Lipids of dystrophic and normal mouse muscle: whole tissue and particulate fractions

K. OWENS* and B. P. HUGHES

Muscular Dystrophy Research Laboratory, Department
of Chemical Pathology, The National Hospital,
Queen Square, London W.C. 1, England

ABSTRACT Myofibrillar, mitochondrial, and microsomal fractions were prepared from normal and dystrophic mouse limb muscle by differential centrifugation and analyzed for phospholipids and cholesterol. Fatty acids and aldehydes of neutral lipids and of phospholipids from whole muscle and particulate fractions were also determined.

Normal microsomes contained more lecithin and less total ethanolamine phospholipids and cardiolipin than mitochondria. The myofibrils had an intermediate phospholipid composition, but their cholesterol–phospholipid ratio was smaller than that of the other two fractions. Except for an increased percentage of phosphatidalethanolamine in the dystrophic mitochondria, only the composition of the dystrophic microsomes differed from normal by containing less lecithin but more total ethanolamine phospholipid, phosphatidalethanolamine, sphingomyelin, and cholesterol.

No significant differences were found in the fatty acid composition of neutral lipid extracts from normal and dystrophic preparations, but there was a significant decrease in the percentage of 22:6 in phospholipids from both dystrophic whole muscle and microsomes (−25% and −37%, respectively), whereas the 20:4 content was unaltered. By contrast, the percentages of 18:0 and total fatty aldehyde increased significantly. Phospholipid extracts from all dystrophic samples showed a significant decrease in 16:0 and an increase in 18:1 as compared with the normal.

SUPPLEMENTARY KEY WORDS   muscular dystrophy ·
neutral lipids   ·   cholesterol   ·   phospholipids   ·
fatty acids and aldehydes · plasmalogens

Hereditary muscular dystrophy of the mouse has been the subject of numerous biochemical studies, but information on changes in tissue lipids is scanty. Some fatty infiltration has been reported (1), although in young animals this appears to be slight (2). There are increases in total lipid (3, 4), triglyceride (5), and free fatty acid (5, 6), but phospholipid does not change significantly (5, 7). As far as individual phospholipids are concerned, it was found (7) that sphingomyelin increased and lecithin decreased in muscle derived from both fore and hind limbs. There is also an increase in total plasmalogen.[1] The purposes of the present investigation were to determine if these phospholipid changes are localized in any subcellular fraction and to study the distribution of cholesterol and total phospholipid fatty acids and aldehydes.

Since there appears to be no information as to whether the increased triglyceride and free fatty acid of the dystrophic muscle have a normal or abnormal composition, we have also studied fatty acids and aldehyde in the neutral lipid of subcellular fractions from normal and dystrophic muscle.

MATERIALS AND METHODS

Dystrophic Mice and Controls

Breeding animals were obtained from the Jackson Laboratories (Bar Harbor, Maine), and litters of F_1 hybrids (57BL Dydy × 129 Dydy) were produced. After weaning, control and dystrophic animals were fed ad

---

lib. a commercial diet (Oxoid Special Breeder Diet; Oxo Ltd., London, England) in powdered form supplemented with corn oil and glycine (8). It is probable that the controls were not genetically homogeneous, but may have been either homozygous normal (DyDy) or heterozygous unaffected (Dydy) individuals.

Removal of Muscle

Animals aged 2–4 months were used in this investigation. They were anesthetized with diethyl ether and killed by decapitation. Muscle from both fore and hind limbs was dissected out, as far as possible free from visible nerve, fat, and connective tissue. In the case of the dystrophic animals, the yield of muscle per animal seldom exceeded 1 g, whereas from normal mice 2–3 g was obtained.

Homogenization of Muscle and Preparation of Cell Fractions

The homogenization procedure was based on that described by Pennington (9). Muscle, 3–4 g, finely chopped with scissors, was homogenized in an ice-cold sucrose–EDTA medium (0.25 M sucrose–1 mM EDTA, pH 7.3, 5 ml/g of tissue) for 3 min at fairly low speed using a Potter-Elvejhem type homogenizer cooled in ice and fitted with a Teflon pestle (clearance about 0.25 mm). The homogenate was diluted with sucrose–EDTA medium to 25 ml/g and centrifuged at 4°C for 10 min at 600–800 g (High Speed 17 refrigerated centrifuge, rotor No. 69181; Measuring and Scientific Equipment Ltd., London, England). The sediment, after washing with sucrose–EDTA medium, was the myofibrillar fraction. A mitochondrial fraction was prepared by centrifuging the combined 600–800 g supernatants at 9000 g for 15 min. The supernatant was again centrifuged at 9000 g for 15 min, and the sediment was discarded. The supernatant solution was centrifuged at 24,000 g for 90 min to yield a microsomal fraction. Although for convenience we shall refer to these fractions as myofibrillar, mitochondrial, and microsomal respectively, they were not homogeneous. For example, the microsomes were contaminated with some mitochondrial material. The fractions were freeze-dried prior to extraction of lipid. The fractionation procedure is illustrated in Fig. 1. A high-speed microsomal fraction was also prepared from the 24,000 g supernatant solution by centrifugation at 104,000 g for 1 hr (Measuring and Scientific Equipment Ltd. Superspeed 50 preparative ultracentrifuge, rotor No. 2408).

Protein Nitrogen

Protein nitrogen was determined. The trichloracetic acid precipitates were digested for 1 hr with 72% perchloric acid at approximately 200°C, and protein nitrogen was determined by Nesslerization using a potassium-free reagent.

Noncollagen Protein

Noncollagen protein was taken to be protein-soluble in dilute alkali and was determined as described by Lilienthal, Zierler, Folk, Buka, and Riley (10).

Succinate: (INT) Oxidoreductase

The activity of succinate oxidoreductase was measured by the method of Pennington (9).

Extraction of Lipids for Phospholipid and Cholesterol Analysis

For the preparation of total lipid, extracts from whole muscle about 0.5 g were frozen in liquid nitrogen and crushed into a pellet using a percussion mortar. The pellet was homogenized in a vortex beaker (Measuring and Scientific Equipment Ltd.) for 3 min with 6–7 ml of chloroform–methanol 2:1 at high speed using a homogenizer fitted with stainless steel blades (Measuring and Scientific Equipment Ltd.). The residue was again homogenized for 3 min with the same solvent. After centrifugation, the supernatant solutions were combined. Additional homogenization of the residue yielded negligible amounts of lipid phosphorus. The combined supernates were evaporated to dryness at 45°C under reduced pressure and treated twice with 4 ml of chloroform–methanol 2:1 containing 4% water in order to precipitate the protein which was bound to lipid (11). The residue was extracted with successive small volumes of dry chloroform–methanol 2:1 and made up to 4 ml in a graduated stoppered centrifuge tube. The chloroform–methanol extract was washed with 0.1 M KCl as described by Folch, Lees, and Sloane Stanley (12). It was then evaporated to dryness under a stream of nitrogen and finally redissolved in 1–2 ml of chloroform–methanol 2:1. The extracts were stored at −20°C prior to thin-layer chromatography or estimation of cholesterol.

Total lipid extracts from subcellular fractions were similarly prepared by homogenizing the freeze-dried preparations in chloroform–methanol 2:1. The extracts were analyzed for phospholipids without further treatment, but there were additional steps for fatty acid and aldehyde studies.

Fatty Acid and Aldehyde Analysis

When a neutral lipid fraction from the total lipid extracts was not required, 0.005% BHT was included in the chloroform–methanol 2:1. At other times, the solvents were simply gassed with N_2 because of the formation of an artifact when neutral lipid extracts containing BHT were methylated. Silicic acid (Mallinckrodt Chemical Works, St. Louis, Mo.) used for column chromatography
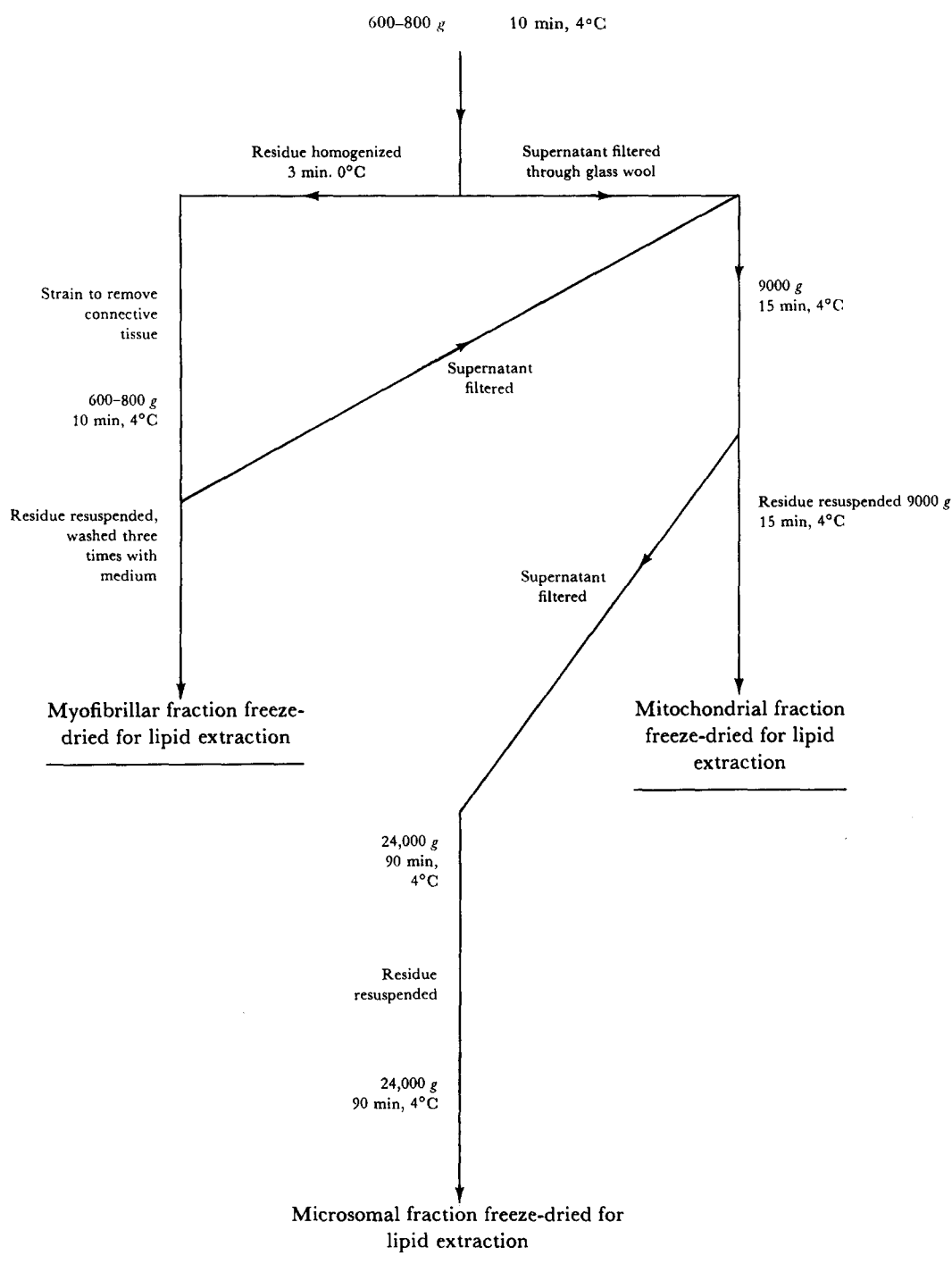
600–800 g 10 min, 4°C

Residue homogenized
3 min. 0°C

Supernatant filtered
through glass wool

Strain to remove
connective
tissue

9000 g
15 min, 4°C

Supernatant
filtered

600–800 g
10 min, 4°C

Residue resuspended,
washed three
times with
medium

Residue resuspended 9000 g
15 min, 4°C

Supernatant
filtered

Myofibrillar fraction freeze-
dried for lipid extraction

Mitochondrial fraction
freeze-dried for lipid
extraction

24,000 g
90 min,
4°C

Residue
resuspended

24,000 g
90 min, 4°C

Microsomal fraction freeze-dried for
lipid extraction

FIG. 1.   Preparation of subcellular fractions from mouse muscle. Finely chopped muscle homogenized 3 min with 0.25 M sucrose–1 mM EDTA pH 7.3, 0°C, 5 ml/g, and then diluted with medium, 20 ml/g.

was sieved, and the 100–240 mesh fraction was refluxed and subsequently rinsed with methanol and then with diethyl ether prior to activation at 150°C for 16 hr. Total lipid extracts, dissolved in chloroform were added to a slurry of the silicic acid (1 g) in chloroform (not more than 0.5 mg of phosphorus per g of silicic acid), and a neutral lipid (nonphosphorus-containing) fraction was eluted with 50 ml of chloroform. This fraction also contained any free fatty acids. The phospholipids were then eluted with 50 ml of methanol; the recoveries of phosphorus were better than 96%. Control eluates from columns on to which no lipid extracts had been added were also prepared for gas chromatography. After concentration under reduced pressure, the eluates were made up to 4 ml with

chloroform–methanol 2:1 and stored at −20°C. A phospholipid fraction was also prepared by thin-layer chromatography. The BHT-containing total lipid extracts were applied to the plate when inside a Perspex box through which a flow of N_2 was maintained, and the neutral lipids were removed using petroleum ether(40°–60°C)–diethyl ether–glacial acetic acid 90:10:1. The origin area was then eluted with methanol. There was agreement in the percentage of polyunsaturated fatty acids in phospholipid extracts prepared by the two methods when these precautions were taken.

Preparation of Fatty Acids from Mouse Diet

Samples of the mouse diet were saponified with 5 N NaOH for 2 hr in sealed N_2-filled ampoules. The contents were washed out, cooled, and then acidified with ice-cold 2 N HCl. The fatty acids were then extracted with petroleum ether at room temperature.

Quantitative Thin-Layer Chromatography of Phospholipids

Two-dimensional thin-layer chromatography was carried out as described by Owens (13), in which plasmalogens are estimated following their hydrolysis to the corresponding lysophospholipids with a mercuric chloride spray reagent.

Phosphorus

Phosphorus was estimated as described by McArdle and Zilkha (14) using 72% rather than 60% perchloric acid for digestion. Digestion was complete in 1 hr at 155–160°C.

Cholesterol

Cholesterol was assayed spectrophotometrically using the FeCl₃–sulfuric acid reagent of Zlatkis, Zak, and Boyle (15) except that ethanol was used as solvent instead of glacial acetic acid.

Total lipid extracts, containing 5–40 μg of cholesterol, were evaporated under a stream of nitrogen and saponified in ethanol (1 ml) containing 10 N NaOH (0.11 ml) for 2 hr at 50°C. 2 ml of water was then added, and the cholesterol was extracted twice with 5 ml of petroleum ether (bp 40–60°C). The extracts were evaporated in a test tube under a stream of nitrogen and then dried over P_2O_5 under reduced pressure. 0.6 ml of FeCl reagent (FeCl₃–6 H_2O, 0.1% w/v), in ethanol was added, and the tubes were stoppered and cooled in ice. 0.4 ml of sulfuric acid (sp gr 1.84) was added slowly to form a lower layer. To prevent a significant temperature rise, the sulfuric acid layer was thoroughly cooled before agitating the tubes vigorously. After 15 min the tubes were removed from the ice bath and kept for a further 90 min at room temperature before reading the optical densities at 560

nm against a solvent blank. Amounts of cholesterol were estimated by reference to standard curves which passed through the origin and were linear up to approximately 45 μg of cholesterol. This procedure avoided the interference from polyunsaturated acids described by Rhodes (16).

Cholesterol and Cholesteryl Ester

Lipid samples containing a minimum of 100 μg of total cholesterol were streaked on to Silica Gel H (E. Merck A. G., Darmstadt, Germany) thin-layer plates with an "Agla" micrometer syringe (Burroughs Wellcome & Co., London, England). Standard samples of cholesterol and cholesteryl palmitate (Sigma Chemical Co., St Louis, Mo.) were similarly applied. The chromatograms were developed in petroleum ether(bp 40–60°C)–diethyl ether–glacial acetic acid 90:10:1 as described by Zeitmann (17). Outer marker lanes containing cholesterol and cholesteryl palmitate were visualized by spraying these areas with iodine dissolved in methanol (1% w/v). The adjacent areas were scraped off into small chromatography columns (10 × 100 mm) fitted with a sintered glass disc (porosity 3), and the lipids were eluted with 20 ml of chloroform which was then evaporated under a stream of nitrogen. Cholesterol was estimated directly, and cholesteryl ester was determined after saponification.

Gas–Liquid Chromatography

Methyl esters and dimethylacetals were prepared from the neutral lipid and phospholipid extracts using 14% BF_3 in CH_3OH (British Drug Houses, Ltd., Poole, Dorset, England) according to the procedure of Morrison and Smith (18), except that the heating at 100°C was performed under N_2 in sealed ampoules. Yields of fatty acid esters were found to be better than 97% as judged by the use of 15:0 and 20:0 as internal standards. The gas chromatograph used was an F. & M. series 810 (F. & M. Scientific Corp., Avondale, Pa.) equipped with hydrogen flame ionization detectors. Dual glass columns (6 ft × ¼ in. I.D.) were packed with acid-washed Celite 545 (60–85 mesh) which had been deactivated with DMCS (18) and coated with PEGS, 6%. Alternatively, acid-washed celite was deactivated with methanolic alkali (19) and coated with Apiezon L (3%). Nitrogen was used as carrier gas at an inlet pressure of 50 psi. Generally, the Apiezon columns were operated isothermally at 185°C, whereas the PEGS columns were run on a temperature program 150 to 188°C at 2°C/min. Fatty acids were identified by comparison of retention times with those of standard methyl esters (99%+ pure) (Sigma Chemical Co.) chromatographed isothermally on polar and apolar stationary phases, and by chromatography after hydrogenation using 5% palladium on barium sulfate in ethanolic solution under slight hy-

drogen pressure for 4 hr. Peak areas were determined by planimetry. The relative molar responses of 15:0, 16:0, 16:1, 18:0, 18:2, and 20:0 methyl esters were found to be identical, whereas the molar response of palmitaldehyde and stearaldehyde dimethylacetals was 0.79 times that of corresponding methyl esters, and peak areas were corrected accordingly.

RESULTS

Noncollagen Protein

Less noncollagen protein (nitrogen $\times$ 6.25) was found in dystropic muscle samples than in normal (Table 1) in agreement with the report of Weinstock, Epstein, and Milhorat (20).

Succinate: (INT) Oxidoreductase

Measurement of succinate:(INT) oxidoreductase activity, which is assumed to be of mitochondrial origin, provided information on the proportion of mitochondria released from the muscle into the supernatant during homogenization, and the degree of contamination of the microsomal fractions with mitochondrial material. Thus comparison of the activity of the whole homogenate with that of the 600–800 g supernate showed that about 40% of the muscle mitochondria was released. Although the average activity of the myofibrillar fraction was low (0.33 μmoles of formazan per mg of nitrogen per min) much of the enzyme activity retained in the 600–800 g residue appeared in this fraction.

The activities of the normal and dystrophic mitochondrial fractions, 2.9 ± 0.2 and 2.5 ± 0.4 μmoles of formazan per mg of nitrogen per min, respectively, did not differ significantly. Taking the mitochondrial fraction as 100%, the amount of mitochondrial material in the normal and dystrophic microsomal fractions was about 14% and 30%, respectively. The value for normal microsomes compares with a figure of 26% for a "heavy" microsome, and 3% for a "light" microsome fraction (calculated from succinate–cytochrome C reductase activity) prepared from rabbit muscle in 0.25 M sucrose (21).

Cholesterol

As had been found by Young, Young, and Edelmann (5), whole dystrophic muscle contained more cholesterol than normal muscle (Table 1), and since this was true regardless of the comparative basis (wet weight, noncollagen protein or lipid phosphorus), this observation was extended using subcellular fractions (Table 2). In agreement with a preliminary report (22), it was found that the cholesterol–phospholipid molar ratio of both dystrophic microsomal fractions was more than double that of the normal, whereas there was no significant difference in the mitochondrial preparations. The much smaller increase observed in the dystrophic myofibrillar fraction might have been due to the presence of segments of intact muscle fiber.

Phospholipids

Differences in the phospholipid composition of normal muscle fractions can be seen in Table 3. The mitochondria contained less lecithin and phosphatidalcholine, but more ethanolamine phospholipid and cardiolipin than the microsomes. The composition of the myofibrillar fraction from normal muscle was intermediate between that of the mitochondria and microsomes with respect to choline and ethanolamine phospholipids and also cardiolipin, but contained less cholesterol. The occurrence of lysolecithin in lipid extracts of these particulate fractions may be an artifact due to phospholipase activity, since there is a negligible amount of lysolecithin in fresh whole muscle. The largest amount of lysolecithin was in the microsomes, possibly because of the lengthier manipulative procedures involved in their preparation. Although the amount of phosphatidalcholine in mouse muscle is small (not more than 2% of the total lipid phosphorus) and is not listed separately, 20–30% of the ethanolamine phospholipid is present as phosphatidalethanolamine; the microsomes contain the highest proportion. The chief differences between normal and dystrophic muscle were in the microsomal fractions, the dystrophic samples containing substantially less lecithin and phosphatidalcholine and more ethanolamine phospholipid than microsomes from normal muscle.

TABLE 1   CHOLESTEROL AND NONCOLLAGEN PROTEIN OF MOUSE SKELETAL MUSCLE*

Tissue	Noncollagen Protein	Cholesterol†	
	mg/g wet wt	μmoles/g wet wt	μmoles/g atom phosphorus
Normal	169.5 ± 28.5 (8)	2.09 ± 0.15 (4)	0.135 ± 0.009 (4)
Dystrophic	123.8 ± 20.2 (5)‡	3.93 ± 0.18 (4)§	0.212 ± 0.005 (4)§

* Values are given as mean ± SD, figures in parentheses indicate number of preparations analyzed.
† Cholesteryl esters were not found.
‡ Difference between normal and dystrophic was significant (t test), 0.01 > P > 0.001.
§ Difference between normal and dystrophic was significant (t test), 0.001 > P.

TABLE 2   CHOLESTEROL IN MOUSE MUSCLE FRACTIONS*

Fraction	Normal	Dystrophic
	μmoles/μg atom lipid phosphorus†	
Myofibrillar	0.093 ± 0.008 (4)	0.136 ± 0.018 (4)‡
Mitochondrial	0.151 ± 0.032 (6)	0.185 ± 0.029 (5)
Microsomal	0.163 ± 0.028 (5)	0.365 ± 0.035 (5)§
High-speed micro-somal	0.166 ± 0.075 (4)	0.372 ± 0.018 (4)§

* Cholesteryl esters were not found.
† Values are given as mean ± SD; figures in parentheses indicate number of preparations analyzed.
‡ Difference between normal and dystrophic preparations was significant (t test), $0.01 > P > 0.001$.
§ Difference between normal and dystrophic preparations was significant (t test), $0.001 > P$.

There was also a marked increase in cardiolipin. Although we were unable to demonstrate any significant changes in phosphatidalcholine, both the microsomes and mitochondria from dystrophic muscle contained more ethanolamine plasmalogen than normal.

Dietary Fatty Acids

Analysis of the mouse diet indicated that it contained about 50% of 18:2 and 2% of 18:3. An essential fatty acid deficiency using this diet fed ad lib. therefore seems unlikely, particularly since the food intake of the dystrophic mouse in proportion to body weight is not below normal (23).

Whole Muscle and Adipose Tissue, Fatty Acids, and Aldehydes

Only small differences were found in the fatty acid and aldehyde content of the neutral lipid fraction from normal and dystrophic whole muscle (24), whereas the fatty acid composition of the phospholipid was significantly different (Table 4). In particular, the decrease in 16:0 and 22:6 and the increase in 18:0, 18:1, and total

aldehyde were all highly significant (Student's "t" test). The results from the fatty acid analysis of adipose tissue phospholipid (Table 5) indicated that substantial differences in fatty acid composition existed between fat cells and normal muscle.

To simplify Tables 4–7, data are only presented for those acids which comprise more than 1% of the total. Since there are no significant differences in the content of 22:4 and 24:1 between any of the preparations, mean values for these acids have been combined even though they are not metabolically related.

Fatty Acids and Aldehyde of Subcellular Fractions

Although the fatty acid composition of the neutral lipids from the myofibrillar, mitochondrial, and microsomal fractions of dystrophic muscle (Table 6) were not significantly different from normal, the contents of 18:0 and 18:2 were different in the various fractions. For example, the myofibrillar neutral lipid contained about half the amount of 18:0 as did the microsomal neutral lipid. The phospholipid extracts from dystrophic samples showed significant differences from normal in fatty acid and aldehyde composition (Table 7), the greatest change being in the amount of 22:6 in the microsomal and, to a lesser extent, mitochondrial fractions. The difference between the 22:6 content of the myofibrillar fractions was not significant. The higher percentage of 18:1 was a characteristic of all dystrophic phospholipid extracts. The greater content of 18:0 observed with dystrophic whole muscle was only reflected to a significant extent in the microsomal phospholipid. Conversely, whereas the lower percentage of 16:0 in dystrophic whole muscle was paralleled by the lower 16:0 content of the myofibrillar and mitochondrial fractions, there was no significant change in the microsomes. In general, with the exception of 18:2, the differences in fatty acid composition were greater between the normal and corresponding dystrophic prep-

TABLE 3   PHOSPHOLIPID CONTENT OF MOUSE MUSCLE FRACTIONS

Constituent	Myofibrils		Mitochondria		Microsomes	
	Normal	Dystrophic	Normal	Dystrophic	Normal	Dystrophic
	*% of total lipid phosphorus**					
Sphingomyelin	2.7 ± 1.3	3.5 ± 0.7	3.3 ± 0.6	4.1 ± 0.9	3.3 ± 0.9	8.3 ± 1.1†
Lecithin plus phosphatidalcholine	52.8 ± 4.0	52.5 ± 2.9	41.4 ± 0.1	39.5 ± 3.9	61.1 ± 3.1	43.1 ± 6.1†
Phosphatidylserine	2.0 ± 1.3	2.3 ± 1.3	3.2 ± 1.0	3.5 ± 1.1	2.5 ± 1.2	5.1 ± 1.1‡
Monophosphoinositide	6.6 ± 1.2	5.9 ± 1.3	5.7 ± 0.7	5.1 ± 0.9	6.8 ± 1.5	6.0 ± 0.6
Total ethanolamine phospholipid	28.6 ± 1.7	27.9 ± 2.4	32.8 ± 1.9	33.3 ± 2.1	21.9 ± 1.7	27.1 ± 1.6†
Phosphatidalethanolamine	7.3 ± 0.8	7.4 ± 0.7	6.3 ± 0.7	9.4 ± 2.0§	6.2 ± 1.3	10.4 ± 1.2†
Cardiolipin	5.4 ± 0.9	5.8 ± 0.7	12.5 ± 2.5	11.5 ± 2.2	2.0 ± 1.0	4.1 ± 0.7‡
Lysolecithin	1.9 ± 2.3	1.4 ± 0.4	1.5 ± 0.5	1.2 ± 1.4	2.6 ± 2.1	5.1 ± 3.0

* Values are given as mean ± SD and are based on the analysis of five preparations except for dystrophic myofibrils where the number was 4. Differences between means of normal and dystrophic values were not significant ($P > 0.05$) unless indicated.
† Difference between means of normal and dystrophic values was significant, $0.001 > P$.
‡ Difference between means of normal and dystrophic values was significant, $0.01 > P > 0.001$.
§ Difference between means of normal and dystrophic values was significant, $0.02 > P > 0.01$.

TABLE 4 MAJOR FATTY ACIDS AND ALDEHYDES OF WHOLE MUSCLE PHOSPHOLIPID

	Normal (5)	Dystrophic (4)
	% of total fatty acid or aldehyde*	
16:0	25.8 ± 1.7	21.2 ± 1.1†
16:1	1.2 ± 0.1	1.5 ± 0.1†
18:0	14.7 ± 1.2	18.6 ± 1.3†
18:1	7.4 ± 0.5	12.1 ± 0.1‡
18:2	11.8 ± 1.0	13.0 ± 0.6
20:4	9.2 ± 0.6	9.6 ± 0.8
22:5	1.9 ± 0.4	1.4 ± 0.3
22:6	23.7 ± 2.0	17.7 ± 0.9†
22:4 plus 24.1	2.2	2.2
Saturated plus monoenoic	49.9	54.4
Polyenoic	49.4	43.3
Aldehydes		
16:0	66.9 ± 4.4	58.4 ± 4.1§
18:0 plus 18:1	33.2 ± 4.4	41.6 ± 4.1§
Total aldehyde	3.5 ± 0.2	4.7 ± 0.2‡

* Values are shown as mean ± SD, except where individual means are combined, and the figure in parentheses denotes the number of determinations. Components less than 1% by weight are not listed.

† Difference between means of normal and dystrophic values was significant (t test), $0.01 > P > 0.001$.

‡ Difference between means of normal and dystrophic values was significant (t test), $0.001 > P$.

§ Difference between means of normal and dystrophic values was significant (t test), $0.02 > P > 0.01$.

arations than between the subcellular fractions or whole muscle. However, the results obtained for 20:4 were unique in that all subcellular preparations from both normal and dystrophic muscle were found to contain a similar percentage of this acid (mean 11%). Both normal and dystrophic whole muscle phospholipid contained slightly less 20:4 (Table 4), but the combined material from the three subcellular fractions did not represent the entire muscle homogenate, by virtue of the removal of connective tissue (Fig. 1).

DISCUSSION

Because there appears to be little available information on the phospholipid composition of skeletal muscle subcellular material, it is pertinent to compare the composition of our normal mouse fractions with those of other tissues and species reported in the literature. Thus the microsomal fraction contains substantially more choline phospholipid and less ethanolamine phospholipid than the mitochondrial fraction and, in this respect, is similar to the microsomes of rat liver, sheep liver, and sheep spleen (25), to microsomes of cultured human heart cells (26), and to ox heart microsomes (27). Cardiolipin is concentrated in the mitochondrial fraction as was also observed in these latter studies (25–27).

With reference to skeletal muscle microsomes, we find that more than 60% of the phospholipid consists of lecithin plus phosphatidalcholine. This is in general

TABLE 5 MAJOR FATTY ACIDS AND ALDEHYDES OF ADIPOSE TISSUE PHOSPHOLIPID

Components*	% of total fatty acid or aldehyde
16:0	17.9 ± 2.4
16:1	1.7 ± 0.2
18:0	19.6 ± 1.0
18:1	14.8 ± 2.6
18:2	21.0 ± 2.8
20:3	1.1 ± 0.4
20:4	14.6 ± 3.1
22:6	5.9 ± 0.8
22:4 plus 24:1	2.7
Saturated plus monoenoic	55.9
Polyenoic	45.9
Aldehydes	
16:0	71.5 ± 5.3
18:0 plus 18:1	28.2 ± 3.7
Total aldehyde	3.9 ± 0.6

* Values from six determinations, mean ± SD except where individual means are combined. Components less than 1% by weight are not listed.

TABLE 6 MAJOR FATTY ACIDS OF THE NEUTRAL LIPID FROM SUBCELLULAR FRACTIONS*

Components	Fatty Acid Distribution†		
	Myofibrils	Mitochondria	Microsomes
	% of total fatty acid		
14:0	3.1 ± 0.5 (4)	2.7 ± 0.6 (5)	3.4 ± 0.5 (6)
16:0	28.2 ± 2.7 (6)	28.5 ± 3.3 (5)	31.9 ± 2.8 (7)
16:1	8.6 ± 1.8 (4)	7.8 ± 1.8 (5)	7.8 ± 1.6 (7)
18:0	5.1 ± 1.0 (4)	7.6 ± 2.1 (5)	10.1 ± 2.5 (7)
18:1	36.1 ± 2.1 (6)	33.8 ± 3.5 (5)	33.7 ± 1.8 (7)
18:2	17.8 ± 3.1 (4)	19.2 ± 2.4 (4)	13.8 ± 1.6 (6)

* Values are all from normal fractions. There were no significant differences (i.e. $P > 0.05$) between normal and dystrophic fractions. Fatty aldehydes were not found.

† Values are shown as mean ± SD, except where individual means are combined, and the figure in parentheses denotes the number of determinations. Components less than 1% by weight are not listed.

agreement with results for a phosphatidylcholine fraction reported by Martonosi (28) and by Drabikowski, Dominas, and Dabrowska (29) in rat and rabbit skeletal muscle microsomes, respectively.

Previous observations (7) and those reported in this paper show that changes occur in the phospholipid and cholesterol content of the skeletal muscle from dystrophic mice. These are reflected by similar, but generally more striking, changes in fractions prepared by differential centrifugation of muscle homogenates. Some of these differences, notably the increase in sphingomyelin and decrease in lecithin, appear to affect only the microsomal fraction. The increase in cholesterol in whole muscle is also more pronounced in the microsomes. By contrast there is an increase in phosphatidalethanolamine in both mitochondria and microsomes.

TABLE 7   MAJOR FATTY ACIDS AND ALDEHYDES IN PHOSPHOLIPIDS FROM MOUSE MUSCLE SUBCELLULAR FRACTIONS*

Components	Myofibrils		Mitochondria		Microsomes	
	Normal	Dystrophic	Normal	Dystrophic	Normal	Dystrophic
	% of total fatty acid or aldehyde					
16:0	25.9 ± 2.0 (7)	22.5 ± 2.3 (5)†	21.9 ± 1.7 (8)	18.1 ± 1.8 (8)‡	25.8 ± 2.9 (8)	23.6 ± 2.2 (9)
16:1	1.7 ± 0.2 (8)	1.7 ± 0.5 (6)	1.7 ± 0.3 (8)	1.6 ± 0.2 (8)	2.1 ± 0.4 (8)	1.5 ± 0.4 (8)
18:0	14.5 ± 3.0 (8)	15.7 ± 0.8 (6)	15.7 ± 1.1 (7)	17.0 ± 1.3 (8)	13.7 ± 1.6 (8)	18.2 ± 2.4 (9)‡
18:1	8.9 ± 0.9 (8)	11.0 ± 1.2 (6)§	10.5 ± 1.4 (8)	14.1 ± 1.2 (8)‡	9.4 ± 1.1 (8)	12.7 ± 1.9 (7)§
18:2	11.6 ± 1.3 (6)	14.0 ± 1.3 (6)	14.9 ± 2.1 (8)	16.1 ± 1.8 (8)	10.5 ± 0.9 (7)	11.8 ± 1.4 (9)†
20:4	11.3 ± 1.2 (7)	11.0 ± 0.9 (6)	10.4 ± 1.4 (7)	10.8 ± 1.1 (8)	13.0 ± 1.8 (7)	13.1 ± 1.3 (8)
22:5	1.5 ± 0.6 (7)	1.6 ± 0.7 (6)	1.5 ± 0.3 (7)	1.2 ± 0.2 (6)	2.3 ± 0.2 (6)	1.3 ± 0.4 (7)‡
22:6	20.0 ± 2.7 (6)	17.8 ± 2.1 (6)	18.9 ± 1.5 (6)	15.8 ± 1.8 (7)§	20.2 ± 2.4 (7)	12.8 ± 1.4 (8)‡
22:4 plus 24.1	2.1	2.3	2.2	2.7	2.7	2.2
Saturated plus mono-enoic	52.4	52.4	51.3	52.6	52.9	57.5
Polyenoic	47.0	47.3	48.8	47.1	49.5	41.9
Aldehydes						
16:0	75.4 ± 2.5 (6)	75.0 ± 4.4 (4)	70.8 ± 3.2 (6)	71.1 ± 4.5 (4)	74.0 ± 3.4 (6)	72.5 ± 2.8 (6)
18:0 + 18:1	24.7 ± 2.5 (5)	25.1 ± 4.4 (4)	29.6 ± 3.3 (5)	28.9 ± 4.5 (4)	26.1 ± 3.4 (6)	27.5 ± 3.3 (6)
Total aldehyde	3.6 ± 0.4 (8)	3.6 ± 0.4 (5)	3.3 ± 0.7 (7)	4.2 ± 0.7 (6)	4.4 ± 0.7 (8)	6.2 ± 1.0 (8)§

* Values are shown as mean ± SD, except where individual means are combined, and the figure in parentheses denotes the number of determinations. Components less than 1% by weight are not listed.
† Difference between means of normal and dystrophic values was significant (t test), $0.05 > P > 0.02$.
‡ Difference between means of normal and dystrophic values was significant (t test), $0.001 > P$.
§ Difference between means of normal and dystrophic values was significant (t test), $0.01 > P > 0.001$.

In discussing the difference between normal and dystrophic muscle microsomal fractions, two factors must be considered. These are cross-contamination and the presence of fat cells in the tissue. Although the microsomes from dystrophic muscle contain considerably more mitochondrial material than normal microsomes and this could in part explain the decrease in lecithin plus phosphatidalcholine, it could not explain the increase in sphingomyelin and cholesterol. In muscular dystrophy of the mouse, an increase in neutral lipid occurs (5). However it is not clear how much of this increase is due to the presence of fat lying between the muscle fibers, and how much to an increase of intrafibrillar neutral lipid. Since there is histological evidence for the presence of considerable amounts of intrafibrillar neutral lipid (6) and little fatty infiltration in dystrophic animals less than 6-months-old (2), much of the increase may be due to intrafibrillar material, but at present it does not appear possible to estimate quantitatively the contributions of the two sources to total neutral lipid. Histological examination of muscle from our animals also showed only slight fatty infiltration. However, McArdle (30) has reported that phospholipids from rat fat have a high sphingomyelin content; if the same is true of mouse fat, it is necessary to consider contamination from fat cells as an explanation of our results. We have found that mouse depot fat phospholipid contains 7–8% sphingomyelin[2] compared with 3% in normal mouse muscle (7) and also that the phospholipid from a microsomal fraction obtained from mouse depot fat by the same procedure which was used to prepare muscle microsomes contains 14% sphingomyelin.[2] However, the lecithin content (44%)[2] of the fat cell microsomal material is about the same as that of dystrophic microsomes. This means that if the difference in lecithin content between normal and dystrophic microsomes were due to fat cell material, then, allowing for the increased amount of mitochondrial lipid, about 75% of the phospholipid in the dystrophic preparation would have to be derived from this source. In this case the expected value for sphingomyelin would exceed 10% compared with the 8% actually observed. Consequently, we consider it unlikely that the presence of fat cell phospholipid can explain our results. This conclusion is supported to some extent by results of the fatty acid analyses.

It, therefore, seems that the increased sphingomyelin and cholesterol and reduced lecithin plus phosphatidalcholine of dystrophic mouse muscle do not result from changes in all cell components which contain large amounts of phospholipids, but only in those which are concentrated in the microsomal fraction. However, the increase in plasmalogen in whole muscle,[1] may result in part from a concomitant alteration in the mitochondria.

To attempt a detailed interpretation of the phospholipid differences between normal and dystrophic whole muscle and between our subcellular fractions would be premature. Nevertheless, because the oxalate-stimulated calcium uptake of muscle microsomes is dependent on phospholipids (31), there may be a connection between our observations and those of Sreter and Gergeley (32),

___
[2] Hughes, B. P. Unpublished results.

who found that the calcium uptake of dystrophic mouse muscle microsomes was diminished.

There are only small differences between the fatty acid composition of the neutral lipid from normal and dystrophic whole muscle (24) and from subcellular fractions (Table 6). Since our neutral lipid fractions also contained the free fatty acids, it is probable that the composition of dystrophic mouse muscle free fatty acid does not differ greatly from normal.

It is interesting that there are considerable differences in the contents of 18:0 and 18:2 of the neutral lipid associated with the various fractions (Table 6). Conceivably, the neutral lipid is a constituent part of the subcellular structures or that the latter show some degree of selectivity in binding material derived from a common pool. The fatty acid and aldehyde analysis confirms the conclusion that fatty infiltration is unlikely to be the cause of the observed phospholipid changes. Thus, although a considerable replacement of normal muscle fibers with fat cells would make the content of 16:0, 18:0, 18:1, and 22:6 approach that found in the dystrophic muscle, the high 18:2 and 20:4 content of adipose tissue phospholipid (Table 5) should produce a considerable increase of these two acids in dystrophic muscle, whereas the observed differences were not significant. In addition, the highly significant increase in dystrophic whole muscle aldehyde (Table 4) could not have been caused by fat cells which have a lower aldehyde content (Table 5).

Changes in the phospholipid fatty acid composition of whole muscle (Table 4) are reflected by corresponding changes in the subcellular fractions. However, in contrast to the phospholipid where, with the exception of the increase in mitochondrial phosphatidalethanolamine, the changes are only in the microsomal fraction, there are significant differences between the phospholipid fatty acids of the normal and dystrophic mitochondrial fractions.

The increase in total fatty aldehyde content of dystrophic mouse muscle (Table 4) was expected because the plasmalogen phosphorus content of dystrophic whole muscle was 2.1% higher than normal[1] and was equivalent to a difference in total fatty aldehyde of 1.2%. The estimates of increased whole muscle plasmalogen content from the mercuric chloride thin-layer chromatographic procedure (13) and from gas–liquid chromatography were thus in agreement.

Although the increase in total aldehyde of whole muscle (Table 4) appeared to be accompanied by a relative increase in the proportion of stearaldehyde to palmitaldehyde, this was not confirmed by the results obtained with subcellular preparations.

Alterations in the degree of cross-contamination of muscle subcellular fractions may account for some of the differences between the fatty acids of normal and dys-trophic fractions, for example, in the case of the myofibrillar fractions. However, this is unlikely to be the sole explanation of the difference between either the normal and dystrophic mitochondria or the normal and dystrophic microsomes. Thus there is no significant difference in phospholipid composition between the normal and dystrophic mitochondrial fractions (Table 6) as would be expected if the fatty acid changes seen here were due to an altered degree of cross-contamination. The decrease in content of 22:6 of the dystrophic microsomal fraction cannot be explained by contamination with mitochondrial material which has a higher content of 22:6. Furthermore, there is no significant change in the content of 18:0 of the mitochondria whereas the content of 18:0 is markedly increased in the dystrophic microsomal fraction.

Although the altered fatty acid composition of the dystrophic microsomal fraction could be due to its changed phospholipid pattern, this explanation does not apply to the mitochondrial fatty acids. In view of the reduced level of 22:6 in the dystrophic samples, it is not clear why the proportion of 20:4 is unchanged. Increased peroxidase activity would be expected to have a more general effect on the level of polyunsaturated fatty acids. Moreover Baker, Bloom, and Blahd (33) failed to find any increase in peroxidase level in dystrophic mouse muscle homogenates; this observation was substantiated in the course of the present work.[3]

Differences have been reported in the ability of dystrophic mouse muscle (34) and denervated rat diaphragm (35) to synthesize and oxidize fatty acids. In the case of the dystrophic mouse, it is possible that the greatly increased free fatty acid content of the muscle (6) exercises complex selective effects on the synthesis of the $n-3$ and $n-6$ series of polyunsaturated fatty acids analogous to the effects of fatty acids on chain elongation in rat liver microsomes (36).

REFERENCES

1. Pearce, G. W., and J. N. Walton. 1963. *J. Pathol. Bacteriol.* **86:** 25.
2. West, W. T., and E. D. Murphy. 1960. *Anat. Rec.* **137:** 279.
3. Kandutsch, A. A., and A. E. Russell. 1958. *Amer. J. Physiol.* **194:** 553.

[3] Owens, K. Unpublished results.

4. McCaman, M. W. 1963. *Amer. J. Physiol.* **205:** 897.

5. Young, H. L., W. Young, and I. S. Edelmann. 1959. *Amer. J. Physiol.* **197:** 487.

6. Susheela, A. K., P. Hudgson, and J. N. Walton. 1968. *J. Neurol. Sci.* **7:** 437.

7. Hughes, B. P. 1965. *In* Research in Muscular Dystrophy. Pitman Medical Publishing Co. Ltd., London, England. 187–194.

8. Coleman, D. L., and W. T. West. 1961. *J. Nutr.* **73:** 273.

9. Pennington, R. J. 1961. *Biochem. J.* **80:** 649.

10. Lilienthal, J. L., Jr., K. L. Zierler, B. P. Folk, R. Buka, and M. J. Riley. 1950. *J. Biol. Chem.* **182:** 501.

11. McIlwain, H., and R. B. Rodnight. 1962. Practical Neuro-chemistry. Churchill (J. & A.) Ltd., London, England. 64.

12. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. *J. Biol. Chem.* **226:** 497.

13. Owens, K. 1966. *Biochem. J.* **100:** 354.

14. McArdle, B., and K. J. Zilkha. 1962. *Brain.* **85:** 389.

15. Zlatkis, A., B. Zak, and A. J. Boyle. 1953. *J. Lab. Clin. Med.* **41:** 486.

16. Rhodes, D. N. 1959. *Biochem. J.* **71:** 26p.

17. Zeitman, B. B. 1965. *J. Lipid Res.* **6:** 578.

18. Morrison, W. R., and L. M. Smith. 1964. *J. Lipid Res.* **5:** 600.

19. James, A. T., A. J. P. Martin, and G. H. Smith. 1952. *Biochem. J.* **52:** 238.

20. Weinstock, I. M., S. Epstein, and A. T. Milhorat. 1958. *Proc. Soc. Exp. Biol. Med.* **99:** 272.

21. Imai, K., T. Omura, and R. Sato. 1966. *J. Biochem. (Tokyo).* **60:** 274.

22. Owens, K. 1969. Abstracts of the 7th Meeting of the Federation of European Biochemical Societies. Madrid, Spain. 1148.

23. Baker, N., M. Tubis, and W. H. Blahd. 1958. *Amer. J. Physiol.* **193:** 525.

24. Owens, K. 1968. *In* Research in Muscular Dystrophy. Pitman Medical Publishing Co. Ltd., London, England. 363–371.

25. Getz, G. S., W. Bartley, D. Lurie, and B. M. Notton. 1968. *Biochim. Biophys. Acta.* **152:** 325.

26. Tsao, S. S., and W. E. Cornatzer. 1967. *Lipids.* **2:** 41.

27. Wheeldon, L. W., Z. Schumert, and D. A. Turner. 1955. *J. Lipid Res.* **6:** 481.

28. Martonosi, A. 1964. *Fed. Proc.* **23:** 913.

29. Drabikowski, W., H. Dominas, and M. Dabrowska. 1966. *Acta Biochim. Pol.* **13:** 11.

30. McArdle, B. 1965. *In* Research in Muscular Dystrophy. Pitman Medical Publishing Co. Ltd., London, England. 196.

31. Martonosi, A. 1963. *Biochem. Biophys. Res. Commun.* **13:** 273.

32. Sreter, F. A., and J. Gergely. 1964. *Biochem. Biophys. Res. Commun.* **16:** 438.

33. Baker, N., A. Bloom, and W. H. Blahd. 1959. *Amer. J. Physiol.* **197:** 483.

34. Lin, C. H., A. J. Hudson, and K. P. Strickland. 1969. *Life Sci.* **8:** 21.

35. Kouvelas, E. D., and K. L. Manchester. 1968. *Biochim. Biophys. Acta.* **164:** 132.

36. Christiansen, K., Y. Marcel, M. V. Gan, H. Mohrhauer, and R. T. Holman. 1968. *J. Biol. Chem.* **243:** 2969.