# Studies on the Lipid Composition of the Fragmented Sarcoplasmic Reticulum of Normal and Dystrophic Chickens[1] (35978)

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Sreter *et al.* (1) discovered that the $Ca^{2+}$ transport function of FSR[3] was impaired in dystrophic animals. Previous work in this laboratory has shown that the $Ca^{2+}$ accumulating ability of the FSR obtained from the breast muscle of dystrophic chickens was more sensitive to phospholipase C digestion than that of the normal controls (2). Our results suggested that perhaps the lipid composition of the FSR is altered in these dystrophic animals. For this reason a detailed analytical study was undertaken on the lipid composition of the FSR of normal and dystrophic chickens.

*Materials and Methods.* All reagents used were of analytical grade. Chickens inflicted with genetically controlled muscular dystrophy were generously supplied free of charge by the Department of Avian Sciences of the University of California, Davies, CA. The FSR was prepared by the method of Martonosi and Feretos (3). The total lipid concentration of the FSR was determined gravimetrically from aliquots of a chloroform–methanol extract (2:1, v:v). The choline-containing phospholipids were separated from the rest of the phospholipids on a silicic acid column by differential elution technique using chloroform–methanol mixtures (from 4:1, v:v to 1:9, v:v). Both choline-poor and choline-rich fractions were subjected to thin-layer chromatography (4). The individual phospholipids were identified against synthetic standards in two solvent systems (chloroform:methanol: water, 93:35:4; and also chloroform:acetic acid:water, 50:50:4), after exposure to iodine vapor. Recovery was 100%. The neutral fat was eluted from the silicic acid column with chloroform and its weight was determined gravimetrically. The total cholesterol concentration of the neutral fat fraction was determined by the method of Zlatkis *et al.* (5). The triglyceride fraction was saponified with 10% KOH in alcohol, and the cholesterol and glycerin were removed with methylene chloride. The acidified (pH 2) residue was again extracted with methylene chloride and the dried residue (fatty acids) was dissolved into bis(trimethylsilyl) trifluoro acetamid containing 12.5% chlorosilane. The individual fatty acids were identified against commercially available highly purified standards with a Gowell Model 320 gas chromatograph (equipped with $H_2$ flame ionization detector) on Gas-Chrom-Q column, in a temperature range of 180–290° using $N_2$ gas as the carrier.

The film pressures of the monomolecular films prepared from the phospholipids of normal and dystrophic FSR, and also from mixtures of these phospholipids with commercially available cholesterol, were measured in a Langmuir trough (Cenco Co., IL) essentially as described by Davies and Rideal (6).

The $Ca^{2+}$ uptake of FSR was measured by the method of Martonosi and Feretos (3) and the partial extraction of cholesterol from FSR was performed by the method of Inesi *et al.* (7).

The ATPase determinations were carried out at 25° in the presence of 0.05 *M* Tris buffer (pH 7.5), 0.005 *M* $Mg^{2+}$ and 0.005 *M* ATP. To measure the "extra ATPase"

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[3] Abbreviations used: FSR = fragmented sarcoplasmic reticulum; ATPase = adenosine triphosphatase; μmoles of $P_i$/mg of prot/min = micromoles of inorganic phosphate liberated per mg of sarcoplasmic protein per minute.

TABLE I. Lipid Composition of Normal and Dystrophic FSR.

| Lipid[a] | Normal | | Dystrophic | |
|---|---|---|---|---|
| | Lipid ($\mu$g/mg of prot) | SD | Lipid ($\mu$g/mg of prot) | SD |
| Cholesterol | 61.00 | ± 8.0 | 164.00 | ±12.00 |
| Triglycerides | 60.80 | ± 5.0 | 126.60 | ± 9.0 |
| Phospholipids (total) | 408.35 | ±38.0 | 406.59 | ±40.0 |
| Sphingomyelin | 37.57 | ± 6.38 | 89.25 | ±15.20 |
| Phosphatidylcholine + lysophosphatidylcholine | 216.55 | ±36.86 | 137.55 | ±23.39 |
| Phosphatidylinositol | 24.18 | ± 4.12 | 23.60 | ± 4.03 |
| Phosphatidylserine | 16.80 | ± 2.87 | 36.83 | ± 6.25 |
| Phosphatidylethanolamine + lysophosphatidylethanolamine | 86.94 | ±14.80 | 96.64 | ±16.46 |
| Cardiolipin | 26.31 | ± 4.46 | 22.72 | ± 3.86 |
| Total phospholipids — cardiolipin | 382.04 | — | 383.87 | — |

[a] The values are averages of 5 experiments.

activity, 0.001 *M* $Ca^{2+}$ was also incorporated into the mixtures. The incubation time was 5 min and the inorganic phosphate liberated was determined by the method of Fiske and Subbarow (8).

*Results.* Table I shows that the lipid composition of normal and dystrophic FSR was significantly different. The dystrophic FSR contained 2.6 times more cholesterol and 2.0 times more triglycerides than the FSR of the normal controls. The cholesterol content of the FSR obtained from the breast muscle of normal chickens was 61 $\mu$g/mg of protein. Fiehn and Hasselbach (9) found about 30 $\mu$g of cholesterol/mg of protein in the FSR of the skeletal muscle obtained from normal rabbits. The total phospholipid concentration was similar in the normal and dystrophic FSR. On the other hand, the composition of phospholipids in the normal FSR was different from that of the dystrophic animals. The FSR of the dystrophic animals contained sphingomyelin and phosphatidylserine in a higher concentration and the phosphatidylcholine + lysophosphatidylcholine in a lower concentration than the FSR of the normal controls. Our results regarding the phospholipid composition of the FSR obtained from the breast muscle of normal chickens compare favorably with those of Martonosi (10), who studied the phospholipid composition of the FSR obtained from the skeletal muscle of rats.

Table II shows the similar fatty acid composition of triglycerides obtained from normal and dystrophic FSR fractions.

It has been shown by several workers (7, 9, 11) that the extraction of cholesterol by diethyl ether treatment may alter the $Ca^{2+}$ activated "extra ATPase" activity and the $Ca^{2+}$ accumulating ability of the FSR. Since in our experiments a significant difference was found in the cholesterol content of the

TABLE II. The Fatty Acid Composition of the Triglycerides Isolated from the FSR Fraction of Normal and Dystrophic Chickens.

| | Normal (% of total) | Dystrophic (% of total) |
|---|---|---|
| Capric acid[a] | 2.22 | 2.27 |
| Lauric acid | 2.59 | 2.40 |
| Myristic acid | 2.02 | 1.34 |
| Palmitic acid | 22.36 | 26.51 |
| Stearic acid | 37.97 | 39.13 |
| Arachidic acid | 17.33 | 16.42 |
| Behemic acid | 4.23 | 2.49 |
| Oleic acid | 2.41 | 1.55 |
| Eicosenoic acid | 1.77 | 1.36 |
| Arachidonic acid | 1.63 | 1.80 |
| Eruic acid | 1.81 | 1.16 |
| Nervonic acid | 3.66 | 3.57 |
| Total | 100.00 | 100.00 |

[a] The average error of these determinations was about ± 10% of the values given.

TABLE III. Effects of Diethyl Ether Treatment on the Cholesterol Content, ATPase Activity, and $Ca^{2+}$ Uptake of the Normal and Dystrophic FSR.

| Conc of ethyl ether (%) | FSR cholesterol extracted (% of total) | | ATPase ($\mu$moles of $P_i$/mg of prot/min) | | | | $Ca^{2+}$ uptake[a] (% of maximal) | |
|---|---|---|---|---|---|---|---|---|
| | | | $Mg^{2+}$ activated | | $Mg^{2+}$ + $Ca^{2+}$ activated | | | |
| | Norm. | Dystr. | Norm. | Dystr. | Norm. | Dystr. | Norm. | Dystr. |
| None | 0 | 0 | 0.72 | 1.25 | 1.02 | 1.27 | 100 | 20 |
| 2.5 | — | — | 0.75 | 1.22 | 1.28 | 1.10 | — | — |
| 5.0 | 8 | 7 | 0.78 | 1.10 | 1.36 | 1.10 | 25 | 10 |
| 7.0 | 9 | 10 | 1.20 | 1.00 | 1.32 | 1.08 | 20 | 5 |
| 10.0 | 10 | 11 | 1.20 | 0.89 | 1.31 | 1.01 | — | — |

[a] 100% $Ca^{2+}$ uptake = 1.8 $\mu$moles of $Ca^{2+}$/mg of sarcoplasmic reticular protein.

normal and dystrophic FSR, we have studied the effects of cholesterol removal on the enzymatic activity and $Ca^2$ accumulating ability of both normal and dystrophic FSR. The results of these experiments are compiled in Table III. As shown, the $Mg^{2+}$ activated ATPase activity of the dystrophic FSR was 43% higher than that of the normal control. On the other hand, the normal FSR increased the $Mg^{2+}$ activated ATPase activity by 30% in the presence of $Ca^{2+}$ (extra ATPase) while the dystrophic FSR showed no such "extra ATPase." Ether treatment increased both the $Mg^{2+}$ activated and the extra ATPase activity of the normal FSR; but it inhibited, rather than accelerated, the activity of the ATPase enzyme in the case of the dystrophic FSR.

We have assumed that the average molecular weight of the mixed phospholipids obtained from the FSR of normal or dystrophic chickens was 810. Using this value and some of the data of Table I, we have calculated that the molar ratio of phospholipid to cholesterol was 2.66 in the case of the normal FSR ($0.9 \times 10^{17}$ molecules of cholesterol/mg of protein; and $2.4 \times 10^{17}$ molecules of phospholipid/mg of protein). On the other hand, in the dystrophic FSR, the phospholipid cholesterol ratio was changed to 1.0 ($2.4 \times 10^{17}$ molecules cholesterol/mg of protein; and $2.4 \times 10^{17}$ molecules of phospholipid/mg of protein).

Demel *et al.* (12) found that the incorporation of cholesterol into phospholipid monolayers may or may not exert a condensing effect on the packing of the phospholipid molecules in the mixed cholesterol–phospholipid films. The nature of phospholipid cholesterol interaction depended upon the fatty acid constituents of the phospholipids (12). The fact that the incorporation of various amounts of cholesterol into phospholipid membranes profoundly modifies the permeability characteristics of these films appears to be well established (13).

For these reasons, we have studied the film pressures of the purified phospholipid fractions of normal and dystrophic FSR in the presence and absence of cholesterol. The results of these experiments are depicted in Fig. 1. As shown, the pressure area curves of the purified normal and dystrophic phospholipids were very similar. The addition of

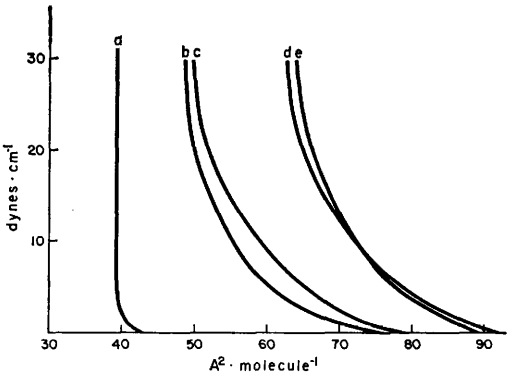


FIG. 1. Film pressure as a function of the film area: (a) 30 $\mu$g of cholesterol; (b) 31.4 $\mu$g of normal FSR phospholipid + 10.0 $\mu$g of cholesterol; (c) 32.3 $\mu$g of dystrophic FSR phospholipid + 8.0 $\mu$g of cholesterol; (d) 31.4 $\mu$g of normal FSR phospholipid; and (e) 32.3 $\mu$g of dystrophic FSR phospholipid.

cholesterol had similar effect on the pressure area curves of both normal and dystrophic phospholipids. From the experiments shown in Fig. 1, we have calculated the mean molecular area for cholesterol and for the phospholipids at 5 dyne/cm film pressure in the cholesterol monolayer (curve a), in the normal FSR phospholipid monolayer (curve e), in the dystrophic FSR phospholipid monolayer (curve d), and in the mixed cholesterol–phospholipid monolayers (curves b and c). The total area available for the films at 5 dyn/cm film pressure was always equal with 38 $\times$ number of cholesterol molecules in the film + 72 $\times$ number of phospholipid molecules in the film (38 $Å^2$ is the area occupied by one molecule of cholesterol and 72 $Å^2$ is the average area occupied by one molecule of phospholipid at the specified pressure). Thus cholesterol had no condensing effect on the phospholipids of normal or dystrophic FSR.

*Discussion.* Several interesting differences were found in the lipid composition of FSR obtained from the breast muscles of normal and dystrophic chickens. The importance of sarcoplasmic reticular protein (or proteins) and phospholipids in the $Ca^{2+}$ transport process appears to be well documented (10, 14). The poor $Ca^{2+}$ uptake of the dystrophic FSR may be explained on the basis of several membrane alterations. Compared to the normal control, the dystrophic FSR contained an increased amount of lipid per milligram of membrane protein (Table I). Excluding the cardiolipin and the triglycerides, the dystrophic FSR contained 4.8 $\times$ $10^{17}$ lipid molecules/mg of membrane protein while the normal FSR contained only 3.3 $\times$ $10^{17}$ lipid molecules/mg of membrane protein. If all lipid molecules were included into the FSR membrane, our results may suggest that the distance was increased between the $Ca^{2+}$ carrier proteins in the dystrophic FSR. Thus, per unit of surface area, the number of $Ca^{2+}$ carriers was reduced in the dystrophic membrane. An alternative explanation would be that the increased lipid content formed a coat on the vesicles of the dystrophic FSR. In this case the thickness of the membrane increased but there need not be any change in the lateral distance between $Ca^{2+}$ carrier proteins. The same reasoning would apply if the extra lipid were inside the vesicles. The decreased phospholipid–cholesterol ratio and/or the altered phospholipid composition of the dystrophic FSR changed the physical properties of the membrane. It is quite possible that these physical and chemical changes in the dystrophic FSR (Table I, Fig. 1) may have brought about significant alterations in the enzymatic and carrier properties of the membrane proteins. Such alterations may have caused the severe curtailment of the extra ATPase activity and $Ca^{2+}$-accumulating ability of the dystrophic FSR (Table III). These enzymatic alterations of the dystrophic FSR may be explained on a different basis. It is possible that the increased amount of lipid in the FSR isolated from dystrophic muscle carried with it more $Ca^{2+}$ throughout the isolation. Such an increase in the endogenous $Ca^{2+}$ content may account for the fully activated ATPase of the dystrophic muscle FSR in the absence of any added $Ca^{2+}$. It would also be consistent with a decreased (apparent) $Ca^{2+}$ uptake (Table III).

It has been shown that a high proportion of cholesterol in membranes may favor the entry of fat (15). Although we did not consider the triglycerides as constituents of the normal or dystrophic FSR, one may not exclude the possibility that the high cholesterol content of the membrane in the case of the dystrophic FSR permitted the penetration of triglycerides into the vesicles. Partial removal of cholesterol destroyed the $Ca^{2+}$ uptake of both normal and dystrophic FSR, perhaps by increasing the leakiness of the membranes (11).

*Summary.* The increased cholesterol content and altered phospholipid composition of the dystrophic FSR seems to support the proposition that membrane alterations may be instrumental in the development of muscular dystrophy [Hsu, Q.-S., and Kaldor, Proc Soc. Exp. Biol. Med. **131**, 1398 (1969); Martonosi, A., Proc. Soc. Exp. Biol. Med. **127**, 824 (1968)].

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