SELECTED ENZYMIC STUDIES OF LIPOFUSCIN GRANULES ISOLATED FROM BOVINE CARDIAC MUSCLE

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THE presence of intracellular particles accumulating with increasing age (i.e., lipofuscin granules) in a wide variety of vertebrate tissues was known as early as the end of the last century. Since then, the use of staining and histochemical techniques, fluorescence microscopy and recently electron microscopy have contributed much information about the properties of these granules *in situ*. Lipofuscin granules have been isolated from human autopsy tissue, viz., heart [17, 20, 26] and spinal cord [3]. Histochemical evidence for esterase activities in lipofuscin granules in a variety of tissues has been demonstrated by Gössner [12], Gomori [11], Pearse [22], Gedigk and Bontke [8], Gedigk and Fischer [9] and Essner and Novikoff [6]. Esterase and cathepsin activities were found in lipofuscin granules isolated from human cardiac muscle by Heidenreich and Siebert [14] and later by Strehler and Mildvan [27]. However, despite the accumulated information on the properties of the lipofuscin granules, an understanding of their origin and function has yet to be attained.

A contributing factor may be the paucity of knowledge concerning the biochemical properties of these granules. Recently, Björkerud [2] worked out a method for the isolation of lipofuscin granules from fresh bovine cardiac muscle. Morphologically homogeneous fractions of lipofuscin granules were prepared according to this method. The present study was undertaken to evaluate the purity of these preparations, from a biochemical standpoint, and to investigate the presence of selected enzymic activities in the lipofuscin granules.

MATERIAL AND METHODS

The material was obtained from cardiac muscle tissue taken from the wall of the left cardiac ventricle of 10–14-year-old cows. The age determinations were done according to Gandal [7].

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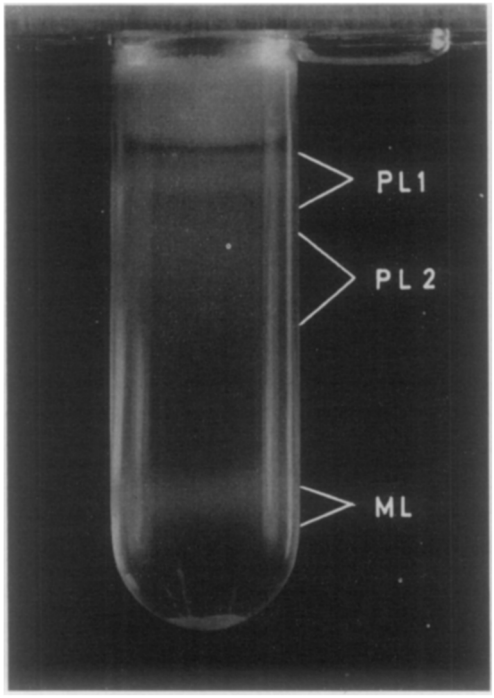

Fig. 1.—Continuous density gradient prepared from 18 and 42 per cent sucrose solutions, charged with prefractionated bovine cardiac muscle homogenate after centrifugation at 90,000 *g* for 50 min. Three zones (pigment layer 1, PL1; pigment layer 2, PL2; mitochondrial layer, ML) and a sediment are found in the gradient (see Material and Methods).

The detailed procedure for the preparative isolation of lipofuscin granules from bovine cardiac muscle is given in [2]. A brief outline of the procedure will be given below. A homogenate was prepared with a glass homogenizer of the TenBroeck type from the minced, fresh tissue in a 22 per cent (w/w) sucrose solution with 0.001 *M* ethylenediaminetetraacetate (EDTA). The homogenate then was mixed with the same volume of a 50 per cent sucrose solution containing 0.001 *M* EDTA. The suspension was centrifuged in an angle centrifuge (Universal Rapid, rotor 6 × 70 ml; Martin Christ, Osterode/Harz, Germany) at 25,000 *g* for 30 min. After centrifugation, a dark brown material was found floating on the supernatant and deposited on the centrifuge wall at the meniscus level during the centrifugation. This material was removed with a minimum admixture of the supernatant and resuspended in a 6 per cent sucrose solution with a glass–Teflon homogenizer. The suspension was centrifuged in a swinging bucket rotor at 730 *g* for 8 min, and the sediment discarded. The supernatant was mixed with a glass–Teflon homogenizer, recentrifuged at 730 *g* for 8 min, and the sediment was discarded. The final supernatant was agitated again with the glass–Teflon homogenizer and layered on a continuous density gradient

prepared from 18 and 42 per cent sucrose solutions. The gradient was centrifuged subsequently in a Spinco L, rotor SW 25.1 at 90,000 *g* for 50 min.

After centrifugation three zones and a sediment were found in the gradient (Fig. 1). The zones (designated as pigment layer 1, PL1; pigment layer 2, PL2; mitochondrial layer, ML) were removed with the help of a slicer [25], each diluted with 6 per cent sucrose solution and centrifuged at 22,000 *g* for 10 min in a Spinco L centrifuge, rotor SW 25.1. The supernatants were discarded. The sediments (PL1′, PL2′, ML′) were treated by either of two alternate ways.

Alternative A.—The sediments (PL1′, PL2′, ML′) each were resuspended in 11 per cent sucrose solution and the suspensions centrifuged at 22,000 *g* for 10 min. The supernatants were discarded. The respective sediments were denoted P1, P2 and M, Alt. A.

Alternative B.—The sediments (PL1′, PL2′, ML′) each were resuspended in 5 ml 40 per cent sucrose solution. Each suspension was layered in the lower part of separate, continuous density gradients prepared from 18 and 42 per cent sucrose solutions. After centrifugation at 90,000 *g* for 50 min, the bulk of the material was found in zones at the upper, middle and lower part of the gradient, containing PL1′, PL2′ and ML′ material, respectively. Each of the zones was removed with the slicer, diluted with 6 per cent sucrose solution and centrifuged at 22,000 *g* for 10 min. The supernatants were discarded. The respective sediments were denoted P1, P2 and M, Alt. B. In the determination of the distribution of ATPase activities the lower parts of the gradients corresponding to the origin were sampled (*vide infra*).

Total nitrogen was determined according to the micromethod of Johnson [16] as given in [28]. All determinations were carried out in triplicate.

Cytochrome oxidase activity was assayed manometrically as described by Potter in [28].

The oxygen uptake was measured by the polarographic method of Davies and Brink [4], using the modification of Hagihara [13]; the chamber was made of Teflon.

ATPase activity was assayed in an incubating medium of the following composition: 25 m*M* tris buffer, pH 7.4; 5 m*M* ATP; 210 m*M* sucrose; 5 m*M* $MgSO_4$; in a volume of 1.2 ml. The tubes were incubated at 37°C for 1 hr. The inorganic phosphate was measured according to [19] and tracer studies were based on the procedure outlined by [18]. In one experiment, ATPase activity was assayed using ATP labeled with ^{32}P in the terminal position (Table IV) according to [24]. In this experiment the samples were incubated in a medium containing 100 m*M* sucrose; 5 m*M* $MgCl_2$; 25 m*M* tris buffer, pH 7.4 and 5 m*M* ATP with 25,000 counts of ^{32}P.

$^{32}P_i$ uptake and $^{32}P_i$–ATP exchange were determined as described by Wadkins and Lehninger [29]. The incubating medium had the following composition: 100 m*M* sucrose; 5 m*M* $MgCl_2$; 25 m*M* tris buffer, pH 7.4; 5 m*M* K_2PO_4, of about 200,000 counts; 0.5 m*M* ADP; 5 m*M* succinate; in a final volume of 1 ml.

The $^{32}P_i$–ATP exchange experiments differed from those of the $^{32}P_i$ uptake by substituting 5 m*M* ATP for the succinate and ADP.

RESULTS

The cytochrome oxidase activities of the particulate fractions P1, P2 and M, Alt. A are given in Table I. On the basis of nitrogen content the cytochrome

oxidase activity of fraction M was 25 and 30 times that of fractions P1 and P2, respectively.

Results from measurements of the oxygen consumption by means of the oxygen electrode of the fractions isolated according to Alt. A are also given in Table I. The oxygen uptake of the M fraction with added succinate on

TABLE I. *Cytochrome oxidase activity and oxygen uptake in fractions isolated according to alternative A.*

		Cytochrome oxidase activity		O_2 uptake by polarographic method	
	γN/100 μl	μAtoms O_2/hr	μAtoms O_2/hr/γN	μAtoms O_2/hr	μAtoms O_2/hr/γN
P1	2γ	0.05	0.025	0.190	0.038
P2	2.5γ	0.05	0.02	0.355	0.057
M	21.5γ	13.1	0.61	3.300[a]	0.154

[a] Plus 0.01 *M* succinate.

the basis of nitrogen was about four and three times that of fractions P1 and P2, respectively. No succinate was added to the P1 and P2 fractions in this experiment. The influence of a series of substrates added to a final concentration of 5 m*M* upon the oxygen uptake of fractions P1 and P2, Alt. A was tested. These substrates were succinate, α-ketoglutarate, L-glutamate, acetoacetate, α-glycerophosphate, β-hydroxybutarate, puruvate and octanoate. No significant change in the oxygen uptake of the P1 and P2 material was observed with these substrates. Furthermore, no effect was seen when ADP and $MgCl_2$ was added independently and in combination with the above-mentioned substrates to a final concentration of 1 m*M* and 5 m*M*, respectively.

TABLE II. *ATPase activity in fractions isolated according to alternative A with and without deoxycholate.*

	Control (μM P_i/γN)	Plus 0.1 % deoxycholate (μM P_i/γN)
P1	0.120	0
P2	0.005	0
M	0.120	0.146

The resulting measurements of oxygen consumption in fractions P1, P2 and M, Alt. A upon the addition of succinate and ADP are shown in Fig. 2. The oxygen uptake of the M fraction was markedly increased by addition of succinate and was further enhanced by ADP, while that of the fractions P1 and P2 did not change.

TABLE III. *ATPase activities exhibited by various fractions before and after purification according to alternative B (cf. text).*

Fraction	PL 1′	PL 2′	ML′	Layer near origin in tubes containing PL 1′	Layer near origin in tubes containing PL 2′	P1	P2
ATPase activity (μMol $P_i/\gamma N$)	0.055	0.170	0.135	0.032[a]	0.104[a]	0	0

[a] Nitrogen determination performed on material precipitated by 10 % TCA.

The oxygen uptake of P1 and P2 fractions declined with storage at 0°C and was not measurable after a period of 6 hr. Moreover, no oxygen uptake was found after heating the material of these fractions at 90°C for 10 min.

The ATPase activities of the fractions P1, P2 and M, Alt. A, on a nitrogen basis, with and without deoxycholate are given in Table II. The activities of P1 and M were of the same order of magnitude while that of P2 was

TABLE IV. *Phosphorylation, exchange of radioactive inorganic phosphate with ATP and ATPase activity in isolated fractions. Alternative A (cf. text).*

		$^{32}P_i$ uptake in presence of succinate		$^{32}P_i$–ATP exchange		ATPase	
Total counts		200,000		200,000		25,000	
	Nitrogen, γ	Counts in aqueous phase	Counts/γN	Counts in aqueous phase	Counts/γN	Counts in isobutanol phase	Counts/γN
P1	2	70	35	290	145	2,610	1,305
P2	5	0	0	120	24	5,130	1,026
M	18	28,300	1.574	15,400	857	6,770	377

considerably lower. The activity of the M fraction was not inhibited by 0.1 per cent deoxycholate, in contrast to the activities of the P1 and P2 fractions which were inhibited.

The distribution of the ATPase activities, following further fractionations of the PL1′ and PL2′ material (for notations, see Material and Methods)

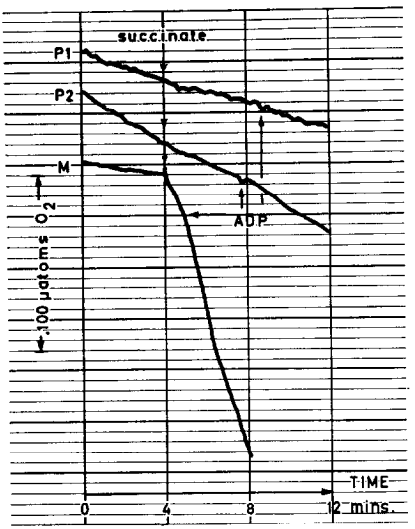

Fig. 2.—Oxygen uptake of fractions P1, P2 and M, Alt. A measured with the polarographic method on an enclosed volume of 0.5 ml sample. The reaction medium had the following composition: 5 m*M* $MgSO_4$; 25 m*M* tris buffer, pH 7.4; 210 m*M* sucrose. Succinate and ADP were added later, as indicated, to a final concentration of 5 m*M* and 1 m*M*, respectively.

according to Alt. B, is given in Table III. ATPase activity was found in the PL1′, PL2′ and ML′ material. After refractionation of these fractions, no activity was detected in the P1 and P2 fractions. In contrast to this, the layer corresponding to the origin of the sample in the gradients showed considerable ATPase activity.

The $^{32}P_i$ uptake, $^{32}P_i$–ATP exchange and ATPase activity, based on nitrogen, in fractions P1, P2 and M, Alt. A of an experiment are given in Table IV. The $^{32}P_i$ uptake of P1 and P2 fractions was negligible, as compared to that of the M fraction. The $^{32}P_i$–ATP exchange of the M fraction was about six and 36 times that of the P1 and P2 fractions, respectively. In contrast, the ATPase activity of the M fraction was smaller than that found in the P1 and P2 fractions. In the P1 fraction this activity was 3.5, and, in the P2 fraction, 2.7 times that of the M fraction.

DISCUSSION

The presence in the M fraction of high cytochrome oxidase activity, succinate- and ADP-stimulated oxygen uptake, $^{32}P_i$ uptake and $^{32}P_i$–ATP exchange shows conclusively the presence of material in this fraction of mitochondrial origin. In previous electron microscopic investigations of the material in this fraction, structures which could be identified as intact mitochondria constituted only a very small part of the material present [3]. The bulk of the material was made up of vesicles or small tubular structures. These structures were composed of membranes with an ultrastructure similar to that found in the occasional intact mitochondrion. Furthermore, the density properties of the particles of the M fraction in density gradients were found to be identical to those of intact bovine heart muscle mitochondria [2]. In view of these findings, together with those of the biochemical properties of the material in the M fraction shown in the present study, it is very probable that the bulk of the material in this fraction was composed of mitochondrial fragments. Furthermore, it has been shown that in frog skeletal muscle the ATPase activity of mitochondrial fragments is not affected by addition of deoxycholate, whereas, the sarcotubular system is inhibited [21]. By inference from these observations, the presence of deoxycholate resistant ATPase activity in the M fraction lends further support to the above conclusion.

On the other hand, mitochondrial enzymic activities in the P1 and P2 fractions, as compared to the M fraction, were very low in the case of cytochrome oxidase and $^{32}P_i$–ATP exchange, or unmeasurable, as in the case of $^{32}P_i$ uptake and deoxycholate resistant ATPase. These findings indicate that the P1 and P2 fractions contain if any, only negligible amounts of mitochondrial material. This conclusion is further confirmed by electron microscopic investigations of these fractions which did not reveal structures that could be identified as mitochondria [2].

As shown in Table II and IV, considerable ATPase activity may be present in the P1 and P2 fractions isolated according to Alternative A. This activity is inhibited by addition of 0.1 per cent deoxycholate (Table II) and is eliminated by further purification of the material in density gradients according to Alternative B (see Material and Methods and Table III). Electron micro-

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Fig. 3.—Electron micrographs of lipofuscin granule fractions isolated from bovine cardiac muscle according to Alternative B (see Material and Methods). The detailed description of the preparation of the material for electron microscopy is given in [3]. (a) fraction P1; (b) fraction P2. ×12,000.

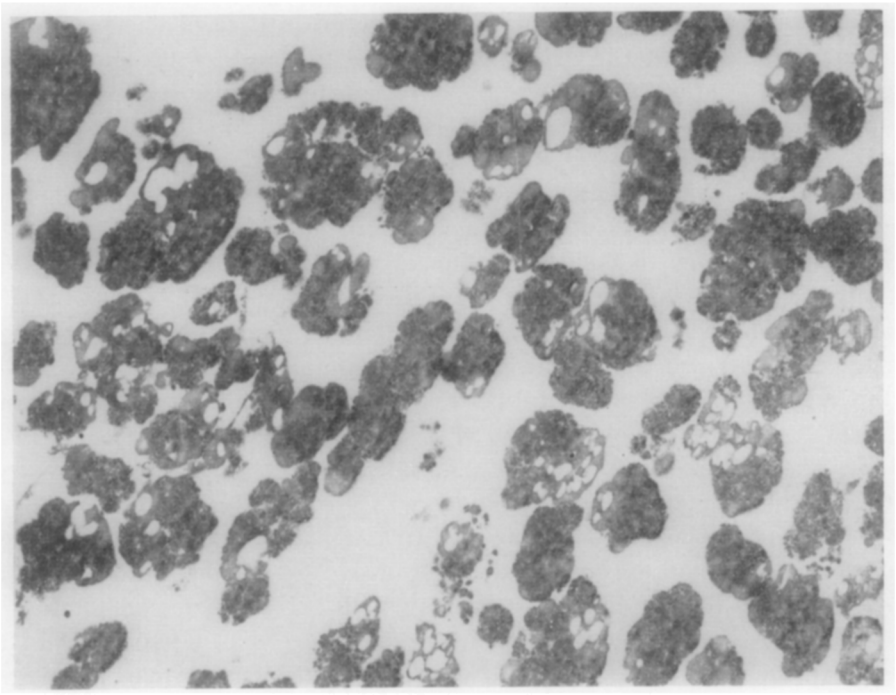

Fig. 3 a.

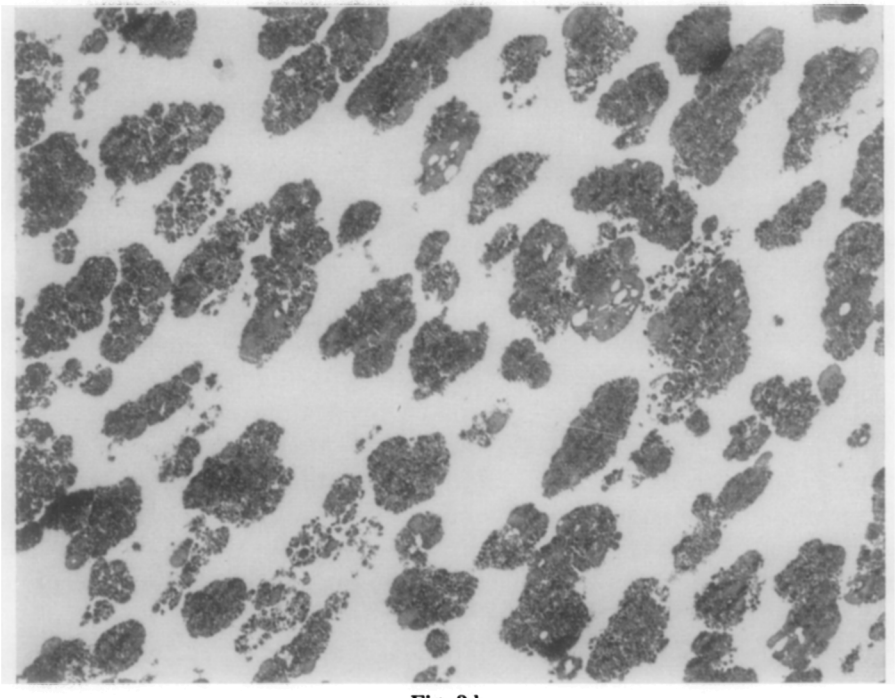

Fig. 3 b.

scopic investigations carried out on the material in P1 and P2 fractions isolated according to Alternative A, showed that the bulk of the material was composed of lipofuscin granules [3]. Between the lipofuscin granules membranous material was found arranged as vesicles. On the other hand, membrane vesicles could not be observed in the fractions isolated according to Alternative B (Figs. 3*a* and *b*). From these results it can be concluded that the purified lipofuscin granules do not contain ATPase activity.

After the last step of the purification according to Alternative B, ATPase activity was traced to the part of the density gradient that corresponded to the position of the sample before centrifugation; i.e., in the 40 per cent sucrose region. However, efforts to study the ultrastructure of the particles present in this region were not successful because of the minute amounts of material. Nevertheless, the parallel elimination of the deoxycholate inhibited ATPase activity and the membrane vesicle material from the lipofuscin granule fractions suggests that the ATPase activity was associated with the membrane vesicle material. This is in agreement with the results presented by Muscatello *et al.* from investigations on frog skeletal muscle [21]. Thus, particulate fractions composed of membranous vesicles which exhibited deoxycholate inhibited ATPase activity were isolated, and evidence was advanced that the membranous material originated from the sarcotubular system, a structure analogous to the endoplasmic reticulum.

In the lipofuscin granule fractions, a considerable oxygen uptake based on nitrogen content was found, as compared to that of the M fraction (Table I). The oxygen uptake of the lipofuscin granule fractions was neither stimulated by addition of the substrates tested (see Results), nor by addition of ADP. These observations indicate that the oxygen uptake of the lipofuscin granule fractions was not caused by mitochondrial respiration. This conclusion is strengthened by the results obtained from the measurements of other mitochondrial activities and the ultrastructural properties of the lipofuscin granule fractions which indicated that the content of mitochondrial material in these fractions was negligible (*vide supra*).

At the present stage, little can be said about the nature of the endogenous oxygen uptake of the lipofuscin granules except that it is not due to mitochondria. The loss of the oxygen uptake by heating or standing indicates that it is dependent on structural organization. Studies in progress presently are concerned with this question and will be published at a later date.

Observations suggesting a mitochondrial origin of lipofuscin granules have been reported in recent electron microscopic studies. Material with an electron microscopic appearance similar to lipofuscin granule material was found

in spatial connection with mitochondria by Hess [15] and Duncan *et al.* [5] in nerve cells; by Poche [23], in heart muscle cells after thyroxin treatment of the animals. Formations in lipofuscin granules which were interpreted as representing mitochondrial structures were found by Ashford and Porter [1] in liver cells of glucagon treated rats; and by Ghosh *et al.* [10] in avian nerve cells.

The results from the present study do not speak in favour of a genetic relationship between mitochondria, as defined by biochemical criteria, and heart muscle lipofuscin granules. This conclusion is strengthened by the results of a study on the electron microscopic characteristics of isolated heart muscle lipofuscin granules [2]. Thus, no structures suggesting the presence of mitochondria, as defined by morphological criteria, were found in the lipofuscin granules.

SUMMARY

Three subcellular fractions have been isolated from fresh, bovine cardiac muscle homogenates of old animals by density gradient and differential centrifugation techniques and the mitochondrial enzyme activities of these fractions were investigated. The "M-fraction", a heavier, membrane fraction, exhibited high cytochrome oxidase activity, substrate- and ADP-influenced oxygen uptake, $^{32}P_i$ incorporation and $^{32}P_i$–ATP exchange. On the basis of these findings, it was concluded that the bulk of this material was of mitochondrial origin.

Cytochrome oxidase activity, substrate- and ADP-influenced oxygen uptake, $^{32}P_i$ incorporation and $^{32}P_i$–ATP exchange were very low or unmeasurable in the two lighter fractions, which contained lipofuscin granules, "P1 and P2". A material containing ATPase activity sensitive to deoxycholate could be separated from the lipofuscin granules by further density gradient purification. It was concluded that these fractions contained negligible amounts of mitochondrial material, and that the ATPase activity probably resides in membranes from the sarcotubular system. However a considerable oxygen uptake, sensitive to aging and heating, was found in the lipofuscin granule fractions.

The results of the present study are discussed in the context of a genetic relationship between mitochondria and lipofuscin granules suggested by other workers.

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