

Changes in adipose tissue of the rat due to early undernutrition followed by rehabilitation

3. Changes in cell replication studied with tritiated thymidine

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1. Well-nourished rats were injected with tritiated thymidine at 15, 22, 28 or 84 d of age. At 1, 6, 11 and 16 d after injection animals from each group were killed, samples of adipose tissue were removed from two subcutaneous sites (abdominal and scapular) and separated, using collagenase (EC 3.4.24.3), into 'fat cell' and 'stromal cell' fractions. The specific (radio)activity of DNA isolated from each fraction was measured. The specific activity of DNA isolated from two 'deep body' sites (perirenal and epididymal) was measured only in the animals injected at 84 d of age.

2. Animals undernourished from birth up to 84 d of age were injected with tritiated thymidine at 22, 28 or 84 d of age. Animals were killed 1 and 11 d after injection, adipose tissue removed, and the specific activity of DNA measured. Other undernourished animals were rehabilitated from 84 to 107 d and injected at 91 d of age with tritiated thymidine. The animals were killed 1, 6, 11 and 16 d after injection, adipose tissue was removed from the subcutaneous and deep body sites and the specific activity of DNA determined as before.

3. In well-nourished animals fat cell replication had largely ceased by 12 weeks of age in the subcutaneous depots. There were differences between the various sites of adipose tissue regarding the period of hyperplastic growth, its timing or rate of replication or both.

4. In undernourished animals replication was slow in the subcutaneous depots compared with well-nourished animals of the same age. Rehabilitation from undernutrition stimulated replication which resulted in higher rates in all four depots examined compared with those in well-nourished animals.

5. The findings are discussed in relation to the concept of a finite period of hyperplasia for adipose tissue.

In a previous paper (Harris, 1980) it was reported that the adipose tissue of Black and White Hooded rats undernourished from birth to 12 weeks of age had fewer observable fat cells at the end of the period of undernutrition than well-nourished animals of the same age. However, after 20 weeks rehabilitation there was no difference in the number of observable fat cells between the rehabilitated rats and those well-nourished throughout the experiment, in any adipose depot examined. These findings are difficult to interpret because of the existence of 'empty' fat cells which, it has been proposed (Widdowson & Shaw, 1973) are fat cells that are devoid of lipid and hence not identifiable by available techniques (Gurr & Kirtland, 1978). The increase in observable fat cells during rehabilitation could result from the filling of pre-existing 'empty' fat cells or the synthesis (replication) and filling of new fat cells.

In order to distinguish between these interpretations cell replication was assessed by measuring the incorporation of tritiated thymidine *in vivo* into the DNA of fat cells and other cell types (stromal cells) in adipose tissue. The principles of using labelled thymidine as an index of DNA synthesis and cell replication have been set out by Cleaver (1967). Few workers have applied this technique to adipose tissue *in vivo*, and these studies were limited to rat epididymal fat pad (Hollenberg & Vost, 1968; Greenwood & Hirsch, 1974; Hayes

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& Lewis, 1975). The assumptions made in interpreting experiments with adipose tissue are discussed by Hollenberg & Vost (1968), Greenwood & Hirsch (1974) and Gurr & Kirtland (1978). One difficulty apparent from these reports is in deciding what level of incorporation of thymidine, expressed as specific activity (disintegrations/min per mg DNA), represents a 'high' or a 'low' level of replication. We have therefore adopted a comparative approach: the specific activity of DNA extracted from different adipose depots has been compared, and changes in specific activity with time noted. Further, the specific activity of adipose tissue DNA has been compared with that of DNA extracted from a section of small intestine, as this is considered to be an organ with a very rapid turnover of cells (Bertalanffy & Lau, 1962; Cleaver, 1967; DeRobertis *et al.* 1965). The objectives of the present investigation were: first, to determine the timing of the replication period of fat cells, especially in scapular and subcutaneous adipose tissue, of well-nourished Black and White Hooded rats; and secondly, to assess the effects of undernutrition and rehabilitation on cell replication.

MATERIALS AND METHODS

Animals. Black and White Hooded rats were reared in litters of three or sixteen and weaned at 21 d of age. Animals reared in litters of sixteen were fed on restricted amounts of stock diet until 12 weeks of age. These animals were regarded as undernourished from birth onwards. At 12 weeks of age they were rehabilitated by being allowed to feed *ad lib.* on stock diet. Rats reared in litters of three were allowed to feed *ad lib.* from weaning, and were regarded as well nourished throughout. Details of these regimes have been described (Harris, 1980).

Experimental procedures. Table 1 summarizes the design of the experiment. [Methyl-7H]-thymidine (The Radiochemical Centre, Amersham, Bucks.) was administered intraperitoneally at a concentration of 33 μC (67 nmol) thymidine/kg body-weight at 10.00 hours. This was followed by unlabelled thymidine, at 167 μmol thymidine/kg body-weight, administered at 6, 24 and 48 h after the labelled thymidine was given. Well-nourished animals were injected with [^3H]thymidine at 15, 22, 28 or 84 d of age. Animals from each of these groups were killed 1, 6, 11 or 16 d after the [^3H]thymidine was given. Details of the number of animals in each group are shown in Table 1. Undernourished rats were injected with [^3H]thymidine at 22, 28 or 84 d of age (during undernutrition), and a further group after 1 week of rehabilitation at 91 d of age. As very little adipose tissue was present in the undernourished animals it was necessary to pool the tissue from a large number of animals and, instead of collecting tissue at 1, 6, 11 and 16 d after the [^3H]thymidine was given, only two 'collections' of tissue were made at 1 and 11 d. Rehabilitated animals were killed at 1, 6, 11 or 16 d after the [^3H]thymidine as before.

The animals were killed as described previously (Harris, 1980) and subcutaneous adipose tissue removed from the abdominal and interscapular sites as previously described (Harris & Widdowson, 1978). Perirenal and epididymal adipose tissue was removed from the 12-week-old well-nourished animals and from the rehabilitated animals. Insufficient material was available at these sites in younger animals for determination of the specific activity of DNA.

A 30 mm length of small intestine was taken from well-nourished animals injected with [^3H]thymidine at 15 d of age.

Techniques. Adipose tissue was digested with collagenase (EC 3.4.24.3) using the technique of Rodbell (1964) and separated into a floating cell fraction ('fat cells') and a sedimenting fraction ('stromal cells'). After washing, the fractions were stored in acetone at -20° before extraction of DNA. DNA was extracted by the method of Hollenberg & Vost (1968) and assayed by the method of Burton (1956) using calf thymus DNA as a standard. Radioactivity associated with the DNA extracts was measured using a Philips PW 4510/01

Table 1. *Experimental design*

Group...	Well-nourished			Undernourished			Rehabilitated		
	Age when [³ H]thymidine administered (d)	Age at death (d)	No. of animals	Age when [³ H]thymidine administered (d)	Age at death (d)	No. of animals	Age when [³ H]thymidine administered (d)	Age at death (d)	No. of animals
15	15	16	9	—	—	—	91	92	11
		21	9	—	—	—		97	9
		26	9	—	—	—		102	6
		31	6	—	—	—		107	6
22	22	23	10	22	23	15			
		28	8	—	—	—			
		33	7	—	33	15			
		38	5	—	—	—			
28	28	29	4	28	29	13			
		34	11	—	—	—			
		39	5	—	39	15			
		44	5	—	—	—			
84	84	85	4	84	85	8			
		90	5	—	—	—			
		95	5	—	95	9			
		100	5	—	—	—			

liquid scintillation counter and specific activity was calculated as disintegrations/min per mg DNA.

Samples of gut were homogenized and DNA extracted according to Zamenhof *et al.* (1964) and specific activity determined as for the cell fractions.

RESULTS

Small intestine

Fig. 1 shows the specific activity (disintegrations/min per mg DNA) of DNA extracted from samples of small intestine from animals injected with [³H]thymidine at 15 d of age and killed 1, 6, 11 or 16 d later. The highest specific activity, 111750 disintegrations/min per mg DNA, was seen in the animals killed 1 d after the [³H]thymidine was given. Thereafter, specific activity declined rapidly. Specific activities of the order of 100000 disintegrations/min per mg DNA therefore represent a 'high' level of incorporation of label into DNA. The rapid decline in specific activity is due to the synthesis of unlabelled DNA. A high level of incorporation followed by a rapid decline is strong indication of a rapid rate of cell replication.

Abdominal fat

Fig. 2 illustrates the specific activity of DNA extracted from the 'fat cell' and the 'stromal cell' fractions of the abdominal tissue in well-nourished animals. Injection of the [³H]-thymidine at 15 d of age resulted in a very high specific activity of stromal DNA (204810 disintegrations/min per mg) 1 d later. The specific activity of the 'stromal cell' fraction declined rapidly with time, suggesting rapid replication. This 'stromal cell' fraction contained the dividing cells that give rise to 'empty' fat cells as well as other cell types. The 'fat cell' DNA also had a high specific activity 1 d after injection of the label to 15-d-old animals. These results indicate that, not only was replication of cells very rapid in the stromal fraction, but part of this replication was due to synthesis of new fat cells, and these filled rapidly and thus appeared in the mature fat cell fraction. The rapid decline in specific activity

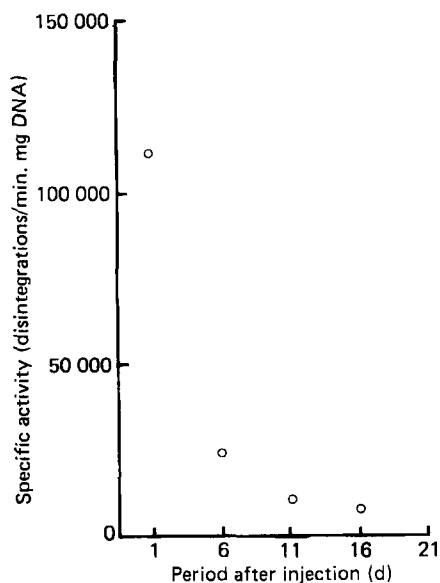


Fig. 1. Specific activity (disintegrations/min per mg DNA) of DNA extracted from the small intestine of animals injected with [^3H]thymidine at 15 d of age and killed 1, 6, 11 or 16 d later.

of the fat cell fraction indicated that new unlabelled cells were being formed and filled, with consequent 'dilution' of the specific activity of the fat cell DNA.

Injection of [^3H]thymidine at 22 d of age resulted in a different labelling pattern. The specific activity of stromal DNA 1 d after the injection was low and declined only slightly with time. Similarly, the specific activity of 'fat cell' DNA was low and remained low, indicating no 'dilution' by unlabelled cells filling and entering the mature fat cell compartment. Over all, these findings indicated that cell replication and filling had almost ceased at 22 d of age. This may have resulted from the trauma of weaning with an associated failure to eat.

In the animals given [^3H]thymidine at 28 d of age the specific activity of DNA isolated from the abdominal site 1 d later was nearly as high as in the 15-d-old animals, as was the rate of filling of fat cells. Both the 'stromal cell' and 'fat cell' fractions followed much the same pattern of specific activities as in the animals injected at 15 d of age. This implies that replication of all cells, including fat cell precursors, was resumed quickly as the animals recovered from weaning, and that the filling of fat cells was again very rapid.

In the animals given [^3H]thymidine at 84 d of age the specific activity of stromal DNA at the abdominal site was low 1 d later compared with that in animals injected at 15 and 28 d of age. The specific activity decreased between 1 and 6 d after the thymidine but thereafter was relatively stable. Part of the initial decline in specific activity is thought to be due to a rapid turnover of connective tissue cells or loss of leucocytes labelled *in situ* (Hollenberg & Vost, 1968; Greenwood & Hirsch, 1974). Although there was some labelling of the 'fat cell' fraction in these animals, the results indicate a slower rate of filling of fat cells than in animals given [^3H]thymidine at 15 or 28 d of age. The findings suggest that a period of very rapid cell replication occurred at the abdominal site between 15 and 28 d of age and, while it cannot be stated at what stage between 28 and 84 d the replication started to decline, it seemed that replication had largely ceased by 84 d of age in well-nourished animals at this site.

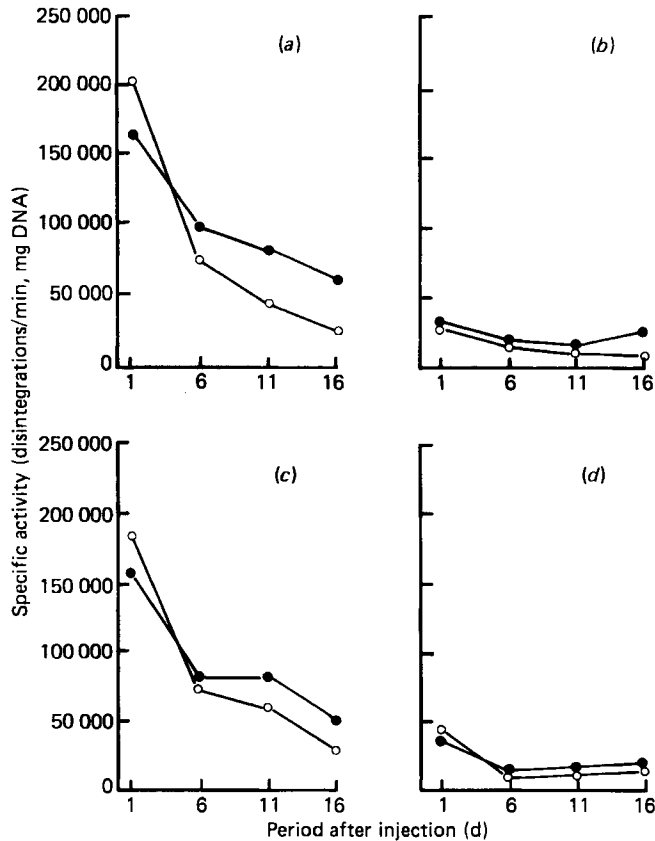


Fig. 2. Specific activity (disintegrations/min per mg DNA) of DNA extracted from 'fat cell' and 'stromal cell' fractions of the abdominal tissue in well-nourished animals injected with [³H]thymidine at (a) 15, (b) 22, (c) 28 or (d) 84 d of age, and killed 1, 6, 11 or 16 d after injection. (●), 'Fat cell' fraction; (○), 'stromal cell' fraction.

Fig. 3 shows the specific activity of DNA in the 'fat cell' and 'stromal cell' fractions of the abdominal site of undernourished and rehabilitating animals. All values for specific activity in the 'stromal cell' fraction during undernutrition were very low compared with those for well-nourished animals. This indicates that cell replication was slow in the undernourished animals at the abdominal site. The values obtained for specific activities of 'fat cell' DNA in the undernourished animals were high, and probably result from a few labelled cells entering a very small pool of mature fat cells. This is a reasonable conclusion, as the DNA content of the 'fat cell' fraction of the undernourished animals was only approximately 2% of that in the well-nourished animals. The 'fat cell' fraction isolated from animals injected with [³H]thymidine at 84 d of age and killed 1 d later had a DNA content below the limits of detection of the method used (2 μg) and hence this value is missing from Fig. 3.

Rehabilitation of the undernourished animals (at 91 d of age) resulted in high specific activities of stromal DNA, higher than in well-nourished animals. Transfer of the label into the mature 'fat cell' fraction was also rapid (indicating rapid filling), with specific activities similar to those in the well-nourished animals injected at 15 d of age. These results suggest that during undernutrition cell replication at the abdominal site was markedly inhibited, with

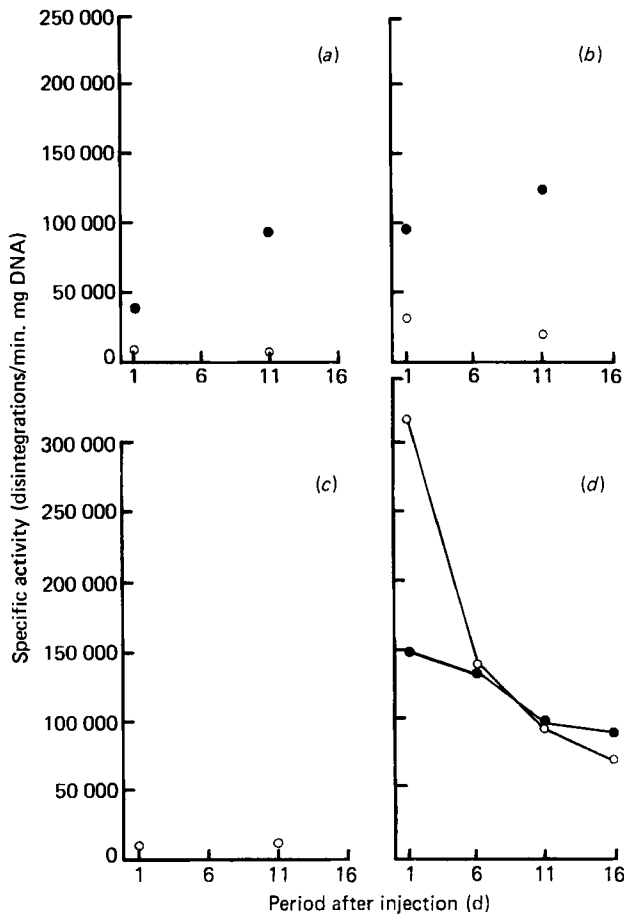


Fig. 3. Specific activity (disintegrations/min per mg DNA) of DNA extracted from 'fat cell' and 'stromal cell' fractions of the abdominal tissue in undernourished animals injected with [^3H]thymidine at (a) 22, (b) 28 or (c) 84 d of age and killed 1 or 11 d after injection, and rehabilitating animals injected with [^3H]thymidine at (d) 91 d of age and killed 1, 6, 11 or 16 d after injection. (●), 'Fat cell' fraction; (○), 'stromal cell' fraction.

only a small extent of fat cell filling. However, on rehabilitation there was a dramatic acceleration of cell replication with an associated rapid filling of fat cells.

Scapular fat

Fig. 4 shows the specific activity of DNA in the 'fat cell' and 'stromal cell' fractions at the scapular site of well-nourished animals. All specific activities were lower than those for the abdominal site at equivalent ages. For example, in the animals given [^3H]thymidine at 15 d of age, the specific activity of stromal DNA in the scapular site was only 32 970 disintegrations/min per mg 1 d later, compared with 204 810 disintegrations/min per mg in the abdominal site. The 'fat cell' fraction showed a slow increase in specific activity followed by a decline. This suggests that the filling of 'empty' fat cells occurred more slowly than in the abdominal site at the same age. In animals given [^3H]thymidine at 22 d of age both stromal replication and fat cell filling were low. Again, this may have been due to the trauma of weaning, although at 28 d of age the stromal uptake of the label was still low and there was

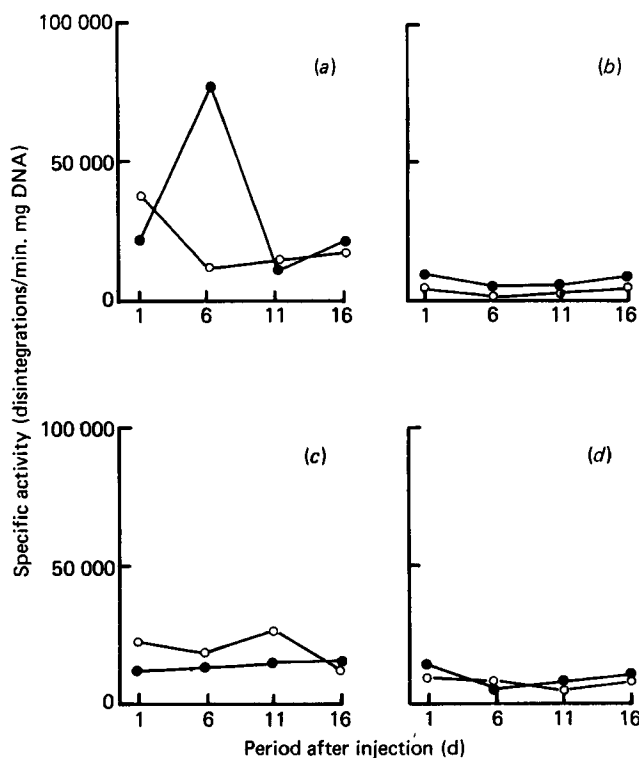


Fig. 4. Specific activity (disintegrations/min per mg DNA) of DNA extracted from 'fat cell' and 'stromal cell' fractions of the scapular tissue in well-nourished animals injected with [^3H]thymidine at (a) 15, (b) 22, (c) 28 or (d) 84 d of age and killed 1, 6, 11 or 16 d after injection. (●), 'Fat cell' fraction; (○), 'stromal cell' fraction.

very little filling of cells and transfer of the label into the mature 'fat cell' compartment. At 84 d of age there was very little uptake of the label by either fraction, again indicating low cell replication and filling. Thus at the scapular site the results suggest that replication had already started to decline by 15 d of age in the well-nourished animals, and had practically ceased by 22 or 28 d of age.

Fig. 5 shows the specific activity of DNA in the 'fat cell' and 'stromal cell' fractions at the scapular site of the undernourished and rehabilitating animals. The specific activity values during undernutrition at this site were similar to those at the abdominal site in undernourished animals. The specific activity of the 'stromal cell' fraction was low throughout, indicating that replication had practically ceased throughout undernutrition. Some specific activity values for the 'fat cell' fraction were relatively high, as in the abdominal tissue. Again, it seemed likely that this was the result of a small amount of label 'swamping' a very small pool of mature fat cells. On rehabilitation the 'stromal cell' fraction at the scapular site showed an increase in specific activity to a value higher than that in well-nourished animals at 15 d of age. This indicates resumption of replication during rehabilitation, although there was not such a large difference in specific activity as seen in the abdominal site between undernourished and rehabilitating animals. The labelling pattern of 'fat cell' DNA at the scapular site of the rehabilitating animals was similar to that in the well-nourished animals given [^3H]thymidine at 15 d of age; a gradual increase in specific activity indicating a slower filling of the newly formed cells than at the abdominal site.

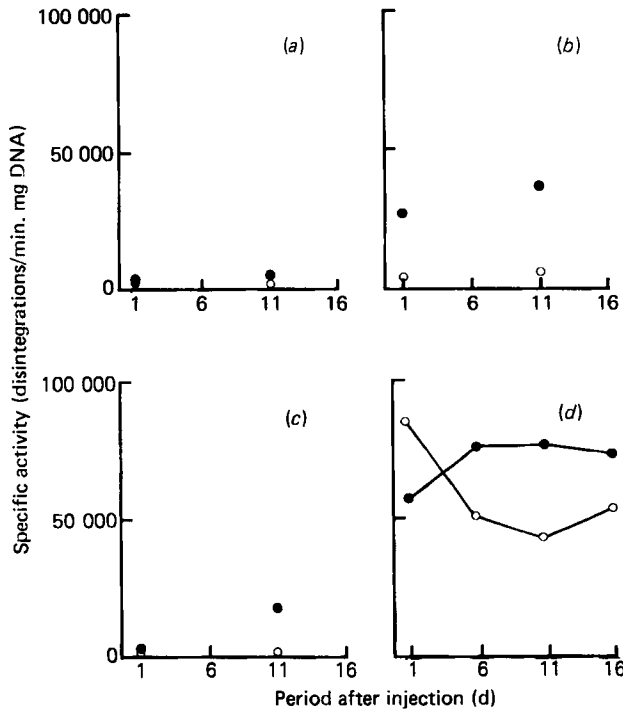


Fig. 5. Specific activity (disintegrations/min per mg DNA) of DNA extracted from 'fat cell' and 'stromal cell' fractions of the scapular tissue in undernourished animals injected with [^3H]thymidine at (a) 22, (b) 28 or (c) 84 d of age and killed 1 or 11 d after injection, and rehabilitating animals injected with [^3H]thymidine at (d) 91 d of age and killed 1, 6, 11 or 16 d after injection. (●), 'Fat cell' fraction; (○), 'stromal cell' fraction.

Perirenal fat

Fig. 6 shows the specific activity of the DNA in the 'fat cell' and 'stromal cell' fractions at the perirenal site of the well-nourished animals given [^3H]thymidine at 84 d of age, and in the rehabilitating animals. The results suggest that cell replication and filling were slow in the well-nourished animals. In the rehabilitating rats the rate of replication and filling was higher than in well-nourished animals of the same age, but still lower than the rates in the two subcutaneous adipose sites during rehabilitation.

Epididymal fat

Fig. 7 shows the specific activity of DNA in the 'fat cell' and 'stroma cell' fractions at the epididymal site of well-nourished animals given [^3H]thymidine at 84 d of age, and in the rehabilitating animals. Stromal replication and filling were slow in the well-nourished animals at 84 d. The specific activity of both 'stromal cell' and 'fat cell' DNA were of a similar order to those found at other sites at the same age. There appeared to be some acceleration of replication in the 'stromal cell' fraction during rehabilitation, although it did not reach the rate shown at the abdominal site.

DISCUSSION

The major results can be summarized as follows.

(1) In well-nourished animals fat cell replication had largely ceased by 12 weeks of age in the subcutaneous depots. There were differences between the various sites of adipose

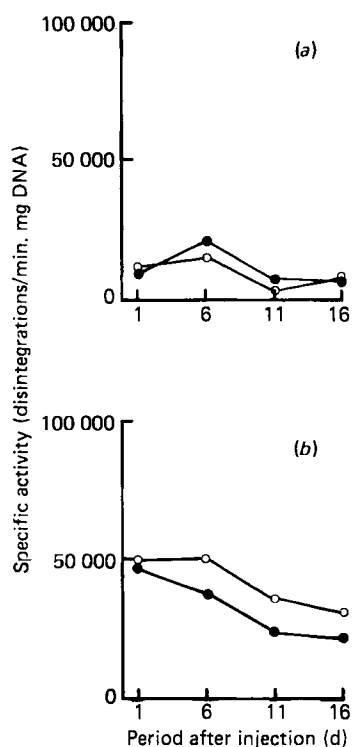


Fig. 6. Specific activity (disintegrations/min per mg DNA) of DNA extracted from 'fat cell' and 'stromal cell' fractions of the perirenal tissue in well-nourished animals injected with [^3H]thymidine at (a) 84 d of age and killed 1, 6, 11 or 16 d after injection, and rehabilitating animals injected with [^3H]thymidine at (b) 91 d of age and killed 1, 6, 11 or 16 d after injection. (●), 'Fat cell' fraction; (○), 'stromal cell' fraction.

tissue regarding the period of hyperplastic growth; its timing or rate of replication or both.

(2) In the undernourished animals replication was slow in the subcutaneous depots compared with well-nourished animals of the same age. Rehabilitation from undernutrition at 12 weeks of age stimulated replication, which resulted in higher rates in all four depots examined compared with well-nourished animals.

Greenwood & Hirsch (1974) showed that the hyperplastic growth phase of epididymal adipose tissue occurred in the early life of the Sprague-Dawley rat, the majority being complete by 5 weeks of age. The present work extends these findings in another strain and shows that fat cell replication is complete in the Black and White Hooded rat before 12 weeks of age in the scapular and abdominal sites and probably in the perirenal depot as well. These findings need confirmation, especially regarding the perirenal fat, as Lemmonier (1972) suggested that hyperplasia occurs in this depot in older animals. Also it has been found recently (J. Kirtland & D. E. Pavey, unpublished results) that the perirenal fat is unusual in being particularly sensitive to dietary composition and to a slimming regimen compared with other sites studied. Although replication occurred in all adipose depots during the early life of the rat, the results suggest that differences exist regarding the precise timing of hyperplasia for different sites as proposed elsewhere (Kirtland & Gurr, 1979). The rate of fat cell replication in the abdominal site was faster than in the scapular site. This could indicate that scapular adipose tissue develops earlier than abdominal tissue,

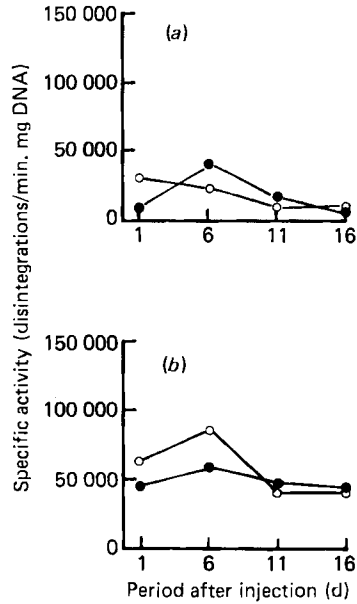


Fig. 7. Specific activity (disintegrations/min per mg DNA) of DNA extracted from 'fat cell' and 'stromal cell' fractions of the epididymal tissue in well-nourished animals injected with [3 H]thymidine at (a) 84 d of age and killed 1, 6, 11 or 16 d after injection, and rehabilitating animals injected with [3 H]thymidine at (b) 91 days of age and killed 1, 6, 11 or 16 d after injection. (●), 'Fat cell' fraction; (○), 'stromal cell' fraction.

which would be in keeping with its role in the production of heat in the newborn rat. However, it is not possible to exclude an alternative interpretation that various adipose sites differ in the rate of replication of fat cells, whilst the total length and timing of the hyperplastic period are similar for all sites. Thus the development of abdominal adipose tissue may occur in a burst of proliferation, whereas that of scapular tissue occurs at a slower rate. Similarly, differences may exist between sites in rate of filling of fat cells. In the abdominal site filling occurred more rapidly than in the scapular site.

The results show that fat cell replication was impaired not only by chronic undernutrition from birth to 12 weeks of age, but also by undernutrition at the time of weaning. The effect of weaning was more marked in the abdominal depot than the scapular fat. This is consistent with the suggestion that abdominal tissue develops later than scapular tissue and therefore may be more vulnerable to undernutrition. The result of increased food intake after weaning, or rehabilitation at 12 weeks of age, was an increased rate of replication at all sites examined. The response was most marked at the abdominal site. Such differences between sites in terms of the timing and susceptibility of replication to nutrition mean that extrapolations made from observations on a single site should be viewed with caution.

The present findings strongly suggest that the period of undernutrition (birth until 12 weeks of age) used in the previous study (Harris, 1980) corresponded to the main phase of fat cell replication at all sites of adipose tissue deposition studied. It seems unlikely that a significant proportion of fat cell synthesis occurred before birth, as Harris (1980) found that the amount of DNA present in various adipose depots of 3-week-old rats was only sufficient to account for the final adult number of observable fat cells if no other types of cells were present. The findings also indicate that the severity of undernutrition used in this and the previous experiment (Harris, 1980) was sufficient to impair fat cell replication significantly.

Further, the present experiment substantiates the suggestion offered (Harris, 1980) to explain why undernutrition failed to alter the number of observable fat cells permanently; rehabilitation resulted in a marked acceleration in replication resulting in a 'catch-up' in cell number. This finding that replication is restarted or 'turned on' at an age when it would normally have ceased contrasts with the work of Winick & Noble (1966), who reported that undernutrition from birth to 3 weeks of age had a permanent effect on the number of cells in all organs examined, although these workers did not include adipose tissue in their study. One interpretation of the findings is that there is no finite period of hyperplasia of adipose tissue, which would therefore be classified wrongly with the non-regenerative organs. This would mean that replication could be initiated at any time in life, not only by a sudden excess of food, but by other circumstances, such as 'times of physiological deposition' of fat as suggested by Brook (1975). This could explain the instances of hyperplastic adult-onset obesity reported by Hirsch & Batchelor (1976), and the findings of Jung *et al.* (1978) that the number of observable fat cells in the body is related to the severity of obesity and not to the age of onset. Alternatively, there may be a finite period of hyperplasia for adipose tissue which is normally confined to the early life of the rat, but with an extent of flexibility in the precise length of the period. The possibility that undernutrition might lengthen, and overnutrition shorten, the hyperplastic period for adipose tissue is discussed in detail elsewhere (Kirtland & Gurr, 1979). Further studies are required using tritiated thymidine to see whether a sudden excess of food in adult animals stimulates fat cell replication and hence whether or not the concept of a finite period of hyperplasia in adipose tissue is tenable.

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