Isotopic labeling of DNA in rat adipose tissue: evidence for proliferating cells associated with mature adipocytes

Barry J. Klyde and Jules Hirsch

Rockefeller University, New York, NY 10021

Abstract The intraperitoneal administration of [^{3}H]thymidine to adult rats resulted in the rapid appearance of label in the adipocyte fraction of collagenase digests of adipose tissue. Low-speed centrifugation followed by freezing and slicing showed the label to be uniformly distributed in the adipocyte fraction. The presence of label in DNA was confirmed by hydrolysis with deoxyribonuclease and by inhibition of incorporation with hydroxyurea. Organelle fractionation revealed that the label was predominantly in nuclei, and radioautography showed that only a few adipocyte nuclei were labeled. The label in the adipocyte fraction could not be reduced by increased collagenase digestion or by trypsin treatment. Mixing of labeled adipocytes with unlabeled stroma did not result in decrease of label and addition of labeled stroma to unlabeled adipocytes did not cause significant transfer of radioactivity. Addition of [^{3}H]thymidine to the collagenase digestion medium of unlabeled adipose tissue resulted in more incorporation by adipocytes than by stroma, suggesting the presence of a very rapidly proliferating cell type associated more with adipocytes than with stroma. In vivo turnover studies of labeled DNA indicated that there are two components in both adipocytes and stroma, a rapidly labeled component with a half-life of only several days and another with a half-life of several months. These experiments suggest that there is a rapidly proliferating cell type in adipose tissue, closely associated with mature adipocytes, that may be an adipocyte progenitor or may have some other unknown function—**Klyde, B. J., and J. Hirsch.** Isotopic labeling of DNA in rat adipose tissue: evidence for proliferating cells associated with mature adipocytes. *J. Lipid Res.* 1979. **20:** 691–704.

Supplementary key words tritiated thymidine · Osborne-Mendel rats · differentiation

It is clear from many lines of evidence (1–5) that adipocyte size is more easily altered than adipocyte number. Indeed, a number of experiments suggest that the number of adipocytes in rats becomes stable at a relatively early age (2, 6). Under some circumstances, however, the number of mature adipocytes can be shown to increase even in the adult rat. Thus, the feeding of a high-fat diet leads to an increase in adipocyte number (7) which persists and is found in all depots tested (8). Furthermore, the surgical removal of adipose tissue from the inguinal area of the rat is followed by the replacement of lost cells (9), indicating the potential for proliferation of fat cells even in the adult. Additionally, cultures of adult human or rat adipose tissue have been shown to contain adipocyte progenitors, which proliferate and later differentiate (10, 11).

Two groups of investigators have used the incorporation of [^{3}H]thymidine into DNA to study turnover of adipocytes and their progenitors. Both Hollenberg and Vost (12) and Greenwood and Hirsch (6) injected labeled thymidine into rats, followed by a "chaser" of unlabeled thymidine to create a brief pulse of exposure to the isotope, and then killed the animals at various points in time intervals after the pulse labeling. Adipocytes were separated from stroma by collagenase digestion, and label in both adipocyte and stromal DNA was determined. Hollenberg and Vost (12) found that the label initially was almost all in the stromal DNA and then appeared in adipocyte DNA several days later, an expected sequence if progenitor cells in the stroma become labeled during the pulse and then fill with lipid over several days. Greenwood and Hirsch (6) interpreted their data, accumulated in rats of various ages, to show no significant adipocyte turnover in adults. In very young rats they found brisk labeling of stroma, with a progressive increase in adipocyte label over many days, presumed to be due to the slow filling with lipid of a group of labeled stromal "pre-adipocytes." However, the interpretations of Greenwood and Hirsch were made difficult because label was present in adipocytes even shortly after the pulse in older animals, where turnover was not believed to occur. It was assumed that such label in the adipocyte fraction was the result of an inevitable contamination with stromal cells which could not be fully separated from adipocytes.

The purpose of this communication is to examine the label found in the adipocyte fraction of adult rats

Downloaded from www.jlr.org by guest, on June 19, 2012

to determine whether it represents stromal contamination of an artifactual type, or if it is of physiological significance.

MATERIALS AND METHODS

Animals

Male rats of the Osborne-Mendel strain were used in most of these experiments because we planned to later study the diet-induced adipocyte hyperplasia which can be so readily induced in this strain (8). The rats were obtained initially 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. Sprague-Dawley rats (Zivic Miller Laboratories, Allison Park, PA) were used in some procedures concerned primarily with methodology. All rats were maintained on Purina Chow, were from 5 months to over 1 yr of age at the time DNA was isolated, unless otherwise noted, and ranged in weight from 400 to 820 g. They were each injected intraperitoneally with 300 μCi of [^{3}H]thymidine (methyl ^{3}H, 20 Ci/mmol, New England Nuclear). If the rats were to be kept longer than 1 day after receiving [^{3}H]thymidine, they were given an intraperitoneal injection of 0.48 mmol of unlabeled thymidine (Sigma) 17 hr after the first injection.

Preparation of isolated fat cells

Rats were anesthetized with ether and decapitated. Epididymal and retroperitoneal fat pads were removed, weighed, rinsed with saline, minced, and then incubated with Krebs-Ringer bicarbonate buffer with albumin using the collagenase digestion method of Rodbell (13), except that the concentration of glucose used was 5.55 mM, the concentration of collagenase (Worthington) was varied from 1 to 3 mg/ml depending on the activity of each lot, and 1–2 g of adipose tissue were used per 3 ml of buffer. After incubation the samples were poured through nylon mesh with either 500 or 250 μm openings (Tetko, Inc., Elmsford, NY); thereafter, the fat cells were separated by flotation. The pink aqueous infranate was aspirated and spun in a tabletop centrifuge to precipitate the stromal fraction. The stromal precipitate was washed twice more by resuspending it in warm buffer and centrifuging. The fat cell layer was washed four times, twice by flotation after the addition of fresh, warm buffer and twice by centrifuging in 17 × 100 mm plastic tubes at 300–400 *g*, at 10–15°C. After the first spin, a pink zone was seen at the fat cell–buffer interface. This was aspirated through Teflon tubing and was not observed after the second spin.

Isolation of DNA

Pooled fat pads from either two or three rats were used for each experiment. DNA was isolated by modifications of the methods of Hollenberg and Vost (12) and Greenwood and Hirsch (6). For total cellular DNA, fat cell and stromal samples were each suspended in 2.5 ml of 1 M NaCl, 25 mM Tris-HCl buffer, pH 7.5, and frozen and thawed three times. All further steps were at 0–5°C, unless otherwise noted. Samples were washed twice with 10 ml of diethyl ether, twice with water-saturated phenol at 10°C (20 ml, then 10 ml), and incubated overnight at 37°C after the addition of 5 ml of 0.5 N KOH. Cold 1 N perchloric acid, 10 ml, was added to each sample and, after standing 20 min in an ice bath, the precipitates were collected by centrifugation. Samples were washed once more with perchloric acid, twice with 95% ethanol, twice with diethyl ether, and then they were air dried. They were then hydrolyzed in 3 ml of 1 N perchloric acid at 70°C for 30 min. After clarifying the samples by centrifugation, 1 ml of the supernatant was added to a vial with Aquasol-2 scintillation cocktail (New England Nuclear) for determination of radioactivity, and 1 ml was used for DNA determination by a modification of the diphenylamine method (14). Radioactivity was measured in a Packard Tricarb liquid scintillation counter at a counting efficiency of 44% for tritium standards prepared with the same counting cocktail and containing the same amount of acid and water as the experimental samples. In some experiments DNA was isolated from nuclei, which were separated by differential centrifugation after homogenization of fat cell or stromal preparations in a Teflon–glass tissue grinder, followed by repeated washing (15). Homogenization of fat cell samples was done at room temperature, because lower temperatures caused the released oil to solidify. All subsequent steps were at 0–5°C unless noted. The solid fat cake present after centrifugation was removed with a spatula. The samples of nuclei were suspended in 2.5 ml of 1 M NaCl, 25 mM Tris-HCl, pH 7.5, and all further steps were the same as for the isolation of total cellular DNA, except that freezing and thawing was omitted and only a single wash with phenol was done.

Student's *t* test was used for data analysis and was corrected for heterogeneity of variance.

RESULTS

When [^{3}H]thymidine is given intraperitoneally to adult rats only a few hours before killing, there is great variability in the specific activities of both stromal

Downloaded from www.jlr.org by guest, on June 19, 2012

and adipocyte DNA. In our hands, rats of the same age given pulses identical in amount and duration and killed at the same time showed considerable variation in DNA specific activities both from animal to animal and from one fat depot to another within the same animal. Thus, for purposes of comparison, the data were normalized by dividing the DNA specific activities of the adipocyte fraction by those of the stromal fraction. A surprising constancy of this ratio was found. Thus, in six separate experiments with pooled fat depots from two or three rats per experiment, injected 1–17 hr before killing, the ratio of DNA specific activities (adipocytes to stroma) was found to be 0.405 ± 0.40 (mean ± SEM) in the epididymal fat depot and 0.547 ± 0.042 in the retroperitoneal depot. While these ratios were fairly constant from animal to animal, for any given animal the actual specific activity of DNA from the epididymal depot, whether from adipocytes or from stroma, always had a higher specific activity than DNA from the retroperitoneal depot, and this was thought to be due to the greater uptake by the epididymal fat pads because of direct exposure to the isotope after intraperitoneal administration. Possible causes of the observed labeling of adipocyte DNA, other than true DNA synthesis, include incorporation into non-DNA compounds, incorporation into DNA via repair rather than synthesis, artifactual incorporation during collagenase digestion, and contamination of adipocytes with DNA fragments from the stromal fraction as well as association of stromal cells with adipocytes, even after collagenase digestion. Experiments were done to systematically test these possibilities. The results suggest that much of the label in the adipocyte fraction is contained in the nuclei of a proliferating cell type in close association with adipocytes.

Adipocyte label is in DNA

A major problem encountered in the use of [^{3}H]-thymidine for the measure of DNA synthesis is that much of the label ends up in compounds other than DNA (16–19). Although we used a rigorous DNA purification procedure that included steps to remove lipid, protein, and RNA contaminants, further studies were done to assure that the adipocyte label was indeed in DNA.

Total cellular DNA was isolated from fat cells and from stroma after in vivo labeling by intraperitoneal injection of [^{3}H]thymidine. A portion of each DNA fraction was treated with DNAase, and the specific activity of the enzyme-treated and untreated portions was compared (**Table 1**). The DNAase-treated samples were not entirely hydrolyzed, although the enzyme was present in excess, possibly because the samples could only be suspended rather than completely dissolved in the buffer. The specific activity was unchanged after DNAase treatment, although only 34–54% of the fat cell DNA remained, indicating that there was so little label in non-DNA compounds that it could not be detected with our methods. The presence of a detectable amount of label in non-DNA compounds would have resulted in an increase in the observed specific activity after partial hydrolysis with DNAase.

Hydroxyurea was administered 1 hr before [^{3}H]-thymidine at a dose of 1000 mg/kg (20) to determine if the observed incorporation of [^{3}H]thymidine following in vivo administration was occurring via syn-

TABLE 1. Treatment of in vivo-labeled DNA with deoxyribonuclease (DNAase)

	Amount of DNA:[a] DNAase-treated/ saline-treated	Specific Activity:[b] DNAase-treated/ saline-treated
Epididymal fat cells	12.4/36.1 = 0.344	256/250 = 1.02
Retroperitoneal fat cells	24.3/58.5 = 0.416	58.5/59.0 = 0.991
Epididymal stroma	47.3/87.8 = 0.539	347/444 = 0.782
Retroperitoneal stroma	91.9/218 = 0.421	95.7/87.5 = 1.09

In vivo-labeled total cellular DNA was prepared from three rats given a pulse of [^{3}H]thymidine 17 hr before death. Each DNA sample was divided in half after the KOH incubation step. After the final ether wash, the precipitated samples were suspended in 5 ml of 50 mM Tris-HCl, pH 8.0, 7 mM $MgSO_4$. One hundred μg of pancreatic deoxyribonuclease type I, low RNAase activity (Worthington), dissolved in saline, was added to half of each sample, while saline alone was added to the other half. After 2 hr of incubation at 37°C, samples were precipitated with cold 1 N perchloric acid, washed with 95% ethanol, then diethyl ether, and treated as usual. As little as 16 μg of enzyme was shown under these conditions to completely hydrolyze 400 μg of calf thymus DNA (Worthington).

[a] μg per total sample.

[b] dpm/μg DNA.

Downloaded from www.jlr.org by guest, on June 19, 2012

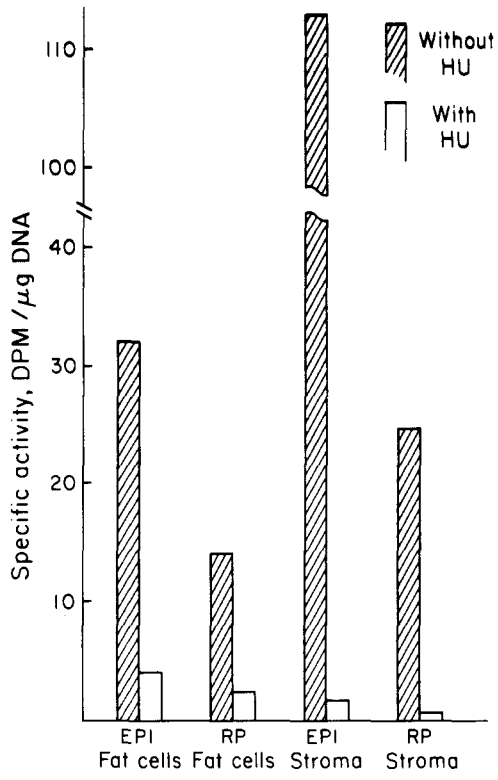

Fig. 1. DNA specific activity after in vivo administration of [^{3}H]-thymidine and hydroxyurea. Four rats were used. All animals received [^{3}H]thymidine 4.5 hr prior to death. Two rats received 1000 mg/kg hydroxyurea (HU) intraperitoneally 1 hr before the [^{3}H]thymidine. Total cellular DNA was isolated. Epi, epididymal; RP, retroperitoneal.

thesis of DNA (**Fig. 1**). There was marked inhibition of labeling, with a suggestion of somewhat greater inhibition in stroma than in adipocytes. In all fractions, more than 83% of the label in DNA thus appears to result from incorporation during DNA synthesis. The specific inhibitors hydroxyurea and cytosine arabinoside (used in a later experiment) inhibit semiconservative DNA replication, but do not inhibit repair replication (21). Thus, in these experiments the major part of ^{3}H labeling is indicative of DNA replication preceding cell division, rather than label incorporation during repair.

Labeled DNA in nuclei

The label in the adipocyte fraction might have resulted from the adherence to adipocytes of stromal DNA fragments or organelles. More extensive washing procedures failed to decrease the specific activity of the fat cell DNA. The use of dinonylphthalate, an oil more dense than fat cells but less dense than aqueous solutions (22), to try to reduce the apparent contamination of the adipocyte fraction with stromal components also did not diminish adipocyte DNA specific activity. Possible contamination of the fat cell fraction with stromal DNA released during collagenase incubation was tested by including DNAase, 2 μg/3 ml, in the collagenase digestion buffer for fat pads removed from rats injected 17 hr previously with [^{3}H]thymidine. The specific activity of fat cell DNA was not reduced.

The most direct evidence that the label associated with the adipocyte fraction is in nuclei came from a study of subcellular fractions. The label in fat cell DNA after in vivo administration of [^{3}H]thymidine was found to be predominantly in nuclei after fractionation of organelles by differential centrifugation (**Table 2**). When total cellular DNA was isolated from the adipocyte and stromal fractions of both the epididymal and retroperitoneal fat depots, as described above, the ratio (adipocyte/stromal) of specific activities was

TABLE 2. Proportions of DNA in different cell fractions, and DNA specific activity

	Proportion of DNA in Different Fractions				DNA Specific Activity, dpm/μg DNA		
	Nuclei	Mitochondria[a]	Microsomes	Total μg DNA	Nuclei	Mitochondria	Microsomes
Expt. 1: 7.5-hr pulse							
Epididymal fat cells	0.964	0.036	0	128	107	400	
Retroperitoneal fat cells	0.709	0.161	0.130	45.8	26.2	88.5	91.7
Epididymal stroma	0.907	0.057	0.036	359	297	491	505
Retroperitoneal stroma	0.929	0.048	0.024	461	78.9	135	162
Expt. 2: 17-hr pulse							
Epididymal fat cells	0.875	0.092	0.033	246	57.4	132	136
Retroperitoneal fat cells	0.820	0.095	0.085	145	8.69	38.8	33.9
Epididymal stroma	0.951	0.026	0.023	854	118	159	315
Retroperitoneal stroma	0.920	0.054	0.026	705	43.3	42.1	72.2

[a] Mitochondria and microsomes were prepared by differential centrifugation, as described by Schneider and Kuff (15). DNA was isolated as from nuclei.

Downloaded from www.jlr.org by guest, on June 19, 2012

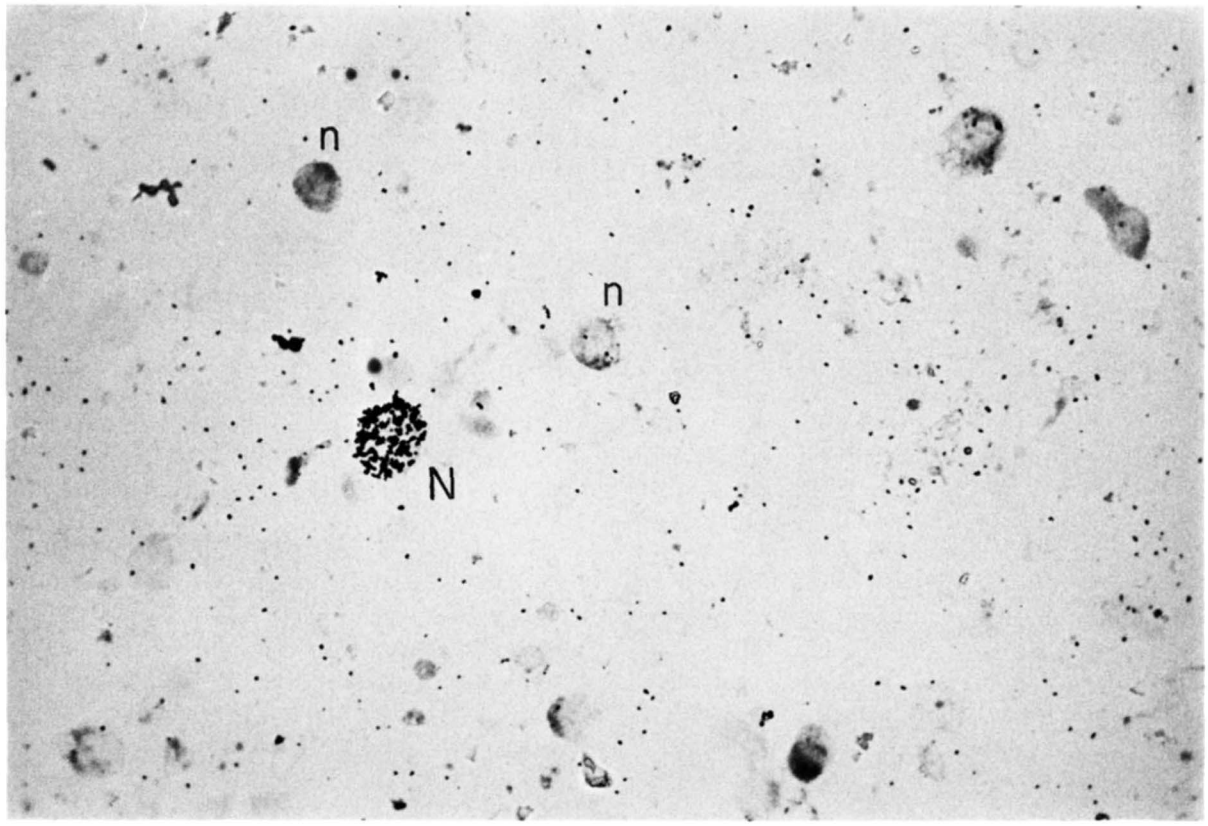

Fig. 2. Radioautography of nuclear preparations from fat cells. Epididymal and retroperitoneal fat pads were removed from three rats (7 months old) injected 12 hr previously with [^{3}H]thymidine; and after collagenase digestion the adipose fractions were pooled. Nuclei for radioautography were prepared by the method of Maggio, Siekevitz, and Palade (31), modified by first doing two low-speed centrifugations (850 *g*, 20 min, 0–5°C) of the samples of adipocyte nuclei in 0.88 M sucrose, 1.5 mM $CaCl_2$, and using a spatula to remove the fat cake above the supernatant prior to ultracentrifugation. The nuclear preparations were resuspended in the same solution, centrifuged onto coverslips, dipped in Kodak NTB-3 emulsion, and exposed for 4 days. Less than 1% of the nuclei were labeled. A representative field, with a single labeled nucleus (N) and several unlabeled nuclei (two of them designated *n*), is shown.

shown above to differ in the different depots, with the ratio in the retroperitoneal depot being greater than that in the epididymal depot. However, the difference was not seen when nuclear DNA was isolated from both depots. In five experiments using two or three rats per experiment, with [^{3}H]thymidine administered 1–17 hr before killing, the ratio in the epididymal depot was 0.384 ± 0.038, and that in the retroperitoneal depot was 0.366 ± 0.063. The reason for the difference in adipocyte to stromal ratio between depots became apparent when DNA was isolated from different organelle fractions (Table 2). The specific activity of DNA from the mitochondrial and microsomal fractions was always higher than that of nuclear DNA in any cellular fraction. Schneider and Kuff (15) made the same observation for rat and mouse liver, and showed that DNA from mitochondria and microsomes had a shorter half-life than nuclear DNA. We found a lower proportion of the total DNA of retroperitoneal fat cells in nuclei, when compared to epididymal fat cells. The larger ratio of specific activities, adipocyte DNA to stromal DNA, for the retroperitoneal fat depot can be explained by the observation that in retroperitoneal fat cells a larger portion of the total DNA is non-nuclear, with a higher specific activity than nuclear DNA, compared to epididymal fat cells.

Radioautography of nuclear preparations from fat cells and stroma confirmed that the label was within nuclei, and showed that it was present in only a small proportion of each fraction (**Fig. 2**). It is tentatively assumed that the labeled nuclei are within cells, rather than being released from the stromal fraction during cell breakage and becoming attached to adipocytes during collagenase digestion.

Binding of labeled cells to adipocytes

The possibility that stromal cells remain attached to adipocytes even after collagenase digestion was tested by increasing either the collagenase concentration or the digestion time. For adipose tissue from adults these changes decreased the yield of adipocytes

ASBMB JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 19, 2012

and increased the amount of oil observed, presumably because of cell breakage, but did not alter the DNA specific activity. For tissue from younger animals these changes also did not alter the specific activity, but the adipocyte yield was increased rather than decreased, probably because of the lesser fragility of the smaller adipocytes.

Trypsin treatment of the fat cell fraction after collagenase digestion was done to see if an adherent cell type, responsible for the uptake of label, could be dissociated from fat cells (**Table 3**). Crude trypsin (1:300, Gibco) was used at a concentration of 0.25% (23). The yield of fat cell nuclear DNA was low, partly because of cell breakage, and a considerable portion of DNA was apparently shifted from nuclei to microsomes. The specific activity of fat cell DNA was not lowered by treatment with trypsin, both in this experiment and in another using 2× crystallized trypsin (Worthington), indicating that labeling of DNA was not occurring in a cell type that could be dissociated from adipocytes by enzyme treatment.

The possibility that the label in DNA of the fat cell fraction resulted from adherence of stromal cells or other stromal components was tested by mixing labeled stroma with unlabeled fat cells. In a first experiment, stroma prepared from rats injected with [^{3}H]thymidine was added to unlabeled fat cells that had undergone collagenase digestion and had been washed twice (**Table 4**). The duration of mixing was brief, with only the few minutes required for the fat cells to separate from the stroma by flotation, and the fat cells and stroma were then washed in the usual way. The specific activity of the fat cell DNA was 5–10% of that of the stromal DNA to which it was exposed, considerably less than the ratio usually seen following in vivo incorporation.

In the second experiment (Table 4) the duration of mixing was much longer; stroma from [^{3}H]thymidine-injected rats was mixed with fat pads from unlabeled rats during the collagenase digestion. Fat cells and stroma were then separated and washed in the usual way. The unlabeled fat cells were exposed to their own, unlabeled, stroma as well as to the labeled stroma added during collagenase digestion. Hence, the lower specific activity of the mixture of labeled and unlabeled stroma rather than that of the added labeled stroma represents the DNA specific activity of stroma that was in contact with the unlabeled fat cells. After mixing during collagenase digestion, the specific activity of fat cell nuclear DNA was 14% of that of the stroma in contact with it, again less than the relative activity seen after in vivo incorporation. Mixing experiments using labeled stroma from young rats and unlabeled fat cells from adults produced similar results.

The mixing experiments above are difficult to interpret because the unlabeled adipocytes may already be associated with unlabeled proliferating cells so that attachment of or replacement by additional cells is not possible, or because the association between mature adipocytes and proliferating cells may be established during adipocyte differentiation.

To test whether the association of label with fat cell DNA was reversible, fat cells from labeled rats were mixed with unlabeled stroma. In the first experiment, stroma prepared from unlabeled rats was added just prior to collagenase digestion of fat pads from rats injected previously with [^{3}H]thymidine to see whether the presence of additional stroma would cause the specific activity of fat cell DNA to decrease (**Table 5**). There was no diminution of DNA specific activity in fat cells exposed to the unlabeled stroma during collagenase digestion, compared to fat cells that had nothing added during preparation. In the labeled animals used in this experiment the pads of the left side appeared to have taken up more label, as evident by

TABLE 3. Trypsin treatment of fat cells after collagenase digestion

	Proportion of DNA in Different Fractions				DNA Specific Activity		
	Nuclei	Mitochondria	Microsomes	Total DNA	Nuclei	Mitochondria	Microsomes
				μg		*dpm/μg*	
Epididymal fat cells	0.089	0.021	0.899	81.1	41.2	674	129
Retroperitoneal fat cells	0.039	0.260	0.701	114	39.6	40.2	34.4
Epididymal stroma	0.671	0.184	0.145	461	343	524	500
Retroperitoneal stroma	0.705	0.161	0.134	594	99.4	124	125

Fat pads were removed from three rats that had received a 17-hr pulse of [^{3}H]thymidine. After collagenase digestion, fat cells were washed once and stroma twice in Krebs-Ringer bicarbonate buffer prepared without calcium, magnesium, or albumin. Fat cells were then incubated for 20 min at 37°C in 0.25% trypsin 1:300 (Gibco) made up in the same buffer. The fat cells were washed once more by flotation, twice by centrifugation, and nuclear DNA was then isolated. The proportion of DNA in the different organelle fractions should be compared with the results shown in Table 2.

Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 4. Effect of mixing radioactive stroma with unlabeled fat cells

	Activity in Cells of Rats Injected with Label dpm/μg DNA	Activity in Unlabeled Cells Mixed with Labeled Stroma, dpm/μg DNA	Relative Activity of Unlabeled Fat Cells as Compared to Stroma in Contact with Them
			%
Experiment 1: Mixing during washing of fat cells			
Epididymal fat cells	208	27.8	5.19%
Retroperitoneal fat cells	56.2	10.1	9.53%
Epididymal stroma	536	—	—
Retroperitoneal stroma	106	—	—
Experiment 2: Mixing during collagenase digestion			
Epididymal fat cells	321	17.1	14.3%
Retroperitoneal fat cells	68.5	—[a]	—
Epididymal stroma	393	120[b]	—
Retroperitoneal stroma	173	27.0	—

Experiment 1: Mixing during washing of fat cells. Fat pads were removed from three adult rats that received a 16-hr pulse of [^{3}H]thymidine and from three noninjected rats. Fat cells and stroma were prepared and radioactive stroma from the pads of the left side of one group of rats were added to unlabeled fat cells, from pads of the left side of the other group, that had been washed twice by flotation through fresh, warm buffer. The labeled stroma and previously unlabeled fat cells were separated by flotation, and each was then washed four times in the usual way. Total cellular DNA was isolated.

Experiment 2: Mixing during collagenase digestion. Fat cells and stroma were prepared from three adult rats that had received a 21-hr pulse of [^{3}H]thymidine, and radioactive stroma from the pads on the left side were added to minced fat pads removed from one side of three noninjected rats. After the collagenase digestion, fat cells and stroma were separated in the usual way, and nuclear DNA was isolated.

[a] Yield of DNA too low for reliable specific activity.

[b] The stroma isolated from unlabeled samples was a mixture of unlabeled stroma and the added radioactive stroma.

the higher DNA specific activity of fat cells of that side, which had been mixed with unlabeled stroma. The DNA specific activity of the stromal fractions of the left side should also have been greater than those of the right, but instead were somewhat lower. This presumably was caused by the unlabeled stromal DNA added to the pads of the left side.

In the second experiment, labeled fat cells were prepared by collagenase digestion, partially washed, and varying amounts of unlabeled stroma were added (**Fig. 3**). Mixing was either brief or was carried out for 45 min at 37°C. The extended mixing caused no decrease in the specific activity of fat cell DNA, regardless of the amount of unlabeled stroma present. These mixing experiments show that the label is irreversibly associated with adipocytes, although unlabeled adipocytes do seem to acquire a small amount of label when mixed with labeled stroma.

Distribution of label in the adipocyte fraction

The presence of either stromal cells or organelles in the adipocyte fraction was tested in an additional way. Hollenberg and Vost (12) found that a "pinkish zone" at the fat cell–buffer interface, present after centrifugation, contained most of the DNA in the fat cell fraction, and they believed this to be stromal contamination. They removed the pink zone by freezing the fat cell column in plastic tubes and then slicing it. In our hands the pink zone was not seen beyond the third of four washes. However, to test the pos-

TABLE 5. Mixing of unlabeled stroma with radioactive fat cells during collagenase digestion

	DNA Specific Activity, dpm/μg	
	Nothing Added	Mixed with Unlabeled Stroma
Epididymal fat cells	169	197
Retroperitoneal fat cells	46.9	53.0
Epididymal stroma	287	282
Retroperitoneal stroma	134	91.0

Stroma was prepared from the epdidymal and retroperitoneal fat pads of three unlabeled 6.5-month-old rats, and was added to the collagenase digestion medium of the pads of the left side of five 4-month-old rats injected 19.5 hr before with [^{3}H]thymidine. Stroma from the unlabeled epididymal pads was added to the radioactive epididymal pads, and stroma from the unlabeled retroperitoneal pads was added to the radioactive retroperitoneal pads. After collagenase digestion, fat cells and stroma were separated and nuclear DNA was isolated. Fat cells obtained after mixing were presumably derived only from labeled rats, since the fat cells from unlabeled rats were removed by washing the stroma prior to mixing. However, the stromal fraction after mixing contained both the labeled stroma originally present and the added unlabeled stroma.

Downloaded from www.jlr.org by guest, on June 19, 2012

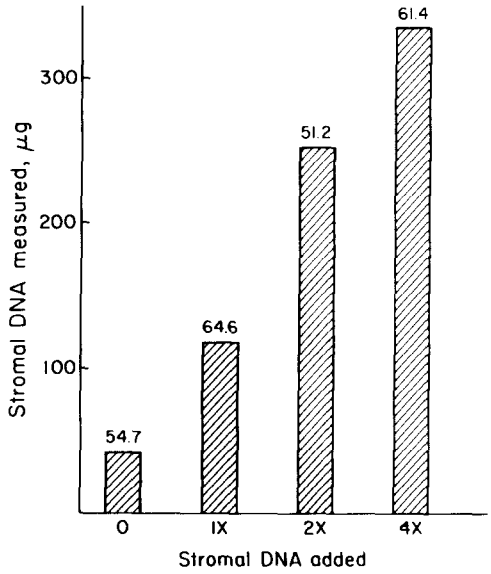

Fig. 3. Mixing of unlabeled stroma with radioactive fat cells after collagenase digestion. Fat cells were prepared from 12 3-month-old rats that had received [^{3}H]thymidine 20 hr previously. Fat cells were washed twice by flotation. The fat cells from different depots were pooled in a plastic beaker, then equal volumes were dispensed into plastic vials using a wide-bore plastic tubing attached to a syringe.

Stroma was prepared from the epididymal and retroperitoneal fat pads of six 6-month-old rats and pooled. Different volumes of the stromal suspension in Krebs-Ringer bicarbonate buffer with albumin were added to different vials (0, 1×, 2×, 4×) and buffer without stroma was added to certain vials so that the contained volume was equal in all vials. The contents of some vials, after swirling, were immediately poured into plastic test tubes for the usual separation and washing of fat cells and stroma (0 min) while the others were first incubated at 37°C for 45 min. Nuclear DNA was isolated.

After 0 min of incubation, the DNA specific activity of fat cells not exposed to added stroma was 88.2, while that of fat cells exposed to 1× added stroma was 55.1. In the figure, the DNA specific activity of fat cells incubated for 45 min after addition of unlabeled stroma is shown above each bar.

sibility that contaminating stromal cells or organelles might be present in the fat cell fraction, adhering to some fat cells and therefore decreasing their buoyancy and causing them to be in the lower part of the fat cell column, a similar freezing and slicing procedure was carried out after the fourth wash (**Table 6**).

Two experiments were done, one with rats injected with [^{3}H]thymidine 1 hr before death, and another with rats injected 4 hr before death. In both experiments, for fat cells from either the epididymal or retroperitoneal fat depots, there was no difference in the specific activity of total cellular DNA from fat cells in the lower third of the fat cell column compared to the upper two thirds. Thus, the uptake of label into fat cell DNA, whether an intrinsic property or a reflection of contamination, has a homogeneous distribution which makes chance contamination by whole cells or organelles from the stromal fraction unlikely.

Hollenberg and Vost (12) also found that, when studies were done in rats younger than those used in our studies, the specific activity of fat cell DNA was only a small proportion of that of stromal DNA shortly after [^{3}H]thymidine injection. We therefore repeated our experiments using both 6-week-old Osborne-Mendel rats, 150–180 g in weight (about the same size as the rats used by Hollenberg and Vost (12)) and 3-month-old rats. Both epididymal and retroperitoneal pads were removed from 3-month-old animals, but in 6-week-old rats the retroperitoneal depot is only a thin streak of tissue, so it could not be used. The collagenase digest of fat pads from the younger animals, poured through nylon mesh with 250-μm openings, had a fat cell DNA specific activity that was a significantly lower proportion of that of stromal DNA compared to experiments where 500-μm mesh, the size we routinely used for larger rats, was employed (**Table 7**). However, use of 250-μm mesh did not give results different from 500-μm mesh in older, larger rats or in 3-month-old rats that had received [^{3}H]thymidine more than 2 weeks previously rather than just a few hours before killing (data not shown), or in 3-month-old rats that had been on a high-fat diet for as short a time as 2 days before label administration (24). Even the smaller mesh has openings large enough to permit passage of single adipocytes from adult rats, so the difference in results with these two mesh sizes is probably due to better retention of aggregates of several cells with the 250-μm mesh.

Rapidity of labeling of adipocytes

The addition of [^{3}H]thymidine to collagenase digestion medium containing adipose tissue from un-

TABLE 6. Distribution of label in a frozen column of fat cells after in vivo [^{3}H]thymidine incorporation

	DNA Specific Activity, dpm/μg	
	1-Hour Pulse	4-Hour Pulse
Epididymal fat cells		
Upper two-thirds	332	59.2
Lower one-third	320	59.1
Retroperitoneal fat cells		
Upper two-thirds	88.7	32.7
Lower one-third	84.6	30.3

Three rats were given a 4-hr pulse, and two rats a 1-hr pulse, of [^{3}H]thymidine prior to death. After collagenase digestion, fat cells were separated and washed in the usual way except that the last centrifugation of fat cells in Krebs-Ringer bicarbonate buffer with albumin was done in $^9/_{16}$″ × 3½″ nitrocellulose test tubes, and the contents were frozen by immersion in dry ice and acetone. The test tubes were then sliced with a Stadie-Riggs blade (Thomas and Co.) so that the lower third of the fat cell column was divided from the upper two-thirds. The divided samples were then processed separately for isolation of total cellular DNA.

Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 7. Comparison of 500-μm nylon mesh with 250-μm nylon mesh in preparing fat cells from 6-week-old and 3-month-old rats

	Ratios of specific Activities, Fat Cell DNA to Stromal DNA		
	500 μ Mesh	250 μ Mesh	
6-Week-old Epididymal	0.561 ± 0.060[a] (4)	0.225 ± 0.054 (4)	$t = 4.16$ $P < 0.01$
3-Month old Epididymal	0.555 ± 0.057 (4)	0.244 ± 0.067 (3)	$t = 3.55$ $P < 0.01$
Retroperitoneal	0.411 ± 0.086 (4)	0.156 ± 0.072 (3)	$t = 2.16$ $P < 0.05$

Collagenase digests of fat pads from groups of nine or ten 6-week-old rats, injected 16–19 hr previously with [^{3}H]thymidine, were poured through either 500 or 250-μm nylon mesh. Digests of pads from groups of two to five 3-month-old rats injected 15–21 hr previously were also poured through either 500-μm or 250-μm nylon mesh. Subsequent washing and preparation of nuclear DNA was done as usual, and DNA specific activities of the fat cell and stromal fractions were determined. Ratios of specific activities obtained with the two meshes (fat cell DNA to stromal DNA) were compared. The number of experiments is shown in parentheses.

[a] Mean ± SEM.

labeled rats was found to result in rapid labeling of both adipocyte and stromal DNA. The in vitro labeling was originally attempted to see if the observed in vivo labeling of fat cell DNA following a pulse of only a few hours might really be due to transfer of labeled DNA precursor from stroma to fat cells during the collagenase digestion. Surprisingly, considerable labeling of fat cell DNA was observed. The specific activity of fat cell DNA was higher than that of stromal DNA (**Fig. 4** and **Tables 8** and **9**), suggesting a greater rate of cell proliferation in the fat cell fraction during the brief period of incubation.

Pretreatment of rats with hydroxyurea, as above, prior to removal of fat pads and collagenase digestion in the presence of [^{3}H]thymidine did not decrease the labeling of fat cell DNA. However, inclusion of 0.5 mM cytosine arabinoside (17) in the collagenase digestion medium decreased the specific activity of fat cell DNA by 59–65% and that of stromal DNA by 63–79% (Fig. 4), showing that incorporation of label from the medium occurred via synthesis of DNA.

The incorporation of label from the medium into DNA was tested by DNAase treatment of the isolated DNA, as above. The specific activity of all DNA fractions isolated after in vitro labeling was found to increase after DNAase treatment, unlike DNA labeled in vivo (Table 1), indicating that a large part of the label was also present in non-DNA compounds (Table 8). An equation can be derived to obtain the amount of label that is non-DNA. The proportion of label in a sample that is in non-DNA, X, can be calculated from the equation:

$$X = \frac{P(R - 1)}{1 - P}$$

where P is the proportion of DNA remaining after DNAase treatment, and R is the ratio of specific activities, activity after treatment to activity without treatment. Using this equation, the four DNA fractions labeled in vitro contained an average of 11% of the label in non-DNA compounds.

Addition of [^{3}H]thymidine to the wash buffer was done to see whether any unincorporated label persisted in the sample (Table 9). An amount of [^{3}H]-thymidine equivalent to that present in the collagenase digestion medium during in vitro incorporation was added to the wash buffer of samples previously incubated in unlabeled collagenase digestion medium. After isolation of total cellular DNA, the specific activity of samples exposed to label in the wash buffer was compared to that of samples having label in the collagenase digestion medium. The DNA from fat cell samples washed with buffer containing [^{3}H]thymidine had 6% of the specific activity of fat cell DNA labeled in vitro, and stromal DNA had 17–24%. Thus, as much as 6% of the radioactivity of in vitro-labeled fat cell DNA may be merely carry-over of label, or else is rapid incorporation into DNA occurring during the brief wash with [^{3}H]thymidine in the buffer.

Labeled thymidine was added to samples just before homogenization to find out whether carry-over of label or rapid incorporation had been responsible for the radioactivity measured subsequently in DNA when [^{3}H]thymidine was added to the wash buffer.

Downloaded from www.jlr.org by guest, on June 19, 2012

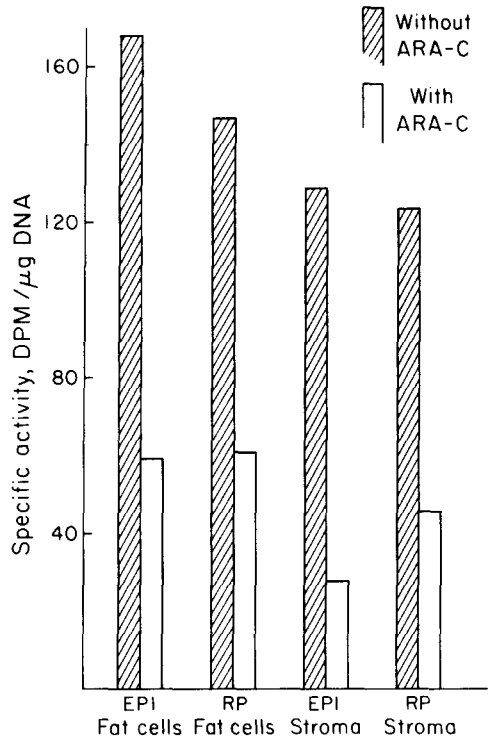

Fig. 4. In vitro incorporation of [^{3}H]thymidine in the presence or absence of cytosine arabinoside. Collagenase digestion of the fat pads from three rats was done in the presence of 3 μCi/ml [^{3}H]thymidine in the incubation medium. The incubation medium for pads from the left side contained, in addition, 0.5 mM cytosine arabinoside (ara-C). Total cellular DNA was isolated. Epi, epididymal; RP, retroperitoneal.

The amount of label added before homogenization was 100 μCi, equivalent to the amount present during in vitro incorporation (Table 9), and the resulting DNA specific activity was 2–3 dpm/μg, about 1–3% of that obtained by in vitro incorporation, suggesting that persistence of unincorporated labeled thymidine does not contribute very much to the observed DNA specific activity when DNA is prepared from isolated nuclei.

TABLE 8. Treatment of in vitro-labeled DNA with deoxyribonuclease (DNAase)

	DNAase Treated/Saline Treated	
	Amount of DNA[a] (DNAase/saline)	Specific Activity[b] (DNAase/saline)
Epididymal fat cells	13.1/487 = 0.270	753/556 = 1.35
Retroperitoneal fat cells	19.4/89.2 = 0.218	472/375 = 1.26
Epididymal stroma	53.6/128 = 0.420	319/268 = 1.19
Retroperitoneal stroma	45.9/144 = 0.320	189/153 = 1.23

In vitro-labeled total cellular DNA was isolated from the fat pads of three rats after the pads were incubated with 3 μCi/ml [^{3}H]thymidine during collagenase digestion. Each DNA sample was divided in half after the KOH incubation step, and then treated as in Table 1.

[a] μg per total sample.

[b] dpm/μg DNA.

TABLE 9. Comparison of labeling after in vitro incorporation of [^{3}H]thymidine with labeling after addition of [^{3}H]thymidine to the wash buffer

	DNA Specific Activity		
	In Vitro Incorporation	[^{3}H]Thymidine in Wash Buffer	Relative Activity (%)
	dpm/μg		
Epididymal fat cells	265	14.6	5.51
Retroperitoneal fat cells	240	13.8	5.74
Epididymal stroma	135	31.9	23.6
Retroperitoneal stroma	89.9	15.0	16.7

For in vitro incorporation, fat pads from two rats were digested with collagenase in the presence of 3 μCi/ml [^{3}H]thymidine. To test for persistence of label after addition of [^{3}H]thymidine to the wash buffer, fat pads from three rats were digested with collagenase, and poured through nylon meshes. Twenty-five ml of warm Krebs-Ringer bicarbonate buffer with albumin containing 3 μCi/ml [^{3}H]thymidine was added to each sample as the fat cells were separating from stroma by flotation. Subsequent steps were as usual for isolation of total cellular DNA. In vitro incorporation occurred during the 60 min of collagenase digestion. Samples washed with buffer containing [^{3}H]thymidine were in contact with the label for about 5 min.

To further test the possibility that the observed in vivo labeling of fat cell DNA might be due to transfer of labeled DNA precursor from stroma to fat cells during the collagenase digestion, two more experiments were done. First, fat depots from rats injected several hours earlier with [^{3}H]thymidine were digested with collagenase in the presence of a large excess of unlabeled thymidine. Second, fat depots from [^{3}H]thymidine-injected rats were incubated with collagenase in the presence of cytosine arabinoside as above. In both experiments the amount of labeling of fat cell DNA was not diminished. Thus, labeling of fat cell DNA after an injection of [^{3}H]thymidine represents DNA synthesis occurring in vivo rather than artifactual incorporation during the collagenase digestion. However, some in vitro synthesis of DNA, as measured by label incorporation, does occur.

In vivo turnover of adipocyte and stromal DNA

The study of DNA turnover in vivo suggests that the adipocyte fraction of adult rats contains a small number of cells that turn over rapidly. Determination of the turnover of DNA in the adipocyte and stromal fractions was done by isolating nuclear DNA at different intervals after [^{3}H]thymidine administration to rats 4–6 months old. Turnover in both adipocytes and stroma, for fractions from both the epididymal and retroperitoneal depots, appears to have two com-

Downloaded from www.jlr.org by guest, on June 19, 2012

ponents. One, which turns over with a half-life on the order of days, probably corresponds to the cells that take up label during in vitro administration or within hours after in vivo administration (**Fig. 5**). The other component has a half-life on the order of months or longer (140 to >14,000 days) and may be radioactivity present in differentiated cells (**Fig. 6**).

DISCUSSION

Adult rats maintained under conditions where adipocyte number appears to be constant during the period of study were shown to incorporate [^{3}H]-thymidine into DNA of the adipocyte fraction. The presence of label in DNA was confirmed by treatment with DNAase, and its incorporation during semiconservative DNA synthesis rather than repair was shown by inhibition with hydroxyurea. The labeled DNA was predominantly in nuclei after organelle fractionation, and radioautography of preparations of adipocyte nuclei showed that the label was only in a small proportion of them. The label in the adipocyte fraction could not be removed by increased collagenase digestion nor by trypsin treatment, and mixing of labeled adipocytes with unlabeled stroma did not cause any loss of label from the adipocytes. The reverse combination, mixing labeled stroma with unlabeled adipocytes, resulted in an exchange of only a small amount of label. Freezing and slicing a column of labeled fat cells after low-speed centrifugation showed that the label was homogeneously distributed.

The addition of [^{3}H]thymidine to the collagenase digestion medium resulted in more rapid incorporation into adipocyte DNA than into stromal DNA, and inhibition by cytosine arabinoside showed that in vitro incorporation was predominantly by syn-

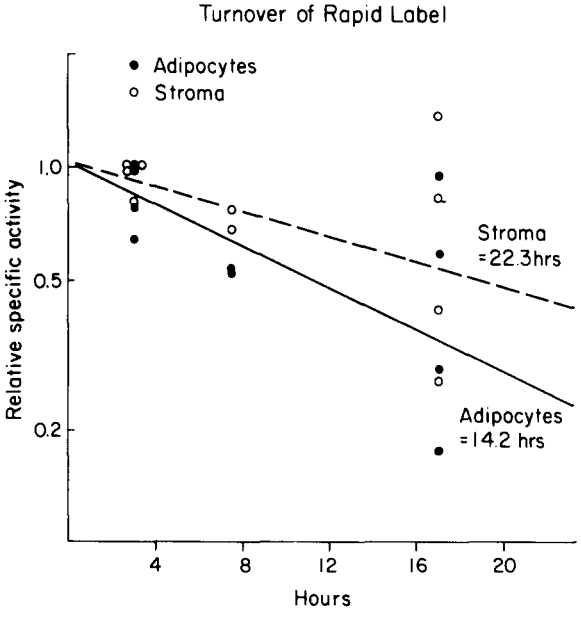

Fig. 5. Turnover of rapid label in adult rats. Rats of age 4 to 6 months were injected intraperitoneally with 300 μCi of [^{3}H]-thymidine and killed after 3, 7.5, or 17 hr. Two rats were used for each experiment, and nuclear DNA was isolated. To permit data from the epididymal and retroperitoneal pads to be plotted on the same graph, the specific activities for each pad and fraction were divided by the highest specific activity at the earliest time point, 3 hr. The curves were drawn using Pearson's regression analysis, and the half-life for the DNA of each fraction is given next to each curve. For the 10 adipocyte DNA points, $r = -0.583$ with $P < 0.05$. For the 10 stromal DNA points, $r = -0.440$ with $P < 0.01$.

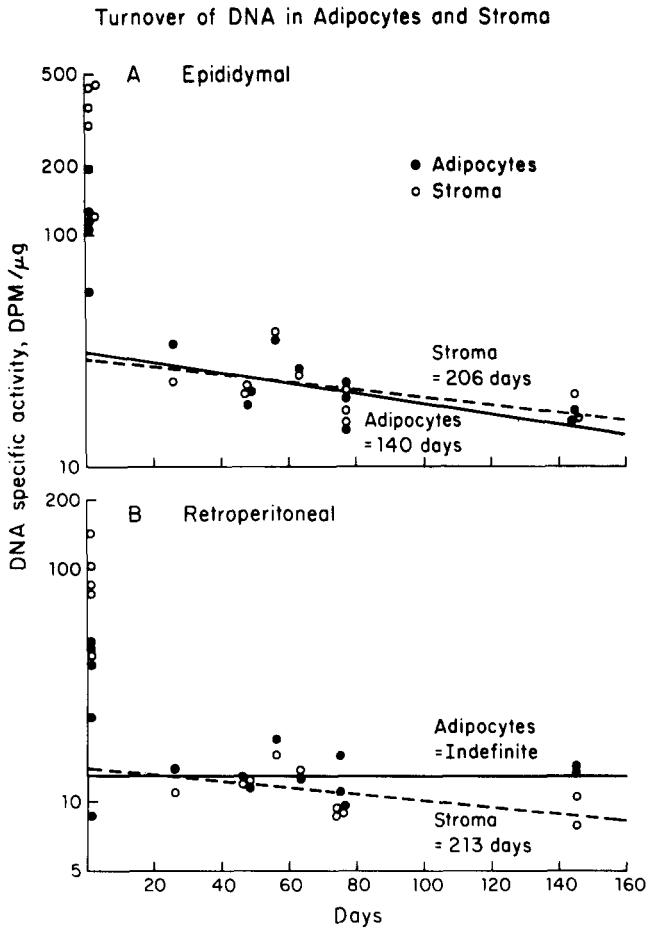

Fig. 6. Turnover of DNA in adipocytes and stroma. Rats of ages 4 to six months were injected intraperitoneally with 300 μCi of [^{3}H]-thymidine, followed by an injection of 0.48 mmol of unlabeled thymidine 17 hr later. Two rats were used for each experiment, and nuclear DNA was isolated. Rats were killed from 26 to 144 days after receiving the label. The curves were drawn using Pearson's regression analysis, and the half-life for the DNA of each fraction is given next to each curve. For epididymal adipocytes, $r = -0.651$ and $P < 0.05$. For epididymal stroma, $r = -0.499$ and $P < 0.1$. For retroperitoneal adipocytes, $r = -0.010$ and P = NS. For retroperitoneal stroma, $r = -0.599$ and $P < 0.05$. Data from the turnover of the rapid label, Fig. 5, are shown on this figure at day 1, for comparison, but were not used in drawing the curves or computing the half-lives.

Downloaded from www.jlr.org by guest, on June 19, 2012

thesis of DNA. Partial hydrolysis with DNAase, however, showed that about 11% of the label was in non-DNA compounds, and addition of [^{3}H]thymidine just to the wash buffer indicated that about 6% of the adipocyte DNA label incorporated in vitro might be due to carryover of label from the incubation medium. Study of the turnover of adipocyte and stromal DNA in vivo revealed two components in both fractions: a rapid component with a half-life on the order of days, and a slow component with a half-life on the order of months or longer. Thus, it appears that a proliferating cell type is closely associated with the adipocytes of adult rats, although its function is unknown.

In our experiments, the specific activity of DNA from the adipocyte fraction of adult rats was found to be almost 40% of that of DNA from the stromal fraction only a few hours after intraperitoneal injection of [^{3}H]thymidine. In contrast, Hollenberg and Vost (12) found that in 150–175 g Wistar rats only a small amount of label was present in adipocyte DNA within a few hours, and its specific activity increased 2–5 days later, apparently due to a shift of labeled DNA from the stromal to the adipocyte fraction, consistent with the lipid-filling of preadipocytes. If the early growth curve of Wistar rats resembles that of Sprague-Dawley and Osborne-Mendel rats in this laboratory, the rats used by these investigators were about 6 weeks old. At this age the initial expansion of the adipocyte pool is still occurring (6). When we used younger animals, 6 weeks old or 3 months old, and passed the collagenase digest of the fat pads through 250-μm mesh rather than the 500-μm mesh that we used for older animals, the specific activity of fat cell DNA was a lower proportion of that of stromal DNA (16–24%), which is closer to the results reported by Hollenberg and Vost (12) (4–6%). However, both mesh sizes gave the same result in older animals, in 3-month-old rats given [^{3}H]thymidine several weeks previously, and in 3-month-old rats that had been fed a high-fat diet for just 2 days prior to label administration (24). It thus seems that in younger animals the proliferating cells are almost all in the stromal fraction, while in adults more of them are in the adipocyte fraction.

The 250-μm mesh has openings sufficiently large to permit the passage of single adipocytes, even from adults. The results obtained using different mesh sizes suggest that in younger animals labeled cells end up in the adipocyte fraction only in aggregates of several cells, since the amount of label is decreased after passage through the smaller mesh. This implies that in the younger animals the labeled cell type is only accidently included in the adipocyte fraction, and that perhaps the use of a mesh with even smaller openings, such as the "fine silk hose" employed by Hollenberg and Vost (12), might reduce the amount of label still further. However, the situation seems different both in adults, where the two mesh sizes give smaller results, and in 3-month-old rats fed a high-fat diet for just 2 days prior to [^{3}H]thymidine injection and then killed the next day. In younger rats it was found that a high-fat diet causes the specific activity of adipocyte DNA to rapidly increase from 16–24% to almost 50% of that of stromal DNA even when 250-μm mesh is used (24). In older rats and in younger rats briefly fed a high-fat diet, the labeled cell type appears to be more closely associated with adipocytes. The increased binding may be due to morphologic changes in cellular relationships caused by increased fat cell size, or perhaps to cellular concentration required for lipid-filling. In 3-month-old rats given [^{3}H]thymidine several weeks previously, the failure of the finer mesh to reduce the DNA specific activity of the adipocyte fraction may mean that the labeled cells have become more tightly bound to adipocytes, or that they have matured into adipocytes during the intervening weeks.

The finding that [^{3}H]thymidine from the collagenase digestion buffer was incorporated into adipocyte DNA was surprising in view of the finding of Hollenberg and Vost (12) who found that washed adipocytes liberated by collagenase digestion failed to incorporate label into DNA, although 1–8% of the label incorporated into DNA of adipose tissue slices was in the fat cell fraction. The experiments with cytosine arabinoside (Fig. 4) and DNAase (Table 8) verified that most of the label was in DNA, but the results of the DNAase experiment together with the results of adding [^{3}H]-thymidine to the wash buffer (Table 9) showed that a small fraction of the in vitro label was not in DNA and might be due to carryover of label from the wash buffer. However, the label in adipocyte DNA was unlikely to be from chance stromal contamination, since in all three experiments the specific activity of adipocyte DNA was greater than that of stromal DNA. The higher DNA specific activity of the adipocyte fraction compared to stroma after in vitro [^{3}H]thymidine administration indicates a higher proliferative index in the cells of that fraction during incubation.

The turnover studies (Figs. 5, 6) suggest that both aidpocyte and stromal fractions have a component which turns over rapidly and another which turns over slowly. The existence of two components may mean that in the adipocyte fraction there are two cell types that take up the labeled precursor independently, proliferating and dying independently, or that one cell type gives rise to the other. The pro-

Downloaded from www.jlr.org by guest, on June 19, 2012

longed half-life depicted in Fig. 6 is consistent with a long-lived, differentiated cell whose number remains fairly constant, such as the mature adipocyte, but may also be that of a quiescent stem cell. The short half-life shown in Fig. 5 may belong to a more rapidly proliferating progenitor cell which then gives rise to mature adipocytes.

The contribution of this measured turnover to the number of adipocytes in adult rats can be calculated, if it is assumed that the turnover is indeed that of adipocyte progenitors which later form mature cells. From Fig. 6, the specific activity of adipocyte DNA levels off at a value on the order of 10 dpm/μg. The specific activity of the [^{3}H]thymidine used was 20 Ci/mmol, so it can be calculated that the 10 dpm represents 1.35×10^8 new molecules of thymidine incorporated per μg of DNA, if it is assumed that the intracellular thymidine pool has the same specific activity. Actually, the intracellular specific activity is probably lower, so this number underestimates the number of new molecules. If there are 1.1×10^{10} nucleotides per cell, and 29% of them are thymidylic acid (T) derived from labeled thymidine, then there are 3.19×10^9 T per cell, and enough T for one new cell would produce 23.6 dpm. The 10 dpm thus represents 0.424 new cells per μg of DNA. If there are 6 pg of DNA per cell, then 1 μg is from 1.67×10^5 cells. Thus, approximately one new cell is formed per 10^5 adipocytes during the 17-hr pulse of label. The epididymal and retroperitoneal fat depots of adult rats have on the order of 10^7 adipocytes each, so at this rate about 100 new cells would be formed in a depot per day, or about 10^4 new cells over 100 days. This number of new cells is an increase of only 0.1%, so small that it would not be detected by the conventional methods for counting adipocytes.

The low rate of turnover of adipocytes in adults is probably more than just the remnant of the vigorous progenitor proliferation seen in young animals, because high fat breeding causes both a dramatic increase in label incorporation into adipocyte DNA (24) as well as a permanent increase in fat cell number (8). Thus, the continued turnover in adults is evidence that the potential for expansion of adipocyte number persists.

The cells in the adipocyte fraction that take up the label may indeed be adipocyte progenitors, or only a portion of them may end up as adipocytes, with the remainder having some other, perhaps supportive, function. The existence of a small proportion of proliferating cells associated with adipocytes after collagenase digestion, and responsible for the uptake of label in that fraction, was confirmed by radioautography of nuclear preparations (Fig. 2).

Studies of adipocyte turnover have been handicapped by an inability to clearly identify adipocyte progenitors morphologically. Cells resembling fibroblasts can be grown from adult human adipose tissue in tissue culture, but these differ from skin-derived fibroblasts in that they contain more glycerol from triglyceride, incorporate [1-^{14}C]glucose into lipid, and begin to acquire large fat droplets (10). They also possess higher activities of lipoprotein lipase and the enzymes of fatty acid synthesis (11). When grown with fatty acids added to the culture medium, adipose tissue-derived cells more quickly come to resemble mature adipocytes, but skin-derived cells retain the appearance of fibroblasts (25).

Clones of cells with the potential to differentiate into adipocytes have been isolated from murine 3T3 fibroblasts, a cultured cell line (26–28). These studies indicate that adipocytes are derived from a fibroblast-like cell, but all fibroblasts do not have the potential to differentiate into adipocytes.

Attempts have been made to use biochemical markers to study adipocyte progenitors. Pilgrim (29) used histochemical localization of alpha-naphthyl acetate esterase activity to study differentiation of fat organs in prenatal and postnatal rats, and Hietanen and Greenwood (30) investigated lipoprotein lipase activity in the adipocyte and stromal fractions of epididymal fat pads of growing rats. The use of such biochemical markers may aid the study of adipocyte progenitors during adipocyte turnover in adults as well as during growth.

The present work shows that proliferating cells are found in close association with mature adipocytes in adult rats. These proliferating cells may be adipocyte progenitors, later forming mature adipocytes, or they may be supportive cells, in intimate association with adipocytes because of some nutritive function. The nature of these proliferating cells can perhaps be assessed by radioautography together with electron microscopic techniques, and by culture methods to determine their potential for differentiation. Additionally, their significance to the organism can be determined by studying them under conditions of adipocyte stress, as in animals on a high-fat diet or subjected to partial lipectomy.

The authors would like to express their gratitude to Dr. Irving M. Faust for many helpful discussions, and to Dr. Ralph Steinman for help with the radioautography. Barry J. Klyde performed this work while a recipient of a Norman Jolliffe Fellowship in Clinical Nutrition and Metabolic Diseases from the Research Corporation. This research was supported by NSF grant PCM 76-09324 and NIH grant 1 R01 AM 20508

Manuscript received 10 July 1978; accepted 5 February 1979.

Downloaded from www.jlr.org by guest, on June 19, 2012

REFERENCES

1. Anderson, D. R., and R. G. Kauffman. 1972. Cellular and enzymatic changes in porcine adipose tissue during growth. *J. Lipid Res.* **14:** 160–168.
2. Hirsch, J., and P. W. Han. 1969. Cellularity of rat adipose tissue: effects of growth, starvation, and obesity. *J. Lipid Res.* **10:** 77–82.
3. Hirsch, J., and J. L. Knittle. 1970. Cellularity of obese and nonobese human adipose tissue. *Federation Proc.* **29:** 1516–1521.
4. Hood, R. L., and C. E. Allen. 1977. Cellularity of porcine adipose tissue: effects of growth and adiposity. *J. Lipid Res.* **18:** 275–284.
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. Greenwood, M. R. C., and J. Hirsch. 1974. Postnatal development of adipocyte cellularity in the normal rat. *J. Lipid Res.* **15:** 474–483.
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. 1978. Diet-induced adipocyte number increase in adult rats: A new model of obesity. *Am. J. Physiol.* **235:** E279–E286.
9. Faust, I. M., P. R. Johnson, and J. Hirsch. 1977. Adipose tissue regeneration following lipectomy. *Science.* **197:** 391–393.
10. Poznanski, W. J., I. Waheed, and R. Van. 1973. Human fat cell precursors: Morphologic and metabolic differentiation in culture. *Lab. Invest.* **29:** 570–576.
11. Van, R. L. R., and D. A. K. Roncari. 1977. Adipocyte precursor replication and differentiation in adults. Abstract, *Second International Congress on Obesity*, Washington, D.C.
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. Rodbell, M. 1964. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239:** 375–380.
14. Abraham, G. N., C. Scarletta, and J. H. Vaughan. 1972. Modified diphenylamine reaction for increased sensitivity. *Anal. Biochem.* **49:** 547–549.
15. Schneider, W. C., and E. L. Kuff. 1969. The association of deoxyribonucleic acid with liver microsomes. *J. Biol. Chem.* **244:** 4843–4851.
16. Schneider, W. C., and A. E. Greco. 1971. Incorporation of pyrimidine deoxyribonucleosides into liver lipids and other components. *Biochim. Biophys. Acta.* **228:** 610–626.
17. Goldsprink, D., and A. L. Goldberg. 1973. Problems in the use of [me-^{3}H]thymidine for the measurement of DNA synthesis. *Biochim. Biophys. Acta.* **299:** 521–532.
18. Morley, C. G. C., and H. S. Kingdon. 1972. Use of ^{3}H-thymidine for measurement of DNA synthesis in rat liver—a warning. *Anal. Biochem.* **45:** 298–305.
19. Bucher, N. L. R., and M. N. Swaffield. 1973. Regeneration of liver in rats in the absence of portal splanchnic organs and a portal blood supply. *Cancer Res.* **33:** 3189–3194.
20. Schwartz, H. S., M. Garofalo, S. S. Steinberg, and F. S. Philips. 1965. Hydroxyurea: Inhibition of deoxyribonucleic acid synthesis in regenerating liver of rats. *Cancer Res.* **25:** 1867–1870.
21. Cleaver, J. E. 1974. Repair processes for photochemical damage in mammalian cells. *Adv. Radiol. Biol.* **4:** 1–75.
22. Gliemann, J., K. Osterline, J. Vinten, and S. Gameltoft. 1972. A procedure for measurement of distribution spaces in isolated fat cells. *Biochim. Biophys. Acta.* **286:** 1–9.
23. Kuchler, R. J. 1977. Biochemical Methods in Cell Culture and Virology. Halsted Press, New York.
24. Klyde, B. J., and J. Hirsch. 1978. Increased cellular proliferation in adipose tissue of adult rats fed a high fat diet. *J. Lipid Res.* **20:** 705–715.
25. Dardick, J., W. J. Poznanski, I. Waheed, and G. Setterfield. 1976. Ultrastructural observations on differentiating human preadipocytes cultured in vitro. *Tissue Cell.* **8:** 561–571.
26. Green, H., and O. Kehinde. 1975. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell* **5:** 19–27.
27. Green, J., and O. Kehinde. 1976. Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell.* **7:** 105–113.
28. Green, H., and M. Meuth. 1974. An established preadipose cell line and its differentiation in culture. *Cell.* **3:** 127–133.
29. Pilgrim, C. 1971. DNA synthesis and differentiation in developing white adipose tissue. *Dev. Biol.* **26:** 69–76.
30. Hietanen, E., and M. R. C. Greenwood. 1977. A comparison of lipoprotein lipase activity and adipocyte differentiation in growing male rats. *J. Lipid Res.* **18:** 480–490.
31. Maggio, R., P. Siekevitz, and G. E. Palade. 1963. Studies on isolated nuclei. I. Isolation and chemical characterization of a nuclear fraction from guinea pig liver. *J. Cell Biol.* **18:** 267–291.

Downloaded from www.jlr.org by guest, on June 19, 2012