Regulation of DNA Synthesis in Fat Cells and Stromal Elements from Rat Adipose Tissue

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ABSTRACT The incorporation of tritiated thymidine into the deoxyribonucleic acid (DNA) of adipose fat and stromal cells was followed under a variety of conditions. After in vitro incubation of adipose slices or up to 2 days after in vivo injection of the isotope, all DNA radioactivity was in the stromal cell fraction. From 2 to 15 days after thymidine injection total tissue DNA radioactivity was constant, while between 2 and 5 days after injection label in fat cell DNA increased markedly. Thus new labeled fat cells, initially collected in the stromal pool, required 2–5 days after completion of DNA synthesis to accumulate sufficient lipid to be harvested in the fat cell fraction. Fasting before thymidine injection practically abolished DNA synthesis in primordial fat cells and reduced less drastically formation of stromal elements. However fasting sufficient to deplete lipid stores by 50% neither destroyed mature fat cells nor impaired their capacity to reaccumulate fat with refeeding. Other studies evaluated the role of new fat cell formation in the process of lipid accretion accompanying refeeding. These experiments indicated that at least during the early phase of rapid weight gain, accumulation of fat was due to deposition of triglyceride in existing cells rather than to accelerated formation of new fat cells. Studies with hypophysectomized rats demonstrated that pituitary ablation variably affected stromal DNA synthesis and nearly abolished the formation and (or) maturation of primordial fat cells. In these animals growth hormone markedly enhanced thymidine incorporation into stromal DNA but had no effect on fat cell precursors. In intact animals the predominant effect of growth hormone was also on the stromal fraction, although an action of the hormone of lesser magnitude on fat cell precursors was also evident.

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INTRODUCTION

While information concerning the accretion and mobilization of adipose lipid has accumulated rapidly over the past decade, there is still surprisingly little known about the regulation of fat cell formation, another mechanism that can influence adipose mass. Hindering work in this area is the difficulty in the histological recognition of primitive fat cells and the fact that only a part of total adipose tissue deoxyribonucleic acid (DNA) is contained in fat cells (1). The information that is available has been derived from the use of radioautography, chemical DNA analysis, and counting of adipose cells. These data indicate that the new fat cells are continually formed in the growing animal (2), that expansion of adipose mass by fat feeding is associated with an increase in total adipose tissue DNA (3), and that in human and in some experimental forms of obesity an increase in adipose cell number accompanies an increase in adipose cell size (4, 5). Although these data suggest an influence of diet on fat cell synthesis, the precise nutritional and hormonal factors that control the formation and maturation of these elements remain to be defined.

In order to approach this problem, tritiated thymidine was injected into rats of varying nutritional states and adipose tissue removed at later times. The cellular constituents of the tissue were separated into two populations, mature fat cells and stromal vascular elements, by collagenase diges-

tion (6), and the specific activity of the DNA of the two cell pools followed in time. This approach permitted observations of the maturation time of fat cells and of the effects of nutritional variations and of growth hormone on DNA synthesis in both cell populations. Other studies were made of the effect of fasting on the cellular integrity of mature fat cells.

METHODS

Male Wistar rats, initial weight 150–175 g and maintained on Purina chow, were used in all studies except those with hypophysectomized animals. In the latter instance, male Sprague-Dawley animals, initial weight 140–160 g, obtained from Charles River Laboratories (Boston, Mass.) were used no sooner than 10 days after hypophysectomy. Both *in vitro* and *in vivo* experiments were performed.

In the in vitro studies, epididymal and lumbar fat pieces from three animals were pooled and sliced, and approximately 2.5 g were added to 12 ml of Eagle's L medium and incubated for varying periods in a Dubnoff shaker at 37°C; the gas phase was 95% O_2 and 5% CO_2. To this medium was added 5% bovine albumin, 200 mg/100 ml of glucose, 0.02 M $NaHCO_3$, 100 U/ml of penicillin, 20 μg/ml of streptomycin, and varying concentrations of unlabeled and tritiated thymidine (thymidine-methyl-^{3}H, [Schwarz Bio Research, Inc., Orangeburg, N. Y.] 6c/mmole). After incubation, the fat pieces were digested with collagenase in the presence of penicillin, streptomycin, and 1.85 mM unlabeled thymidine, and the specific activity of DNA in fat cells and stromal cells were determined as later described.

In the in vivo studies, two schedules were used for intraperitoneal administration of tritiated thymidine. In some studies, a single injection of 22 μc/rat was followed in 1 hr by administration of 80 μmoles of unlabeled thymidine; animals were sacrificed 20 min after the second injection. In other experiments, each rat received a total of 30 μc of labeled thymidine in two injections given at 10 and 11 p.m., and animals were sacrificed at varying times, the earliest being 12 hr after injection of the isotope. In experiments in which animals were followed over days, 25 μmoles of unlabeled thymidine was administered daily beginning 12 hr after isotope injection.

Animals in groups of three were anesthetized with ether and exsanguinated by aortic puncture. Epididymal and lumbar fat from the three animals was rinsed in bicarbonate buffer, pooled minced, and then the fat cells and stromal elements were separated by the collagenase method of Rodbell (6). Despite repeated washes of the fat cells, a pinkish zone, undoubtedly representing stromal fractions, was consistently evident at the fat cell buffer interface after centrifugation. This zone was found to contain most of the DNA in the fat cell fraction. While it is unlikely that this contamination would influence experiments concerned with carbohydrate and lipid metabolism of free fat cells, it would completely vitiate studies of fat cell DNA synthesis. Several additional procedures were therefore introduced to reduce stromal contamination. After collagenase digestion, fat cells and stroma were separated by 40-sec centrifugation in a clinical centrifuge; the stroma was aspirated and saved. The fat cells were redispersed in warm buffer-albumin and filtered through a single layer of fine silk hose; the hose retained many fine strands of tissue and virtually no lipid, and the residue was transferred to the stromal pool. The filtered fat cells were then washed twice more in buffer-albumin followed by a third wash in buffer alone; after each wash stromal sediment was added to the stromal pool. While the filtering procedure removed most of the visible stromal elements, when the fat cells were transferred with buffer to a 77×10 mm plastic tube and spun for 1 min, a faint thin grayish-pink line was usually evident at the fat cell buffer interface. This interfacial zone was removed from the fat cell pool by freezing the entire fat cell column with a Cryokwik spray (International Equipment Co., Needham Heights, Mass.) and by slicing the frozen cell column 1–2 mm above and just below the interface. The upper 30–40 mm of fat cells were processed separately from the interfacial and stromal elements. An indication of fat cell breakage and hence of potential nuclear loss from fat cells was obtained by measuring the amount of oil produced during the entire procedure. This amount rarely exceeded 5% of the triglyceride recovered from the fat cell fraction.

The fat cell and stromal fractions were separately homogenized in 15 ml of cold acetone using a motor-driven Teflon pestle. After they were filtered, the precipitates were defatted with 100 ml of cold acetone followed by 50 ml of ethyl ether and then digested overnight in 6 ml of 0.5 N KOH at 37°C. DNA was precipitated in the cold from the digests by addition of perchloric acid to 0.5 N, collected by centrifugation, and washed twice more with 5 ml of 0.5 N perchloric acid. The DNA in the sediment was then hydrolyzed by treating twice with 1–2 ml of 0.5 N perchloric acid at 80°C and aliquots of the pooled extracts taken for radioactive counting and for DNA determination by the diphenylamine reaction, with calf thymus DNA as standard (7). The acetone-ether washes were saved, evaporated, brought to volume with acetone, and aliquots taken for radioactive counting and triglyceride determination (8).

A number of studies were performed to determine the adequacy of the lipid extraction, washing, and digestion procedures. The final cold perchloric acid washes were devoid of radioactivity and, when tritiated thymidine was added to unlabeled tissue at the time of homogenization, all radioactivity was recovered in the washes and none in the hot perchloric acid extracts. When radioactive triolein was added at the time of homogenization, all radioactivity was in the acetone-ether washes and none in the hot perchloric digests. The efficiency of the two hot perchloric acid extractions was established by finding that a third extraction increased recovery of radioactivity

by only 6%. Radioactivity in the residual pellet, dissolved by boiling in 2 N KOH, was inconsequential. It was also found that the specific activity of DNA was the same in both the first and second hot perchloric acid extracts.

The procedure described produced a distribution of DNA between fat and stromal cells quite different than that reported by Rodbell (1). In the present study fat cells contained 15%, interfacial cells 1%, and stromal cells 84% of the recovered DNA. As it has been previously shown that with the collagenase method total recovery of whole tissue DNA is not achieved (1), data concerning absolute content of chemical DNA in the various cell fractions must be interpreted with caution. Nonetheless the procedure led to very reproducible recoveries of fat cell and stromal DNA; in four studies in which the adipose tissue pool was divided into two aliquots of equal weight before collagenase digestion, the difference between the two aliquots in fat cell DNA content ranged from 3–9%, in stromal DNA from 2–10%. Recoveries of radioactive DNA in the two cellular pools were equally reproducible.

In some experiments, the specific activity of liver DNA was determined 80 min after injection of tritiated thymidine. The liver pieces were homogenized in 0.25 M sucrose containing 0.002 M $CaCl_2$ and spun at 800 *g* for 15 min. The nuclear pellet was resuspended in sucrose-$CaCl_2$ and the centrifugation repeated. The washed pellet was dissolved in KOH and radioactive and chemical DNA determined as previously noted. In one experiment, the specific activity of liver DNA was obtained as described and was compared to that noted when liver was homogenized in acetone and carried through the same procedures used for fat and stromal cells; the specific activities were identical.

In a number of experiments, plasma obtained at the time of exsanguination was used for determination of plasma water radioactivity. Plasma water was prepared from whole plasma by microsublimation (9). In other studies the nature of the radioactivity present in the acetone-ether washes of fat cells was characterized by thin-layer chromatography and by saponification (8).

The radiochemical purity of each batch of thymidine-^3H was established as > 99%, by the supplier (Schwarz Bio Research, Inc.) using radioautography of paper chromatograms run in two different solvent systems; also, microsublimation of aqueous thymidine-^3H resulted in negligible recovery of radioactivity in the volatile fraction. The bovine growth hormone used in these studies was obtained from the National Institutes of Health, Bethesda, Md. (NIHGH B 13). Radioactivity was counted in a Nuclear-Chicago liquid scintillation spectrometer by methods previously described (8) and was converted to disintegrations per minute by using an external standard and by correcting for quenching by channel ratio techniques.

RESULTS

In Vitro incorporation of tritiated thymidine into adipose tissue DNA. When adipose slices were incubated with tritiated thymidine and the slices subsequently digested with collagenase, almost all of the DNA radioactivity was in the stromal cells (Table I). Fat cell DNA usually contained 1–2% of the radioactivity recovered in DNA, an amount that was probably due to residual contamination of this fraction with stroma. Incorporation of thymidine was nearly linear during 3 hr of incubation, and extent of incorporation was independent of whether the medium was Eagle's L containing 5% albumin and glucose, or Krebs-Ringer bicarbonate buffer with albumin only.

When isolated fat cells were incubated with tritiated thymidine, no radioactivity was recovered in DNA. Isolated stromal elements did incorporate thymidine, but extent of incorporation was irregular possibly because of damage to the stromal cells produced by the collagenase or the washing procedures.

Table II indicates the effect of medium concentration of thymidine and of prior fasting of the animals on incorporation of thymidine. In these studies adipose slices were incubated with tritiated thymidine and radioactivity in the DNA of subsequently isolated stromal cells determined. Above a medium thymidine concentration of 5 μmoles/liter, incorporation was nearly independent of the

TABLE I

*In Vitro Incubation of Adipose Slices with Tritiated Thymidine. DNA Radioactivity in Fat Cells and Stromal Cells**

Expt.	Duration of incubation	Thymidine incorporation‡		% Total DNA radioactivity in stromal cells
		Stromal cells	Fat cells	
	Min			
1	60	513	40	99
	180	1348	204	92
2	180	1270	80	98
3	180	862	93	97
4	180	2090	95	99

DNA, deoxyribonucleic acid.

* All samples were incubated in Eagle's L medium. In experiments 1–3, the specific activity of thymidine in the medium was 29.4 μc/μmole at a medium concentration of 5 μmoles/liter. In experiment 4, the specific activity of thymidine was 5.9 μc/μmole and the medium concentration, 250 μmoles/liter.

‡ μμmoles of thymidine incorporated/mg of DNA.

TABLE II

*Effect of Medium Concentration of Thimidine and of Fasting on In Vitro Incorporation of Thymidine into Adipose Tissue Stromal DNA**

Expt.	Medium concentration of thymidine	Thymidine incorporation‡	
		Fed	48-hr fasted
	μmoles/liter		
1	5	1485	—
	250	2080	—
2	5	1270	41
3	250	2090	95

DNA, deoxyribonucleic acid.

* In all experiments adipose slices were incubated for 3 hr. In experiment 1, the medium was Krebs–Ringer bicarbonate, in experiments 2 and 3, Eagle's L; all media were supplemented with albumin and glucose.

‡ μμmoles of thymidine incorporated/mg of stromal DNA.

quantity of thymidine added, for a 50-fold increase in medium concentration led to less than a 2-fold increment in incorporation. Tissue obtained from 48-hr fasted animals incorporated less thymidine than did tissue from fed animals, and this difference was as marked at a medium thymidine concentration of 250 μmoles/liter as at 5 μmoles/liter. If it is assumed that the high medium thymidine concentration increased the size of the intracellular thymidine pool, the latter finding suggests that the reduction in incorporation noted with tissue from fasted rats was not due to label dilution in an expanded nucleotide pool. If this had been the case incubation with a high concentration of thymidine would have reduced differences between fed and fasted groups in nucleotide pool size and thus would have reduced differences in extent of incorporation.

Although incorporation of tritiated thymidine into the DNA of fat cells was small irrespective of whether adipose slices or free fat cells were used, consistently, with both preparations, radioactivity was recovered in the acetone-ether extracts of these cells. It was also noted that when adipose slices were incubated in Krebs-Ringer bicarbonate, the presence of glucose increased the radioactivity of the acetone-ether washes 40-fold, while, as prevoiusly noted, glucose had no effect on recovery of radioactivity in stromal DNA. The nature of the radioactivity in acetone-ether was determined by thin-layer chromatography; 75% of the label migrated with either triglyceride (57%), free fatty acid (4%), or diglyceride (13%), while only 21% remained at the origin, the site where thymidine was recovered in the same system. When the triglyceride was eluted and saponified, the saponified fraction contained 90% of the radioactivity all of which ran with free acid on thin layer. Hence the bulk of the radioactivity in acetone-ether appeared to be in glyceride fatty acid. The influence of medium glucose on extent of incorporation of label into this fraction suggested that tritium derived from thymidine had been incorporated into fatty acids synthesized during incubation. Tritium could have been made available for incorporation into fatty acids by formation of tritiated water or by production of other thymidine metabolites such as β-aminoisobutyric acid (10) which could conceivably serve as fatty acid precursors. Tritiated water was in fact produced during incubation but only when adipose slices were present; after 3 hr of incubation 1.5% of the added radioactivity was present in medium water.

Specific activity of adipose tissue and liver DNA 80 min after injection of tritiated thymidine. In the first series of in vivo experiments, labeled thymidine was administered to fed, fasted, and refed rats and adipose samples and, in some instances, liver removed 80 min later. The data derived from these studies, shown in Fig. 1, are expressed per unit of total tissue DNA. As was found in the in vitro experiments, 48 hr of fasting markedly depressed thymidine incorporation into adipose tissue DNA. When 48-hr fasted rats were refed for 3 days, values similar to those observed in fed animals were obtained, while the specific activity of adipose DNA derived from animals refed for 5 days was usually higher than in continuously fed animals. In fed animals, no correlation was noted between the specific activities of adipose and liver DNA; however, as with fat tissue, fasting reduced incorporation of thymidine into liver DNA. In conformity with the results of the incubation studies, when fat tissue was removed from fed animals 60 min after injection of labeled thymidine and digested with collagenase, almost all of the DNA radioactivity was present in the stromal fraction (Table III).

Radioactivity was also present in the acetone-ether washes of the adipose samples removed after thymidine injection (Fig. 2). The radioactivity

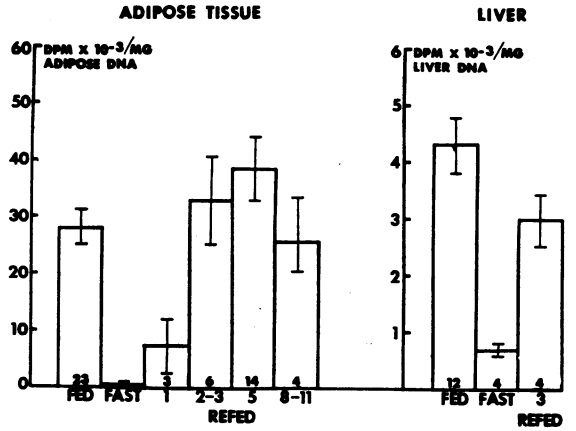

FIGURE 1 Specific activity of adipose and liver DNA 80 min after thymidine injection. Heights of bars and brackets represent means and standard errors of the mean, respectively. Data are expressed per unit of total tissue DNA, and the number of experiments in each series is shown at the foot of the columns. In the fasted series, all groups were fasted for 48 hr except one in which this interval was 18 hr; in the adipose tissue experiments the fasted data were derived from five groups. In the refed experiments all animals were starved for 48 hr before the start of refeeding. The duration of the refeeding interval in days is shown below the various columns.

present in these washes fell with fasting, increased markedly when fasted animals were refed for 2–3 days, and then returned toward levels seen in fed animals with longer periods of refeeding. This pattern is of course very reminiscent of the changes in lipogenesis induced in adipose tissue by fasting and refeeding. Thin-layer chromatography of an acetone-ether extract of tissue obtained from a 5 day refed group revealed that 75% of the radioactivity travelled with triglyceride, 9% with diglyceride, 10% with free fatty acid, and 6% remained at the origin. When the triglyceride was saponified, 80% of the radioactivity was in the saponified fraction. Thus, as in the in vitro studies, most of the radioactivity recovered in acetone-ether had the characteristics of glyceride fatty acid.

Specific activity of fat cell and stromal DNA varying times after injection of tritiated thymidine. It was evident from the preceding studies that during brief in vitro or in vivo exposure of adipose tissue to tritiated thymidine, radioactivity was incorporated only into stromal DNA. These results were anticipated since there is adequate histological evidence that mature fat-filled adipose cells do not display mitotic activity (11). It was considered likely however that among the labeled stromal elements were new, primordial fat cells which would only be collected in the fat cell pool after differentiation and accumulation of lipid. Thus experiments were designed to follow fat cell and stromal cell DNA radioactivity over a longer period of time. In these studies, simultaneous injection of many animals with tritiated thymidine was followed by sacrifice of groups of three rats at various times from 12 hr to 15 days later. These data are shown in Fig. 3; 0 time values represent tissues excised 12 hr after injection.

TABLE III

DNA Radioactivity in Fat Cells and Stromal Cells 60 min after Injection of Tritiated Thymidine

Expt.	DNA radioactivity*		DNA specific radioactivity‡	
	Stromal cells	Fat cells	Stromal cells	Fat cells
1	36.60	0.28	69.0	2.5
2	49.25	0.44	89.5	5.1

Animals in groups of three were injected with 30 μc of labeled thymidine/rat and fat removed 1 hr later.
* dpm × 10^{-3}.
‡ dmp × 10^{-3}/mg of DNA.

During the first 2 days of the study, radioactivity in stromal DNA fell markedly. Thereafter a gradual decline in stromal specific activity was

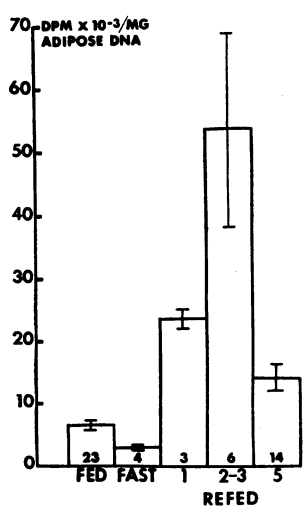

FIGURE 2 Radioactivity in acetone-ether washes of adipose tissue removed 80 min after thymidine injection. Data are expressed per unit of total tissue DNA. Means, standard errors of the mean, numbers of experiments, and duration of refeeding are as shown in Fig. 1.

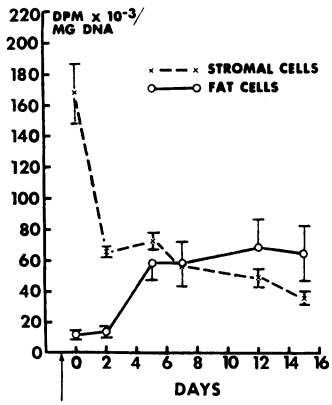

FIGURE 3 Specific activity of fat cell and stromal DNA varying times after thymidine injection. Data are expressed per unit of fat cell or stromal DNA. The arrow indicates the time of thymidine injection which was 12 hr before 0 time. Six groups of animals were used at each period except at days 12 and 15 when five groups were sacrificed. Brackets indicate standard errors of the mean.

observed which was due in part to an increase in unlabeled stromal DNA that occurred with weight gain. While the reason for the abrupt decline in stromal radioactivity is not known, it may have been due to rapid turnover of a fraction of the cellular elements of this pool or to migration from the tissue of cells such as leucocytes and macrophages labeled in situ. At 0 time only 1% of the total DNA radioactivity was in fat cells, a proportion which was identical with that noted in the in vitro and short-term in vivo experiments and which probably represents the extent of stromal contamination of the fat cell pool.

The appearance of radioactivity in fat cell DNA followed a pattern quite distinct from that of the stromal cells. The specific activity of adipocyte DNA rose slightly from 0 to 2 days, markedly from 2 to 5 days, and slowly thereafter. It was clear that this change in specific activity was due to redistribution of radioactivity between fat cell and stromal DNA and not to net accretion of label in DNA over the experimental interval. As shown in Table IV, after the initial decline in total DNA radioactivity that occurred between 0 and 2 days, radioactivity in the total cellular pool remained constant up to 15 days. Thus the increase in fat cell radioactivity that was evident at 5 days was due to a diversion of DNA label from the stromal into the fat cell pool, a process that was essentially complete 7 days after injection of the isotope. It is of interest that of the radioactivity present in stromal DNA 12 hr after thymidine administration, only about 10% was ultimately recovered in the fat cell pool, and that there was no correlation between the stromal specific activity at 0 time and fat cell specific activity 7–15 days later.

These data are entirely consistent with the concept that within the stromal fraction was a pool of primordial fat cells of variable size, and that these cells required 2–5 days after completion of DNA synthesis to accumulate enough lipid to be harvested in the fat cell fraction. It was also evident that estimates of DNA synthesis in primitive fat cells could be made on the basis of the specific activity of the fat cells collected at least 7 days after thymidine administration, a period sufficient for maturation of these elements.

When tissue derived from 48-hr fasted rats was used it was found that the minimum amount of cellular lipid necessary to allow collection of cells in the fat pool was 0.010–0.015 μg/cell, whereas the average lipid content of the fat cells used in the experiments shown in Fig. 3 was 0.080 μg/cell.[1]

[1] These calculations were based on the assumption that the DNA content per cell was 7×10^{-6} μg.

TABLE IV

*Radioactivity in Adipose DNA Varying Times after Thymidine Administration**

Days after injection...	0	2	5	7	12	15
Total DNA radioactivity‡	35.91 ± 4.41	18.15 ± 1.86	20.04 ± 2.26	19.57 ± 3.87	19.01 ± 1.93	17.25 ± 2.42
% Total DNA radioactivity in fat cells	1.0 ± 0.3	3.4 ± 1.3	11.2 ± 2.5	14.6 ± 3.0	14.4 ± 2.6	22.7 ± 8.1

DNA, deoxyribonucleic acid.

* Total DNA radioactivity represents sum of radioactivity in fat cell and stromal fractions. Six groups of rats were used at each interval except at days 12 and 15 when five groups were sacrificed. Standard errors are indicated.

‡ dpm × 10^{-3}.

It is of interest that Hirsch, using rats of similar size but entirely different methods of fat cell collection and sizing, also found the mean lipid content per cell to be 0.080 μg (4), while Goldrick who employed the collagenase system followed by repeated fat cell washes was also able to collect fat cells containing as little as 0.01 μg of lipid per cell (12).

Effect of fasting and short periods of refeeding on thymidine incorporation into fat cell and stromal DNA. While the 80-min in vivo studies had demonstrated that fasting markedly diminished incorporation of thymidine into adipose DNA and that a short period (2–3 days) of refeeding did not increase total DNA synthesis above that seen in fed animals, these experiments did not provide data as to the possible differential behavior of fat cells and stromal elements under these conditions. In particular the possibility had not been excluded that hyperplasia and more rapid maturation of primordial fat cells had accompanied the rapid weight gain seen during the first several days of refeeding. To explore this problem experiments analogous to those shown in Fig. 3 were performed with, in this instance, fasted and fasted-refed rats.

In the fasted series, animals were starved for 36 hr, labeled thymidine administered, and the rats kept without food until the initial group was sacrificed 12 hr after thymidine injection. The rest of the animals were then allowed to feed ad lib. and were sacrificed at varying times. To study the effects of refeeding, rats were fasted for 48 hr and then refed chow. 36 hr after the start of refeeding, the time at which the per cent of increase in rat weight was most rapid, all animals in this series were injected with labeled thymidine, and 12 hr after the injection the first group of refed rats was sacrificed. Adipose tissue was obtained from other groups at later times. The data from these studies compared to the results obtained in fed animals that had been matched for weight and injected simultaneously with the refed animals are shown in Fig. 4. 0 time values were derived from tissues removed 12 hr after thymidine administration.

In the experiments with fasted animals, radioactivity in fat cell DNA was barely detectable even when tissue was removed 2 wk after thymidine injection, hence 2 wk after completion of fasting. This interval was sufficient to permit complete

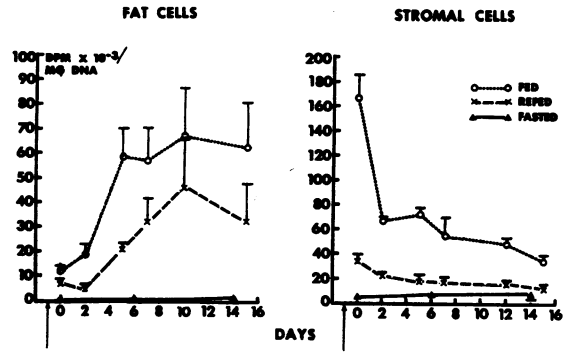

FIGURE 4 Effect of fasting and of refeeding on incorporation of thymidine into fat cell and stromal DNA. Data are expressed per unit of fat cell or stromal DNA. The number of groups in the fed series has been indicated in Fig. 3. In the refed series five groups of animals were used at each time, while in the fasted series one group was sacrificed at 0 time, one at 6 days, and five at 14 days. The arrow indicates the time of thymidine injection, the brackets the standard errors.

restoration of lipid stores as estimated by lipid content of the tissues removed and lipid content per fat cell and hence was presumably sufficient to ensure complete harvesting of all labeled cells that had differentiated into adipocytes. Incorporation of thymidine into stromal DNA was also markedly reduced by fasting, but this reduction was not as complete as noted with fat cells; consistently, the specific activity of stromal DNA exceeded that of fat cell DNA.

Although 36 hr of refeeding restored to a considerable extent incorporation of thymidine into fat cell DNA and had a similar but less marked effect on the stromal fraction, the rate of DNA synthesis in fat cells and stroma of refed animals was always less than in fed rats. Furthermore, maturation of fat cells occurred no more rapidly in the refed than in the fed series; in both instances radioactivity in fat cell DNA increased markedly between day 2 and day 5 of the study.

From the data obtained in the refed experiments it was possible to assess the contribution of new fat cell formation to the process of lipid repletion that occurred during the refeeding interval. It was obvious that, relative to continuously fed animals, refeeding for 36 hr had not induced hyperplasia of primordial fat cells nor more rapid maturation of these elements. Furthermore it was evident that during the first 2 days of refeeding, an interval during which weight gain and lipid accumulation

was rapid, any new fat cells formed would not have matured and thus would not have contributed significantly to the lipid stores of the tissue. Hence during this interval lipid repletion must have been due entirely to accretion of fat by existing but depleted fat cells. Between the 2nd and 4th day of refeeding (day 0–day 2 of the study), the lipid content of the excised tissues and the lipid content per fat cell increased from 30 to 60% of fed values. While it is not certain that the lipid deposited during this interval was also in existing cells predominantly, it is likely that this was so. Because of the time required for maturation of new fat cells, only those cells formed during the first 24 hr of refeeding could conceivably have been in the fat cell pool by the end of the 4th refeeding day (day 2 of the study). As new fat cell formation was negligible at the end of the fasting period and still less than that seen in fed animals 36 hr after refeeding, it is unlikely that synthesis of new fat cells had contributed in a major fashion to the rapid accretion of lipid that occurred not only during the first 2 days of refeeding but also during the ensuing 48 hr interval. The possibility that hyperplasia of primordial fat cells was a significant feature later in refeeding remains to be explored.

Radioactivity in acetone-ether washes after thymidine injection. As in the in vitro and short-term in vivo experiments, radioactivity was consistently present in the acetone-ether washes of fat cell fractions derived from tissues excised 12 hr or longer after thymidine administration (Table V). Once again, most of this radioactivity had the characteristics of glyceride fatty acid, and incorporation of label into this fraction was greater in refed than in fed animals.

In a series of continuously fed animals, radioactivity in fat cell lipid was followed up to 25 days after thymidine injection, and these data, expressed per milligram of fat cell DNA, are shown in Fig. 5. Radioactivity in this fraction increased up to 10 days after isotope administration and thereafter gradually declined; the decline was due both to a reduction in recovered radioactivity and to the increase in DNA content of the fat cell pool that occurred with weight gain. As intravenously injected thymidine disappears very rapidly from the circulation (10), it was evident that the continued labeling of adipose lipid must have been due to persistence in the circulation of a tritiated

TABLE V

Radioactivity in Acetone-Ether Washes of Fat Cells 12 hr after Thymidine Administration

	Fed	36-hr refed
dpm × 10^{-3}/mg of fat cell DNA	513	1030
% Distribution of radioactivity*		
CE	2	tr.
TG	93	99
FFA	2	tr.
DG and Chol	2	tr.
PL	1	tr.
% of TG radioactivity in saponified fraction	71	97

* DNA, deoxyribonucleic acid.

* CE, cholesterol ester; TG, triglyceride; FFA, free fatty acid; DG, diglyceride; Chol., cholesterol; PL, phospholipid.

derivative of thymidine that could be incorporated into adipose tissue fatty acid. Irrespective of the route by which tritium entered fat cell lipid, net accumulation of label was essentially complete 10 days after injection. Thus abrupt reductions in adipose lipid radioactivity induced over a brief period after this time must have been due to changes in the rate of fat mobilization. In later studies use was made of this fact to estimate extent of lipid depletion induced by fasting.

In searching for a tritiated thymidine metabolite that could be incorporated into adipose lipid, attention was focused on production and turnover of tritiated water which is known to accumulate

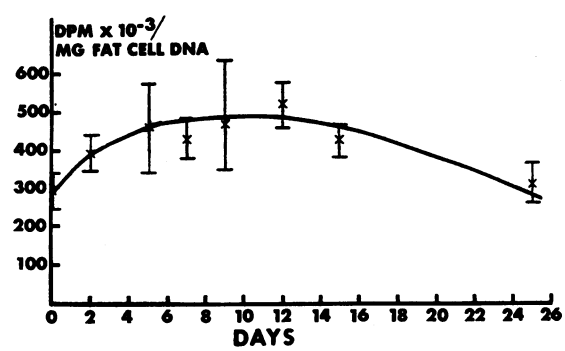

FIGURE 5 Radioactivity in acetone-ether washes of fat cells varying times after thymidine injection. The data were derived from fed animals, and the number of groups used at the varying times were: 0 time, nine; 2 days, nine; 5 days, seven; 7 days, eight; 9 days, five; 12 days, seven; 15 days, eight; 25 days, five. Brackets represent standard errors.

shortly after injection of tritiated thymidine (10). Fig. 6 indicates the change in plasma water radioactivity from 12 hr (0 time) to 15 days after administration of labeled thymidine. If it is assumed that newly formed tritiated water had equilibrated with total body water by 0 time, it was evident that over the 12 hr subsequent to thymidine injection, 40–50% of the administered radioactivity had been incorporated into body water. This figure is very similar to that obtained in man (10). Radioactivity in plasma water declined exponentially at the same rate in fed and refed animals and 10 days after injection, the time at which net accretion of radioactivity in adipose lipid had ceased, plasma water contained only 10% of the radioactivity present 12 hr after isotope injection.

In a few observations made in animals fasted before thymidine administration and then allowed to feed, a similar disappearance rate of tritiated water was observed. Although very little radioactivity (or lipid) was recovered in the acetone-ether washes of fat cells obtained from these animals before feeding was started, when tissues were removed varying times during the feeding period, radioactivity in adipose lipid was not only demonstrable but usually greater than in samples taken from fed animals at the same time. Thus the rebound in lipogenesis produced by refeeding resulted in more extensive incorporation of tritium, possibly derived from tritiated water, into newly formed lipid. However, as shown in Fig. 4, despite the presence of tritiated water throughout the period of observation of the fasted group and despite enhanced incorporation of label into adipose lipid, very little radioactivity was recovered in fat cell DNA. Furthermore no increase in stromal DNA radioactivity was evident over the period of observation. These data provided convincing evidence that tritium derived from tritiated water was not incorporated into the DNA of either cellular pool.

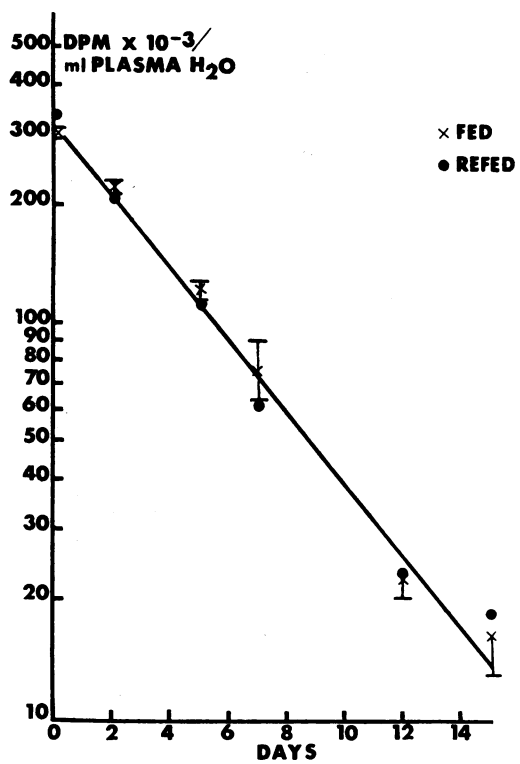

FIGURE 6 Radioactivity in plasma water varying times after thymidine injection. At each of days 0, 2, and 5 the fed series was comprised of four groups, at days 7, 12, and 15, of two groups. The animals in the refed series had been fasted for 48 hr and fed for 36 hr before thymidine injection (given 12 hr before 0 time), and this series was comprised of three groups at days 0, 2, and 5, two groups at days 7 and 12, and one at day 15. Brackets indicate standard errors of the fed series.

Effect of fasting on mature fat cells. From the data obtained in refed animals it was concluded that repletion of adipose lipid during at least the early stages of refeeding had been accomplished by refilling of existing cells. This conclusion implied that those cells depleted of fat by starvation had survived and had remained capable of lipid accumulation with refeeding. This assumption was examined in the next study.

Fed rats were injected with tritiated thymidine and divided into two groups, one of which was fed continuously for 25 days before sacrifice. The other group was fed for 10 days, a period long enough to ensure maturation of fat cells, fasted for 2 days, and then fed again for 13 days. The final feeding interval was sufficient to restore lipid content of excised tissues and lipid content per fat cell to values seen in continuously fed animals and hence should have been long enough to allow complete harvesting of previously depleted fat cells. If fasting had destroyed depleted fat cells with loss of DNA or had significantly reduced the capacity of these cells for subsequent lipid accretion, recovery of radioactivity in fat cell DNA would have been substantially less in this group. Alternatively if these cells had retained their in-

tegrity and their ability to reestablish activities of those enzymes involved in lipid synthesis and assimilation, fat cell DNA radioactivity would have been comparable in the two series.

As shown in Table VI, the two groups of animals displayed very similar values not only for lipid content but also for radioactivity and specific activity of fat cell and stromal cell DNA. However, radioactivity in fat cell lipid was 50% less in the fasted than in fed animals, a value which should approximate the extent of lipid depletion produced during the fasting period. While variation between groups precluded detection of small changes, it is evident that fasting sufficient to reduce lipid stores by one-half produced neither extensive destruction of fat cells or stromal elements nor significant impairment in the capacity of depleted fat cells to accumulate lipid with refeeding.

Effect of growth hormone on thymidine incorporation in fat cell and stromal DNA. Growth hormone is known to exert a variety of effects on adipose tissue metabolism; these include enhancement of fat mobilization (13), ultimate inhibition of fatty acid synthesis (14), and increased in vitro incorporation of thymidine into the DNA of adipose pieces when the hormone is given before removal of the tissue (15). While the effects of this agent on free fatty acid release and lipogenesis are undoubtedly due to alterations in fat cell function, it is not known which of the cellular elements of the tissue responds to the hormone with an increased rate of DNA synthesis. Indeed it would appear paradoxical that a hormone that acts to reduce adipose lipid would also have a pronounced effect on the rate of multiplication of those cells destined to store fat. An effect of growth hormone on increasing growth of supporting and vascular tissue would be a more understandable phenomenon. A series of experiments were therefore performed to determine whether this hormone stimulates DNA synthesis in both the fat cell and stromal fractions of adipose tissue or whether it has a more discriminatory effect.

In these studies growth hormone was administered over 36 hr to fed hypophysectomized rats, fed intact animals, and intact animals fasted during the period of hormone administration. Thymidine was injected into control and growth hormone–treated groups 6 hr after the last dose of hormone, and animals were killed no sooner than 8 days and more commonly 12–16 days after isotope administration. The results are shown in Fig. 7. In the control hypophysectomized group, incorporation of thymidine into stromal DNA was variable, while consistently little if any radioactivity was recovered in fat cell DNA. When growth hormone was given to hypophysectomized animals, DNA synthesis in the stromal fraction was markedly increased, but the hormone had no apparent effect on the incorporation of thymidine into the DNA of fat cell precursors. A differential effect of the hormone on stromal and fat cell DNA content was also evident. Despite the fact that growth hormone had been given 1–2 wk before tissue sampling, the quantity of DNA extracted from the stromal pool of the treated animals was significantly greater than that obtained from control rats. No such difference was evident in fat cell DNA content.

It was thought possible that the reduction in DNA radioactivity noted in fat cells of hypophysectomized animals was due to the smaller amount of food consumed by these animals as compared to normal rats. However several facts were not in keeping with this interpretation. The lipid content per fat cell in both the control and hormone-

TABLE VI

*Effect of Fasting on Mature Fat and Stromal Cells**

	Fed	Fasted
Radioactivity in fat cell DNA‡	7.0 ± 1.3	8.5 ± 0.9
Radioactivity in stromal DNA‡	25.2 ± 6.9	27.8 ± 6.8
Specific activity in fat cell DNA§	30.2 ± 5.6	38.2 ± 4.7
Specific activity in stromal DNA§	19.2 ± 6.1	19.9 ± 6.9
Radioactivity in fat cell lipid‡	64.1 ± 8.8	33.8 ± 4.0‖
Radioactivity in fat cell lipid/mg of fat cell DNA	287.5 ± 44.8	156.6 ± 17.4‖

DNA, deoxyribonucleic acid.

* Means and standard errors of six experiments are shown. Fasted series represents those animals starved for 2 days beginning 10 days after thymidine injection and refed for 13 days before sacrifice.

‡ dpm × 10^{-3}.

§ dpm × 10^{-3}/mg of DNA.

‖ Fed vs. fasted $P < 0.01$.

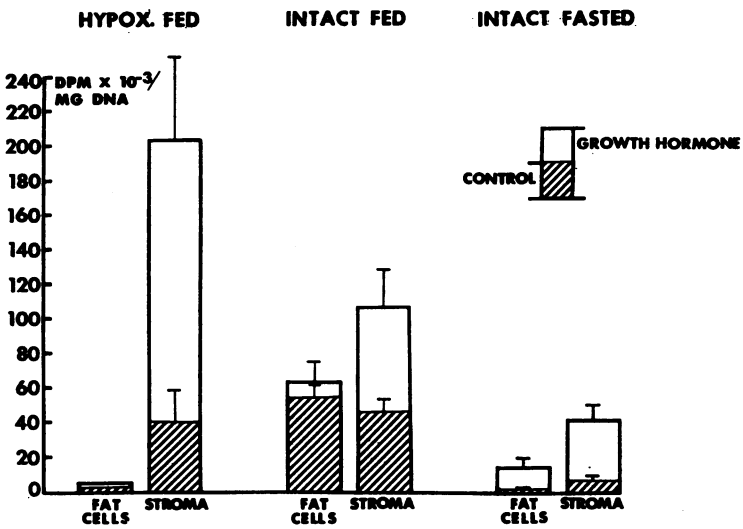

FIGURE 7 Effect of growth hormone on incorporation of thymidine into fat cell and stromal DNA. Data are expressed per unit of fat cell or stromal DNA; the height of the entire bar represents the results derived from the growth hormone experiments, the hatched area the control studies. Brackets indicate standard errors. All growth hormone–treated rats received 3.0 mg of bovine growth hormone I-P in divided doses over 36 hr and were given tritiated thymidine 6 hr after the last growth hormone injection. Control animals were either not injected or given saline.

In the hypophysectomized series (*hypox. fed*) there were eight control and five growth hormone–treated groups. Adipose tissue was removed 8–16 days after thymidine injection. In the intact fed series there were 30 control and 9 growth hormone–treated groups, and in this series tissue was removed 12–16 days after isotope administration. In the intact fasted series 13 control and 9 growth hormone–treated groups were used; the hormone was administered during the 2 day fast, and feeding was begun 12 hr after thymidine injection. Tissue was obtained 12–16 days after the beginning of the refeeding period. The differences between the control and growth hormone groups were significant ($P < 0.01$) in the following instances: hypophysectomized stroma, fed intact stroma, fasted intact fat cells and stroma.

treated hypophysectomized animals was 0.06–0.07 μg, a value similar to that obtained in intact rats of the same weight and much greater than seen in fasted intact rats. Moreover in control hypophysectomized animals, the specific activity of stromal DNA far exceeded that of fasted intact animals. Hence inadequate nutrition was probably not the major reason for the absence of radioactivity in the DNA of fat cells derived from hypophysectomized rats, and it would appear more reasonable to attribute this finding to an effect of hypophysectomy on fat cell formation and (or) maturation.

While a specific action of growth hormone on stromal proliferation was the most obvious explanation of the different effects of the hormone on fat cell and stromal DNA radioactivity, it was evident that pituitary ablation may have abolished a fat cell response to growth hormone that would have been demonstrable in the presence of normal pituitary function. To explore this possibility a second series of experiments was performed in fed intact rats. In this instance once again growth hormone clearly stimulated thymidine incorporation into stromal but not fat cell DNA. Even this experiment was not considered as providing definitive evidence that the hormone only affects stromal DNA synthesis, for it was possible that DNA formation in primordial fat cells was progressing maximally in untreated animals. As it has been shown that prior administration of

growth hormone to fasted rats enhances the subsequent in vitro incorporation of thymidine into DNA of adipose slices (15), a third series of intact animals was given the hormone during a 48 hr fasting period. In this instance growth hormone produced a small but significant increase in DNA radioactivity extracted from fat cells removed 12–16 days after isotope injection; however, this increment was only one-third that noted in the stromal fraction. Thus it is evident that growth hormone stimulated DNA synthesis in both stromal cells and fat cell precursors, although the major effect of the hormone was undoubtedly on the stromal pool. It was also clear that removal of the pituitary resulted in an inhibition of fat cell formation and (or) maturation that was not reversed by a brief period of growth hormone treatment.

DISCUSSION

The results of these experiments demonstrate the feasibility of studying DNA synthesis in both the fat cell and stromal elements of adipose tissue under in vivo conditions. To approach this problem it was found necessary to purify the fat cell fraction by methods somewhat more elaborate than the multiple washes usually employed. In experiments done before the introduction of these additional procedures, when adipose tissue was removed within 12 hr of thymidine injection, a considerable fraction of the radioactive DNA of the tissue was recovered in the fat cell fraction, a finding also reported by others (16). The known lack of mitotic activity displayed by lipid-containing adipose cells casts serious doubt on the validity of these observations and suggested the possibility of residual stromal contamination despite repeated fat cell washes. This apparent paradox was easily resolved when the fat cell pool was purified more thoroughly.

In this study thymidine incorporation into DNA has been used as an index of synthesis of new DNA; it is recognized, however, that it could also have been a function, at least in part, of DNA repair. However, if the latter process had significantly influenced the results, it is likely that all cells would have been labeled initially, not just the stromal fraction. It was also assumed that the extent of thymidine incorporation was a measure of the rate of new cell formation, and while no definite proof of this assumption is available, it would appear to be a reasonable inference since polyploidy is not a feature of the cellular constituents of adipose tissue (3). A further assumption inherent in the study was that radioactivity recovered in DNA had been introduced solely during the process of thymidine incorporation. Mention has already been made of one finding that supports this assumption; in fed and fasted animals followed for 2 wk after injection of the isotope, no net accretion of DNA label was evident despite persistent circulation throughout the period of at least one radioactive thymidine metabolite, tritiated water. Finally it must be appreciated that all studies of DNA synthesis which involve incorporation of a labeled precursor into DNA are based on the assumption that changes in radioactivity in product reflect alterations in rates of synthesis and not in specific activity of intracellular precursors. In the present study the failure of changes in medium thymidine concentration to abolish differences in DNA radioactivity between fed and fasted rats and the increase produced by growth hormone in the chemical DNA content of stroma suggest that, at least in these instances, the isotopic data did in fact reflect changes in rates of DNA formation. A more direct approach to this problem could not be made in this study for the first requirement, separation of primordial fat cells from the rest of the stromal fraction, could not be accomplished. Furthermore it was evident that since collagenase digestion altered thymidine flux across subsequently isolated stromal cells, accurate measurement of nucleotide pools in these cells was impossible.

The picture of the kinetics of the cellular elements of adipose tissue that emerges from these experiments contains several features of interest and a number that are unexplained. It is evident that of the DNA synthesized in adipose tissue during the first 12 hr after thymidine injection, one-half was lost from the tissue during the next 48 hr by unknown mechanisms and that only 10% was ultimately recovered in cells containing fat. Maturation of fat cells occurred rapidly, the interval between completion of DNA synthesis and significant lipid accumulation being between 2 and 5 days, and it is clear that the processes of differentiation and maturation of primordial fat cells were not hastened by refeeding after a period of

fasting. As in other tissues (17, 18) fasting depressed DNA synthesis in fat cells and stromal elements, and it is of some interest that this effect was more profound in the fat cell fraction.

The demonstration that mature fat cells were not destroyed by an acute fast of duration sufficient to reduce lipid stores by 50% is consonant with the observation that in obese man, fasting does not reduce the number of existing fat cells but merely empties them (4). It is also clear that fat cells depleted of lipid by fasting retained the capacity for reaccumulation of triglyceride when refeeding was begun, and that the rate of refilling of existing cells was more rapid than the rate of maturation of new fat cells. Because of this fact, lipid repletion during at least the early stages of refeeding was due entirely to refilling of existing fat cells and was not significantly influenced by new fat cell formation. Thus while it is very likely that protracted under- and over-nutrition will influence adipose stores by affecting both the production of fat cells and the lipid content per cell, it is probable that abrupt short-term changes in adipose mass predominantly reflect changes in cellular lipid content. Hausberger, using principally histological techniques, has previously reached a similar conclusion (5).

The data derived from the experiments with hypophysectomized and growth hormone–treated animals can be considered as providing only introductory information on pituitary control of fat cell formation and maturation. It is evident that pituitary ablation almost completely abolished formation and (or) maturation of fat cells, and that this effect was not influenced by brief treatment with growth hormone. The specific consequences of hypophysectomy that produce this phenomenon remain to be defined, and it is not clear as to whether removal of the pituitary resulted in a reduction in the rate of DNA synthesis in primordial fat cells or in arrest of cellular differentiation.

There is less ambiguity in the interpretation of the growth hormone studies; in all systems used, administration of this hormone produced a marked increase in DNA synthesis in the stromal fraction and in intact animals caused a lesser augmentation of DNA formation in cells destined to become adipocytes. The abolishment by growth hormone of the inhibitory effect of fasting on DNA synthesis, a phenomenon first described by Murakawa and Raben (15), is particularly intriguing and remains completely unexplained. It would be of obvious interest to determine whether protein and RNA synthesis are involved in this hormone effect.

The differences between fat cells and stromal elements in responsiveness to growth hormone was one of several observations that suggested that DNA synthesis in the two cellular pools was controlled independently, at least to some extent. In fed animals, the ultimate recovery of radioactive DNA in fat cells was unrelated to radioactivity present in stromal DNA 12 hr after injection of isotope. Furthermore nutritional variations appeared to influence more significantly incorporation of thymidine into primordial fat cell rather than stromal DNA. In relation to the latter phenomenon it is pertinent to note that in those forms of human and experimental obesity associated with hyperinsulinemia, increases in fat cell number have been described (4, 5). Obviously consideration must be given to the possibility that alterations in nutrition produced changes in fat cell DNA synthesis due to fluctuations in insulin action on primitive fat cells. Indeed it would not be surprising if such a control system was found to exist, for it is well established that insulin in vitro can enhance DNA synthesis in mammary gland explants (19).

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