Evaluation of Alternative Methods to Prepare Porcine Adipocytes for Measurement with an Electronic Particle Number and Size Determination Apparatus

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ABSTRACT: Experimental investigations with mammalian adipose tissue require a determination of adipocyte number as a basis for expression of metabolic and growth data. Determination of cell size is also important in adipose tissue because the fivefold or greater variation in adipocyte diameter in most growing and adult mammals precludes simple determination of cell number to interpret the biological observations. There are two approaches to determine adipocyte size and number: microscopic methods and electronic particle counter methods. Microscopic methods use embedded sections, frozen sections, or isolated cells, whereas electronic particle number and size instrumental methods use adipocytes released from fixed tissue fragments or adipocytes fixed after isolation. The advantage of the electronic approach is that it evaluates thousands of particles, although the standard fixative, osmium, is quite toxic. Consequently, we evaluated a number of alternative fixation methods to prepare isolated porcine adipocytes for number and size determination by electronic instrumentation. Fixation in 3, 4, or 5% glutaraldehyde or in 4% formaldehyde were not acceptable procedures for porcine adipocytes. The 4% glutaraldehyde fixation procedure was acceptable for isolated rat adipocytes (Stewart and Kaplan, 1993); porcine adipocytes seem to be much more susceptible to breakage using these procedures than rat adipocytes. We also added urea or Triton X-100 to glutaraldehyde- and osmium-fixed cells to decrease clumping and adhesion of individual cells; none of these additions was beneficial. Ability to store samples would improve the logistics for these time-consuming analyses. Samples of osmium-fixed adipocytes were stored in osmium, in .9% NaCl (saline) after removal of osmium, in 8 M urea after osmium removal with saline, or in .01% Triton X-100 after osmium removal with saline. Storage in urea or Triton was inappropriate because of irreversible clumping of individual cells. Storage in osmium was acceptable for at least 30 d, and storage in saline was marginally acceptable. The variability of the size determination process for osmium-fixed adipocytes was evaluated.

Key Words: Adipocyte Size, Adipocyte Number, Pigs, Adipocyte Fixation, Adipocytes

Introduction

Experimental exploration of properties of adipocytes requires the determination of cell number, cell size, and sometimes size distribution. In a given tissue from an individual mammal, adipocytes are not uniform in size, and diameters are distributed over a fivefold or greater range. There are essentially two distinct approaches to determine adipocyte number and size; one uses microscopic evaluation and the other uses an electronic particle number and size determination apparatus. The electronic methods have an advantage over microscopic methods in that they evaluate thousands of particles, whereas microscopic methods evaluate from 50 to 300 particles. All electronic particle number and size methods relate to the original studies of Hirsch and Gallian (1968) wherein small fragments of adipose tissue are fixed in
osmium to preserve their integrity during the preparative handling and counting procedures. In recent years, many studies of adipocyte metabolism have used isolated adipocytes rather than tissue slices or fragments. The osmium fixation method may be applied to isolated cells to prepare them for number and size evaluation (Cushman and Salans, 1978). Recently, glutaraldehyde fixation rather than osmium fixation has been used for isolated rat adipocytes (Stewart and Kaplan, 1993). Because glutaraldehyde is not as toxic as osmium, we investigated the use of glutaraldehyde and formaldehyde fixation for isolated porcine adipocytes. In addition, we determined the conditions for storage of osmium-fixed isolated adipocytes before particle counting/sizing and the variability of the osmium fixation procedure for number and size determination.

Methods

Crossbred castrated male pigs (Dekalb terminal cross offspring from predominantly Yorkshire × Landrace sows mated with predominantly Duroc × Hampshire boars) were obtained from Texas A&M University, College Station. The average weight was 18.1 kg (SD = 4.6 kg). Pigs were killed by captive bolt pistol coupled with exsanguination. The animal procedures were approved by the Institutional Animal Care and Use Committee, Baylor College of Medicine. Subcutaneous adipose tissue was removed from the dorsal neck region (combined outer and middle layers), placed in 0.15 M NaCl, 5.6 mM glucose, 25 mM HEPES buffer (pH adjusted to 7.4 at 22°C) at approximately 35°C, and transported to the laboratory. Tissue slices (approximately .5 mm thick) were prepared, incubated with collagenase in Krebs-Ringer bicarbonate buffer containing glucose under a 5% CO2 in oxygen medium, and the released cells were washed with fresh buffer to remove collagenase. The cells isolated from 1-g tissue slices were suspended in a final total volume of 3 mL of Krebs-Ringer bicarbonate buffer containing glucose. Methods for collagenase digestion of porcine adipose tissue slices to yield isolated adipocytes were previously detailed (Mersmann and McNeel, 1992).

Fixation. An aliquot of a pool of isolated cells obtained from a single animal was fixed in osmium at 37°C for 4 h and then at room temperature for the next 14 to 20 h (.5 mL suspended cells + 3 mL of 3% osmium in .05 M collidine buffer, pH 7.1). Other aliquots from the same pool were fixed in a similar fashion but osmium was replaced by 3%, 4%, or 5% glutaraldehyde in .05 M collidine buffer, pH 7.1, or by 4% formaldehyde in collidine buffer. After 18 to 24 h of fixation, the fixative was removed by filtration through a 250-μm nylon net and particles were trapped on a 10-μm nylon net. Cells were suspended in 15 mL of .9% NaCl (saline); after approximately 4 h the saline was removed and replaced by another 15 mL of saline. After 1 to 4 h, the cells were again filtered through a 250-μm net and trapped on a 10-μm net; the cells were then suspended in .01% Triton X-100 in saline for immediate counting and sizing (unless otherwise indicated). Aliquots of osmium- and of 4% glutaraldehyde-fixed adipocytes were rinsed in saline as indicated above, then suspended in .01% Triton-saline or in 8 M urea overnight, and finally filtered and suspended in Triton-saline for counting and sizing.

In this approach, osmium-fixed cells from an individual animal were the standard or control condition against which other conditions were compared. There were two to four animals represented for each fixation condition (n = 2 to 4 in a repeated measures design with osmium fixation as the standard condition). Multiple aliquots (vials) of adipocytes from each animal were fixed using each of several different fixatives. There were two replicate number/size determinations for each vial; if the average diameter agreed within 1 μm, the two values were averaged, whereas if they did not agree, a third determination was made and the one that did not agree was discarded. For each fixative from a given animal, two vials were counted and sized and if the mean diameter for each vial agreed within 1 μm, the average of the mean diameters for the two vials was used; if the mean diameters did not agree within 1 μm, a third vial was counted and sized, and the one that did not agree was discarded.

Storage. Selected conditions were investigated regarding the effect of storage on cell number and size. Immediately after isolation, an aliquot of adipocytes from a pool was fixed in osmium overnight. The next day, the fixed cells were washed in saline, suspended in .01% Triton X-100 in saline, and counted and sized. These immediately fixed cells were considered the control or standard condition (0 d storage) to which various storage conditions were compared. Other aliquots from the same pool of adipocytes from the same animal were also fixed in osmium overnight immediately after isolation. They were then stored in a) osmium (remained in the osmium after 18 to 24 h), b) saline after post-osmium washing in saline, c) 8 M urea after post-osmium washing in saline, or d) .01% Triton-saline after post-osmium washing in saline. Cells were stored in each of these solutions for 7, 30, or 165 d. At the end of the storage period, they were washed with saline, filtered through a 250-μm nylon net, trapped on a 10-μm nylon net, and suspended in Triton-saline for counting and sizing. Aliquots of non-fixed cells (.5 mL) were also frozen in liquid nitrogen immediately after isolation and stored at −70°C for 7 d; frozen cells were rapidly thawed at 37°C, fixed in osmium overnight, washed in saline, filtered, suspended in Triton-saline, and counted and
sized. Three pigs were used for these experiments (n = 3) in a repeated measures design with cells fixed in osmium immediately after isolation (0 d storage) as the standard condition.

Electronic Instrument. Particle number and size were determined with a Coulter Multisizer particle size analyzer, model 0646 with AccuComp® software and a 400-µm aperture (Coulter Electronics, Hialeah, FL). The nominal particle diameter range for this aperture is 8 to 240 µm. Computer analysis distributes the particles into 256 channels (from 0 to 285 µm) of increasing diameter; the usable portion is set by the nominal range (8 to 240 µm) and the practical range is usually set with a lower limit of 20 or 25 µm to avoid excessive artifacts. The following variables were used to describe the distribution of particles according to size: mean diameter15 = the mean diameter beginning at 14.54 µm, mean diameter20 = the mean diameter beginning at 20.14 µm, 50%20 = the size of the particle representing 50% of the distribution of the total number of particles beginning at 20 µm diameter = the median, 75%20 and 90%20 = the size of the particles representing 75% and 90%, respectively, of the distribution of the total number of particles beginning at 20 µm diameter. The 50%20, 75%20, and 90%20 variables were used because the size distribution was not normal but in all cases contained some indication of a biphasic distribution.

Statistical Analysis. For the fixation data, analysis of variance for repeated measures was used to compare osmium fixation (the standard condition) with each of eight other treatments (3%, 4%, and 5% glutaraldehyde, 4% formaldehyde, 4% glutaraldehyde + urea, 4% glutaraldehyde + Triton, osmium + urea, and osmium + Triton). The effect of method (use of data beginning at 15 µm vs data beginning at 20 µm) and the interaction between treatment and method were also assessed. This analysis was repeated for each of five particle size variables (diameter15, diameter20, 90%20, 75%20, and 50%20). The analysis was done using a GLM procedure to fit the standard repeated measures model, \( y = u + \text{pig} + \text{treat} + \text{method} + \text{treat x method} + e \) (MINITAB, Minitab, Inc., State College, PA).

For the storage experiments, two-way analysis of variance was used to compare each storage condition over time with the control (0 d storage). Pig and condition served as factors in the model. Fisher’s LSD was used to compare the individual conditions.

For analysis of the variability, variance components analysis was used to analyze the variance contribution made by pig, vial (replicate sample from same pig), pour (replicate analysis from same vial), and experimental error. Each of these factors was treated as random in the model \( y = u + \text{pig} + \text{vial} + \text{pour} + e \). MINITAB’s ANOVA procedures with EMS (expected mean squares) subcommand was used to estimate the variance components.

Textual conclusions are indicated as statistically significant at \( P < .05 \) with trends indicated as \( P < .1 > .05 \).

Results and Discussion

General Discussion. Cellularity of adipocytes is an essential component for comprehension of the modulation of adipose tissue growth during animal growth or after genetic, nutritional, pharmacological, or physiological manipulation. In most mammals, growth of adipose tissue is by hyperplasia, hypertrophy of existing cells, or a combination of both. Determination of adipocyte size, mandatory to evaluate hypertrophy, is a difficult task. There are two distinct approaches, microscopic and electronic particle size/number analysis.

Microscopic methods measure adipocyte diameter of paraffin-embedded or frozen sections of tissue or diameters of isolated fixed or unfixed cells. They do not require highly specialized or costly equipment (unless data are processed with digital imaging systems coupled with some automation and complex software programs). Microscopic methods suffer from the small number of cells evaluated (from as few as 50 to a maximum of 300). Electronic particle size and number analysis measures adipocytes obtained from fixed tissue or adipocytes isolated by collagenase digestion of the tissue with fixation after isolation. The basis for the electronic methods is the work of Hirsch and Gallian (1968), who validated the use of osmium fixation coupled with determination of size and number by an electronic particle counter/sizer. These methods require expensive equipment and usually complex computer programs. They have the additional disadvantage that only particles are measured; thus, artifacts such as electronic noise, broken cells, debris from the isolation process, and small adhering cells measured as one large cell are included in the population of particles. There is no way to distinguish the quality of the preparation evaluated or to exclude artifacts from the measurement. The advantage of the method is the very large sample size; literally thousands of particles per sample are evaluated and observer bias in selection of which particular cells to include or exclude from the small sample used in microscopic methods is eliminated. Techniques for measurement of adipocyte cellularity have been critically evaluated and reviewed (Hirsch and Gallian, 1968; Sjostrom et al., 1971; Stern and Conrad, 1975; Cushman and Salens, 1978; Gurr and Kirtland, 1978; Cartwright, 1987).

For mammalian adipocytes, particularly those from agricultural species such as cattle, pigs, and sheep with very large and fragile cells, there are difficulties in releasing the osmium-fixed cells from the tissue matrix in which they are embedded. The approach of Etherton et al. (1977) to treat the fixed tissue with
urea in order to release the cells is a major contribution toward the use of particle counter methods in agricultural species. A number of variables connected with determination of adipocyte size and number in particles isolated by urea treatment of osmium-fixed porcine adipose tissue slices with subsequent electronic particle analysis have been evaluated (Mersmann and MacNeil, 1986). The question of small cells and artifacts in this technique was thoroughly discussed, as was the possibility of detecting multiple populations of cells in the size distribution.

There has been one report of successful electronic measurement of unfixed, isolated rat adipocytes (Maroni et al., 1990); this approach has not been attempted with bovine, ovine, or porcine adipocytes. Speculatively, this approach may not be fruitful with agricultural species because of the large size attained by adipocytes in these species (leading to extreme fragility and cell breakage) and by the notorious adhesion of cells ("stickiness") encountered by investigators who study adipose tissue in these species (D. C. Rule, University of Wyoming and S. B. Smith, Texas A&M University, personal communications; Mersmann, unpublished observations).

Fixation (Figure 1 and Table 1). Osmium fixation is the accepted standard method for preparation of adipocytes for number and size evaluation with an electronic particle analyzer; the diameter of adipocytes prepared from osmium-fixed tissue and electronically sized is the same as the diameter determined by optical microscopy of frozen sections of adipose tissue (Sjöstrom et al., 1971). Fixation using 3%, 4%, or 5% glutaraldehyde was not acceptable for preservation of porcine adipocytes to be counted and sized with an electronic particle counter. The mean diameter (beginning at 20 μm, Table 1 or 15 μm, data not presented) was less for all concentrations of glutaraldehyde fixed than for osmium fixation. The diameter differences of approximately 6.4 μm between osmium fixation and any of the glutaraldehyde concentrations translate to a large-volume difference of approximately 13,000 μm³ (diameter of 39.8 μm = volume of 33,000 μm³ and diameter of 33.4 μm = volume of 19,509 μm³); the mean size of glutaraldehyde-fixed particles was only 59% of the osmium-fixed particles. The major decrease in volume by glutaraldehyde fixation is readily observed in Figure 1, in which the volume has been calculated from the average diameter 20. Variables that represent the distribution of particle size (75%20 in Table 1 as well as data not presented for 50%20 and 90%20) all indicate the particles preserved with glutaraldehyde were smaller than those preserved with osmium. Fixation with formaldehyde produced results similar to those observed with glutaraldehyde fixation.

We attempted to improve both osmium and glutaraldehyde fixation by incubation with urea or Triton X-100 after removal of the fixative and washing with saline. Urea has been used to solubilize adipose tissue slice collagen (Etherton et al., 1977), so the premise was that urea would reduce the adhesion of isolated adipocytes by removal of any remaining collagen fibers associated with the cell surface (see Mersmann et al., 1975 for electron micrographs). Etherton et al. (1977) also added Triton to the final medium to reduce the adhesion of adipocytes when counted and sized with an electronic particle counter (we routinely suspended fixed adipocytes in saline containing 0.01% Triton immediately before the count/size measurements). For glutaraldehyde-fixed particles, urea increased the particle size (diameter and volume) toward that of osmium-fixed cells. Triton numerically increased the particle size of glutaraldehyde-fixed cells, but to a lesser extent than urea. Urea increased the particle size of osmium-fixed particles and Triton tended to increased particle size. Observation of the urea- or Triton-treated osmium-fixed particles in the vials and during handling for preparation to count and size indicated considerable clumping.

### Table 1. Effect of method of fixation on size distribution of adipocytes

<table>
<thead>
<tr>
<th>Fixative</th>
<th>n</th>
<th>Diameter 20</th>
<th>75%20</th>
<th>90%20</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>Mean SD</td>
<td>Mean%20</td>
<td>SD</td>
</tr>
<tr>
<td>OS</td>
<td>5</td>
<td>39.8±3.3</td>
<td>51.0±6.3</td>
<td></td>
</tr>
<tr>
<td>3% G</td>
<td>3</td>
<td>33.4±1.0</td>
<td>37.1±1.4</td>
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</tr>
<tr>
<td>4% G</td>
<td>4</td>
<td>32.9±2.7</td>
<td>36.4±4.6</td>
<td></td>
</tr>
<tr>
<td>5% G</td>
<td>3</td>
<td>34.0±1.6</td>
<td>38.1±3.4</td>
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</tr>
<tr>
<td>4% F</td>
<td>3</td>
<td>32.3±1.1</td>
<td>35.3±2.7</td>
<td></td>
</tr>
<tr>
<td>4% G-U</td>
<td>2</td>
<td>39.4±5.6</td>
<td>46.6±12.3</td>
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</tr>
<tr>
<td>4% G-T</td>
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<td>35.7±1.2</td>
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<td></td>
</tr>
<tr>
<td>OS-U</td>
<td>2</td>
<td>46.0±3.9</td>
<td>59.8±6.0</td>
<td></td>
</tr>
<tr>
<td>OS-T</td>
<td>2</td>
<td>43.3±4.9</td>
<td>54.5±6.6</td>
<td></td>
</tr>
</tbody>
</table>

*Abbreviations: OS = osmium, G = glutaraldehyde, F = formaldehyde, U = 8 M urea, T = .01% Triton.

The mean diameter 20 and the size of the particle (μm) representing 75% of the distribution of the total number of particles beginning at 20 μm (75%20) are indicated. Data expressed as mean and SD in μm. Each variable in a column was compared to OS so that values in a column with superscripts different from c (i.e., d or e) differ from OS at P < .05. The superscript “e” represents P < .1 compared to c.
Figure 1. Mean volume of isolated adipocytes fixed by various methods. The mean diameter beginning at 20 μm was used to calculate the mean volume ($v = \pi d^3/6$) for each fixation condition. No error terms are indicated for these derived numbers; the SD for the mean diameter beginning at 20 μm is indicated in Table 1. Abbreviations: Os = osmium fixation; G = glutaraldehyde fixation (3%G, 4%G, 5%G); F = formaldehyde fixation; U = urea treatment after osmium (Os-U) or 4% glutaraldehyde (G-U) fixation; T = Triton X-100 treatment after osmium (Os-T) or 4% glutaraldehyde (G-T) fixation.

We fixed isolated adipocytes in osmium and stored them for 7, 30, and 165 d in osmium, in saline after removal of osmium with saline, in 8 M urea after removal of osmium with saline, or in saline containing .01% Triton X-100 after removal of osmium with saline. There were few major changes ($P < .05$) in particle size with storage, but there were a number of numerical trends ($P > .05 < .2$) worth mentioning because they were consistently observed for most size criteria and size distribution variables. When compared to standard osmium-fixed cells (no storage), the storage in osmium for up to 165 d, evaluated by particle size, was acceptable ($P > .1$). The particles stored for 165 d tended to be numerically smaller, by all size criteria, than those stored for 0, 7, or 30 d (Table 2). The calculated volumes (Figure 2) indicate the extent of the changes in adipocyte size by the various storage conditions. Osmium-fixed cells stored in saline tended to be smaller than the standard osmium-fixed cells with no storage. Cells stored in saline had more variability (not statistically different to invalidate analysis of variance) than those stored in osmium, as evidenced by the SD for each size variable, which was 2 to 3 times greater than for osmium-stored cells (Table 2 and data not presented). The 165-d saline-stored adipocytes were the largest of the saline-stored group; this may be random variation because we have not observed clumping of these adipocytes with both agents. Thus, clumping obviated the suggested improvement in the methods for glutaraldehyde- or osmium-fixed cells.

Osmium fixation for approximately 24 h remains the method of choice for isolated porcine adipocytes. Shorter fixation times may be acceptable but were not studied.

Storage (Figure 2 and Table 2). It would be convenient to store samples for number and size determination when animal experimental design includes collection of samples over a period of days or even weeks. The electronic particle procedure requires a concerted effort to process and evaluate samples. Consequently, a more efficient approach would be to store samples so they could be processed together at a later time. However, it has been observed by several workers that cattle (D.C. Rule; S.B. Smith, personal communications) and pig (Mersmann, unpublished data) adipose tissue fragments or slices cannot be stored in osmium for extended periods of time. Long-term storage in osmium results in a semi-amorphous mass that cannot be further processed to yield useful data. Osmium-fixed porcine adipose tissue slices may be stored in osmium or perhaps in saline for 30 d with only modest changes in size distribution (Mersmann and MacNeil, 1986). There are no data regarding longer storage times and no data on storage of isolated adipocytes fixed in osmium.
Figure 2. Mean volume of isolated adipocytes fixed in osmium and stored in various solutions. The mean diameter beginning at 20 μm was used to calculate the mean volume \( (v = \pi d^3/6) \) for each storage condition and time of storage. No error terms are indicated for these derived numbers; the SD for the mean diameter beginning at 20 μm is indicated in Table 2. Abbreviations: Os = stored in osmium fixative; S = stored in saline after removal of osmium with saline; U = stored in 8 M urea after removal of osmium with saline; T = stored in Triton X-100 after removal of osmium with saline. Storage times were 0 = fresh cells fixed in osmium overnight, washed in saline, suspended in .01% Triton for counting and sizing; 7 d = stored in osmium, saline, urea, or Triton for 7 d, then storage solution removed with saline, and cells suspended in .01% Triton for counting and sizing; 30 d = same as 7 d but stored for 30 d in indicated solution; 165 d = same as 7 d but stored for 165 d in indicated solution.

Table 2. Effect of storage of osmium-fixed adipocytes on particle size

<table>
<thead>
<tr>
<th>Variable and storage medium</th>
<th>Storage time</th>
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<tr>
<td></td>
<td>OD</td>
<td>7 d</td>
<td>30 d</td>
<td>165 d</td>
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<tr>
<td>Diameter 20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osmium</td>
<td>48.0</td>
<td>47.5</td>
<td>48.6</td>
<td>45.6</td>
</tr>
<tr>
<td>Saline</td>
<td>44.4</td>
<td>45.1</td>
<td>47.1</td>
<td>4.6</td>
</tr>
<tr>
<td>Urea</td>
<td>45.0</td>
<td>47.4</td>
<td>41.8</td>
<td>2.9</td>
</tr>
<tr>
<td>Triton</td>
<td>50.1</td>
<td>51.4</td>
<td>45.7</td>
<td>2.6</td>
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<tr>
<td>75% 20</td>
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<td></td>
</tr>
<tr>
<td>Osmium</td>
<td>66.1</td>
<td>65.9</td>
<td>67.1</td>
<td>62.0</td>
</tr>
<tr>
<td>Saline</td>
<td>60.1</td>
<td>61.6</td>
<td>64.0</td>
<td>7.3</td>
</tr>
<tr>
<td>Urea</td>
<td>60.8</td>
<td>65.0</td>
<td>52.8*</td>
<td>4.8</td>
</tr>
<tr>
<td>Triton</td>
<td>69.1</td>
<td>70.7</td>
<td>61.0</td>
<td>4.2</td>
</tr>
<tr>
<td>Cell number b</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Osmium</td>
<td>6,174</td>
<td>4,746*</td>
<td>4,669*</td>
<td>4,367*</td>
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<tr>
<td>Saline</td>
<td>4,423*</td>
<td>4,368*</td>
<td>3,218*</td>
<td>576</td>
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<tr>
<td>Urea</td>
<td>2,714*</td>
<td>3,503*</td>
<td>2,044*</td>
<td>864</td>
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<tr>
<td>Triton</td>
<td>4,470*</td>
<td>4,944*</td>
<td>2,824*</td>
<td>517</td>
</tr>
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</table>

The mean diameter 20 and the particle size (μm) representing 75% of the distribution of the total number of particles beginning at 20 μm (75% 20) are presented. Data are expressed as the mean with the pooled SD and the P-value for the analysis. *P < .05 when a single storage variable was compared to the 0-d storage value.

The cell number was determined beginning with particles 20 μm in diameter (6,174 = 617,400 particles per .5 mL aliquot of adipocytes isolated from 1 g of tissue and suspended in 3 mL total volume; extrapolation to cells per gram of tissue yields 3,704,400 cells/g).
samples. Samples stored in urea tended (P < .16 for all variables) to be smaller than the osmium-fixed cells with no storage. We have visually observed clumping in preparations stored in urea. Samples stored in Triton for 7 or 30 d tended (P < .2) to be larger than standard osmium-fixed cells with no storage. Clumping was extensive in these preparations. Cells stored in Triton for 165 d tended to be smaller than standard cells as indicated by most variables. The clumping with urea and especially with Triton was not just “stickiness” that could be reversed by washing with saline, but a tight adhesion forming sheet-like masses that could not be disassociated using saline washing techniques.

Cell number data obtained from particles >20 μm diameter (Table 2) indicate a significant loss of particles in all storage groups (> 6,000 in standard osmium-fixed cells with no storage reduced to < 5,000 in stored groups). The urea groups had major losses to <4,000, as did the Triton group stored for 165 d. It is not clear why all groups had a loss of particles. However, these observations imply that stored samples should not be used for direct cell number determinations. Because the size did not change, in cells stored in osmium for 7 and 30 d, cell number may be calculated from the mean diameter of these stored cells; number = \( \frac{10^{12} \mu m^3}{\text{mean volume, where } 10^{12} \mu m^3 = \text{approximate volume of } 1 \text{ g of tissue assuming density of } 1 \text{ and mean volume } = \pi d^3/6 \text{ where } d = \text{mean diameter.} \) (There was no effort to maximize adipocyte yield during these studies. Consequently, the direct cell number on fresh osmium-fixed cells accounts for only 22% of the tissue space [footnote b, Table 2]; this direct number should not be extrapolated to a gram of tissue but is valid for the cell number in an aliquot of cell suspension.)

Isolated adipocytes were frozen in liquid nitrogen as a possible approach to storage. The size of frozen adipocytes (isolated cells frozen in liquid nitrogen, stored at -70°C, rapidly thawed, fixed in osmium, and prepared for number/size determination) was much less than that of fresh adipocytes (isolated cells immediately fixed in osmium then processed for number/size determination) was much less than that of fresh adipocytes (isolated cells immediately fixed in osmium then processed for number/size determination); the mean fresh vs mean frozen, pooled SE for diameter = 38.1, 2.6 μm (P < .05), for calculated cell volume 57,906 vs 13,171 μm³, and for cell number = 617,400 vs 223,200, 666 cells per .5 mL suspension (P < .005). The decrease in cell size and number after freezing suggests freezing caused considerable cell rupture.

We conclude that storage in osmium is acceptable for at least 30 d but probably not for 165 d. Storage in saline does not seem to be as favorable as storage in osmium because of the numerically lower size and the increased variability. Storage in urea or Triton is unacceptable because of cell clumping. Freezing is not acceptable because of excessive cell breakage.

**Variability.** Detailed examination of the variability in the data from the five pigs represented by osmium-fixed cells (Table 1) indicated 83% of the variability was explained by animal variation, < 1% variation was explained by the replicate vials from each pig, essentially zero variation was explained by the replicate counting determination on each vial, and 13% of the variation was unexplained. A second analysis of variation in the methodology used data from Akanbi and Mersmann (1996); this data included four pigs with five adipose tissue depots sampled per pig. Again, replicate vials and replicate determinations on a single vial each explained a small component of the variation (< .1% each). (It should be noted that the method of analysis, which is an analytical chemistry approach that eliminates divergent data, precludes large variation in vials from the same pig or replicate analyses on the same vial. The fact that aliquots from a pool of cells was used for vials also reduced vial variability.) After pooling the very small and nonsignificant variation from vial and determination with error, the animal variation explained 22.1% (P = .1) and the depots explained 2.4% (P = .38) of the variation, whereas the animal × depot interaction explained 70.4% (P < .001) of the variation, leaving 5.1% unexplained as error. When three of the depots were analyzed together (outer subcutaneous from dorsal neck region, middle subcutaneous from dorsal neck region, and ham from the lateral central region), the variation for depot was approximately zero, that for animal = 21.1% (P = .24), that for animal × depot = 72.0% (P = .001), with 7% as error. For the other two depots (mesenteric and perirenal), variation for depots = 16.9% (P = .008), variation for animals = 78.7% (P = .002), variation for animal × depot = 1.3% (P = .009), with 3.2% as error. The variation between sampling sites (i.e., depots) will depend on the breed, the age of the animals, the nutritional status, the environment, and so on. Depot variability in cell size has been documented at a large number of sites in the pig (Mersmann and MacNeil, 1986), as have the disparate growth rates of depots (Mersmann and Leymaster, 1984).

It should be noted that fixation of adipose tissue pieces, fragments, or slices in osmium as used in many studies (e.g., Etherton et al., 1977; Mersmann and MacNeil, 1986) has more vial-to-vial variation than that of isolated adipocytes. The adipocytes are a homogeneous pool of cells with varying size, so a well-mixed pool sample should essentially reproduce any other sample, as we demonstrated by the small variability between vials. On the other hand, individual tissue fragments or slices can have considerable variation in the size distribution of adipocytes contained in the selected portion (see Mersmann et al., 1975 for light micrographs of adipose tissue sections and Mersmann and MacNeil, 1986 to illustrate this point).

**Size Distributions, Artifacts, and Small Cells.** Limits on the detectable cell or particle size are set by the
electronic particle number and size determination instrument. First, the size of the aperture determines the limits of the particles included in the analysis (e.g., a 400-μm aperture has a nominal size range of 8 to 240 μm, whereas a 280-μm aperture has a nominal size range of 5.6 to 168 μm). There is considerable variation in the distribution of cell sizes (obtained with a 400-μm aperture). With relatively young pigs (<40 kg), we often observe a distribution with some small particles < 30 μm and a large number of particles > 30 μm (Figure 3A). We seldom observe a distribution with only a small number of particles <30 μm and mostly with particles >30 μm (Figure 3B). In many studies, regardless of age, we commonly observe a distribution with a large number of particles < 30 μm and with a second population of particles > 30 μm (Figure 3C).

With our current instrument, we have used a 400-μm aperture for most studies and have set the lower limit for data accumulation by the instrument computer program to approximately 15 μm. Although smaller sizes are detectable with a 400-μm aperture, our experience indicates the data for particle sizes from 8 to 15 μm contain considerable noise not filtered out by background subtraction. Although small cells are present, many of the particles represented at sizes < 30 μm are artifacts of free lipid droplets, cellular debris from broken cells, and collagen particles not digested by collagenase when cells are isolated or solubilized by urea when tissue slices or thin pieces are fixed in osmium and subsequently treated with urea to free the particles (see Mersmann et al., 1973, 1975; Mersmann and MacNeil, 1986). The problem of small cells and artifacts in the electronic particle analyzer methodology has been thoroughly discussed when associated with the use of osmium-fixed tissue fragments or slices counted and sized with a particle analyzer (Mersmann and MacNeil, 1986).

As a compromise, we have usually set the lower size limit to 20 μm for data analysis of most of our studies with porcine adipocytes (Figures 3 and 4). Occasionally, if the distribution is markedly skewed to the left because of excessive numbers of small particles, perhaps resulting from excessive cell breakage, it may be necessary to use an instrument cutoff of 25 μm or even 30 μm to yield meaningful data.

When cells from very young animals are sized (Mersmann, 1986), a smaller aperture must be used, e.g., a 280-μm aperture yielding a nominal size range of 5.6 to 168 μm; we collect data from 10 (9.6) μm or in slightly older animals use a practical cutoff of 15 (14.8) μm for data analysis.

After fixation, cells are routinely trapped on a 10-μm net in this laboratory (Mersmann and MacNeil, 1986). However, the original method of Hirsch and Gallien (1968) used a 25-μm net to trap fixed particles; most investigators have continued to use a 25-μm net. Figure 4A represents a distribution of osmium-fixed cells trapped on a 10-μm net and Figure 4B represents cells from the same pool (same animal used to prepare a large pool of cells) trapped on a 25-μm net. There were fewer small (<30 μm) and more large (30 to 60 μm) particles in the distribution that used the 25- compared to the 10-μm net (Figure 4B and 4A, respectively). The 25-μm net probably eliminates an excessive number of small particles that are cells. Because there is no experimental approach to eliminate the artifacts in this technique, we have taken the compromised position to trap particles with a 10-μm net and to begin most size distributions at 20 μm. The choice of 20 μm does not imply there are no cells < 20 μm present but is only a practical, partial solution to a difficult problem. Finally, we would add,
Figure 4. Effect of net size used to trap particles in preparation for counting and sizing. A = particle size distribution for particles trapped on a 10-μm net. B = particle size distribution for particles trapped on a 25-μm net. Both samples were from the same pool of isolated adipocytes. The dotted line indicates the 20-μm diameter lower limit used in most experiments. D = diameter (μm) beginning at 20 μm, 75% = the size of the particle representing 75% of the total distribution beginning at 20 μm diameter, and V = the volume (μm³, calculated from the average diameter 20 as V = πd³/6). P determined from a two-tailed t-test was < .01 for each variable (D, 75%, and V).

In spite of the problems with the semi-automated electronic particle number and size determinations, particle counters have the advantage that they eliminate the bias inherent in the choice of which cells are sized, as well as the limitation of the very small numbers imposed by microscopic approaches to adipocyte size determination.

**Implications**

Metabolic studies of adipose tissue as well as evaluation of adipocyte growth require determination of adipocyte number and size. The electronic particle analyzer has been used to measure thousands of adipocytes in a single sample. Alternative methods of adipocyte fixation were tested to obviate the toxicity of osmium, the standard fixative for adipocytes. With isolated adipocytes, glutaraldehyde and formaldehyde were not acceptable fixatives for these procedures; osmium remains the fixative of choice for porcine adipocytes. Storage of fixed adipocytes would be advantageous to improve the logistics for the cumbersome fixation, counting, and sizing processes. Isolated adipocytes fixed in osmium could be stored for at least 30 d, storage in sodium chloride was marginally acceptable, and storage in either urea or Triton was unacceptable.

**Literature Cited**


