

Cellularity of adipose tissue in fetal pig

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Abstract Adipose tissue cellularity was studied in the 85-day old Large-White pig fetus. The aim of this work was to count the adipose cells of forming tissue in an animal species which could be a possible model for studying adipose tissue in humans. Using a morphometric method with electron microscopy, mean triglyceride volume per cell was determined independently of mean cell volume. This method is suitable for counting adipose cells in the early stage of differentiation whatever their size and lipid inclusion volume. Site-by-site dissection of adipose tissue was not feasible in the 85-day old fetus and adipose cell number was computed by dividing total carcass triglyceride volume by mean triglyceride volume per cell. The carcass triglyceride seemed to originate only from adipose cells. The mean total carcass triglyceride volume per fetus (1.84 g) was low but, owing to the low mean triglyceride volume per cell ($180.28 \mu\text{m}^3$), the adipose cell number (11.15×10^9) was relatively important, as it represented about 27% of the extramuscular adipose cell number in the Large-White adult pig (41×10^9).—**Desnoyers, F., G. Pascal, M. Etienne, and N. Vodovar.** Cellularity of adipose tissue in fetal pig. *J. Lipid Res.* 1980. **21**: 301–308.

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The quantitation of the adipose mass of an organism that will allow study of the normal or pathological development of the adipose tissues is determined by different methods. In the last 10 years, it has been observed that adipose tissue activity depends on the number of cells. Thus the determination of adipose mass, by estimating the number of adipose cells and their volume, may provide useful data.

Studies have been carried out on several animal species (1–7) and on humans (8–13) with the intention of determining the role of adipose cell number increase (hyperplasia) and adipose cell volume augmentation (hypertrophy) during adipose tissue development in relation to the subject's age.

Most authors agree that adipose cell volume can vary with age, and that it is a factor causing adipose mass development in adults. Nevertheless, the question of the period of adipose cell proliferation still remains unsolved. According to many authors, proliferation would be limited, with a normal diet, to a

period immediately after birth and the time span would vary with the species and the adipose tissue site in the organism.

Other studies report that in some species, especially in the rat (7, 11), the number of adipose cells in adult animals varies with the nutrition received immediately after birth, while in other species such as pigs, nutritional manipulation after birth does not affect the number of extramuscular adipose cells (5, 14, 15). However, some work has shown that high fat diet may induce the adipose cell number to increase in adult rats and mice either in the adipose tissue of certain sites (16) or in the whole of the adipose tissue (17).

Owing to the methods used, the investigators who observed an increase in the number of adipocytes during growth or in adulthood never stated whether the augmentation was due to true proliferation at that stage, or whether it resulted from the filling of pre-existing preadipocytes, or from adipose cells having too low a lipid volume to be counted.

In order to contribute to the solution of this problem, a technique must be used permitting the adipose cells to be counted whatever their developmental stage and their lipid inclusion volume.

We have formerly used a autoradiographic method to determine the intensity of rat adipose cell proliferation when adipose tissue is forming (18). We now estimate the number of adipose cells at an early stage of adipose tissue formation by the morphometry with electron microscopy. For these determinations Delesse's principle (19) was applied, as explained and developed for histological studies in other reports (20–23).

We chose to work with the pig fetus because the slow development of the adipose tissue at the prenatal stage in that species seemed to provide the best possibilities for studying structural and ultrastructural adipose cell development and because this species could be a model for studies of human adipose tissue. Fetuses, 85-days old, were used, first, because that age corresponds approximately to the half-time between the appearance of the first adipose cells in the

TABLE 1. Diet composition (/100 g)

Barley	86.0 g
Soya	10.0 g
Mineral premix ^a	3.6 g
Vitamin premix ^b	0.4 g

^a Mineral premix (%): calcium phosphate dibasic, 78.0; calcium carbonate, 11.6; sodium chloride, 9.0; ferrous sulphate, 0.45; zinc sulphate, 0.7; manganese dioxide, 0.15; cupric sulphate, 0.10.

^b Vitamin premix (%): vitamin D₃ (100,000 UI/g), 0.25; riboflavin, 0.10; ascorbic acid, 0.10; nicotinamide, 0.20; calcium pantothenate, 0.25; vitamin A (50,000 UI/g), 2.0; vitamin B₁₂ (100 mg/kg), 2.0; choline (25%), 20.0; methionine, 10.0; anhydrous glucose, 65.10.

pig teguments and birth, and second, because at that stage lipid inclusion volume in many cells is low as compared to cell volume, thus facilitating a study using electron microscopic morphometry.

MATERIAL AND METHODS

Animals

We used eight 85-day old male fetuses, sampling two random fetuses per litter from Large-White sows fed a balanced diet (Table 1). The fetuses were taken either by operation or after slaughter of the sows. They were weighed and then killed.

Preliminary study

Preliminary observation (24) has shown that, under our experimental conditions, adipose tissue appears at day 50 of gestation and that site-by-site dissection of 85-day old fetus adipose tissue for biochemical measurement is not feasible. Intramuscular adipose tissues are not present at that stage and no significant triglycerides have been detected by either extraction or histological observation of muscle and bone cells. This indicates that triglycerides extracted from the carcass (body without viscera) originate from the adipose cells. It has also been observed that the size of adipose cells and lipid inclusions does not significantly differ from one site to another, even between the visceral and the tegumentary areas, as is the case later during growth.

Adipose tissue preparation

Adipose tissues were taken from different tegumentary sites: the neck, interscapular, dorsal, ventral, and inguinal regions, and from visceral sites: pericardium, epididymis, and kidney. They were taken site by site essentially with the intention of using them for comparison in future studies in older animals.

The tissues were fixed and prepared by the usual techniques (25, 26) for microscopic observation of

semi-thin and thin sections. Total carcass lipids were extracted by the method of Folch, Lees, and Sloane Stanley (27), and the triglycerides were separated from the phospholipids (28).

Sampling

Since the validity of the results obtained by electron microscopic morphometry is directly related to the representativeness of the studied samples, we wanted to be sure that the samples were indeed representative. In the 85-day old fetus, preliminary observation has shown that tegumentary adipose tissue must be oriented perpendicularly to the tegument so as to present the whole depth of the tegumentary adipose tissue. The orientation does not affect the results on visceral adipose tissue.

For these investigations, three pieces of adipose tissue were sampled at each site in the eight fetuses. The tegumentary pieces were taken as far as the muscular sheet. Each piece was cut in 10 blocks, making 240 blocks for each site in the eight fetuses. Fifty blocks per site were then randomly chosen, and a total of 400 blocks were prepared. One or two semi-thin sections were cut from each block to estimate the topographical aspect of adipose cell distribution and to orient the tegumentary adipose tissue so that the thin sections were perpendicular to the tegument. In order to avoid observation of the same cell several times, the micrographs were taken from only one section per block. The surfaces of the 2000 cells and of their lipid inclusions were measured.

Morphometric determination

These determinations were based on Delesse's principle by which the ratio between a section of a solid body constituent area (S') and a body area (S) is directly proportional to the ratio between the constituent volume (V') and the body volume (V):

$$\frac{S'}{S} = \frac{V'}{V}$$

As long as the sections are thin, the particles are well-defined in shape and randomly distributed on the section, and their size is randomly distributed, this principle permits section area measurement instead of volume determination. Adipose cells were considered as spheres. Owing to the physical properties of triglycerides, the lipid inclusion was spherical, and its size and distribution in young adipose cells corresponded to Delesse's principle.

Adipose cell diameter and area. The surface of a single section randomly taken from each cell (2000 cells) was ascertained by superimposing a grid with meshes corresponding to an area of $1 \mu\text{m}^2$ surface with the

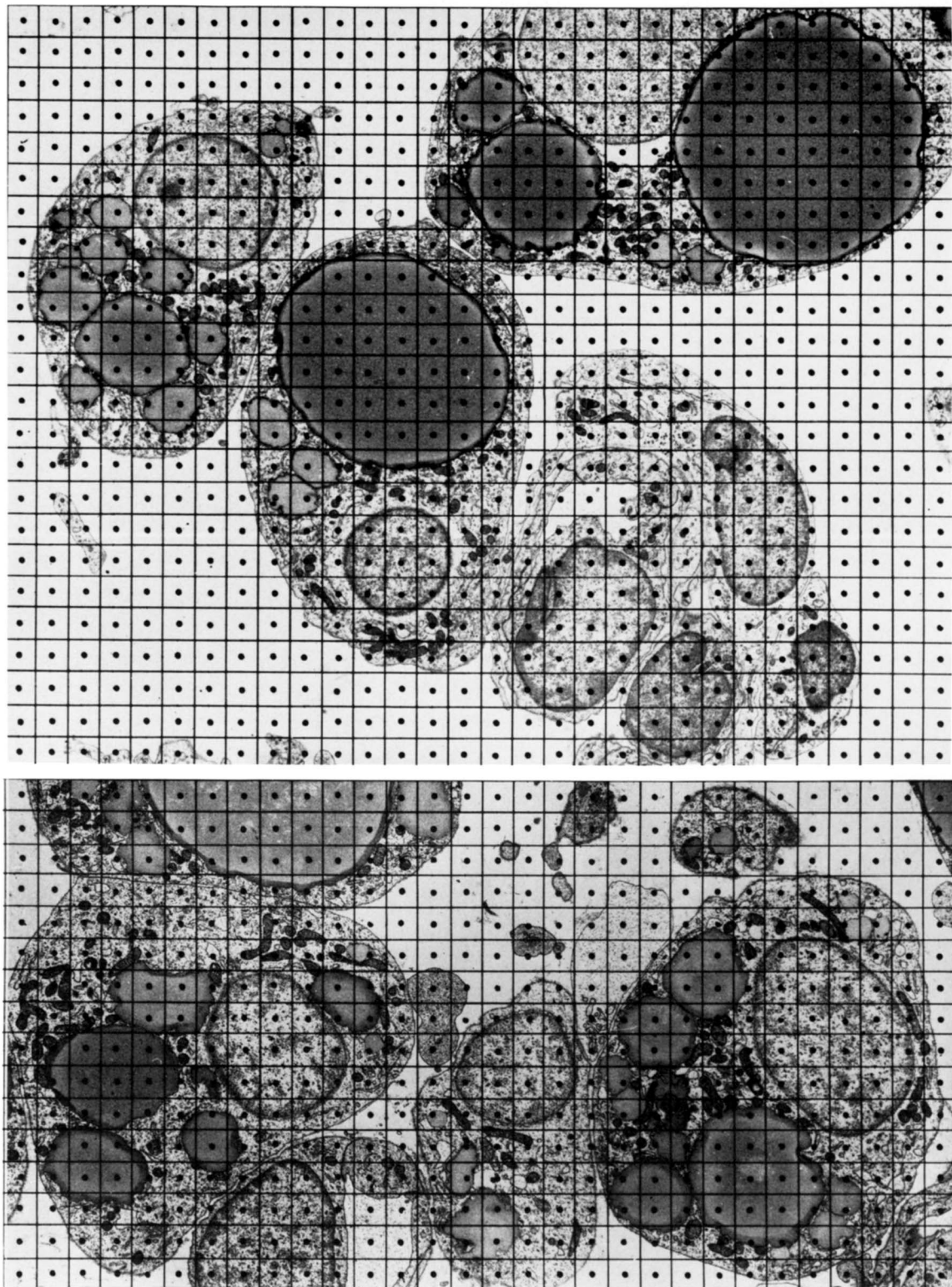


Fig. 1. Determination of adipose cell and lipid inclusion volumes. The mesh of the superimposed grid corresponds to a $1 \mu\text{m}^2$ surface of the tissue studied ($\times 5,000$).

enlargement used (**Fig. 1**). The diameter, d , of each of these cell sections was computed from the measured surfaces. Owing to their wide dispersion, the

diameters, d , were arranged in classes whose number had to be between 10 and 16 (22). From this histogram, the mean diameter, \bar{d} , of the whole of the cell section

TABLE 2. Fetal weight and carcass lipid analysis

Fetus No.	Weight	Water	Dry Matter	Lipids	Tri-glycerides	Phos-pholipids	Triglyceride Weight/Fetus
	g			%			g
1	503.7	87.98	12.04	1.11	0.41	0.70	2.06
2	435.2	88.26	11.74	1.08	0.39	0.69	1.70
3	420.6	87.79	12.21	1.12	0.42	0.70	1.77
4	497.7	87.23	12.77	1.11	0.41	0.70	1.99
5	416.2	87.87	12.13	0.99	0.38	0.61	1.58
6	414.8	87.80	12.20	1.07	0.42	0.65	1.74
7	466.3	86.96	13.04	1.12	0.41	0.71	1.91
8	498.1	87.75	12.25	1.09	0.40	0.69	1.99
Mean	456.58	87.71	12.30	1.09	0.41	0.68	1.84
± SEM	± 13.92	± 0.15	± 0.14	± 0.02	± 0.005	± 0.012	± 0.06

was computed. Mathematical proof has shown that the mean diameter, \bar{d} , of the random sections of the sphere with D equatorial diameter is equal to $(\pi/4)D$ (22, 23). Therefore, the mean diameter, \bar{d} , of the adipose cell section was related to the mean equatorial cell diameter, \bar{D} , by $\bar{d} = (\pi/4)\bar{D}$ in which $\bar{D} = (4/\pi)\bar{d}$. The mean cell area was obtained by the formula $S = \pi\bar{D}^2/4$.

Lipidic inclusion diameter, area, number, and volume. The number of lipid inclusion profiles for each adipose cell section studied was determined. Lipid inclusion mean equatorial diameter, \bar{D} , was computed by the previously described method, and the mean surface of the lipid inclusion was calculated by the relation $S = \pi\bar{D}^2/4$. Mean lipid inclusion volume, V_i , was obtained by the formula $V_i = \pi\bar{D}^3/6$. The mean number of lipid inclusion profiles, \bar{n} , per adipose cell section was obtained by dividing the total number of lipid inclusion profiles counted on the cell sections by the total number of cell sections.

From the mean number of lipid profiles, \bar{n} , per adipose cell, the mean number of lipid inclusions per cell, N_v , was obtained by the relation of Weibel and Gomez (20):

$$N_v = \frac{1}{\beta} \times \frac{\bar{n}^{3/2}}{\rho^{1/2}}$$

(β , coefficient of form = 1.38 for the sphere; ρ , ratio between the mean surface occupied by the lipid profiles (mean profile surface of a lipid inclusion \times mean number of lipid inclusion profiles, \bar{n} , per cell section) and the mean surface of the cell section). The mean triglyceride volume per cell, V_c , was obtained by multiplying the mean volume of a lipid inclusion, V_i , by the mean number of lipid inclusions per cell, N_v . $V_c = V_i \times N_v$.

Adipose cell number. The number of fetus adipose cells was the quotient of total carcass triglyceride volume (weight of extracted triglycerides divided by

triolein density: 0.915) divided by mean triglyceride volume per cell.

RESULTS

Fetal weight and carcass lipid analysis (Table 2)

At 85 days each fetus weighed between 414.8 g and 503.7 g, with a mean weight of 456.58 g (± 13.94 g SEM). The extracted carcass triglycerides weighed between 1.58 g and 2.06 g, depending on the fetus; this gave a mean value of 1.84 g (± 0.17 g SEM) per fetus and corresponded to 0.41% of the mean fetal weight. The mean triglyceride volume per fetus (triglyceride weight divided by 0.915) was about $2.01 \times 10^{12} \mu\text{m}^3$.

These results showed that, in our conditions, fetal weight as well as triglyceride weight per fetus was rather homogeneous, although the fetuses were selected at random from four different litters.

Morphological observations

Topographic distribution of cell aggregates, with or apparently without lipids, was relatively comparable at all sampling levels on semi-thin sections of tegumentary adipose tissue, oriented perpendicularly to the tegument surface and extending to the muscle layer. A conjunctive lamina separated the tegumentary adipose layer into an external and an internal layer. The conjunctive lamina was present in the whole tegument but differed according to the level; it was uniform in the dorsal, interscapular, and neck regions, but relatively distended and irregular in depth in the inguinal and ventral areas.

The aspect of adipose cell aggregates was usually comparable in all sites but sometimes the adipose cells were more developed in the internal layer. Aggregate distribution in visceral adipose tissue was somewhat

TABLE 3. Adipose cell and lipid inclusion numerical data

No. of Fetuses	Body Weight (g)	Triglyc. Weight (g)	Triglyc. Volume $\times 10^{12} \mu\text{m}^3$	Adipose Cell Diameter μm	Lipid Incl. Diameter μm	Lipid Incl. Number per Cell	Lipid Vol. per Cell μm^3	Adipose Cell Number $\times 10^9$
8	456.6 ± 39.42	1.84 ± 0.17	2.01	12.15	3.26	9.94	180.28	11.15

different according to the site, and thus pericardiac aggregates formed a layer while perirenal aggregates were clustered. At that stage of development, the aggregates were usually composed of a similar number of cells and were generally separated from one another. The cells, with or apparently without lipid inclusions, were all grouped around one or more blood capillaries which were formed or forming. These data, obtained from semi-thin section observation, permitted us to better understand adipose tissue development at that stage and to consider it in the thin section preparation and orientation.

Electron microscopic examination of the cells from aggregates at different developmental stages showed their specific ultrastructural characteristics, such as the basement lamina, the endoplasmic reticulum, and the mitochondria. At the onset of lipogenesis, electron microscopy showed very small lipid inclusions which were invisible under a light microscope; the lipid volume, however, could only be estimated by electron microscopic morphometry. Adipose lipid inclusions were often numerous, except at the beginning of lipogenesis, and lipid volume per cell was, on the whole, very much lower than cell volume.

Lipid inclusions were more numerous in cells of the external tegumentary layer than in cells of the internal tegumentary layer, even if they were of the same size and at a presumed comparable stage of development. Visceral adipose tissue cells, owing to their cytoplasmic components, seemed to develop faster than tegumentary adipose tissue cells.

Morphometric data: number of adipose cells (Table 3)

Adipose cell diameter and volume. On electron micrographs (enlarged $\times 5000$) of adipose tissue sections from various visceral and tegumentary sites of the 85-day old pig fetus, we determined the section surface of 2000 adipose cells taken at random using the method explained above. The diameters, d , computed from measured cell section surfaces, ranged between 3 and 25 μm . Owing to this wide dispersion, the whole of the \bar{n} values were distributed into 11 classes. Diameters of less than 5 μm were assigned to the first class, and the succeeding classes were grouped in increments of 2 μm , so that the 11th and last class included values higher than 23 μm and reaching 25 μm . Using this

distribution, we computed that the mean diameter, \bar{d} , of the 11 classes was 9.54 μm .

The mean cell diameter, \bar{D} , considered as being the equatorial section diameter of a spherical, medium-sized cell, was 12.15 μm . This value was obtained from the transformation of the mean section diameter, \bar{d} , using the formula:

$$\bar{D} = \frac{4\bar{d}}{\pi} \quad \text{thus} \quad \bar{D} = \frac{4}{3.14} \times 9.54 = 12.15 \mu\text{m}.$$

As shown in the histogram (Fig. 2), the class having the highest frequency was that with a diameter, d , of about 8 μm and comprising 470 of the 2000 cell sections studied. According to the formula $V = \pi\bar{D}^3/6$ the mean volume of the 2000 adipose cells, computed from the mean cell diameter, \bar{D} , was 939.13 μm^3 .

Diameter, volume and number of lipid inclusions. In the 2000 sections studied, 7360 lipid inclusion profiles, or a mean of 3.68 lipid inclusion profiles per cell section, were counted. These profiles were unequally distributed among the cell sections. As shown on the histogram (Fig. 3), sections having one or two lipid inclusion profiles were the most numerous. Sections presenting more than 10 lipid inclusion profiles were rare, while about 170 sections out of the 2000 studied had no lipid inclusion profiles.

As for the cell section diameters, the diameter, d , values of the 7360 lipid inclusion profiles were computed from the surface measurements of each lipid inclusion profile; these values ranged between 0.5 and 12.5 μm . To determine the mean diameter, \bar{d} , of the

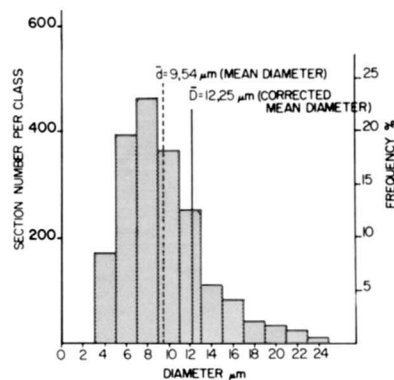


Fig. 2. Distribution of adipose cell section diameters observed on random sections of 85-day old pig fetuses (2,000 sections).

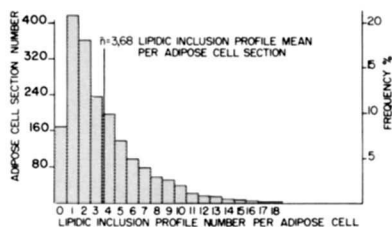


Fig. 3. Distribution of lipid inclusion profiles per random adipose cell sections of 85-day old pig fetuses.

whole of the 7360 lipid inclusion profiles, the diameter values were divided into 12 classes of 1 μm interval (**Fig. 4**). Lipid inclusions were more numerous in the first class with diameters ranging between 0.5 and 1.5 μm , and represented 35% of all the lipid inclusion profiles. Mean diameter, \bar{d} , of the lipid inclusion profiles, was 2.56 μm , after correction ($\bar{D} = 4/\pi \times \bar{d}$), a value of 3.26 μm was obtained for the mean diameter, \bar{D} , considered as being the equatorial section diameter of a medium-sized lipid inclusion.

The mean volume of the lipid inclusion, computed from the mean diameter, \bar{D} , and using the formula $V = \pi\bar{D}^3/6$, was 18.14 μm^3 .

The mean number of lipid inclusions per adipose cell was obtained from the formula $Nv = 1/\beta \times \bar{n}^{3/2}/\rho^{1/2}$. By introducing the numerical data above (β , coefficient of form = 1.38; \bar{n} , mean number of lipid inclusion profiles per cell section = 3.68; ρ , ratio between the mean surface occupied by the lipid inclusion profiles of a cell section = 18.94 μm^2 , and mean surface of a cell section = 71.48 μm^2), we obtained 9.94 for the mean number of lipid inclusions per adipose cell.

Number of adipose cells per fetus. The mean volume of each lipid inclusion was 18.14 μm^3 and the mean number of lipid inclusions computed per cell was 9.94; thus the mean triglyceride volume per cell of the 2000 cells studied in the 85-day old pig fetus was 180.28 μm^3 . The number of adipose cells in a fetus was obtained by dividing the mean triglyceride volume per carcass ($2.01 \times 10^{12} \mu\text{m}^3$) by the mean triglyceride volume per cell (180.28 μm^3) which gave 11.15×10^9 adipose cells per 85-day old fetus.

DISCUSSION

The adipose tissue cellularity of young animals cannot be determined with the methods actually in use (29–31) because a large part of the adipose cells is too small to be counted, owing to the low or negligible lipid volume. Thus, some studies have emphasized (32, 33) the different methods in current use that only show apparent adipose tissue cellularity; they are thus

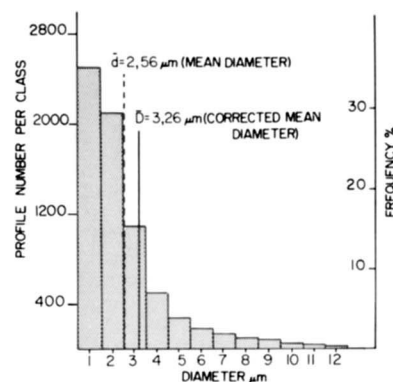


Fig. 4. Distribution of lipid inclusion profile diameters observed on random section of 85-day old pig fetuses (2,000 sections).

unsuitable for ascertaining at what moment adipose cell number truly increases. They are also unsatisfactory for estimating the influence, at different ages, of various factors, such as nutrition, on the increase of cell number.

Electron microscopic morphometry, discussed here and illustrated with results on 85-day old fetal pig adipose tissue, appeared to contribute a solution to the problem of adipose tissue cellularity in the young animal, and eventually to the counting of young adipose cells in growing or in adult adipose tissues. Consequently, we were able to study problems such as the increase in adipose cell number and the role of various factors leading to variation in the rhythm of cellularity kinetics.

The adipose tissue samples used for morphometric study must be representative if satisfactory and repeatable results are to be obtained. Previous studies (34–37) and preliminary investigations showed that the angle of the section had to be considered for tegumentary adipose tissue section in the 85-day old pig fetus. Owing to the nature of adipose tissue, site-by-site dissection was not feasible at that stage of development, and we were obliged to estimate whole body cellularity. Nevertheless, as adipose cell size and lipid volume were comparable in different sites at that stage, and since triglyceride appears to originate only from adipose cells, the results should not be significantly different from those that would have been obtained if site-by-site analysis had been possible.

The volume of the adipose cell nucleus was relatively important compared to the cellular volume, so that some sections showing mostly nuclear profiles made us wonder if the number of lipid inclusions could be underestimated. Nevertheless, we thought that random sections outside of the nucleus were numerous enough to restore the balance, provided that a large number of sections were studied. Since the shape of the lipid

inclusions and the random distribution of their size and number were consistent with Delesse's principle, the morphometric method was as suitable for determining lipidic inclusion volume as for any other intracytoplasmic particle.

During the preliminary study, surface measurement of cells and lipid inclusions, for diameter computation, seemed easier and more suitable than direct diameter measurement. The mean diameter of 85-day old pig adipose cells was only 12.15 μm , showing that adipose cell size at that stage was very reduced and that the usual methods for determining adipose tissue cellularity were impractical since they did not include cells with a diameter of less than 25 μm .

In spite of the very early appearance of adipose cells during fetal life, at about day 50 of pregnancy, the mean amount of total carcass triglyceride (1.84 g) was relatively low, representing only 0.39% of the mean fetal weight.

The mean number of adipose cells estimated by the method used was about 11.15×10^9 in the 85-day old pig fetus, which is apparently large as compared to the number of adipose cells observed in adult pigs by several authors. Lee, Kauffman, and Grummer (15) reported an extramuscular adipose cell population of about 30×10^9 in Poland China \times Duroc pigs, while Hood and Allen (5) found 56×10^9 and 46×10^9 , respectively, in Hampshire \times Yorkshire and Minnesota 3 \times 1. Large-White pigs had an extramuscular adipose cell population of about 41×10^9 (37). Comparing these various results to ours on the 85-day old fetal pig, it appears that at that developmental stage, extramuscular adipose cells represent about 27% of the extramuscular population in adults.

In conclusion, electron microscopic morphometry seems to be a satisfactory method for dealing with the problem of developmental adipose tissue cellularity and the proliferation of adipose cells according to the subject's age and nutrition.

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