphosphopyridine nucleotide.

TABLE I: ATPase Activities of Skeletal Muscle Mitochondria from Thyrotoxic Rats.[a]

Incubation Conditions	μmoles of P_i/mg of Protein per hr			
	No Mg^{2+}, No DNP	+Mg^{2+}, No DNP	No Mg^{2+}, +DNP	+Mg^{2+}, +DNP
	Expt I			
Basic ATPase medium − albumin	77.5	74.0	126.0	104.0
+Oleate	21.0	40.5	28.0	50.0
+PMB	3.0	24.5	3.5	29.0
+Oligomycin	4.5	5.0	3.0	4.5
Basic ATPase medium + albumin (0.12%)	14.5	29.0	125.0	91.0
+Albumin (0.24%)	12.5	29.0	105.0	80.0
+PMB	2.0	26.5	2.5	28.0
+Azide	5.0	7.0	6.5	7.5
+Oligomycin	2.5	3.0	2.5	4.5
+Atebrin	2.0	2.5	2.0	3.0
+Fluoride	15.5	30.5	39.0	34.5
	Expt II			
Basic ATPase medium + albumin (0.12%)	25.5	35.5	144.0	78.0
Basic ATPase medium − albumin with Tris-HNO_3 as buffer in place of Tris-HCl	44.0	52.5	73.5	68.5
+$AgNO_3$ (0.04 μmole)	2.5	52.0	2.0	61.5
+$AgNO_3$ (0.12 μmole)	2.0	53.0	3.0	58.0

[a] Mitochondria isolated and made up finally in mannitol (0.25 M)–heparin (0.50%)–EDTA (0.01 M).

TABLE II: ATPase Activities of Skeletal Muscle Mitochondria from Alloxan Diabetic Rats.[a]

Incubation Conditions	μmoles of P_i/mg of Protein per hr			
	No Mg^{2+}, No DNP	+Mg^{2+}, No DNP	No Mg^{2+}, +DNP	+Mg^{2+}, +DNP
Basic ATPase medium + albumin (0.12%)	10.5	26.0	120.0	101.5
+PMB	2.0	25.0	3.0	28.0
+NEM	3.0	22.0	3.5	40.0
+Azide	5.0	11.0	10.5	11.5
+Oligomycin	2.0	4.0	2.0	3.5
+Atebrin	2.0	5.0	2.0	2.5
+Fluoride	12.0	25.0	31.0	29.5
Basic ATPase medium − albumin with Tris-HNO_3 as buffer in place of Tris-HCl	72.0	75.0	105.0	85.0
+$AgNO_3$ (0.006 μmole)	3.5	55.0	2.5	62.0

[a] Mitochondria isolated and made up finally in mannitol (0.25 M)–heparin (0.50%)–EDTA (0.01 M).

cubes. Corn oil (5%) was added to the feed to reduce nasal irritation due to the powdered diet. Food and water were available *ad libitum*. The animals were considered to be thyrotoxic when they had shown growth arrestment for at least 1 week.

Other young male, Hooded rats were made diabetic by the intraperitoneal injection of a 3% solution of alloxan following a 36-hr starvation period. Then, daily subcutaneous injections of protamine zinc insulin (Connaught Research Laboratories, Toronto, Ontario) were given until the growth rate of the animals had returned to normal in order to permit repair of any systemic alloxan damage. Only those rats having blood glucose values in excess of 300 mg % on the morning of tissue sampling were considered to be diabetic. The diabetic rats received Purina rat cubes and water

TABLE III: The Effects of Added Thyroxine on the Various ATPase Activities of Skeletal Muscle Mitochondria.[a]

Incubation Conditions	μmoles of P_i/mg of Protein per hr			
	No Mg^{2+} No DNP	+Mg^{2+} No DNP	No Mg^{2+} +DNP	+Mg^{2+} +DNP
Basic ATPase medium − albumin	88.0	90.5	200.0	165.0
+thyroxine (5×10^{-4} M)	22.5	31.5	45.0	51.0
Basic ATPase medium + albumin (0.12%)	30.0	41.0	170.5	144.5
+thyroxine (5×10^{-4} M)	30.0	31.0	90.0	90.0
+Mg^{2+} (5 mM) + thyroxine (5×10^{-4} M)	30.0	50.5	90.0	102.5

[a] Thyroxine, as a suspension in water, was added to the incubation medium prior to the addition of the mitochondria.

TABLE IV: The Effects of Added Steroids on the Various ATPase Activities of Skeletal Muscles Mitochondria.[a]

Incubation Conditions	μmoles of P_i/mg of Protein per hr			
	No Mg^{2+} No DNP	+Mg^{2+} No DNP	No Mg^{2+} +DNP	+Mg^{2+} +DNP
Basic ATPase medium + albumin (0.12%)	21.5	33.0	91.0	61.5
+Cortisol acetate (5×10^{-5} M)	22.0	34.0	85.0	58.0
+Deoxycorticosterone (5×10^{-5} M)	20.5	29.5	87.0	59.0
+NEM	3.0	21.0	4.5	50.0
+Oligomycin	2.0	3.5	2.0	4.5

[a] Mitochondria isolated and made up finally in mannitol (0.25 M)–heparin (0.50%)–EDTA (0.01 M).

ad lib. throughout the treatment period. Untreated control rats received the same diet and water *ad lib.*

Isolation of Mitochondria. Mitochondria were isolated in the cold from the skeletal muscle of the hind legs of untreated, thyrotoxic, and diabetic rats. The technique employed was that outlined in Dow (1967) and involved the use of 0.50% heparin in the isolation medium. The mitochondria were suspended finally in mannitol (0.25 M)–EDTA (0.01 M)–heparin (0.50%) (330 units/ml of medium, crystalline sodium heparin, Fisher Scientific Co.). The mitochondria were used within 3 hr after isolation.

Determination of Protein Content of Mitochondria. The protein content of the mitochondria was determined by the method of Gornall *et al.* (1949).

Determination of ATPase Activities of Mitochondria. The Mg^{2+}-stimulable and DNP-stimulable ATPase activities, as well as spontaneous activity, were measured as inorganic phosphate released during a 15-min incubation at 37° in the following medium: KCl (0.075 M), Tris-HCl (pH 7.4, 0.050 M), mannitol (0.050 M), EDTA (0.0005 M), antimycin A (2.5 μg/ml in ETOH), plus and minus albumin (0.06 or 0.12%), plus and minus $MgCl_2$ (0.0015 M), plus and minus 2,4-dinitrophenol (DNP) (0.0001 M), Na_2ATP (0.006 M), and mitochondrial protein (0.07–0.10 mg), in a total volume of 1.5 ml. The technique was that outlined in Dow (1967).

Inhibitors, at the following concentrations, were tested for their effects on the various mitochondrial ATPase activities: PMB = 3.5×10^{-3} M, NEM = 5×10^{-4} M, $AgNO_3$ = 4×10^{-6} M, oligomycin = 6 μg/ml, sodium azide = 0.01 M, KF = 0.005–0.040 M, atebrin = 8×10^{-3} M, and sodium oleate = 2×10^{-5} M.

Polarographic Determination of Respiratory Control. Oxidation rates and respiratory control indices were measured polarographically at 37°. ADP:O ratios were also determined. Oxygen utilization was measured in the following medium: KCl (0.015 M), KPO_4 buffer (pH 7.4, 0.03 M), Tris-HCl (pH 7.4, 0.025 M), sucrose (0.045 M), mannitol (0.010 M), $MgCl_2$ (0.005 M), EDTA (0.007 M), albumin (0.20%), glucose (0.02 M), cytochrome *c* (0.015 mM), DPN (0.50 mM), substrate (0.010 M), mitochondrial protein (0.18–0.23 mg), and ADP (0.372-μmole aliquots), in a total volume of 2.0 ml. The technique was that outlined in Dow (1967).

Results

ATPase Activities of Mitochondria from Thyrotoxic and Diabetic Rats. Tables I and II show that neither

the characteristics nor the rates of the various ATPase activities of mitochondria from thyrotoxic and diabetic rats are different from those of mitochondria from untreated rats, as reported in Dow (1967). These tables also show that the characteristics of the various ATPase activities of mitochondria from treated rats are not altered. As with mitochondria from untreated rats (Dow, 1967), ATP is hydrolyzed spontaneously unless the ATPase sites are protected by albumin. The Mg^{2+}- and DNP-stimulable ATPase activities show differential sensitivity to albumin, sulfhydryl reactants, and respiratory inhibitors. As with untreated rats this would appear to indicate that the divalent cation stimulable ATPase involves an independent enzyme site in these mitochondria. Activation of the Mg^{2+}-ATPase site results in masking of the DNP-stimulable, respiratory-chain coupling site to free access to $AgNO_3$. Again as with untreated rats, this would appear to indicate that Mg^{2+}-ATPase activity is inhibitory to the respiratory-chain-linked energy-transfer site. The various ATPase activities show similarities in behavior since all are inhibited completely by the flavin inhibitor, atebrin, and by sodium azide and oligomycin, inhibitors of energy-chain-linked phosphorylation. However, as was suggested in Dow (1967), such a similarity of inhibitor effects does not necessarily implicate the Mg^{2+}-ATPase mechanism in the energy-coupling apparatus of the respiratory chain.

Table III shows the effect of the addition of 5×10^{-4} M thyroxine *in vitro* on the ATPase activities of mitochondria from untreated rats. Contrary to the findings of Chance and Hollunger (1963) and Bronk (1965) with liver mitochondria, it is apparent that thyroxine did not affect the Mg^{2+}-stimulable ATPase activity significantly but it was inhibitory to the DNP-stimulable respiratory-chain-coupling ATPase. This was true both in the presence and in the absence of albumin in the incubation medium. Absorbance measurements appeared to indicate that the thyroxine precipitates on the mitochondrial membrane since a momentary increase in absorbancy was quickly followed by a pronounced thyroxine-dependent mitochondrial swelling (to be published). Incubation of the mitochondria in the presence of deoxycorticosterone (5×10^{-5} M) or of cortisol acetate (5×10^{-5} M), both of which have been shown to enhance the latent ATPase activity of liver mitochondria (Blucher and White, 1960), also failed to affect the Mg^{2+}-ATPase of these mitochondria (Table IV). However, by contrast with thyroxine, these steroids also failed to affect the respiratory-chain-coupling ATPase activity. The *in vitro* addition of pork amorphous insulin (Zn^{2+} free and glucagon free) (6×10^{-5} M), as the hexamer, failed to affect the ATPase activities of mitochondria from normal or alloxan diabetic rats.

Oxidative and Energy-Coupling Activities of Mitochondria from Thyrotoxic and Alloxan Diabetic Rats. Because the Mg^{2+}-stimulable ATPase activity involves an independent site in these mitochondria and because an active Mg^{2+}-ATPase is inhibitory to the respiratory-chain-linked energy-transfer site it is apparent that a true measure of respiratory-chain-coupling potential

TABLE V: Rat Skeletal Muscle Mitochondria Isolated in Mannitol–Heparin–EDTA and Incubated in the Presence of Albumin and Excess EDTA.[a]

Substrate	Untreated Rats				Thyrotoxic Rats				Alloxan Diabetic Rats			
	Q_{O_2} before ADP	Q_{O_2} after ADP	RI	ADP:O	Q_{O_2} before ADP	Q_{O_2} after ADP	RI	ADP:O	Q_{O_2} before ADP	Q_{O_2} after ADP	RI	ADP:O
Glutamate	1.4 ± 0.9	43.6 ± 7.7	31.1	2.94	1.5 ± 0.5	46.1 ± 10.0	30.7	2.68	1.5 ± 0.7	39.5 ± 7.0	26.3	2.83
Pyruvate + malate	8.1 ± 2.4	40.6 ± 9.7	5.0	4.14	10.5 ± 2.6	52.4 ± 9.1	5.0	3.58	7.4 ± 0.9	46.2 ± 6.5	6.2	3.74
α-Ketoglutarate + malonate	1.5 ± 0.4	22.3 ± 2.8	14.9	4.18	3.8 ± 1.0	27.0 ± 5.4	7.1	4.11	1.5 ± 0.8	20.3 ± 1.5	13.5	4.02
Succinate + amytal	18.9 ± 5.1	30.9 ± 5.1	1.6		22.9 ± 5.4	30.1 ± 10.3	1.3		19.0 ± 1.2	24.8 ± 4.2	1.3	

[a] The values represent the averages for four separate experiments, each experiment being comprised of tissue from three rats. Incubation conditions were those outlined in the Methods section. Glutamate = 0.010 M; pyruvate = 0.010 M + malate = 0.0025 M; succinate = 0.020 M + amytal = 3 mM; α-ketoglutarate = 0.010 M + malonate = 0.010 M; Q_{O_2} = microgram-atoms of O per milligram of protein per hour; RI = respiratory index = Q_O after ADP:Q_{O_2} before ADP; ADP:O = micromoles of ADP per microgram-atoms of O.

TABLE VI: Skeletal Muscle Mitochondria Isolated without Heparin and Incubated in the Presence of Albumin and Excess EDTA.[a]

Substrate	Untreated Rats			Thyrotoxic Rats			Alloxan Diabetic Rats		
	Q_{O_2} before ADP	Q_{O_2} after ADP	RI	Q_{O_2} before ADP	Q_{O_2} after ADP	RI	Q_{O_2} before ADP	Q_{O_2} after ADP	RI
Glutamate	1.6	19.3	12.1	2.7	24.0	8.9	2.2	24.9	11.2
α-Ketoglutarate + malonate	2.4	17.1	7.1	2.1	14.0	6.8	2.0	17.2	8.4
Pyruvate + malate	8.0	25.5	3.2	11.7	17.6	1.5	8.7	25.3	2.9
Succinate + amytal	8.6	15.2	1.8	7.1	7.4	1.0	6.6	10.9	1.7
L-Glycero-P	8.2	9.4	1.2	5.7	5.7	1.0			

[a] Incubation conditions were those outlined in the Methods section. Glutamate = 0.010 M; pyruvate = 0.010 M + malate = 0.0025 M; succinate = 0.020 M + amytal = 3 mM; α-ketoglutarate = 0.010 M + malonate = 0.010 M; Q_{O_2} = microgram-atoms of O per milligram of protein per hour; RI = respiratory index = Q_{O_2} after ADP:Q_{O_2} before ADP; ADP:O = micromoles of ADP per microgram-atoms of O.

could be obtained only in the absence of an operating Mg^{2+}-ATPase. Consequently, these conditions were employed for the measurements of oxidation and coupling activity. Albumin was added to the incubation medium because of the coupling factor effect on ATPase activity.

Table V shows oxidation rates, respiratory indices, and coupling activity of mitochondria from untreated, thyrotoxic, and diabetic rats. It is apparent that mitochondria from treated rats, when isolated in the presence of heparin and incubated in the presence of albumin and excess EDTA, showed very tight coupling, very high respiratory activity, and theoretical ADP:O ratios for all substrates tested. These values were not significantly different from the very high values for mitochondria from untreated rats.

Table VI shows a set of data for mitochondria isolated in the absence of heparin. As was shown by Dow (1967) for mitochondria from untreated rats, the omission of heparin has resulted in much lower respiratory activities. It also yielded a very much smaller volume of heavy, intact mitochondria, particularly from alloxan diabetic rats.

Thyroxine, at a concentration of 5×10^{-4} M, was added to the incubation medium containing albumin and mitochondria from untreated rats. The thyroxine was added either as a suspension in water or dissolved in albumin solution at pH 7.4. The addition of thyroxine in either form resulted in complete inhibition of the respiratory stimulation that normally results from the addition of ADP as high-energy acceptor. Thyroxine at a concentration of 5×10^{-5} M failed to show an effect. *In vitro* addition of pork amorphous insulin (Zn^{2+} free and glucagon free) (6×10^{-5} M), as the hexamer, failed to affect coupling activity of mitochondria from normal or alloxan diabetic rats.

Discussion

The lower oxidative activity of mitochondria from thyrotoxic and diabetic rats, when isolation was performed in the absence of heparin, confirms the finding for mitochondria from untreated rats (Dow, 1967) that heparin acts to prevent loss of structural integrity. When isolation was carried out in the presence of heparin, the ATPase, respiratory, and coupling activities of mitochondria from thyrotoxic and from alloxan diabetic rats were not significantly different from the very high activities obtained for mitochondria from untreated rats. Owing to the fairly wide variation in the respiratory activities of mitochondrial samples from untreated rats, the slightly higher average rate for mitochondria from thyrotoxic rats is of doubtful significance. It certainly does not account for the two- to threefold rise in basal metabolic rate which was observed for the thyrotoxic rat in this laboratory (Dow and Allen, 1961).

It was suggested by Tata *et al.* (1963) that mitochondrial respiration and phosphorylation on the one hand and tightness of coupling on the other may respond differently according to the degree of hyperthyroidism or thyroxine intoxication. Several groups of workers have shown an uncoupling effect of large doses of thyroxine or of thyroxine *in vitro* on oxidative phosphorylation (see Tata *et al.* 1963). However, when a change in basal metabolic rate was effected by doses of thyroxine small enough to have no effect on growth rate, Tata *et al.* (1963) failed to show any alteration of P:O ratios or of respiratory control indices of liver or muscle mitochondria. An increase in both the respiratory and phosphorylative capacities of mitochondria from such hypermetabolic rats was observed but the stimulated rates were significantly less than those

reported here for untreated animals when heparin was employed to prevent loss of structural integrity of the mitochondria. With mitochondria isolated in the presence of heparin, hyperthyroidism failed to affect the very high oxidative or energy-coupling capacity, even as a secondary effect relative to growth arrestment.

As with mitochondria from untreated rats the findings demonstrate the nonidentity of the two ATPase activities found in these mitochondria, but they indicate no aberration in the ATPase activities. Activation of the Mg^{2+}-ATPase by thyroxine *in vitro* has been reported for liver mitochondria by Chance and Hollunger (1963) and Bronk (1965). Thyroxine *in vitro* failed to affect the Mg^{2+}-ATPase of skeletal muscle mitochondria although it did inhibit the respiratory-chain-coupling site. Such an *in vitro* effect appears to indicate a mechanism of action different from the uncoupling action observed with liver mitochondria. Indeed, the observance of an effect of thyroxine *in vitro* without any effect of the hormone being shown by mitochondria from thyrotoxic rats would appear to indicate that thyroxine acts by binding to the outer membrane of the mitochondrion. Swelling studies with thyroxine *in vitro* (to be published) may help to elucidate this mechanism.

In vitro addition of pork amorphous insulin failed to affect ATPase activities or coupling in mitochondria from untreated or alloxan diabetic rats although an *in vitro* effect of insulin on the swelling of liver mitochondria has been reported (Lehninger and Neubert, 1961). The findings fail to show an effect of insulin *in vitro* or *in vivo* on energy metabolism although an uncoupling of energy transfer had been suggested as the mechanism of insulin action (Randle and Smith, 1957, 1958; Krahl, 1957).

The findings show that when skeletal muscle mitochondria are isolated and subsequently tested under very carefully controlled conditions, neither thyrotoxicosis nor alloxan diabetes causes any aberration in oxidation, oxidative phosphorylation, or ATPase activities.

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