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The Respiratory and Adenosinetriphosphatase Activities of Skeletal-Muscle Mitochondria

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The study of the functions and enzymic nature of isolated mitochondria has been carried out mainly on particles obtained from liver. Kidney preparations have received some attention, but only recently have attempts been made to study mitochondria isolated from other tissues, such as, for example, from brain (Brody & Bain, 1952) and from mammary gland (Moore & Nelson, 1952).

A number of the oxidative systems, now usually associated with intact mitochondria, have been studied in muscle for several decades. Keilin & Hartree (1938, 1939) and Stern (1939) carried out early work on the nature of preparations from heart muscle which showed succinic oxidase activity and which were particulate in nature, but it is only very latterly that detailed studies have been made on heart-muscle particles isolated to correspond as closely as possible to their natural state in the cell (Harman & Fiegelson, 1952*a*; Slater & Cleland, 1953). Skeletal muscle has received even less attention, although recently communications have appeared on the enzymic properties of insect flight-muscle mitochondria (Watanabe & Williams, 1951; Sacktor, 1953), and the adenosine triphosphatase (ATPase) activity of the particulate components of rabbit skeletal muscle has been investigated by Perry (1952*a*).

Skeletal muscle is quantitatively by far the most important tissue for the oxidation of many metabolites, and for this reason alone its mitochondrial system merits study. This apparent neglect is no doubt due to the fact that the resting respiration rate of skeletal muscle from many animals is very low and that, in contrast to liver, the cells contain fewer granules, myofibrils being the dominant feature of the muscle cell. Paul & Sterling (1952) have prepared cyclophorase preparations from the skeletal muscle of various species, and report that many are poor in oxidative activity and that in general a correlation exists between granule count and oxidative activity. Cyclophorase preparations from muscle may, however, be very poor in mitochondria because this preparation contains, as main contaminant, the myofibrillar fraction which may account for 90–60 % of the total nitrogen, depending on the type of muscle.

We have investigated the oxidative activities of granules from back and leg muscle of the rabbit,

rat- and hamster-leg muscle, and pigeon-breast muscle. The last preparation was found most suitable for our purpose and was used for most of the studies reported here. These studies constitute a preliminary approach to the investigation of the metabolism of adenosine triphosphate (ATP) in the skeletal-muscle cell with special reference to the organization of these processes in relation to the intracellular components.

A preliminary account of this work was presented at the Second International Congress of Biochemistry (Chappell & Perry, 1952).

EXPERIMENTAL

Granule preparation. All stages of the preparations and fractionations were carried out in the cold room at 1–2°. Breast muscle from a pigeon killed by decapitation was removed as quickly as possible and cooled in ice-cold 8·5% (w/v) sucrose. The muscle was blotted with filter paper, cut into small cubes and passed through a previously chilled Latapie mincer. The mince was then suspended in 9 vol. of 8·5% sucrose, comminuted in the Potter-Elvehjem all-glass homogenizer, and centrifuged for 5 min. at 600 *g* to remove myofibrils, nuclei and cell debris. An appreciable proportion of the granules with oxidative activity was brought down in the myofibrillar fraction, but in routine preparations this fraction was discarded for reasons of speed and convenience. The turbid supernatant was decanted into 50 ml. plastic centrifuge tubes and centrifuged for 10 min. at 3500 *g* in the Servall angle centrifuge. This procedure sedimented out the mitochondria, which were resuspended in 8·5% sucrose with the aid of a pipette, and washed once or twice by repeating the high-speed centrifugation. The actual volume used for the final resuspension varied with the enzymic assay to be carried out, but usually the mitochondria from 200 ml. of 10% suspension were resuspended in 20 ml.

Similar procedures were employed for the preparation of granules from hamster- and rat-leg muscles, and from heart muscle. In the case of rabbit back and leg muscle the yield was so poor that it was necessary to resuspend in fresh 8·5% sucrose solution the residue obtained after the low-speed centrifugation. The supernatants thus obtained were combined, and the granules sedimented by centrifugation at 8000 *g* for 15 min.

Fractionation of pigeon-breast muscle suspensions. A 10% pigeon-breast suspension, prepared as described above, was passed through cheese cloth to remove any fibrous material or pieces of unbroken tissue. Enzymic assays were carried out simultaneously on the following fractions within 90 min. of the death of the pigeon. (i) *Whole suspension (untreated)*; original suspension stored at 0° and not centrifuged. (ii) *Whole suspension (centrifuged and resuspended)*; 50 ml. suspension centrifuged for 5 min. at 600 *g* followed by resuspension with the Potter-Elvehjem homogenizer. Repeated twice. (iii) *Mitochondria*; 50 ml. suspension processed as described for fraction (ii) but with the difference that the supernatant was removed after each centrifugation. Fresh 8·5% sucrose was added to the residue to bring the volume back to 50 ml. before resuspension. The combined supernatants were centrifuged at 3500 *g* for 10 min. to sediment the mitochondria, which were washed once and dispersed in 50 ml. 8·5% sucrose. (iv) *Residue*; fraction remaining after the removal of successive supernatants for the preparation of mitochondria as described in (iii). Resuspended in a final volume of 50 ml. 8·5% sucrose. (v) *Supernatant*; 50 ml. suspension processed as described in (ii). After the third centrifugation the supernatant was removed, the mitochondria were sedimented as in (iii) and the clear supernatant was used directly for enzymic assay. An undiluted supernatant was used, since dilution affects the activity of some enzymes. Some experiments were carried out using the combined diluted supernatants obtained after removal of the mitochondria as described in (iii). The results with the supernatant prepared in this way were essentially the same as those obtained with the undiluted supernatant.

Adenosine triphosphatase activity. ATPase activity was determined in the presence of 0·025 M-glycylglycine-NaOH buffer, pH 7·4, 0·3 ml. ATP (0·04—0·05 M-Na salt), and $MgCl_2$, $MgSO_4$ or $CaCl_2$ as activators. Tonicity was varied by the addition of 1·0 M-sucrose. The final volume was 2·0 ml. in all cases. Incubations were carried out at 17 and 30°. The reaction was started by addition of the mitochondrial suspension and stopped with 1·0 ml. 20% (w/v) trichloroacetic acid. Phosphate was estimated by the method of Fiske & Subbarow (1925).

Manometric experiments. These were carried out at 17 and 30° in Warburg manometers of approximately 20 ml. gas space, which had been calibrated by the method of Dickens (1951). KOH (0·2 ml., 20%, w/v) was added to the centre well and a roll of Whatman no. 40 (starch-free) filter paper inserted, as recommended by Dixon (1951). The mitochondrial suspension was added directly to the main vessel, and readings were begun 5 or 10 min. after immersion in the bath, the shorter equilibration time being used at the lower temperature. Readings were taken at 5 min. intervals without bringing the manometers to rest.

Measurement of oxidative phosphorylation. Experiments were carried out in Warburg manometers at 17°. The medium consisted of 0·025 M-glycylglycine-NaOH buffer, pH 7·4; 0·005 M-$MgSO_4$, 5 μmoles ATP, 30 μmoles potassium phosphate buffer, pH 7·4, 40 μmoles sodium α-ketoglutarate, 60 μmoles glucose, 0·005 M-NaF. Tonicity was varied by the addition of sucrose and KCl. Finally 0·2 ml. hexokinase in 1% (w/v) glucose was added immediately followed by the mitochondrial suspension. The total volume was 4·0 ml. Trichloroacetic acid (0·5 ml., 20%, w/v) was added from the side bulb after 5 min. equilibration in the control flasks, and 5–25 min. later in the experimental flasks. Phosphate estimations were carried out on the trichloroacetic acid filtrate.

Succinic dehydrogenase activity. Succinic dehydrogenase activity was determined in Warburg manometers at 30° in the presence of 0·001 M-methylene blue, 0·025 M-sodium succinate and 0·15 M-phosphate buffer, pH 7·4. The total volume was 4·0 ml. The enzyme was added directly to the main compartment of the manometer vessel.

Hexokinase activity. Hexokinase activity was measured by following the disappearance of the inorganic phosphate P liberated in 7 min. by N-HCl at 100° when the enzyme was incubated with glucose and ATP. These estimations were carried out in a medium corresponding as closely as was possible in composition to that used for the oxidative phosphorylation experiments.

Adenosine triphosphate. ATP was prepared as a routine in this laboratory as the barium salt. The method used was based on that of Needham (1942) and included treatment with 8-hydroxyquinoline to remove heavy-metal impurities.

Barium ATP was converted into the sodium salt by the procedure of Bailey (1942), the solution adjusted to pH 6·5 and 10 ml. samples stored at −15°. Dr K. Bailey (personal communication) has shown, using ion-exchange columns, that such ATP preparations may contain as much as 15% adenosine diphosphate on a molar basis. Using hexokinase it has been confirmed that these preparations usually contain 85–90% ATP. The ratio of labile P to total P is an unsatisfactory criterion for the purity of ATP preparations; for example, a mixture containing 15% ADP would give a ratio of 0·65, whereas for pure ATP the ratio is 0·67. It seems likely that most ATP preparations made in a similar manner will contain adenosine diphosphate (ADP) unless special measures have been taken to remove it (see Cohn & Carter, 1950).

Hexokinase. Hexokinase was prepared from yeast supplied by the Ark Yeast Co., using the method of Kunitz & McDonald (1946).

Triphosphopyridine nucleotide (TPN). This was prepared by the method of Warburg, Christian & Griese (1935).

Cytochrome c. Cytochrome *c* was prepared by the method of Keilin & Hartree (1945).

Carboxylic acids. Citric and succinic acids were A.R. preparations, the fumaric acid was supplied by Gentosan and the malic acid by Light and Co. Ltd. Pyruvic and α-ketoglutaric acids were made by Mr B. R. Slater by the methods of Robertson (1942) and Friedman & Kosower (1946), respectively. The pyruvic acid was stored in the refrigerator as a 0·3 M-solution of the sodium salt adjusted to pH 5·0. Immediately before use this stock solution was diluted to 0·1 M and the pH brought to 7·4.

Removal of heavy-metal impurities with 8-hydroxyquinoline. All reagents were tested for heavy-metal impurities by shaking their aqueous solutions with a very small volume of $CHCl_3$ containing 0·5% 8-hydroxyquinoline. If the $CHCl_3$ phase darkened, heavy-metal impurities were presumed to be present. These were removed as follows. A concentrated solution of the reagent was brought to about pH 7 if stable at this pH, extracted twice with 0·5% 8-hydroxyquinoline in $CHCl_3$ solution, washed with two portions of $CHCl_3$ and the reagent recrystallized or precipitated with ethanol. It was found necessary to treat the succinic and fumaric acids in this way.

RESULTS

General properties of pigeon-breast mitochondria. Mitochondrial preparations in 0·25 M-sucrose were obtained as pale biscuit-coloured suspensions which microscopic examination showed to consist of spherical bodies 1–2 μ. in diameter, completely unaggregated and moving freely on the slide. These preparations consisted mainly of the large granular components of the muscle and will be referred to as mitochondria in view of the association with them of practically all the oxidative activity of whole pigeon muscle. High magnification phase contrast was the most satisfactory method of microscopic observation, for under these conditions it was not necessary to use stain, which tended to aggregate the preparations. When viewed with the electron microscope the granules appeared as spherical bodies which varied considerably in size, the largest being approximately 2 μ. in diameter.

In the visible region the mitochondrial suspensions showed a strong absorption spectrum containing bands corresponding to cytochromes *a*, *b* and *c*. These bands were particularly conspicious after reduction with succinate or dithionite. Myoglobin could not be detected in the preparations. Fig. 1 shows the cytochrome spectrum of the mitochondria together with that of yeast for comparison (these photographs were kindly taken by Dr R. Hill and Dr H. E. Davenport).

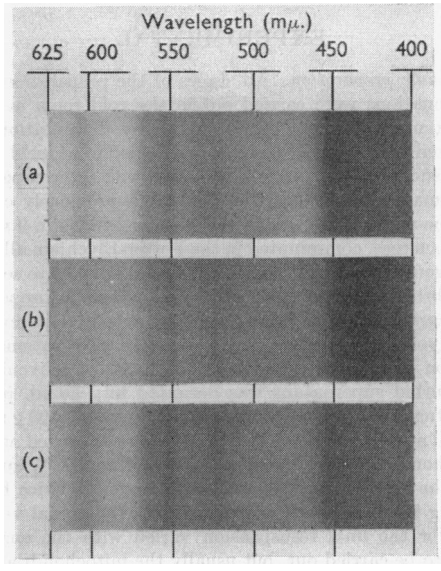

Fig. 1. Absorption spectra: (*a*) and (*c*) suspension of pigeon-breast mitochondria in the presence of succinate; (*b*) compressed baker's yeast.

The mitochondria appear to maintain their spherical shape when stored for as long as 10 days at 0°, and even after repeated freezing and thawing microscopically apparent changes could not be readily detected.

Oxidation of Krebs's cycle intermediates. In general the initial rates of oxidation of citrate, α-ketoglutarate, succinate, fumarate and malate by different mitochondrial preparations were remarkably constant. Table 1 shows the initial rates of oxidation at 30° of these tricarboxylic acid cycle intermediates. It will be noted that the rate of oxidation of citrate was substantially lower than that obtained with the other acids. The view that TPN might be a limiting factor in the system was supported by the observation that the addition of this co-enzyme increased the rate of citrate oxidation; for example, in one experiment 0·015% TPN increased the oxygen uptake from 112 to 194 μl. O_2/mg. N/hr.

Table 1. *Oxidation of Krebs's cycle intermediates by pigeon breast muscle mitochondria at 30°*

(Each manometer vessel contained 50 μmoles of substrate, 0·005 M-$MgCl_2$, 5×10^{-5} M-cytochrome *c*, 0·01 M-potassium phosphate buffer pH 7·4, 10 μmoles ATP and 1·0 ml. mitochondrial suspension. Total vol., 4·0 ml. The experiments were commenced 60 min. after the death of the animal.)

Substrate	Initial rate of oxygen uptake (μl./mg. N/hr.) Prep. 1	Prep. 2
Citrate	100	120
α-Ketoglutarate	350	370
Succinate	470	450
Fumarate	225	220
D-Malate	265	290

Table 2. *Oxidation of pyruvate by pigeon-breast mitochondria at 30°*

(The conditions were as in Table 1. 0·0125 M-sodium pyruvate, 0·00125 M-sodium malate added as indicated below.)

Substrate	Initial rate of oxygen uptake (μl./mg. N/hr.) Prep. 1	Prep. 2
Pyruvate	0	0
Malate	100	100
Pyruvate + malate	300	280

Pyruvic oxidase activity. At 30° pyruvate was not oxidized by pigeon-breast mitochondria at a measurable rate. In the presence of 'sparker' concentrations of malate (0·001 M) the rate of oxygen uptake became considerably higher than that obtained with the 'sparker' malate alone (see Table 2). This oxidation of pyruvate, however, fell off sharply until after 25–30 min. there was virtually no oxygen uptake in excess of that due to the 'sparker' malate. Varying the sucrose concentration in the manometer pot from 0·063 to 0·30 M did not alter the rate of pyruvate oxidation when 0·001 M-fumarate was used as a 'sparker', nor was there any change in the rate at which inactivation took place.

At 17° the oxidation of pyruvate was far less labile and the rate was maintained for periods up to 80 min. The results of experiments at this temperature are presented in Table 3 and they clearly demonstrate the need for low concentrations of malate for the oxidation of pyruvate by pigeon-breast mitochondria. It will be seen from Fig. 2 that both Mg^{2+} and ATP were essential additions for full activity of the pyruvic oxidase system, whereas cytochrome *c* had no effect either on the initial rate of oxidation or on the maintenance of this rate. The succinic oxidase system of the mitochondria showed a similar insensitivity to added cytochrome *c*. Freezing and thawing the mitochondria six times

Table 3. *Pyruvate oxidation by pigeon-breast mitochondria at 17°*

(Each manometer contained 0·005 M-$MgSO_4$, $2{\cdot}5 \times 10^{-6}$ M-cytochrome *c*, 10 μmoles ATP, 10 μmoles potassium phosphate pH 7·4, 0·01 M-glycylglycine-NaOH buffer pH 7·4, 40 μmoles potassium pyruvate and 5 μmoles sodium malate were added as indicated below, followed by 1 ml. of mitochondrial suspension. Total vol., 4 ml. Readings commenced after 7·5 min. equilibration.)

Substrate	Initial rate of oxygen uptake (μl./mg. N/hr.) Prep. 1	Prep. 2	Prep. 3
Pyruvate	49	—	60
Malate	30	34	46
Pyruvate + malate	218	200	224
No substrate	18	—	28

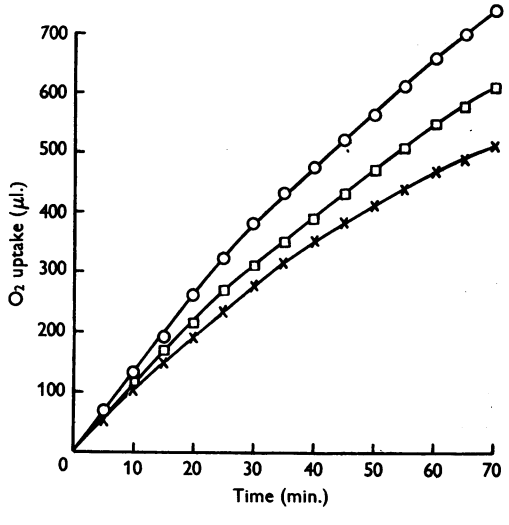

Fig. 2. The effect of cytochrome *c*, Mg^{2+}, and ATP on the malate-'sparked' oxidation of pyruvate by washed pigeon breast muscle mitochondria at 17°. Conditions as in Table 3. O—O, complete system with or without cytochrome *c*; □—□, Mg^{2+} omitted; ×—×, ATP omitted or Mg^{2+} and ATP omitted.

increased the requirement for magnesium ions and ATP, but still no increase in the rate of pyruvate oxidation could be demonstrated on the addition of cytochrome *c*.

Localization of the succinic and pyruvic oxidase systems. In all tissues so far examined almost the whole of the intracellular oxidative activity has been found to be associated with the large granule fraction.

Skeletal muscle is unique in that although it is capable of high oxidative activity, the dominant features of the cytoplasm are myofibrils, and the granules lying between these structures, the sarcosomes, are arranged in apparently regular fashion

Table 4. *Localization of oxidative activity in pigeon-breast suspensions*

(For details of fractionation, see Methods. Assays were carried out at 17° in the presence of 0·005 M-$MgSO_4$, 40 μmoles potassium phosphate pH 7·4, 0·05 M-glycylglycine pH 7·4, 5 μmoles ATP, 2×10^{-5} M-cytochrome *c* and 0·25 M-sucrose. In the case of the succinoxidase experiment, 40 μmoles sodium succinate were added, and for the pyruvic oxidase, 40 μmoles sodium pyruvate and 5 μmoles sodium malate. Total vol., 4·0 ml.)

Fraction	Pyruvic oxidase activity			Succinoxidase activity		
	(μl. O_2/ ml./hr.)	(μl. O_2/ mg. N/hr.)	Activity (%)	(μl. O_2/ ml./hr.)	(μl. O_2/ mg. N/hr.)	Activity (%)
Whole suspension						
(*a*) Untreated	130	46	—	—	—	—
(*b*) Suspended, centrifuged, not separated	110	39	(100)	160	123	(100)
Residue (myofibrils, nuclei, etc.)	16	14	14·5	2	2·5	1·3
Mitochondria	90	170	82	84	298	52·5
Supernatant	—	—	—	9	10·5	5·6
Recovery in all fractions	—	—	96·5	—	—	59·5

and in close association with them. In view of this close association it was of interest to determine the pattern of the intracellular distribution of oxidative activity in this tissue. Table 4 contains the summarized results of experiments on the distribution of pyruvic and succinic oxidase activities in a suspension of pigeon-breast muscle which was initially strained through muslin to remove the coarse fibrous tissue particles. It is clear that the major portion of the activity of the suspension with respect to both systems was associated with the mitochondrial fraction. The residue, which consisted mainly of myofibrils contaminated with nuclei, stroma, etc., and the supernatant, in which the glycolytic enzymes are to be found, contained little activity. When the results are expressed as μl. O_2/mg. N/hr. the concentration of both oxidase systems on the mitochondria is made very evident.

Respiratory granules from other skeletal muscles. Granules with respiratory activity have been obtained from rabbit-, rat- and hamster-skeletal muscle but have been studied less extensively than pigeon-breast mitochondria. Considerable variation was obtained in the yields from different muscles, the relative abundance of granules being as follows: pigeon-breast > hamster-leg > rat-leg > rabbit-back and rabbit-leg muscles. Thus 15–21 % of the total nitrogen of pigeon-breast muscle is accounted for by the mitochondrial fraction sedimented at 3500 *g*, whereas in rabbit muscle the granules require 8000 *g* to be sedimented in any quantity and then only make up 3–4 % of the total nitrogen of this tissue.

The succinic oxidase activity of the granules obtained from rat- and hamster-leg muscle was essentially the same as those of pigeon-breast mitochondria when compared on a nitrogen basis (see Table 1 for assay conditions). Rabbit-muscle granules had lower succinic oxidase activity; for example, two assays carried out at 20 and 30° gave oxygen uptakes of 24 and 93 μl./mg. N/hr. respectively. The preparation from rabbit muscle required more extensive manipulation than was needed for the granule preparation from other muscles, and microscopic examination indicated the presence of occasional myofibrils.

Rabbit skeletal-muscle myofibrils, prepared as described in an earlier communication (Perry, 1952*b*), were completely without succinic dehydrogenase or oxidase activity.

It proved impossible to obtain an oxidation of pyruvate by rat leg-muscle granules under conditions in which pigeon-breast mitochondria showed rapid oxygen uptake.

Oxidative phosphorylation. Attempts to show an uptake of inorganic phosphate with adenylic acid or creatine as acceptor and either α-ketoglutarate or glutamate as substrate were uniformly unsuccessful. Frequently an increase in inorganic phosphate occurred during the incubation due to the fact that the added ATP was hydrolysed. When incubations were carried out at 17° some improvement was obtained, for although there was little uptake of inorganic phosphate after periods of 15 min. at least over the equilibration period there was a definite fall in the inorganic phosphate level in the manometers.

Rat-liver mitochondria prepared in the same way as the pigeon-breast preparations and assayed using the same solutions, behaved differently in that they showed a marked uptake of inorganic phosphate. The uptake of phosphate in both preparations was completely prevented by 5×10^{-5} M-2:4-dinitrophenol (DNP). This difference in apparent phosphorylating ability can be explained partly by the relative ATPase activity of the mitochondria of pigeon breast and liver. When freshly prepared, rat-liver mitochondria had only

Table 5. *Effect of 2:4-dinitrophenol on oxidative phosphorylation and ATPase activity of rat-liver and pigeon breast muscle mitochondria*

(All experiments were carried out simultaneously. Each incubation mixture contained 0·005 M-$MgCl_2$, 2×10^{-5} M-cytochrome *c*, 40 μmoles sodium glutamate, 10 μmoles potassium phosphate pH 7·4, 40 μmoles sodium adenylate, 0·005 M-NaF, 0·25 M-sucrose, 0·05 M-glycylglycine-NaOH buffer pH 7·4, and 5×10^{-5} M-2:4-dinitrophenol (DNP) where indicated. Total vol., 4·0 ml. ATPase assays were carried out in 0·25 M-sucrose. Incubations at 17° throughout.)

Source of mitochondria	O_2 consumption (by extrapolation) (atoms/ml./15 min.)		Inorganic P esterified (moles/ml./15 min.)		ATPase activity* (moles inorganic P liberated/ml./5 min.)	
	−DNP	+DNP	−DNP	+DNP	−DNP	+DNP
Rat liver	5·7	11·4	9·3	−2·0	4·5	24·5
Pigeon breast	7·3	3·3	2·8	−2·0	23·0	24·0

* There was no fluoride present in the incubation medium in these assays.

Table 6. *Oxidative phosphorylation by pigeon-breast mitochondria with α-ketoglutarate as substrate and glucose and hexokinase as phosphate acceptor system*

(For experimental details see Methods section. The mitochondria were normally not washed after sedimentation except in preparation 5 when they were washed once.)

Preparation no.	Preparation medium	Further additions	Time interval (min.)	Oxygen uptake (μatoms)	Inorganic P uptake (μmoles)	P:O ratio
1	0·25 M-sucrose	None	5–10	6·4	1·6	0·25
			5–15	11·8	2·5	0·21
		0·15 M-KCl	5–10	6·8	4·1	0·60
			5–15	11·9	7·2	0·61
2		0·15 M-KCl	5–10	4·1	7·4	1·85
			5–15	8·2	12·3	1·50
3		0·25 M-sucrose	5–10	6·4	13·3	2·10
			5–15	12·9	20·5	1·67
			5–20	16·6	25·7	1·55
4	0·25 M-sucrose + 0·001 M-ethylenediaminetetra-acetic acid	0·25 M-sucrose + 0·01 M-ethylenediaminetetra-acetic acid	5–10	2·5	5·0	2·00
			5–15	5·2	7·9	1·50
			5–20	6·4	9·2	1·45
			5–30	10·0	13·2	1·30
5		0·25 M-sucrose + 0·01 M-ethylenediaminetetra-acetic acid	5–15	8·9	6·6	0·74

one-fifth to one-sixth the ATPase activity of a pigeon-breast preparation obtained under similar conditions and showing a comparable oxygen uptake with α-ketoglutarate as substrate (Table 5).

The use of hexokinase and glucose as a phosphate-acceptor system produced a definite uptake of inorganic phosphate when α-ketoglutarate was used as substrate and incubation was carried out at 17°. Table 6, summarizing the results of a number of experiments carried out to demonstrate oxidative phosphorylation, illustrates the following features. (i) As has already been shown with heart-muscle mitochondria by Slater & Cleland (1952), the incorporation of 0·001 M-ethylenediaminetetraacetic acid (Versene) in the 0·25 M-sucrose used in preparing the pigeon-breast mitochondria had a beneficial effect on the P:O ratio. (ii) Higher P:O ratios were obtained when the medium in the manometer vessels was made isotonic by the addition of sucrose. (iii) The addition of 0·01 M-ethylenediaminetetraacetic acid to the medium used for the demonstration of oxidative phosphorylation produced little if any increase in the uptake of inorganic phosphate. (iv) Washing the granules tended to destroy their power of oxidative phosphorylation.

The adenosine triphosphatase activity of pigeon-breast mitochondria. Mitochondrial preparations from pigeon-breast muscle readily liberated inorganic phosphate from ATP at pH 7·4. From microscopic examination and a study of the characteristics of the enzyme it was obvious that the ATPase activity could not be attributed to myofibrillar contamination. The evidence strongly suggested that the production of inorganic phosphate from ATP was due to an enzyme similar to that already reported to be present on granules

isolated from rat and rabbit muscle (Perry, 1952*a*), and comparable to the ATPase invariably found to be present in mitochondria isolated from other tissues (Kielley & Kielley, 1951). At pH 7·4 the enzyme was activated by magnesium ions and to a less degree by calcium ions, although at the higher concentration of calcium the activity was quite marked. Fig. 3 shows a typical ion activation curve from which it will be seen that under the specified

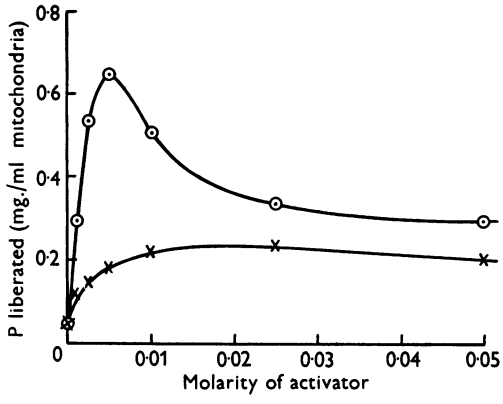

Fig. 3. The effect of Mg^{2+} and Ca^{2+} on the ATPase activity of pigeon-breast-muscle mitochondria at pH 7·4. ⊙—⊙, Mg^{2+}; ×—×, Ca^{2+}. Incubation at 30°.

conditions, 0·005 M-magnesium chloride produced maximal activation, whereas with calcium chloride no sharp maximum was obtained. Pigeon-breast preparations did not liberate phosphate from β-glycerophosphate at pH 7·4, but showed slight pyrophosphatase activity at this pH. In these properties the pigeon-breast mitochondria closely resembled the granules isolated from rabbit muscle.

Under the conditions of the experiments reported above, the mitochondria showed no latent ATPase activity (Kielley & Kielley, 1951) and progress curves were linear for incubation periods up to 15 min. The q_P values (μl. P/mg. N/hr.) ranged from 2300 to 1400, the lower values usually being obtained with those mitochondrial preparations which had been washed several times with 0·25 M-sucrose (Table 7). Preliminary experiments have shown that by no means all the magnesium-activated ATPase of myofibril-free granule suspensions obtained from pigeon-breast muscle is associated with the mitochondrial fraction, for even after centrifugation for 1 hr. at 140 000 *g* some ATPase activity remained in the supernatant.

Attempts to demonstrate on the mitochondria a latent ATPase such as has been reported for liver (Kielley & Kielley, 1951) were uniformly unsuccessful when the preparation was carried out in 0·25 M-sucrose. In attempts to increase the ATPase activity of freshly prepared pigeon-breast mitochondria the following procedures were employed: treatment with 5×10^{-5} M-DNP; freezing followed by thawing, repeated six times; incubation at 30° for 2 hr.; dispersion in the Waring Blendor; ageing by storing at 0°. In one case only, out of many experiments, was an activation obtained. In this case the increase was about 15% after repeated freezing and thawing, but it was not considered significant. When 0·001 M-ethylenediaminetetraacetic acid was introduced in the 0·25 M-sucrose used for the isolation of the mitochondria, it was apparent that ageing at 0° and treatment with DNP produced an increase in the ATPase of these preparations. Before the latter effect could accurately be interpreted as an example of latent ATPase activity, it was necessary to carry out some preliminary investigations on the effects of both ethylenediaminetetraacetic acid and DNP on the mitochondrial enzyme.

Ethylenediaminetetraacetic acid itself inhibited the ATPase activity of pigeon-breast mitochondria. The magnesium-activation curve of the enzyme (Fig 4) undergoes significant modification in the presence of 0·01 M-ethylenediaminetetraacetic acid; the activity is considerably reduced, the maximum is flatter and occurs at a higher magnesium-ion concentration than it does in the absence of the chelating agent. Even at high magnesium-ion concentrations, when the molarity of the latter ion is 2·5 times as great as the ethylenediaminetetraacetic acid there is still considerable inhibition. It was necessary to establish that, in the presence of ethylenediaminetetraacetic acid, DNP did not activate the ATPase activity of aged mitochondria, i.e. those with no latent ATPase activity. Fig. 5 clearly shows that increasing the ethylenediamine-

Table 7. *The activity of pigeon-breast mitochondrial ATPase*

(The incubation medium contained 0·005 M-$MgSO_4$, 12–15 μmoles ATP, 0·05 M-glycylglycine-NaOH buffer pH 7·4, 0·25 M-sucrose, 0·1 ml. mitochondrial suspension. Total vol., 2·0 ml. Incubation at 17°.)

Preparation no.	Times washed	Mg. N/ml. suspension	Moles inorganic P liberated/min./ mg. N	μl. P/mg. N/ hr.
1	None	1·92	1·69	2270
2	Once	2·10	1·32	1770
3	Twice	2·08	1·06	1420
4	Twice	1·76	1·07	1440

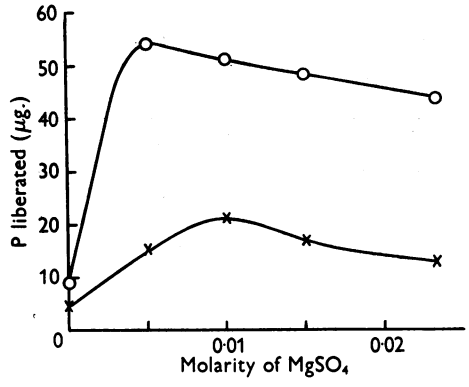

Fig. 4. The effect of Mg^{2+} on the ATPase activity of pigeon breast muscle mitochondria at pH 7·4. Incubation at 17° in 0·25 M-sucrose. O—O, in the absence of added ethylenediaminetetraacetic acid; × — ×, with 0·01 M-ethylenediaminetetraacetic acid.

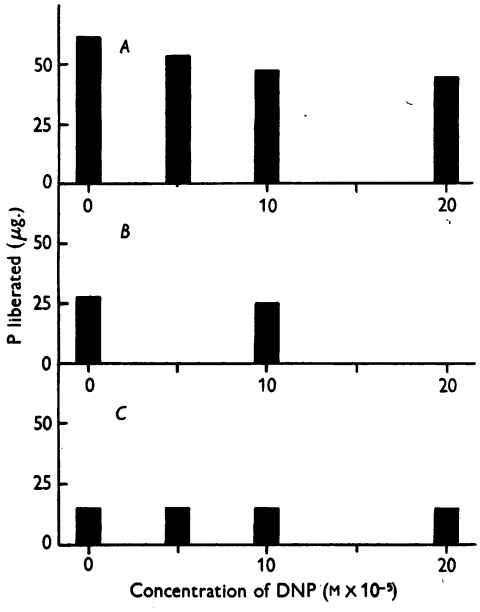

Fig. 5. The effect of DNP on the Mg^{2+} activated ATPase of pigeon-breast mitochondria in the presence of ethylenediaminetetraacetic acid at pH 7·4. The histograms represent ATPase activity with increasing DNP concentration: *A*, in absence of ethylenediaminetetraacetic acid; *B*, 0·005 M-ethylenediaminetetraacetic acid; *C*, 0·01 M-ethylenediaminetetraacetic acid. The enzymic assays were carried out simultaneously with samples of the same mitochondrial preparation. Incubations at 16° in the presence of 0·005 M-$MgSO_4$ and 0·25 M-sucrose.

tetraacetic acid concentration in an incubation mixture of otherwise fixed composition, induced a marked inhibition of the ATPase. Addition of DNP to incubation mixtures which already contained ethylenediaminetetraacetic acid produced little or no drop in activity, whereas DNP did cause an inhibition which progressively increased with concentration, when there was no ethylenediaminetetraacetic acid present during the test.

Mitochondria prepared in the ethylenediaminetetraacetic acid-sucrose medium and tested within 1 hr. of the death of the pigeon, liberated phosphate at rates which were markedly increased when DNP was added to the incubation mixture. In the experiment illustrated in Fig. 6, which compares the effect

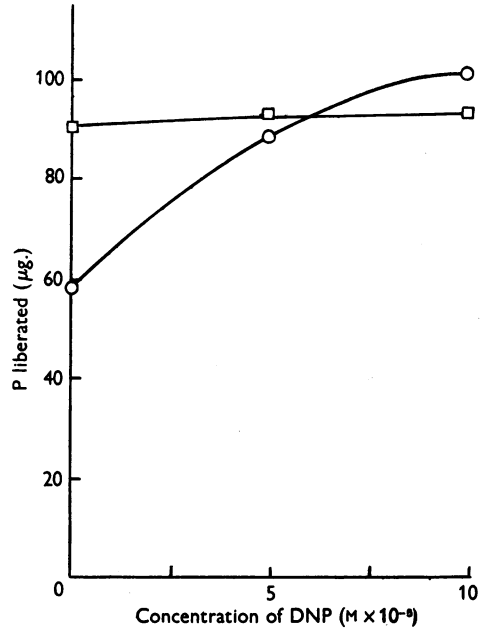

Fig. 6. Comparison of the effect of DNP on the ATPase activity of fresh and aged pigeon-breast mitochondria at pH 7·4. O—O, mitochondria 45 min. after death of animal; □—□, after 24 hr. at 0°. Incubation at 17° with 0·005 M-$MgSO_4$ and 0·25 M-sucrose.

of DNP on fresh and aged preparations, no ethylenediaminetetraacetic acid was added to the incubation mixture other than that which was associated with the mitochondrial suspension in 0·001 M-ethylenediaminetetraacetic-acid-sucrose medium (final concentration of ethylenediaminetetraacetic acid in the incubation mixture would be 5×10^{-5} M). If ethylenediaminetetraacetic acid was added directly to the incubation mixture, DNP still produced activation but much less than that obtained in the absence of ethylenediaminetetraacetic acid. Washing the mitochondrial preparation with ethylenediaminetetraacetic-acid-sucrose medium completely abolished the latent ATPase activity.

A further example of the sensitivity of the ATPase activity of pigeon-breast mitochondria to the nature

of the environment is illustrated in Fig. 7. Here is shown the effect of varying the tonicity of the incubation medium by the addition of sucrose, and it will be noted that activity is at minimum in 0·2M-sucrose. With fresh preparations in the presence of DNP and with aged preparations in the absence or presence of DNP, the effect is abolished.

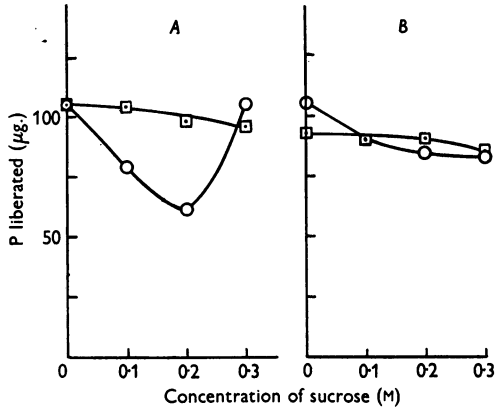

Fig. 7. Effects of varying the tonicity of the incubation medium with sucrose on the ATPase activity of pigeon-breast mitochondria at pH 7·4. *A*, preparation 1 hr. after death; O—O, no DNP; ⊡—⊡, 5×10^{-5}M-DNP. *B*, preparation after 30 hr. at 0°; O—O, no DNP; ⊡—⊡, 5×10^{-5}M-DNP.

It was not possible to explain the increased liberation of phosphate in the presence of DNP, which was observed in the ATPase experiments, by the uncoupling of oxidative phosphorylation taking place with endogenous substrate. These mitochondrial preparations had negligible oxygen uptake in the absence of Krebs's cycle intermediates.

DISCUSSION

It is clear that in skeletal muscle the myofibrils had no oxidative function so far as pyruvic and succinic acids were concerned. The aerobic breakdown of these substances and the other Krebs's cycle intermediates was associated mainly with the large granule fraction of pigeon-breast muscle. These granules are very similar to the mitochondria of liver and kidney in their behaviour with the Krebs's cycle intermediates and are in our opinion best referred to as mitochondria. Heidenhain (1911) distinguished between A (anisotropic) and I (isotropic) granules in his scheme for skeletal muscle, but although the A granules appear dominant in the more active types of muscle the relation between these two types is by no means clear. It seems convenient and logical to reserve the term sarcosome as a general one for muscle granules, other than glycogen or neutral-fat particles, without implying any particular reference to their location or function (cf. Slater & Cleland, 1953). As a result of the investigations on heart muscle by Harman & Feigelson (1952*b*) it could be concluded that in this tissue the so-called I granules are produced by the breakdown of mitochondria which takes place during fixation. It seems difficult, however, to explain in this way the very regular arrangement of I granules seen by the earlier histologists in many other types of striated muscle. The pigeon-breast mitochondria studied in this communication most likely correspond to the so-called A granules.

Striated muscles of different types and from different species show considerable variation in the size and abundance of their sarcosomes. In pigeon-breast muscle the mitochondria prepared as described in this communication may account for 20% of the total nitrogen of the muscle, which is about 5 times the contribution made by the granules to the total nitrogen content of mixed rabbit-skeletal muscle. The low oxidative activity of rabbit-muscle suspensions can be correlated with the lower granule content and suggests that this tissue must be largely anaerobic in function.

Fresh pigeon-breast mitochondria prepared in 0·25M-sucrose had a much higher magnesium ATPase activity than fresh liver mitochondria obtained under similar conditions and of similar oxidative ability. No doubt the failure to obtain satisfactory P:O ratios with such preparations from pigeon-breast muscle was related to this fact. The possibility that this high ATPase activity is a feature of the intact muscle mitochondrion cannot be completely ruled out, but if special precautions were taken during the preparation, e.g. by incorporation of ethylenediaminetetraacetic acid in the medium, the enzymic behaviour of pigeon-breast mitochondria more closely resembled that of preparations from liver. The efficiency of oxidative phosphorylation *in vitro* was increased and the ATPase activity was dependent on the tonicity of the medium in which it was measured (cf. Potter & Recknagel, 1951). It is possible that the inhibitory action of ethylenediaminetetraacetic acid on the mitochondrial ATPase is one of the factors responsible for the increased stability and improved P:O ratios obtained with preparations made in the presence of this reagent.

Mitochondria in skeletal muscle are arranged within the myofibrillar lattice of the cell and the tissue has to be subjected to considerable mechanical disruption to achieve their isolation in any quantity. It may be that isolation thus involved limited structural damage, perhaps at the point of attachment of the mitochondrion to the myofibril. It is debatable whether such an attachment exists, but the work of Harman & Feigelson (1952*b*) suggests that in certain heart muscle some mitochondria are

closely associated with the A band. For this association to be maintained during contraction and relaxation of the myofibrils some type of attachment must be visualized.

It can be concluded from this study that the main aerobic production of ATP in pigeon-breast muscle is extra-myofibrillar and almost entirely associated with the large sarcoplasmic granules, the mitochondria. The mitochondria possessed ATPase activity, but if analogies can be drawn from the work with liver (Kielley & Kielley, 1951), and there are indications from the present study that they can, probably this enzymic activity is concerned with the general mitochondrial function and bears little on the mode of direct energy liberation for contraction. The transport of the energy-rich phosphate from the site of aerobic formation, the mitochondrion, to the contractile system where it is utilized, the myofibril, is a special problem on which our investigations are now focused.

SUMMARY

1. Granules with respiratory activity have been prepared from the skeletal muscles of various animals. The respiratory granules, mitochondria, of pigeon-breast muscle have been studied in some detail.

2. Pigeon-breast mitochondria readily oxidized citrate, α-ketoglutarate, succinate, fumarate, and malate at 30°.

3. At 17° pigeon-breast mitochondria oxidized pyruvate at very slow rates, but low concentrations of malate or fumarate catalytically increased these rates.

4. Myofibrils from rabbit-skeletal and pigeon-breast muscles had no oxidative activity.

5. Oxidative phosphorylation has been demonstrated with pigeon-breast mitochondria. The highest P:O ratios were obtained when preparations were carried out in the presence of 0·001 M-ethylenediaminetetraacetic acid and incubations in the presence of isotonic sucrose.

6. Pigeon-breast mitochondria contain an ATPase which is activated to a greater extent by magnesium than calcium.

7. The magnesium-activated adenosine triphosphatase activity of mitochondria freshly prepared in the presence of ethylenediaminetetraacetic acid is increased by 2:4-dinitrophenol and by ageing at 0°.

8. The magnesium-activated adenosine triphosphatase activity of mitochondria prepared with ethylenediaminetetraacetic acid is dependent on the tonicity of the incubation medium. Minimum activity was obtained when the medium contained 0·2 M-sucrose.

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