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Research Article

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Effect of Early Nutrition on the Development of Rat Epididymal Fat Pads: Cellularity and Metabolism

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ABSTRACT The effect of infantile nutritional levels on adipose tissue cellularity and metabolism was studied in two groups of Sprague-Dawley rats. Caloric intake was varied during the suckling period by manipulating litter size immediately after birth; however, all animals had free access to food after weaning. The epididymal fat pads of animals raised in small litters were heavier than those of their paired siblings raised in large litters. Initially, the differences in pad weight were accounted for primarily by differences in total cell number; however, at 20 wk both cell number and cell size contributed equally. The rate of glucose incorporation into CO_2 and triglyceride during *in vitro* incubations was the same for both groups if expressed on a per cell basis; therefore total tissue incorporation was greater in animals with more cells. The results support the hypothesis that early nutritional experiences can effect permanent changes in the cell number and size of the epididymal fat depot and that total cell number is important in the total metabolism of this organ. These findings and the fact that extreme human obesity is accompanied by similar alterations in cellularity and metabolism indicate that early nutritional experiences should be studied further as a guide to the etiology of obesity in man.

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INTRODUCTION

The size of the adipose depot in man and animals is ultimately dependent upon the number and size of its constituent cells. Thus, in the course of the growth and development of an individual the final dimension achieved by this or any other organ in the body will be modified by factors that exert their effect on cell division and/or cellular enlargement. It has been demonstrated in other organ systems that the degree to which either of these mechanisms is modulated by nutritional factors depends in part on the age of the animal. The earlier in life that they exert their influence, the greater the likelihood that permanent alterations in body and organ size will occur (1-3). Comparable studies of the cellular growth and development of adipose tissue have not as yet been reported. Consequently, little is known about the factors that determine total adipose cell number or size in the adult animal.

The recent development of a new technique for sizing and counting adipose cells from the fat depots of man and animals has, however, now made it feasible to perform studies of the cellular development of this organ (4). In the present report we have utilized this method to examine the effect of early nutrition on adipose cell number and size in the epididymal fat pad of Sprague-Dawley rats.

Caloric intake was varied during the suckling period by manipulating litter size in a manner similar to that described by Kennedy and Widdowson and McCance (5, 6). Male rats born of the same

mother but reared by different females, in litters of different sizes, were sacrificed at varying time intervals after weaning and the total adipose cell number and cell size of each epididymal fat pad determined. The incorporation of uniformly labeled glucose- ^{14}C into $^{14}\text{CO}_2$ and tissue triglycerides during *in vitro* incubations was also studied.

The results indicate that early feeding experiences profoundly affect the total cell number and cell size of the epididymal fat pad. Significant differences in glucose metabolism were observed in cells of different sizes when specific activity was related to the amount of fat in the incubation flasks. However, these differences were largely eradicated when the data was expressed on a per cell basis. Thus, early feeding experiences can modify the total metabolism of an adult organ, whereas metabolism per cell is unaffected.

METHODS

Experimental design. Sprague-Dawley rats were used in all experiments. At birth, the litters of a large number of mothers were redistributed to give some mothers a litter of four (Group I) and others, a litter of twenty-two (Group II). Each male in group I was matched with a male born of the same mother but raised in a litter of twenty-two. After weaning (21 days), all animals had free access to regular laboratory chow. Six previously matched male animals in each experimental group were sacrificed at 5, 10, 15, and 20 wk of age. Both epididymal fat pads were removed intact for determination of wet weight, lipid content, adipose cell size and number, and glucose metabolism.

Determination of adipose cell number and size. The left and right epididymal fat pads were removed after the animals were sacrificed, and separate determinations were made for each. Upon removal, the wet weight of the whole pad was obtained after washing three times in saline to remove adherent debris. The pad was then placed in a beaker of saline at 37°C . Tissue fragments of approximately 5×2 mm were obtained by mincing. After mixing thoroughly, aliquots of tissue were removed and placed on a tared dry Nitex filter¹ and the wet weight determined. Cell size and number were obtained by method III described by Hirsch and Gallian (4). With this method, filter and tissue are placed in a flask containing 25 ml of a 2% solution of osmium tetroxide in collidine buffer and incubated at 37°C for 48 hr. Individual intact osmium-fixed cells separate from the tissue matrix during this time interval and are separated from supporting tissue and debris by washing with saline through a 250μ Nitex filter. The free cells are collected on a 25μ sieve and then suspended in a 400 ml

¹ Cut from Nitex nylon screen, mesh size 202μ , obtained from Tobler, Ernst, and Traber, Inc., New York.

beaker containing a known amount of saline. The cells are then counted in a Coulter counter and the total number of cells in the sample determined.

Another sample of tissue was placed on a tared filter and its wet weight determined. The filter and sample were then placed in 30 ml of a chloroform-methanol (2:1) solution and kept overnight. 10 ml of water was added and a two-phase system obtained. The upper phase was removed and anhydrous Na_2SO_4 was added to the remaining chloroform phase. Aliquots were then taken for determination of carboxyl ester bonds (7). Cell size (average lipid content per cell) was calculated by the following equation:

$$\text{lipid content/cell} = \frac{\text{per cent of lipid} \times \text{wet weight of sample.}}{\text{total number of cells in sample}}$$

The total number of adipose cells in each pad was calculated as follows:

$$\text{total cell number in epididymal pad} = \frac{\text{total wet weight of pad} \times \% \text{ lipid.}}{\text{lipid/cell}}$$

***In vitro* incubation of adipose tissue.** Tissue fragments were placed into 20-ml plastic flasks containing 2 ml of Krebs-Ringer bicarbonate buffer, pH 7.4 at 37°C . Each milliliter of buffer also contained 1 mg of glucose and a tracer dose of uniformly labeled glucose- ^{14}C ($0.5 \mu\text{C}/\text{mg}$). The flasks were capped with rubber stoppers from which a plastic cup containing a smaller glass cup was suspended by means of a wire. The flasks were then gassed for 5 min with 95% O_2 :5% CO_2 and incubated in an Eberbach water shaker bath (37°C at 72 cycle/min) for 2 hr. At the end of the incubation period, $\frac{1}{2}$ ml of hyamine hydroxide was injected through the rubber stopper into the glass cup and 1 ml of 6 N H_2SO_4 into the medium. $^{14}\text{CO}_2$ was collected in the hyamine during an additional hour of shaking. The caps were then removed and the glass cups placed in 18 ml of counting solution containing phosphor (0.4% PPO, 0.01% POPOP) in toluene. The tissue fragments were washed to remove adsorbed label and placed into 20 ml of isopropanol:heptane:1 N sulfuric acid (4:1:0.1) and extracted overnight. Appropriate amounts of water and heptane were added to form a two-phase system, and aliquots were taken from the upper (heptane) phase for lipid determination and another aliquot for determinations of ^{14}C activity (7, 8). All determinations of ^{14}C were performed in a Packard Liquid Scintillation spectrophotometer (89% efficiency).

The incorporation of glucose into $^{14}\text{CO}_2$ and triglyceride was calculated both as cpm/ μg of tissue triglyceride and as cpm/adipose cell, using cell size data obtained from other tissue samples of the same pad as described above. All incubations were performed in duplicate.

RESULTS

Body weight and epididymal fat pad weight. The growth curves of the two groups of animals are presented graphically in Fig. 1. The results

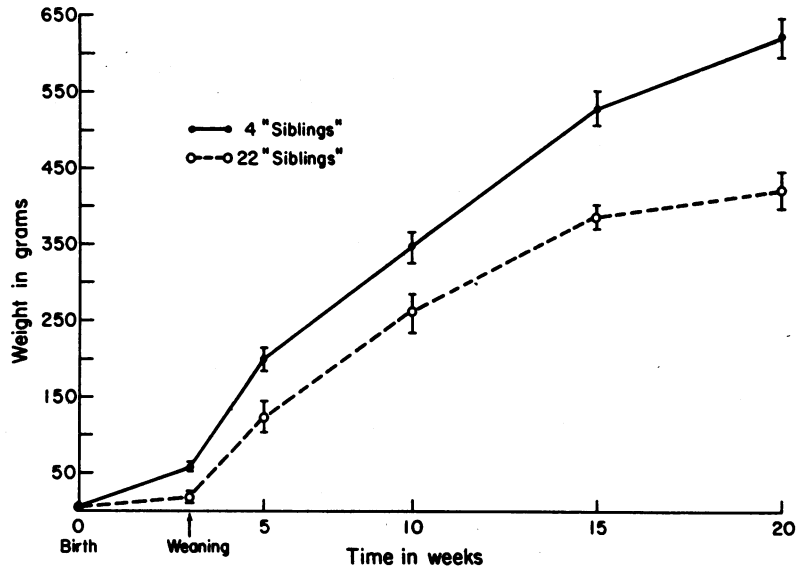


FIGURE 1 Effect of litter size on body weight of rats. Each point represents the mean weight of six animals \pm the standard error of the mean.

are similar to those described earlier by other investigators (1-3). Significant differences in body weight are seen at weaning and continue throughout the length of the study. This occurs despite the fact that all animals had free access to food after the suckling period.

The rate of growth of the epididymal fat pads for each group is presented in Fig. 2. In both groups, pad weight increases with age; however, in animals raised in small litters, this increase is more dramatic. Differences in the weights of epididymal fat pads are seen at all age levels, but do

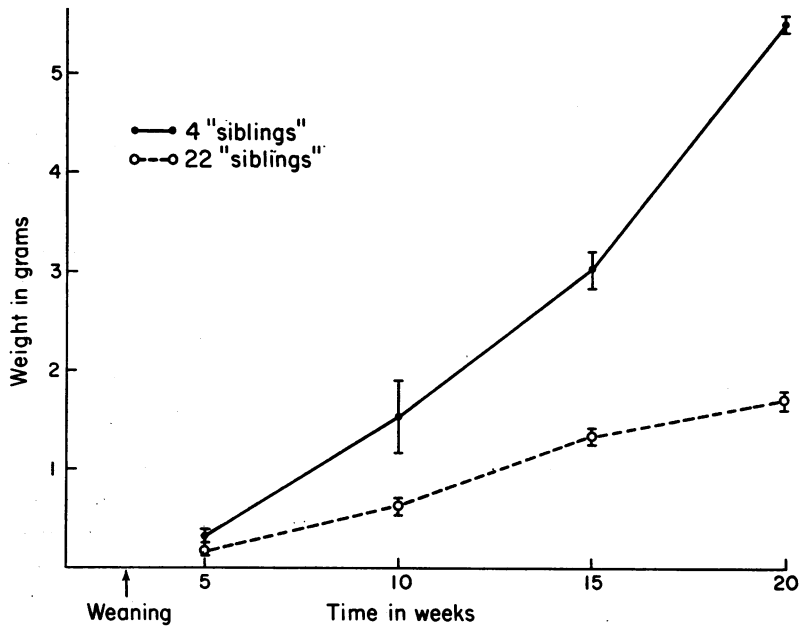


FIGURE 2. Effect of litter size on wet weight of rat epididymal fat pads. Each point represents the mean of duplicate determination in six animals \pm the standard error of the mean.

not reach levels of significance until rats are 10 wk of age.

A comparison of Figs. 1 and 2 indicates that the differences observed between the epididymal fat pads of the two groups is greater than that observed in total body weight, especially at 15 and 20 wk of age. This was confirmed by calculation of the ratio of epididymal fat pad weight to total body weight in each animal. The average ratios, pad weight, and total body weight for each group at 5, 10, 15, and 20 wk of age are shown in Table I.

In both groups of animals the ratios increase with age, but at 10 wk group I animals have significantly higher ratios. By 20 wk of age, the difference in ratios is even more marked. Thus, the epididymal fat depots of animals raised in small litters are both absolutely and relatively larger than those of their corresponding siblings raised in large litters.

Adipose cell number and size. The average cell number and size for each group of animals are listed in Table II. At all ages studied, animals raised in large litters have fewer and smaller cells

TABLE I
Comparison of Body Weight, Epididymal Fat Pad Weight, and Ratio of Pad/Body of Rats Raised in Litters of Four (Group I) and Litters of Twenty-Two (Group II)

Age	No. of animals	Body wt	Epididymal fat pad weight*	Weight of pad/body × 100
		g	g	
5 wk				
Group I	6	192 ± 22‡	0.313 ± 0.07§	0.16§
Group II	6	139 ± 13	0.187 ± 0.05	0.12
10 wk				
Group I	6	349 ± 35	1.517 ± 0.35	0.42
Group II	6	250 ± 14	0.625 ± 0.07	0.25
15 wk				
Group I	6	471 ± 8	3.082 ± 0.17‡	0.65
Group II	6	375 ± 25	1.321 ± 0.08	0.35
20 wk				
Group I	6	624 ± 15‡	5.489 ± 0.04‡	0.87‡
Group II	6	421 ± 18	1.677 ± 0.09	0.39

Each value represents the mean ± SEM.

* Average value of both epididymal fat pads. Significance levels were calculated by the paired Student's *t* test.

‡ Significant *P* value *P* < 0.01.

§ Not significant.

|| Significant *P* value *P* < 0.05.

TABLE II
Comparison of Adipose Cell Number and Size in Epididymal Fat Pads of Rats Raised in Litters of Four (Group I) and Litters of Twenty-Two (Group II)

Age	No. of animals	Adipose cell number	Adipose cell size
		× 10 ⁴	μg of lipid/cell
5 wk			
Group I	6	2.716 ± 0.313*	0.1047 ± 0.01‡
Group II	6	2.135 ± 0.283	0.0668 ± 0.04
10 wk			
Group I	6	5.326 ± 0.267§	0.2313 ± 0.03*
Group II	6	3.165 ± 0.315	0.1605 ± 0.05
15 wk			
Group I	6	5.425 ± 0.154§	0.5365 ± 0.05*
Group II	6	3.900 ± 0.279	0.2807 ± 0.02
20 wk			
Group I	6	6.935 ± 0.340§	0.6392 ± 0.01§
Group II	6	3.947 ± 0.171	0.3751 ± 0.01

Each value represents the mean ± SEM of both epididymal fat pads. Significance levels were calculated by the paired Student's *t* test.

* Significant *P* value *P* < 0.05.

‡ Not significant.

§ Significant *P* value *P* < 0.01.

than those raised in small litters. The differences observed in cell number are statistically significant at all time intervals; however, differences in cell size do not reach levels of significance until the 10th wk of age.

The relative contribution of cell number and cell size to the size of epididymal fat depots is presented in Fig. 3. Here cell number is plotted along the ordinate and cell size along the abscissa. The rectangle generated by these values depicts the size of the fat depot at each time interval. The total area represents the depot of animals raised in small litters, and the cross-hatched area represents the depots of animals raised in large litters. The stippled portions of the bar represent the difference in the size of the two depots.

One can see from the shape of the rectangles that both groups have qualitatively similar developmental patterns. Early in life (5–10 wk) the height of the bar is greater than the base, indicating a relatively greater contribution of cell number. As the animals age, the shape of the bar approaches a square, indicating a more equal participation of cell size.

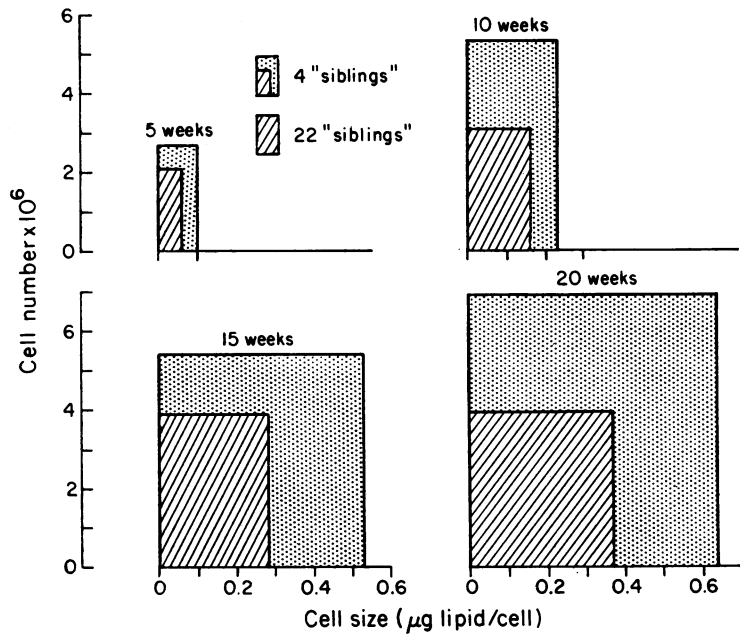


FIGURE 3 Relative contribution of cell number and cell size to the size of the epididymal fat pad of rats raised in different litter size.

These results are similar to those reported for other organ systems in the rat, in which early development is due primarily to cell division, and secondarily to hypertrophy. Once cell number is established, further growth is achieved by an increase in cell size alone (10–12). In the present study, total cell number reached a plateau in group

II animals at 15 wk, whereas the cell number of group I animals continued to increase throughout the study. In both groups cell size continued to increase throughout the 20 wk of the study.

It can also be seen in Fig. 3 that during the first 10 wk differences in the size of the depot

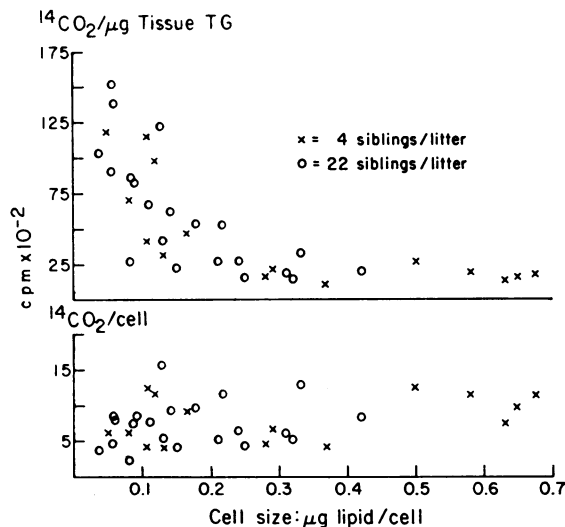


FIGURE 4 Uniformly labeled glucose-¹⁴C incorporation into ¹⁴CO₂ during in vitro incubations of rat adipose tissue removed from animals raised in different litter sizes. Each point represents the average of four determinations for each rat.

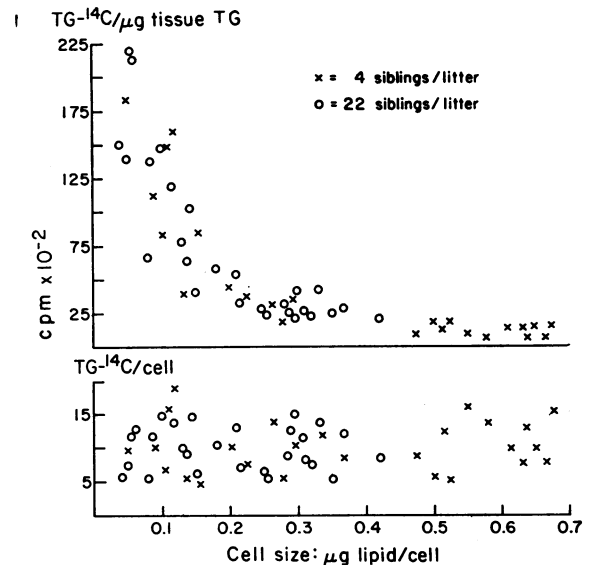


FIGURE 5 Uniformly labeled glucose-¹⁴C incorporation into tissue triglyceride during in vitro incubations of rat adipose tissue removed from animals raised in different litter sizes. Each point represents the average of four determinations for each rat.

(stippled area) were due primarily to differences in cell number. Later in life the differences in size encountered in the epididymal pads of the two groups were achieved by a combination of differences in cell size and number of approximately equal magnitude.

In vitro incubations of epididymal fat pads with uniformly labeled glucose- ^{14}C . The incorporation of uniformly labeled glucose- ^{14}C into $^{14}\text{CO}_2$ and tissue triglyceride is presented in Figs. 4 and 5, respectively. In the upper panel of each figure the data are plotted as cpm/ μg of tissue triglyceride. When specific activity is calculated in this manner the rate of conversion of glucose into CO_2 and triglyceride appears to decrease as cell size increases, suggesting that animals with larger cells are less active metabolically. However, if the specific activity of $^{14}\text{CO}_2$ and triglyceride is calculated on a per cell basis, as shown in both lower panels, one can see that each cell converts glucose at comparable rates, regardless of size.

This latter formulation is more meaningful since the fat contained in the cell is not involved in the metabolic activity of the cell. Thus no real difference in glucose metabolism is seen between the cells of the two groups of animals, regardless of size.

DISCUSSION

The increase in the size of fat depots found in extremely obese human adults is due primarily to an increase in adipose cell number and, to a lesser extent, to an increase in cell size (9). A more striking finding, however, is the apparent constancy of adipose cell number in these subjects even after marked weight reduction. Indeed, the decrease in the fat depot that is produced as a result of caloric restriction (600 cal/day) is achieved almost exclusively by a decrease in adipose cell size, without any significant change in cell number.

A similar constancy of adipose cell number is encountered in the study of epididymal fat pads of Sprague-Dawley rats.² When adult rats are subjected to periods of caloric deprivation (starvation or semistarvation), the loss of body weight that results is rapidly made up when ad lib. feedings are reinstated (2). Recovery is so complete that body weight is restored to levels that are indistinguishable from those of littermates

² Hirsch, J. To be published.

whose food intake has not been restricted. Analyses of adipose cell number and size in the epididymal fat pads of the deprived adult animals reveal a parallel decrease in adipose cell size without any significant change in total adipose cell number. Upon refeeding, the weight of the fat depot is rapidly restored by an increase in cell size to values that are equivalent to those of their nondeprived littermates. Furthermore, overfeeding during adult life produces an increase in the weight of the epididymal fat pad by cellular enlargement alone.

Thus, in both man and rat, adult adipose cell number is unaffected by dietary manipulation, and changes in the size of the fat depot are mediated solely by changes in cell size. In addition, decreases in adipose cell size in the adult are not permanent and are quickly restored to predeprivation levels when ad lib. feedings are reestablished.

Permanent changes in body and individual organ size have, however, been reported in the rat when litter size is altered immediately after birth (1, 2, 5). The differences in body weight produced by this procedure are never completely overcome despite free access to food after the animals are weaned. The reasons for the persistence of this effect are not entirely clear. It has been suggested that food intake is altered by differences in litter size due to varying levels of competition for the mother's milk. The permanent change in body size produced by caloric deprivation during this period of early development has been attributed to its effect on cell division, while a similar intervention later in life affects cellular enlargement (3). In the former case, recovery is not possible after cell division has ceased, whereas cellular hypertrophy can occur throughout life of the animal.

Studies of the growth and development of a variety of organs in the rat tend to support this hypothesis, since most organ systems show similar developmental patterns (10-12). Early in life there is a rapid increase in cell number which then reaches a plateau at varying time intervals, depending upon the organ under study. The increase in cell size is less dramatic but continues throughout the life of the rat and finally accounts for all of the animal's growth once adult life is reached. Observations of the growth and development of adipose tissue from a large number of

untreated rats have revealed the same sequence of events. The epididymal fat pad increases in weight throughout the life of the animal. During the first 10 wk adipose cell number rises rapidly and then appears to level off without any significant increase for as long as 30 wk of age. On the other hand, adipose cell size continues to increase throughout the time periods studied.

In the present study animals raised in different litter sizes displayed dissimilar developmental patterns. Those raised in small litters had significantly heavier epididymal fat pads by the tenth week of life, and the differences became more marked with time. In both groups cell size increased throughout the study; however, the increase was more dramatic in animals raised in small litters. In addition, in animals raised in large litters the early increase in adipose cell number was not as rapid, nor was the number attained as great as in those raised in small litters. Indeed, while cell number appeared to plateau between the 10th and 15th wk in the former group, cell number continued to increase throughout the study in the latter. Initially, the differences observed in the weight of the fat pads were due primarily to changes in adipose cell number; however, by the 20th wk increases in both cell number and cell size contributed to the heavier fat pads.

Although these findings are consistent with the concept that the observed differences were mainly due to differences in infantile caloric intake, one cannot attribute all the changes observed solely to differences in caloric intake during the suckling period. Pups raised in litters of varying sizes have markedly different early social and feeding experiences that could alter later feeding patterns and contribute to changes in energy balance. The continued increase in differences in cell size and number observed after weaning might have resulted from differences in caloric intake during this period. However, no attempt was made in this or the previously reported studies to measure food intake or physical activity. Therefore, one can only state at the present time that no obvious differences in feeding behavior such as that seen in hypothalamic lesioned animals was noted. Apart from differences in body size, the casual observer could not determine the origin of a particular animal. Only future studies, in which food intake, activity, and other parameters of behavior are

carefully measured, can determine the extent to which differences in these factors contribute to the changes observed. However, the fact that dietary manipulation in untreated animals failed to effect permanent changes after the age of 10 wk indicates that some time limit does exist during which levels of food consumption can effectively alter adipose tissue cellularity.

Winick and Noble have reported similar age-dependent cellular responses in the heart, kidney, and muscle of rats subjected to caloric deprivation during different periods of development (3). They found that deprivation early in life resulted in permanent changes in cell number while deprivation later in life produced reversible changes in cell size. Their results differ somewhat from those presented in the present report, since they found no change in the cell size of animals raised in different litter sizes. However, this discrepancy may be due to the uniqueness of the adipose cell, which is exquisitely sensitive to changes in caloric intake. In addition, in the present study cell size is based on fat content, and not protein/DNA ratios which are more meaningful measures of cellular hypertrophy. Therefore, one cannot deduce from our observations whether the differences in adipose cell size are due merely to a "stuffing" of the cell with triglyceride or to a combination of changes in lipid content and cell mass.

Whatever the mechanism, the fact that differences in lipid content per cell are produced by early dietary manipulation raises the possibility of concomitant changes in lipid metabolism. It was, therefore, of interest to study *de novo* glucose lipogenesis during *in vitro* incubations of epididymal fat pads taken from each group of animals. The results of these experiments could be interpreted in two ways, depending upon the specific activity used for calculating the conversion of glucose into tissue triglyceride. If one calculates the data in the customary manner, that is, as counts per minute of glucose-¹⁴C incorporated per microgram of tissue triglyceride, there appears to be a decrease in the rate of incorporation with increasing age and cell lipid content. In addition, animals in group I appear to incorporate less glucose at each age level studied. This interpretation is misleading, for if one expresses the data on a per cell basis it can be seen that the incorporation of glucose proceeds at equal rates regardless of

cell size, age, or previous dietary history. Hence, total glucose lipogenesis is dependent upon the total number of cells present and is greater in the heavier animals with the more numerous cells. The findings do not, however, provide a metabolic basis for the differences found in individual cell lipid content, and further studies of other parameters of lipid metabolism (e.g., free fatty acid uptake and release and hormonal responses) are indicated.

The striking similarity between these results and those previously reported for obese and non-obese human subjects merits further comment (9). In both studies the "obese" populations display relatively larger fat depots that are characterized by permanent increases in adipose cell number and size. In addition, *in vitro* incubations of adipose tissue with radioactive glucose indicate that total adipose cell number is important in the total metabolism of the adipose organ in both man and rat. Since the present findings clearly show that early nutritional experiences can permanently modify adipose cell number and size in the rat, it is postulated that similar nutritional experiences in man are of prime importance in producing the hyperplasia and hypertrophy of adipose tissue found in obese subjects. Indeed, the inadequacy of current dietary approaches to the problem of obesity may be due to their inability to effect any permanent changes in adipose tissue cellularity in adult life. The treatment of this disorder may, therefore, lie in its prevention early in life through the control of factors that influence adipose cell division and enlargement.

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