Very small fat cell populations: mammalian occurrence and effect of age[1]

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Abstract A normal Gaussian distribution of fat cell diameters has been found in the adipose depots of most animals and man. In our early work we observed very small fat cells in isolated cell preparations of normal adult rat epididymal pads and frequency distributions of measured fat cell diameters were bimodal, indicating the existence of a separate population of very small fat cells in addition to the normal adipocyte population. We investigated in detail the age- and weight gain-associated changes in adipocyte size distribution from a unimodal distribution in very young animals to a bimodal distribution in maturing heavier animals. Bimodality in the epididymal depot was fully established by 14 weeks of life and persisted without detectable change to 28 months of age. Several other depots representing deep abdominal and subcutaneous adipose tissue in adult rats and in the adult form of male guinea pigs, female C57 BL/6J lean mice, rabbits, and cats also have been found to contain bimodal adipocyte populations. Our results show that in normal adipose depots of several mammalian species a separate population of very small fat cells exists in addition to the usual adipocyte population and we conclude this is a characteristic morphologic feature of adult mammalian adipose tissue.—**DeMartinis, F. D., and A. Francendese.** Very small fat cell populations: mammalian occurrence and effect of age. *J. Lipid Res.* 1982. **23:** 1107–1120.

Supplementary key words adipocytes • preadipocytes • bimodal distribution • adipose cell size • fat depots • Fischer 344 rat • lean C57 BL/6J mouse • guinea pig • rabbit • cat

Morphological studies on size changes in adipocytes during the normal growth and development of adipose tissue (2–4) and the expression of adipocyte cellularity in mature organisms (5, 6) are obviously relevant to the problem of obesity.

Determinations of adipocyte size and number by a variety of methods (7–9) have demonstrated a normal Gaussian distribution of fat cell diameters in adipose depots of both man and animals (7–13). However, a few investigators have found an additional population peak of smaller diameter cells, chiefly in adipose tissue obtained from farm animals noted for excessive weight gain (14–20) or from obese animals (21–25). This second population of smaller fat cells may be a usual morphological component of normal adipose tissue or these cells may be a characteristic induced by the obese condition.

This report addresses itself to resolving this question. We show that a very small fat cell population usually exists in normal adipose tissue of several species. What the function of this population is in adipose tissue of the normal or obese organism remains unclear but leads to interesting questions.

METHODS

Cell terminology

Several published studies deal with metabolic and other differences between "small" fat cells and "large" fat cells. These cells are derived from young, lean animals ("small" cells) and older, fatter animals ("large" cells) (26) or are adipocytes of different mean diameters obtained by differential flotation techniques from a general population of fat cells (27). Nevertheless, they represent in part or in whole the normal adipocyte population. To avoid confusion with existing terminology we define the very small fat cell (VSFC) population as the population in a bimodal distribution of adipocytes having the smaller mean cell size. In our study the VSFC population consists of fat cells approximately 8–35 microns in diameter. We refer to the classic adipocyte population as normal or large fat cells (LFC).

Animals

Caesarian-originated barrier-maintained male rats of the Fischer 344 strain were obtained from the Charles River Breeding Laboratories approximately 2 weeks prior to killing and maintained as previously described

Abbreviations: VSFC, very small fat cells; LFC, large fat cells, the normal adipocyte population.

[1] A part of this work has been presented at the 1976 Fall Meeting of the American Physiological Society and was published in abstract form (1).

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(12). For the study of the epididymal adipocyte population as a function of age, the rats were killed at the following ages expressed in weeks: 6, 9, 11, 14, 52, 105, and 120. Other adipose depots (perirenal and inguinal) were studied in rats 11 to 14 weeks old.

Male guinea pigs (Hartley strain) were obtained from Perfection Breeders, Inc. at 1, 16, and 52 weeks of age. They were killed at 11–15 days, 16–20, and 52–54 weeks old, respectively.

Lean female mice of the C57BL/6J strain (+/+ or Ob/+) were obtained from Jackson Memorial Laboratories and killed at 11 and 14 weeks of age.

Rabbit and cat fat samples were obtained from mature females that were used as controls in other experiments or subjected to in vivo experimentation of short duration.

Preparation of fat cell suspensions

All animals were fed ad libitum. Rabbits were anesthetized with pentobarbital and cats were anesthetized with alpha-chloralose. Rats, guinea pigs, and mice were killed by decapitation. Subcutaneous and paired adipose depots from all species were excised and isolated fat cells were prepared by the technique of Rodbell (28) with modifications described earlier (12). The tissue was minced and digested in 15 ml of a medium containing 2.7 mg of glucose, 6500–7000 units (50 mg) of collagenase (Type 1, Worthington), and 0.2 g of bovine serum albumin (Fraction V, fatty acid free, Sigma) dissolved in Krebs Ringer bicarbonate solution, pH 7.4, and gassed with 95% O_2–5% CO_2 continually. Incubation took place at 37°C with gentle swirling on a gyrotory shaker water bath (New Brunswick Scientific) for 60 min. At the end of the incubation the suspension was filtered through 150-micron nylon mesh to remove undigested vascular and stromal elements but was not processed further to minimize selective disruption or loss of cells.

Eight percent glutaraldehyde was added slowly with mixing to fix the filtrate when it was at room temperature. The final concentration of glutaraldehyde was 4%. Samples not measured the same day as prepared were covered and kept at room temperature. These were resuspended by gentle swirling. Fixation preserved the morphological relationships among the various adipocytes throughout the time necessary for sizing of the fat cell populations. Preliminary experiments established that glutaraldehyde fixation had no detectable effect on the diameter of either the very small or large fat cell populations. Furthermore, successive sizing of identical preparations showed no change in either population distribution up to 21 days. Other experiments have shown that neither the mesh size nor the incubation time used exerted any significant effects on the mean adipocyte diameter or on the distribution of diameters of the LFC or VSFC populations (data not presented).

Staining of adipocytes

The fixed cell suspensions were thoroughly mixed by gentle swirling and one drop was quickly removed with a plastic pipette to a small plastic beaker. To this were added two drops of saline and ten drops of an aqueous crystal violet stain (25 mg/100 ml). Mixing was achieved by swirling. After 5 min aliquots of the stained suspension were transferred to a sizing well as previously described (12). A siliconized cover slip was placed over the well and gently pressed down to remove air. Stromal-vascular cells and other debris sedimented to the bottom of the well while the isolated fat cells and free lipid droplets floated to the top. For staining with Nile Blue Sulfate and Oil-Red-O, equal volumes of each stain (29) and cell suspension were mixed in the same manner. After 5 min an aliquot was prepared for microscopic examination.

Identification and sizing of adipocytes

The results of preliminary staining experiments revealed that free lipid droplets were present in every collagenase digest. Although quantification was not made, our impression was that more than 80–90% of them were smaller than 40 microns in diameter. Consequently, staining of the cytoplasm and nucleus was the key to visualization of the VSFC. Initially, the Nile Blue Sulfate–Oil-Red-O staining procedure was employed to verify the presence of a lipid inclusion within the cytoplasmic mass. However, for routine use crystal violet staining alone was sufficient.

We used the following criteria to identify VSFC with diameters below approximately 25 μm: flotation to the undersurface of the cover slip, an approximately spherical shape, the presence of a clear, spherical inclusion representing stored lipid, a stained cytoplasm, and a stained nucleus. The primary means of identification was the detection of stained cytoplasm at the periphery of the cell either in a circumferential band or in signet ring configuration. Cells appeared well stained due to the violet-colored, thicker cytoplasmic layer surrounding the lipid inclusion. Compared to the stained adipocytes the free lipid droplets appeared lighter in color and had different refractive qualities, despite the continued presence of stain in the suspending medium. The difference was readily distinguishable with a little practice.

It was often possible to visualize the nucleus and when observable (**Fig. 1A**) it confirmed the identification. Two major conditions impeded nuclear recognition in cells below approximately 20–25 μm. When abundant

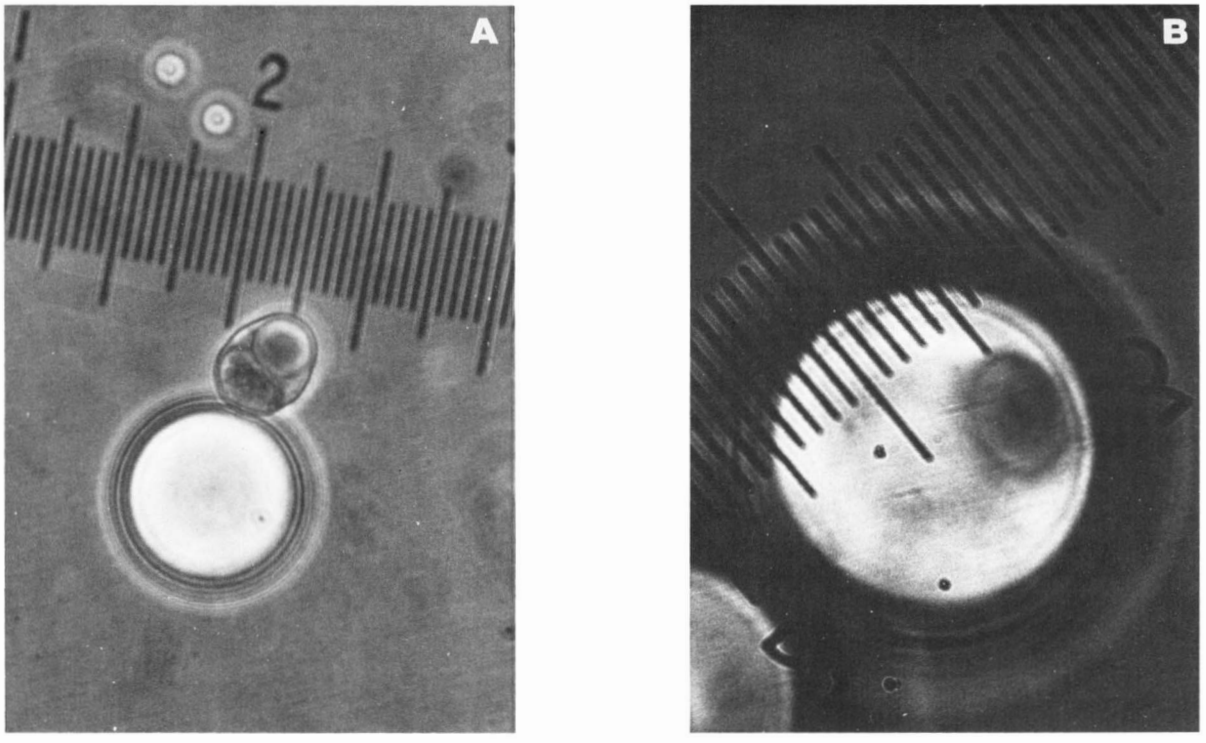

Fig. 1. A, Photograph of a very small fat cell of approximately 13 μm diameter showing both a nucleus and lipid inclusion. A larger free lipid droplet sits near the nucleus. B, Photograph of the nucleus in a very small fat cell of approximately 30 μm diameter. The perimeter of the cell extends from the edge of the line labeled with the number 3 over to the eighteenth grid mark. The cells were obtained from the epididymal depots of a 52-week-old rat and were stained with crystal violet as described in the text. In each photograph the distance between grid marks is 1.65 μm.

cytoplasm surrounded the nucleus the similarity of staining intensity of each under these staining conditions made positive identification of the nucleus difficult. The other circumstance occurred when the nucleus was located on the lower half of the cell. To visualize the nucleus meant looking through the lipid inclusion, which, due to its small size and great curvature, created a lens effect that distorted the light traversing the inclusion. The result allowed us to see the stain color but not the morphological details. Both of these effects were greatly attenuated in cells with larger diameters.

The criteria for identification of VSFC larger than approximately 25 μm diameter and for the adipocytes of the LFC population were: flotation to the undersurface of the cover slip, a spherical shape, a stained cytoplasm, and stained nucleus. These larger adipocytes generally had a signet ring appearance and a light lavender tint over the entire cell, with the cell perimeter and nucleus more intensely colored. At these diameters the cytoplasmic rim became too thin to be detected and confusion of free lipid droplets for cells greatly increased. The primary means of identification, therefore, had to be visualization of the cell's nucleus (Fig. 1B).

Nearly all the very small fat cells were unilocular. Occasionally what appeared to be a bi- or multi-locular cell, due to the presence of two or three large lipid inclusions, could be seen. Whether they were truly multilocular could not be determined as we were not able to rigorously exclude the possibility of two or three unilocular cells remaining adherent. The occurrence of these cells was extremely low and they have not been included in any of the data presented.

The microscopic method of DiGirolamo, Mendlinger, and Fertig (9) was used to measure cell diameters. All work was done by phase microscopy using a Zeiss photomicroscope with a micrometer disc inserted in the eyepiece. For total fat cell population studies on the rat epididymal fat cells as a function of age, cells were sized in intervals of 4.12 μm. For all other studies a different micrometer disc with an interval of 3.12 μm was used. As the micrometer scale was moved across the field, all floating cells whose centers fell within the scale's bounds were sized after a proper focus was obtained. For each animal the diameters of 300 cells were plotted in class intervals (either 4.12 μm or 3.12 μm) to construct a general population histogram showing the distribution of all cell sizes in the adipocyte population.

For greater definition of the VSFC population, an additional 100 adipocytes having a diameter of 35 μm

TABLE 1. Changes with age in the VSFC and LFC populations in the epididymal depot of the Fischer 344 rat.

Age	Body Weight	Fat Cell Population: Diameter 35 μm or Less — Class Interval with Median Value (μm)	Fat Cell Population: Diameter 35 μm or Less — % (Range)[e]	Fat Cell Population: Diameter Greater Than 35 μm — Mean ± S.E.M. (μm)
weeks	*g*			
6	83.0 ± 6.8[a] (6)[b]	27.1–28.7[c]		35.8 ± 3.8[f]
9	173.7 ± 8.0 (6)	22.1–23.8[d]	12.3 (4–23)	56.4 ± 1.9[g]
11	203.4 ± 14.6 (5)	20.5–22.1	10.3 (4–23)	62.5 ± 7.3
14	251.4 ± 16.2 (5)	13.9–15.6	6.3 (3–15)	80.2 ± 4.4
52	371.4 ± 13.8 (5)	13.9–15.6	6.6 (4–13)	90.7 ± 2.2
104	375.6 ± 24.2 (5)	13.9–15.6	5.6 (4–7)	92.8 ± 6.7
120	333.7 ± 38.4 (6)	13.9–15.6	5.9 (3–11)	81.8 ± 4.5

[a] Mean ± S.D.
[b] Number of animals.
[c] For this age the average population number was 128. The class interval containing the median value was that interval in which the 64th cell occurred.
[d] For this and all subsequent ages the average population number was 100 and the class interval containing the median value was that interval in which the 50th cell occurred.
[e] The mean percentage values were calculated from data on the average populations of 300 cells; the range was calculated from each individual 300 cell population.
[f] The mean ± S.E.M. value was calculated using the entire 300 cell population of each animal.
[g] For this and all subsequent ages the average values were calculated from data on the population of cells remaining after excluding cells 35 μm or less in diameter.

or less were measured using a magnification of 500×. The histogram was plotted in class intervals of 1.65 μm (or 1.25 μm). For the 6-week-old rats and the 1-week-old guinea pigs separate measurements of 100 cells were not performed. Instead, all those cells below 35 μm in diameter found in the initial series of 300 cells were used. The average number of cells obtained by this method for the 6-week-old rat was 128; for the 1-week-old guinea pig, 130.

For each group of animals the number of fat cells in similar class intervals was treated as a single variable and the mean and standard deviation were calculated. The average value obtained for each class interval was used to construct a histogram representing the group.

Statistical analysis

The Goodness of Fit Chi Square test was used to analyze the average or individual histograms of the VSFC populations. The observed distribution from approximately 10 to 35 μm was divided into three equal segments. For the VSFC sized in intervals of 1.65 μm, there were five class intervals per segment and the midpoints of the first and last class intervals in each segment were as follows: 9.9 to 16.5, 18.2 to 24.8, and 26.5 to 33.1 μm. For the VSFC sized in intervals of 1.25 μm, there were seven class intervals per segment and the midpoints of the first and last class intervals in each segment were: 10.0 to 17.5, 18.8 to 26.3, and 27.5 to 35.0 μm. Comparison was made to a uniform distribution of cell diameters. A P value of 0.05 or less was considered significant.

RESULTS

Initial studies revealed a bimodal characteristic in frequency distribution histograms of 300 diameters obtained from the general population of adipocytes found in epididymal pads of 3-month-old rats. With an indication of the existence of two populations of fat cells in this depot, the studies were extended to determine the age at which bimodality became established and whether it was modified with advancing age.

A detailed breakdown of the ages of the Fischer 344 rats used in these investigations appears in **Table 1.** The body weight of the animals increased up to 52 weeks of age, remained stable to 104 weeks, and declined thereafter in rats living to 120 weeks of age. Histograms of the total population of epididymal adipocytes at the various ages are shown in **Fig. 2A.** At 6 weeks of age a distribution of diameters with a single peak of 35 μm was obtained. At age 9 weeks the histogram of cell sizes had become skewed to the left with a suggestion of a peak in the very small size range and this was the case for the 11-week-old rats as well. By 14 weeks it was clear that a bimodal distribution of fat cell sizes had devel-

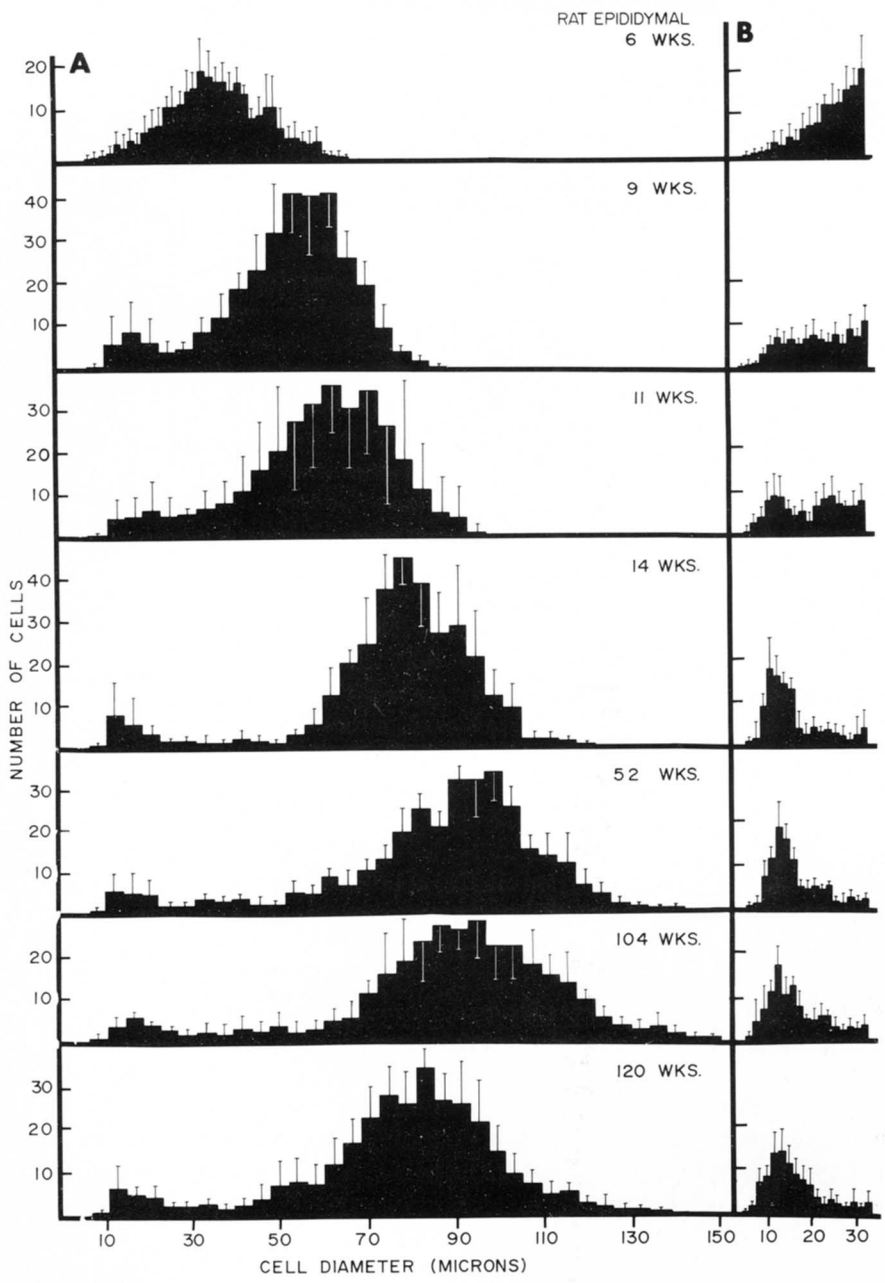

Fig. 2. A, Histograms of the average distribution of adipocyte diameters from the epididymal depots of the Fischer 344 rat as a function of age. The average histogram for each age was constructed by sizing 300 fat cells from each of five or six rats. Each class interval represents the mean ± S.D. of the number of cells in that interval and is 4.12 μm wide except at 6 weeks of age where it is 1.65 μm. B, Histograms of the average distribution of adipocyte diameters between zero and 35 μm. The average histogram for each age was constructed by sizing 100 fat cells 35 μm or less in diameter from each of five or six rats. For the 6-week-old rats the portion of the histogram between zero and 35 μm depicted in Fig. 2A was utilized and had an average of 128 cells. Each class interval represents the mean ± S.D. of the number of cells in that interval and is 1.65 μm wide. Statistical significance of the average VSFC distribution: at 6 weeks, $P < 0.001$; 9 weeks, $P > 0.05$; 11 weeks, $P > 0.05$; 14 weeks, $P < 0.001$; 52 weeks, $P < 0.001$; 104 weeks, $P < 0.001$; and 120 weeks, $P < 0.001$.

oped and this was maintained virtually without change up to 120 weeks of age.

The population dynamics of the cells under 35 μm in diameter is more readily followed in Fig. 2B. Here an additional 100 VSFC were sized at greater microscopic magnification. These results revealed a marked change in the distribution of very small adipocyte diameters within the first 3 months of life. At 6 weeks of age there was a statistically significant ($P < 0.001$), progressive increase in frequency as adipocyte diameter in-

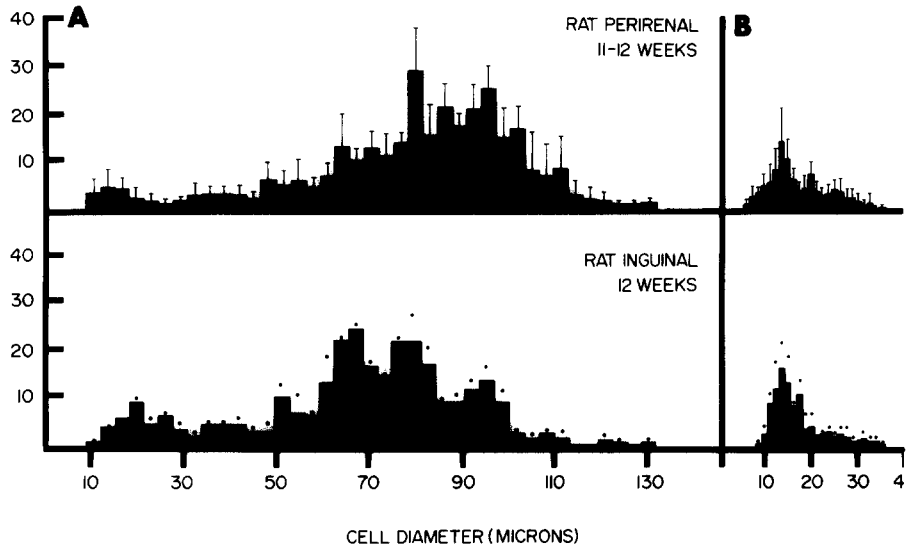

Fig. 3. A, Histograms of the average distribution of adipocyte diameters from the perirenal and inguinal depots of the Fischer 344 male rat. The average histogram for the perirenal depots was constructed by sizing 300 fat cells from each of five rats and each class interval represents the mean ± S.D. of the number of cells in that interval. The inguinal depot histogram is an average of two rats. The dot placed above each class interval represents the greater of the two values for that interval. Each interval is 3.12 μm wide. B, Histograms of the average distribution of adipocyte diameters between zero and 35 μm. The average histograms shown were constructed by sizing 100 fat cells 35 μm or less in diameter from each of five rats for perirenal tissue and two rats for the inguinal pads. Each class interval for the perirenal depots represents the mean ± S.D. of the number of cells in that interval. For the inguinal tissues the dot above each class interval represents the greater of the two values for that interval. Each class interval is 1.25 μm wide. Statistical significance of the average VSFC distribution: perirenal, $P < 0.001$; inguinal, $P < 0.001$.

creased. This was no longer true by 9 weeks ($P > 0.05$). In the 11-week-old rats a peak seemed to be emerging in the small size classes but it was not significant ($P > 0.05$). By 14 weeks of age, a distinct population of VSFC had become evident and was found to be present at all subsequent ages up to 120 weeks of age. At each of these ages the statistical significance for this distribution of diameters was $P < 0.001$.

The changes in the average adipocyte size within each population with age may be seen in Table 1. Fat cells with a diameter greater than 35 μm showed a doubling of their mean cell size by 14 weeks of age with a less rapid increase in diameter from then to 52 weeks of age. Between 52 and 104 weeks, the mean diameter remained essentially unchanged, while in the period of 104 to 120 weeks of age a moderate reduction in mean diameter occurred.

Due to the skewed distribution for the fat cells with diameters of 35 μm or less, the class interval containing the median value has been listed for each age, since it gives a better indication of the central tendency of the data than the arithmetic mean. Although the 6-week-old rat lacks a bimodal distribution, the median value of those cells 35 μm or less in diameter is included for comparative purposes. Up to the age of 14 weeks the median diameter of the VSFC population became progressively smaller. Beyond that age it remained stable. To more clearly indicate the magnitude of this shift the entire range of very small fat cell sizes (6–35 μm) was divided into two equal segments of 6–20 and 21–35 μm and the percentage of the very small fat cells occurring in the 6–20 μm segment was calculated for every age. At 6 weeks of age, the percentage was 15; at 9 weeks, 34%; at 11 weeks, 43%; and at 14 weeks, 75%. It ranged between 70 and 74% for subsequent ages.

An appreciation of the relative magnitudes of the two populations may be gained from Table 1 where the percentage of VSFC in the total population of adipocytes is listed for each age. It averaged at 12% for rats 9 weeks old, subsequently declining to 6% for 14-week-old animals and remaining at that level thereafter.

Other adipose depots in additional groups of adult male rats were also found to exhibit a bimodal characteristic and **Fig. 3** depicts the results obtained when perirenal and inguinal depots were examined. In each instance a population of VSFC was identified in the general population histogram while the VSFC population histogram presented a well-defined peak that was statistically significant ($P < 0.001$). The median diameter of the VSFC for each depot (**Table 2**) was close to that previously found for the epididymal pad. This was true also for the percentage of VSFC in the perirenal adipocyte population, while that for the inguinal depot seemed slightly higher.

TABLE 2. VSFC and LFC populations in the perirenal and inguinal depots of the Fischer 344 male rat.

			Fat Cell Population		
			Diameter 35 μm or Less		Diameter Greater Than 35 μm
Age	Body Weight	Depot[c]	Class Interval with Median Value[d] (μm)	% (Range)[e]	Mean ± S.E.M.[f] (μM)
weeks	*g*				
11	239.0 ± 22.3[a] (5)[b]	P	13.1–14.4	7.8 (4–12)	82.9 ± 5.3
12	242.5	I	13.1–14.4	13.6	74.6 ± 15.5[a]
	286.0		16.9–18.1	14.0	73.3 ± 16.6[a]

[a] Mean ± S.D.
[b] Number of animals.
[c] P = perirenal, I = inguinal.
[d] See footnote *d* in Table 1.
[e] See footnote *e* in Table 1.
[f] See footnote *g* in Table 1.

Whether other species would show the existence of bimodality in their adipocyte populations was investigated next with data for the guinea pig epididymal adipose depot as a function of age shown in **Fig. 4.** At 1–2 weeks of age the general population histogram showed a unimodal distribution of adipocytes with a mean diameter of 40 μm (**Table 3**). The VSFC population histogram showed a progressive increase in frequency as adipocyte diameter increased. This distribution of sizes was statistically significant ($P < 0.001$). By 16–20 weeks of age the average diameter of the fat cells had doubled and the histogram displayed a skew to the left but without evidence of bimodality. This was supported by the VSFC population histogram which had a relatively uniform frequency distributed across all diameters ($P > 0.05$). There was no indication of bimodality in the leftward skew of the general histogram at age 52–54 weeks. The average histogram of the small-sized cells suggested that a separate population of VSFC was present but this distribution of cell diameters was not statistically significant ($P > 0.05$). Closer examination revealed that three of the five individual histograms comprising this group had a statistically significant distribution suggesting a VSFC peak ($P < 0.025$). The median diameter of these cells in the guinea pig epididymal pad at this age was smaller than those at earlier ages (Table 3) but somewhat larger than that of the VSFC population of the rat epididymal depot at a similar age (Table 1). In percent of the general population, the VSFC population of the guinea pig epididymal adipose tissue (Table 3) was somewhat less than noted for the rat epididymal pad (Table 1).

Female C57BL/6J (+/+ or OB/+) mice also evidenced VSFC populations in their adipose depots. Histograms for the parametrial and perirenal fat pads of an 11- and a 14-week-old mouse are given in **Fig. 5.** In the 11-week-old mouse the general adipocyte histograms for the two depots did not show much of a leftward skew nor any indication of bimodality, but histograms for the smaller sized fat cells revealed such a population peak existed in the parametrial pad at this age ($P < 0.001$) but had not formed yet in the perirenal pad ($P > 0.05$). In the 14-week-old mouse, both types of histograms showed that a VSFC population existed in each depot and each VSFC population histogram was statistically significant ($P < 0.001$) in its size distribution. The median diameter of these populations in each depot (Fig. 5) was comparable to that found for the rat epididymal pad (Table 1) except for the perirenal tissue in the 11-week-old which was larger. The percentage of VSFC in the adipocyte population (Fig. 5) was similar to those for the rat (Tables 1 and 2).

Very small fat cell populations were found as well in several depots of adult female cats and rabbits (**Fig. 6**). In the cat only the general adipocyte histogram for the subcutaneous depot showed a skew to the left and a suggestion of a VSFC population, while in the rabbit neither of these were evident for any depot. Histograms of the VSFC population in the cat perirenal and subcutaneous depots that indicated population peaks were statistically significant ($P < 0.005$). The VSFC histogram for the rabbit perirenal depot also was significant ($P < 0.025$) while that for the parametrial depot, which suggested a peak was present, failed to reach significance ($P > 0.05$). The rabbit subcutaneous tissue VSFC histogram was significant ($P < 0.001$) for a peak near the 35 μm diameter limit.

In both species the median diameters of the VSFC

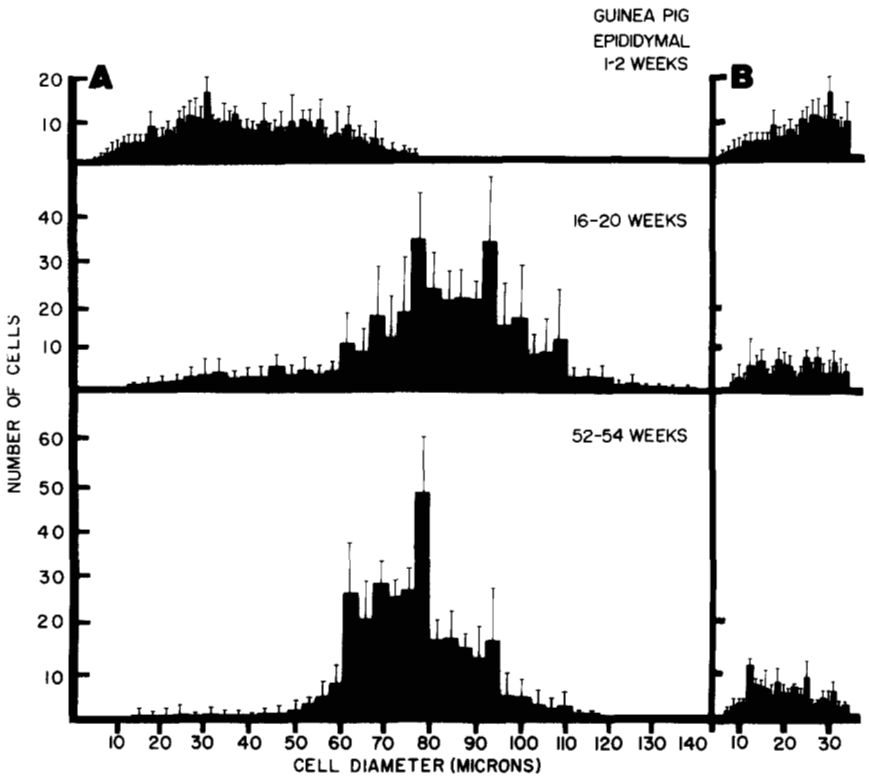

Fig. 4. A, Histograms of the average distribution of adipocyte diameters from the epididymal depots of guinea pigs up to 1 year of age. The average histogram for each age was constructed by sizing 300 fat cells from each of five or six guinea pigs. Each class interval represents the mean ± S.D. of the number of cells in that interval and is 3.12 μm wide except at 1–2 weeks of age where it is 1.25 μm. B, Histograms of the average distribution of adipocyte diameters between zero and 35 μm. The average histogram for each age was constructed by sizing 100 fat cells 35 μm or less in diameter from each of five or six guinea pigs. For the 1- to 2-week-old animals the portion of histogram between zero and 35 μm depicted in Fig. 4A was utilized and had an average of 130 cells. Each class interval represents the mean ± S.D. of the number of cells in that interval and is 1.25 μm wide. Statistical significance of the average VSFC distribution: 1–2 weeks, $P < 0.001$; 16–20 weeks, $P > 0.05$; and 52–54 weeks, $P > 0.05$.

populations were larger (Fig. 6) than that of the rat (Tables 1, 2) but the rabbit subcutaneous VSFC median diameter was considerably larger than those of the other depots and reflected the absence of a very small fat cell peak. In percent of population, the VSFC populations (Fig. 6) are close to that noted for the rat epididymal pad (Table 1) or smaller.

DISCUSSION

The present experiments provide direct evidence that bimodal distributions of adipocyte diameters exist in each of several adipose depots taken from animals of normal body weight representing several mammalian orders. Development of this bimodality is due to the emergence of a population of smaller fat cells of approximately 8–35 microns in diameter in addition to the classic adipocyte population (35–150 microns). We refer to the population of smaller cells by the trivial name of very small fat cells to distinguish them from the usual adipocytes. They cannot be called small fat cells as this term already has been used in another context (26, 27).

Several lines of evidence support the conclusion that the population of fat cells of lesser diameter are truly adipocytes. *1*) Observations with light microscopy indicate they are cells that contain a single, large, spherical inclusion in the cytoplasm. *2*) A nucleus often could be identified within the cell. *3*) The inclusion stains with Oil-Red-O. *4*) The cells float. *5*) Electron microscopy studies indicate a cellular architecture similar to that of classic adipocytes.[3]

There is ample evidence that adipocytes as small in diameter as those found in this study exist as a natural component of adipose tissue. Unilocular fat cells as small as 10–20 μm in diameter have been observed in intact white adipose tissue by investigators using light micros-

[3] Francendese, A., and F. D. DeMartinis. Unpublished observations.

TABLE 3. Changes with age in the VSFC and LFC populations in the epididymal depot of the guinea pig

		Fat Cell Population		
		Diameter 35 μm or Less		Diameter Greater Than 35 μm
Age	Body Weight	Class Interval with Median Value (μm)	% (Range)[e]	Mean ± S.E.M. (μm)
weeks	*g*			
1–2	205.7 ± 23.3[a] (6)[b]	25.6–26.9[c]		40.3 ± 3.3[f]
16–20	904.0 ± 118.4 (6)	23.1–24.4[d]	3.6 (0–8.6)	84.8 ± 7.7[g]
52–54	1363.0 ± 67.0 (5)	19.4–20.6	1.4 (0–6)	77.3 ± 4.9

[a] Mean ± S.D.
[b] Number of animals.
[c] For this age the average population number was 130. The class interval containing the median value was that interval in which the 65th cell occurred.
[d] See footnote *d* in Table 1.
[e] See footnote *e* in Table 1.
[f] See footnote *f* in Table 1.
[g] See footnote *g* in Table 1.

copy (8, 21, 22, 30). Electron microscopic evidence of their existence in intact depots (16) and as small adipocytes in collagenase digests (12) is also available.

Data exist showing that a distinct and separate population of small diameter fat cells within adipose tissue is a real phenomenon and not a consequence of the particular method employed by this laboratory. A bimodal distribution of fat cell diameters has been noted by several investigators using the osmium tetroxide method (14, 18–20, 23–25), by two groups employing thick, frozen, or fixed thin sections of adipose tissue (21, 22), and by other investigators (15–17) using methylene blue-stained preparations from collagenase digests.

Bimodal adipocyte populations have been found in interfascicular fat depots of bovine muscle (14), in subcutaneous backfat of obese cattle (18, 20), in porcine subcutaneous backfat (15–17), in subcutaneous fat of the posterior abdominal area of the female guinea pig (21), and in the perirenal, subcutaneous (22), parametrial, and epididymal (22, 23) adipose tissue of the C57 BL/6J (ob/ob) obese mouse but not in its lean counterpart (23). Subcutaneous and retroperitoneal depots in the female Zucker (fa/fa) obese rat (24) and the epididymal depot in the male (25) have been found to contain two populations of fat cells, but only unimodal distributions were noted in the lean controls.

From these data the impression gained is that bimodality is most likely to be found in adipose depots of farm animals noted for excessive weight gain or in genetically obese animals. Our findings clearly refute that impression. The Fischer 344 rat strain is not noted for excessive gains in body weight and has a growth curve that is consistently below that of the common Sprague-Dawley rat strain. We have shown that the lean C57BL/6J mouse had two populations of adipocytes in at least two depots and that the guinea pigs, cats, and rabbits we used had body weights that were in the normal range.

These findings of a separate population of very small fat cells in a large number of species, in various anatomical locations, in both sexes, in lean as well as obese states, in early maturity, and at advanced ages lead us to suggest that a VSFC population is an integral characteristic morphologic feature of all adipose tissue in the adult mammalian organism.

With these results it now has been demonstrated that at least three separable pools of cells, whose function is related in some manner to triglyceride storage, may be found within adipose tissue. Fibroblast-like cells termed preadipocytes, obtained from the stromal fraction of rat adipose tissue, may be grown in culture and exhibit many of the biochemical characteristics of adipocytes (31–34). In this laboratory we have cultured preadipocytes from the epididymal depots of 3-month-old Fischer 344 rats (31). Such cultures secrete lipoprotein lipase (31), use very low density lipoprotein as a substrate, and store large amounts of cellular triglyceride (33). Thus, preadipocytes, very small fat cells, and adipocytes exist simultaneously in the 3-month-old Fischer rat epididymal adipose tissue. Both preadipocytes (35–37) and a distinct VSFC population[4] have also been detected in adult human adipose tissue. We suggest that the coexistence of these various cell pools in adult fat depots is a general phenomenon.

The normal development of fat tissue in the rat be-

[4] DeMartinis, F. D., A. Francendese, and D. Bartuska. Unpublished observations.

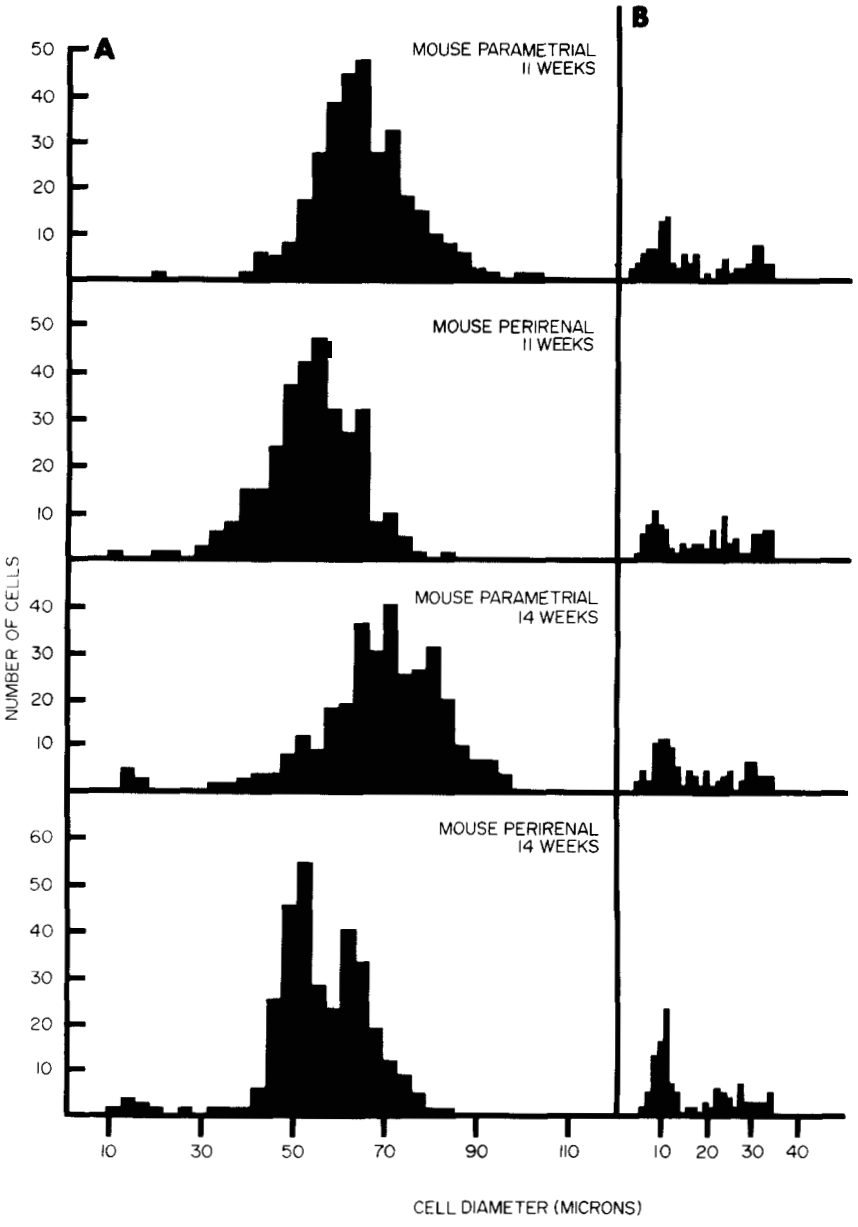

Fig. 5. A, Histograms of the distribution of adipocyte diameters from the parametrial and perirenal depots of C57 BL/6J (+/+ or ob/+) female mice at 11 and 14 weeks of age with body weights 20.9 and 24.1 g, respectively. Each histogram was constructed by sizing 300 fat cells from the depot indicated. The class intervals are 3.12 μm wide. The mean diameter (±S.D.) of the fat cell population greater than 35 μm and the percentage of VSFC in the total adipocyte population were: 11 weeks parametrial, 63.4 ± 10.1, 0.3; 11 weeks perirenal, 53.7 ± 8.4, 5.6; 14 weeks parametrial, 69.1 ± 11.4, 2.6; and 14 weeks perirenal, 55.8 ± 8.5, 3.3. B, Histograms of the distribution of adipocyte diameters between zero and 35 μm. Each histogram was constructed by sizing 100 fat cells 35 μm or less in diameter from the depot indicated. Each class interval is 1.25 μm wide. The class intervals with the median value and the statistical significance of the individual VSFC distributions were: 11 weeks parametrial, 13.1–14.4, $P < 0.001$; 11 weeks perirenal, 19.8–20.6, $P > 0.05$; 14 weeks parametrial, 14.4–15.6, $P < 0.001$; and 14 weeks perirenal, 11.9–13.1, $P < 0.001$.

gins with a distribution of fat cell diameters that is unimodal (Fig. 2). This corresponds in time to the phase of rapid fat cell proliferation of 5–6 weeks duration in which some lipid filling occurs (3, 4). The subsequent rapid lipid filling phase over the next 6–8 weeks results in a marked increase in adipocyte diameters (3, 4) and it is during this period that a bimodal distribution progressively becomes established (Fig. 2). This pattern of bimodality development in adipocyte populations occurs as well in the guinea pig (Fig. 4), pig (15–17), cattle (20), Zucker (fa/fa) obese rat (24, 25), and C57 BL/6J (ob/ob) obese mouse (23). In the Fischer rat epididymal

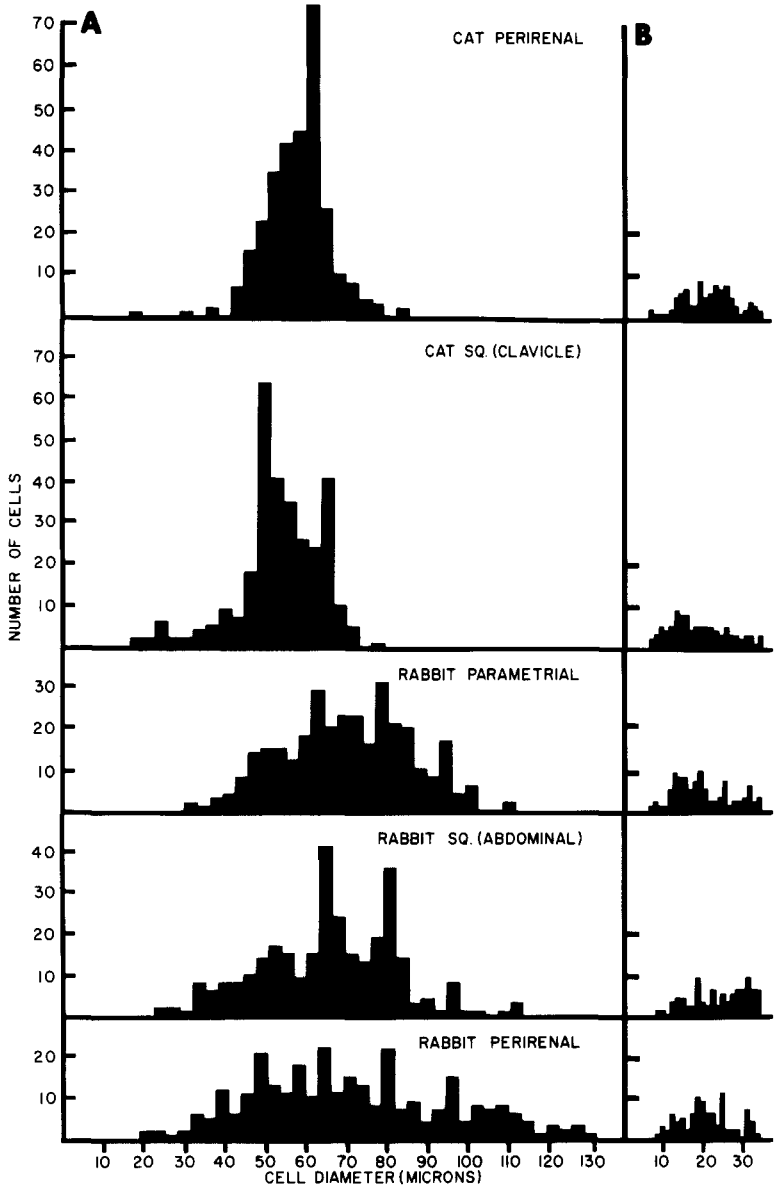

Fig. 6. A, Histograms of the distribution of adipocyte diameters from the perirenal, subcutaneous, and parametrial depots of cats and rabbits. Each histogram was constructed by sizing 300 fat cells from the depot indicated. The subcutaneous depot for the cat is from the dorsal area near the clavicles while that for the rabbit is from the abdominal area. Each class interval is 3.12 μm wide. The body weight (kg), mean diameter (±S.D.) of the fat cell population greater than 35 μm, and the percentage of the VSFC in the total adipocyte population were: cat perirenal, 3.0, 59.7 ± 7.4, 0.6; cat subcutaneous, 2.8, 53.6 ± 7.6, 7.6; rabbit parametrial, 2.3, 71.1 ± 15.4, 1.0; rabbit subcutaneous, 2.3, 66.4 ± 14.9, 6.3; and rabbit perirenal, 2.3, 73.9 ± 22.0, 6.0. B, Histograms of the distribution of adipocyte diameters between zero and 35 μm. Each histogram was constructed by sizing 100 fat cells 35 μm or less in diameter from each depot indicated. Each class interval is 1.25 μm wide. The class interval with the median value and the statistical significance of the individual VSFC distributions were: cat perirenal, 20.6–21.9, $P < 0.005$; cat subcutaneous, 16.9–18.1, $P < 0.005$; rabbit parametrial, 18.1–19.4, $P > 0.05$; rabbit subcutaneous, 26.9–28.1, $P < 0.001$; and rabbit perirenal, 20.6–21.9, $P < 0.025$.

pad, once the VSFC population is established, it is maintained without significant change up to 120 weeks of age. This represents a major portion of the life span of these animals since a longevity study of male rats of the Fischer 344 strain kept under conditions identical with those in our study revealed that the mortality rate is very low before 52 weeks of age, is only 10% at 104 weeks, but is 50% at 120 weeks (38). Although the normal adipocyte population fluctuated in mean diameter during the 14- to 120-week period, it was without apparent effect on the VSFC population. The age-related changes in mean diameter of the large fat cell popula-

tion seen in this study confirm similar results already reported (12).

We chose a diameter of 35 microns as the dividing line between the adipocyte populations since the lowest frequency of cells occurred here in the 14-week-old rat, the first age evidencing a clear indication of a bimodal population. We have adhered to this limit for the purposes of this exploration but claim no special distinction for it. In some of our results alteration of the nadir to a somewhat smaller diameter seems possible, but whether such a change would contribute to a better understanding seems doubtful. After its development in the rat, the epididymal VSFC population stabilized at an average value of 6% of the total adipocyte population. We found values of similar magnitude in other depots and in other species, indicating that when the VSFC population is fully developed in an organism of normal body weight it is a relatively minor component of the adipose depot. In much of the reported data on bimodal populations (14–25), the nadir between the populations occurs at a larger diameter. Since most of these studies utilized the osmium fixation technique of Hirsch and Gallian (7) where cells below 25 microns were discarded, it is not surprising that bimodality would be discovered primarily when the nadir occurred at a greater dimension. What is interesting is that not only is the nadir displaced upwards but the percentage of VSFC found in the general adipocyte population is usually increased as well, and these results seem to occur principally in those animals predisposed to excessive weight gain with adipocyte hypertrophy and hyperplasia (14–25). This led some investigators (14, 18, 21) to suggest that the bimodal distribution of adipocyte diameters should be interpreted as an indication of the hyperplasia occurring in these animals.

Indeed, the very small fat cells may represent newly generated fat cells filling up with lipid. In this model the assumption is made that some preadipocytes have been committed to develop fully into adipocytes and that the VSFC represent a stage in this development. If the persistence of the VSFC population throughout the rat's adult life indicated a continuing hyperplasia, then an increase in adipocyte numbers with the passage of time might be expected to result. Although we did not evaluate fat cell numbers in these experiments, there are several reports in the literature on the number of adipocytes in the various fat depots of the normal ad-libitum, chow-fed rat as a function of age. There is no significant increase in either the epididymal or retroperitoneal depot of the Sprague-Dawley rat between 3 to 6 months of age (2–4, 39). In the lean Zucker rat, the inguinal and epididymal fat pads from 15 to 33 weeks of age (40, 41) and the retroperitoneal, parametrial, and subcutaneous depots when examined from 14 weeks up to a year of age (40, 41) showed no increase in fat cell number, but another study demonstrated an increase in the epididymal pad between 6 months and 1 year of age (42). Using the Fischer 344 rat it was shown previously that fat cell numbers in the epididymal pad rose significantly between 6 months and 1 year of age and again after 2 years of age (12). A later study indicated that no change in adipocyte number took place in the epididymal pad but a significant increase in the perirenal pad occurred between 6 and 18 months of age (43). These results indicate that increases in fat cell number with age occasionally do occur in rats whose caloric intake has not been altered experimentally. The incorporation of labeled thymidine into the adipocyte fraction of adult rats on a chow diet (4, 44) may also be another indication of fat cell generation.

If the presence of a VSFC population in adipose depots represents a process of ongoing hyperplasia, then our data suggest that normal adipose tissue never stops generating additional fat cells. In those depots where adipocyte numbers did not increase, the generation of new VSFC may have occurred at very low levels, too low despite the length of time involved to have resulted in increases detectable by present methods of enumeration. Based on these considerations, we may envision in this model a trickle of VSFC being generated as a normal function of adipose tissue under conditions of normal caloric intake, which would in some manner, under conditions of increased caloric intake, be suitably amplified to an appropriate degree.

An alternative speculation is that the VSFC are preadipocytes that have stored a limited amount of triglyceride but are prevented from continuing additional lipid accumulation as a result of some process regulating the number of adipocytes within the adipose depot. VSFC have the capacity to mobilize their triglyceride stores[3] and, since the quantity of lipid stored in these cells is small, it is conceivable they may completely delipidate under appropriate circumstances. Clark and Clark (45) observed a similar phenomenon during their studies of lipid deposition within fibroblast-like cells in vivo in adult rabbits. Accumulation of lipid over 6–8 days yielded cells comparable in size and appearance to VSFC (see Figs. 3–5, ref. 45) that subsequently delipidated completely in about 24 hours and then began to re-fatten.

Unlike the previous alternative in which the VSFC represented cells already committed to develop into adipocytes, the preadipocytes in this model may cycle through the VSFC stage but the VSFC are restrained (except for a trickle, perhaps) from proceeding onward to the adipocyte stage. Presumably, when an increase in fat cell number within a depot is necessary, greater numbers of VSFC somehow would be triggered to com-

plete their development, enabling them to increase their storage of TG beyond their present limitation.

Other alternative explanations for the VSFC include the possibilities that (3) they may operate chiefly to support adipose tissue functions other than that of triglyceride storage, (4) they may be true adipocytes incapable of developing fully as a consequence of some cellular defect, and (5) they may result from large adipocytes that have lost nearly all of their stored lipid. The role of these cells raises many questions as well as the possibility that understanding their true nature will add significantly to our knowledge of the development and growth of adipose tissue.

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