Lipids of the Serum and Connective Tissue of the Rat and Rabbit

By Nancy L. Noble, Ph.D. and Robert J. Boucek, M.S., M.D.

The lipid profile of connective tissue, isolated from the rat and the rabbit, by the sponge-implantation technique, is presented. This tissue is rich in cholesterol, phospholipid, and neutral fat. Several sex, age, and species differences in the concentration of its lipids are reported. Simultaneous determination of serum lipids indicates that there is no significant correlation between the lipid values of the serum and connective tissue. The relationship of these findings in connective tissue, the chief constituent of the intima, to the problem of atherosclerosis is discussed.

The intima of the arterial system, a connective tissue structure, increases in thickness with age and is the site of fatty atheromatous changes. It is not known whether these phenomena are related in the early stages since pathologists who have studied tissue removed at this phase of thickening of the intimal coat of the arterial tree failed to report stainable lipids. Atherosclerotic intimal tissue is available for analysis, and search has been made for changes in the "ground substance" of this connective tissue. However, until recently, connective tissue equivalent to that in the normal or early abnormal state of the intima has not been available in sufficient quantity to permit chemical analysis. The development of the sponge-implantation technic has made possible the isolation of connective tissue from the rat and the rabbit. This tissue has been found to be rich in lipids, and the types and relative concentrations of its lipid fractions form the basis of this report.

Questions have been justly raised as to the existence of correlation between the concentration of lipids in the serum and in the tissues. The filtration or imbibition concept of atherosclerosis has been developed because of the similarity in the chemical composition of the lipids of the serum and the aorta in rabbits fed cholesterol. However, it is quickly recognized that experimentally induced atherosclerosis in animals is at best a crude technic for simulating such clinical conditions as xanthomatous disease, biliary cirrhosis, or nephrosis. In these pathological states characterized by tremendous increases in the lipids of the serum, the imbibition concept of atherosclerosis may be well founded; however, the majority of patients with atherosclerosis do not fall into this category. The previously described method for the procurement of connective tissue and the subsequent analysis of its lipids affords a unique departure in the study of atherosclerosis from the classical studies of serum lipids.

It is the purpose of this paper to report analyses for the various lipid constituents of serum and connective tissue of the rat and the rabbit, the lack of correlation between, the effect of age and sex upon, and certain species differences of these values.

Methods

Connective tissue was obtained from male and female Sprague-Dawley rats and from male and female albino rabbits by the previously described connective tissue-sponge biopsy technic. The rats and rabbits were fed ad libitum stock diets of Purina Laboratory Chow and Rockland Rabbit Ration respectively. Two polyvinyl sponges were implanted in the majority of the rats studied and ten sponges were placed in each of the rabbits. The tissue-sponge specimens were removed after different time intervals of implantation (14 to 140 days).

After removal of the connective tissue-sponge specimen, it was immediately frozen and weighed. The weight of fresh tissue was obtained by sub-

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TABLE 1.—Tissue Lipids (Per Cent Dry Weight)

<table>
<thead>
<tr>
<th>Description</th>
<th>Total Fatty Acids (A)</th>
<th>Ester Fatty Acids (B)</th>
<th>B/A</th>
<th>Total Cholesterol (A)</th>
<th>Ester Cholesterol (B)</th>
<th>Choline-Containing Phospholipid (A)</th>
<th>Ester Phospholipid (B)</th>
<th>Total Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Heart</td>
<td>0.83</td>
<td>0.53</td>
<td>0.77</td>
<td>7.67 (8)</td>
<td>6.93 (8)</td>
<td>4.58</td>
<td>3.20</td>
<td>19.20</td>
</tr>
<tr>
<td>Rat Liver</td>
<td>11.38</td>
<td>3.06</td>
<td>1.52</td>
<td>7.42</td>
<td>9.12 (17)</td>
<td>4.58</td>
<td>3.20</td>
<td>19.20</td>
</tr>
<tr>
<td>Rabbit Heart</td>
<td>10.02</td>
<td>1.77</td>
<td>0.98</td>
<td>5.22</td>
<td>7.14 (18)</td>
<td>4.58</td>
<td>3.20</td>
<td>19.20</td>
</tr>
</tbody>
</table>
| Rabbit Connective Tis-

tracting the weight of the dry sponge (weighed prior to implantation) from the weight of the tissuesponge specimen. The frozen tissue-sponge block was placed in a very cold metal mortar with a tight-fitting pestle and the tissue-sponge crushed by hard blows with a steel hammer on the pestle. The crushed specimen was then cut into small pieces with a cold blade and repounded. It was found that a homogeneous mixture of tissue and sponge was obtained after alternately crushing and quartering the specimen three times. The homogeneity of the mixture was demonstrated by the agreement of determinations of both phospholipids and cholesterol on duplicate aliquots from the same crushed specimen.

The crushed tissue-sponge sample was extracted with hot 3:1 ethanol-diethyl ether and aliquots of the extract were taken for determinations of total and ester cholesterol, total fatty acids, and total choline-containing phospholipids. Cholesterol, phosphorus, and fatty acid analyses were made by methods previously described. The total phospholipid concentration was calculated on the basis of lipid phosphorus concentration in a methanol extract of the dried alcohol-ether aliquot. The choline-containing phospholipids were separated in the methanol extract from the non-choline-containing phospholipids by the adsorption method of Taurog and associates, and the concentration of choline-containing phospholipids was calculated from the phosphorus concentration in the eluate. The non-choline-containing phospholipid concentration was obtained by the difference between total phospholipid and choline-containing phospholipid concentrations.

The total lipid value in table 1 is a sum of the concentrations of total phospholipid, total cholesterol, fatty acid of ester cholesterol, and neutral fat.

The polyvinyl sponge is chemically inert, and blank determinations on portions of the sponge demonstrated it to be free of any substances which would interfere with lipid analyses.

Blood was obtained from the heart of the rat and from the ear of the rabbit. Total and ester cholesterol of the serum were determined by the direct chloroform extraction method. Analyses for total phospholipid, choline-containing, and non-choline-containing phospholipid were made on an ethanol-diethyl ether extract of the serum in a manner similar to that described above.

The lipid values for the tissues are expressed as grams per 100 grams of fresh tissue (tissue-sponge weight minus dry sponge weight) or as per cent of dry weight in tables 1 and 3. Since the connective tissue obtained by the implantation technique contains 90 per cent water, dry weight of the tissue was calculated on this basis. Lipid values in the serum are expressed as grams per 100 ml. of serum.

RESULTS

Connective tissue of the rat and rabbit is rich in lipids, cholesterol, phospholipid, and neutral fat. Table 1 summarizes the various lipid values of several tissues of the rat and rabbit studied in our laboratory and of other tissues from these species and the beef reported in the literature. The mean values for the lipids of the connective tissue of the rat and rabbit given in the table were obtained by averaging all the results for each species, both male and female. The concentration of neutral fat, Phospholipid fatty acid = 0.65 total phospholipid, Neutral fat fatty acid = total fatty acid - (phospholipid fatty acid + ester cholesterol fatty acid), Neutral fat = 1.053 neutral fat fatty acid.
**Table 2—Lipids of Connective Tissue (Per Cent Dry Weight)**

<table>
<thead>
<tr>
<th>Animal, Sponge Age</th>
<th>Total Fatty Acids</th>
<th>Phospholipids</th>
<th>Cholesterol</th>
<th>Neutral Fat</th>
<th>Total Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Choline-Containing</td>
<td>Cephalin</td>
<td>Total</td>
<td>Ester</td>
</tr>
<tr>
<td>Male Rat 14-140 days</td>
<td>9.59 ±0.53*</td>
<td>4.50 ±0.29</td>
<td>3.18 ±0.20</td>
<td>7.78 ±0.19</td>
<td>1.29 ±0.24</td>
</tr>
<tr>
<td>Female Rat 14-140 days</td>
<td>13.18 ±1.20</td>
<td>4.67 ±0.20</td>
<td>2.13 ±0.22</td>
<td>7.74 ±0.24</td>
<td>1.76 ±0.33</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td>&lt;0.70</td>
<td>&gt;0.90</td>
<td>0.90</td>
<td>&lt;0.50</td>
</tr>
<tr>
<td>Male Rabbit 14-153 days</td>
<td>18.21 ±6.00</td>
<td>2.85 ±0.28</td>
<td>3.10 ±0.20</td>
<td>6.01 ±0.19</td>
<td>1.20 ±0.25</td>
</tr>
<tr>
<td>Female Rabbit 14-119 days</td>
<td>19.25 ±10.38</td>
<td>2.48 ±0.32</td>
<td>2.40 ±0.24</td>
<td>4.88 ±0.84</td>
<td>1.42 ±0.25</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td>&lt;0.70</td>
<td>&lt;0.70</td>
<td>&lt;0.70</td>
<td>&lt;0.70</td>
</tr>
</tbody>
</table>

* Standard error of mean.
† Number of animals.
‡ Probability of a chance occurrence of the difference from the control.

The mean total fatty acid value of 11.38 per cent of the dry weight of the connective tissue of the rat is higher than that noted in the liver of that species. The total cholesterol concentration in connective tissue is striking. Mean values of 3.08 Gm. per cent in rat connective tissue and 1.77 Gm. per cent in rabbit tissue are higher than any other reported tissue, with the exception of beef brain and lungs. On the other hand, concentration of cholesterol esters of connective tissue of the rat and rabbit, 1.52 Gm. per cent and 1.31 Gm. per cent respectively, is higher than any other reported tissue. It should be noted that the concentrations of phospholipid in the connective tissue of the rat are of the same order as the values for the heart and liver and are much lower than the levels reported for beef liver and brain. Connective tissue of the rabbit has a significantly lower total phospholipid concentration than the connective tissue of the rat. This species difference is due to the smaller concentration of choline-containing phospholipid in the connective tissue of the rabbit since the cephalin concentrations in the connective tissue of the two animals are essentially the same.

The concentrations of total lipids in connective tissue of the rat and of the rabbit are similar, and when compared with other tissues, this value is exceeded only in beef brain. In table 2, the lipid profile of connective tissue is compared in male and female rats and in male and female rabbits. The connective tissue of the female rat has a significantly higher concentration of total lipids than the connective tissue of the male rat. It was found that this larger total lipid value in the female rat is due to significantly higher concentrations of neutral fat (p < 0.05) and of total cholesterol (p < 0.05). On the other hand, there are no significant differences between the various lipid fractions of the connective tissue of the male and female rabbit.

* The probability (p) of a chance occurrence of the difference from the control was taken from Fisher's table of t (Fisher, R. A., Statistical Methods for Research Workers, ed. 11. London, Oliver and Boyd, 1960).
Several species differences are apparent (table 3). There is a significantly higher concentration of neutral fat in the male rabbit connective tissue than in the male rat. Furthermore, the male rabbit connective tissue has a significantly lower concentration of total phospholipid \((p < 0.02)\) than the tissue of the male rat, and this is due to a smaller concentration of choline-containing phospholipid in the tissue of the male rabbit \((p < 0.01)\) than in the male rat tissue. It appears that these lipid differences also exist between the female rat and female rabbit. The concentrations of total phospholipid \((p < 0.001)\) and of choline-containing phospholipid \((p < 0.01)\) are significantly less in the rabbit.

The results of the determinations carried out on specimens of sera are given in table 3, and the values are of the same order as those reported in the literature. The serum of the female rat contains significantly greater concentrations of cholesterol, both total \((p < 0.02)\) and esterified \((p < 0.02)\) and of total phospholipid \((p < 0.001)\) and of choline-containing phospholipid \((p < 0.01)\) than the serum of the male rat. The only significant differences between the sera of the male and female rabbit are found in the phospholipid fractions. The female rabbit has greater concentration of total phospholipid \((p < 0.001)\), of choline-containing phospholipid \((p < 0.02)\), and of cephalin \((p < 0.01)\) in the serum than does the male rabbit. In addition to these sex differences in serum lipids, there are species differences. The serum of the male rat contains significantly higher concentrations of total \((p < 0.02)\) and ester \((p < 0.001)\) cholesterol, of total \((p < 0.001)\) and non-choline-containing \((p < 0.05)\) phospholipid than does the male rabbit serum. However, no significant differences in the lipids of the serum were found in the two female species.

An attempt was made to correlate the lipid values of the serum with lipid values of

### Table 3.—Lipids of Serum and Connective Tissue (Gm.%)

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Serum</th>
<th>Connective Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male Rat</td>
<td>Male Rabbit</td>
</tr>
<tr>
<td>Cholesterol Ester</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.048</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>±0.003</td>
<td>±0.005</td>
</tr>
<tr>
<td>Total</td>
<td>0.045</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>±0.003</td>
<td>±0.006</td>
</tr>
<tr>
<td>Phospholipid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline-Containing</td>
<td>0.063</td>
<td>0.054</td>
</tr>
<tr>
<td>Cephalin</td>
<td>±0.005</td>
<td>±0.001</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>±0.005</td>
<td>±0.021</td>
</tr>
<tr>
<td></td>
<td>(15)</td>
<td>(4)</td>
</tr>
<tr>
<td>Total</td>
<td>0.129</td>
<td>0.074</td>
</tr>
<tr>
<td></td>
<td>±0.008</td>
<td>±0.004</td>
</tr>
<tr>
<td>Neutral Fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>±0.006</td>
<td>±0.054</td>
</tr>
<tr>
<td></td>
<td>(21)</td>
<td>(4)</td>
</tr>
<tr>
<td>Total Lipids</td>
<td>1.566</td>
<td>2.337</td>
</tr>
<tr>
<td></td>
<td>(20)</td>
<td>(7)</td>
</tr>
</tbody>
</table>

* Probability of a chance occurrence of the difference from the control.
† Standard error of the mean.
‡ Number of animals.
LIPIDS OF SERUM AND CONNECTIVE TISSUE

Correlation of the age of connective tissue with the concentration of lipids of the tissue showed significant changes in several lipid fractions with age. In the female rat, there is a highly significant increase in total lipids of the connective tissue as the tissue ages from 14 to 140 days \((r = 0.75, p < 0.001)\), and this increase is due to significant increments in concentrations of neutral fat \((r = 0.64, p < 0.01)\) and total cholesterol \((r = 0.68, p < 0.05)\). Similar changes in the concentration of these lipids with the age of the tissue were found in the connective tissue of the male rat. The concentration of total lipids is positively and significantly correlated with tissue age \((r = 0.75, p < 0.001)\), and again this rise in total lipids can be accounted for by increases in concentrations of total cholesterol \((r = 0.77, p < 0.001)\) and of neutral fat \((r = 0.66, p < 0.01)\).

In both the male and the female rat, the significant increase in total cholesterol is due to an increase in the esterified fraction. Free cholesterol concentration does not change significantly with the age of the connective tissue in the male \((r = 0.25, p < 0.50)\) or female \((r = 0.43, p < 0.10)\) rat, but ester cholesterol concentration rises very significantly \((r = 0.82, p < 0.001)\). Total phospholipid concentration does not change with the age of connective tissue in either the female rat \((r = 0.30, p < 0.30)\) or the male rat \((r = 0.44, p < 0.10)\). However, the concentration of cephalin in the connective tissue increases significantly with the age of the tissue in the female \((r = 0.44, p < 0.05)\) and male \((r = 0.71, p < 0.001)\) rat but no significant change occurs in the concentration of choline-containing phospholipids.

The male rabbit data revealed a total lipid concentration increase with age \((r = 0.87, p < 0.02)\), and this rise is due solely to an increase in neutral fat.

**DISCUSSION**

Comparison of the concentrations of lipids in the connective tissue of the albino rat and white rabbit with the concentrations in other tissues indicates that connective tissue is a very lipid-rich structure. The reported lipid values for connective tissue (table 1), which were averaged for a species regardless of the age of the tissue or the sex of the animal, might be even greater than other tissues if comparison were made on a similar sex and age basis since there are both significant increases in concentrations of neutral fat and cholesterol with the age of the tissue and significant sex differences.

Earlier work on the lipids of the heart and liver of the albino rat indicates that several lipid constituents of these tissues increase in concentration as the animal ages. Thus, the changes with age in the neutral fat and cholesterol concentrations of connective tissue may not be unique to this structure.

Because of this high concentration of lipids in connective tissue, which is the chief constituent of the intima of the vascular wall, and because of the implication of serum lipids in atherosclerosis, it was of interest to ascertain the degree of correlation between lipid values of the serum and of connective tissue. Correlative studies showed that there is no significant relationship between the concentrations of total phospholipid and total cholesterol in the serum and in the connective tissue. The total cholesterol concentration in the tissue of the rat increases significantly with the age of the tissue, and yet the concentration in the serum does not change. On the other hand, neither the total phospholipid concentration of the serum nor of the serum changes significantly. Furthermore, there appears to be no consistent relationship between the observed differences in concentrations of total cholesterol and total phospholipid in the serum and the tissue of the two species. For example, the concentrations of total cholesterol and cephalin in the serum of the male rat are significantly greater than in the serum of the male rabbit, yet there is no
significant difference in tissue total cholesterol or total phospholipid concentration of the two species. The concentrations of choline-containing phospholipid in the sera of the male rat and rabbit are not different, but the connective tissue of the male rat has a significantly greater concentration of these phospholipids than the tissue of the male rabbit.

This lack of correlation between serum and connective tissue lipid values in the normal rat and rabbit suggests that synthesis and storage of phospholipid, cholesterol, and neutral fat must occur in the latter tissue in order to account for the large quantities of these tissue lipids in comparison with serum values. It would seem that such large amounts of lipids in the connective tissue can not result from imbibition or filtration of serum lipids alone. Most tissues are able to synthesize cholesterol, and *in vitro* synthesis of cholesterol from acetate in the aorta, primarily in the intima, has been demonstrated. Synthesis of phospholipid is also widespread in the animal organism.

The significant increase in the concentration of neutral fat in connective tissue with time, in both the male and the female rat, is associated with a significant increase in cephalin concentration, while total phospholipid does not rise significantly. Thus, the concentration of the choline-containing phospholipids, lecithin and sphingomyelin, remains relatively unchanged or even becomes slightly less with the age of the tissue, and the ratio of lecithin-sphingomyelin to cephalin is decreased. This ratio decreases significantly in the connective tissue of the female rat with age of the tissue \( r = -0.40, p < 0.05 \), but there is no significant change in the male rat.

This direct relationship between the concentrations of neutral fat and cephalin, or in other words, the inverse relationship between concentrations of neutral fat and choline-containing phospholipids, has been noted in the intima and in other tissues under different experimental conditions. On choline-deficient diets, rats are unable to synthesize normal amounts of the phospholipids containing choline and lipid deposits occur in the intima of the aorta and coronary arteries. Such preparations exhibit an increase in non-phospholipid fatty acids in the liver, i.e., neutral fat, and this concentration of neutral fat is decreased and lecithin is increased following feeding of choline supplements.

The significance of this relationship between choline-containing phospholipids and neutral fat of connective tissue in the problem of atherosclerosis can not now be realized. However, it is interesting to note that the connective tissue of the male rabbit has a significantly lower concentration of choline-containing phospholipids and a significantly greater concentration of neutral fat than the male rat. This observation parallels the reported relationship between susceptibility of certain species to atherosclerosis and their phospholipid metabolism. For example, the rat, relatively immune to atherosclerosis, has been shown to possess a greater ability to form lecithin from free ethanolamine than the chick or guinea pig, atherogenic species.

In the problem of atherosclerosis, the ratio of cholesterol to phospholipid in the serum is thought by some to be more significant than the absolute values of these two lipids. As the connective tissue ages, total cholesterol and ester cholesterol increase in concentration, but total phospholipid concentration does not change significantly. The ratio of cholesterol to phospholipid in connective tissue increases significantly with age in the female rat \( r = 0.56, p < 0.01 \) and in the male rat \( r = 0.49, p < 0.01 \).

**Summary**

Connective tissue of the albino rat and rabbit is rich in the lipids, cholesterol, phospholipid, and neutral fat. Its high concentrations of cholesterol and cholesterol ester are outstanding for animal tissue.

Species and sex differences in the concentrations of lipids in connective tissue are noted. The concentrations of neutral fat and cholesterol increase significantly with the age of the tissue.

There is no significant correlation between lipid values in the serum and in connective tissue of the rat or the rabbit.

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