Very small fat cells. II. Initial observations on basal and hormone-stimulated metabolism[1]

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Abstract Very small fat cells (<35 μm diameter) and normal large fat cells (>40 μm diameter) were isolated from adult Fischer 344 rat epididymal adipose depots. These very small fat cell preparations were free from normal, large fat cells (40–130 μm diameter) and stromal-vascular elements. Examination by electron microscopy and lipid analysis showed a similarity in overall organization and composition to normal, large fat cells. Incubations with [U-^{14}C]glucose showed that the very small fat cell preparations oxidized glucose in proportion to both cell number and time. These preparations also responded to insulin, increasing [U-^{14}C]glucose oxidation in a manner similar to normal large fat cell preparations (i.e., 2- to 4-fold increases in CO_2 production with insulin stimulation). The very small fat cells also incorporated radiolabeled glucose into lipids; but, unlike normal large fat cells, insulin failed to stimulate this process. Glycerol release from very small fat cells was stimulated by lipolytic hormones in a manner similar to these responses in co-isolated large fat cells. —**Francendese, A., and F. D. DeMartinis.** Very small fat cells. II. Initial observations on basal and hormone-stimulated metabolism. *J. Lipid Res.* 1985. **26:** 149–157.

Supplementary key words epididymal adipose depot • fat cell size • fat cell number • insulin • catecholamines • glucagon • Fischer 344 rat

We have previously described the occurrence of very small fat cells (VSFC) in collagenase digests of adult rat epididymal fat depots (1). These smaller fat cells bore an organizational resemblance to the larger, commonly described adipocyte (40–130 μm diameter). Subsequently, we showed that they presented as a discreet population of fat cells, 8–35 μm in diameter, producing a bimodal population distribution in several adipose depots of various mammalian species (2). Recent reports from other laboratories have confirmed these observations (3) in regard to their organizational appearance and their bimodal distribution in collagenase digests of epididymal adipose tissue.

The present report examines the metabolic activities of very small fat cell preparations and comparisons are made with co-isolated normal large fat cells in regard to basal and hormonally stimulated glucose metabolism and lipolysis, ultrastructure, and lipid content.

METHODOLOGY

Animals

Cesarean-originated barrier-maintained male rats of the Fischer 344 strain were obtained from the Charles River Breeding Laboratories approximately 2 weeks prior to being killed and were maintained as previously described (1). Animals were killed in the fed state between 10 AM and 11 AM by decapitation. For metabolic studies on lipolysis, animals 12 months of age or 350–400 g body weight were used. For studies on the metabolism of glucose in isolated cell preparations, animals 6 months of age or 250–325 g body weight were used.

Preparation of various cell fractions for metabolic studies

Heterogeneous isolated fat cell preparations from epididymal tissue were obtained by using a collagenase technique as previously described (1). The digests (containing undigested material, large fat cells, very small fat cells, and stromal-vascular elements)[3] were filtered through a 150-μm opening nylon screen into a 125-ml plastic separatory funnel. The cell digests were gently mixed and allowed to stand for 15 min, and the infranatant was slowly

Abbreviations: VSFC, very small fat cells; LFC, large fat cells; LFCM, manipulated large fat cells.

[1]This work is part of an original thesis submitted in partial fulfillment of the requirements for the Ph.D. degree at the Medical College of Pennsylvania, October, 1977 (A. F.).

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[3]All of these preparations contained a "liposome-like" contaminant: spherical floating objects that were particularly resistant to removal. These objects tended to float but became dispersed at the slightest disturbance. They stained positively with Oil Red O, suggesting that they contained lipid. No tendency to congeal into a single globule was observed (as one would expect of free triglyceride), and they maintained their spherical shape and size distribution (0.5 μm to 4 μm diameter, mean ~1 μm) through various manipulations. Freeze-thawing caused them to

drained off and discarded. The residual fat cell "cake" was washed 5 times (with 5 min of separation time between washes) using 5 ml of Krebs-Ringer bicarbonate solution containing 1.5% albumin (KRB 1.5A). The residual fat cell population in the funnel was free of very small fat cells and stromal-vascular components and is referred to in the text as the large fat cell (LFC) fraction. The LFC fraction had mean cell diameters of ~90 μm (individual cell diameters ranged from 40 to 125 μm) for 6-month-old animals and ~107 μm (individual cell diameters ranged from 40 to 130 μm) for 12-month-old animals.

The pooled infranatants contained VSFC, some residual normal size fat cells, and the stromal-vascular fraction. We used the device pictured in **Fig. 1** to screen against fat cells larger than 30 μm. It was manufactured from a 50-ml plastic syringe barrel with the cannula end removed. The barrel was fitted with a 25-mm plastic Buchner funnel bottom equipped with a plastic retaining ring to hold a piece of 30-μm nylon mesh screen in place. A three-way plastic Luer-lock valve was attached to the funnel bottom and connection was made to a reservoir containing Krebs-Ringer bicarbonate (KRB) with 1.5% albumin. Fluid from the reservoir was made to flow into the barrel to remove air trapped beneath the screen and to provide a 5–10-ml volume of fluid above the screen. The infranatant fraction was added gently, stirred gently with a plastic spoon, allowed to stand for 45 sec, and then slowly drained through the screen (care being taken not to allow the air-surface interface to touch the screen). The residual infranatant fraction was washed 5 times (with 45 sec standing time) with 5 ml of KRB–1.5% albumin buffer solution. The end product of this screening procedure contained no fat cells larger than 35 μm in diameter. The stromal-vascular component was removed from this very small fat cell fraction by gentle centrifugation (10 min, 800 *g*). Final preparations of very small fat cells were harvested from the surface of these centrifugations and diluted with KRB containing sufficient albumin to reach a 4% final concentration. These are referred to in the test as very small fat cell fractions (VSFC) and for all preparations

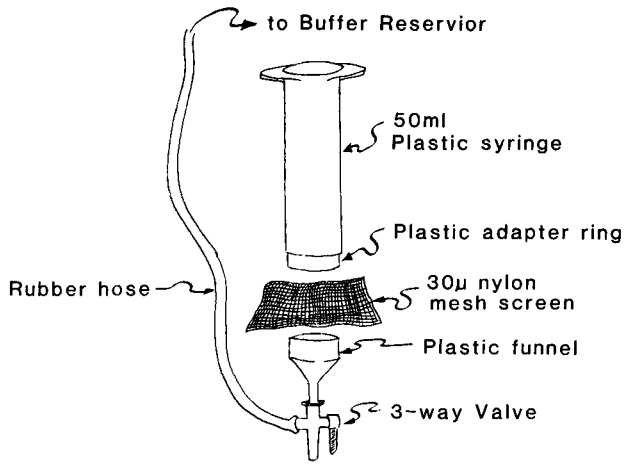

Fig. 1 Very small fat cell separator. The separator was constructed as illustrated. Pooled infranatants from crude isolated fat cell cakes (see text) were gently placed into the separator which was initially filled with 5 to 10 ml of albumin solution from the reservoir. The cell suspension was gently stirred with a plastic spoon and the cells were allowed to stand for 45 sec and then slowly drained through the screen. Analysis of the cell size and number distribution, both remaining in the chamber and collected as the filtered infranatant, showed that five washes were sufficient to remove 90+% of the original VSFC fraction present while eliminating all fat cells above 35 μm in diameter, provided that care was taken not to allow the air-fluid interface to come into contact with the 30-μm screen (data not shown). Subsequent removal of stromal-vascular elements was accomplished by mild centrifugation of the collected fractions as described in the text.

they exhibited a population distribution statistically identical to that found in the original collagenase preparations.

A portion of the LFC preparations was forced through 30-μm opening nylon mesh screens and centrifuged at 1000 rpm in a clinical centrifuge for 10 min. These roughly handled large fat cells are referred to in the text as manipulated large fat cells (LFCM). The size distributions of LFC and surviving LFCM were statistically identical (data not presented).

Metabolic incubations

Cell preparations of VSFC, LFC, and LFCM were obtained from the same collagenase digests and subjected to one or more of the following incubation procedures.

To determine [U-^{14}C]glucose metabolism, the methodology of Rodbell (4) was generally followed. Cells of all preparation types were incubated in plastic 20-ml scintillation vials containing a final volume of 2 ml of Krebs-Ringer bicarbonate buffer with cells such that each ml of the incubant contained 40 mg (4%) of albumin, 5.56 μmol of unlabeled glucose, 0.75 μCi of [U-^{14}C]glucose, and 0 or 1.0 mU of recrystallized bovine pancreatic insulin (Grade B, Lot. No. 101174, Calbiochem Co., Los Angeles, CA). Vials were gassed with 5% CO_2–95% O_2, and fitted with a rubber stopper and a plastic hanging well (Kontes Glass Co., Vineland, NJ) containing a rolled 1 cm × 5 cm strip of paper cut from Whatman No. 1 filter paper. Incubations were allowed to proceed at 37°C for various times

disappear, while homogenization increased their numbers. No cytoplasmic or nuclear organization was visible under the light microscope or in electron microscopy preparations. In the electron microscope, they appeared to be spherical droplets coated with an osmophilic shell. Some of them excluded the vital dye Nigrosin, while others did not. In aqueous polymer systems (31) they behaved as if they possessed surface charge properties, exhibiting partiality for certain polymer layers, which could not be attributed to density alone. Samples isolated via aqueous polymer systems showed no metabolic activity ([^{14}C]glucose oxidation to CO_2). All attempts to remove them completely were unsuccessful, but the final VSFC preparation procedure used for analytic work seemed to be less contaminated than most other preparations. They seemed to resemble the "liposomes" from adipose tissue described by Angel (32), and the possible uncontrollable variability that they might introduce into the lipid determinations prompted us to limit our compositional analysis. Although the absolute quantitative validity of the lipid analysis of the VSFC preparations is questionable because of the presence of these "liposomes," the analysis was useful in obtaining approximate and reasonable figures on VSFC composition.

and were terminated by acidification of the medium. Hyamine hydroxide was added to the hanging wells to trap evolved CO_2 (4). Standard liquid scintillation techniques were used to estimate the radioactivity trapped as $^{14}CO_2$ in the hanging wells.

Identical cell preparations were also used for studies involving the incorporation of [U-^{14}C]glucose into total lipid. These were similar to the CO_2 incubations except that 1.75 μCi/ml of label was used and the vials were capped with a normal stopper. These reactions were terminated by the addition of 5 ml of CH_3OH with subsequent total lipid extraction following the procedure of Bligh and Dyer (5). Radioactivity was determined in dried aliquots of the total lipid extract.

Cell preparations monitored for lipolytic activity via glycerol release were handled in a similar manner with the following exceptions. The media contained only unlabeled glucose (5.56 mM); the reactions were terminated by the addition of 1 ml of 10% trichloroacetic acid; and norepinephrine, epinephrine, glucagon, or a KRB-4% albumin blank was added immediately prior to the addition of the cell preparation. Glycerol blanks and standards were made up in incubation medium containing no cells. The glycerol content was assayed in the deproteinated supernatant of blanks, standards, and cell preparations using a spectrophotometric method (6).

For each cell preparation, 5 × 1-ml aliquots were randomly taken for the determination of cell numbers using a DNA analysis method that followed the procedure of Hinegardner (7).

Insulin (recrystallized bovine insulin, Calbiochem Co.) and glucagon (crystalline pork glucagon treated with dithiothreitol to destroy insulin, Eli Lilly Laboratories, Indianapolis, IN) were prepared for use according to the method of Parrilla, Goodman, and Toews (8). Epinephrine and norepinephrine (Sigma Chemical Co., St. Louis, MO) were prepared immediately prior to use according to procedures established to minimize degradation (9).

Microscopic size determination

Fat cell diameters were determined microscopically as previously described (1) except that the cells were stained with crystal violet to visualize cytoplasm and nuclei, and Oil Red O to visualize lipid (2). The diameters of 300 fat cells of all sizes were determined to estimate the relative distribution of VSFC versus LFC. An additional 100 VSFC were sized to obtain an estimate of their population distribution independent of LFC.

Electron microscopy

VSFC preparations were placed in a plastic beaker and mixed 4:1 with a solution of 5% glutaraldehyde yielding an effective glutaraldehyde concentration of 1%. The cells were fixed overnight at ambient temperature. The following day the cells were osmicated by the addition of 2% OsO_4 in 0.1 M Na-cacodylate buffer in a 1:1 ratio with the fixed preparations. The osmication was allowed to proceed for about 3 days. The osmicated cells were centrifuged at 400 *g* in an International clinical centrifuge for 10 min. The supernatant was removed, and the cells were resuspended and washed three times with fresh 0.1 M cacodylate buffer.

Washing, dehydration, and embedding were all carried out in 15-ml Teflon centrifuge tubes by alternate centrifugation at 400 *g*, removal of supernatant, and resuspension in fresh solutions. Samples were dehydrated in graded aqueous ethanolic solutions and embedded in Epon 812 by standard procedures. Sections were cut with a diamond knife on a Sorvall ultramicrotome and stained with Reynolds' lead citrate and uranyl acetate. Viewing was accomplished with a Phillips 300 electron microscope.

Lipid extraction and analysis

Aqueous cell fractions were subjected to total lipid extraction by the method of Folch, Lees, and Sloane Stanley (10). Lipid extracts were taken to dryness and redissolved in 15 ml of $CHCl_3$. Duplicate 1-ml aliquots were removed for the estimation of total lipid phosphorus content using the method of Bartlett (11). Total triglycerides were measured by the method of Van Handel and Zilversmit (12), and total cholesterol was determined by the method of Rudel and Morris (13). Both the triglyceride and cholesterol determinations were performed after phospholipids had been adsorbed to activated diatomaceous earth (12).

Samples of the total lipid extracts were also subjected to thin-layer chromatography on pre-coated Silica Gel plates, along with triglyceride (triolein), fatty acid (oleic acid), and cholesterol standards. Plates were developed in hexane–ethyl ether–glacial acetic acid 85:25:1 (vol/vol), air dried, visualized with iodine vapors, and quantified by the H_2SO_4 charring method of Marsh and Weinstein (14).

Linear regression analysis, means, *t*-tests, and other statistical manipulations of the data were performed on a Wang 600 series calculator equipped with a STAT-ROM. Data are generally expressed as the mean value ± standard error of the mean (SEM) unless otherwise indicated. *P* values of less than 0.05 were considered statistically significant where such comparisons are appropriate.

RESULTS

Morphologic observations

Plots of the distribution pattern of isolated adipocytes from whole epididymal collagenase digests from the rats used for this study exhibited a pattern of bimodality as previously reported (2). No changes in either the size distribution or the relative numbers of cells below 35 μm diameter were detected with changes in the age or weight of the animals used (data not presented).

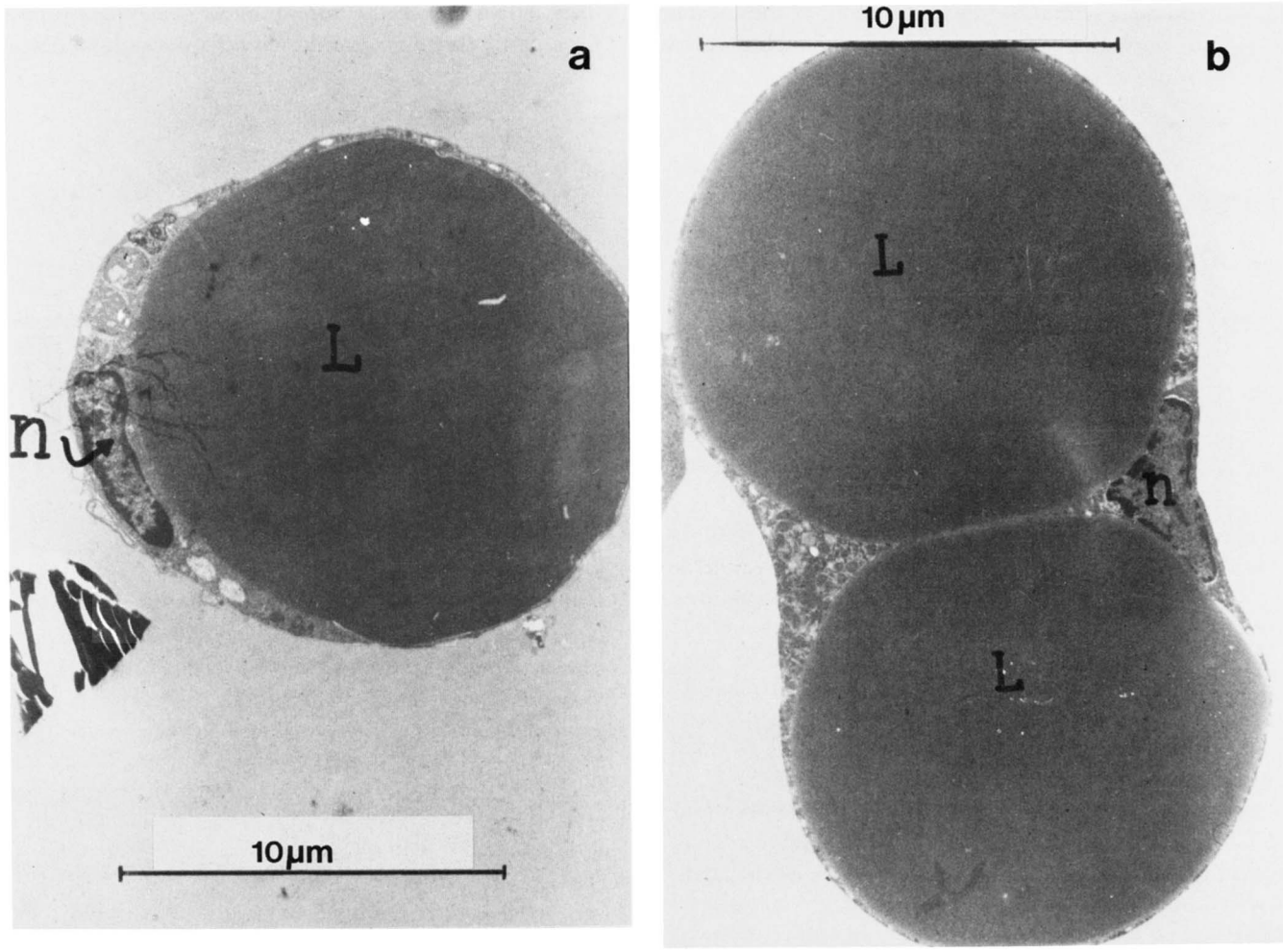

Fig. 2 a, Electron micrograph of a unilocular very small fat cell. Cells of this type were the predominant type seen in the VSFC preparations described in the text. Cells were apparently intact and devoid of stromal-vascular elements and debris. L, lipid droplets; n, nucleus. A single dominant lipid drop is apparent and the resemblance of these cells to normal adult fat cells is striking. Several micrographs were subjected to morphometric analysis of volumes as described in the text. Results indicate that, on the average, these cells contain approximately 45 ± 4.6% (SEM) of their volume as lipid. b, A bilocular very small fat cell. L, lipid droplet; n, nucleus. The presence of two major lipid droplets of approximately equal size caused these cells to take on a dumbbell-like shape. The nucleus, as we commonly observed in these bilocular cells, is nestled at the junction of the two droplets. This particular cell appears to have numerous mitochondria in its cytoplasm. Cells of this type and of the multilocular variety (not shown) were seen infrequently in the VSFC preparations.

Light microscopy showed the cells <35 μm in diameter to be spherical in shape with an eccentrically located nucleus and a lipid positive, centrally located vacuole or, more infrequently, vacuoles, a morphology identical to that reported in unseparated populations of these cells (1–3). Their resemblance to normal size adipocytes was also supported by electron microscopic observations. In all preparations examined, the VSFC were devoid of stromal-vascular type cells and cellular debris. Three types of cells were in evidence. The most numerous (greater than 90%)[4] were of the unilocular variety (**Fig. 2a**) which look like miniature, mature fat cells. They possessed a large central vacuole of electron-dense material surrounded by a relatively thin rim of cytoplasm and an eccentrically placed nucleus—typical features of mature adipocytes (15, 16). The cell membranes appeared to be intact and there was no gross evidence of cellular damage from the separation procedure. Two other cell types were observed in lesser numbers, a bilocular and a multilocular type. Bilocular cells (Fig. 2b) contained two lipid-filled vacuoles of approximately equal size. The nucleus was

[4]Routine analysis of the relative distribution of the VSFC fraction cells into unilocular, bilocular, and multilocular categories was not undertaken. A few epididymal preparations were examined with the aid of a calibrated Vicker's Split Image Sizing device which used a considerably higher resolution than was used for the bulk of these studies (1 Vicker's size interval = 0.5 μm). In all of these examinations less than 5% of the cells were bilocular and less than 1% were multilocular. It is entirely possible that these relative distributions may vary with animal age and nutritional status. In a subsequent study (unpublished) mesenteric fat exhibited higher frequencies of bi- and multilocular varieties compared to epididymal fat, suggesting that anatomical location might influence the relative distribution of the various forms of VSFC.

TABLE 1. Lipid composition of normal versus very small fat cells

Cell Preparation[a]	Lipid Class	μg Lipid per Cell	μg Lipid per μg Triglyceride	μg Lipid per μg DNA
Literature values for large fat cells	Total glycerides[b]	0.5–1.0	1	$(72–140) \times 10^3$
	Phospholipids	$(0.15–0.8) \times 10^{-3}$	$(0.3–0.8) \times 10^{-3}$	21.5–115
	Total cholesterol	$(0.05–0.5) \times 10^{-3}$	$(0.1–0.5) \times 10^{-3}$	7.2–72
Large fat cells (LFC)	Total glycerides[b]	0.59	1	$(84.4 \pm 5.9) \times 10^3$
	Phospholipids	0.17×10^{-3}	0.28×10^{-3}	24.0 ± 2.5
	Total cholesterol	0.06×10^{-3}	0.22×10^{-3}	18.3 ± 4.3
VSFC	Total glycerides[b]	0.66×10^{-3}	1	94 ± 20
	Phospholipids	0.06×10^{-3}	0.10	9.0 ± 0.2
	Total cholesterol	0.01×10^{-3}	0.02	2.0 ± 0.4

Data in the last column (μg lipid per μg DNA) are those originally obtained from triplicate determinations on parallel preparations from three 12-month-old rats (mean values ± SEM). Values in the other columns are derived from these and are presented to facilitate comparison with literature values.

[a]Cell preparations are as described in the methodology. Literature values appear in their originally published form as either μg lipid per cell (19) or μg lipid per μg triglyceride (20, 21). As presented here, the data are extrapolated to cover the range of fat cell sizes from 4 μm to 100 μm diameter.

[b]Thin-layer chromatography of both LFC and VSFC lipid extracts showed the total glyceride component to be predominantly triglyceride (95 + % triglyceride for the LFC and VSFC preparations).

most often nestled between the two droplets. A very thin line of cytoplasm separated the two droplets with no visible organelles. The multilocular variety (not shown) contained one or more larger vacuoles and numerous smaller ones. The cytoplasm was dense with mitochondria and they were generally larger and less spherical than the unilocular variety. These multilocular cells may be preadipocytes (17, 18).

All three cell types exhibited numerous small, clear vesicles at their cell borders. Such vesicles are common features of regular size fat cells and are believed to be associated with lipolysis (16). Finger-like projections from the cell border not adjacent to the lipid droplet were often seen, and clear vacuoles were often associated with these projections. They may be related to the normal adipocyte's ability to phago- or pinocytose (15). Mitochondria were of the even cristaed, rounded type described in normal adipocytes (15).

Several electron micrographs of unilocular VSFC between 8 and 35 μm in diameter were examined in the following way: diameter measurements for the whole cell and the lipid inclusion were made and the theoretical volumes occupied by each were calculated.[5] The percentage of the total volume of these cells occupied by the single droplets ranged from 21 to 66% with a mean of 45.3 ± 4.6% (SEM).

Lipid analysis

Several cell preparations were analyzed for cholesterol, phospholipid, and triglyceride content (**Table 1**). The lipid data for the large cell preparations fell within reported values (19–21), with triglycerides being the major lipid component. While the triglyceride values obtained would appear to be somewhat lower than expected for cells of this size (~107 μm diameter), the results are in very close agreement with previously reported triglyceride contents (1) of cells derived from the Fischer 344 rat. The VSFC preparations had less triglyceride both on an absolute and a per cell basis, but triglycerides remained the dominant lipid component. Phospholipid and cholesterol were also much lower in the VSFC preparations than in the large cell preparations, but much higher when expressed on a per μg triglyceride basis. This reflects the changing relationship between triglyceride droplet size and the total cellular lipid component. When expressed on a per μg DNA basis, both phospholipid and cholesterol are less in the VSFC preparations, suggesting less total cellular lipid and in amounts appropriate to their diminutive size. It has been reported that a constant triglyceride:cholesterol ratio is found in the normal-sized fat cell population regardless of the size (20), a fact attributable to the enormous bulk of triglyceride normally associated with fat cells. Such relationships cannot be extrapolated to include the VSFC since they contain relatively small lipid droplets and a relative amount of membrane (with its associated cholesterol and cholesterol esters) which would tend to obscure such relationships.

[5]We caution the reader that such analyses are fraught with technical limitations, the chief limitation being the problem of the plane through which the cuts were taken. By limiting our spatial analyses only to those sectioned cells that contained a nucleus, we reasoned that the distribution of cuts through the cell would be Gaussian and random, with a range from tangential to those through the actual diameter. The true diameter of the sections in question could then be very crudely estimated as approximating 2d (observed). Such measurements are indeed crude and inherently inaccurate. They were undertaken to obtain estimates of the relative lipid storage capacities of these cells and to crudely match the morphological appearance with subsequent chemical analysis. The results gave good agreement.

Thin-layer chromatography of total lipid extracts of VSFC preparations showed their lipid component to be composed of glycerides (tri-, di-, and mono-), phospholipids, cholesterol, and trace amounts of free fatty acids. Subsequent analysis gave mean percent ratios of 90:8:2 (total glycerides:phospholipid:total cholesterol). The total glyceride component was 95+% triglyceride.

The amount of triglyceride per μg of DNA in the VSFC preparations remained relatively constant for different VSFC preparations, and therefore, it is probably a close approximation of the amount of triglyceride present in the VSFC. Assuming that these numbers can be taken as an approximation of the triglyceride content of the VSFC[5] and assuming the density of triglyceride is the same in both VSFC and LFC preparations, and has a value of 0.916 grams/cm[4] as previously determined (1, 22), the average diameter of a VSFC lipid droplet can be calculated.[6] Such calculations yield a mean lipid droplet diameter of 11.4 ± 0.25 μm for the VSFC, or approximately 40 to 50% of the total volume of the VSFC as determined by light microscopy. This is in good agreement with the volume occupied by the lipid droplet independently estimated from electron micrographs (~45%).

Metabolism of [U-^{14}C]glucose

The quantity of [U-^{14}C]glucose oxidized to CO_2 was proportional to the number of very small fat cells and oxidation proceeded at a constant rate for at least 90 min (data not shown). Additions of insulin (1 mU per ml) caused a 2- to 4-fold increase in glucose oxidation (**Fig. 3**), which also proceeded at a constant rate for at least 90 min. Such responses are characteristic of normal, metabolically active isolated fat cells (4, 23).

A comparison of basal and insulin-stimulated glucose oxidation in very small fat cells and co-isolated (from the same fat depot) large fat cells appears in **Fig. 4**. A similar dose of insulin on an equivalent number of cells elicited a greater maximum response in large cell preparations than in the VSFC. Nevertheless, the degree of stimulation above basal value was similar in VSFC and LFC (236 and 296%, respectively), suggesting an intact insulin response and a similarity of response between the two cell types. Portions of the LFC preparation were deliberately manipulated to produce alterations in insulin response characteristic of damaged fat cells (24). Both the LFC and the manipulated LFC preparations showed about the same maximum response to insulin. However, the manipulated

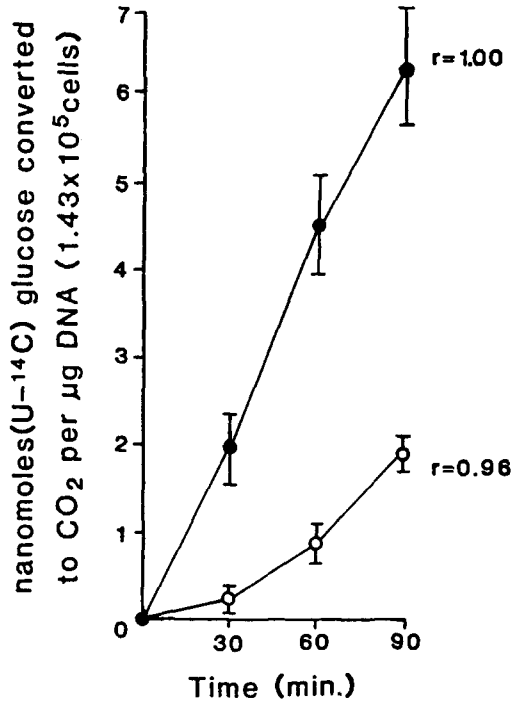

Fig. 3 Basal and insulin-stimulated oxidation of [U-^{14}C]glucose to CO_2 in VSFC preparations. Cell preparations were incubated at 37°C for various times with (●) and without (○) 1 mU of insulin per ml. Each point is the mean of triplicate incubations from five different VSFC preparations (± SEM). [U-^{14}C]Glucose incorporation was linear with time in both basal (r = 0.96) and stimulated (r = 1.00) preparations for at least 90 min. The presence of insulin significantly increased glucose oxidation.

LFC preparations showed a much elevated basal rate of glucose oxidation with a dramatic decrease in percent stimulation. Such findings are not dissimilar to those reported by Gliemann (24) in similar kinds of experiments. The results from the VSFC preparations (i.e., low basal values and large percent increases above basal) tend to support the contention that these cells are not damaged by the separation procedure.

The incorporation of labeled glucose into total lipids was also examined in both LFC and VSFC derived from the same fat depots. The results are summarized in **Table 2**. VSFC incorporated about half as much labeled glucose into lipids than did LFC on a per cell basis. VSFC failed to significantly increase labeled glucose incorporation into lipids in response to insulin stimulation while LFC did ($P < 0.05$). It can be concluded that these cells incorporate significant amounts of glucose carbon into their lipid fraction, albeit at a lower per cell rate than LFC preparations.

Basal and hormone-stimulated lipolytic activity

In addition to their lipogenic activities, normal adipocytes exhibit basal and hormone-stimulated lipolytic activities. Glycerol release is a commonly accepted indicator

[6] $$d^3 = \frac{6}{\pi}\left[\frac{10^{12}\ \mu m^3}{0.916\ \mu g\ \text{triglyceride} \times 10^6}\right][X\ \mu g\ \text{triglyceride}]$$

where d equals diameter of the lipid droplet and X equals the chemically determined triglyceride content per cell.

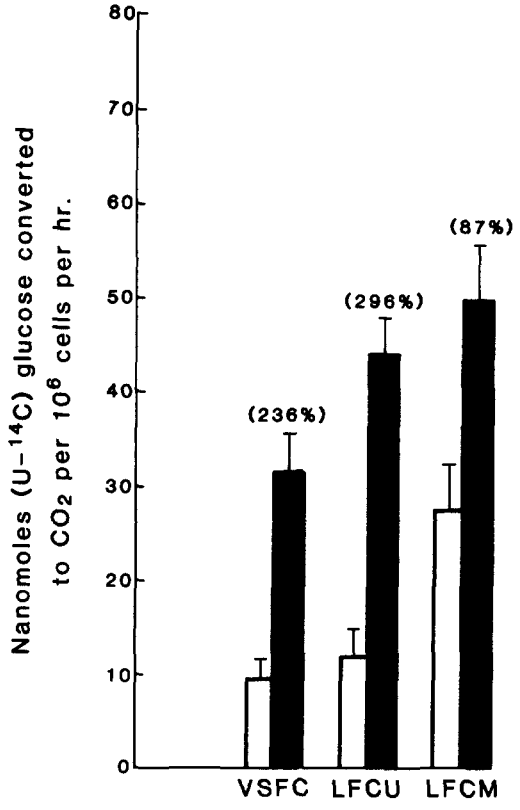

Fig. 4 A comparison of VSFC and LFC [U-^{14}C]glucose metabolism and the effects of insulin stimulation. VSFC, fresh LFC (normal fat cells), and LFCM (manipulated normal fat cells, see text) were co-prepared from the same collagenase digests. Cells were incubated for various periods of time with (■) and without (□) 1 mU of insulin per ml. Each bar is the mean of duplicate incubations of five different cell preparations. Results were linear with time and, while absolute values were different, the percent stimulation by insulin in the VSFC and intact LFC was statistically identical.

of the lipolytic rate in LFC (25) and the hormone-sensitive triglyceride lipase of LFC is known to respond to a host of hormones, most notably epinephrine, norepinephrine (26), and glucagon (27).

Paired cell preparations of VSFC and LFC were examined in regard to their basal lipolytic rates, as well as their response to norepinephrine, epinephrine, and glucagon. These results are summarized in **Table 3**. Basal and hormone-stimulated glycerol release was proportional to VSFC cell number and proceeded linearly for at least 60 min (data not shown). On a per cell basis, VSFC released less glycerol than LFC, both basally and in response to norepinephrine, epinephrine, or glucagon stimulation ($P < 0.01$ for all VSFC/LFC comparisons). VSFC preparations did respond significantly ($P < 0.05$) to hormonal stimulation by the catecholamines, suggesting the presence of a hormone-sensitive triglyceride lipase. Glucagon stimulation was significantly depressed in both VSFC and LFC, which is not surprising considering the age (12 months) of the animals (28, 29) from which the cells were derived.

DISCUSSION

During routine microscopic analysis of collagenase digests of rat epididymal adipose depots, we consistently observed significant numbers of adipocyte-like cells that were considerably smaller than the normally described adipocyte (1). Preliminary observations on their appearance under light and electron microscopy led us to speculate that they were indeed adipocytes. Additional studies by ourselves (2) and others (3) have confirmed these initial findings.

In the current study preparations of these very small fat cells were obtained by subjecting collagenase digests of epididymal fat depots of mature rats to both differential bouyancy flotation (30) and physical screening for cell size. Both light and electron microscopic evaluations showed these VSFC preparations to be free of stromal-vascular contaminants and normal large fat cells. Electron microscopic evaluations showed the VSFC to be composed primarily of unilocular cells with the occasional appearance of a bi- or multi-locular cell. All these cell types had morphologies identical to normal fat cells (15, 16) or cell types in the normal developmental chain of fat cell growth (17, 18).

Microscopic estimation of lipid content showed them to be approximately 45% lipid, and theoretical calculations based on chemical analysis of lipid content were in good agreement with this figure. Like normal large fat cells, the lipid content of the VSFC preparations was primarily triglyceride; however, their smaller size and greater total cellular lipid to lipid vacuole ratio yielded higher compositional percentages of phospholipid and total cholesterol than that encountered in normal large fat cells (19–21).

Our primarily metabolic data on the VSFC preparations support the following conclusions. *1*) The VSFC preparations are metabolically viable, demonstrating positive correlations of product formation with cell number and time for both glucose incorporation into CO_2 and glycerol release. *2*) The VSFC preparations respond to hormonal stimulation in a manner consistent with the view that they are indeed adipocytes, i.e., insulin stimulated glucose oxidation and catecholamines stimulated

TABLE 2. [U-^{14}C]Glucose converted to total extractable lipids[a]

	VSFC	LFC
	nmol per 10^6 cells per hr (± SEM)[b]	
Basal	62.3 ± 0.3	120.0 ± 0.4
Insulin (1 mU per ml)	66.7 ± 0.3	175.0 ± 0.7

Data are expressed as mean nmol of [U-^{14}C]glucose converted to total extractable lipids per 10^6 cells per hr ± SEM.

[a]Total extractable lipids are 99 + % triglyceride for LFC, 90% triglyceride for VSFC.

[b]Data were gathered from triplicate incubations of four paired VSFC, LFC preparations from four 6-month-old rats.

TABLE 3. Effect of lipolytic hormones on glycerol release by VSFC and LFC preparations

Condition	VSFC	LFC
	μmol glycerol released per 10^6 cells per 30 min	
Basal	0.58 ± 0.10	0.87 ± 0.05
Norepinephrine (10^{-4} M)	0.91 ± 0.05 (57%)[a]	2.69 ± 0.14 (209%)
Epinephrine (10^{-4} M)	0.93 ± 0.06 (60%)	2.63 ± 0.14 (202%)
Glucagon (2 μg per ml)	0.65 ± 0.10 (12%)	1.09 ± 0.04 (25%)

Data are expressed as mean μmol of glycerol released per 10^6 cells per 30 min ± SEM from triplicate incubations on two separate preparations of paired VSFC, LFC cell fractions obtained from pooled epididymal fat depots from 12-month-old rats. Glucose concentration was 5.56 mM.
[a]Values in parentheses are mean percent stimulation above basal value.

glycerol release. *3*) The VSFC preparations also incorporated glucose into lipids.

These cells are unlike the traditional adipocyte in the following ways. *1*) They are much smaller in size (1–3). *2*) On a per cell basis they are less metabolically active in their utilization of glucose (both basally and insulin-stimulated) for CO_2 and total lipid production and in their basal and hormonally stimulated release of glycerol. *3*) Glucose incorporation into lipids does not appear to be stimulated by the presence of insulin.

While these VSFC appear to be less metabolically active than normal large fat cells when the data are expressed on a per cell basis, quite the reverse is true if the metabolic data are expressed on either a per unit surface area or per unit volume basis. Viewed in these ways, which normalize for their diminutive size, the VSFC are far more active than normal large fat cells in both glucose carbon incorporation and lipid mobilization. In the current investigation, no attempt was made to partition the glucose carbon labeling of the triglyceride into glycerol and fatty acid moieties. It would be of some interest in future experiments to determine whether de novo fatty acid synthetic capabilities are maintained in these cells at a time when their larger counterparts have lost this ability (23, 24).

In a preceding paper (2) we have described the population distribution of the very small fat cells and presented evidence that the pan-mammalian occurrence of these cells seems likely; yet the origin and physiological role of the VSFC remain an enigma. In the current work we have given sufficient morphological and metabolic data to justify the inclusion of the VSFC in the general family of fat cells.

The authors wish to thank Drs. E. J. Masoro and H. A. Bertrand for their advice and encouragement during the early stages of this work. Our thanks also to Drs. Walter Rubin and Bruce Schwartz for their advice and access to their electron micrography facilities, and to Drs. Susan K. Fried, Marci Greenwood, P. Johnson, George Rothblat, and Julian Marsh for their comments on the manuscript. And finally, thanks to Ms. Monica Fink and Ms. Evelyn Buzzy for typing the manuscript. This work was supported in part by NIH grant no. HD-06267, USPHS training grant no. GM-2008, and USPHS fellowship no. AG-05161. This work was performed under the auspices of the U.S. Department of Energy.

Manuscript received 27 March 1984.

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