Studies on the compartmentation of lipid in adipose cells. II. Cholesterol accumulation and distribution in adipose tissue components

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Abstract
Adipose tissue was shown to contain 0.6-1.6 mg of cholesterol per gram wet weight. When expressed per unit of protein or organ mass, fat tissue contains more cholesterol than most other organs or membranes. The cholesterol content of fat tissue increased with the age and weight of the rat. Over 95% of adipose tissue sterols was cholesterol, and most of it was free. In young (150-165 g) rats two-thirds of fat tissue cholesterol was in collagenase-derived adipocytes while in older rats (450-480 g) 90% of fat tissue cholesterol was in adipocytes and the remainder was in stromal-vascular elements. Age-related differences in subcellular cholesterol distribution were also observed. The cholesterol/phospholipid molar ratios of purified plasma membrane fractions from small and large fat cells were identical (0.22-0.25), thus resembling muscle and liver membranes. 7.5 days after intravenous administration of [4-14C]cholesterol the specific activity of adipose tissue cholesterol exceeded that of plasma cholesterol. At 28 days the specific activity of adipose and muscle cholesterol exceeded that of plasma three- to fivefold. The t_{1/2} disappearance of adipose tissue cholesterol was approximately 27 days, which is consistent with its function as a slowly turning over storage pool.

Thus, fat tissue is a major cholesterol storage organ. This may well account for the marked expansion of the slowly exchangeable cholesterol pool (pool B) observed in obesity.

Supplementary key words cholesterol metabolism - cholesterol turnover - obesity - plasma membrane - membrane composition - membrane cholesterol - cholesterol storage

There is considerable indirect evidence suggesting that adipose tissue is important in cholesterol storage in the mammalian species. After intravenous injection of 4C-labeled cholesterol to terminal patients and analysis of the specific activity of adipose tissue cholesterol at necropsy, equilibration with plasma cholesterol occurred within a 3-4-wk interval; this indicates dynamic turnover and exchange of adipose tissue cholesterol with that of the circulating pool (1). After the intravenous infusion in rats of chylomicrons labeled with 14C-labeled cholesteryl esters, up to 3% of the infused isotope was taken up by adipose tissue within the first 60 min; by 24 hr this increased to 5% of the administered dose, suggesting net accumulation of cholesterol in adipose tissue (2-4). On analysis of plasma cholesterol turnover curves after intravenous administration of 14C-labeled cholesterol, Nestel, Whyte, and Goodman (5) observed that the disappearance curve of radioactive cholesterol conformed to a two-pool model (6) consisting of a rapidly exchangeable pool (pool A) and a slowly exchangeable pool (pool B). They also demonstrated that the size of the slowly exchangeable pool correlated positively with body weight and particularly with excess body weight. It has been further demonstrated that endogenous cholesterol production in obesity is increased (5, 7), perhaps due to cholesterol synthesis in the enlarged adipose organ (5, 8).

These studies implied but did not prove that adipose tissue is in some way involved with storage, exchange, and/or synthesis of cholesterol. For this reason a study was initiated to determine by direct chemical analysis the amount, subcellular location, and chemical character of adipose tissue cholesterol. Results of preliminary studies (9, 10) are confirmed in this report and show that adipose tissue is a major cholesterol storage organ.
MATERIALS AND METHODS

Animals, diet, and preparation of adipose tissue fractions

Male Wistar rats raised on Purina chow were used throughout the study. The animals had free access to food and tap water up to the time of killing. The total lipid content of the chow was examined on two occasions and found to be 4.3 and 5.4 g/100 g dry weight. Analysis of the nonsaponifiable fraction revealed a total ferric chloride-sulfuric acid-reactive sterol content of 60 and 77 mg/100 g dry weight, respectively. Of this material, 82.2%, as measured chemically, cochromatographed with [4-14C]cholesterol on neutral lipid TLC. As determined by GLC, 36% of the feed sterols was cholesterol, 15% was campesterol, 49% was β-sitosterol, and there was a trace amount of a high molecular weight sterol.

The animals were stunned with a sharp blow to the head and killed by cervical dislocation. Epididymal fat pads were excised and weighed, taking care to remove all tissue distal to the major vessel just cephalad to the epididymis. Isolated fat cells were prepared by collagenase digestion of epididymal fat as described by Rodbell (11), with minor modifications (12, 13). After separation of free cells from the stromal–vascular sediment and shreds of undigested tissue, the isolated adipocytes were washed three times with 0.25 M sucrose to remove extraneous proteins.

Mitochondrial and microsomal fractions were obtained by preparative ultracentrifugation of cell homogenates as previously described (12, 13). Adipose cell ghosts were prepared by treatment of isolated cells with hypotonic buffer as described by Rodbell (14). The yield was approximately 0.3–0.6 ml of packed ghosts starting from 22–24 g of adipose tissue.

Mitochondrial and microsomal fractions were obtained from the adipose cell ghosts by an adaptation of Neville's procedure for preparation of rat liver cell membranes (9, 15). In brief, the ghosts were made up to a volume of 1.0 ml with 0.001 M NaHCO₃ and homogenized in a 2-ml Penbrook homogenizer with three strokes. The homogenate was then added to a 50-ml graduated cylinder containing 28.2 ml of 69% sucrose. The volume was made up to 50 ml with double-distilled water and the cylinder was inverted 20 times to ensure mixing. 8.1-ml portions of the solution were distributed into each of six cellulose nitrate tubes, and the solutions were then overlaid with 4.1 ml of 42.3% sucrose. The tubes were centrifuged at 90,000 g for 2 hr in a Spinco model L2-65B ultracentrifuge, using an SW-40 titanium rotor. After centrifugation, the floating layers were removed with a spatula and pooled in a centrifuge tube containing 8.0 ml of 0.001 M NaHCO₃. The final volume of 12.5 ml was made up with 0.001 M NaHCO₃, and the mixture was centrifuged for 10 min at 25,000 g. The supernate was decanted and the packed sediment was dispersed with 2 ml of 0.001 M NaHCO₃ delivered through a syringe fitted with a 22-gauge needle. 5 ml of a sucrose gradient (37% to 3%) was placed over a 4-ml cushion of 50% sucrose, and the 2-ml dispersion was overlaid. This gradient was centrifuged at 550 g for 1 hr, the tube was sliced just above the 50% sucrose cushion, and the opaque layer was taken as the membrane-rich fraction. The preparation of ghosts and the isolation of plasma membrane fraction were carried out at 0–4°C.

Preparation of human isolated fat cells

Isolated fat cells were prepared from subcutaneous adipose tissue of two women, aged 41 and 46, and five men, aged 27–61, undergoing either open heart surgery for valve replacement or aortocoronary bypass surgery. 5–7 g of subcutaneous fat was removed from the lower end of the midternal incision (i.e., upper midabdominal fat) and incubated with collagenase as described above. None of the patients was obese, diabetic, hypothyroid, hypertensive, or on hypolipidemic drugs.

Calculation of total body adipose cholesterol

To make this calculation it was assumed that adipose tissue represents 7.08% of body mass (16). The validity of this number for total dissectable fat was confirmed in the following study. Excision of total body adipose tissue by blunt dissection of four rats weighing 208 g, 221 g, 480 g, and 520 g revealed adipose masses of 7.6%, 6.4%, 7.0%, and 8.1% of body weight, respectively. These values must be regarded as minimal numbers since adipose tissue embedded in muscle and invisible to the naked eye could not be included in the dissectable mass.

Chemical analysis of lipids

Intact tissue fragments were homogenized in 10 ml of chloroform–methanol 2:1 using a Potter-Elvehjem homogenizer; isolated cells were extracted with 10 ml of chloroform–methanol 2:1 by mixing with a Vortex agitator. The extract was filtered through prewashed Whatman no. 1 filter paper, which was then rinsed twice with chloroform–methanol 2:1. The filter paper was saved for subsequent analysis of the cellular or tissue protein content.

The chloroform–methanol extracts were washed once with 0.74% KCl and three times with pure solvents upper phase containing 0.74% KCl (17). Total adipocytes or adipose tissue lipid was measured gravimetrically in tared vessels. The total lipid content of adipose cell ghosts and the purified plasma membrane fraction was

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determined by the oxidimetric method of Bragdon (18), which is based on the reduction of $\text{K}_2\text{Cr}_2\text{O}_7$. The sensitivity of this method was increased fivefold by reducing the final volume to 5 ml thus permitting accurate measurement of 50 $\mu$g of lipid. To measure total cholesterol, portions of lipid extracts were saponified as described by Abell et al. (19) except that saponification was carried out at 58–60°C for 120 min rather than at 37–40°C for 55 min. Nonsaponifiable lipids were extracted into petroleum ether (bp 30–60°C), and total cholesterol was measured by the colorimetric procedure of Chiamori and Henry (20), using a ferric chloride–acetic acid–sulfuric acid reagent. By reducing the final volume to 2 ml, accurate measurement of 5–10 $\mu$g of cholesterol was achieved.

Cholesteryl esters were measured by dissolving a portion of the dried lipid extract (approximately 20 mg) in 2.5 ml of 95% ethanol–ether 3:1 to which 0.5 ml of 0.5% digitonin in 95% ethanol was added (20). After standing for 20 min at room temperature, the solvents were evaporated with a stream of nitrogen and the residue was dispersed in 5 ml of petroleum ether and brought to a boil by immersion in a 60°C water bath. The mixture was allowed to stand 10–15 min at room temperature to allow the precipitate to settle; the supernatant solution, which contained cholesteryl ester, was removed. The residue was extracted a second time with petroleum ether, and the supernatant solutions were combined, evaporated, and analyzed for cholesterol as described above. Recovery of [4-$^{14}$C]cholesterol added to the nonsaponifiable fraction of adipose lipid and carried through digitonide precipitation procedure and pyridine extraction was 78.9 ± 0.4% (mean ± SEM, n = 4).

In the presence of large amounts of glyceride (e.g., lipid extracts of adipose tissue or cells), precipitation of free cholesterol by digitonin was variable and incomplete, resulting in spuriously high values for esterified cholesterol. Therefore, the cholesteryl ester content of adipose tissue and isolated cells was determined by the following indirect approach. Free cholesterol content was measured by analyzing an eluate of the cholesterol–diglyceride spot after TLC of a portion of the lipid extract (21) (see below). Recovery of [4-$^{14}$C]cholesterol (added to the original total lipid extract) from thin-layer plates was 86–97%. The total cholesterol content was determined by chemical assay of a separately prepared nonsaponifiable fraction of the lipid extract. The difference between these two measurements was taken to represent the cholesteryl ester content.

Phospholipids were measured by determining inorganic phosphorus after digestion of total lipids with sulfuric acid and 30% hydrogen peroxide according to the method of Bartlett (22); the value for inorganic phosphorus was multiplied by 25. Recovery of 2,3-dihexadecyl-1-glycerophosphorylcholine (equivalent to 2 $\mu$g of phospholipid P) added to a lipid extract of adipose tissue containing 4.8 $\mu$g of phospholipid P was 88.5 ± 0.7% (mean ± SEM, n = 3).

Neutral lipid separation was accomplished on thin-layer plates coated with silica gel H as described previously (21). The cholesterol–diglyceride area was vacuumed into a semimicrocolumn plugged at one end with a small wad of prewashed glass wool, and the lipids were eluted with 8 ml of chloroform. The total sterols were then separated by argentation TLC according to the procedure of Subbiah (23). Briefly, silica gel H was dispersed in 12% silver nitrate at a ratio of 1:2.3 and the plates were activated for 60 min at 110°C. The plates were developed by ascending chromatography for approximately 40 min, using chloroform–acetone 98:2 as a solvent mixture. The plates were sprayed with a solution of dichlorofluorescein (0.2% in ethanol) and the spots were visualized under ultraviolet light and marked off. Cholesterol, cholestanol, and lanosterol were separated, and each of the corresponding areas was vacuumed into a semimicrocolumn and eluted with 8–12 ml of chloroform. It was not possible to count directly scappings from the silver nitrate plates because of interfering chemiluminescence.

Recovery of radioactivity when labeled cholesterol was carried through these purifications was 88.2 ± 2.1% (mean ± SD, n = 3), and recovery of mass (50–100 $\mu$g) measured simultaneously was 88.5 ± 1.7% (mean ± SD, n = 3). Chemical analysis of cholesterol obtained by argentation TLC of nonsaponifiable lipids of adipose tissue (corrected for losses as indicated by recovery of added [4-$^{14}$C]cholesterol) indicated that more than 90% of adipose sterol (i.e., ferric chloride–sulfuric acid-reactive material in the NSF) was indeed cholesterol. Recovery of [4-$^{14}$C]cholesterol added to epididymal adipose tissue at the time of lipid extraction and carried through saponification, neutral lipid TLC, and argentation TLC was 73 ± 5.8% (mean ± SD, n = 6). Corrections for these losses were made in the final calculation.

**Protein and DNA measurements**

Tissue and isolated cell proteins were determined by digestion of residues trapped on the filter paper after filtration of the lipid extracts. The filter papers together with the protein residues were cut into shreds and digested in 0.5 N NaOH for 60 min at 80°C in a shaking water bath. The slurry was transferred to a centrifuge tube and spun to sediment cellulose fragments; an aliquot of the supernatant solution was assayed for protein by the procedure of Lowry et al. (24), using bovine serum albumin as a standard. DNA was measured in the res-
idue obtained after adipose tissue or isolated cells were extracted with acetone and ether; the procedure of Burton (25) was used.

**Determination of cholesterol in stromal–vascular fractions of adipose tissue**

In the adipocyte isolation procedure (11, 12), varying amounts of partly digested tissue fragments remain and must be taken into account in the calculation of cholesterol distribution between cells and stromal–vascular fractions. An estimation of the cholesterol associated with the stromal–vascular fraction of these tissue remnants was achieved by measuring total cholesterol content of the partially digested tissue fragments and subtracting that amount of cholesterol associated with fat cells in the tissue fragments.

In these experiments (Table 3) the distribution of cholesterol between fat cells and stromal elements was determined as follows. After collagenase digestion, the partly digested fragments were removed, extracted with chloroform–methanol 2:1, and analyzed for cholesterol and total lipid content. The remaining material was centrifuged at 300 g for 15 sec, and three layers appeared: (a) floating fat cells, (b) an interphase of Krebs-Ringer bicarbonate–albumin buffer containing stromal and vascular elements, and (c) a sediment of dense stromal and vascular elements (pellet I). The isolated cells were analyzed for cholesterol and total lipid; the interphase was recentrifuged at 700 g for 12 min and the sediment (pellet II) was combined with pellet I and extracted with chloroform–methanol for determination of cholesterol and total lipid.

The cholesterol associated with the fat cells and the stromal–vascular component of partly digested tissue fragments was calculated using the following equations.

1. Cholesterol in fat cells of partly digested tissue fragments = cholesterol content of isolated fat cells (mg/g cell lipid) × total lipid in tissue fragments

2. Cholesterol content of the stromal–vascular component of the tissue fragments = total cholesterol in tissue fragments – cholesterol associated with the fat cells in the tissue fragments (from equation 1)

The cholesterol associated with the stromal–vascular elements of the whole tissue was calculated by combining that found in pellets I and II with that calculated to be in the stromal elements of the partially digested fragments (equation 2).

GLC of adipose tissue sterols was kindly performed by Dr. A. Kuksis, Best Institute, University of Toronto, and carried out on an F & M model 402 biomedical gas chromatograph (Hewlett-Packard, Avondale, Pa.). Approximately 2 μg of mixed silyl ethers in 1 μl of reaction mixture was injected. The column (180 cm X 2 min i.d., glass U tube) was packed with 3% EGSS-X on Gas-Chrom Q (100–120 mesh); the carrier gas was helium, 40 ml/min. The temperatures were: on column injector, 230°C; detector, 260°C; and column, 230°C (isothermal).

Residues of lipid extracts, column eluates, or silica gel scrapings were counted in 15 ml of Bray's scintillation mixture (26). Radioactivity was measured in a Beckman LS-250 liquid scintillation spectrometer. Counting efficiency was 64%. [4-14C]Cholesterol was purchased from New England Nuclear Corp., Boston, Mass. (sp act 19.3 μCi/μmole) and was purified twice by TLC before use. The solvents, inorganic materials, and unlabeled substrates were reagent grade. Solutions were made up with double-distilled water (glass).

**RESULTS**

**Chemical form of adipose tissue cholesterol**

Almost all the sterol in adipose tissue was cholesterol, as shown by gas–liquid chromatographic analysis of a nonsaponifiable fraction prepared from adipose tissue of 200-g rats (Fig. 1). GLC analysis of sterols from adipose tissue of 490–570-g rats was qualitatively identical with the profile shown in Fig. 1 but differed in that cholesterol made up a greater proportion (over 97%) of the total sterol mass in the older animals. This confirms and validates the methods used for purification of adipose cholesterol (see Materials and Methods). A small amount of lanosterol and two unidentified compounds were present, but these represented less than 5% of the total...
sterol mass. Measurement of the distribution between free and esterified forms revealed that more than 75% of adipose tissue and adipocyte cholesterol was free and that membrane-associated sterols were mainly free. Over 90% of human adipocyte cholesterol was in the free form (Table 1).

**Cholesterol content of adipose tissue, kidney, muscle, and liver**

The cholesterol content of white adipose tissue was compared with that of kidney, muscle, and liver. It is apparent that the concentration of cholesterol in adipose tissue per milligram of protein exceeded that of all tissues studied in both 200-g (7.5 wk) and 600-g (36 wk) rats (Fig. 2). It is also evident that, with age, adipose tissue cholesterol concentration trebled, whereas levels in kidney, liver, and muscle remained constant (Fig. 2). This indicates a selective and preferential accumulation of cholesterol in the adipose bed. To determine the size and distribution of cholesterol within each tissue pool, the results were expressed in terms of organ mass (Fig. 3). It is evident that if the size of an organ increases with age its cholesterol content would also increase. This was indeed the case for adipose tissue, liver, and muscle, which increased in mass as the animals grew. However, the degree of increase in adipose tissue cholesterol was proportionally greater than that of liver and muscle (Fig. 3). Kidney mass did not increase during the interval studied.

**Lipid composition of intact adipose tissue and isolated cells**

Since accumulation of cholesterol in adipose tissue was relatively rapid, it was important to determine whether this was a function of the lipid-storing adipocyte or of other tissue constituents such as capillary endothelial cells, fibroblasts, red blood cells, etc. Accordingly, experiments were carried out to compare the cholesterol content of isolated fat cells with that of intact pads in relation to age (Tables 2a and 2b). The expected increase in glyceride content of adipose tissue and isolated cells with respect to age is evident (Table 2a, column A, and Table 2b, column F). The cholesterol content in intact adipose tissue expressed per gram wet weight or per gram of lipid (Table 2a, columns B and C) and isolated cells expressed in terms of cell lipid (Table 2b, column G) also increased with age and weight, indicating that cholesterol accumulation in fat tissue and isolated adipocytes is not simply a function of the mass of stored triglyceride. It is also noted that the phospholipid content of both adipose tissue and isolated cells decreased with age (Table 2a, column D, and Table 2b, column H), a finding consistent with and explained by expansion of the glyceride storage pool rather than a reduction in membranous structural elements. Finally, it is apparent that the cholesterol/phospholipid molar ratio in intact tissue as well as isolated cells increased (Table 2a, column E, and Table 2b, column I), suggesting that cholesterol accumulation in adipocytes was not strictly a function of membrane mass.
TABLE 1. Chemical form of adipose cell cholesterol

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Origin</th>
<th>% Free Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated cells</td>
<td>400-g rats</td>
<td>76.4 ± 7.5*</td>
</tr>
<tr>
<td>Isolated cells</td>
<td>100-g rats</td>
<td>75.6±</td>
</tr>
<tr>
<td>Adipocyte ghosts</td>
<td>200-g rats</td>
<td>90.8±</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>200-g rats</td>
<td>88-100±</td>
</tr>
<tr>
<td>Human isolated</td>
<td>Subcutaneous tissue</td>
<td>93 ± 8.5*</td>
</tr>
</tbody>
</table>

* Mean ± SD (n = 6).
† Average of two analyses from pooled cells obtained from 12 rats.
‡ Ghosts pooled from 24 rats.
§ Three membrane preparations; no esters were found in two of the preparations.

**Proportion of adipose tissue cholesterol in fat cells**

A comparison of the cholesterol/lipid ratios in intact tissue and in isolated cells suggests that the cholesterol content of isolated fat cells was significantly less than that of intact tissue only at 6 wk of age (Table 2a, experiment 1, vs. Table 2b, experiment 4) and not in the other two groups. However, in Table 2a, experiment 3 (last three animals), the right epididymal fat pads were analyzed in duplicate and the left pads were combined and subjected to collagenase digestion for isolation of intact cells (corresponds to Table 2b, experiment 6c). Here the cholesterol/lipid ratio in isolated cells (1.3 mg/g lipid) was lower than that of tissue (1.4–2.0 mg/g lipid).

If it is assumed that all the glyceride in fat tissue is in fat-storing adipocytes, the data suggest that over 85% of the cholesterol in fat tissue is in adipocytes. This reasoning was confirmed by an independent analysis of the distribution of cholesterol in tissue fractions after collagenase digestion of epididymal fat. The results of the latter study (Table 3) show that the proportion of total tissue cholesterol associated with the fat cell is high and varies with age. In the younger animals approximately 65% of tissue cholesterol was in isolated fat cells, and in older rats (450–480 g) this proportion increased to 90%.

**Subcellular distribution of cholesterol**

To determine where cholesterol accumulated within the fat cell, subcellular analyses were carried out. Initially, the partition of cholesterol between the lipid stores (floating oil fraction) and the aqueous infranate after brief centrifugation of fat cell homogenates was measured (Table 4). It is evident that in 160-g rats (7 wk old) about 50% of cholesterol was associated with the lipid stores and that with increasing age (and hence fat cell size) the proportion of total cell cholesterol in the oil phase increased to 87% in cells obtained from animals weighing 570–700 g. These results suggest but do not prove that cholesterol accumulation in fat tissue occurs primarily in the lipid storage vacuoles of adipocytes.

**Cholesterol content of organelles, ghosts, and purified plasma membranes**

Further direct evidence to support the conclusion that most of the adipocyte cholesterol is in the lipid droplet was obtained from studies on the cholesterol/phospholipid molar ratios of various organelle fractions (Tables 5–7). In Table 5 the cholesterol/phospholipid molar ratios of the bulk lipids, mitochondria, microsomes, and plasma membrane fractions are compared with the ratio for the total cell. It is apparent that the cholesterol/phospholipid ratios of membrane elements are small compared with that of the whole cell. This can be explained by the preferential localization of cholesterol in the storage pool (Table 4) and of phospholipid in organelle fractions; this is consistent with the high cholesterol/phospholipid molar ratio of the bulk lipid phase (Table 5).

In Table 6 the lipid composition of adipose cell ghosts adds further credence to the view that a significant portion of adipocyte cholesterol is not associated with membrane fragments but is localized in the lipid storage droplet. The cholesterol/phospholipid molar ratio of adipose cell ghosts is approximately one-half that of in-
fractions were prepared from 200-g and 550-g animals. Ratios of adipose cell ghosts were similar to those observed in plasma membranes of animals of corresponding size (Table 7). Accordingly, purified plasma membrane content of adipocyte membrane was related to the age of animals of the same size (Table 7).

It is apparent that no differences could be found with respect to lipid content, cholesterol/protein ratio, phospholipid content, or cholesterol/phospholipid molar ratios. This leads to the conclusion that adipocyte growth did not significantly alter adipocyte membrane cholesterol content at a time when the stored cholesterol pool was expanding. This suggests that structural cholesterol and stored cholesterol represent functionally and anatomically distinct cholesterol pools. The similar cholesterol/ phospholipid molar ratios in plasma membranes of young and old animals, despite a sixfold increase in total cell cholesterol/phospholipid ratio, are significant in one other context. They indicate a nonrandom distribution of cell cholesterol during the homogenization and preparative isolation of various organelles and membrane fractions.

**Cholesterol uptake and turnover in adipose tissue**

The experiments in Figs. 4 and 5 were designed to determine whether cholesterol incorporation could be demonstrated in adipocytes incubated in vitro. While
TABLE 2b. Lipid composition of white adipose cells in relation to age

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Subgroup</th>
<th>Age of Rats</th>
<th>Weight Range</th>
<th>No. of Rats</th>
<th>Glyceride</th>
<th>Cholesterol</th>
<th>Phospholipid</th>
<th>Chol/Pho Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>a</td>
<td>6-7</td>
<td>91-105</td>
<td>8</td>
<td>86.8</td>
<td>0.78</td>
<td>5.1</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>110-150</td>
<td>5</td>
<td>111.4</td>
<td>0.71</td>
<td>4.9</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>121-154</td>
<td>8</td>
<td>105.9</td>
<td>0.67</td>
<td>3.4</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>102-122</td>
<td>10</td>
<td>96.3</td>
<td>0.57</td>
<td>3.3</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM (n = 4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>99.5±4.7</td>
<td>0.66±0.04</td>
<td>3.98±0.85</td>
<td>0.34±0.02</td>
</tr>
<tr>
<td>5</td>
<td>a</td>
<td>7-10</td>
<td>200-216</td>
<td>6</td>
<td>156</td>
<td>0.87</td>
<td>2.7</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>189-225</td>
<td>6</td>
<td>153</td>
<td>0.93</td>
<td>3.1</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>162-175</td>
<td>4</td>
<td>144</td>
<td>0.98</td>
<td>3.0</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>163-201</td>
<td>4</td>
<td>132</td>
<td>1.10</td>
<td>3.3</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM (n = 5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>139.4±6.2</td>
<td>0.91±0.04</td>
<td>3.0±0.15</td>
<td>0.60±0.02</td>
</tr>
<tr>
<td>6</td>
<td>a</td>
<td>26-36</td>
<td>580</td>
<td>1</td>
<td>566</td>
<td>1.45</td>
<td>1.1</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td>b</td>
<td>530</td>
<td>1</td>
<td>342</td>
<td>1.60</td>
<td>1.6</td>
<td>1.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>475-737</td>
<td>3</td>
<td>742</td>
<td>1.30</td>
<td>0.9</td>
<td>2.42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>450-480</td>
<td>3</td>
<td>406</td>
<td>1.30</td>
<td>2.5</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM (n = 4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>498 ± 77</td>
<td>1.41 ± 0.07</td>
<td>1.4 ± 0.3</td>
<td>2.14 ± 0.25</td>
</tr>
</tbody>
</table>

Isolated fat cells were prepared from rats of different ages as shown. Each subgroup represents a pool of cells obtained from both epididymal fat pads of 1-10 rats (except for experiment 6c, in which cells from the left pad only were obtained; see legend of Table 2a). Most analyses were done on duplicate aliquots of cells. A progressive rise in cholesterol content per gram lipid, and in the cholesterol/phospholipid molar ratio, in respect to rat age was observed. With respect to parameters F, G, H, and I, the results obtained in rats 7-10 wk old (expt. 5) were significantly different from those in the 6-7-wk-old rats (P < 0.05) and the 26-36-wk-old rats (P < 0.01).

It is evident that uptake of [4-14C]cholesterol was very rapid (Fig. 5) and linearly related to medium concentration (Fig. 4) and that glucose plus insulin had no apparent effect (see legend), these experiments do not differentiate between net uptake of cholesterol and isotope exchange.

TABLE 3. Cholesterol distribution in adipose tissue

<table>
<thead>
<tr>
<th>Rat Age and Weight</th>
<th>Tissue Fraction</th>
<th>Lipid stores</th>
<th>Organelles</th>
<th>% distribution of cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5 wk</td>
<td>155-165 g</td>
<td>50.6a</td>
<td>49.4</td>
<td>65.1* 69.7* 90.2 ± 2.2*</td>
</tr>
<tr>
<td>8 wk</td>
<td>195-220 g</td>
<td>58.1</td>
<td>41.9</td>
<td>69.7 90.2 ± 2.2</td>
</tr>
<tr>
<td>22 wk</td>
<td>450-480 g</td>
<td>84.4 ± 5.6*</td>
<td>78.5 ± 1.8*</td>
<td>90.2 ± 2.2 ± 2.2*</td>
</tr>
</tbody>
</table>

Measurement of stromal-vascular cholesterol is described in Materials and Methods. The proportion of total adipose tissue cholesterol associated with fat storing adipocytes increased with age of animal.

* Epididymal fat from six rats was pooled.

In an effort to demonstrate the dynamic character of adipose cholesterol in vivo, studies were undertaken in which the specific activity die-away curves of plasma and adipose tissue cholesterol were compared over a

TABLE 4. Subcellular distribution of cholesterol in isolated adipose cells

<table>
<thead>
<tr>
<th>Rat Age and Weight</th>
<th>Cell Fraction</th>
<th>Lipid stores</th>
<th>Organelles</th>
<th>% distribution of cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5 wk</td>
<td>155-165 g</td>
<td>50.6a</td>
<td>49.4</td>
<td>65.1* 69.7* 90.2 ± 2.2*</td>
</tr>
<tr>
<td>8 wk</td>
<td>195-220 g</td>
<td>58.1</td>
<td>41.9</td>
<td>69.7 90.2 ± 2.2</td>
</tr>
<tr>
<td>22 wk</td>
<td>450-480 g</td>
<td>84.4 ± 5.6*</td>
<td>78.5 ± 1.8*</td>
<td>90.2 ± 2.2 ± 2.2*</td>
</tr>
</tbody>
</table>

Isolated adipocytes were homogenized in 0.25 M sucrose and centrifuged at 300 g for 30 sec. The upper oil phase, which represents the bulk storage pool, and the lower aqueous phase containing membranous organelles, enzymes, and polar cytoplasmic constituents were separated and analyzed for cholesterol. The proportion of total adipocyte cholesterol associated with the lipid storage phase increased with age of the rat.

a Adipocytes from six rats were pooled.

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which together constitute a major fraction of the slowly results of all treatments were identical and the data were therefore equilibration of plasma and adipose tissue cholesterol separated by incubation, cells and medium were separated by centrifugation, and activities of both adipose tissue and muscle cholesterol, occurred after 7-8 days; and that: adipose tissue cholesterol is rapidly labeled within 24 hr of intravenous administration of \([4-\text{C}]\)cholesterol; \(b\) the crossover point indicating isotopic equilibration of plasma and adipose tissue cholesterol occurred after 7-8 days, and \(c\) within 4 wk the specific activities of both adipose tissue and muscle cholesterol, which together constitute a major fraction of the slowly exchangeable pool, exceeded the specific activity of serum cholesterol by factors of 3-5.

To determine the extent of cholesterol uptake by adipose tissue and muscle, the proportions of injected isotope found in adipose tissue and muscle in the experiment were calculated; the data are shown in Table 8. It is evident that, although adipose tissue contained 3.9% of the injected \(^{14}\text{C}\) within 24 hr, this declined to 0.85% at day 28, in contrast to muscle, which was found to contain 11.3% of the injected material at the end of the experiment.

**DISCUSSION**

The cholesterol content of rat adipose tissue in relation to the mass of stored glyceride is small, 0.6-1.6 mg/g wet weight of tissue. Similar levels have been observed in other studies on fat from rat (28, 29) and humans (30-33). When the results were expressed in terms of total organ mass (Fig. 3), adipose tissue contained at least one-half the amount of cholesterol found in muscle. It should be emphasized that calculation of total adipose organ activity is based on two assumptions: \(1\) epididymal fat is a representative tissue of the entire bed, and \(2\) the total visible and dissectable adipose tissue is a gross underestimation (perhaps by 50%) of total adipose organ mass. Thus, the values obtained for total adipose organ composition or activity (Fig. 3 and Table 8) probably underestimate the quantitative and functional significance of the findings. When the results were expressed in terms of active cellular mass, i.e., per milligram of protein rather than wet weight of tissue, it became apparent that fat tissue contains higher concentrations than muscle, liver, or kidney. Furthermore, with growth the cholesterol concentration in fat tissue per milligram of protein increased selectively compared with liver, kidney, and muscle, which remained relatively constant (Fig. 2). Additionally, the cholesterol/phospholipid molar ratio of whole adipose cells exceeded that of any
known tissue or biological membrane studied, including myelin and RBC ghosts (27, 34). If phosphatidic concentration in fat cells is proportional to the lipoprotein membrane content, the finding of cholesterol/phospholipid molar ratios of 2.4 for the whole cell and 0.25–0.35 for isolated membrane fractions can be taken as evidence that cholesterol accumulates in fat cells beyond that required for synthesis and turnover of structural elements and supports the conclusion that most of the adipocyte cholesterol is associated with the central oil droplet. Because growth of adipose tissue after birth till age 15 wk occurs by hyperplasia of fat cells and thereafter primarily by an increase in cell size (35), it was not surprising that the pattern of cholesterol accumulation in fat tissue would reflect the relative proportion of fat cells to stromal elements (RBCs, WBCs, mast cells, fibroblasts) (11) and that this proportion would be affected by animal age (Table 3). These age-related anatomical changes affected tissue compartmentation of cholesterol such that approximately 65% of the cholesterol was in fat cells of rats 6–8 wk old (150 g), and by age 16 wk (400 g) the proportion increased to 90%. Compartmentation of cholesterol at the subcellular level was also demonstrated. In cells of 150-g rats, one-half the cholesterol was associated with the storage droplet and the remainder was structural, in contrast to fat cells from mature rats where 87% of cell cholesterol was in the storage pool. Thus, adipose tissue cholesterol is distributed among a heterogeneous mixture of cellular and subcellular pools, and this distribution is affected by age. It is noteworthy that the cholesterol/phospholipid molar ratio of adipocyte lipids from mature animals far exceeded that of any mammalian membrane system (27, 34, 36). Since high cholesterol/phospholipid molar ratios in membranes are generally associated with structural stability and rigidity (37), it was important to determine whether the cholesterol content of fat cell membranes differed from other tissues or changed with age, as the difference might explain one or more well-known age-related changes in adipose tissue metabolism. These studies revealed that the cholesterol/phospholipid ratios of mitochondria, microsomes, and plasma membranes were not different from those of liver or muscle organelles (27) and that purified plasma membrane fractions showed no differences in cholesterol content, phospholipid content, or cholesterol/phospholipid molar ratios. It is therefore concluded that differences between young and old animals with respect to adipose tissue metabolism, e.g., insulin sensitivity (38), cannot be attributed to gross alterations in plasma membrane cholesterol content.

The present study shows that adipose tissue readily accumulates 14C-labeled cholesterol after it is injected.

### Table 5: Cholesterol/phospholipid molar ratio in adipose cell fractions

<table>
<thead>
<tr>
<th></th>
<th>Total cell</th>
<th>Bulk lipid</th>
<th>Mitochondria</th>
<th>Microsomes</th>
<th>Plasma membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molar ratio</td>
<td>2.35 ± 0.19</td>
<td>8.3 ± 1.6</td>
<td>0.22 ± 0.02</td>
<td>0.32 ± 0.05</td>
<td>0.22 ± 0.04</td>
</tr>
</tbody>
</table>

Isolated adipose cells were homogenized in 0.25 M sucrose and separated by preparative ultracentrifugation into organelle fractions. The cholesterol/phospholipid molar ratio for total cell exceeded that of corresponding organelles and purified plasma membranes.

* From epididymal fat of rats weighing 647 ± 64 g. Values are means ± SEM (n = 4).

† Data taken from Table 7.

### Table 6: Lipid composition of adipose cell ghosts

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Weight Range</th>
<th>No. of Rats</th>
<th>Glyceride</th>
<th>Cholesterol</th>
<th>Phospholipid</th>
<th>Chol/Phos Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td></td>
<td>(mg/mg protein)</td>
<td>(µmoles/mg protein)</td>
<td>(µmoles/mg protein)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>160–190</td>
<td>11</td>
<td>0.31</td>
<td>0.16</td>
<td>0.53</td>
<td>0.29</td>
</tr>
<tr>
<td>2</td>
<td>198–230</td>
<td>12</td>
<td>0.21</td>
<td>0.26</td>
<td>0.76</td>
<td>0.34</td>
</tr>
<tr>
<td>3</td>
<td>168–222</td>
<td>12</td>
<td>0.67</td>
<td>0.29</td>
<td>0.75</td>
<td>0.39</td>
</tr>
<tr>
<td>4</td>
<td>180–200</td>
<td>12</td>
<td>0.39</td>
<td>0.19</td>
<td>0.68</td>
<td>0.29</td>
</tr>
<tr>
<td>Mean</td>
<td>± SEM (n = 4)</td>
<td></td>
<td>0.40 ± 0.1</td>
<td>0.23 ± 0.03</td>
<td>0.68 ± 0.05</td>
<td>0.33 ± 0.05</td>
</tr>
</tbody>
</table>

Ghosts from rats 7–10 wk old were prepared by osmotic lysis of isolated adipose cells.

### Table 7: Lipid composition of plasma membrane preparation in relation to rat age

<table>
<thead>
<tr>
<th>Age Range</th>
<th>Total lipid (mg/mg protein)</th>
<th>Cholesterol (moles/mg protein)</th>
<th>Phospholipid (moles/mg protein)</th>
<th>Cholesterol/phospholipid molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5–10 wk</td>
<td>1.21 ± 0.06a</td>
<td>0.20 ± 0.03</td>
<td>0.86 ± 0.09</td>
<td>0.24 ± 0.01</td>
</tr>
<tr>
<td>26–36 wk</td>
<td>1.19 ± 0.09b</td>
<td>0.26 ± 0.07</td>
<td>1.16 ± 0.25</td>
<td>0.22 ± 0.04</td>
</tr>
</tbody>
</table>

Amounts of total lipid, cholesterol, and phospholipid of purified plasma membrane were not affected by age.

* Means ± SEM (n = 7).

† Means ± SEM (n = 3).
intravenously and that on a semilog plot of specific activity vs. time, loss of the label from fat tissue was almost linear over the next 27 days, with an apparent \( t_{1/2} \) of disappearance of 27 days. It should be pointed out that the relationship between adipose tissue and plasma cholesterol specific activities must be interpreted with caution because of the heterogeneous nature and number of structural and cellular pools that are analyzed together by whole tissue extraction. Nevertheless, the results are entirely consistent with those of Avigan, Steinberg, and Berman (39), who reported on cholesterol specific activities in a number of rat tissues other than adipose tissue after oral administration of [4-\(^{14}\)C]cholesterol. While equilibration of liver, red blood cells, and small intestine with circulating cholesterol was rapid (40), adipose tissue did not equilibrate with plasma until day 7.5 (Fig. 5). A lag of 7–8 days also occurred before muscle and kidney cholesterol specific activity equaled that of plasma (39). A similar finding has been reported in man (1), where isotopic equilibration between plasma and fat tissue took 30 days and resembled that of muscle rather than liver or intestines. These findings are entirely consistent with the suggestion that adipose tissue, like muscle, contains a large pool of cholesterol that exchanges slowly, hence conforming more closely to the kinetics of pool B rather than pool A (40).

It is worth noting that after day 7.5 the specific activity-time curves diverge, such that by day 28 the specific activities of adipose and muscle cholesterol exceeded the specific activity of plasma cholesterol by factors of 3–5 (Fig. 6). This finding has been previously observed in three baboons (41) examined approximately 10 wk after intravenous injection of \([^{14}\)C]cholesterol. The presence of high specific activity pools of cholesterol in adipose tissue and muscle implies compartmentation. Whether this segregation of isotopic sterol in adipose tissue is based in whole or in part on anatomical considerations at the cellular or subcellular level, or whether there is a stabilizing reservoir due to physical–chemical interactions with cellular glyceride, cannot be discerned from the data reported here. It is also worth pointing out that the accumulation of radiocholesterol in adipose tissue and muscle is probably not a function of route of administration because the cholesterol turnover calculated from a two-pool model was the same for rats injected with lipoprotein-bound or particulate cholesterol (42).

The finding of high specific activity cholesterol pools in adipose tissue and muscle is particularly relevant to a number of recent reports concerning the effects of hypolipidemic agents on the slope of plasma cholesterol specific activity–time curves. Horlick, Kudchodkar, and Sodhi (43) and Miettinen (44) have reported that chlorophenoxyisobutyric acid (Atromid S) or nicotinic acid causes a rise or a flattening in the plasma specific activity–time curve and they have attributed this effect to either inhibition of de novo synthesis of sterol or mobilization of one or more high specific activity storage pools. The data reported in this paper lend credence to the latter possibility since both adipose tissue and muscle, which together make up the bulk of the cholesterol storage pool, contained cholesterol with a specific activity many times that of plasma cholesterol.

It follows from the present study that correct interpretation of a number of clinical investigations has been hindered because the existence of a stable cholesterol storage pool in fat tissue was not appreciated. Its greatest impact will be on (a) the interpretation of size, distribution, and turnover of cholesterol pools in intact...
man, where adipose tissue normally represents 15–25% of total body mass, as well as in obesity, where the expansion of total exchangeable cholesterol is primarily a function of pool B (5), the slowly exchangeable pool of cholesterol, and on (b) the interpretation of studies on the hypocholesterolemic effect of polyunsaturated fat, which is presumed to cause a redistribution of plasma cholesterol into tissue storage sites (45). Finally, it has been observed that certain individuals on high cholesterol diets sequester cholesterol outside the plasma compartment. It is possible that fat tissue might serve as an important buffer mechanism by taking up cholesterol from the plasma during brief periods of increased dietary load. Recent work from our laboratory supports this view since it was shown that the cholesterol content of adipose tissue is affected by dietary cholesterol content (46).

In ending this discussion it is interesting to speculate about a number of possible mechanisms which may account for the accumulation of cholesterol in fat tissue. The simplest explanation would be one of lipid–lipid interaction in which adipose glyceride acts like a sink for cholesterol by virtue of hydrophobic interaction (47). Accumulation of vitamin D₃ in adipose tissue may be cited as an example of lipid–lipid interaction determining the distribution of lipid-soluble sterols (48). It is possible too that a mechanism such as this could account for the age-related increase in adipose cholesterol since cholesterol accumulation parallels glyceride accumulation to some extent. While lipid–lipid interaction may be a major determinant in directing net flow of cholesterol into and out of fat tissue, additional factors probably play a role. For example, the composition of stored glyceride, which is known to vary with animal age (49), and dietary lipid composition could affect the amount of cholesterol taken up by fat tissue from the plasma since the solubility of cholesterol in glyceride is related to degree of saturation of glyceride fatty acids (47). Other considerations, such as the size, surface area, and number of lipid droplets within the fat cell may also modulate the flow or storage capacity of cholesterol in the fat cells. It is also possible that cholesterol in the lipid storage droplet is there as an obligatory constituent of that phase, either to maintain the glyceride as a latent storage pool or to facilitate emulsification at the surface of the lipid droplet during the lipolytic process (50). Finally, the role of sterol-binding protein (51) in cholesterol accumulation in fat tissue should be mentioned as an additional suggestion. Since adipose tissue is able to synthesize cholesterol de novo (52, 53), it probably contains such a protein. Whether enough exists to bind sufficient cholesterol to account for a significant proportion of the large amount of cholesterol found in fat tissue remains to be studied.

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