The Isolation of Skeletal Muscle Mitochondria Showing Tight Coupling, High Respiratory Indices, and Differential Adenosine Triphosphatase Activities*

Dorothy S. Dow

ABSTRACT: Skeletal muscle mitochondria, isolated in the presence of heparin and incubated in the presence of albumin and excess EDTA, show oxidative activity and respiratory indices which are much higher than have been observed by other workers. Isolation of these mitochondria in the absence of heparin yielded granules in which the Mg^{2+}–ATPase had lost its oligomycin sensitivity and the 2,4-dinitrophenol (DNP) stimulable adenosine triphosphatase (ATPase) activity and the respiratory activity were very significantly reduced. Mitochondria isolated in the presence of heparin and EDTA contain a respiratory-chain-coupling ATPase site which permits spontaneous adenosine triphosphate (ATP) hydrolysis. The observed spontaneous ATPase activation and respiratory inhibition are caused presumably by free access to water and not by fatty acid anions since free fatty acids were found to inhibit the ATPase activities of these mitochondria. The inclusion of bovine serum albumin in the incubation medium converts these mitochondria from the open form to a closed system, one which is accessible only to the lipid-soluble DNP vehicle for ATPase activation. The findings also indicate that the divalent-cation-stimulable ATPase activity in skeletal muscle mitochondria involves an independent enzyme site and that such an ATPase activity is inhibitory to the respiratory-chain-linked energy-transfer site.

Fresh, intact liver mitochondria show tight coupling and thus respire at very low rates unless an acceptor of high-energy phosphate is present (Lardy and Wellman, 1952). Muscle mitochondria, however, have generally shown rather high endogenous respiration and low stimulation by uncoupling agents, both of which are usually attributable to high intrinsic ATPase[1] activity. Whereas the 2,4-dinitrophenol (DNP)-stimulable ATPase activity, which represents respiratory-chain-coupling activity, and the Mg^{2+}-stimulable activity occur as alternative enzymic features in liver mitochondria, all muscle mitochondria, even if fresh and giving high P:O ratios, exhibit a high Mg^{2+}-stimulable activity (Azzone and Carafoli, 1960). Although the same laboratory has presented evidence that the Mg^{2+}–ATPase of fresh muscle mitochondria is not related to the respiratory-chain phosphorylating system, other work has contradicted this since oligomycin, a respiratory-chain phosphorylation inhibitor, prevents the Mg^{2+}–ATPase reaction (Lindberg *et al.*, 1961).

Azzone and Carafoli (1960) have shown that the high ATPase of muscle mitochondria is not a limiting condition for obtaining good P:O ratios and respiratory indices in the presence of efficient trapping systems. However, Chance and Williams (1955) suggest that preparations which require a very reactive trapping system to compete with ATPases are not representative of the physiological situation and do not have as high phosphorylation efficiency.

The results of the present experiments establish the separate identity of the two ATPase activities found in skeletal muscle mitochondria and show that the divalent-cation-stimulable activity is inhibitory to the respiratory-chain energy-coupling site. The findings show that the lower respiratory and coupling activities observed by other workers are the result of loss of structural integrity during isolation, loss of a soluble coupling factor during incubation, and inhibition due to divalent-cation-stimulable ATPase activity.

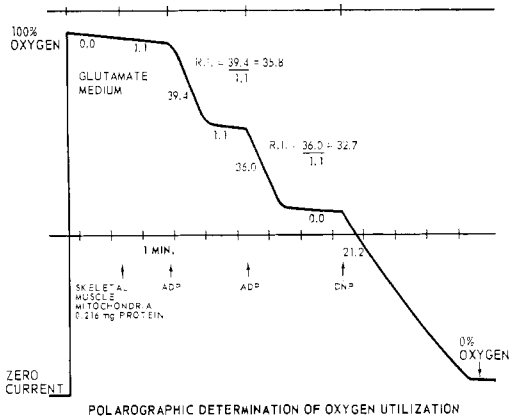
Experimental Procedures

Treatment of Animals. Young male, hooded rats received a diet of Purina rat cubes and water *ad lib.*

Isolation of Mitochondria. Mitochondria were isolated in the cold from the skeletal muscle of the hind legs. Fat and connective tissue were cut away and a total of 12–15 g of washed muscle from three rats was weighed into cold isolation medium (pH 7.4) (0.21 M mannitol–0.07 M sucrose–0.01 M EDTA). The muscle was thinly chopped with a McIlwain tissue slicer (H.

* Contribution No. 242 from the Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Ontario, Canada. *Received September 20, 1966.*

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



POLAROGRAPHIC DETERMINATION OF OXYGEN UTILIZATION

FIGURE 1: Rates of oxygen utilization expressed here as Q_{O_2} (microgram-atoms of oxygen per milligram of mitochondrial protein per hour were measured polarographically employing the following medium with glutamate as substrate: KCl (0.015 M), $K.PO_4$ buffer (pH 7.4) (0.030 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), glutamate (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 respiratory index (RI) represents the ratio between Q_{O_2} after and before the addition of phosphate acceptor (ADP).

Mickle Mill Works, Gomshal, Surrey, England) (McIlwain and Buddle, 1953) and then passed through a Delepine tissue press (Arthur H. Thomas Co., Philadelphia, Pa.) into a ten-times volume of cold isolation medium containing Tris buffer (pH 7.4) and approximately 0.50% (330 units/ml of medium) crystalline sodium heparin (Fisher Scientific Co.). The resulting tissue suspension was homogenized gently in ice with a loose, Tri-R (Tri-R Co., Jamaica, N. Y.), groundglass and Teflon homogenizer and then with a Thomas Teflon homogenizer "C."

The homogenized sample was centrifuged in the cold for 5 min at 500g. After filtration to remove lipids, the supernatant fraction was centrifuged for 10 min at 12,000g in a Servall-refrigerated centrifuge. The white layer of the mitochondrial pellet was removed by gentle shaking of the tube with a small volume of medium and the brown mitochondrial pellet was resuspended in 12 ml of isolation medium containing 0.50% heparin. The sample was centrifuged at 2400g for 5 min. The heavy mitochondrial pellet was suspended finally in 0.25 M mannitol–0.01 M EDTA–0.50% heparin.

Mitochondria were also isolated and suspended finally in the presence of 0.1% albumin, instead of heparin. Mitochondria were isolated using a modification of the proteinase procedure developed by Chance and Hagihara (1961). The modification involved a decrease of the first proteinase incubation from 20 to 10 min because the tissue was finely comminuted with a tissue slicer prior to the enzyme incubation. All mitochondria were used for *in vitro* tests 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 a medium consisting of KCL (0.075 M), Tris-HCl (pH 7.4) (0.05 M), mannitol (0.050 M), EDTA (0.0005 M), antimycin A (2.5 μg/ml in ETOH), plus or minus albumin (0.06% or 0.12%), plus or minus $MgCl_2$ (0.0015 M), plus or minus DNP (0.0001 M), Na_2-ATP (0.006 M), and mitochondrial protein (0.07–0.10 mg) in a total volume of 1.5 ml. Antimycin A was added to prevent the involvement of endogenous substrate in phosphate esterification; it also promoted a minor stimulation of spontaneous ATP hydrolysis. 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).

The ATPase reaction was terminated by the addition of ice-cold 10% TCA and the tubes were immediately chilled in an ice bath. ATPase activity was measured as inorganic phosphate released, according to the method of Sumner (1944). The ATPase activities were found to be linear over a 15-min period of incubation. The rate of liberation of inorganic phosphate from ADP in the presence of DNP was found to be negligible, in agreement with Lardy and Wellman (1953) for liver mitochondria.

Polarographic Determination of Respiratory Control of Mitochondria. Oxidation rates and respiratory control indices were measured at 37° in a Gilson Polarograph, Model KM, which employs a vibrating platinum electrode (Gilson Medical Electronics, Middleton, Wis.). Rates of oxygen utilization were measured in a medium as outlined under Figure 1. Respiratory indices were measured as the ratio of respiratory activity after and before the addition of ADP as high-energy phosphate acceptor. ADP:O ratios were also determined. The solubility of oxygen in isotonic salt medium was taken as 0.39 μg-atom of O/ml at 37°.

Results

The Protective Action of Albumin against Spontaneous ATP Hydrolysis. It is evident from Table I that in the absence of added albumin, the rates of the spontaneous and Mg^{2+}-stimulable ATPase activities were nearly as high as that of the DNP-stimulable activity. The inclusion of albumin in the incubation medium very significantly reduced the spontaneous and Mg^{2+}-stimulable activities without affecting the DNP-ATPase

TABLE I: ATPase Activities of Skeletal Muscle Mitochondrial from Untreated Rats.[a]

Incubn Condn (%)	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	88.0	90.5	200.0	165.0
+Albumin (0.12)	30.0	41.0	180.5	144.5
+Albumin (0.12) − antimycin	15.5	20.0	194.0	132.8
	Expt II			
Basic ATPase medium	110.0	92.0	149.0	128.5
+Albumin (0.06)	17.0	34.0	136.0	122.0
+Albumin (0.12)	15.0	32.5	113.0	90.5
+PMB	2.5	30.0	3.5	31.5
+DOC	3.0	31.0	2.5	30.5
+Albumin (0.06) + PMB	2.5	28.0	3.5	30.0
+Albumin (0.12) + PMB	3.5	30.0	3.5	31.0
	Expt III			
Basic ATPase medium				
+ Albumin (0.06)	39.5	53.0	120.0	105.0
+ Albumin (0.12)	17.5	35.5	123.0	98.5
+ Albumin (0.06) + PMB	3.5	31.0	5.5	33.5
+ Albumin (0.06) + NEM	3.0	30.0	5.0	57.0
+ Albumin (0.06) + oleate	28.5	46.5	33.5	65.0
+ Albumin (0.06) + oligomycin	3.0	4.5	2.5	5.5
+ Albumin (0.06) + azide	4.0	6.0	5.5	6.0
+ Albumin (0.06) + atebrin	1.5	2.0	2.0	2.5

[a] Mitochondria were isolated in mannitol (0.25 M), heparin (0.50%), and EDTA (0.01 M), buffered with Tris, and were made up finally in mannitol (0.25 M), heparin (0.50%), and EDTA (0.01 M).

significantly. It would appear that ATP can be hydrolyzed spontaneously unless the ATPase sites are protected by albumin. However, raising the concentration of the albumin beyond an optimum level caused some inhibition of the DNP-stimulable activity.

Table I also shows that free fatty acid was not responsible for spontaneous ATPase activation. Labilization of the mitochondrial lipid due to the inclusion of 0.025% DOC in the medium resulted in complete inhibition of the spontaneous and DNP-stimulable ATPase activities, but the Mg^{2+}-stimulable activity was not affected. Higher levels of DOC were inhibitory only to the Mg^{2+}–ATPase. It is apparent that ATPase was not activated by the free fatty acids released by DOC, although aged rat liver mitochondria have shown such an effect for the Mg^{2+}–ATPase (Potter *et al.*, 1953). The addition of free fatty acid as sodium oleate also inhibited the DNP–ATPase, even in the presence of albumin. Free fatty acid has not activated the Mg^{2+}-stimulable ATPase activity although the albumin concentration was not sufficient completely to prevent spontaneous ATP hydrolysis. Indeed, oleate was inhibitory to the Mg^{2+}–ATPase at all levels tested between 10 and 1100 μmoles/g of protein. It would appear that the observed beneficial effect of albumin against spontaneous hydrolysis is not due to its ability to bind free fatty acids.

The Differential ATPase Activities of Skeletal Muscle Mitochondria Isolated in the Presence of Heparin. Table I shows that the presence or absence of albumin has not affected the PMB-resistant Mg^{2+}–ATPase activity. Consequently, it would appear that a Mg^{2+}-stimulable ATPase site is resistant to attack by sulfhydryl reactants. The addition of Mg^{2+} to the DNP incubation medium resulted in the same degree of PMB-resistant Mg^{2+}–ATPase activity as when Mg^{2+} was present alone and this activity was inhibitory to the DNP-stimulable activity. But the spontaneous and DNP-stimulable ATPases were completely susceptible to PMB. It would appear, then, that the spontaneous activity is related to the DNP-stimulable, respiratory-chain-coupling activity. The sulfhydryl reactant NEM duplicated the differential effects of PMB. By contrast, both the Mg^{2+}– and DNP–ATPase activities of rat liver mitochondria are susceptible to sulfhydryl reactants (Siekevitz *et al.*, 1958; Lardy and Wellman, 1953).

The differential behavior of the ATPase activities is further emphasized by titration of the free sulfhydryl groups with $AgNO_3$ (Table II). Although the DNP-

TABLE II: The Effect on the ATPase Activities of Skeletal Muscle Mitochondria of Titration of Free Sulfhydryl Groups with $AgNO_3$.[a]

Incubn Condn	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%) with Tris-HNO_3 as buffer in place of Tris-HCl	17.5	34.0	67.0	58.0
Basic ATPase medium–albumin with Tris-HNO_3 as buffer in place of Tris-HCl	36.0	56.5	66.5	63.5
+$AgNO_3$ = 0.001 μmole	36.5	56.0	67.5	65.0
+$AgNO_3$ = 0.002 μmole	27.5	54.0	62.5	64.0
+$AgNO_3$ = 0.003 μmole	12.0	56.0	28.0	64.5
+$AgNO_3$ = 0.004 μmole	8.0	55.5	7.5	61.0
+$AgNO_3$ = 0.005 μmole	6.0	56.0	6.5	60.0
+$AgNO_3$ = 0.006 μmole	2.0	55.5	2.5	60.0

[a] Mitochondria were isolated in mannitol (0.25 M), heparin (0.50%), and EDTA (0.01 M), buffered with Tris, and were made up finally in mannitol (0.25 M).

TABLE III: The Effect of Graded Levels of KF on the Various ATPase Activities of Skeletal Muscle Mitochondria.[a]

Incubn Condn (M)	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 (0.12%)	30.0	35.0	125.0	77.0
+KF (0.005)	34.0	38.0	103.0	70.0
+KF (0.01)	35.0	34.0	95.0	55.5
+KF (0.02)	33.0	32.0	52.5	42.5
+KF (0.03)	34.0	32.0	42.0	39.0
+KF (0.04)	32.5	31.0	39.0	37.5
	Expt II			
Basic ATPase medium + albumin (0.12%)	35.0	41.0	103.0	74.0
+PMB	2.0	17.5	3.0	18.0
+KF (0.04)	30.0	33.0	30.5	32.0
+KF (0.04) + PMB	2.5	17.0	2.0	19.0

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

stimulable and spontaneous ATPase activities were completely inhibited by this sulfhydryl reactant, both were only very slowly influenced by $AgNO_3$ in the presence of an active Mg^{2+}–ATPase. It would appear that activation of the Mg^{2+}–ATPase site results in masking of the DNP-stimulable, respiratory-chain-coupling site.

A differential behavior of the ATPase activities was also found toward respiratory inhibitors. Table III shows that KF, which forms complexes with several metal–enzyme systems, inhibited the DNP–ATPase activity without affecting the PMB-resistant, Mg^{2+}-stimulable activity. Ca^{2+}, which caused complete inhibition of respiration, also caused inhibition of the DNP–ATPase but did not inhibit the Mg^{2+}-stimulable activity (Table IV), although this has been shown for liver mitochondria (Kielley and Kielley, 1953). These findings would appear to implicate a respiratory-chain transfer in the DNP-stimulable ATPase activity but not in the Mg^{2+}–ATPase.

Although the Mg^{2+}- and DNP-stimulable ATPase activities showed a differential sensitivity to albumin, to sulfhydryl reactants, and to respiratory inhibitors, nevertheless they showed similarities in behavior since

TABLE IV: The Effects of Mg^{2+} and Ca^{2+} Concentrations on ATPase Activities.[a]

Incubn Condn (mM)	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%)				
Mg^{2+} (1.5)	23.0	28.5	127.5	109.5
Mg^{2+} (1.5) + PMB	3.0	25.0	3.5	25.5
Mg^{2+} (5.0)	23.0	56.5	127.0	90.0
Mg^{2+} (5.0) + PMB	3.5	52.5	2.5	55.5
Mg^{2+} (8.0)	23.5	45.5	127.5	87.5
Mg^{2+} (8.0) + PMB	3.0	45.5	3.0	48.0
Mg^{2+} (1.5) + Ca^{2+} (2.0)	26.0	67.0	38.5	71.0
Mg^{2+} (1.5) + Ca^{2+} (2.0) + PMB	3.5	33.5	3.5	40.0
Mg^{2+} (1.5) + Ca^{2+} (4.0)	29.0	74.0	41.0	82.5
Mg^{2+} (1.5) + Ca^{2+} (4.0) + PMB	4.0	37.0	3.5	43.5
Mg^{2+} (5.0) + Ca^{2+} (2.0)	29.0	58.0	41.0	71.5
Mg^{2+} (5.0) + Ca^{2+} (2.0) + PMB	4.0	41.5	4.5	45.5
Mg^{2+} (5.0) + Ca^{2+} (4.0)	29.0	57.5	40.5	63.5
Mg^{2+} (5.0) + Ca^{2+} (4.0) + PMB	3.5	43.5	4.0	48.0

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

TABLE V: ATPase Activities of Skeletal Muscle Mitochondria from Untreated Rats.

Isolation Condn	Incubn Condn	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
A[a]	Basic ATPase medium + albumin (0.12%)	14.5	48.0	56.5	45.5
	+NEM	2.5	27.5	3.0	30.5
	+Oligomycin	3.0	28.0	3.5	25.5
B[b]	Basic ATPase medium + albumin (0.12%)	22.5	54.0	68.5	66.5
	+NEM	3.0	29.5	3.5	36.5
	+Oligomycin	4.0	27.0	3.0	26.5

[a] Mitochondria isolated in mannitol (0.21 M), sucrose (0.07 M), EDTA (0.01 M), and Tris (0.07 M), then finally made up in mannitol (0.25 M). [b] Same as *a* except that isolation medium also contained 1% albumin.

both were inhibited completely by the flavin inhibitor (atebrin), sodium azide (which reacts with hemoproteins), and the oxidative phosphorylation inhibitor (oligomycin) (see Table I).

Contrary to the findings of Azzone *et al.* (1961), the rate of the Mg^{2+}–ATPase was doubled when the Mg^{2+} concentration was increased from 1.5 to 5.0 mM. Mg^{2+} (8.0 mM) was inhibitory (Table IV). The increase in activity with higher concentrations of Mg^{2+} is indeed due to a PMB-resistant Mg^{2+}–ATPase. However, such an increase in Mg^{2+} concentration does not activate the ATPase as effectively as does Ca^{2+} at the same concentration. However, PMB largely inhibited Ca^{2+} activation of the ATPase. A divalent cation concentration of 5 mM saturates the ATPase site since the superimposition of Ca^{2+} no longer activates the Mg^{2+}–ATPase in the presence of this concentration of Mg^{2+}. It would appear that the Mg^{2+}–ATPase site in skeletal muscle mitochondria is an independent, divalent-cation-stimulable site.

The ATPase Activities of Mitochondria Isolated in the Absence of Heparin. It is apparent in Table V that the DNP-stimulable ATPase activity of mitochondria isolated in the absence of heparin is considerably less than that shown by mitochondria isolated in the presence of heparin throughout. Also, the PMB-resistant Mg^{2+}–ATPase of such mitochondria is not susceptible to oligomycin, in contrast to the suscepti-

TABLE VI: The Oxidative and Energy-Coupling Activities of Skeletal Muscle Mitochondria.

Substrate	A. Mitochondria Isolated in Presence of Heparin[a]				B. Mitochondria Isolated in Absence of Heparin[b]			
	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	1.6	19.3	12.1	2.65
Pyruvate + malate	8.1 ± 2.4	40.6 ± 9.7	5.0	4.14	8.0	25.5	3.2	2.78
α-Ketoglutarate + malonate	1.5 ± 0.4	22.3 ± 2.8	14.9	4.18	2.4	17.1	7.1	2.54
Succinate + amytal	18.9 ± 5.1	30.9 ± 5.1	1.6		8.6	15.2	1.8	

[a] Skeletal muscle mitochondria isolated in mannitol–heparin–EDTA and incubated in the presence of albumin and excess EDTA. [b] Skeletal muscle mitochondria isolated without heparin and incubated in the presence of albumin and excess EDTA. Incubation conditions were those outlined under Figure 1; glutamate (0.10 M); pyruvate (0.10 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; and ADP:O = micromoles of ADP:microgram-atoms of O.

bility of heparin-treated mitochondria. Isolation of the mitochondria in the presence of albumin did not significantly improve the DNP-stimulable ATPase activity and did not yield an oligomycin-sensitive Mg^{2+}–ATPase. Mitochondria isolated in the presence of heparin but suspended finally in its absence showed a pronounced reduction in DNP–ATPase activity (compare Tables I and II). On the other hand, mitochondria suspended in heparin can be kept for hours with no significant loss of activity.

The Oxidative and Energy-Coupling Activities of Skeletal Muscle Mitochondria Isolated in the Presence of Heparin. It appeared that the Mg^{2+}-stimulable ATPase activity involved an independent site in skeletal muscle mitochondria and that an active Mg^{2+}–ATPase was inhibitory to the respiratory-chain-linked energy-transfer site. Consequently, it appeared that a true measure of respiratory-chain-coupling potential could only be obtained in the absence of an operating Mg^{2+}–ATPase. Measurements of oxidative activity confirmed this postulation. The inclusion of Mg^{2+} in the incubation medium did not increase oxidation rates but high ATPase activity under these circumstances caused inhibition of oxidation. The inclusion of 7 mM EDTA with 5 mM Mg^{2+} in the incubation medium permitted maximal rates of oxidation. The inclusion of low levels of EDTA alone promoted the slow development of an ATPase activity, presumably due to the leakage of bound Mg^{2+} from the mitochondria.

Figure 1 shows the controlling effect of successive additions of ADP on the respiration of mitochondria incubated in a medium containing 5 mM Mg^{2+} and 7 mM EDTA. The omission of albumin from the incubation medium or the addition of albumin after the addition of the mitochondria resulted in failure of respiratory stimulation. When albumin was added to the medium prior to the addition of the mitochondria, the rate of oxidation of substrate was markedly stimulated by the addition of phosphate acceptor and the initial low rate of oxidation was resumed as soon as the ADP concentration was no longer able to maintain full coupling activity. Consequently, these conditions were employed for the measurements of oxidation and coupling activity.

Mitochondria isolated in the presence of heparin and incubated in the presence of albumin and excess EDTA showed very tight coupling and very high respiratory activity (Table VI). They oxidized glutamate and pyruvate plus malate at very high rates while α-ketoglutarate (in the presence of malonate) and succinate (in the presence of amytal) were oxidized at very good but lesser rates. The rate of β-hydroxybutyrate oxidation was extremely low. Glutamate and α-ketoglutarate showed tight coupling and theoretical ADP:O ratios but pyruvate, although it showed a theoretical ADP:O ratio, reflected the uncoupled oxidation of succinate in its lower respiratory index. Succinate oxidation was almost completely uncoupled, in agreement with Tata *et al.* (1963).

The Oxidative and Energy-Coupling Activities of Mitochondria Isolated in the Absence of Heparin. It is apparent in Table VI that the omission of heparin from the isolation medium has resulted in much lower respiratory activities. Also P:O ratios of the two substrates which involve substrate level phosphorylation (pyruvate and α-ketoglutarate) were significantly below theoretical values. The technique yielded a much smaller volume of heavy, intact mitochondria.

Mitochondria isolated and made up finally in 0.10% albumin showed good respiratory control but oxidative activities were not nearly as high as for mitochondria isolated in the presence of heparin.

The use of proteinase for the isolation of the mitochondria resulted in a poor separation of the heavy,

intact mitochondria because of gel formation. Consequently, ATP was used in the isolation medium to obtain a more effective packing. However, the considerable amount of insoluble residue in the biuret reaction mixtures and the variation in respiratory activities indicated significant contamination of the mitochondrial precipitate with inert collagen protein.

Discussion

Heparin as a Possible Inhibitor of Mitochondrial Lipoprotein Lipase. The fairly rapid decline in oxidative activity which occurs when the mitochondrial pellet is suspended finally in the absence of heparin and the lower oxidative activity of mitochondria isolated in the absence of heparin would appear to support the findings of Ito and Johnson (1964) that decrease in respiratory control is due to the loss of structural integrity. A possible lipoprotein nature of the ATPase has been suggested (Pullman *et al.*, 1960). The beneficial effect of heparin on respiratory activity would be explained by the finding that heparin, a polyanion, acts as a substrate–competitive inhibitor of lipoprotein lipase (Korn, 1962). Phospholipase activity of mitochondria has been demonstrated (Scherphof and Van Deenen, 1965; Rossi *et al.*, 1965). Activity of this enzyme has been shown to destroy the oligomycin sensitivity of the mitochondrial ATPase (Kagawa and Racker, 1966); longer exposure to phospholipase results in inactivation of the respiratory chain (Ambe and Crane, 1959). Isolation of skeletal muscle mitochondria in the absence of heparin yielded granules in which the Mg^{2+}–ATPase had lost its oligomycin sensitivity and the DNP-stimulable ATPase activity and the respiratory activity were very significantly reduced.

Albumin as a Mitochondrial Coupling Factor. It is apparent that these mitochondria contain a respiratory-chain-coupling ATPase site which permits spontaneous ATP hydrolysis. This hydrolysis would appear to be caused by accessibility to water since it is not caused by fatty acid. The inclusion of albumin in the incubation medium converts these mitochondria from the open form to a closed system, one which is accessible only to the lipid-soluble DNP vehicle for ATPase activation. Yoshida *et al.* (1965) and Machinist and Crane (1965) have shown a closing action of the polycation, protamine, which has been shown to combine with acidic phospholipids of mitochondria. A soluble protein has been obtained from mitochondria (Pullman and Monroy, 1963; Machinist and Crane, 1965) which was shown to combine with the mitochondrial ATPase to yield an ATPase–inhibitor complex which did not react with water but was capable of coupling oxidation to phosphorylation. Such a closing effect was supported by the early work of Lardy (1951). Recently, Lee and Ernster (1965) have suggested that some of the so-called coupling factors may stimulate oxidative phosphorylation by inhibiting the hydrolytic breakdown of a nonphosphorylated intermediate.

The Differential ATPase Activities of Skeletal Muscle Mitochondria. The present findings demonstrate differential behavior for the Mg^{2+}- and DNP-stimulable ATPase activities of skeletal muscle mitochondria and thus indicate independent enzyme sites. This is confirmed by the fact that Mg^{2+}–ATPase activity is inhibitory to respiratory-chain-linked energy transfer. It would appear then that the Mg^{2+}–ATPase is not a part of the enzyme sequence involved in the DNP–ATPase, as suggested for liver mitochondria by Potter *et al.* (1953) and Siekevitz *et al.* (1958). A similarity of effects by oxidative phosphorylation inhibitors does not necessarily implicate the energy-coupling apparatus of the respiratory chain in the Mg^{2+}–ATPase mechanism.

Like the DNP–ATPase activity, the Mg^{2+}–ATPase is inhibited completely by the flavin inhibitor (atebrin). We must consider the possibility then that both reactions involve reduced flavoprotein as an intermediate. The findings would appear to support the postulation of Ernster (1961) that the P_i–ATP exchange involves an actual electron shuttle between DPN and flavin, with reduced flavoprotein acting as an intermediate phosphate transmitter. However, since respiratory inhibitors had no effect on the Mg^{2+}–ATPase it would appear that an electron shuttle within the respiratory chain is not involved in this ATPase. An alternative postulation involving a flavoprotein that is not a member of the electron-transport chain in the Mg^{2+}–ATPase mechanism (Azzone *et al.*, 1961) would appear to fit the present findings. Indeed, the finding that Ca^{2+} activates the Mg^{2+}–ATPase site more effectively than does Mg^{2+} would appear to indicate that this is a divalent-cation-stimulable site, in common with kinase reactions in general.

The Oxidative and Energy-coupling Activities of Skeletal Muscle Mitochondria. Skeletal muscle mitochondria, isolated in the presence of heparin and incubated in the presence of albumin and excess EDTA, showed oxidative activity and respiratory indices which were much higher than have been observed by other workers. All of these workers employed a glucose–hexokinase trap for ATP as well as albumin in the incubation medium (Chappell and Perry, 1953; Azzone and Carafoli, 1960; Azzone *et al.*, 1961; Kiessling, 1962; Marcus and Manery, 1963; Tata *et al.*, 1963; Blanchaer, 1964). However, P:O ratios found by these workers approached theoretical values for respiratory-chain-coupled substrates. P:O ratios were found to be relatively less susceptible to changes in physical state of the particles than was oxidative capacity (Slater and Cleland, 1953). But P:O ratios for the two substrates that involve substrate level phosphorylation were found by these workers to be significantly below theoretical values. Heart sarcosomes have also been found consistently to show low P:O ratios for these substrates (Slater and Cleland, 1953). Theoretical P:O ratios were found for all substrates with skeletal muscle mitochondria isolated in the presence of heparin and incubated in the presence of albumin and excess EDTA.

The findings show that loss of structural integrity, possibly due to phospholipase activity during isolation, loss of a soluble-coupling factor during incubation,

and inhibition resulting from divalent-cation-stimulable ATPase activity are the factors responsible for the lower respiratory and coupling activities of skeletal muscle mitochondria observed by other workers.

Acknowledgment

The author wishes to thank Mr. Charles Paul for sharing the technical challenges of this work, Mr. Leonard Carter for assuming the responsibility of the animal experiments, and Mr. Harold Costin for assisting with the animal experiments.

References

Ambe, K. S., and Crane, F. L. (1959), *Science 129*, 98.
Azzone, G. F., and Carafoli, E. (1960), *Exptl. Cell Res. 21*, 447.
Azzone, G. F., Carafoli, E., and Muscatello, U. (1960), *Exptl. Cell Res. 21*, 456.
Azzone, G. F., Eeg-Oloffson, O., Ernster, L., Luft, R., and Szabolcsi, G. (1961), *Exptl. Cell Res. 22*, 415.
Blanchaer, M. C. (1964), *Am. J. Physiol. 206*, 1015.
Chance, B., and Hagihara, B. (1961), *Proc. 5th Intern. Congr. Biochem., Moscow*, V-3.
Chance, B., and Williams, G. R. (1955), *J. Biol. Chem. 217*, 429.
Chappell, J. B., and Perry, S. V. (1953), *Biochem. J. 55*, 586.
Ernster, L. (1961), *Proc. 5th Intern. Congr. Biochem., Moscow*, V-115.
Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.
Ito, T., and Johnson, R. M. (1964), *J. Biol. Chem. 239*, 3201.
Kagawa, J., and Racker, E. (1966), *J. Biol. Chem. 241*, 2461.
Kielley, W. W., and Kielley, R. K. (1953), *J. Biol. Chem. 200*, 213.
Kiessling, K. H. (1962), *Exptl. Cell Res. 28*, 145.
Korn, E. D. (1962), *J. Biol. Chem. 237*, 3423.
Lardy, H. A. (1951), *in* Phosphorus Metabolism, Vol. I, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., Johns Hopkins, p 484.
Lardy, H. A., and Wellman, H. (1952), *J. Biol. Chem. 195*, 215.
Lardy, H. A., and Wellman, H. (1953), *J. Biol. Chem. 201*, 357.
Lee, C. P., and Ernster, L. (1965), *Biochem. Biophys. Res. Commun. 18*, 523.
Lindberg, O., Low, H., Conover, C., and Ernster, L. (1961), *Symp. Biol. Structure Function, Stockholm*, II-3.
Machinist, J. M., and Crane, F. L. (1965), *J. Biol. Chem. 240*, 1788.
Marcus, G. J., and Manery, J. F. (1963), *Can. J. Biochem. Physiol. 41*, 2529.
McIlwain, H., and Buddle, H. L. (1953), *Biochem. J. 53*, 412.
Potter, V. R., Siekevitz, P., and Simonson, H. C. (1953), *J. Biol. Chem. 205*, 893.
Pullman, M. E., and Monroy, G. C. (1963), *J. Biol. Chem. 238*, 3762.
Pullman, M. E., Penefsky, H. S., Datta, A., and Racker, E. (1960), *J. Biol. Chem. 235*, 3322.
Rossi, C. R., Sartorelli, L., Tato, L., Barretta, L., and Siliprandi, N. (1965), *Biochim. Biophys. Acta 98*, 207.
Scherphof, G. L., and Van Deenen, L. L. M. (1965), *Biochim. Biophys. Acta 98*, 204.
Siekevitz, P., Low, H., Ernster, L., and Lindberg, O. (1958), *Biochim. Biophys. Acta 29*, 378.
Slater, E. C., and Cleland, K. W. (1953), *Biochem. J. 53*, 566.
Sumner, J. B. (1944), *Science 100*, 413.
Tata, J. R., Ernster, L., Lindberg, O., Arrhenius, E., Pedersen, S., and Hedman, R. (1963), *Biochem. J. 86*, 408.
Yoshida, H., Frijisawa, H., and Ohi, Y. (1965), *Can. J. Biochem. 43*, 841.