A MEMBRANE PREPARATION FROM INSECT FLIGHT MUSCLE SARCOSOMES

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Summary

A membrane fraction was prepared from the sarcosomes of the thoracic muscles of *Lucilia cuprina* by the action of 0·5% sodium deoxycholate. The sarcosome membranes contained 23% of lipid extracted with solvents in three separate fractions. Acid hydrolysis of the defatted membranes liberated 14 amino acids which were estimated semiquantitatively, but no sugars or amino sugars could be detected. The proportion of phospholipid contained in the sarcosome and sarcosome membrane lipids was less than that reported for the lipids of other mitochondria. The breakdown of sarcosomes by deoxycholate was accompanied by loss of lipid components which were not phospholipid in nature. Most of the phospholipids and sterols of sarcosomes formed part of the membrane structure.

I. Introduction

Insect flight muscles are of interest because they can convert chemical energy into mechanical work at an extremely rapid rate. They can maintain very fast rates of contraction and this is matched by an extremely high rate of respiratory metabolism. The structure and function of insect flight muscles has been described by Pringle (1957) and their basic physiology has been reviewed by Boettiger (1960) and by Sacktor (1961).

Sarcosomes were separated as large interfibrillar cytoplasmic bodies from insect muscle (Kölliker 1888) before mitochondria were isolated from vertebrate cells. However, it remained for Watanabe and Williams (1951) to conclude that, because of their several points of biochemical similarity, flight muscle sarcosomes were the mitochondria of this highly specialized tissue. Since then, many investigations have confirmed the occurrence of oxidative phosphorylation in insect flight muscle sarcosomes and have shown that sarcosomes also contain many of the cytochromes and enzyme systems present in vertebrate mitochondria. Nevertheless, Estabrook and Sacktor (1958) have shown that cytochrome c_1 is absent from flight muscle cytochromes and is apparently replaced by a different pigment. Birt (1961) has demonstrated that experimental techniques can greatly influence the behaviour of preparations. It is well established that in mitochondria the membranes carry most of the enzyme systems responsible for the various oxidative and other reactions.

Insect flight muscle sarcosomes exhibit a membrane structure of internal cristae enclosed in an external double membrane and these are similar in structure to mammalian mitochondria (Chapman 1954; Edwards and Ruska 1955). This supports the biochemical evidence that sarcosomes are the mitochondria of muscle.

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Hitherto, except for a study by Watanabe and Williams (1953), attention has usually been concentrated on the enzymes carried in the membrane system rather than on the nature of the sarcosome membrane itself. Particularly in so active a system as the sarcosomes of insect flight muscle, the permeability and other properties of the membranes may assume importance. This paper reports an investigation into the chemical nature of sarcosome membranes prepared from the thoracic muscles of the sheep blowfly, *Lucilia cuprina* (Wied.).

II. Methods

(*a*) *Isolation of Sarcosomes*

Flies, taken from a laboratory culture of *L. cuprina*, were used when 7–8 days old because Levenbook and Williams (1956) found that, in *Phormia*, the size of sarcosomes increased during the first week of adult life. The blowflies were immobilized by cooling and the wings, legs, head, and abdomen removed. It was necessary to ensure that the gut was withdrawn from the thorax with the abdomen in order to avoid contamination of the sarcosome preparation with gut parasites. The thoraces thus obtained, which consisted mainly of flight muscle, were bisected into chilled 0·5M sucrose buffered at pH 6·8 with 0·025M phosphate buffer. The muscle was dispersed by the propeller of a laboratory stirrer, passed through muslin to remove cuticular debris, and then filtered through a coarse sintered-glass filter. Any fibrils and debris remaining were removed by centrifugation at 150 *g* for 5 min. The sarcosomes were collected by further centrifugation at 3000 *g* for 5 min and were resuspended in buffered 0·5M sucrose medium.

For those experiments in which intact sarcosomes were analysed, a suspension was centrifuged at 60,000 *g* for 10 min to produce a compact pellet which was washed in 0·25M sucrose, then slurried in the same medium, and centrifuged at 60,000 *g* for 20 min. The sarcosome pellet was dried on absorbent paper, dispersed in distilled water, and freeze-dried.

(*b*) *Preparation of Sarcosome Membranes*

The best conditions for treatment of sarcosomes with sodium deoxycholate were selected from preliminary tests in which preparations were observed by phase-contrast and by electron microscopy. The technique finally selected was to treat sarcosomes suspended in buffered 0·5M sucrose with 0·5% sodium deoxycholate at 0°C for 30 min. The resulting membranes were collected by centrifugation at 60,000 *g* for 20 min, resuspended in water, and sedimented again. To collect samples for analysis, the membranes were suspended in water and freeze-dried.

(*c*) *Analytical Methods*

Sarcosome and sarcosome membrane preparations were separated into lipid and "defatted" membrane fractions which were analysed separately.

(i) *Lipid Extraction.*—Three lipid fractions were isolated. None of the fractions corresponded to any single chemical class:

Lipid I: Preparations were refluxed with methanol to disrupt lipoprotein complexes (Reichert 1944) and the methanol evaporated under reduced pressure. The lipid was removed by repeated extraction six times with dry ether. No further lipid was extracted by dry chloroform.

Lipid II: After removal of lipid I, preparations were extracted twice with methanol–chloroform (1 : 1 v/v), the extract dried under reduced pressure, and then re-extracted twice with chloroform. The chloroform-soluble material constituted lipid II.

Lipid III: Residues after removal of lipids I and II were treated at room temperature for 5 min with formic acid–ethanol comprising 1N formic acid in aqueous (50% v/v) ethanol. Insoluble material was removed by centrifugation and the extract dried under reduced pressure. Lipid III was obtained by further extraction twice with chloroform of the material soluble in formic acid–ethanol.

(ii) *Analysis of Defatted Membrane Fraction.*—After removal of lipid, membrane preparations were subjected to acid hydrolyses and were analysed for amino acids and for carbohydrates and amino sugars. For amino acids, hydrolysis of 5-mg samples was carried out at 105°C for 14 hr with constant-boiling HCl in a sealed tube. Amino acids in the hydrolysate were detected by paper chromatography using the methods of Hackman and Lazarus (1956). Estimates of relative concentrations were made by comparison of spot intensities obtained with known mixtures of amino acids constructed after preliminary trials. Tests for carbohydrate and amino sugar components of defatted membranes were made after hydrolysis of 20-mg samples in 2N HCl for 2 hr under reflux at 100°C. Ionophoresis was carried out in acetate buffer at pH 5 on Whatman No. 3 paper in an apparatus similar to that of Foster (1952, 1957). Development was with aniline hydrogen phthalate and ninhydrin. Standards used were an artificial amino acid mixture and glucosamine. Ascending paper chromatography was by the methods of Smith (1962), with isopropanol–water as solvent and development with either benzidine, phloroglucinol, or dinitrosalicylic acid, or with aniline hydrogen phthalate which was according to the method of Partridge (1949). Glucose and fructose were used as standards.

(iii) *Analysis of Lipid Fractions.*—Total nitrogen was estimated by a micro-Kjeldahl method on sarcosome membranes as well as on membrane lipids. Phosphorus analyses were by the method of Rhodes (1955). Cholesterol was determined colorimetrically by the method of Brown (1959) which includes a saponification step.

III. Results

Sarcosomes appear under phase-contrast microscopy as dense, rounded particles 2–3 μ diameter when suspended in buffered 0·5M sucrose medium. Electron micrographs of osmic acid-stained sections revealed the typical structure of internal cristae surrounded by a limiting membrane. In more dilute media, the sarcosomes were distended and the phenomena described by Williams and Watanabe (1953) could be observed. Craston and Manery (1962) have found that a similar concentration of

sucrose preserves the chemical and morphological integrity of pigeon breast muscle mitochondria better than other concentrations tested.

When sarcosomes were treated with sodium deoxycholate and the membrane fraction collected, the preparations were light red to brown in colour. Sections of these, examined in the electron microscope, showed only vesicular material which appeared to be derived from cristae and were similar in appearance to electron micrographs of membrane preparations from rat liver mitochondria published by Watson and Siekevitz (1956). The characteristic structure pattern of sarcosomes was destroyed. Such observations were used to ensure that complete disruption of sarcosomes to sarcosome membranes was achieved.

The methods used produced consistent membrane preparations. The membrane yield (expressed as percentage dry weight of sarcosomes) for the four separate membrane preparations was, respectively, 34·9, 36·5, 30·2, and 40·6 (av. 35·8), and their percentage total nitrogen contents were, respectively, 8·4, 9·7, 10·1, 8·7 (av. 9·2). These preparations were combined for subsequent extraction of lipids. The percentage lipid contents of sarcosome membranes and of intact sarcosomes of the three lipid fractions isolated as described in Section II(*c*)(i) are as follows:

	Membranes	Sarcosomes
Lipid I	22·2	17·7
Lipid II	5·9	2·3
Lipid III	5·7	2·8
Total	33·8	22·8

The amino acids detected in the hydrolysate of the defatted membrane fraction after hydrolysis in constant-boiling hydrochloric acid for 16 hr at 105°C, together with their relative molar proportions, are listed in the following tabulation:

Amino Acid	Relative Molar Proportion	Amino Acid	Relative Molar Proportion
Glycine	1·5	Lysine	0·5
Alanine	1·5	Arginine	0·5
Valine	1·0	Histidine	0·1
Leucine	1·5	Phenylalanine	0·15
Serine	0·3	Tyrosine	0·3
Aspartic acid	1·25	Proline	0·2
Glutamic acid	1·0	Hydroxyproline	0·3

Different preparations gave similar results to the example shown. In the search by paper ionophoresis and paper chromatography for sugars and amino sugars, the only compounds detected, other than amino acids, were glucose and fructose. These amounted together to less than 0·1% of the defatted membrane and can be attributed to traces of sucrose remaining from the method of preparation.

Analytical results for the three lipid fractions of sarcosome membranes and of sarcosomes are given in Table 1. The lipid fractions II and III from both membranes and sarcosomes appear to be similar. No phospholipid was detected in lipid I. Nitrogen was not detected in any of the lipids extracted from membranes. The

membrane material remaining after extraction of lipid III contained 0·2% phosphorus, some of which may have been phospholipid not freed from the membrane under the conditions used.

IV. Discussion

The morphological changes induced by the action of sodium deoxycholate on the sarcosomes of blowfly flight muscle structure are similar to those which occur with rat liver mitochondria. The analyses of the sarcosome membranes indicate they are lipoprotein in nature. The amino acids detected after hydrolysis show no unexpected features. A similar result for amino acids from the so-called "structural protein" of beef heart mitochondria has been obtained by Criddle *et al.* (1962). The major difference is that cystine, present in the beef heart mitochondrial protein, was not observed in the sarcosome membrane hydrolysate. The proteins remaining in sarcosome membranes are no doubt numerous and comprise various enzyme systems and structural units. It is possible that some components corresponding to part of the structural protein of other mitochondria were removed in the treatment with deoxycholate even though no butanol or detergent was used in the preparation of the sarcosome membranes.

Table 1

CHOLESTEROL, PHOSPHORUS, AND NITROGEN CONTENT OF THE THREE LIPID FRACTIONS OBTAINED FROM L. CUPRINA SARCOSOME MEMBRANES AND SARCOSOMES

	Membrane Lipid Fractions*			Sarcosome Lipid Fractions*		
	I	II	III	I	II	III
Cholesterol (%)	0·8	6·1	4·0	3·4	5·9	4·5
Phosphorus (%)	0	0·9	0·4	0	1·2	0·3
Nitrogen (%)	0	0	0	n.d.	n.d.	n.d.

* Isolated as described in Section II(c)(i).

The lipids extracted from sarcosome membranes account for one-third of the dry weight of the membranes. In intact sarcosomes, the 23% lipid extracted from *Lucilia* sarcosomes may be compared with 29% lipid found in sarcosomes from *Phormia regina* muscle (Watanabe and Williams 1951). In other mitochondria, lipids constitute similar proportions but the bulk (90%) of the lipid is phospholipid (Slater 1957; Green and Hatafi 1961) and much of the phospholipid, e.g. lecithin and cephalin, contains nitrogen. The lipids extracted from the sarcosome membranes and the sarcosomes of *Lucilia* flight muscle contain much less phospholipid, even if it is assumed that all the phosphorus determined in sarcosome membranes after extraction of lipids I, II, and III was phospholipid. In addition, no nitrogen was detected in any of the lipids extracted from the membranes. The phospholipid

present in the sarcosome membranes may be similar to the nitrogen-poor, cardiolipin-like compound found to be present in substantial proportions in the lipids of rat liver mitochondria by Getz *et al.* (1962).

In its action to break down the sarcosomes to form the membrane fraction, the sodium deoxycholate probably interacts with lipid materials. It is capable of forming "choleic acids" with most types of lipids and is unlikely to act on the peptide chains of proteins (Turner 1960). Although deoxycholate is used to solubilize structural protein in mitochondria (Green *et al.* 1961), it is then used in conjunction with butanol and anionic detergents. However, the loss of some protein material from sarcosomes when converted to membranes is likely as some thoracic muscle lipoproteins, phosphoproteins, and other protein complexes are relatively water-soluble (Price and Lewis 1959). On the present results, from 100 g of *L. cuprina* sarcosomes, there could be extracted 22·8 g of lipid made up of 17·7, 2·3, and 2·8 g of lipids I, II, and III respectively. In comparison, the 35·8 g of membranes derived from 100 g of sarcosomes yielded a total of 12·1 g of lipid comprising 7·9, 2·1, and 2·0 g of lipids I, II, and III. The amounts of lipids II and III were thus comparable in both sarcosomes and membranes and the lipids had similar cholesterol and phosphorous contents. The main loss of sarcosomal lipid on conversion to membranes was in the non-phospholipid, least strongly bound moiety comprising lipid I. An infrared spectrum of this material indicated the presence of carbonyl compounds. Although no more detailed information is available, the lipid removed from the sarcosomes on treatment with sodium deoxycholate probably includes sterols, waxes, fatty acids, and some of the lipids included in the biochemical processes of the sarcosomes. The phospholipids and cholesterol which remain in the membrane may be structural lipids incorporated in the lipoprotein matrix of the membrane.

It has been shown above that some chemical differences exist between the lipids from flight muscle sarcosomes and those of rat liver mitochondria. On the present results, it is not possible to relate these differences directly to any biochemical properties. Chemical differences in the lipoproteins of the membrane structures could also be the basis of differences in the physicochemical properties of the membranes. Membrane properties, such as permeability, and the physical arrangement of the chemical entities of the membrane merit study alongside more purely biochemical investigations to understand the special nature of insect flight muscle sarcosomes.

V. References

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