Postnatal development of adipocyte cellularity in the normal rat

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Abstract It has been shown by several investigators that adipocyte number is stable in mature human beings and several species of rodents. Although the number of new cells appearing in the adipose depot can be measured histometrically and by Coulter counting of osmium-fixed cells, such methods do not distinguish between "lipid filling" of preexistent adipocytes and synthesis of new adipocytes. The experiments reported here using in vivo injection of [^{3}H]thymidine show that synthesis of new adipocytes in the Sprague-Dawley rat continues after birth and ceases before sexual maturity. Furthermore, during the second and third postnatal weeks, a "bed" of preadipocytes is synthesized. Preadipocytes may take as long as 30 days to appear as mature adipocytes.

Supplementary key words adipose tissue · preadipocytes · [^{3}H]thymidine · adipose DNA

The details of the regulatory mechanisms whereby normal mature mammals maintain constancy of body weight remain largely unknown. In any pathological disturbance of body weight regulation, it is the degree of caloric storage, primarily in the adipose depot, that changes (1). Depot size changes as the size and/or number of adipocytes present in it change (2–5). In mature man as well as in rats and mice, depot cellularity is known to be stable (5–9). In juvenile-onset obesity in man and in some strains of obese rats and mice, there is hyperplasia of the adipose depot (7–9), but in all cases of obesity, hypertrophy occurs (3, 4, 7–9). Studies have shown that in obese man or animals, reduction of the depot size does not reduce cell number (5, 7). In the rat epididymal fat pad, a depot commonly used in studies of adipose tissue metabolism, growth, and development, there is a continual rise in DNA content that persists until 12 wk of age (10). Similar results are reported when the number of adipose cells is counted by the method of Hirsch and Gallian (11) or by visual estimation (12). In mice, a similar phenomenon is noted, except that cell number stops increasing around 40–60 days of age (9).

In both rats and mice, it appears by both DNA determination and cell counting methods that during the postnatal, prepubertal growth period a maximum number of adipose cells is attained and further growth is a result of cell enlargement and not cell proliferation (5, 11). Thus, various manipulations such as severe starvation, chronic starvation, and hypothalamically induced obesity have no lasting effects on cell number in the adult (5, 13, 14). At 6–12 wk postpartum, when cell number is still increasing as determined by conventional methods, starvation appears to halt cell addition, but refeeding results in a return of the cell number to control levels. Only when the manipulation occurs during the preweaning period is there an ultimate reduction in cell number of the epididymal pad compared with ad lib. controls (5). Lemonnier (15), who reported an increase in adult cell number in rats fed a high fat diet, also found the greatest effect when the nutritional manipulation was started before weaning. These studies could certainly be interpreted to mean that in normal epididymal fat a predetermined cell number exists in the depot that, when reached, permits no further cell addition but only cell size fluctuations. It should be remembered however, as indicated by Peckham, Entenman, and Carroll (16), that an increase in total tissue DNA content, but not necessarily adipocyte DNA, can accompany the normal process of adipocyte hypertrophy associated with the organism's maturation. Furthermore, Rakow, Beneke, and Vogt (14) and Rakow et al. (17) also showed that chronic starvation and refeeding resulted in fluctuation in the number of connective cells and not in the number of fat cells.

Hollenberg and Vost (18) reported that, when adipose tissue was removed from rats in their postweaning, prepubertal growth period 0–2 days after an injection with [^{3}H]thymidine, less than 1% of the radioactivity was present in the adipocyte fraction. The specific activity of the adipocyte fraction rose for 2–5 days and more slowly thereafter. A similar effect in in vitro cultured adipose tissue from rats at weaning was also demonstrated; there was a slight rise in DNA specific activity of the adipocyte fraction after 3 days (19). These studies (18, 19) have

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been interpreted to mean that, within the stromal elements of the adipose tissue, there are primordial adipose cells that give rise to new adipocytes during this period of prepubertal growth. In keeping with this idea, Pilgrim (20) studied the development of the subcutaneous adipose tissue in pre- and early postnatal rats and found that the proliferative index measured by [^{3}H]thymidine radioautography was highest in cells that he suggested were preadipocytes.

In an effort to delineate more precisely those periods in rat epididymal fat pad development when de novo adipocyte DNA synthesis is occurring as contrasted with "lipid filling" of already existent cells, we determined the changes in adipose cell number by Coulter counting of osmium-fixed cells and simultaneously monitored the de novo cell synthesis by determining the specific activity changes in isolated stromal–vascular and adipocyte fractions of adipose tissue after a pulse injection of [^{3}H]thymidine.

METHODS

All animals were male Sprague-Dawley rats supplied by the Holtzman Co. They were fed Purina lab chow ad lib. and housed in a temperature-controlled room with a 12-hr light–dark cycle. Rats were killed by decapitation. Rats from 9 days to 5 months of age were injected intraperitoneally with 40 μCi of [^{3}H]thymidine/100 g of body weight followed 1 hr later by 80 μmoles of unlabeled thymidine per 100 g of body weight. For an initial reference point, groups of animals at each age were killed 1 hr after the second thymidine injection. Other rats received a daily injection of the unlabeled thymidine for 10 days after the injection of the labeled material, and they were killed serially at various times after injection. Two patterns of sequential postinjection killing were used. In one series of experiments, rats were killed at 0, 1, 2, 3, 5, 8, 10, 17, and 120 days after injection. This pattern was used in order to determine a more exact course of labeled adipocyte appearance after injection. In another set of experiments, three time points after injection were selected: zero time, 1 to 2 wk postinjection, and 3 to 4 wk postinjection. The ages of the rats at injection were selected to fall into the following categories: preweaning (9 and 15 days of age), weaning (22 days of age), early postweaning and prepubertal (28, 35, and 49 days of age), and postpubertal (3 and 5 months of age).

Cells were prepared according to the method described by Rodbell (21), except that albumin was not used. In addition, cells and stroma were separated by flotation and washed separately, four times for cells and three times for stroma. This procedure yielded cell preparations devoid of a "pink" lower layer as described by Hollenberg and Vost (18) and a stromal preparation essentially free from lipid droplets. Such adipocyte preparations appear devoid of stromal contamination at the light microscopic level. After the washing procedure, cells and stroma were each brought to a known volume in warm buffer, and aliquots were taken for DNA determination, lipid extraction, and scintillation counting.

It was determined in pilot studies that 800–1200 mg of adipose tissue was needed to prepare cell suspensions yielding sufficient cells and stroma for accurate specific activity determinations, especially in young rats; therefore, the number of rats used for an assay depended on the age of the rats and ranged from 60 rats per pool for 9-day-old rats to 4 rats per pool for 5-month-old rats. In every experiment of this type, two separate groups of rats were used. The epididymal pads from each group were separately pooled.

DNA determinations

The following modifications of the method of Kissane and Robins (22) were used. Cells and stroma were separated as described above, frozen, and thawed. Samples were then extracted twice with ethyl ether and centrifuged, and the ether phase was carefully removed. Samples were then precipitated twice with 5 ml of cold 5% trichloroacetic acid and centrifuged. The samples were then washed twice with 95% ethanol and twice with ethyl ether. After careful drying with air at room temperature, two aliquots were incubated for 90 min at 37°C in phosphate buffer, pH 7.4, containing 0.37 mg/ml DNase (Worthington Biochemical Corp.). The reaction was stopped by the addition of 0.2 ml of cold 20% trichloroacetic acid followed by two 5-ml washes of ethanol and two 5-ml washes of ethyl ether. The DNase treatment procedure was used to provide an adequate control for nonspecific fluorescence in the samples. Standards were prepared from a stock solution of 0.5 mg/ml DNA (calf thymus) in 1 N NH_4OH, and half of these were also treated with DNase. All samples were visually inspected for dryness.

A 30% aqueous solution of diaminobenzoic acid–HCl (Aldrich Chemical Co.) was freshly made before each set of determinations. It was then mixed with 1 g of activated charcoal (Norit) and filtered through one layer of no. 1 Whatman filter paper. 0.2 ml of this filtered solution was added to each sample. The samples were mixed vigorously with a Vortex mixer, placed in a water bath at 60°C for 15 min, remixed in a Vortex mixer, and returned to the water bath for 15 min. The tubes were then diluted to 3 ml with 0.6 N perchloric acid. Samples were mixed again with a Vortex mixer and read in a Farrand Spectrofluorometer with the excitation wavelength at 410 nm and the emission wavelength at 520 nm.

DNA was calculated by subtracting the fluorometric readings of DNase-treated samples from those of untreated

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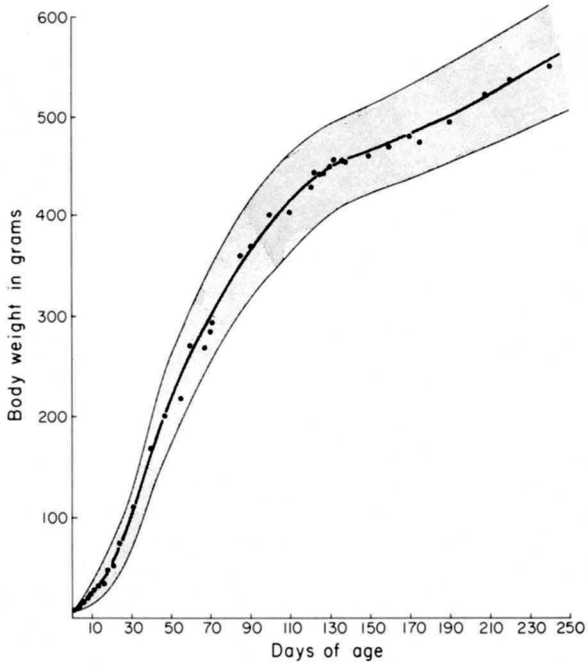

Fig. 1. Normal body weight curve for Sprague-Dawley rats. Each data point represents the mean body weight of 10–24 rats. The shaded gray area represents the mean ± SD.

samples and comparing the differences with a standard curve, which was always linear from 0.5 to 50 μg of DNA. All solutions for this assay were made with glass-distilled water. When specific activity was being measured, duplicate samples were extracted exactly as those for DNA determination, dissolved in hot NH_4OH, and counted in dioxane Cab-O-Sil (New England Nuclear). Samples were counted in a Packard Tri-Carb refrigerated scintillation counter. Counting efficiency was calculated from a prepared quench curve.

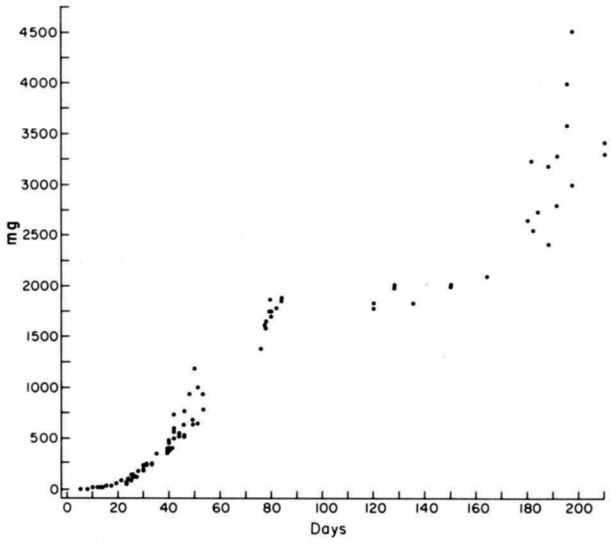

Fig. 2. Epididymal fat pad development. Each data point represents the mean value, or the pooled value, for 4–12 rats.

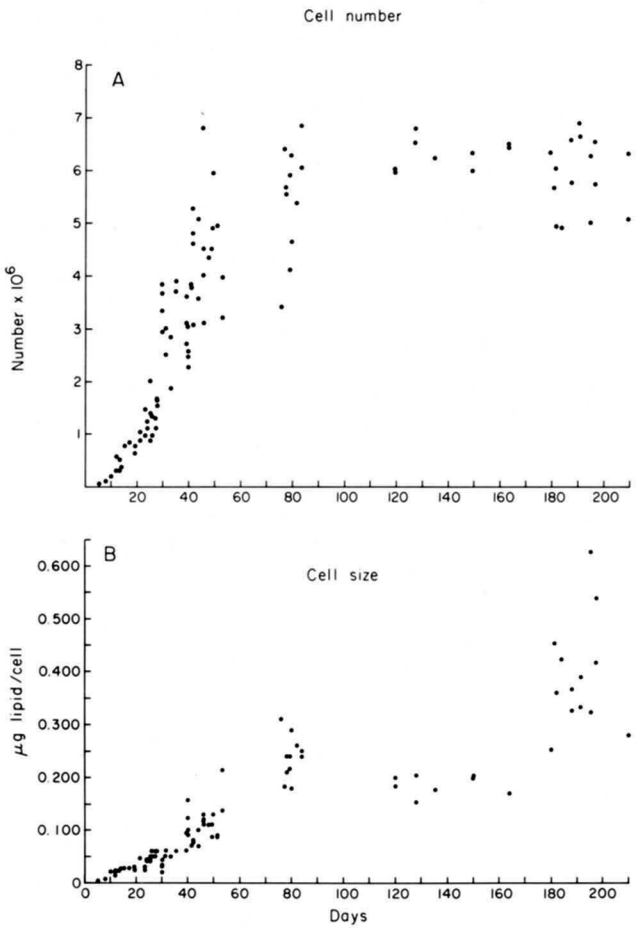

Fig. 3. Cellularity of rat epididymal fat pad. For both cell number and cell size, each data point represents the mean pooled value for 4–12 rats.

Cell counting and sizing

Animals from 5 days to 9 months of age were killed and their epididymal fat pads were removed for cell size determination as described by Hirsch and Gallian (11). In this method, a small weighed piece of tissue is fixed in osmium tetroxide. The fixed cells are separated on nylon sieves, washed, and counted in a Coulter Counter to give the number of cells in a known wet weight of tissue. A separate lipid determination is made on another known wet weight of tissue. Cell size is expressed as micrograms of lipid per cell. A 280-μm (diameter) aperture or 400-μm aperture was used in the Coulter Counter depending upon the range of cell sizes. In this procedure, cells with a diameter below 25 μm or above 250 μm are not counted.

RESULTS

Fig. 1 is the normal growth curve for the many Sprague-Dawley rats used in these experiments. The shaded area on either side of the mean line represents two stan-

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TABLE 1. Mean body weights, cell number, and cell size of rats at time of killing

Age at Start of Experiment	Days Since Injection	Age at Killing	Body Weight	Cell Number	Cell Size
days		*days*	*g*		*μg lipid/ cell*
9 (60)	0	9	25	229,725	0.008
" (30)	15	24	66	1,245,297	0.042
" (16)	30	39	138	3,562,972	0.053
15 (40)	0	15	38	398,071	0.042
" (20)	15	30	99	3,743,875	0.026
" (8)	30	45	181	4,986,732	0.074
22 (20)	0	22	57	1,571,768	0.021
" (20)	1	23	67	1,698,357	0.046
" (20)	2	24	75	1,338,937	0.049
" (16)	3	25	78	1,664,274	0.061
" (16)	5	27	89	1,792,152	0.034
" (16)	8	30	117	1,420,895	0.065
" (12)	10	32	142	2,621,681	0.105
" (10)	17	39	182	5,027,558	0.073
" (6)	90	112	414	6,027,049	0.340
28 (16)	0	28	99	3,135,236	0.036
" (12)	7	35	150	4,794,204	0.044
" (6)	21	49	218	4,932,331	0.090
35 (16)	0	35	134	2,804,224	0.087
" (16)	1	36	159	2,890,572	0.093
" (12)	2	37	159	2,377,262	0.140
" (12)	3	38	178	3,837,684	0.120
" (10)	5	40	187	3,544,207	0.118
" (10)	8	43	199	4,700,783	0.098
" (8)	10	45	229	3,583,748	0.175
" (8)	17	52	251	4,299,413	0.542
" (6)	90	125	439	6,319,214	0.341
49 (12)	0	49	215	3,942,174	0.125
" (10)	6	54	247	4,842,078	0.127
" (8)	27	86	357	4,440,470	0.119
3 mo (8)	0	3 mo	327	5,823,600	0.243
" (8)	15	+15 days	369	4,700,587	0.195
" (6)	32	+32 days	389	6,633,734	0.179
5 mo (6)	0	5 mo	454	6,291,132	0.342
" (6)	1	+1 day	468	5,642,311	0.452
" (6)	2	+2 days	469	5,440,629	0.359
" (6)	3	+3 days	465	4,658,651	0.427
" (6)	5	+5 days	481	6,132,452	0.351
" (6)	8	+8 days	474	6,733,064	0.361
" (6)	10	+10 days	489	5,611,817	0.470
" (6)	17	+17 days	488	6,096,617	0.416
" (4)	90	+90 days	535	6,227,047	0.564

Each value is the mean obtained from two pools of tissue. The number of animals in each pool is indicated in parentheses.

dard deviations from the mean. From this typical growth curve it can be seen that these animals grew continuously throughout life, but the rate of growth slowed at around 112 days of age. The growth of the rat epididymal fat pad is shown in **Fig. 2**. There is an exponential increase in pad weight from 5 days (the earliest measurable point) until 70–80 days of age, then pad weight stabilizes until 180 days of age. After 180 days of age there is a further increase in pad weight, which appears to be increasing at 210 days of age. The contributions of cell number and cell size to this pattern of growth as determined by the Coulter counting method are presented in **Fig. 3**.

When cell number is determined by this technique, cells are very rapidly added from the earliest measurable point (5 days) and continue to be added until 70–80 days of age. Cell size, as determined by counting and lipid determination, changes very little through 21 days of age and then shows a steady increase until 50–70 days of age, when there is a stable cell size until approximately 180 days of age. After 180 days of age, cell size increases again, and this cell hypertrophy occurs at the same time as the pad weight increase. Therefore, one can account for the pad growth by increases in cell number up to 60–80 days of age and increases in cell size throughout the life-span

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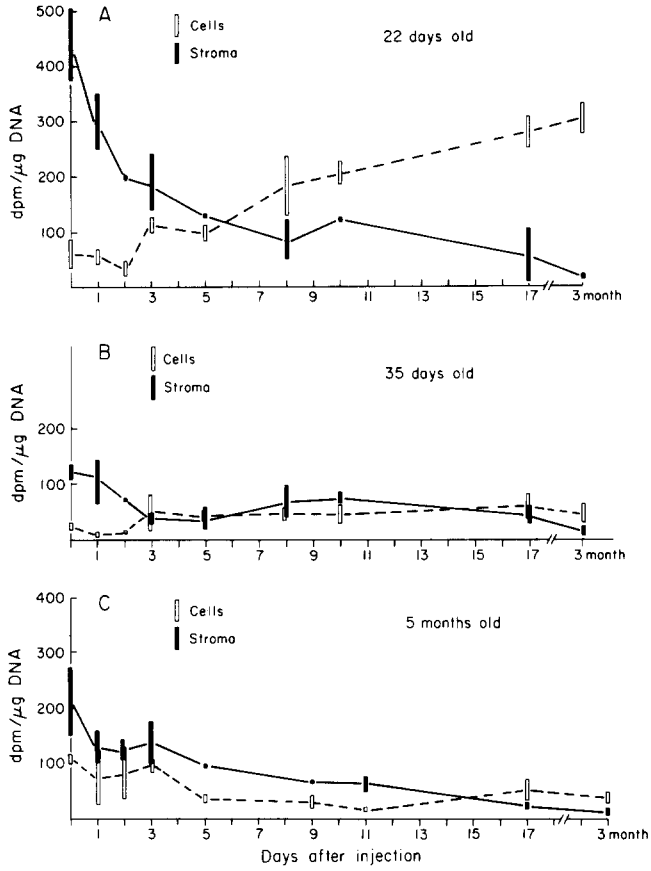

Fig. 4. In vivo injection of [^{3}H]thymidine; specific activity of adipocyte and stromal fractions. Open bars and dotted lines represent the specific activity determined at various times postinjection in the isolated adipocyte fraction. Closed bars and solid lines represent the specific activity determined in the stromal–vascular fraction. Bars represent the ranges of the two pools sampled at each time point.

studied. The late burst in pad weight growth as shown in Fig. 2 is exclusively the result of adipocyte hypertrophy. In all of the injection experiments, rats gained an expected amount of weight during the course of the experiment and showed typical adipose cell numbers and cell sizes for their ages at the time they were killed. **Table 1** gives the mean body weight, mean cell size, and mean cell number for the rats at the age at which they were killed. A comparison of these data to those illustrated in Figs. 1 and 3 indicates that the values are within the expected range.

When methyl [^{3}H]thymidine is injected in vivo and the adipocyte cell fraction and stromal–vascular fraction are separated from epididymal tissue taken from rats killed at sequential time points after the injection, the specific activity of the DNA in both fractions can be monitored over time. Due to the inherent difficulties in obtaining sufficient tissue, as discussed in the Methods section, we used separate duplicate pools of tissue. The range of the data from these two pools is indicated in **Figs. 4** and **5**. Although such data are not easily analyzed by standard statistical methods, it can be established by inspection of the data that the range of values between pools was not so large as to obscure marked increases in adipocyte and decreases in stromal specific activities over time.

Data from all experiments is presented in three blocks. First, the changes in specific activity of adipocyte and stromal fractions during the more detailed studies of rats 22 days, 35 days, and 5 months old are depicted in Fig. 4. Then, in a similar presentation, the specific activity changes for the experiments with three time points are shown in Fig. 5. Finally, the total accumulation of radioactive thymidine into adipocyte DNA over the experimental period is presented in **Figs. 6**, **7**, and **8**. Four basic patterns

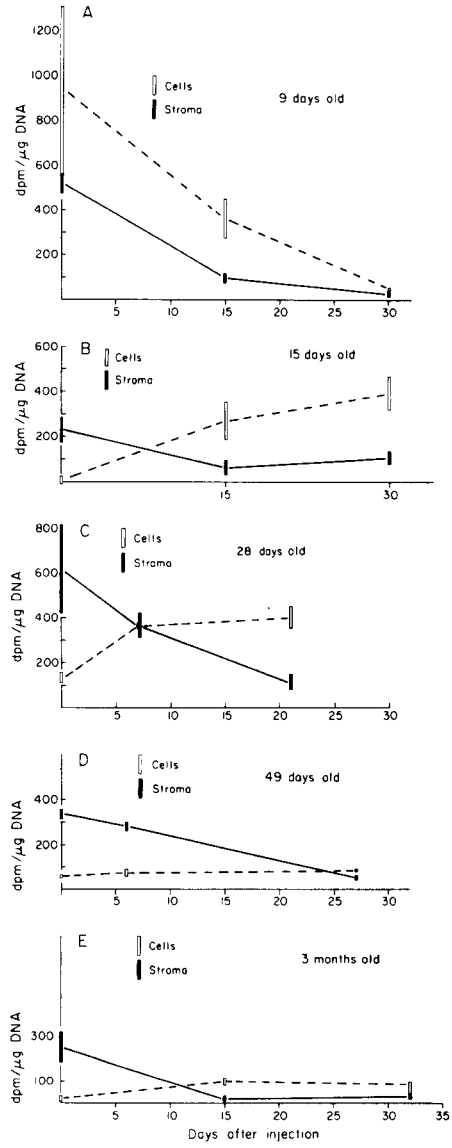

Fig. 5. In vivo injection of [^{3}H]thymidine; specific activity of adipocyte and stromal fractions. Open bars and dotted lines represent the specific activity determined at various times postinjection in the isolated adipocyte fraction. Closed bars and solid lines represent the specific activity determined in the stromal–vascular fraction. Bars represent the ranges of the two pools sampled at each time point.

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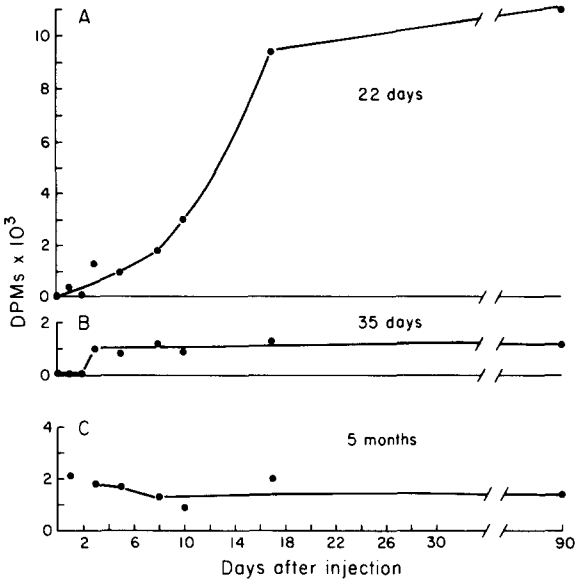

Fig. 6. Total dpms in adipocyte DNA for 22-day-old, 35-day-old, and 5-month-old rats. Each data point represents the mean value for the two pools sampled at each time.

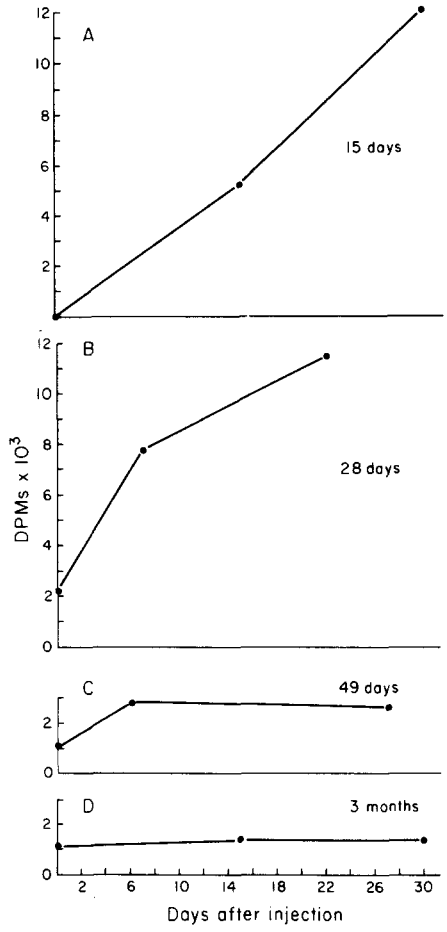

Fig. 7. Total dpms in adipocyte DNA for 15-day-old, 28-day-old, 49-day-old, and 3-month-old rats. Each data point represents the mean value for the two pools sampled at each time.

are immediately apparent in these results: (*a*) in postpubertal rats injected at 3 and 5 months of age, no significant change in adipocyte specific activity occurs (Figs. 4*C* and 5*E*); (*b*) in 35- and 49-day-old rats, a slight increase in specific activity occurs over the experimental period (Figs. 4*B* and 5*D*); (*c*) in rats 15, 22, and 28 days old, the initially low specific activity rises markedly during the postinjection period (Figs. 4*A*, 5*B*, and 5*C*); (*d*) in 9-day-old rats, a very high specific activity 1 hr after injection decreases with time (Fig. 5*A*).

Even with the careful separation and washing procedures employed, some contamination of the adipocyte fraction with stromal cells undoubtedly occurs and renders interpretation complex. By 2–3 days after injection, in all cases, even in 5-month-old animals, some residual label appears to be incorporated into adipocytes. In 5-month-old rats the incorporated label decreases with time (Fig. 4*C*). In 3-month-old rats the slight increase in specific activity seen at 15 days postinjection is absent at 31 days postinjection (Fig. 5*E*). In any event, when these data are expressed as total counts in adipocyte DNA (Figs. 6*C* and 7*D*), at 3 and 5 months of age there is no increase in total radioactivity throughout the experiment. At these ages, Coulter counting of osmium-fixed cells showed no increase in adipose cell number during the course of the experiment (Table 1). These data are, therefore, consistent with the concept that, in the postpubertal rat, adipose cell number is constant in the epididymal pad and is the result of little or no de novo adipose cell proliferation. It seems reasonable, therefore, that the appearance of any label in these postpubertal rats represents stromal contamination. In contrast, in the 35-day-old rats a slight increase in specific activity of the adipocyte fraction is definite and is maintained throughout the experimental period (Fig. 4*B*). In the 49-day-old rats the slight increase in specific activity is less obvious (Fig. 5*D*). Yet, in both 35- and 49-day-old rats, when the data are expressed as total dpms in adipocytes there is a small increase in total counts 3–6 days postinjection that is maintained throughout the experimental period (Figs. 6*B* and 7*C*) compared with no net accumulation of radioactivity in the 3- and 5-month-old rats. Coulter counting of osmium-fixed cells of 35- and 49-day-old rats showed increases in cell number (Table 1)

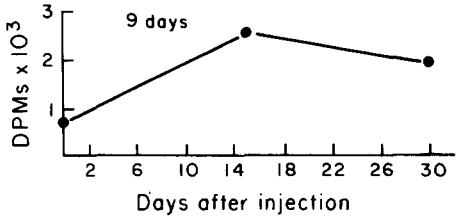

Fig. 8. Total dpms in adipocyte DNA of 9-day-old rats. Each data point represents the mean value for the two pools sampled each time.

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during the postweaning, prepubertal period, indicating that some new adipocyte synthesis is occurring. The newly synthesized cells require 3–6 days to differentiate and to fill with enough lipid to be collected in the adipocyte fraction.

In 22-day-old (Fig. 4*A*) and 15- and 28-day-old (Fig. 5 *B* and *C*) rats a striking change in specific activity of the adipocyte fraction occurs during the course of the experiment. In 22-day-old rats the definite change in specific activity of adipocyte DNA at 3 days continues to increase, reaching, by 17 days after injection, a level three times greater than at the initial time point. This increased specific activity is maintained at 3 months postinjection (Fig. 4*A*). In 28-day-old rats (Fig. 5*C*) there was a 2.5-fold increase by 6 days postinjection that was maintained at 21 days postinjection. In 15-day-old rats there was a threefold increase in specific activity at 15 days postinjection and a fourfold increase in specific activity by 30 days postinjection (Fig. 5*B*). Clearly, in these 15-, 22-, and 28-day-old rats, radioactive thymidine is incorporated into "preadipocyte" DNA, and cells thus labeled require considerable lengths of time to fill with lipid and appear as mature fat cells.

If we consider total radioactivity in adipocytes, a pattern emerges that further substantiates the concept that preadipocytes are rapidly proliferating in 15-, 22-, and 28-day-old rats (Figs. 6*A* and 7, *A* and *B*). At 15 days of age, the rise in total counts at 15 days postinjection is followed by a further increase in total counts by 30 days postinjection (Fig. 7*A*). At 22 days of age, there is a rise in total counts that continues to increase at 17 days postinjection and remains constant at 90 days postinjection (Fig. 6*A*). At 28 days, the fourfold increase in total counts at 6 days postinjection is followed by a 50% further increase at 21 days postinjection (Fig. 7*B*). During these experimental periods, Coulter counting of osmium-fixed cells shows marked increments in adipose cell number (Table 1). These data are therefore consistent with the idea that during the preweaning and early postweaning period the increases in epididymal pad adipose cell number shown by conventional counting techniques reflect very active de novo cell synthesis. In addition, cells synthesized during this period can remain as lipid-depleted "preadipocytes" and take as long as 30 days to appear as lipid-filled "mature" adipocytes.

Rats 9 days of age show a strikingly different pattern. Even at the initial time point they show a variable but clear increase in specific activity in the adipocyte fraction that declines over the course of the experiment (Fig. 5*A*). The expression of the data as total counts in adipocytes indicates that a real incorporation of label into adipocyte DNA occurred even in the 1-hr pulse time in the tissue and remained throughout the experiment (Fig. 8). Coulter counting of osmium-fixed cells during the experimental period shows a 15.5-fold increase in cell number. The data therefore suggest that at 9 days very rapid proliferation and differentiation are taking place. Consequently, the rapid addition of unlabeled cells to the adipocyte fraction results in a decline of specific activity.

DISCUSSION

The results presented confirm and clarify the hypothesis outlined and previously proposed from this laboratory for the development of adipose tissue in the rat epididymal fat pad (11, 23). It was suggested that the rat epididymal fat pad showed an increase in adipocyte number from birth to 12–14 wk of age. Once attained, this adipocyte number could not be changed. There are at least three possible ways by which the growth and development of the epididymal pad as measured by Coulter counting of osmium-fixed cells could be explained. One possibility is that all of the predetermined adipocytes are present at birth. The progressive appearance of adipocytes until maturity, demonstrated by various counting techniques, would then be the result of lipid filling of already existent cells. This type of development is typically found in nervous tissue. In the other extreme of tissue development that could account for a fixed adult adipocyte number, cells are continually proliferating and being removed from the tissue, but the net effect is to build up and then maintain a fixed mass. The hematopoietic system and skin epithelium are tissues characterized by this type of cell mass maintenance. The third possibility is that at birth a few mature cells are detectable, some "preadipocytes" (non-lipid filled) cells are present, and some proliferative cells are present. Proliferative cells continue to divide until some point before maturity, after which no further proliferation occurs, and further development is a consequence of lipid filling. In both postnatal proliferative models, it is possible that at a critical point during postnatal development a burst of proliferation provides a bed of nondifferentiated but predetermined adipocytes to be filled with lipid as the organism matures and requires a larger caloric storage depot. The specific activity studies reported here delineate these processes in detail and distinguish between new cell synthesis and lipid filling of predetermined but already existent cells. The experiments support the third concept, i.e., some proliferation after birth and before maturity.

Several schemes have been presented to explain the normal cellular growth and development of mammalian tissue. They all have the following characteristics in common (24, 25). (*a*) There are three compartments: proliferative, differentiating, and mature; (*b*) cells in the proliferative compartment are made at different rates and at different times, and the rate depends upon a variety of influences, such as hormones, temperature, age, and dietary intake;

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(*c*) cells leave the proliferative compartment and enter the differentiating compartment where they may stay for varying lengths of time, depending again on the presence of stimuli, such as hormones, substrate availability, or temperature changes; (*d*) cells that enter the mature compartment may continue to accumulate differentiation products, enlarge, or die and be lost from the population; and (*e*) there are several feedback loops that can act to control proliferation or differentiation that are derived from within the system itself.

These concepts are useful as a framework when postulating an interpretation of our results. A basic principle of this experimental design is that a single injection of [^{3}H]thymidine followed by an injection of unlabeled thymidine in vivo acts as a pulse, is available to cells synthesizing DNA for only a brief period of time, and is then rapidly cleared from the circulation (25). Accordingly, our data would fit the following developmental pathway.

In the 9-day-old rat, proliferation is extremely rapid. This is supported by both the specific activity study (Fig. 5*A*) and the rapid increases in adipose cell number seen by Coulter counting of osmium-fixed cells (Table 1). The total counts in adipocyte DNA data (Fig. 8) are consistent with the hypothesis that at 9 days of age the process of proliferation → differentiation → maturity is uninhibited and proceeds rapidly. The decline in specific activity after 15 days is interpreted to mean that after several days unlabeled cells in large numbers begin to appear in the mature compartment and the addition of the unlabeled DNA dilutes the label present in the adipocyte fraction. This interpretation seems all the more likely when it is seen that all the counts are accumulated in the 9-day-old rat by 15 days postinjection with no change at 30 days postinjection. Considering the steep decline of specific activity from 0 to 15 days postinjection, it seems likely that an earlier sampling point might well have shown that the total counts in adipocyte DNA had plateaued before 15 days postinjection.

At 15, 22, and 28 days of age, the data support the idea that proliferation is very high but differentiation relatively slow. The rise in specific activity after the initial time point shows that some cells labeled in the proliferative compartment by the pulse were added to the mature compartment by 5–15 days postinjection. The maintenance or slight increase in specific activity at later time points, while the number of cells in the maturation compartment is increasing (see cell number curve, Fig. 3), means that new label is being added continually. A look at the total counts in adipocyte DNA confirms this. The best interpretation of these data is that when the pulse of [^{3}H]thymidine was administered a large number of immature cells in the stroma were labeled; it then took as long as 30 days postinjection (in 15-day-old rats) for these cells to appear in the maturation compartment. These findings suggest that in the third and fourth weeks after birth, proliferation and differentiation are not synchronous, i.e., cells remain in the differentiating compartment for considerable lengths of time. Whether the time spent in the differentiating compartment is regulated by an internal factor generated by the maturation compartment, by systemic influences, such as hormone levels, or by environmental influences, such as diet, is not known.

At 35 and 49 days a much slower rate of proliferation is evident, but the time cells spend in the differentiation compartment is once again shortened. This can be seen more clearly in Figs. 6 and 7. In this situation, the total counts in adipocyte DNA rise 3–6 days postinjection and then remain constant with no further increase throughout the experimental period. Such data are consistent with the concept that the majority of new cell synthesis is over by the fifth postnatal week. In mature rats 3 and 5 months of age, when cell number is stable as measured by other methods, (4, 5) there is no change in either specific activity or total counts in DNA over the experimental period, supporting the concept that the stable adipose cell number in mature organisms is a result of little or no net turnover of adipocytes.

In all of the experiments, the specific activity of the stromal–vascular fraction declined over the experimental period. This finding has been reported by others (18) and is thought to be the result of at least three processes: (*a*) migration or loss from the tissue of leukocytes, macrophages, etc., labeled in situ; (*b*) differentiation of preadipocytes; and (*c*) the addition of new DNA from synthesis of new vascular and supportive elements as adipocytes enlarge. The small amount of residual stromal–vascular label is presumably associated with connective and supportive tissue cells, which are known to be dividing throughout development.

In these experiments, typically, a small percentage of label even at the initial sampling point was associated with the adipocyte fraction, although such preparations appeared free from contamination at the light microscopic level. This initial value is therefore thought to be the result of inevitable contamination in the preparation of adipocyte and stromal–vascular fractions. Particular efforts were made to reduce the level of contamination in 5-month-old animals, including an unsuccessful attempt at freezing and slicing the polyethylene tubes in which cells were isolated. Perhaps the enormously increased connective cells and collagen network of tissue from older rats makes the collagenase digestion less complete and stromal–vascular cells or fragments adhere more easily. At any rate, because we were primarily interested in changes, the initial time point was considered as the basal contamination.

These investigations are consistent with the findings of other investigators. The demonstrations that fat cell num-

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ber remains constant in mature humans, rats, and mice (5, 8, 9, 14) throughout the adult life-span, even when body weight fluctuates, can now be said to be the result of little or no turnover of adipocytes. The increase in adipose cell number until 12–16 wk of age in the rat (5) and 50–60 days in the mouse (9) can now be divided into three periods. In the rat there is a highly proliferative period up to 4 wk after birth, a predominantly lipid-filling period with some proliferation until around puberty (60–80 days), and a period of lipid filling during maturity. In other words, the cell number curves determined by use of osmium fixation and Coulter counting or by histometric means are similar, but not coincident with, the patterns suggested by changes in specific activity. The greater portion of new cells are made in the epididymal pad either before or shortly after weaning. Further cells counted come primarily from lipid filling of cells existent at weaning.

Our findings are also consistent with the in vivo experiments of Hollenberg and Vost (18) and the organ culture experiment of Frohlich, Vost, and Hollenberg (19). They found, in both cases, an increase in the specific activity of the adipocyte fraction at various times after exposure to the [^{3}H]thymidine. In both experiments, judging by the body weights given, the rats were postweaning and prepubertal, and, therefore, their tissue should show some adipocyte proliferation. Although our experiments were confined to a study of the epididymal fat depot, Hollenberg and Vost (18) used pooled epididymal and inguinal fat. Their findings, in addition to our own preliminary result, suggest that our findings could reasonably be extended to other fat depots.

The interesting report by Lemonnier (15) that feeding a high fat diet to lean Zucker female rats produced an increase in adipose cellularity of the retroperitoneal depot will require a similar in vivo labeling study to determine whether de novo cell synthesis or lipid filling of preexistent cells is occurring in the retroperitoneal depot during the course of feeding the high fat diet.

Our findings help to provide a basis for understanding the nutritional effects found by Knittle and Hirsch (23) and Hirsch and Han (5) in rats and by Rakow et al. (13) in mice. Hirsch and Han (5) were unable to reduce adult cell number by acute starvation to inanition as early as 6 wk after birth or by chronic starvation after 15 wk of age. Both of these manipulations reduced depot weight dramatically but, upon refeeding, the finally attained adult cell number was not significantly different from ad lib.-fed controls. The studies by Rakow et al. (13, 14, and 17) on chronic starvation and refeeding in mice provided similar results. Only the severe undernutrition and stunting in early postnatal life that occurs by raising rats in large litters reduced adult adipose cell number (23). The explanation for these findings suggested by Knittle and Hirsch (23) is now confirmed, i.e., only effects early on in postnatal development in normal rats can change adult adipocyte number by interfering directly with adipocyte proliferation in the epididymal pad. The mechanism whereby adipocyte proliferation is regulated is not known, but one possible regulator during development could be that the absolute amount of lipid presented to the tissue for assimilation determines the rate of lipid filling; i.e., during rapid postnatal organismal growth, the rate of the incoming lipid determines the degree of lipid deposition in preformed fat cells. The rate of departure of cells from the differentiating compartment may in some way serve as a feedback signal, regulating more proliferation. In the genetically obese Zucker rat, early overnutrition results in an additional hyperplastic effect, suggesting once again that during the preweaning period cellularity is flexible (26).

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REFERENCES

1. Keys, A., and J. Brožek. 1953. Body fat in adult man. *Physiol. Rev.* **33:** 245–325.
2. Reh, H. 1953. Die Fettzellgrosse beim Menschen und ihre Abhängigkeit vom Ernährungszustand. *Virchows Arch. Pathol. Anat. Physiol. Klin. Med.* **324:** 234–242.
3. Bjorntorp, P. 1972. Disturbances in the regulation of food intake. *Advan. Psychosom. Med.* **7:** 116–147.
4. Hellman, B., and C. Hellerström. 1961. Cell renewal in the white and brown fat tissue of the rat. *Acta Pathol. Microbiol. Scand.* **51:** 347–353.
5. Hirsch, J., and P. W. Han. 1969. Cellularity of rat adipose tissue: effects of growth, starvation, and obesity. *J. Lipid Res.* **10:** 77–82.
6. Hirsch, J., J. L. Knittle, and L. B. Salans. 1966. Cell lipid content and cell number in obese and nonobese human adipose tissue. *J. Clin. Invest.* **45:** 1023. (Abstr.)
7. Hirsch, J., and J. L. Knittle. 1970. Cellularity of obese and nonobese human adipose tissue. *Federation Proc.* **29:** 1516–1521.
8. Johnson, P. R., L. M. Zucker, J. A. F. Cruce, and J. Hirsch. 1971. Cellularity of adipose depots in the genetically obese Zucker rat. *J. Lipid Res.* **12:** 706–714.
9. Johnson, P. R., and J. Hirsch. 1972. Cellularity of adipose depots in six strains of genetically obese mice. *J. Lipid Res.* **13:** 2–11.
10. Enesco, M., and C. P. LeBlond. 1962. Increase in cell number as a factor in the growth of the organs and tissues of the young male rat. *J. Embryol. Exp. Morphol.* **10:** 530–562.
11. Hirsch, J., and E. Gallian. 1968. Methods for the determination of adipose cell size in man and animals. *J. Lipid Res.* **9:** 110–119.

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12. Hellman, B., L. Thelander, and I. B. Täljedal. 1963. Postnatal growth of the epididymal adipose tissue in yellow obese mice. *Acta Anat.* **55:** 286–294.
13. Rakow, L., G. Beneke, W. Mohr, and I. Brauchle. 1970. Comparative morphological and chemical investigations on white and brown adipose tissue of the mouse after chronic starvation and refeeding. *Beitr. Pathol.* **141:** 349–373.
14. Rakow, I., G. Beneke, and C. Vogt. 1971. Changes in collagen content of white adipose tissue in starved–refed and obese mice. *Beitr. Pathol.* **144:** 377–388.
15. Lemonnier, D. 1972. Effect of age, sex, and sites on the cellularity of the adipose tissue in mice and rats rendered obese by a high-fat diet. *J. Clin. Invest.* **51:** 2907–2915.
16. Peckham, S. C., C. Entenman, and H. W. Carroll. 1962. The influence of a hypercaloric diet on gross body and adipose tissue composition in the rat. *J. Nutr.* **77:** 187–197.
17. Rakow, L., G. Beneke, W. Mohr, and I. Brauchle. 1971. Investigations on cell proliferation in white adipose tissue of albino mice obesified by administration of aurothioglucose. *Pathol. Microbiol.* **37:** 261–274.
18. Hollenberg, C. H., and A. Vost. 1968. Regulation of DNA synthesis in fat cells and stromal elements from rat adipose tissue. *J. Clin. Invest.* **47:** 2485–2498.
19. Frohlich, J., A. Vost, and C. H. Hollenberg. 1972. Organ culture of rat white adipose tissue. *Biochim. Biophys. Acta.* **280:** 579–587.
20. Pilgrim, C. 1971. DNA synthesis and differentiation in developing white adipose tissue. *Develop. Biol.* **26:** 69–76.
21. Rodbell, M. 1964. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239:** 375–380.
22. Kissane, J. M., and E. Robins. 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.* **233:** 184–188.
23. Knittle, J. L., and J. Hirsch. 1968. Effect of early nutrition on the development of rat epididymal fat pads: cellularity and metabolism. *J. Clin. Invest.* **47:** 2091–2098.
24. Lehmiller, D. J. 1971. Approaches to the study of molecular and cellular renewal. *In* Cellular and Molecular Renewal in the Mammalian Body. I. L. Cameron and J. D. Thrasher, editors. Academic Press, New York. 1–24.
25. Cameron, I. L. 1971. Cell proliferation and renewal in the mammalian body. *In* Cellular and Molecular Renewal in the Mammalian Body. I. L. Cameron and J. D. Thrasher, editors. Academic Press, New York. 45–85.
26. Johnson, P. R., J. S. Stern, M. R. C. Greenwood, L. M. Zucker, and J. Hirsch. 1973. Effect of early nutrition on adipose cellularity and pancreatic insulin release in the Zucker rat. *J. Nutr.* **103:** 738–743.

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