Increased cellular proliferation in adipose tissue of adult rats fed a high-fat diet

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Abstract The feeding of a high-fat diet to adult rats was shown to increase the incorporation of [^{3}H]thymidine into DNA of the adipocyte and stromal fractions. After only 2 days on a high-fat diet there was a marked increase in the incorporation of label. When a 2-week period was interposed between [^{3}H]thymidine administration and determination of DNA specific activity, the greatest increase in incorporation of label was found after 1 week on the diet, when incorporation increased 6-fold or more in both adipocytes and stroma and subsequently decreased to stabilize at a level two or three times that of chow-fed rats in the adipocyte fraction. Rats labeled when young and later placed on a high-fat diet showed a decrease in DNA specific activity in both adipocytes and stroma, confirming that cellular proliferation had occurred in both fractions. The specific activities of both stromal and adipocyte DNA were very similar at all time points studied. An attempt to increase the difference in specific activities by waiting many weeks after [^{3}H]thymidine injection before isolating DNA was not successful. This may be because the total amount of DNA in the stromal and adipocyte fractions increases in parallel on the diet. The significance of these findings in terms of the normal turnover of adipose tissue DNA and the responsiveness to diet is discussed.—**Klyde, B. J., and J. Hirsch.** Increased cellular proliferation in adipose tissue of adult rats fed a high-fat diet. *J. Lipid Res.* 1979. **20:** 705–715.

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Adipocyte number in adult rats (1), pigs (2, 3), and humans (4, 5) has been thought to remain constant, with increased caloric intake reflected by adipocyte hypertrophy rather than hyperplasia. However, Johnson and Hirsch (6) found that in mice made obese by gold-thioglucose treatment the adipocyte number in the retroperitoneal pad was increased. In addition, Lemonnier (7) showed that feeding a high-fat diet to 5-month-old rats caused an increase in adipocyte number in the perirenal fat pads after 7 months on the diet. Faust et al. (8) found that the diet-induced increase in adipocyte number in adult rats also occurred in other fat depots, and they demonstrated that the increased adipocyte number persisted even after animals were returned to an ordinary chow diet. Thus, adipose tissue, even in adult rats, can show an increase in the number of lipid-filled cells. It is not known whether these cells are created by the filling of progenitor cells already present, or whether the adipocyte number is increased by proliferation of progenitors. In the preceding report (9) we showed that adult rats on a regular diet incorporated [^{3}H]thymidine into the adipocyte fraction as well as the stromal fraction. The adipocyte label appeared to be not merely stromal contamination, but was more likely due to proliferation of cells closely associated with adipocytes. In the present work we show that in adult rats a high-fat diet causes a considerable increase in the DNA specific activity of both the adipocyte and stromal fractions, suggesting that the diet-induced increase in cell number is a consequence of cellular proliferation rather than the filling of existing preadipocytes.

MATERIALS AND METHODS

Animals

Male rats of the Osborne-Mendel strain were obtained from a colony maintained at the National Institutes of Health, courtesy of Clarence R. Reeder and, when these were no longer available, from Camm Laboratories (Wayne, NJ). At age 3 months, rats were put on a high-fat diet containing 55% fat (10) and controls animals were maintained on Purina Chow. Weight gain was found to be most consistent when the animals were kept in wire mesh cages without wood shavings; when the rats were kept in ordinary plastic cages, wood shaving became mixed with the diet, leading to decreased intake.

Isolation of DNA

Animals were injected intraperitoneally with 300 μCi of [^{3}H]thymidine (methyl-^{3}H, sp act 20 Ci/mmol, New England Nuclear), followed 17 hr later by an injection of 0.48 mmol of unlabeled thymidine (Sigma) if the experiment was to last more than 1 day after administra-

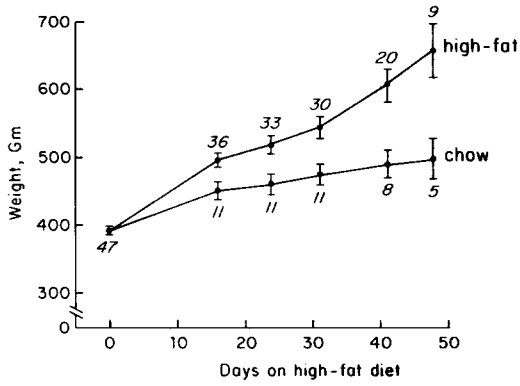

Fig. 1. Body weight curve for rats injected with label after different periods of time on a high-fat diet. The mean ± SEM is shown. Rats were 3 months old when begun on the diet. The number of rats weighed is shown above or below each time point.

tion of label. Some animals were labeled at a young age and then placed on the high-fat diet at a later date. In most instances, labeling was done while the animals were on the high-fat diet. The epididymal and retroperitoneal fat depots were removed and subjected to collagenase digestion. Fat cells and stroma were separated and total cellular DNA or nuclear DNA was isolated as previously described (9).

Adipose cellularity

The number of adipocytes in each fat depot was determined by counting the cells after fixing with osmium tetroxide (11). In the analysis of data the value of Student's *t* was corrected for heterogeneity of variance.

RESULTS

To determine if the rate of new adipocyte formation varied with the length of time on the high-fat diet, animals fed the diet for different periods of time were injected with [^{3}H]thymidine, followed after 17 hr by an injection of unlabeled thymidine. Two weeks later total cellular DNA was isolated from fat cells and stroma of the epididymal and retroperitoneal fat depots. The interval of 2 weeks between [^{3}H]thymidine administration and DNA isolation was selected because Hollenberg and Vost (12) had shown that during the first week after isotope administration label was shifted from the stromal to the adipocyte fraction, perhaps the result of newly formed cells filling with lipid, a process that appeared to be complete by 7 days. **Fig. 1** shows the body weight curves for animals on high-fat and chow diets used in this experiment. **Table 1** shows the number of adipocytes in different depots. In this study the number of adipocytes increased considerably in both the epididymal and retroperitoneal depots in the animals on a high-fat diet, as compared to chow-fed controls which were killed at various times throughout the experiment. Adipocyte number in chow-fed rats appeared to remain constant. Weight gain was seen within a few days after beginning the diet, but an increase in adipocyte number was not apparent until after 5 weeks. The high fat-induced increase in adipocyte number has been investigated more thoroughly in our laboratory (8).

We found that in rats injected after 1 week on the high-fat diet the specific activity of DNA of the fat cell and stromal fractions of both depots studied was greater by 6- or 7-fold than that of chow-fed rats (**Fig. 2**). Rats injected with [^{3}H]thymidine after a longer time on the high-fat diet also had a higher specific activity of DNA of the fat cell and stromal fractions, when compared to chow-fed rats, but never as great as at 1 week. For this experiment specific activity was determined on total cellular DNA, while in later experiments DNA was prepared from nuclei. The majority of total cellular DNA is in nuclei, so the diet-induced increase in label incorporation probably reflects synthesis of nuclear DNA. For each depot and time point, the specific activity of fat cell DNA was not very different from that of stromal DNA, although for rats on the diet for just 1 week stromal DNA seemed to have a greater specific activity than fat cell DNA, whereas in rats injected after longer time periods on the diet this was reversed. Thus, the stromal fraction of the epididymal and retroperitoneal fat depots formed new cells under the influence of a high-fat diet, as evidenced by increased DNA synthesis as judged by [^{3}H]thymidine incorporation.

The increased incorporation of [^{3}H]thymidine into fat cell DNA of rats on a high-fat diet, as compared

TABLE 1. Adipocyte number for rats on the high-fat diet for different periods of time

Diet When Killed	Fat Depot Adipocyte Number × 10^6	
	Epididymal	Retroperitoneal
Chow[a]	8.11 ± 0.444 (8)[b]	7.61 ± 0.911 (8)
3 weeks high fat	9.43 ± 2.44 (3)	8.00 ± 0.962 (3)
4 weeks high fat	8.28 ± 0.434 (3)	9.49 ± 0.838 (3)
5 weeks high fat	13.6 ± 1.97 (3)	12.0 ± 2.73 (3)
6 weeks high fat	15.7 ± 1.62 (3)	16.7 ± 3.92 (2)
8 weeks high fat	23.0 ± 4.89 (2)	29.4 ± 0.915 (2)
9 weeks high fat	16.5 ± 7.01 (2)	27.6 ± 9.34 (2)

Samples were taken for determination of adipocyte number at the time fat depots were removed for isolation of DNA. Adipocyte number was not obtained in all DNA isolation experiments.

[a] Adipocyte number in chow-fed rats did not appear to change during the period of this study. Data from chow-fed rats killed after different intervals is therefore pooled.

[b] Mean ± SEM. The number of animals is in parentheses.

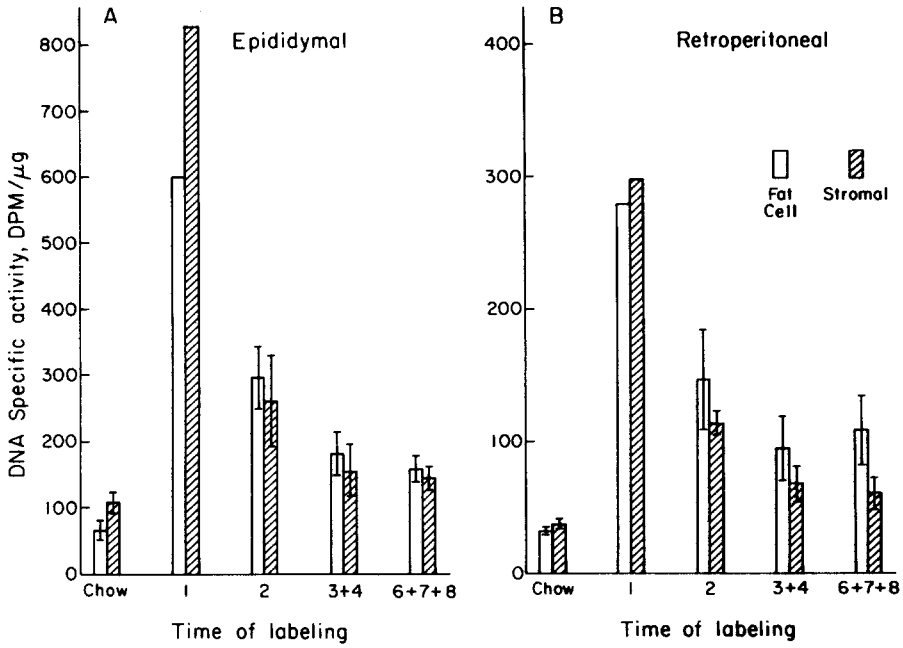

Fig. 2. Incorporation of [^{3}H]thymidine after different periods of time on a high-fat diet. Rats received [^{3}H]thymidine after being on the high-fat diet for different periods of time. Two weeks after injection, total cellular DNA was isolated from fat cells and stroma. Each experiment required two or three rats. Of the four experiments done with chow-fed rats, two were done with rats injected at the time the high-fat-fed rats had been on the diet for 3 weeks, one at 5 weeks, and one at 7 weeks. Of the ten experiments done with fat-fed rats, one was done after 1 week on the high-fat diet, two at 2 weeks; two each at 3 weeks and 4 weeks; and one each at 6, 7, and 8 weeks. The mean ± SEM is shown. Data for the epididymal fat depot is shown in *A*, and for the retroperitoneal fat depot in *B*. A comparison of the DNA specific activity in the fat cell and stromal fractions for rats injected after 2–8 weeks on the high-fat diet showed that for the retroperitoneal fat depot, Student's $t = 1.88$, with $P < 0.04$, one-tailed, but for the epididymal depot the difference was not significant.

to chow-fed controls, suggested that de novo cell formation occurred in the adipocyte fraction as well. However, the similarity between the specific activity of fat cell DNA and that of stromal DNA for a given depot and time point suggested that the label in fat cell DNA was derived from the stromal fraction. The specific activity of fat cell DNA from the retroperitoneal depot was significantly greater ($P < 0.04$, Fig. 2) than that of stromal DNA after 2–8 weeks on the high-fat diet, but for the epididymal depot the difference in DNA specific activities was not statistically significant. Further experiments were done to find out if the label

TABLE 2. Comparison of the fat pad weight of rats on the high-fat diet for a short time with that of chow-fed rats

	Chow	High-Fat for 3 Days	Chow	High-Fat for 7 or 8 Days
		g	*g*	*g*
Epididymal:	3.51 ± 0.21[a]	5.33 ± 0.31	3.17 ± 0.13	5.37 ± 0.31
	$t = 4.64$		$t = 6.49$	
	$P < 0.00008$[b]		$P < 0.00002$	
Retroperitoneal:	2.70 ± 0.20	4.44 ± 0.22	1.89 ± 0.093	4.47 ± 0.29
	$t = 5.77$		$t = 8.45$	
	$P < 0.000006$		$P < 0.00004$	

Two lots of 22 animals were used. Both lots were 3 months old, but body weight and fat pad weight of chow-fed rats differed slightly between lots, so comparisons are made only within each lot. In one lot, 12 rats were fed the high-fat diet for 3 days prior to death and were compared to the remaining 10 chow-fed rats. In the other lot, nine rats were fed the high-fat diet for 7 or 8 days prior to death and were compared to the remaining 13 chow-fed rats.

[a] Mean ± SEM.

[b] *P* value, one-tailed.

TABLE 3. Effect of brief high-fat feeding on the yield of nuclear DNA from fat cells and stroma

	Amount of Nuclear DNA Per Rat, μg		
	3 Days High Fat	Chow	
Epididymal fat cells	10.0 ± 0.95 (4)	9.33 ± 0.35 (3)	$t = 0.606$ NS
Retroperitoneal fat cells	10.0 ± 1.2	8.03 ± 0.16	$t = 1.68$ NS
Epididymal stroma	61.2 ± 2.5	51.9 ± 1.5	$t = 2.95$ $P < 0.02$
Retroperitoneal stroma	69.9 ± 2.7	57.7 ± 4.2	$t = 2.57$ $P < 0.03$
	7–8 Days High Fat	Chow	
Epididymal fat cells	30.8 ± 3.5 (3)	15.8 ± 3.0 (4)	$t = 3.27$ $P < 0.02$
Retroperitoneal fat cells	19.2 ± 4.1	18.8 ± 6.4	$t = 0.0552$ NS
Epididymal stroma	73.5 ± 5.3	52.8 ± 5.3	$t = 2.69$ $P < 0.03$
Retroperitoneal stroma	85.2 ± 3.4	46.9 ± 2.1	$t = 10.1$ $P < 0.0001$

Data are from the same experiments as Table 2. Two to five rats were used for each experiment, and the data are presented as amount of nuclear DNA per rat, mean ± SEM, and Student's t and p, one-tailed. The number of experiments is in parentheses.

in fat cell DNA was evidence of de novo cell formation or merely of stromal contamination.

Comparison of the fat pad weights of chow-fed animals and of animals fed the diet for just a short time showed that only 3 days on the diet was sufficient to significantly increase the pad weight (**Table 2**). A comparison of the yield of nuclear DNA per rat also showed an effect after only 3 days on the diet (**Table 3**). The amount of nuclear DNA from the stromal fraction of both the epididymal and retroperitoneal pads was significantly increased compared to chow-fed rats from the same lot. This increase in stromal DNA was also seen after 7 or 8 days on the diet, and there was a significant increase in the amount of DNA from epididymal fat cells as well. Thus, the early increase in fat pad weight without an accompanying increase in the amount of fat cell DNA suggests that the existing fat cells enlarged after only 3 days on the high-fat diet. An increase in fat cell number was not apparent until rats had consumed the high-fat diet for 5 weeks (Table 1).

Our previous work (9) had shown that within a day after administration of [^{3}H]thymidine to chow-fed adult rats, considerable label was present in the adipocyte fraction. It was unlikely that this label was within mature fat cells, because Hollenberg and Vost (12) had shown that at least 2–5 days was required after [^{3}H]thymidine injection of young rats for label to appear in the adipocyte fraction. We had concluded that the label in adult rats was in another, proliferating cell type, intimately associated with mature fat cells. To see whether such rapid labeling of the adipocyte fraction was influenced by the high-fat diet, rats fed the diet for 2 days or for 6 or 7 days were injected with [^{3}H]thymidine and nuclear DNA was isolated from fat cells and stroma 15–21 hr later.

Six or seven days on the diet caused a significant increase in the specific activity of fat cell DNA within 1 day of [^{3}H]thymidine administration compared to that of chow-fed rats, 14-fold for the epididymal pad and 8-fold for the retroperitoneal pad, with lesser increases in the specific activity of the stromal fractions (**Fig. 3**). In this and later experiments, specific activity was determined for nuclear DNA. Two days on the diet also caused increases, but they were not as large. The increases in fat pad weight and DNA content show the extraordinarily rapid tissue response to a high-fat diet and thus help to explain why an increase in DNA synthesis could be seen after so short a time. Specific activity, a measure of DNA synthesis, was determined on DNA prepared from nuclei. Thus, the more rapid turnover of non-nuclear DNA fractions did not contribute to the observed dietary influence on DNA synthesis (9).

To further study the influence of the high-fat diet on the rapid labeling of adipocyte DNA, incorporation

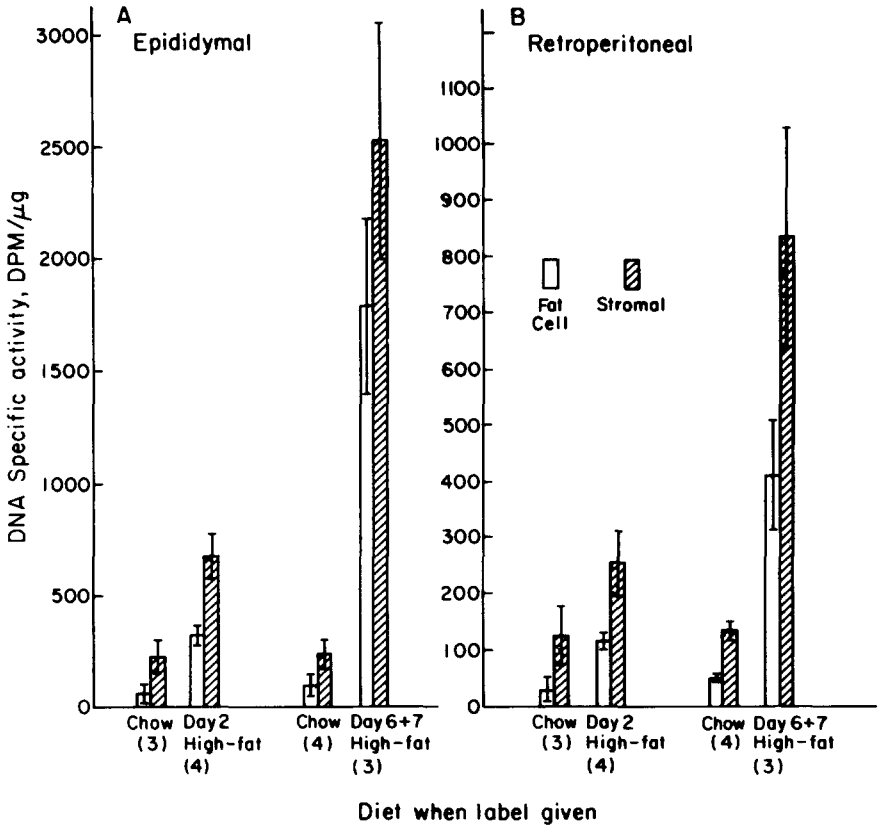

Fig. 3. Incorporation of [^{3}H]thymidine during a short pulse in animals fed chow or a high-fat diet. Three-month old rats were placed on a high-fat diet for 2 days or for 6 or 7 days prior to administration of [^{3}H]thymidine, and were continued on the diet until killed 15–21 hr later. Chow-fed rats were injected at the same times as high-fat-fed rats. Nuclear DNA was isolated from fat cells and stroma. Each experiment was done with 2–5 rats and the number of experiments in each group is shown on the figure in parentheses. The mean ± SEM is shown. Data from the epididymal depot is shown in *A*, and from the retroperitoneal depot in *B*. Data from the chow-fed animals whose DNA specific activity was determined together with that of rats on the high-fat diet for 2 days is shown separately because in these rats the fat cell separation procedure was altered by pouring the collagenase digest through 250-μm mesh rather than 500-μm mesh. Values for Student's *t* and *P* (one-tailed) comparing high-fat with chow-fed animals are as follows for epididymal fat cells, retroperitoneal fat cells, epididymal stroma, retroperitoneal stroma, respectively. For 2 days on the diet: 5.16 ($P < 0.002$), 3.65 ($P < 0.008$), 3.30 ($P < 0.02$), 1.69 ($P < 0.08$); for 6 or 7 days on the diet: 4.26 ($P < 0.03$), 3.69 ($P < 0.04$), 4.27 ($P < 0.03$), 3.59 ($P < 0.04$).

was carried out in vitro. Fat pads removed from rats fed the high-fat diet for 3 days and from chow-fed rats were subjected to collagenase digestion in the presence of added [^{3}H]thymidine as previously described (9). In vitro labeling of both adipocyte and stromal DNA was considerably increased in adipose tissue from high-fat-fed rats, compared to chow-fed rats (**Table 4**). The influence of the high-fat diet on [^{3}H]thymidine incorporation was greater in the adipocyte fraction, with a 5- to 6-fold increase in labeling, compared to the stromal fraction, with only a 2- to 3-fold increase in labeling.

A similar comparison of the diet-induced increase in [^{3}H]thymidine incorporation in the adipocyte and stromal fractions can be made for the data on rapid labeling in vivo shown in Fig. 3. For animals fed the diet for 2 days when injected with [^{3}H]thymidine, the specific activity of adipocyte DNA was increased 4- to 5-fold, while that of stromal DNA was increased only 2- to 3-fold. For animals injected after 6 or 7 days

TABLE 4. Comparison of in vitro [^{3}H]thymidine incorporation by adipose tissue from high-fat-fed and chow-fed rats

	High Fat	Chow	Ratio
	dpm/μg DNA		
Epididymal adipocytes	2780	456	6.10
Retroperitoneal adipocytes	3390	665	5.10
Epididymal stroma	1960	831	2.36
Retroperitoneal stroma	2240	805	2.78

Four rats, 5 months old, were used. Two were fed the high-fat diet for 3 days before being killed, and two were maintained on chow. Adipose tissue was removed, and [^{3}H]thymidine was present during the 60-min collagenase digestion as previously described (9). Adipocytes and stroma were separated and nuclear DNA was prepared.

TABLE 5. Ratio of specific activities, fat cell DNA to stroma DNA, in 3-month-old rats fed a high-fat diet for a short time compared to chow-fed rats

	Ratio of DNA Specific Activities		
	High Fat	Chow	
Experiment 1			
High-fat for 2 days prior to [^{3}H]thymidine; 250-μm-mesh			
Epididymal	0.490 ± 0.051 (4)[a]	0.244 ± 0.067 (3)	$t = 2.99$[b] $P < 0.02$
Retroperitoneal	0.488 ± 0.071 (4)	0.156 ± 0.072 (3)	$t = 3.19$ $P < 0.02$
Experiment 2			
High-fat for 6 or 7 days prior to [^{3}H]thymidine; 500-μm mesh			
Epididymal	0.707 ± 0.016 (3)	0.555 ± 0.057 (4)	$t = 2.22$ $P < 0.04$
Retroperitoneal	0.492 ± 0.016 (3)	0.411 ± 0.086 (4)	$t = 0.924$ NS
Comparison of experiments with 250-μm mesh with experiments with 500-μm mesh			
Epididymal	$t = 3.50$ $P < 0.01$	$t = 3.55$ $P < 0.01$	
Retroperitoneal	$t = 0.0587$ NS	$t = 2.16$ $P < 0.04$	

In experiment *1*, high-fat-fed rats were on the diet for 2 days prior to [^{3}H]thymidine injection, while in experiment *2* rats were first on the diet for 6 or 7 days. Fat pads were removed 15–21 hr after [^{3}H]thymidine administration. In experiment *1*, collagenase digests of fat pads were passed through 250-μm mesh, while in experiment *2*, 500-μm mesh was used. Two to five rats were used in each experiment.

[a] Mean ± SEM; number of experiments is in parentheses.

[b] Students t; P value, one-tailed.

on the diet, the specific activity of adipocyte DNA was increased 8- to 14-fold, while for the stromal fraction the increase was only 6- to 10-fold. Thus, the diet-induced increase in rapid labeling of the DNA of adipose tissue, whether it occurs during a 1-hr incubation in vitro or within a day after injection of [^{3}H]-thymidine in vivo, is greater in the adipocyte fraction than in the stromal fraction.

The size of the openings in the nylon mesh used to retain cell aggregates after collagenase digestion appeared to be important in determining the amount of label in the adipocyte fraction when pads were removed from 3-month-old rats that had been injected with [^{3}H]thymidine within the previous day. Use of a larger mesh size, 500-μm, resulted in more label in the adipocyte fraction, suggesting that the larger mesh permitted the passage of aggregates containing labeled cells that were not adipocytes (9). For older rats the mesh size did not seem as important. In the in vivo rapid labeling using rats fed the high-fat diet and those fed chow (Fig. 3), some experiments were done with 250-μm mesh and some with 500-μm mesh. The ratio of DNA specific activities, adipocytes to stroma, was influenced by the mesh size in chow-fed rats (**Table 5**); however, in high-fat-fed rats the ratio was affected by the mesh size in only one of the two fat depots studied. The effect of the mesh size for rats on the high-fat diet is not clear, because the groups being compared also differed in the number of days on the diet.

We tried two ways to bypass the problem of possible stromal contamination causing label to be present in adipocyte DNA. One was to inject rats with [^{3}H]-thymidine when very young (33 days) and to look for a subsequent decrease in adipocyte DNA specific activity after the animals had been on a high-fat diet, as evidence for de novo adipocyte formation. The other was to inject rats after 8 weeks on the high-fat diet and then wait a long period of time before isolating DNA, 3 more weeks on the high-fat diet and then 8 weeks after switching back to chow, so that the DNA specific activity in the presumably more rapidly turning over stromal fraction would have fallen more than that of the adipocyte fraction. The rats were not continued on the high-fat diet for the additional 8 weeks, because the further increase in adipocyte number thus

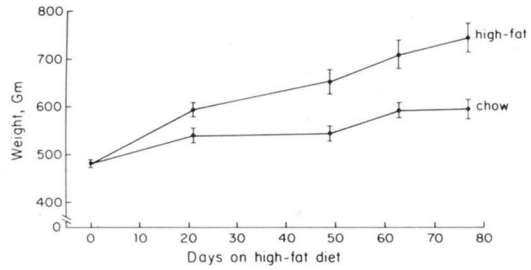

Fig. 4. Body weight curve for rats injected with label at 33 days or 5 months of age. The mean ± SEM is shown. Ten animals received the high-fat diet and eight were maintained on chow.

induced would have decreased the specific activity of adipocyte DNA. In addition, collagenase digestion of adipose depots of rats on the high-fat diet for more than a few weeks gave a poor yield of adipocytes and much oil, presumably because the larger adipocytes were more fragile. Resumption of chow feeding permitted the adipocytes to decrease in size. It had previously been shown in our laboratory (8) that the increased adipocyte number produced by placing rats on a high-fat diet did not diminish when the animals resumed a chow diet.

The body weight curves for these rats are shown in **Fig. 4,** and the number of adipocytes in each depot is shown in **Table 6.** The rats used in this experiment were larger at age 3 months and chow-fed animals had a greater fat cell number than those used in the previous experiment in which cell number was determined (Fig. 1, Table 1), possibly because they were obtained from different suppliers. However, in both experiments, high-fat feeding produced similar increases in body weight and adipocyte number.

The rats injected with [^{3}H]thymidine at age 33 days showed a decrease in the DNA specific activity of both the adipocyte and stromal fractions after being on a high-fat diet for 11 weeks, as compared to chow-fed rats also injected at age 33 days (**Fig. 5**). This suggested that there was de novo cell formation in both the fat cell and stromal fractions as a consequence of the high-fat diet. Rats receiving [^{3}H]thymidine after 8 weeks on the diet, followed by a considerable period of time prior to DNA isolation, showed increased incorporation of label into both fat cells and stromal DNA relative to chow-fed controls, but only in the epididymal depot was the specific activity of fat cell DNA significantly greater than that of stromal DNA ($P < 0.04$, Fig. 5).

TABLE 6. Adipocyte number for rats fed chow or high-fat diets

Diet	Epididymal	Retroperitoneal
	Fat depot adipocyte number × 10^6 [a]	
Chow	13.6 ± 0.90 (8)	16.3 ± 2.0 (8)
High fat[b]	21.7 ± 1.5 (10)	39.6 ± 3.6 (10)
	$t = 4.36$	$t = 5.60$
	$P < 0.0003$	$P < 0.00003$
High fat[c]	19.0 ± 1.3 (4)	32.5 ± 4.2 (4)
	$t = 3.43$	$t = 3.99$
	$P < 0.004$	$P < 0.002$

[a] Results are means ± SEM. Values are given for student's t and P one-tailed. The number of rats in each study is in parentheses.

[b] Ten 3-month-old rats were fed the high-fat diet for 11 weeks, and then chow for 8 weeks. Six of these rats received [^{3}H]thymidine after 8 weeks on the high-fat diet; four had received [^{3}H]thymidine when they were 33 days old.

[c] These four rats, a subset of the group of 10 rats, were administered [^{3}H]thymidine at age 33 days.

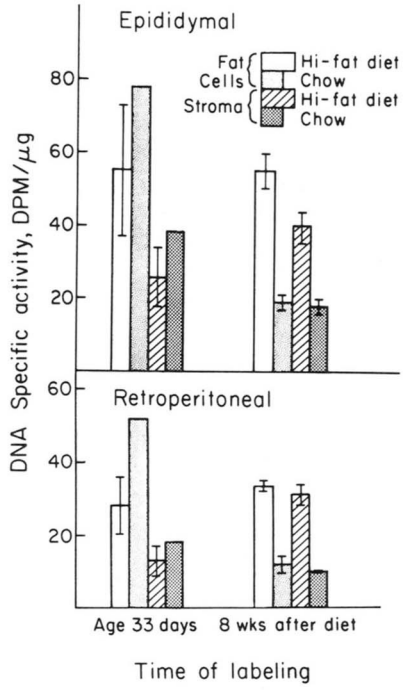

Fig. 5. Comparison of label distribution in adult rats receiving [^{3}H]thymidine at 5 months and at 33 days of age. At age 33 days, 6 of 18 rats were each injected with 500 μCi of [^{3}H]thymidine, which was not followed by an injection of unlabeled thymidine. At age 3 months, four of the labeled and six of the unlabeled rats were begun on a high-fat diet, while the rest remained on chow. After 8 weeks on the high-fat diet, the six unlabeled rats were given 300 μCi of [^{3}H]thymidine followed 17 hr later with 0.48 mmol of unlabeled thymidine. The six unlabeled rats on chow were injected at the same time. The rats fed the high-fat diet were kept on it for a total of 11 weeks and then switched to chow for the last 8 weeks prior to isolation of nuclear DNA. Two rats were required for each DNA isolation. Thus, for rats injected when young there were two determinations of DNA specific activity from high-fat-fed animals and one from chow-fed animals, while for rats injected as adults there were three determinations each from high-fat-fed and chow-fed animals. The mean ± SEM is shown. A comparison of the DNA specific activity in the fat cell and stromal fractions of rats receiving [^{3}H]thymidine after 8 weeks on the high-fat diet showed that, for the epididymal fat depot, Student's $t = 2.41$, with $P < 0.04$ (one-tailed), but for the retroperitoneal depot the difference was not significant.

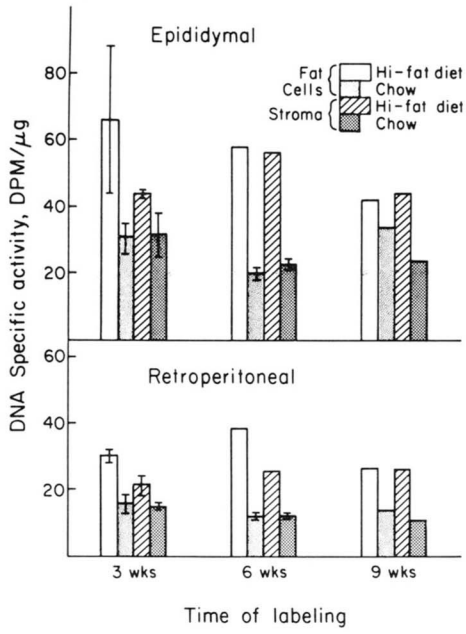

Fig. 6. Comparison of rats receiving [^{3}H]thymidine after different periods of time on the high-fat diet, but DNA not isolated until many weeks later. Rats were begun on the high-fat diet at age 3 months and were injected with [^{3}H]thymidine after either 3, 6, or 9 weeks on the diet. Chow-fed animals received [^{3}H]thymidine at the same times. Unlabeled thymidine, 0.48 mmol, was administered 17 hr after the labeled thymidine. All animals fed the high-fat diet were kept on it for 11 weeks, then put on restricted chow intake for 5 days, followed by chow ad libitum for 1 week. Nuclear DNA was then isolated from fat cells and stroma. Each DNA isolation experiment required two rats. For high-fat-fed animals, two experiments were done at 3 weeks, and one each at 6 weeks and 9 weeks. For chow-fed animals, two experiments were done at 3 weeks, two at 6 weeks, and one at 9 weeks. The mean ± range is shown.

Further experiments were done in which we waited a longer period of time between [^{3}H]thymidine administration and isolation of fat cell and stromal DNA, in an attempt to increase the difference in the specific activity of DNA from the two fractions. In a group of rats to be maintained on the high-fat diet for 11 weeks, some were injected with label after 3, 6, or 9 weeks (**Fig. 6**). Chow-fed rats were injected at the same time. After 11 weeks on the diet, the rats were put on restricted chow intake for 5 days to decrease adipocyte size and reduce cell breakage during collagenase digestion, and then were given chow ad libitum for 1 week. The previous study in Fig. 2 showed that the rate of incorporation of label into fat cell and stromal DNA was fairly constant between 3 and 8 weeks after beginning the diet. This study showed that, despite different intervals between injection of label and termination of the diet (2–8 weeks), the specific activities of fat cell DNA and stromal DNA remained close, indicating continued cell proliferation in both fractions while the rats were fed the high-fat diet.

Cellular proliferation in both the fat cell and stromal fractions was confirmed by looking at the amount of DNA in the two fractions for the rats used in the studies shown in Figs. 5 and 6 (**Table 7**). In both studies, rats were on the high-fat diet for 11 weeks. Two rats were used for each experiment, and nuclear DNA was isolated from both fractions of the epididymal and retroperitoneal depots. High-fat feeding caused an increase of about 40% in the amount of DNA in both the fat cell and stromal fractions of the epididymal pad,

TABLE 7. Amount of DNA in the fat cell and stromal fractions of high-fat-fed and chow-fed rats

	High-Fat	Chow	Ratio, High-Fat to Chow
	μg of DNA		
Epididymal fat cells	137 ± 15.7	95.2 ± 8.02	1.44
	$t = 2.35$		
	$P < 0.02$		
Retroperitoneal fat cells	180 ± 48.9	97.8 ± 8.56	1.84
	$t = 1.66$		
	NS		
Epididymal stroma	372 ± 19.4	258 ± 14.2	1.44
	$t = 4.73$		
	$P < 0.0002$		
Retroperitoneal stroma	752 ± 29.0	388 ± 15.4	1.94
	$t = 11.1$		
	$P < 0.$ (extremely small)		

The data are from the same rats used for experiments depicted in Figs. 5 and 6. Each determination of DNA specific activity in the fat cell and stromal fractions of the epididymal and retroperitoneal depots required two rats. Nine experiments were done with rats on the high-fat diet for 11 weeks, and nine with chow-fed rats of the same age. Nuclear DNA was isolated. The mean amount of DNA in μg ± SEM is given with the value for student's t and P, (one-tailed). For the amount of DNA per rat, the values in the table should be divided by two.

and more than 80% in both fractions of the retroperitoneal pad. The parallel increase in DNA content in both fractions helps to understand why the DNA specific activity in both fractions should be so similar.

DISCUSSION

These studies show that the feeding of a high-fat diet to adult rats causes an increase in the incorporation of [^{3}H]thymidine into the DNA of both the adipocyte and stromal fractions. The permanent increase in adipocyte number induced in adult rats by feeding this diet (8) is thus probably caused by proliferation of progenitor cells rather than lipid-filling of existing preadipocytes.

Hollenberg and Vost (12) had shown that 2–5 days is required for the filling of newly formed adipocytes as determined by transfer of label from the stromal fraction to adipocytes after [^{3}H]thymidine administration. When rats were injected with label at different times after beginning the high-fat diet (Fig. 2), we therefore waited 2 weeks between administration of [^{3}H]thymidine and removal of fat pads for the determination of DNA specific activity in the fat cell and stromal fractions. After 1 week on the diet the rate of DNA synthesis was increased by 6-fold or more in both the adipocyte and stromal fractions compared to chow-fed rats, but it subsequently decreased. In rats fed the high-fat diet for 3–8 weeks, the rate of DNA synthesis seemed to stabilize at a new, higher level, about two or three times that in chow-fed rats for the adipocyte fraction. Most importantly, after 5 weeks on the diet (Table 1), there was a startling increase in the specific activity of DNA of the adipocyte fraction long before additional adipocytes were seen.

A major difficulty was that the DNA specific activity of the adipocyte fraction often seemed to parallel that of the stromal fraction, suggesting that label in the adipocyte fraction might not represent DNA synthesis there, but instead be due to stromal contamination. However, the specific activity of fat cell DNA from the retroperitoneal pad was found to be significantly greater than that of stromal DNA in rats injected with [^{3}H]thymidine 2–8 weeks after the start of the diet, making stromal contamination unlikely as the cause of fat cell DNA labeling (Fig. 2). In the epididymal pads from rats injected with [^{3}H]thymidine after 8 weeks on the diet and 11 more weeks elapsing before removal of fat pads, fat cell DNA also had a significantly greater specific activity than that of stromal DNA (Fig. 5).

An additional approach to confirm new DNA formation was to label rats when young (33 days old) and look for a decrease in DNA specific activity after high-fat feeding compared to chow-fed rats. The high-fat diet was found to cause a decrease in the DNA specific activity of both adipocytes and stroma of rats labeled when young, relative to chow-fed rats (Fig. 5), indicating that diet-induced cellular proliferation occurred in both fractions. Parallel increases in nuclear DNA content in the adipocyte and stromal fractions of a given fat pad (Table 7) explain why the high-fat diet caused similar changes in DNA labeling of the two fractions.

In related work we found label in DNA of the adipocyte fraction within a day after [^{3}H]thymidine administration to chow-fed adult rats, and this label was interpreted as being in the nuclear DNA of a small number of proliferating cells intimately associated with mature adipocytes. Labeling of adipocyte DNA was so rapid that it could be shown to occur during a 1-hr incubation in vitro in the presence of added [^{3}H]thymidine (9). In the present work, high-fat feeding caused this rapid labeling of DNA of the adipocyte fraction to increase after as short a time as 2 days on the diet (Fig. 3). Three days of the high-fat diet (2 days before injection plus 1 day after) also caused a significant increase in fat pad weight (Table 2) and in the amount of nuclear DNA in the stromal fraction (Table 3). In vitro incorporation of [^{3}H]thymidine by DNA of the adipocyte and stromal fractions was also increased by prior feeding of the high-fat diet (Table 4). The high-fat diet caused a greater increase in the rapid labeling of the DNA of the adipocyte fraction than that of the stromal fraction, both in vivo and in vitro. The greater effect of the diet on labeling of DNA of the adipocyte fraction suggests that such labeling has physiological significance and is not merely artifactual. Moreover, after in vitro labeling the specific activity of adipocyte DNA was higher than that of stromal DNA in tissue from high-fat-fed rats, making contamination from stroma an unlikely explanation for the labeling of adipocyte DNA.

The diet-induced increase in the rapid labeling of the adipocyte fraction, seen within a time too short for the appearance of mature adipocytes from newly formed cells, suggests that these adipocyte-associated proliferating cells play an important role in the eventual appearance of additional adipocytes. The adipocyte-associated cells may even be adipocyte progenitors.

The adipocyte fraction from adult human tissue has been found to contain cells that proliferate in culture and that differ from skin fibroblasts in incorporating 16-fold more [^{14}C]glucose into lipid in the presence of insulin (13). The occurrence of possible adipocyte progenitors, both in the adipocyte fraction (perhaps identical to the proliferating cells that we found in vivo)

and in the stromal fraction (14, 15) suggests that these different locations may have some physiologic significance. It is possible that the proliferating progenitors closely associated with mature adipocytes interact with them and that this interaction results in a direct relationship between adipocyte size and progenitor proliferation—when adipocyte size is small, progenitor turnover is minimal, but as the lipid content of adipocytes increases, the proliferation of progenitors is stimulated. Additional evidence for this postulated relationship between adipocyte size and progenitor proliferation is the observation that adipocyte hyperplasia in rats fed a diet high in fat or sucrose is seen only after a maximal adipocyte lipid content of about 1.5 μg per cell is reached (8). The progenitors isolated from the stromal fraction may be a different stage of the same cell type or they may be a different type entirely, one which may have a different early role but later also differentiates into an adipocyte.

The study of differentiation in other tissues has provided models that may help interpret experiments with adipocytes and their progenitors and suggest additional approaches. Adipocyte-associated progenitors may be equivalent to the renewing stem cells of spermatogonia, which frequently divide and later differentiate, while progenitors in the stromal fraction may resemble the reserve stem cells of spermatogonia, which function as a backup to provide additional progenitors when needed (16). Adipocyte progenitors located in the different fractions may also differ in their proliferative capacity and their commitment to differentiation, as do different progenitors of the erythroid series; early erythroid progenitors have high proliferative capacity but their number does not vary in anemia or polycythemia, while late erythroid progenitors will divide only several times before differentiating and their number is very responsive to physiological need (17, 18). Perhaps the adipocyte-associated progenitors are equivalent to the late erythroid progenitors, since both display responsiveness to the physiological situation; just as the number of late erythroid progenitors is increased by anemia or erythropoietin administration, the adipocyte-associated proliferating cells appear to be increased by feeding a high-fat diet. Cell culture systems for the differentiation of adipocytes and erythroid cells suggest that, for both, the commitment to differentiation is a random event. For example, the murine fibroblast cell line 3T3 converts to adipocytes at a frequency determined by the clone and the medium composition (19), and the Friend murine erythroleukemia cell line can be induced to form late erythroid progenitors with certain agents that appear to act in a stochastic manner, a constant fraction of the cell population remaining uncommitted to differentiation during each cell division cycle (20). These fundamental similarities to other tissues suggest that adipocyte differentiation could be studied using similar approaches, but the special problems encountered with this tissue emphasize that new approaches must be developed as well. A better understanding of adipocyte differentiation and regulation of adipocyte number must await morphologic and biochemical study of adipocyte progenitors.

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REFERENCES

1. Hirsch, J., and P. W. Han. 1969. Cellularity of rat adipose tissue: effects of growth, starvation, and obesity. *J. Lipid Res.* **10:** 77–82.
2. Anderson, D. R., and R. G. Kauffman. 1972. Cellular and enzymatic changes in porcine adipose tissue during growth. *J. Lipid Res.* **14:** 160–168.
3. Hood, R. L., and C. E. Allen. 1977. Cellularity of porcine adipose tissue: effects of growth and adiposity. *J. Lipid Res.* **18:** 275–284.
4. Hirsch, J., and J. L. Knittle. 1970. Cellularity of obese and nonobese human adipose tissue. *Federation Proc.* **29:** 1516–1521.
5. Salans, L. B., E. S. Horton, and E. A. H. Sims. 1971. Experimental obesity in man: Cellular character of the adipose tissue. *J. Clin. Invest.* **50:** 1005–1011.
6. Johnson, P. R., and J. Hirsch. 1972. Cellularity of adipose depots in six strains of genetically obese mice. *J. Lipid Res.* **13:** 2–11.
7. Lemonnier, D. 1972. Effect of age, sex and site on the cellularity of the adipose tissue in mice and rats rendered obese by a high fat diet. *J. Clin. Invest.* **51:** 2907–2915.
8. Faust, I. M., P. R. Johnson, J. S. Stern, and J. Hirsch. Diet-induced adipocyte number increase in adult rats: A new model of obesity. *Am. J. Physiol.* **235:** E279–E286.
9. Klyde, B. J., and J. Hirsch. 1979. Isotopic labeling of DNA in rat adipose tissue: evidence for proliferating cells associated with adipocytes in adults. *J. Lipid Res.* **20:** 691–704.
10. Faust, I. M., P. R. Johnson, and J. Hirsch. 1977. Surgical removal of adipose tissue alters feeding behavior and the development of obesity in rats. *Science.* **197:** 393–396.
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.
12. 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.

13. Dixon-Shanies, D., J. Rudick, and J. L. Knittle. 1975. Observations on the growth and metabolic functions of cultured cells derived from human adipose tissue. *Proc. Soc. Exp. Biol. Med.* **149:** 541–545.
14. Poznanski, W. J., I. Waheed, and R. Van. 1973. Human fat cell precursors: Morphologic and metabolic differentiation in culture. *Lab. Invest.* **29:** 570–576.
15. Dardick, I., W. J. Poznanski, I. Waheed, and G. Setterfield. 1976. Ultrastructural observations on differentiating human preadipocytes cultured in vitro. *Tissue and Cell.* **8:** 561–571.
16. Clermont, Y. 1972. Kinetics of spermatogenesis in mammals: Seminiferous epithelium cycle and spermatogonial renewal. *Physiol. Rev.* **52:** 198–236.
17. Clarke, R. J., and D. Houseman. 1977. Characterization of an erythroid precursor cell of high proliferative capacity in normal human peripheral blood. *Proc. Natl. Acad. Sci. USA* **74:** 1105–1109.
18. Nienhuis, A., and E. J. Benz, Jr. 1977. Regulation of hemoglobin synthesis during the development of the red cell. *N. Engl. J. Med.* **297:** 1318–1328, 1371–1381, 1430–1436.
19. Green, H., and O. Kehinde. 1976. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell.* **7:** 105–113.
20. Gusella, J., R. Geller, B. Clarke, V. Weeks, and D. Houseman. 1976. Commitment to erythroid differentiation by erythroleukemia cells: A stochastic analysis. *Cell.* **9:** 221–229.