Improved techniques for studies of adipocyte cellularity and metabolism[1]

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Summary Two methods are described for the study of adipose tissue cellularity and metabolism. In the first, 8 M urea was used to liberate osmium tetroxide-fixed adipocytes from the connective tissue matrix. In the smaller-sized cell ranges there was a significant reduction in apparent adipocyte number of rat, pig, and beef adipose tissue with 8 M urea treatment. This was attributed to solubilization of connective tissue debris that was counted as adipocytes in samples isolated without urea. There was no effect on the larger cell-size fractions with 8 M urea treatment. Eight molar urea had no effect on fixed adipocyte retention of radioactivity. The second method entailed the use of hydrogen peroxide to volatilize the black, osmium tetroxide–fatty acid complex of osmium tetroxide-fixed adipocytes, containing radioactivity, resulting in colorless lipid suitable for liquid scintillation counting. This latter technique permits incubation of unfixed adipose tissue slices with a radioactive substrate, followed by fixation with osmium tetroxide and subsequent separation of the adipocytes, by screening, into the desired size ranges. Adipocytes in various size fractions can then be counted, sized, and then decolorized with hydrogen peroxide in order to quantitate the amount of radioactivity within the adipocytes. There was no loss of radioactivity from the fixed cells with hydrogen peroxide treatment.

Supplementary key words cell counting technique · urea · decolorization · lipogenesis

The Coulter Counter and radioactive substrates have been extensively utilized in the study of adipose tissue cellularity and metabolism. Use of the Coulter Counter permits rapid counting and sizing of a large number of adipocytes that have been fixed with osmium tetroxide (2–4). The principle reason for utilizing fixed adipose tissue slices instead of isolated adipocytes has been the observed tendency of adipocytes (especially large cells) to rupture during collagenase preparation and handling (2, 5).[2] Therefore, it is advantageous to fix adipocytes in the connective tissue matrix to prevent cell rupture. However, the determination of cell size and number of fixed adipocytes liberated from the connective tissue is limited by certain technical problems. With the Coulter Counter it is difficult to accurately ascertain cell size, and particularly cell number, for small adipocytes when connective tissue debris from the isolation procedure is present. This debris problem has been circumvented, to some extent, by the use of a nylon screen with mesh openings of 25–30 μm to trap larger cells and allow the debris to pass into the filtrate (2–4). In our laboratory, it routinely appeared that not all the debris was being rinsed away, regardless of the species. In addition, adipocytes smaller than the filter pore size are lost and this presently limits the application of the Coulter Counter in quantitating adipocytes of diameters less than 20–30 μm. Associated with the debris problem has been the tedious problem of liberating fixed adipocytes from the tissue slice by rinsing with copious amounts of water and massaging with a glass stir rod. In view of the potential contribution of connective tissue debris to adipocyte number and the loss of small adipocytes, other methods were investigated that would quantitatively liberate the adipocytes from the connective tissue without having any deleterious effect on adipocyte cellularity. In a system free of connective tissue debris, adipocyte cellularity could then be more accurately determined by the Coulter Counter. Results of those experiments are presented in portions of this paper.

In an effort to study metabolic changes associated with cell size, Bjorntorp and Karlsson (5) first attempted to isolate adipocytes of different sizes. Briefly, their method involved the incubation of human adipose tissue slices with collagenase to liber-

[1] A preliminary report of this work was presented (1) at the Tenth Meeting of the Midwestern Society of the American Society of Animal Science; Ames, Iowa, June 3–4, 1976.

[2] Etherton, T. D., and C. E. Allen. Unpublished data.

ate free adipocytes. The free cells were separated by flotation in dialysis tubing filled with Krebs-Ringer bicarbonate buffer at 37°C. Considering the time involved in preparation of free adipocytes with collagenase and the rupture of larger cells (5), it would be desirable if a technique were available for separation of adipocytes without breakage after incubation with a radioactive substrate.

Gliemann and Vinten (6) recently described a method for measuring incorporation of D-[U-^{14}C]glucose by single fat cells. After incubation, the fat cells were fixed with osmium tetroxide and a single cell was placed in Bray's scintillation fluid for quantitation of radioactivity. In a similar fashion Hansen, Nielsen, and Gliemann (7) measured glucose incorporation in 500–2000 osmium-fixed cells. In the latter study, the fixed cells were fractionated by filtration into different diameter ranges prior to quantitation of radioactivity. However, neither of these methods is adaptable to a larger number of cells, per sample, because of color quenching due to the presence of osmium tetroxide. In both studies, it was not possible to measure glucose incorporation into glyceride-glycerol and fatty acids. In addition, one cannot determine adipocyte cellularity with a Coulter Counter in conjunction with the determination of radioactivity present in different sized cells with such a small number of adipocytes present.

Portions of this report describe a technique developed for the incubation of thin adipose tissue slices with a radioactive substrate, followed by separation of osmium tetroxide-fixed adipocytes into discrete size groups. Adipocytes in the different size fractions were counted and sized with a Coulter Counter. The osmium-fixed adipocytes were then decolorized with hydrogen peroxide in order to quantitate the amount of radioactivity present and its distribution into glyceride-glycerol and fatty acids.

Materials and methods

Male Sprague-Dawley rats (ARS/Sprague-Dawley, Madison, WI) weighing approximately 500 g were killed and the epididymal fat pads were removed. Pigs weighing 90 kg were killed for cellularity studies and samples of the middle layer of subcutaneous adipose tissue opposite the first thoracic vertebra were obtained. Rat and pig adipose tissue utilized for in vitro incubation with D-[U-^{14}C]glucose was placed in 0.154 M NaCl at 37°C immediately post-excision. Beef subcutaneous adipose tissue samples were taken dorsal to the 13th rib and 13 cm lateral to the midline. Beef carcasses had approximately 5 cm of backfat at the point of sampling.

Several proteolytic enzymes (collagenase, papain, ficin, bromelain) were investigated in preliminary experiments in an attempt to solubilize the connective tissue matrix of osmium-fixed adipose slices and obtain a suspension of adipocytes free of debris. However, none of the enzymes displayed any significant capacity to solubilize the connective tissue in the osmium-fixed slices. Subsequent experimentation demonstrated that 8 M urea solubilized the connective tissue matrix and produced a cell suspension devoid of debris.

Urea-isolated adipocytes. Adipose tissue slices (<500 μm thick) of 100–150 mg were rinsed twice in 37°C 0.154 M NaCl. Rinsing was done to minimize the amount of free lipid released from adipocytes ruptured during slicing. If the free lipid was not rinsed away, subsequent fixation with osmium tetroxide resulted in aggregates of fixed adipocytes entrapped by fixed, free lipid and the urea was not able to penetrate the slice or aggregate. Thus, adipocyte number would be underestimated due to the adipocytes entrapped in the fixed lipid. The rinsed adipose slices were then fixed in osmium tetroxide by a modification of the method of Hirsch and Gallian (2). A tissue slice was placed in a 2.5 × 5.0 cm vial (scintillation counting vial) containing 3 ml of 50 mM collidine–HCl buffer, pH 7.4, and 5 ml of 3% osmium tetroxide in collidine buffer was added. Adipose tissue slices were left in the fixative for 72–96 hr at room temperature in a ventilated fume hood. After fixation, the osmium–collidine buffer was removed from the scintillation vial and 10 ml of 0.154 M NaCl was added. The addition of 0.154 M NaCl for 24 hr appeared to facilitate the urea-solubilization of the connective tissue, although this was highly variable among samples. After 24 hr the 0.154 M NaCl was removed and 10 ml of 8 M urea in 0.154 M NaCl was added. Following the addition of 8 M urea, samples were occasionally swirled by hand and left at room temperature. Within 24–48 hr, fixed adipocytes had been liberated from the adipose tissue slice, as evidenced by swirling of the sample and observing the suspension of adipocytes devoid of connective tissue debris. Subsequent microscopic evaluation confirmed the absence of debris.

Fixed and urea-isolated adipocytes were separated into different size ranges by successive filtering through nylon mesh screens with pore diameters of 153, 102, 63, and 20 μm, and collecting the cells retained on the respective screens. Non-urea treated adipose tissue slices were rinsed with copious amounts of distilled water on a 253 μm screen to liberate fixed adipocytes (2). These liberated adipocytes were collected on the above nylon screens as previously described. Adipocytes that were isolated with 8 M

urea and then transferred to 0.154 M NaCl or water had a marked tendency to adhere to each other (clumping) and, as such, could not be accurately counted or sized (**Fig. 1**). However, the intense cell–cell interaction was not observed while in 8 M urea or in the non-urea treated samples, suggesting that the cell surface characteristics were altered by urea treatment. The cellular clumping could not be overcome by rapid stirring while using the Coulter Counter. However, the cellular clumping was eliminated by rinsing the fixed adipocytes through the successive screens with 0.01% Triton X-100 in distilled water (v/v), pH 10. When the different sized cells on the respective screens were rinsed into beakers for eventual sizing and number determination, 0.01% Triton X-100 in 0.154 M NaCl, pH 10, was used. The rinse volume was approximately 30 ml, which was sufficient to prevent clumping when the electrolyte, 0.154 M NaCl, was added to a final volume of 300 ml. Osmium-fixed adipocytes in the respective fractions were then sized and counted using a Coulter Counter (Model B). Adipocytes in the 20–63 μm fraction were counted and sized with the use of a 280 μm aperture. In adipocyte fractions greater than 63 μm in diameter, size and number were determined with a 400 μm aperture.

Adipocyte number was determined at 10-μm diameter intervals. Diameter distributions were then calculated by pooling cell number in each 10-μm diameter range for both the 280 and the 400 μm apertures. Total adipocyte number was ascertained and the percentage of adipocytes in each 10-μm diameter range was determined. Diameter distributions were then calculated and plotted as a histogram with each histogram bar representing the average percentage of adipocytes present in a 10-μm diameter range. All adipocyte number determinations were corrected for coincidence of counting.

Decolorization of osmium-fixed adipocytes. Osmium-fixed adipocytes are black and, in the organic solvent system used for quantitating radioactivity, extreme color quenching occurs. Hydrogen peroxide had previously been utilized to decolor radioactive samples to increase counting efficiency (8). Experiments were undertaken to determine if H_2O_2 would decolorize osmium-fixed fat cells, without volatilizing any radioactivity. The method developed is described herein.

Following excision, adipose tissue was placed in 37°C 0.154 M NaCl. Slices approximately 400–500 μm thick were cut and rinsed thoroughly in warm saline (0.154 M). Slices of 100–150 mg were then incubated in 3 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing one-half the recommended Ca^{2+} concentration with 10 μmol of glucose and 0.1 IU of insulin per ml. One μCi of D-[U-^{14}C]glucose (Amersham/Searle Corp., Arlington Heights, IL) was included in each incubation flask. Details of the incubation procedure have been previously described (9).

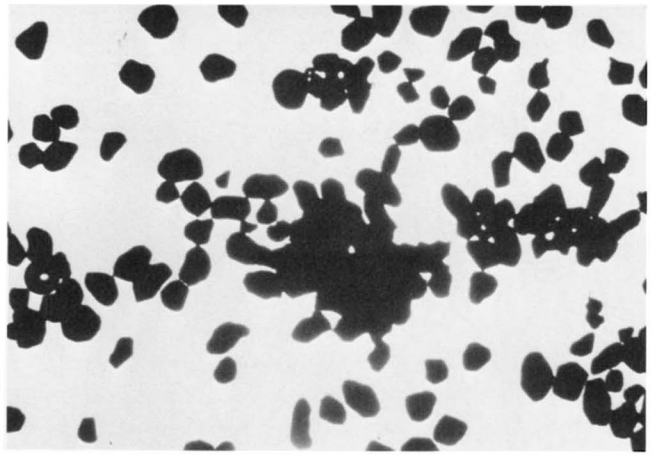

Fig. 1. Clumping of urea-isolated pig adipocytes in absence of Triton X-100. Cell fraction >63 μm; magnification 9.6×.

Techniques for extraction of lipids from unfixed adipose tissue slices after incubation have been described (9). Adipose tissue cellularity was determined from urea-isolated, osmium fixed adipose slices. After the fixed adipocytes in different size ranges were counted and sized, the remainder of the cells in that fraction were collected on a 20 μm nylon screen. The cells collected on the screen were then rinsed into a glass scintillation vial with water. The water was then evaporated in a sand bath at 60°C until approximately 1 ml remained. Then one ml of H_2O_2 (30%) was added and heating (in a ventilated fume hood) was continued to volatilize osmium tetroxide from the adipocytes. A second or third addition of H_2O_2 was made, if needed, to completely volatilize all of the osmium tetroxide, indicated by absence of color in the sample and a white lipid residue in the vial. The water in the samples was evaporated until approximately 1 ml remained. The contents of the scintillation vial were then rinsed into a 2.5 × 9.0 cm glass vial with 19 ml of chloroform–methanol–water 1:2:0.8 (v/v/v). The scintillation vial was rinsed with 5 ml of chloroform and 5 ml of distilled water, yielding a biphasic mixture that was allowed to equilibrate for 24 hr. Techniques for subsequent extraction of lipid and quantitation of incorporation of radioactivity into fatty acids, glyceride-glycerol, and total lipid have previously been described (9), with the following modifications. The total lipid fraction, suspended in chloroform, was transferred to a 200-ml round-bottom flask and evaporated to dryness with a Buchi rotary evaporator. The total lipid fraction was resuspended in 6 ml of chloroform. A 2 ml aliquot was

evaporated to dryness in a scintillation vial and 10 ml of toluene scintillation fluid (6 g of 2,5-diphenyloxazole, 75 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene and 1000 ml of toluene) was added. This fraction represented incorporation of D-[U-^{14}C]glucose into total lipid. Another 2 ml aliquot was utilized for determination of the relative incorporation of D-[U-^{14}C]glucose into glyceride-glycerol and fatty acids as previously described (9). The glyceride-glycerol fraction was counted in 10 ml of PCS solubilizer (Amersham/Searle Corp., Arlington Heights, IL). The esterified fatty acids, now in the form of methyl esters, were counted in toluene scintillator.

In experiments where total glucose incorporation was measured, the water remaining after H_2O_2 treatment was evaporated, the residue was then dissolved in toluene scintillator and incorporation of D-[U-^{14}C]-glucose was determined by liquid scintillation counting.

Differences between two treatments were determined by using a simple *F*-test (10).

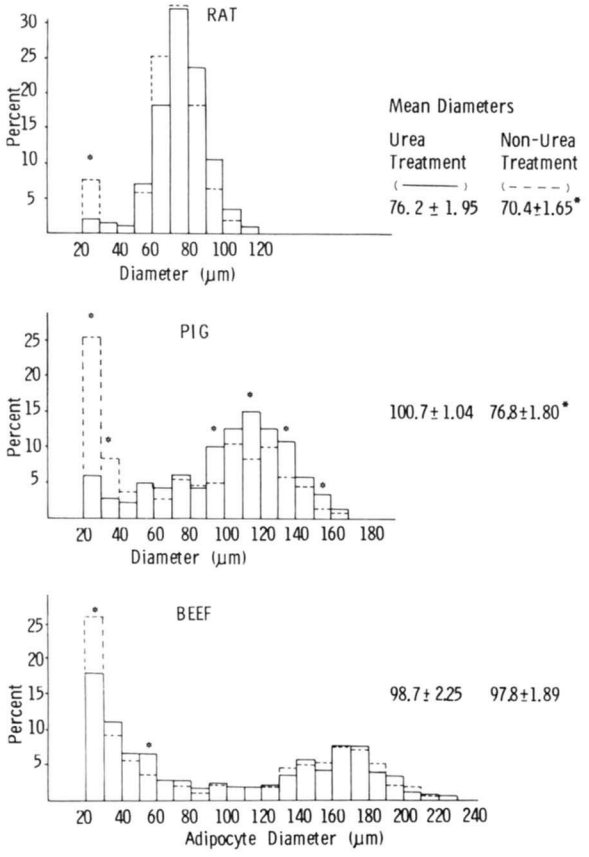

Fig. 2. Diameter distribution histograms representing urea and non-urea treated samples. Asterisk denotes significant differences ($P < 0.05$) between treatments within respective histogram bars, and between means (x̄D) for average cell diameter. Each distribution is for one animal replicated six times.

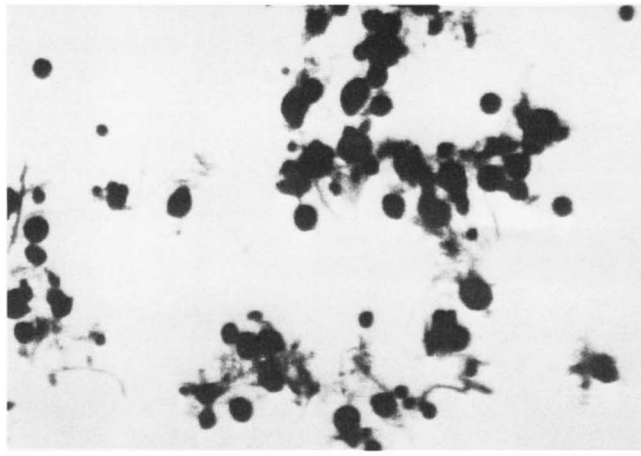

Fig. 3. Pig adipocytes (20–63 μm) isolated in absence of 8 M urea. Magnification 16.1×.

Results and discussion

Urea-isolated adipocytes. Treatment of fixed adipose tissue slices with 8 M urea to liberate fixed adipocytes had a significant effect upon the respective diameter histograms (**Fig. 2**). In all three species there was a marked reduction in the apparent number of cells in the 20–30 μm range with 8 M urea treatment. The greater apparent number of cells in the 20–30 μm fraction in samples isolated without urea was due to connective tissue debris being counted as adipocytes by the Coulter Counter. Eight molar urea treatment solubilized the connective tissue. Examination of fixed and non-urea isolated pig adipocytes in the size range of 20–63 μm revealed that there was a significant amount of debris (**Fig. 3**). During screening, all of the debris passed through the larger sized screens, accumulated on the 20 μm screen (even though profusely rinsed) and was counted as adipocytes re-

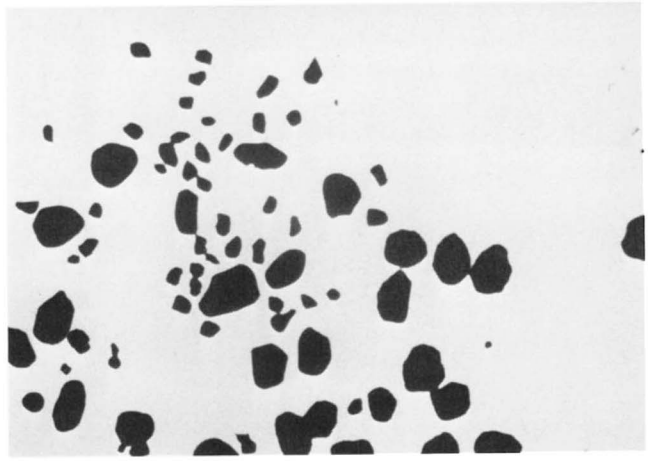

Fig. 4. Pig adipocytes (20–153 μm) isolated with 8 M urea in presence of Triton X-100. Magnification 14.4×.

TABLE 1. Effect of urea isolation of adipocytes on number in rat adipose tissue

Cell Fraction	Without Urea[a]	With 8 M Urea	
20–63 μm	217,900 ± 18,400[b]	147,300 ± 14,200	$P < 0.025$
63–102 μmm	1,729,600 ± 93,800	1,805,700 ± 95,200	NS[c]
Overall	1,947,500 ± 103,600	1,958,000 ± 100,800	NS

[a] Number of fixed adipocytes per gram of adipose tissue was determined by manual liberation of cells from the connective tissue matrix.
[b] Mean ± SEM, comparisons made from same animal with six replications.
[c] NS, means do not differ significantly ($P > 0.05$).

sulting in an erroneously high cell count. Following urea isolation, very little debris was apparent in the samples (**Fig. 4**).

In Fig. 2 the decrease in the percentage of small cells with 8 M urea treatment was reflected by an increased percentage of larger cells, especially for the pig. Since the numbers in Fig. 2 are presented as a percent, a change in actual cell number within one range of the histogram will be reflected by a change in the percentage of all ranges. Pig adipocytes isolated without urea had a markedly lower average cell diameter, which was the result of debris in the 20–40 μm cell range. With urea treatment the connective tissue debris was solubilized and therefore not counted; hence, the upper cell size ranges increased as a percent to reflect this diminished number of counts in the small cell range. Due to the absence of connective tissue debris with 8 M urea treatment, there was a significant increase in average cell size when compared to non-urea treated samples in both pig and rat adipose tissue. However, there was a greater effect in the pig, which can be attributed to the larger observed quantity of connective tissue in pig subcutaneous adipose tissue when compared to the rat epididymal pad or beef subcutaneous adipose tissue. A biphasic diameter distribution of beef adipocytes was apparent in both urea and non-urea isolated samples. This is in agreement with a previous report (11) that in obese beef animals there is a biphasic adipocyte diameter distribution.

Studies were undertaken to determine if urea isolation resulted in cell breakage or loss. Data presented in **Table 1** indicate that there was no significant effect of urea isolation on adipocyte number per g of adipose tissue in the 63–102 μm fraction. However, there was a decrease in apparent cell number with urea isolation in the 20–63 μm cell fraction, indicative of a loss of debris, which was also observed in comparisons between Figs. 3 and 4. Similar results were obtained with pig and beef adipose tissue.

Decolorization of osmium-fixed adipocytes. Comparisons were made with osmium fixed and unfixed adipose tissue slices that had been incubated with D-[U^{14}C]-glucose to determine if there were any volatile ^{14}C-labeled lipid compounds lost after H_2O_2 treatment of fixed adipocytes (**Table 2**). Since there were no significant differences between unfixed and fixed adipose tissue for incorporation of glucose into total lipid, glyceride-glycerol, and fatty acids, one can conclude that removal of osmium tetroxide with H_2O_2 did not cause any loss of radioactivity. This result with adipose tissue is in contrast to the reported loss of some amino acids that were volatilized when subjected to H_2O_2 treatment in an effort to decolorize the sample (12).

Treatment of fixed adipose tissue slices with 8 M urea, to liberate osmium-fixed adipocytes, had no effect on the overall retention of radioactivity that was incorporated by the adipocytes (**Table 3**). On a per cell basis, the nmol of glucose incorporated was equivalent in the 63–102 μm fraction (Table 3). Adipocytes 20–63 μm in diameter isolated with urea

TABLE 2. Effect of hydrogen peroxide volatilization of osmium tetroxide from adipose tissue on retention of radioactivity in total lipid, glyceride-glycerol, and fatty acids[a]

	Total Lipid		Glyceride-Glycerol		Fatty Acids	
Treatment	Rat	Pig	Rat	Pig	Rat	Pig
Unfixed adipose slice	950 ± 38	1746 ± 36	359 ± 12	511 ± 25	534 ± 26	1116 ± 60
Osmium-fixed adipose slice[b]	858 ± 32 NS[c]	1594 ± 30 NS	343 ± 16 NS	526 ± 15 NS	540 ± 35 NS	933 ± 86 NS

[a] All values are nmol of D-[U^{14}C]glucose incorporated per g adipose tissue during a 2 hr incubation. Mean ± SEM from same animal replicated six times.
[b] Following incubation and fixation, adipocytes were isolated with urea and osmium tetroxide was volatilized with H_2O_2 prior to lipid extraction.
[c] NS, means do not differ significantly ($P > 0.05$).

TABLE 3. Effect of urea isolation on retention of radioactivity by different size rat adipocytes[a]

Treatment	20–63 μm	63–102 μm	Overall
Without urea	30 ± 3[b]	330 ± 19	296 ± 16
With 8M urea	57 ± 8	330 ± 16	309 ± 25
	$P < 0.01$	NS[c]	NS

[a] Values are nmol of D-[U-^{14}C]glucose incorporated per 10^6 cells for a 2 hr incubation.
[b] Mean ± SEM, from same animal replicated six times.
[c] NS, means do not differ significantly ($P > 0.05$).

had a significantly higher apparent incorporation of glucose, per 10^6 cells, when compared with adipocytes isolated without 8 M urea. This reflects dilution of radioactivity in the non-urea treated slices by the connective tissue debris. This debris contributed little to glucose uptake, yet was counted as adipocytes. This resulted in a higher cell count than actual and, therefore, a lower calculated glucose incorporation per cell.

Application of the urea technique eliminates manual liberation of fixed adipocytes from the tissue slice. In addition, the suspension of fixed adipocytes is essentially free of all connective tissue debris. However, treatment of fixed adipocytes with 8 M urea results in severe clumping if the cells are not rinsed and suspended in a solution containing Triton X-100. In the absence of Triton X-100 rinsing, this clumping appeared to be less severe in adipocytes from the rat when compared to either pig or beef adipocytes. Ongoing investigations indicate that it may be possible to use the urea isolation of adipocytes for quantitating adipocytes less than 20 μm in diameter.

Hydrogen peroxide in conjunction with mild heat was utilized to decolorize the black, osmium-fixed adipocytes. The decolorization eliminated color quenching. Thus, it is possible to incubate tissue slices with a radioactive substrate, fix the slice, determine adipocyte cellularity, and then quantitate the radioactive lipid present. These two methods have proven very useful in ongoing studies investigating the independent effects of cell size and animal age on adipocyte metabolism.

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