Respiratory Granules of Heart Muscle

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Work in a number of laboratories has established that the respiratory activity of liver and kidney cells resides in the mitochondria (Hogeboom, Claude & Hotchkiss, 1946; Kennedy & Lehninger, 1949; Harman, 1950). No comparable study has been made with heart muscle, although much of our knowledge of respiratory enzymes, particularly of the cytochrome system, has come from studies of particulate preparations obtained from heart muscle.

In the present paper, it will be shown that the sarcoplasmic granules possess the highest and probably all the respiratory activity. Like the mitochondria of other tissues, these granules catalyse not only the oxidation of a number of intermediary metabolites but also the phosphorylation of adenosinediphosphate (ADP) to adenosinetriphosphate (ATP). The Keilin & Hartree (1938, 1947) heart-muscle preparation, which contains the complete cytochrome system and actively oxidizes succinate and reduced diphosphopyridine nucleotide (DPNH), but does not bring about oxidative phosphorylation, is derived from the disintegration of these granules.

The granules present in the sarcoplasm of striated muscles have been the subject of intensive investigation by cytologists for more than a century. In a study parallel to the present investigation and which is published elsewhere (Cleland & Slater, 1953), the extensive literature on these granules, much of it dating back to the last century, is reviewed. On the basis of their granules, striated muscles may be divided into two classes which exactly parallel the physiological muscle types. Those with granules at the level of the I (isotropic) bands are muscles with intermittent activity such as the skeletal muscle of vertebrates and invertebrates, the metabolism of which is largely anaerobic during activity. Muscles with granules at the level of the A (anisotropic) bands are found in situations requiring sustained activity, such as the flight muscles of birds, bats and insects, and the heart muscle of vertebrates. These muscles, which are predominantly aerobic in their metabolism, are the tissues with the highest cytochrome content (Keilin, 1925). The A granules, particularly in insect muscle (Watanabe & Williams, 1951), are larger and more numerous than the I granules.

The other paper already mentioned (Cleland & Slater, 1953) gives reasons why the term 'sarcosome', first proposed by Retzius (1890), is preferred to 'mitochondria' for describing these granules. It also describes in detail the morphology of heart-muscle sarcosomes both in the intact fibre and after separation from the other constituents of the fibre. Special consideration is given to the isolation of sarcosomes in a form as near as possible to that found in the intact cell. The effects of the tonicity of the medium and of ageing on the morphology were also studied. The effect of tonicity of the medium on the respiratory activity of the isolated sarcosomes is described in the following paper (Slater & Cleland, 1953*a*). Preparations isolated by the usual methods are unstable; factors promoting this instability and a method of overcoming it are described in further papers (Slater & Cleland, 1952, 1953*b*).

METHODS

Isolation of sarcosomes

The following procedure is for rat heart; for cat or pig heart the same method was used except for a proportional scaling of quantities and for the fact that the heart was removed under ether anaesthesia.

The rat was killed by a blow on the head, the thorax opened and the heart removed. After trimming away the great vessels and pericardium, the heart (0.5–0.8 g.) was blotted free of blood with filter paper and chopped with a sharp knife. These operations were carried out as rapidly as possible. The chopped heart was stirred for about 2 min. in 10 ml. suspending medium (see below) at 0°, the fluid was decanted and the heart muscle was ground in an ice-cooled glass mortar with 1.5 ml. suspending medium and 0.5–1.0 g. acid-washed sand until finely comminuted (2–3 min.). The mixture was transferred with 10 ml. ice-cold suspending medium to a 15 ml. centrifuge tube in an ice bath.

Centrifugation was performed in a Servall SS1 centrifuge kept at between 0° and 5° in a deep-freeze cabinet (–15°). The first centrifugation was for 3.5 min. at 700 *g* (maximum reached at end of 3.5 min.). The sediment was thoroughly dispersed in 6 ml. suspending medium and recentrifuged at the same speed. The combined supernatants were centrifuged at 7500 *g* for 7.5 min. (not including 5 min. required to reach this speed), the sediment suspended in 10 ml. medium and recentrifuged. The sediment was suspended in 1–2 ml. medium by a brief but vigorous shaking in a glass-stoppered measuring cylinder.

A number of different media were used in the present and related investigations for isolating the sarcosomes from cat, rat, rabbit, pig or guinea pig heart. In this and subsequent papers, the different preparations will be designated according to the suspending medium, as follows:

Phosphate-saline. 0.051 M-KCl, 0.043 M-phosphate, pH 7.1-7.3.

Saline. 0.135 M-KCl, 0.02 M-phosphate, pH 7.4.

Saline-Versene. 0.115 M-KCl, 0.02 M-phosphate, 0.01 M-Versene (ethylenediaminetetraacetic acid, Schwarzenbach & Ackermann, 1947), pH 7.4.

Isotonic sucrose-Versene. 0.28 M-sucrose, 0.01 M-Versene, pH 7.4 or 0.30 M-sucrose, 0.005 M-Versene, pH 7.4.

Isotonic sucrose. 0.32 M-sucrose.

Hypertonic sucrose. 0.88 M-sucrose.

The pH was measured with the glass electrode, standardized with potassium hydrogen phthalate and phosphate-citrate buffers.

The phosphate-saline medium, which was used in a previous study (Slater, 1950*a*) and is similar to that used by Ochoa (1944), is in effect considerably hypotonic because HPO_4^{2-} , the major anion of the phosphate buffer at pH 7.1-7.3 enters the sarcosomes and is not, therefore, osmotically active (Cleland, 1952). The saline and saline-Versene media are approximately isotonic with blood. Versene was added to give greater morphological stability to the granules (Cleland & Slater, 1953) and to prevent the inactivation of the α -ketoglutaric oxidase system which occurs when the preparation is exposed for only a few minutes to temperatures of 15° or greater (Slater & Cleland, 1952, 1953*b*).

Enzyme activities

Activity of the α -ketoglutaric oxidase system. This was measured at 25° as described in the following paper (Slater & Cleland, 1953*a*), using the isotonic reaction mixture containing fluoride. The activity is expressed as Q_{O_2} ($\mu\text{l. O}_2/\text{mg. protein/hr.}$).

Activity of succinic oxidase system. This was studied under the conditions optimal for the Keilin & Hartree heart-muscle preparation (Slater, 1949*a*), except that the temperature was 25° instead of 38°. The activity is expressed as Q_{O_2} .

Succinic dehydrogenase. This was measured either manometrically (Slater, 1949*a*) or spectrophotometrically by determining the rate of reduction of potassium ferricyanide, under the conditions described by Slater & Bonner (1952). A unit of succinic dehydrogenase is defined as that amount of enzyme required to produce a change of optical density of 0.01 per min. at 20°, under these conditions. The specific activity equals the number of units per mg. protein.

Cytochrome oxidase. This was measured at 25° at only one level of cytochrome *c*. Otherwise conditions were those found optimal by Slater (1949*b*). The activity is expressed as Q_{O_2} . At a fixed concentration of cytochrome *c*, the rate of O_2 uptake is not directly proportional to the concentration of enzyme preparation (Slater, 1949*b*). In fractionation experiments, therefore, it was necessary to select volumes of the different fractions which gave approximately the same rate of O_2 uptake.

Myokinase. This was measured at 25° by the procedure described previously (Slater, 1953). This is the activity under the conditions of measuring oxidative phosphorylation (isotonic medium, containing fluoride) and is very much below the full activity, since the reaction mixture contains two strong inhibitors of myokinase—fluoride and

adenylic acid. The activity is expressed as Q_P ($\mu\text{l. P/mg. protein/hr.}$) (Bailey, 1942).

ATP-ase. This was also measured at 25°, under the conditions for measuring oxidative phosphorylation, by the procedure described previously (Slater, 1953). This method measures the decrease of the reactive phosphorus groups ($\sim\text{P}$) of ATP after the addition of ATP to the enzyme preparation in phosphate buffer, containing MgCl_2 (0.005 M). The activity is expressed as Q_P . No attempt was made to vary the Mg concentration, nor to study the Ca-activated ATP-ase as distinct from the Mg-activated, since the main purpose of measuring the ATP-ase activity was in connexion with its possible interference in the method of measuring the yield of oxidative phosphorylation. Any other reaction leading to loss of $\sim\text{P}$, e.g. the hexokinase reaction, would be estimated by this method of measuring ATP-ase activity. However, the production of hexosemonophosphate or hexosediphosphate during the incubation was found to be negligible.

Determination of protein contents

The protein content of sarcosomal preparations or of fractions obtained from the heart muscle was determined by the biuret method as follows. A sample of the suspension containing 1-4 mg. of protein was treated in a centrifuge tube with trichloroacetic acid (TCA) to a final concentration of 5% (w/v). After centrifugation, the supernatant was removed and the precipitate mixed with 2 ml. 50% ethanol (v/v) containing 1 drop 40% (w/v) TCA. The supernatant was again removed after centrifugation and the residue stirred with 2 ml. 96% ethanol. After standing, centrifugation and removal of the supernatant, the residue was stirred with 0.9 ml. water and 4 ml. biuret reagent (Gornall, Bardawill & David, 1949) were added. After standing overnight, the solution was clarified, if necessary, by centrifugation and the optical density at 540 m μ . was measured in a spectrophotometer, using a blank consisting of 1 ml. water and 4 ml. biuret reagent in the reference cell. A standard curve constructed with egg albumin (standardized by Kjeldahl N) showed no change over a period of more than a year.

The washed TCA precipitate was allowed to stand overnight to allow the protein to dissolve in the alkaline biuret reagent as completely as possible. The optical density with the egg albumin was unchanged over this period. Protein derived from the Keilin & Hartree preparation dissolved completely in the reagent in a few minutes. The sarcosomal preparations usually contained a very small precipitate which did not dissolve, while the fibril fraction obtained during the preparation of the sarcosomes contained a considerable residue. The residue was probably mainly collagen; thus, the protein contents in this paper refer to the non-collagen fraction.

The protein content of sarcosomal preparations determined in this way agreed with that obtained by the Nessler method after acid digestion (LePage, 1949), and with the fat-free dry weight, determined gravimetrically as previously described (Slater, 1949*a*).

RESULTS

Fractionation of heart muscle

Grinding of heart-muscle mince with sand in a mortar, a procedure used for many years, is a suitable method of separating the granules from the

fibrillar elements. Such treatment causes sufficient damage to allow a large proportion of the granules to be separated from the fibres without breaking the latter too much. Separation of the granules from the much larger fibril fragments, connective tissue and nuclei by differential centrifugation is then a relatively simple procedure. This is the basis of the methods used by Keilin & Hartree (1938) to obtain heart-muscle preparations suitable for investigations of the cytochrome system and by Ochoa (1944) and Slater (1950*a*) for studies of oxidative phosphorylation.

Tables 1 and 3 describe experiments, with rat and cat heart respectively, in which the different fractions separated by the differential centrifugation described above were examined. Samples were also taken of the homogenate after grinding with sand and, in the rat-heart experiment, of the combined supernatants after the two low-speed centrifugations (called first supernatant in Table 2). Table 2 shows the distribution of the cytochrome oxidase and succinic dehydrogenase after the high-speed centrifugation of the first supernatant. The supernatant after sedimentation of the cat-heart sarcosomes was further centrifuged, at the maximum speed of the centrifuge for 30 min., in order to collect the particulate matter which is not sedimented by the usual procedure. This fraction will be referred to as 'microsomes', without prejudice as to its true nature which will be discussed later. The supernatant of Table 1 corresponds to the 'microsomes' + supernatant in Table 3.

Tables 1 and 3 clearly show that the highest specific activities of the three respiratory enzyme systems tested (the α -ketoglutaric oxidase system, cytochrome oxidase and succinic dehydrogenase) were found in the sarcosomes. The considerable proportion of cytochrome oxidase found in the residue (31 % in the rat experiment, 24 % in the cat experiment) is very likely due to sarcosomes found by microscopic examination to be contained in this fraction. The amount will no doubt vary somewhat from preparation to preparation depending upon the degree to which the muscle fibres are broken up.

There was also a considerable proportion of the cytochrome oxidase and succinic dehydrogenase in the supernatant obtained from the centrifugation at 7500 *g* for 7.5 min. Table 2 shows that the succinic dehydrogenase and cytochrome oxidase activities are distributed in the same proportion in the sarcosomal fraction and in the supernatant. Since much of the cytochrome oxidase in this supernatant can be sedimented by prolonged centrifugation (Table 3), it is probably due to granules not sedimented by the usual procedure. The specific activity of both the α -ketoglutaric oxidase system and of cytochrome oxidase in the sediment obtained by the prolonged high-speed centrifugation was, however,

considerably lower than in the sarcosome fraction. The 'microsome' fraction, then, probably consists of sarcosomes (possibly partly damaged, see Cleland & Slater, 1953) contaminated with non-respiratory granular material. The higher centrifugal forces used in previous studies (Slater, 1950*a*), namely, 9000 *g* for 15 min., probably sediments the latter granules to some extent. The yield was thereby increased, but since the object of this and related investigations was to obtain a homogeneous sarcosomal preparation rather than the maximum yield, the present procedure is preferred.

If it can be assumed that the sarcosomes are the only respiratory constituent of the muscle and that the cytochrome oxidase activity of the sarcosomes in the three fractions is the same, the percentages of the total protein of the muscle in the various constituents of the heart muscle can be calculated. Table 1 gives: residue (consisting of myofibrils, connective tissue, nuclei), 49 %; sarcosomes, 23 %; 'soluble' proteins (including sarcoplasmic proteins and microsomes), 28 %. The values calculated from Table 3 are 58, 15 and 27 % respectively. Another fractionation of cat heart gave 60, 20 and 20 % respectively; in this fractionation, the succinic oxidase system was distributed in approximately the same proportion as the cytochrome oxidase.

The distribution of the α -ketoglutaric oxidase system in the rat-heart fractionation was considerably different from that of the cytochrome oxidase. A large part of the activity (64 %) remained in the residue and only 28 % was obtained in the sarcosomes, compared with 31 and 44 % respectively of the cytochrome oxidase (Table 1). The use of different suspending media did not significantly alter either the yield of sarcosomes from rat heart or the specific activity of the α -ketoglutaric oxidase system (Table 4). The discrepancy between the distribution of cytochrome oxidase and of the α -ketoglutaric oxidase system was found also in the fractionation of cat heart (Table 3), but it was less marked.

A large part of the ATP-ase activity was found in the supernatant and only 16 % in the sarcosomes (Table 1). The ATP-ase activity of the soluble fraction may be due to a truly soluble ATP-ase or, more likely, it is associated with the damaged sarcosomes. Kielley & Kielley (1951), Hunter & Spector (1952) and Potter & Simonson (1952) have found that liver mitochondria possess significant ATP-ase activity only after ageing or other treatments which might be expected to alter the morphology of the mitochondria. The low ATP-ase activity of the myofibrillar fraction is surprising, since it is usually considered that most of the muscle ATP-ase is in this fraction. This may indicate a difference between heart and skeletal muscles, since Perry (1951) found that the myofibrils con-

Table 1. *Centrifugal fractionation of rat-heart muscle*

(Rat-heart muscle (1.6 g., two hearts) minced, ground with sand and saline-Versene solution and fractionated according to the procedure described in Methods. All sediments were resuspended in saline-Versene solution. H (homogenate), mixture immediately before first centrifugation. R (residue), sediment after two centrifugations at 700 g. S (sarcosomes) sediment after two centrifugations at 7500 g. Sup. (supernatant), supernatant from final centrifugation. In all measurements, final Versene concentration was 0.002 M. Cytochrome c concentration in cytochrome oxidase measurement was 8×10^{-5} M.)

Protein	H	R	S	Sup.	R + S + Sup.
mg.	214	130	26	70	226
% of homogenate	100	61	12	33	106
α -Ketoglutaric oxidase system					
Q_{O_2}	23	24	52	—	—
% of homogenate	100	64	28	—	92*
Cytochrome oxidase					
Q_{O_2}	360	184	1300	135	—
% of homogenate	100	31	44	12	88
Succinic dehydrogenase					
Specific activity	—	—	19.0	1.9	—
ATP-ase					
Q_P	144	75	190	220	—
% of homogenate	100	32	16	50	98
Myokinase					
Q_P	> 16†	3.7	6.6	149	—
% of homogenate†	100†	4	1	95	100†

* No analysis of supernatant.

† Substrate exhausted; Q_P calculated from total enzyme found in three fractions is 37.

‡ Total enzyme found in three fractions used for this calculation.

Table 2. *Distribution of cytochrome oxidase and succinic dehydrogenase in 'high-speed' centrifugation of first supernatant (see Table 1)*

	Cytochrome oxidase		Succinic dehydrogenase	
	Q_{O_2}	% of 1st supernatant	Specific activity	% of 1st supernatant
1st supernatant	367	100	5.5	100
Sarcosomes	1300	88	19.0	85
Supernatant	135	24	1.9	23
Total: sarcosomes + supernatant		112		108

Table 3. *Centrifugal fractionation of cat-heart muscle*

(Cat-heart muscle (4.8 g.) minced, ground with sand and saline-Versene solution and fractionated according to the procedure described in Methods. The combined supernatants from the two centrifugations at 7500 g were recentrifuged at 14 300 g for 30 min. Otherwise, procedure as in Table 1. H, R, S and Sup. as in Table 1. M ('microsomes'), sediment after centrifugation at 14 300 g.)

Protein	H	R	S	M	Sup.	R + S + M + Sup.
mg.	—	420	62	33	164	679
% of H*	100	62	9	5	24	100
α -Ketoglutaric oxidase system						
Q_{O_2}	12	7	70	19	—	—
% of H	100	36	55	8	—	99†
Cytochrome oxidase						
Q_{O_2}	220	85	1590	580	49	—
% of H	100	24	66	13	6	109
Myokinase						
Q_P	—	0.5	2.5	1.8	61	—
% of H*	100	1.5	1.2	0.5	97	100

* Total found in all fractions used in this calculation.

† No analysis of supernatant.

Table 4. *Comparison of different media for the isolation of sarcosomes from rat-heart muscle*

(The yield of protein is calculated to be equivalent to one rat heart. Saline, saline-Versene and isotonic sucrose media as defined in Methods; washed Versene preparation was isolated in saline-Versene and the sarcosomes then washed twice with 100 vol. saline to remove Versene and finally suspended in saline (see Slater & Cleland, 1952). Figures after $\pm$ are standard errors of mean calculated from pooled standard deviation of series.)

	Isolation medium			
	Saline	Saline-Versene	Washed saline-Versene	Isotonic sucrose*
Yield of protein				
No. of preparations	16	7	4	11
Mean (mg.)	13.8 $\pm$ 0.68	13.7 $\pm$ 1.03	10.0 $\pm$ 1.36	13.8 $\pm$ 0.82
Activity of α -ketoglutaric oxidase system				
No. of preparations	15	10	4	10
Mean Q_{O_2}	53 $\pm$ 3.4	54 $\pm$ 4.1	59 $\pm$ 6.5	58 $\pm$ 4.1

* Those prepared with and without Versene in isolation medium included together.

tributed at least 97 % of the total ATP-ase activity of a collagenase-treated rabbit psoas muscle suspension. However, Perry obtained indications that the granular ATP-ase is destroyed during the treatment with collagenase, and in a more recent paper (Perry, 1952) has found that granules can account for 20-25 % of the ATP-splitting activity of the whole rabbit psoas muscle. It must be emphasized that the conditions used in the present study might not be the most suitable for the study of ATP-ase itself. They were chosen to reproduce those of experiments on oxidative phosphorylation and were considerably different from the conditions of Perry's experiments. Since the medium contained Versene, the concentration of calcium ions would be very low, while the concentration of magnesium ions was much greater than that found to be optimal by Perry (1951). Another difference which might prove important was the presence of 0.03 M-inorganic phosphate in our reaction mixture. Except for this addition, our conditions were not very different from those used by Kielley & Meyerhof (1950) with muscle or Swanson (1951) with liver.

Practically all (95-97 %) of the myokinase activity was in the supernatant. It seems clear, then, that myokinase is in the soluble sarcoplasm of the muscle. The low activity of the residue and sarcosomes shows that the washing procedure effectively removed the soluble proteins. The Q_p values for myokinase given in Tables 1 and 3 are far below the maximum activity of the myokinase, since they were measured in the presence of two strong inhibitors, fluoride and adenylic acid (see Methods). The use of isotonic sucrose instead of saline as the isolation medium did not alter the small residual myokinase activity in the sarcosomes (Slater, 1953).

Keilin and Hartree heart-muscle preparation

The Keilin & Hartree heart-muscle preparation consists of the granular material which is isolated from water-washed heart muscle by grinding with

sand, followed by differential centrifugation. There are the following important differences between this method and the method described in the present paper: (1) The heart-muscle mince is washed for several hours at room temperature with tap water. (2) The washed mince is extracted with strongly hypotonic solution (0.01 M-phosphate buffer, pH 7.3). (3) The granules in the first supernatant are collected, in the Keilin & Hartree procedure, not by high-speed centrifugation but by acidification to pH 5.5 in the cold, followed by a low-speed centrifugation. However, granules isolated by high-speed centrifugation have essentially the same properties (Slater, 1949a).

The prolonged treatment under hypotonic conditions would be expected to cause extensive morphological change in the granules. The calcium content of Cambridge tap water (0.003 M) might contribute to this process (Cleland & Slater, 1953). Nevertheless, the granules isolated by the Keilin & Hartree procedure possess a very high activity for the oxidation of succinate (Keilin & Hartree, 1949) and DPNH (Slater, 1950b). The granules contain the complete cytochrome system in a high state of organization; the activity of the cytochrome *c* in this preparation is unusually high (Keilin & Hartree, 1940; Slater, 1950c). The Keilin & Hartree preparation is unable to oxidize many substrates (e.g. α -ketoglutarate) which are oxidized by the preparations of intact sarcosomes, neither is it able to bring about oxidative phosphorylation.

Differential centrifugation. Horse-heart mince was washed with tap water and ground in a mechanical mortar with 0.02 M-phosphate buffer, pH 7.3, and sand as described by Keilin & Hartree (1947). The sand homogenate was first centrifuged in the International centrifuge for 20 min. at 1500 rev./min., using the 250 ml. bottles, according to the method of Keilin & Hartree (1947). (Centrifugation for 3 min. at 700 g in the Servall centrifuge (cf. Methods, Isolation of sarcosomes) gave the same results.)

The supernatant (called the first supernatant in Table 5) from this centrifugation is essentially the

Table 5. *Fractionation of Keilin-Hartree heart-muscle preparation*

(H (homogenate), sand homogenate prepared according to Keilin & Hartree (1947). Sup. (supernatant), first supernatant after centrifugation in International centrifuge.)

	Protein		Succinic oxidase system			Succinic dehydrogenase	
	% H 100	% Sup. —	% H 100	% Sup. —	Q_{O_2} 68	% Sup. —	Specific activity —
Homogenate							
Fractionation in International centrifuge							
Sedimented at 1500 rev./min., 20 min.	87.4*	—	41*	—	32	—	—
Supernatant	12.6	100	59	100	320	100	10.1
Fractionation of 1st supernatant in Servall centrifuge							
Sedimented at 10400 g, 15 min., washed†	—	39.3	—	49.4	402	66.6	17.1
Sedimented at 14300 g, 30 min.	—	15.6	—	21.2	436	28.2	18.2
Not sedimented	—	43.1	—	20.8	154	22.6	5.3
Total	—	98.0	—	91.4	—	117.4	—

* Since it was difficult to take a sample of this fraction for analysis, these values have been calculated by difference.

† By resuspending and centrifuging at 10400 g for 15 min.

Keilin-Hartree preparation, since the next step in the Keilin-Hartree procedure is merely to concentrate the protein by precipitation with acid, followed by a low-speed centrifugation and resuspension of the sediment. Table 5 shows that the first supernatant contains approximately 59 % of the total activity of the succinic oxidase system of the homogenate, while the specific activity is increased about five-fold. The enzymic activity of the sediment from the low-speed centrifugation is probably due to aggregated granules or granules still remaining in the fibrils (see below). The first supernatant was then subjected to fractionation by differential centrifugation in the Servall centrifuge shown in Table 5. The following conclusions may be drawn: (1) Approximately one-half the activity of the succinic oxidase system was not sedimented at 10400 g for 15 min. More than 20 % of the total succinic oxidase activity was not sedimented after 30 min. at 14300 g. (2) The two sediments had about the same specific activity with respect to the complete succinic oxidase system and succinic dehydrogenase; these values were about 30 % higher than those of the first supernatant (cf. Slater, 1949a). The fraction not sedimented had a lower specific activity, probably due to the presence of soluble proteins. (3) The ratio of the specific activities of the complete succinic oxidase system and of the succinic dehydrogenase component was not appreciably changed during fractionation.

The fractionation shown in Table 5 was carried out with 0.02M-phosphate buffer, pH 7.3, as suspending and washing medium. No appreciable difference was found when the medium was made isotonic by the addition of potassium chloride, before the high-speed centrifugations.

If the distribution of activity shown in Table 5 is compared with that in the fractionations shown in

Tables 1-3, it is apparent that the particles of the Keilin & Hartree heart-muscle preparation are considerably smaller than the sarcosomes isolated by the procedures described earlier in this paper. The fractionation of the Keilin & Hartree preparation suggests that particles of differing size are present, but it is difficult to establish this with certainty by a fractionation of the type shown in Table 5. If this is so, the smallest particles show no separation of succinic dehydrogenase from the other components of the succinic oxidase system.

Morphology. Horse heart obtained from the slaughterhouse some hours after death of the animal proved difficult to stain specifically for sarcosomes. As far as could be judged, however, their shape and distribution within the fibres was similar to that described for cat and rat heart (Cleland & Slater, 1953). After the water treatment, no demonstration of the sarcosomes could be made, although intercalated disks and nuclei were readily recognizable, while the myofibrillar striations were, if anything, rather clearer than in the original mince.

There was not appreciably more rupture of the fibres in the mince after washing with water and grinding with sand than was usual in the normal sarcosome preparation. In fragments of all sizes the striations were readily recognizable. Quite large amounts of a very fine granular material arise in the homogenate at this stage, which are most probably derived from the sarcosomes. A myofibrillar origin is ruled out by the fact that no comparable degree or extent of comminution of the myofibrils could be seen. Denatured sarcoplasmic proteins would not give such a homogeneous picture, and the majority of sarcoplasmic proteins are in any case removed during the water washing. The strong affinity of these granules for cresyl violet after osmic acid fixation is further evidence for their

sarcosomal origin, since sarcosomes also show a high affinity for this dye. Furthermore, under conditions of high hypotonicity and mechanical agitation, sarcosomes can be converted into granules similar to those at present under discussion (Cleland & Slater, 1953).

The sediment from the low-speed centrifuging shows two layers. The lower consists of relatively large fragments of fibre and much sand. The upper layer consists of large amounts of small fragments of fibre together with some sarcosomal fragments. In the supernatant from this centrifugation only sarcosomal fragments could be recognized.

The initial high-speed centrifuging (Table 5) failed to remove all the particulate material, even some of the larger granules, described below, remaining in suspension. The centrifugation at 14 300 *g* for 30 min. removed a proportion of these, together with some of the smaller granules and some irregularly shaped bodies provisionally identified as small myofibrillar fragments.

In the sediment from the centrifugation at 10 400 *g* for 15 min. only sarcosomal fragments were identified. Two types of granule could be seen in this fraction. One type present in small numbers was a spherical, refractile, relatively osmiophilic granule of similar size to a small sarcosome. The centrifugal and staining properties of these granules, as well as their size, argue against their being the lipid granules from the muscle (Bullard, 1916), although such an origin cannot be completely excluded. The other granules, which greatly predominate, are much smaller, less refractile and less osmiophilic than the above. They exhibit a strong tendency to agglutinate both in fresh and osmic acid fixed suspensions. It was impossible to assign a shape to granules as small as these since they appeared to be below the limit of microscopic resolution, and it was not possible to decide whether any given object was single or multiple. Apparently single components do, however, seem to be spherical, although elongated bodies which seem to consist of strings of the spherical components were also seen.

DISCUSSION

Respiratory activity of sarcosomes

Harman (1950) included heart muscle in his study of the association of 'cyclophorase' with mitochondria. He found that 'mitochondria' were the only recognizable pre-existent structural unit in heart-muscle 'cyclophorase' preparations and concluded therefore that, in heart muscle as well as in other tissues, mitochondria were the active components of 'cyclophorase'. However, since a 'cyclophorase' preparation consists of the total sedimentable fraction of the homogenate, its major constituent would be myofibrillar in origin. It was obviously

desirable, therefore, to separate the granules from the myofibrils by the method previously used (Slater, 1950*a*) and to determine the relative contributions of these muscle constituents to the respiratory activity of the whole muscle.

It is clear from the fractionations described in Tables 1-3 that the sarcosomes have the highest respiratory activity. These experiments, and the morphological examination of the fractions obtained, are consistent with the view that all the respiratory activity of the cell is in the sarcosomes. In this respect, the sarcosomes correspond to the mitochondria in liver and kidney cells. Watanabe & Williams (1951) showed that this was also the case in blowfly thoracic muscle; the specific cytochrome oxidase activity of the isolated sarcosomes was twice that of the whole muscle.

After the completion of our work, Harman & Feigelson (1952) described the separation of heart-muscle mitochondria from the myofibrillar fraction and showed that they possessed respiratory activity. They did not determine the distribution of the respiratory activity in the different fractions. The identification of the respiratory granules of muscle with the sarcosomes or mitochondria has also been strengthened by Paul & Sperling (1952) who have shown a correlation between 'cyclophorase' (respiratory) activity and mitochondrial density of different types of striated muscle.

Neither our own nor other work, however, excludes the possibility that the myofibrillar elements or nuclei might contain some respiratory activity, since we have not prepared this fraction free from sarcosomes.

The sediment from the low-speed centrifugation contains a considerable proportion of the sarcosomes still inside the fibres. These unseparated sarcosomes appear to possess a considerably higher activity of the α -ketoglutaric oxidase system relative to the cytochrome oxidase activity than do the isolated sarcosomes. This may indicate that even the most satisfactory procedure found for isolating the sarcosomes in the present study does not preserve the complete activity of the α -ketoglutaric oxidase system, although a wide range of suspending media all gave sarcosomes of approximately the same α -ketoglutaric oxidase activity (Table 4).

In one respect at least, isolated heart-muscle sarcosomes may differ from isolated liver mitochondria. They contain only 1 % of the myokinase of the homogenate. It is probable that the myokinase of the muscle is entirely in the sarcoplasm, since it would be reasonable to expect that 1 % of a sarcoplasmic constituent would remain in the sarcosome preparation after a single washing. On the other hand, Kielley & Kielley (1951) have found that 72 % of the myokinase activity of a liver homogenate was found in the mitochondria and only 6 %

in the supernatant. Both Barkulis & Lehninger (1951) and Kielley & Kielley (1951) have found that liver myokinase is readily liberated into solution by mechanical damage of the mitochondria. Perry's (1952) finding that myokinase is closely associated with skeletal-muscle sarcosomes might indicate a difference between the heart-muscle (A) sarcosomes and skeletal-muscle (I) sarcosomes. However, since Perry has not determined the percentage of the total myokinase of the muscle which is found in the sarcosomes, it is possible that his preparations, like ours, contain only a small residual fraction of the total activity.

Keilin-Hartree heart-muscle preparation

The fractionation described in Table 5 and the morphological observations show that the Keilin & Hartree preparation consists of particles derived from the disintegration of sarcosomes. Since even the smallest particles contain a high specific activity for the aerobic oxidation of succinate, they must contain all the components of the succinic oxidase system. Stern (1939) concluded from a study with the analytical ultracentrifuge that the particles of the Keilin & Hartree preparation were about 0.05μ . in diameter. However, Stern used the relatively inactive preparation described by Keilin & Hartree in 1938 and it is not certain whether the much more active preparation used in this laboratory since 1940 and described by Keilin & Hartree in 1947 would give the same results. The sarcosomes are very much larger (of the order of $1-2 \mu$.).

In an earlier paper (Slater, 1949*a*), the Keilin & Hartree preparation was regarded as being a solution of some subcellular structure or macromolecules, rather than unspecific fragments of the mass of the protoplasm. We can now conclude that this preparation is derived from a structure which is contained in the sarcosomes, which probably contain all the activity of the succinic oxidase system in the cell. In the Keilin & Hartree procedure, the sarcosomes are subjected to highly hypotonic conditions for several hours at room temperature, followed by a severe mechanical treatment. It is shown elsewhere (Cleland & Slater, 1953) that this treatment causes swelling of the sarcosomes, followed by fragmentation of the insoluble cortex or membrane to give small particles. It seems likely, then, that the Keilin & Hartree preparation consists of fragments of the sarcosomal membrane. Many enzymes which are present in the sarcosomes are lost during this procedure, e.g. malic dehydrogenase, fumarase, α -ketoglutaric dehydrogenase and the enzymes concerned in oxidative phosphorylation. These enzymes may be present within the body of the sarcosome which dissolves under hypotonic conditions (Cleland & Slater, 1953) and

are thus removed into the supernatant when the fragmented sarcosomes are centrifuged.

Hogeboom & Schneider (1950) have shown that treatment of liver mitochondria with sonic vibrations liberates 60 % of the nitrogen into solution and the particulate matter requires very high centrifugal speeds for sedimentation. This particulate matter appears to be similar to the Keilin & Hartree preparation, so far as its constitution of enzymes is concerned.

Green (1951) refers to preparations similar to that of Keilin & Hartree as microsomes. This terminology is undesirable, since microsomes have been recognized by cytologists as distinct from mitochondria for a very long time. Hogeboom, Schneider & Pallade (1948) obtained a microsomal fraction, containing 20 % of the nitrogen of the liver cell, which differed markedly from mitochondria, e.g. these particles possessed no succinic oxidase activity. The enzyme distribution in the microsome fraction of the oyster egg is also different from the distribution in the mitochondria or yolk granules (Cleland, 1951). Although the particles of the Keilin & Hartree preparation are similar in size to the microsomes, their high respiratory activity distinguishes them sharply from liver microsomes. The fractionation described in Table 3 gave evidence that a small amount of particulate material, not possessing cytochrome oxidase activity, might be present in heart muscle. This material might correspond to the microsomes isolated by Hogeboom *et al.* (1948) from liver. It does not appear to be present in the extract obtained in the Keilin & Hartree procedure, since the specific activity of the fraction obtained at the highest centrifugal speed was no lower (it was in fact slightly higher) than that obtained at the lower speed (Table 5).

It is significant that all except some of the early steps of the hydrogen-transferring system of the sarcosomes are present in the insoluble fraction of the sarcosomes. Succinic dehydrogenase, diaphorase and the entire cytochrome system are contained in these particles and constitute an integrated system of hydrogen-transferring enzymes which transfer the hydrogen atoms from succinate or DPNH, in successive steps, to oxygen (Slater, 1950*b*). In the sarcosomes, some of these hydrogen-transferring steps are coupled with the esterification of inorganic phosphate. This oxidative phosphorylation is lost in the Keilin & Hartree preparation, probably because some phosphorylating enzymes are not part of the insoluble fraction of the sarcosomes, but are either washed away or inactivated when the sarcosomal membrane is broken. But there is no reason to believe that the remaining non-phosphorylating hydrogen-transferring system differs fundamentally from the phosphorylating system in the intact sarcosome. It seems likely that

the Keilin & Hartree preparation could be made to phosphorylate if the missing phosphorylating enzymes were replaced. This would be analogous to the reactivation of a cytochrome *c*-deficient heart-muscle preparation by soluble cytochrome *c* (Keilin & Hartree, 1949; Tsou, 1952) or the activation of the DPNH oxidase activity of the preparation by diaphorase (Slater, 1950b). Experience with these reactivations suggests that relatively large amounts of phosphorylating enzymes would be required to evoke oxidative phosphorylation in the Keilin & Hartree preparation.

Green & Beinert (1951) have recently reported the restoration of the ability of a preparation, somewhat resembling the Keilin & Hartree preparation, to oxidize all the members of the Krebs cycle by the addition of a coenzyme mixture and a soluble fraction. This particulate preparation of Green & Beinert differs from that of Keilin & Hartree in that some phosphorylation accompanies the oxidation of succinate ($P:O = 0.4$). It probably represents an intermediate stage in the disintegration of the sarcosomes. The activation of the oxidative phosphorylation reactions in this preparation has not yet been achieved, with the exception of one step of α -ketoglutaric oxidation, which has been obtained in solution by Kaufman (1951) and Littlefield & Sanadi (1952).

The Keilin & Hartree preparation is eminently suitable for studying the respiratory chain itself, uncomplicated by phosphorylation. It is much more satisfactory than sarcosomal preparations for studying the succinic oxidase system, since the fumarate is not further oxidized. In sarcosomal preparations, fumarate is further oxidized and the activity measurements are complicated by the powerful inhibitory effect of oxaloacetate, the product of this oxidation, on succinic dehydrogenase (Das, 1937; Keilin & Hartree, 1940; Pardee & Potter, 1948). The Keilin & Hartree preparation contains more cytochrome *c* than sarcosomes isolated with salt-containing solutions (Slater & Cleland, 1953a).

It should be understood that in the above discussion, the terms 'soluble' and 'insoluble' have been used in a relative sense only. Thus several components of the relatively insoluble Keilin & Hartree heart-muscle preparation have been brought into solution by special means. In the case of cytochrome *c*, this has been achieved by the relatively mild procedure of stirring water-washed heart muscle with salt solutions (Tsou, 1952). Straub (1939) obtained diaphorase from this preparation and, more recently, Morton (1950) prepared succinic dehydrogenase by disruption of the phospholipin-protein linkage with butanol. Smith & Stotz (1950) have highly purified cytochrome oxidase by treatment of the particles with crude

proteolytic enzyme preparations in the presence of bile salts.

The suggestion, from morphological evidence, that the system of enzymes which comprise the respiratory chain is situated in the sarcosomal membrane is supported by other considerations. First, isolated sarcosomes are able rapidly to react with both oxidized and reduced cytochrome *c*, which being a protein would not be expected to penetrate a membrane. Secondly, succinate which is rapidly oxidized by heart-muscle sarcosomes has been shown not to penetrate the membrane (Cleland, 1952). These observations are easily explained if it is assumed that cytochrome *c* and succinate react with components which are situated on the surface of the sarcosome.

SUMMARY

1. A method of isolating the sarcosomes, free from other components of the heart muscle, in about 50–65% yield is described.

2. A fractionation of heart muscle into three fractions—residue (myofibrillar fragments, connective tissue and nuclei), sarcosomes and sarcoplasm—was carried out. An intermediate fraction containing both sarcosomes and microsome-like material was also obtained.

3. The highest specific activity of respiratory enzymes (α -ketoglutaric oxidase system, cytochrome oxidase and succinic dehydrogenase) was in the sarcosome fraction. Assuming that this is the only component of the muscle which contains these respiratory enzymes, it was calculated that the total protein was distributed in the fractions as follows: residue, 49–60%; sarcosomes, 15–23%; sarcoplasm + microsomes, 20–28%.

4. Myokinase was found almost entirely in the sarcoplasm, which also contained a considerable proportion of the magnesium-activated adenosinetriphosphatase.

5. A number of different suspending media was employed without significantly altering the yield of sarcosomes, the activity of the α -ketoglutaric oxidase system or the small residual myokinase activity.

6. From morphological and enzymic studies of the Keilin & Hartree heart-muscle preparation, it is concluded that this preparation consists of small particles derived from the fragmentation of the sarcosomal membrane.

7. These particles contain all except some of the early steps of the hydrogen-transferring system of the sarcosomes, but have lost many of the more soluble proteins which are found in the sarcosome. It is suggested that these proteins are in the body of the sarcosome and are liberated into the medium in a soluble form when the sarcosome membrane is broken.

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