

CYTOLOGICAL RESPONSES OF BROWN FAT TISSUE IN COLD-EXPOSED RATS

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ABSTRACT

In young adult laboratory rats exposed to cold (6°C) the brown adipose tissue undergoes time-dependent increases in cellularity, vascular supply, and total mass. These changes are largely complete after 16 days in the cold and concurrent generally with the development of a thermoregulatory state not greatly dependent upon shivering. Histologically the brown fat changes from a tissue having both unilocular and multilocular fat cell types to one having almost exclusively the latter. During the first 6 to 12 hours in cold, the multilocular cells lose their lipid vacuoles and decrease in size, but these features are restored to normal by 24 hours. Cell proliferation, as estimated by the DNA synthetic index method (using tritiated thymidine autoradiography), appears in the reticuloendothelial cells of the brown fat at 1 day of cold exposure, becomes maximal at 4 days, and returns to the control level by 16 days. In animals injected with tritiated thymidine on the 3rd day of cold exposure and then maintained for 1 or more additional days in the cold, autoradiographs indicate that new brown fat (multilocular) cells arise by cytogenesis from reticuloendothelial progenitor cells and not by proliferation of existing brown fat cells. Throughout this and subsequent periods, cells of the epididymal white adipose tissue slowly decrease in size. Because a thermogenic role in cold acclimation has been established for the brown fat, the reported changes are regarded as adaptive responses to a cold environment.

INTRODUCTION

The physiological significance of brown adipose tissue has remained relatively obscure (8) until its recent emergence as a thermogenic effector system in cold-exposed rats (15-17, 20) and in hibernating mammals during arousal (14, 19). In view of findings with rats during cold acclimation that both mass (11, 15, 21) and respiration (15-17, 21) of brown adipose tissue from all of the various sites appeared to increase, two main questions arose: first, does this increase in mass represent an increase in cell number, and if so, secondly, does the cytogenesis entailed simply represent mitotic activity of brown fat cells or a process involving

cellular differentiations? Finally, it was surmised from earlier work (9, 11, 13) that during the response to cold there should appear changes in the relative amount of white adipose tissue associated with the brown adipose tissue; hence an attempt was made to assess this effect on the nature and time-rates of change in the cytologic patterns of response to cold.

METHODS

Young adult male rats of the Long-Evans strain were maintained in individual cages under constant conditions of lighting (12-hour light cycle, with 15 watt

night light). Food and water were freely given. A group of animals taken from 4 litters was maintained at 26°C until the start of the experiment at 103 days of age. The group was then divided into a control group (26°C) of 4 animals and an experimental group scheduled for cold exposures (6°C) of from 6 hours to 16 days. Other conditions were comparable between experimental and control groups. Additional rats were used for longer exposure times.

One hour prior to killing, each rat was injected intraperitoneally with tritiated thymidine (specific activity, 3 c/mmole) at a dose level of 1 μ c/gm of body weight (*cf.* references 2, 3). In another experiment on the cytogenesis of brown fat cells, two rats were exposed to cold for 72 hours, injected with labeled thymidine, and then killed after another 24 and 96 hours.

All animals were killed at 0830 to 0900 hours in the

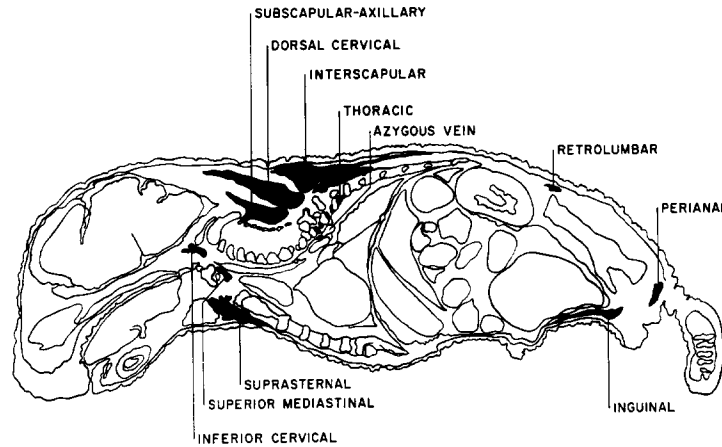


FIGURE 1 Drawing made from a projected slide, showing the anatomical distribution of brown fat from a parasagittal section of newborn mouse. $\times 3.5$.

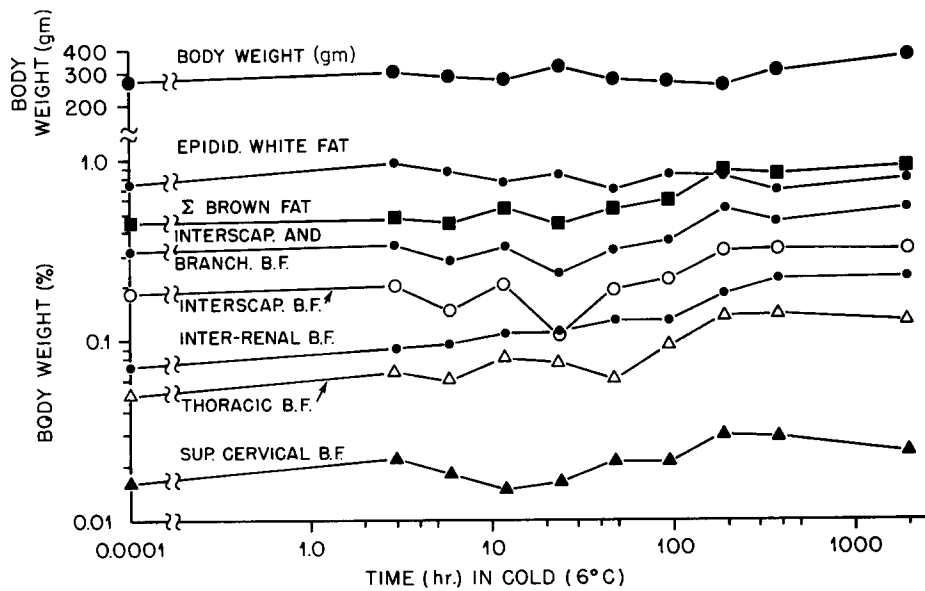


FIGURE 2 Change in the proportional mass of brown fat (*B.F.*) (expressed as the per cent of body weight) from different body sites as a function of time that rats were cold-exposed. Each point is the average of 4 rats. The proportional mass of the epididymal fat pad and the gross body weight are also shown.

room of residence by a high cervical blow followed by decapitation. Tissues were immediately removed from the body and fixed for 3 days in Bouin-Hollande solution. Brown fat was removed from the following regions: interscapular, superior cervical, axillary and subscapular, intrathoracic (around the azygous vein and thymus), interrenal, and periadrenal. Skin was taken from the back over the region of the inter-

Another group of 40 young adult rats was used to determine the weight changes of epididymal white fat pad and also the brown fat from various body sites. In this series, 4 animals were killed at each of the above time intervals of cold-exposure, and the respective tissue excised and immediately weighed. Additionally, a series of newborn mice and rats was fixed and sectioned for study.

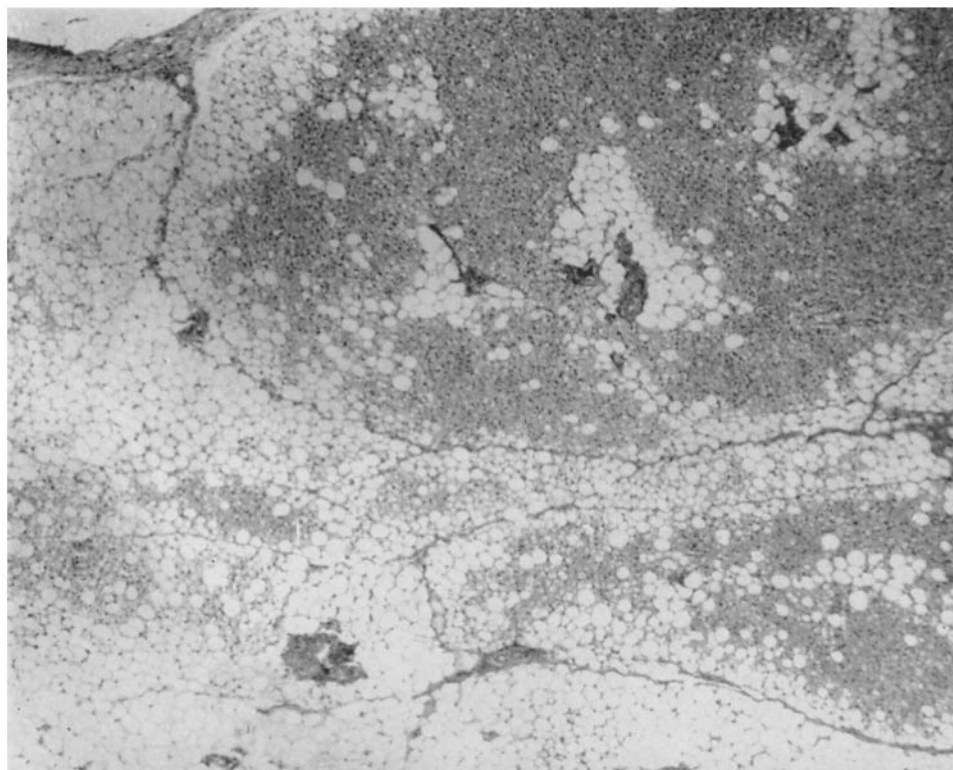


FIGURE 3 Histological section of the interscapular brown fat gland taken from a young adult rat maintained at 27°C. The lobules are separated by connective tissue trabeculae, and both the multi- and unilobular, as well as transitional fat cell types, are apparent. Masson's trichrome stain. $\times 100$.

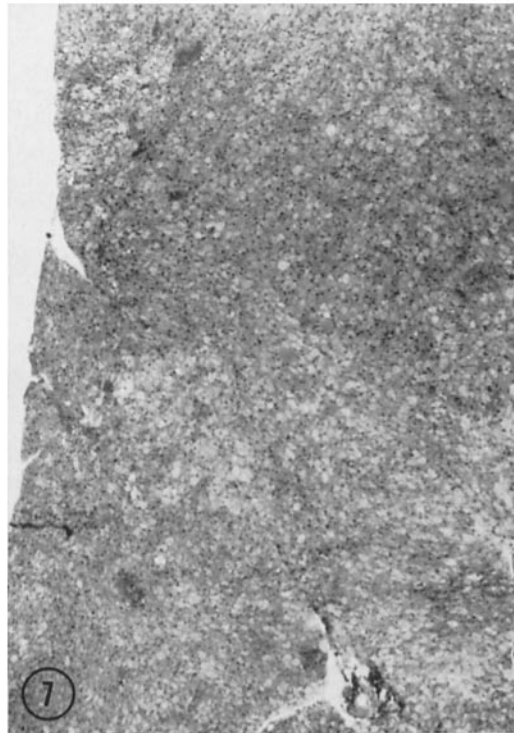
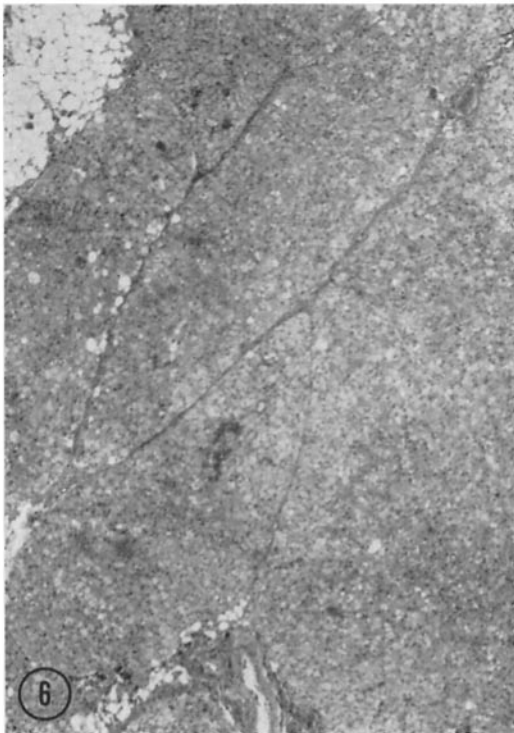
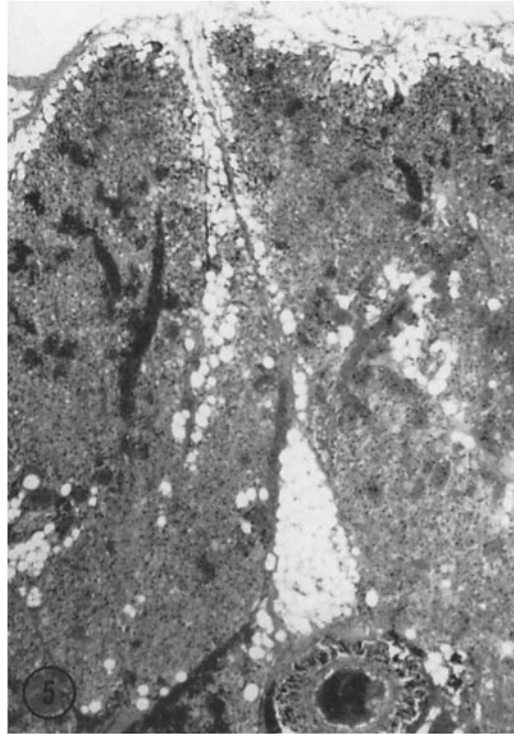
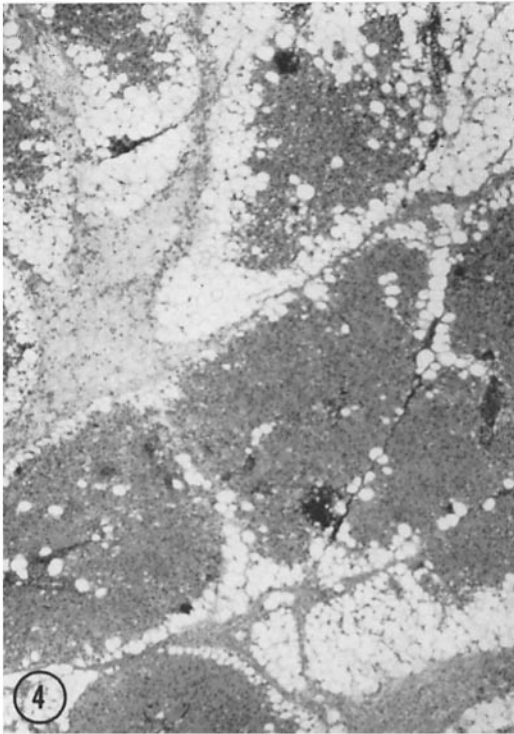
scapular gland. The epididymal fat pad was also removed and similarly fixed.

The tissues were processed for histology by double embedding in nitrocellulose-paraffin. Sections $5\ \mu$ thick were stained with periodic acid-Schiff reaction (PAS), hematoxylin and eosin, Masson's trichrome method, and thionin. Another set of PAS-prestained material was autoradiographed by the coating technique (10), exposed for 3 weeks, developed, and then stained with hematoxylin. In appropriate material, cell size was measured by planimetry of uniformly projected photomicrographs and the incidence of H^3 -labeled DNA counted directly in randomly selected microscopic fields.

RESULTS

Mass Changes and Anatomical Distribution

Brown fat is distributed in a number of different regions in the young adult rat, as in many other mammals (*cf.* references 1, 4, 12), and it is especially prominent in the neonatal stages. No differences were found in the anatomical distribution of brown fat between rats and mice. A parasagittal section of a newborn mouse is shown in Fig. 1 to illustrate the extent and topography of brown fat in neonatal mice and rats. Notably, the



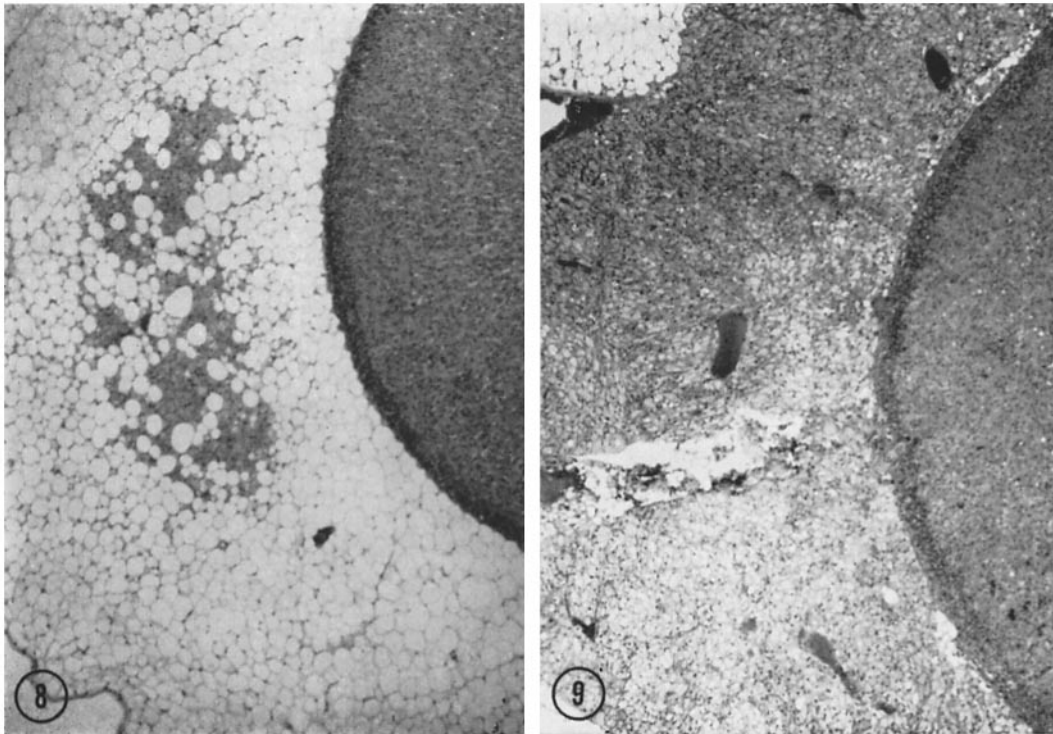


FIGURE 8 Section from control rat, showing adrenal region with associated adipose tissue. Only a small number of multilocular (brown fat) cells appear among the unilocular cells Masson's trichrome stain. $\times 100$.

FIGURE 9 Section from 16-day cold-exposed rat; same periadrenal area as in Fig. 8. Note the increase in proportion of multilocular fat cells. Masson's trichrome stain. $\times 100$.

volume of brown fat in the newborn is relatively greater than in the adult, although the topologic distribution is essentially similar in both. In the neonatal stage, however, this volume and distribu-

tion may take on a particular significance in thermoregulation, as has been suggested elsewhere (17, 18).

In all regions the brown fat tissue is found

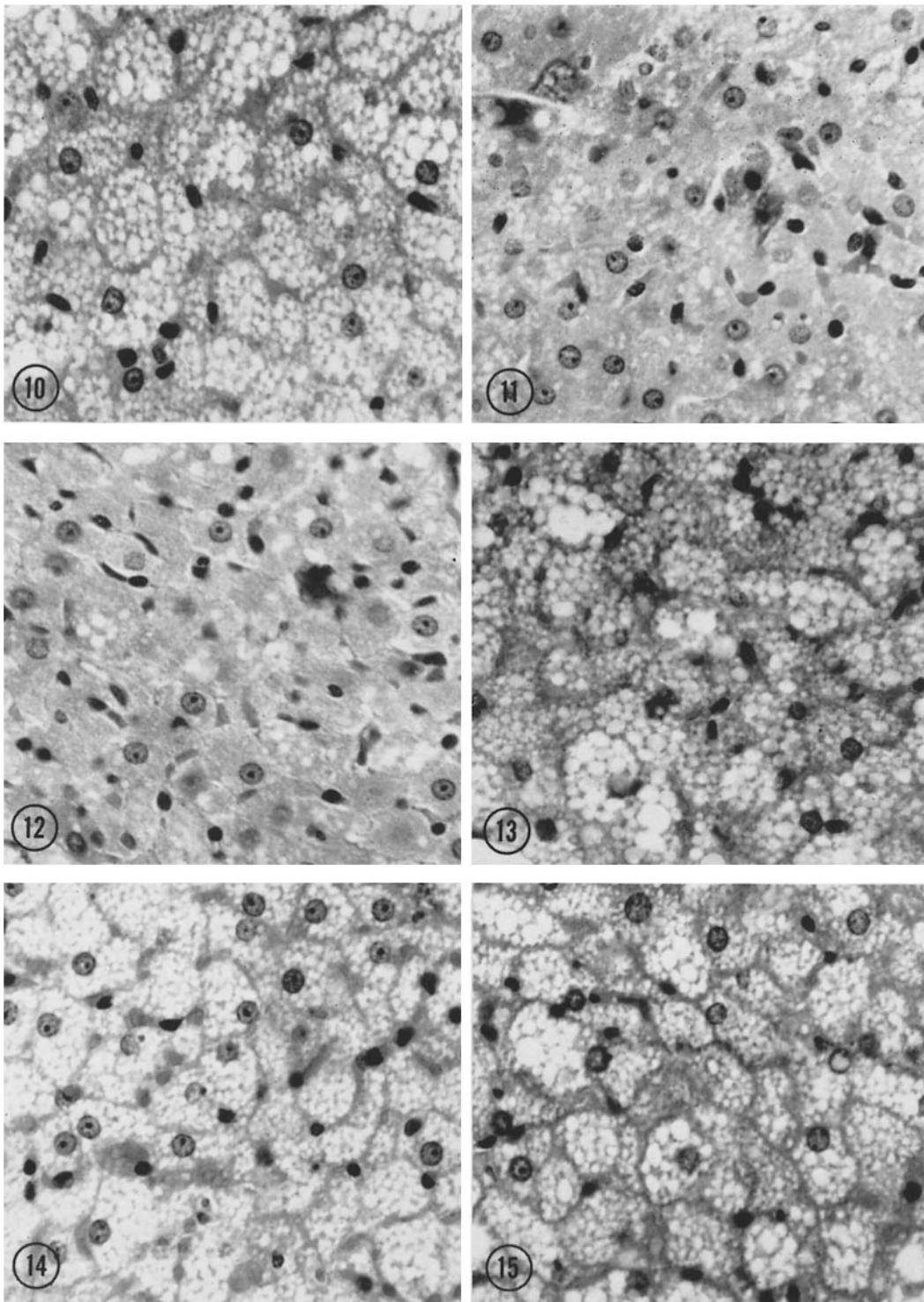
Figs. 4 to 7 show interscapular brown fat gland from a series of rats cold-exposed (6°C) for various times. All Masson's trichrome stain. $\times 100$.

FIGURE 4 From animal exposed 12 hours to cold. The transitional cell forms between multi- and unilocular types are here much less apparent than in control animals.

FIGURE 5 From animal exposed 48 hours to cold; the unilocular (white fat) cells have decreased considerably. The dark areas indicate vascular development and corresponding engorgement of the tissue with blood at this time.

FIGURE 6 From animal exposed 8 days to cold; the unilocular cells have nearly disappeared and the gland is composed almost entirely of multilocular (brown fat) cells.

FIGURE 7 From a 60-day cold-exposed animal; the unilocular fat cells are absent and only the multilocular (brown fat) cell type persists.



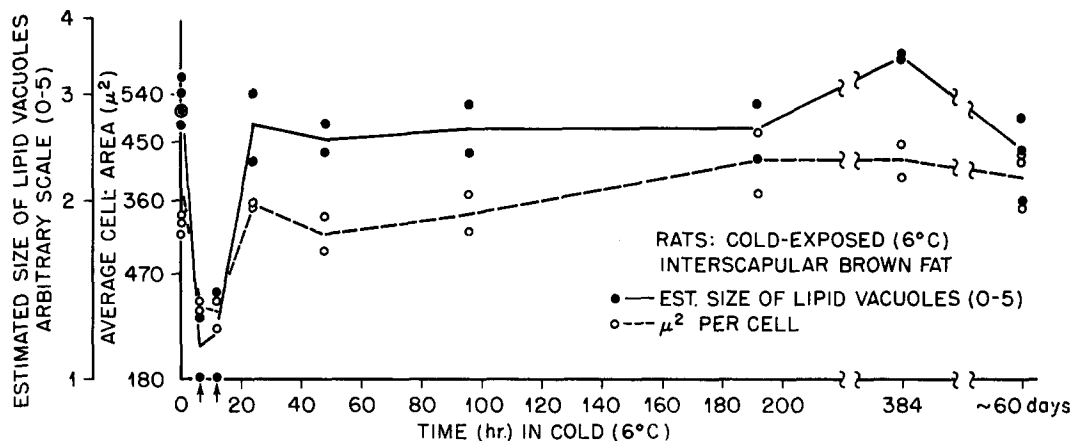


FIGURE 16 Changes in size of lipid vacuoles (based on an arbitrary scale from 0 to 5) and areas of multilocular brown fat cells as a function of time in cold. Each point on the graph represents one animal.

closely associated with major blood vascular channels and is itself highly vascularized (*cf.* references 4-7, 17, 22).

The per cent body weight of brown fat in the major sites in the adult rat during cold exposure (Fig. 2) shows significant increases by the end of 192 hours in the cold, with the maximal growth rate having appeared uniformly, in all sites, between this and the preceding 96 hour time point (see below). Concurrently the pattern of per cent change in the epididymal white adipose is generally toward the negative.

Histological Findings

Although most of the quantitative estimates of cell parameters were made on the interscapular gland, an examination of the other areas of brown fat indicated that all of these areas were responding similarly during the exposure to cold. In a typical histological section from an animal maintained at 26°C (Fig. 3), the brown fat gland contains two types of fat cells, one with many lipid

vacuoles (multilocular), the other with a single large lipid vacuole (unilocular). The latter cells were indistinguishable from the typical unilocular fat cell seen normally in the white adipose depots elsewhere; *e.g.*, in epididymal fat. In the interscapular pads the trabeculae which separate each lobule of the brown fat are heavily flanked by white fat (unilocular) cells, which also invade the lobular areas and appear as islands among the multilocular brown fat cells (Fig. 3). Appearing in the region between the trabecular and central lobular portions of the gland are possibly transitional fat cells with two or three lipid vacuoles per cell. When the animals were exposed to 6°C for 12 hours (Fig. 4), these transitional cells disappeared and a clear distinction could be drawn between brown fat cells and the unilocular cells. At this stage the unilocular cells appear as normal white fat cells except for an apparent diminution in size, but after 24 to 48 hours of the cold treatment (Fig. 5) most of them have disappeared both along the trabeculae and within the lobules. Also,

FIGURES 10 to 15 Cytological changes taking place in the brown fat cells during cold exposure. All tissues are from the interscapular gland. Masson's trichrome stain. $\times 750$.

Figs. 10 to 15 are from animals subjected to various times in the cold: Fig. 10 (0 hour) shows the typical appearance of brown fat from a rat maintained at 26°C; Figs. 11 and 12 (12 hours) show a diminution in size and quantity of lipid vacuoles within the cells; note also the corresponding decrease in cell size. Fig. 13 (24 hours) marks the reappearance of normal size lipid vacuoles and normal cellular dimensions. These parameters are unchanged during further exposure to cold, *e.g.*, Fig. 14 (48 hours) and Fig. 15 (60 days).

the gland is characteristically engorged with blood at this time (Fig. 5).

This use of the unilocular white fat cells within the gland evidently continues, so that in rats exposed 4 and 8 days in the cold (Fig. 6) these cells have almost disappeared from both trabecular and intralobular regions. Thus in the glands seen after cold exposures of the rats for 16 and 60 days the transformation appears completely established (Fig. 7). A similar sequence in response to cold is observed in brown fat elsewhere in the body; thus, in sections of brown fat from the periadrenal region, the tissue of the control animal (Fig. 8) shows an histological appearance similar to that

turned essentially to normal values where it remained throughout all of the periods of more prolonged cold exposure (*cf.* Figs. 11 to 16).

In the same series, the cell area decreased from a mean of $360 \mu^2$ in the controls to a mean of $234 \mu^2$ at both 6 and 12 hour exposures; but with 24 hour cold exposure, mean cell size was restored to a value of $351 \mu^2$. The coincidence of this change with that of the lipid vacuoles suggests that cell size varies with lipid vacuolar size, and it is true that for each animal the coefficient of correlation between these two estimates was always positive, but not significantly so in most cases. More impressive is the coincidence of reduced cell size

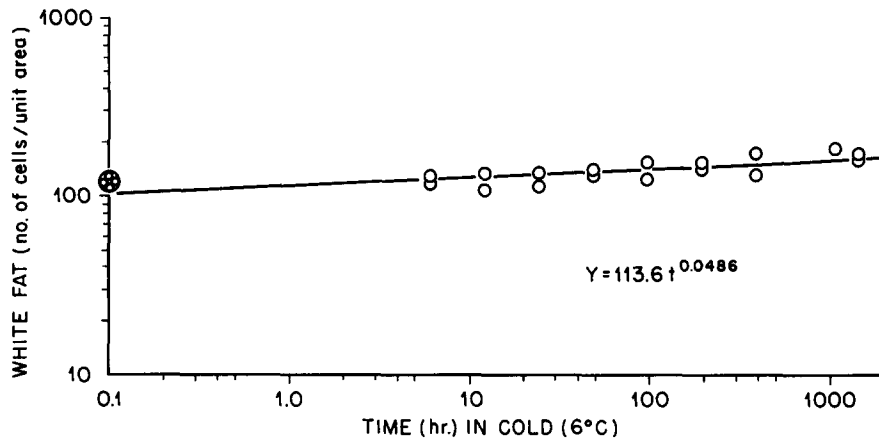


FIGURE 17 Graph showing relationship between the log of the number of epididymal white fat cells per unit area and the log of treatment time (18 observations). The last three values on the right of the graph are not included in the regression analysis.

of the interscapular gland, while in the animal exposed to cold for 16 days (Fig. 9) there is an obvious increase in the ratio of brown to white fat cells.

Cytological Findings

In the normal animal maintained at 26°C , the brown fat cells are typically multilocular, showing numerous lipid granules in the cytoplasm and a centrally placed nucleus (Fig. 10). For these cells, the size of the lipid vacuoles was estimated, on an arbitrary scale of 0 to 5, where each arbitrary unit is equivalent to a vacuole diameter of about 0.75μ , to give a mean diameter of 3, (Fig. 16). In animals exposed to cold for 6 hours, this diameter had decreased to 1.2 and remained so through the 12 hour period. However, by the 24 hour period, the size of the lipid vacuole had re-

turned essentially to normal values where it remained throughout all of the periods of more prolonged cold exposure (6 and 12 hours) and its subsequent restitution within 24 hours to a quasi-permanent state of normality (Figs. 14 to 16).

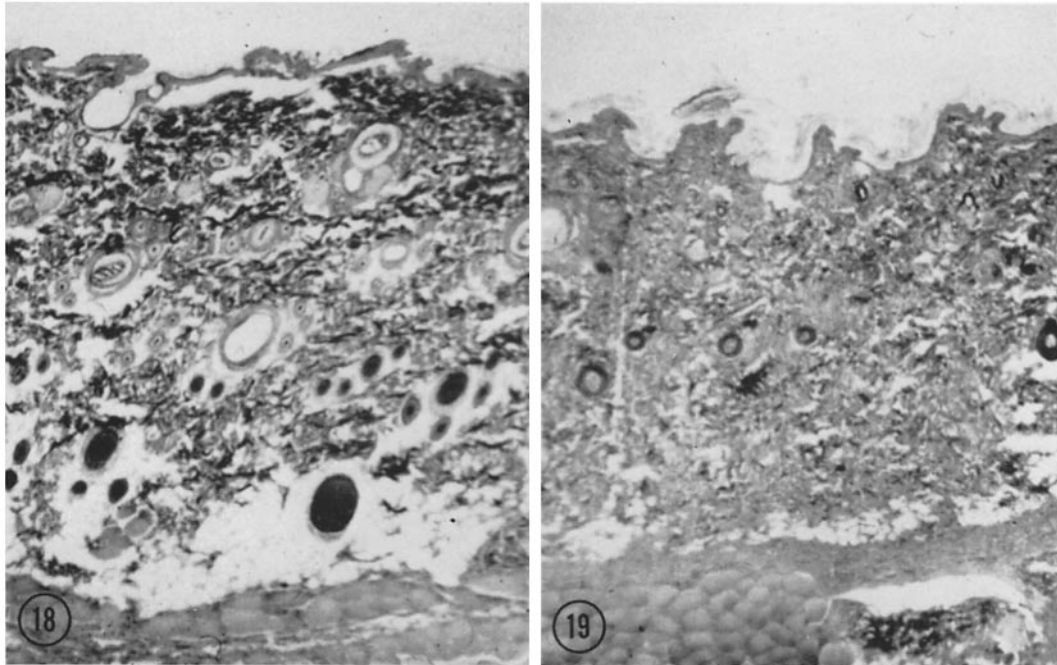
In epididymal depot fat from this series it was found that white adipose cells were also decreasing in size as a function of time in cold, as determined from counts of cells/unit area in standard projections of the sections (Fig. 17). Planimetric measurements confirmed this conclusion; the mean area of the epididymal white fat cell was $644 \mu^2$ for control animals as compared with $432 \mu^2$ in rats exposed to cold for 8 days. Examination of sections from the mid-dorsal skin of these animals suggests that the subcutaneous fat layer attenuates appreciably during the first 16 days of cold exposure (Figs. 18, 19) but may regain this fat with more

prolonged exposure. Judging by the slow rate of utilization appearing concurrently in the epididymal fat, this may represent some displacement of depot fat toward the periphery during prolonged cold.

Cell Proliferation and Differentiation in Brown Fat Tissue

Normally, cells involved in synthesis of deoxyribonucleic acid (DNA) will divide shortly after

in the autoradiographs of sections stained with PAS-hematoxylin in which one can easily distinguish the labeled endothelial cells within the capillary bed (Figs. 20 and 21). A plot of the number of such labeled cells from the tissue of rats exposed to cold for different times gives a clear indication that proliferative activity of the reticuloendothelial cells in this tissue begins within 24 hours and passes through a maximum at about 96 hours (Fig. 24). Thereafter the rate decays to

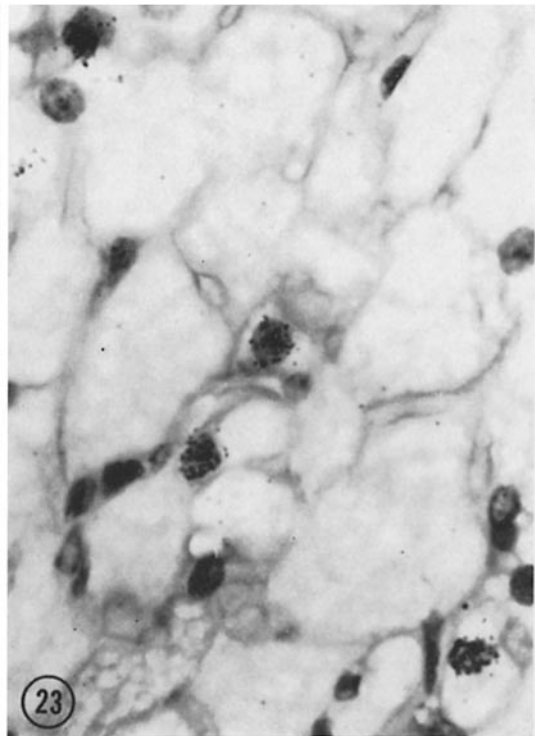
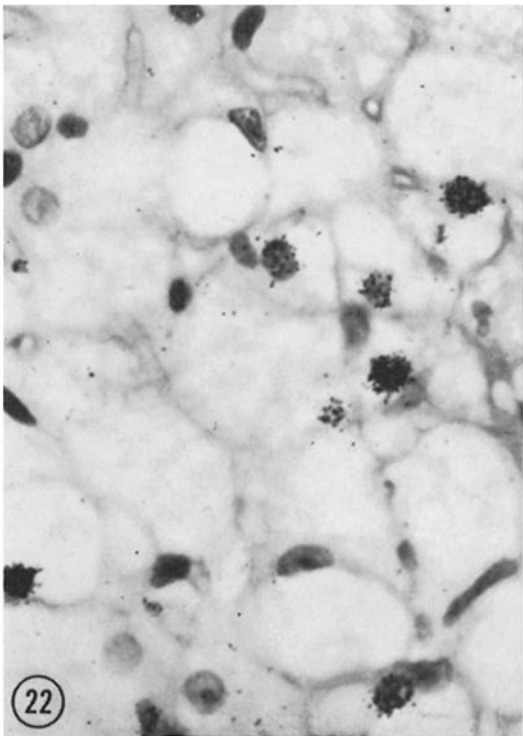
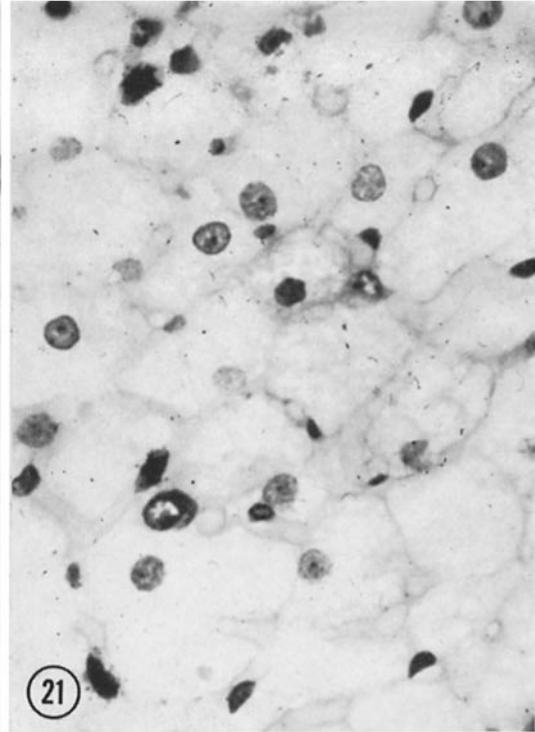
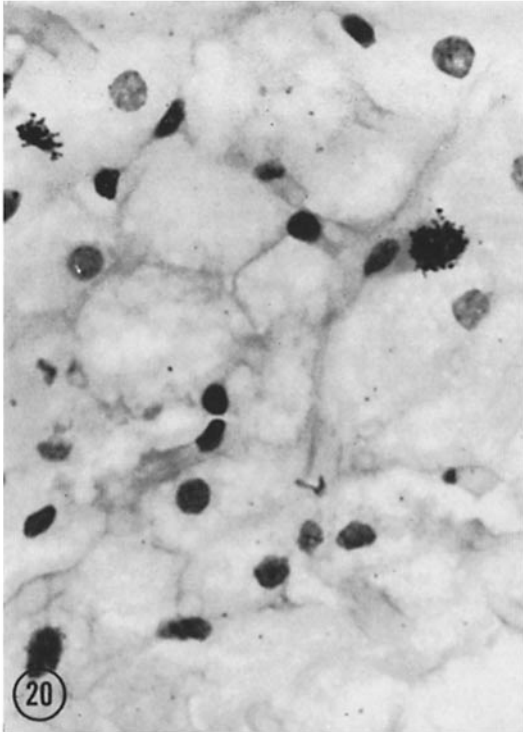


FIGURES 18 and 19 Skin from the mid-dorsal region of a control (Fig. 18) and a 16-day cold-exposed animal (Fig. 19). Masson's trichrome stain. Note the greater abundance of fat in the subcutaneous layer of control animal. $\times 100$.

replication of the required DNA complement; thus autoradiographic analysis of the number of cells involved in DNA synthesis shortly after injection of tritiated thymidine (1 hour) affords a measure of the proliferative activity of the cells within any given tissue region (2, 3). We never saw mitosis of a brown fat cell. However, inspection of autoradiographs from animals given H^3 -thymidine 1 hour before killing revealed that the DNA synthesis evidently occurs in the reticuloendothelial cells lying within the expanding vascular spaces of the tissue but never in brown fat cells (Figs. 20 and 21). This is especially apparent

reach control levels by the 8th to 16th day of cold exposure.

Whether brown fat cells derive from reticuloendothelial precursors was tested as follows: Two rats were exposed to cold for 72 hours and then given the usual injection of tritiated thymidine but kept in the cold and not killed until 24 and 96 hours later, respectively. Under these conditions the autoradiographs showed labeling of brown fat cells (Figs. 22 and 23). From these data and the apparent absence of any mitotic figures in brown fat cells, we conclude that the latter are normally derived from reticuloendothelial progenitors.



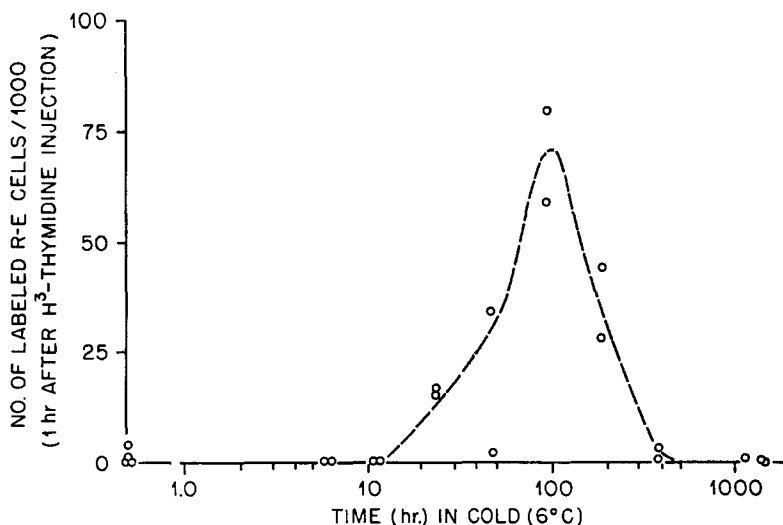


FIGURE 24 Graph of the number of labeled reticuloendothelial cells (*R-E*) per 1000 cells in tritiated thymidine autoradiograph of the brown fat gland plotted against the log of time that the individual rats were cold exposed.

CONCLUSIONS

Earlier work in this laboratory (15, 17, 21) has shown that during cold acclimation the metabolic heat production of brown adipose tissue in the rat is probably increased by a factor of 6 or more owing to its increase both in tissue weight and in metabolism *in vitro*. When the animal is exposed to cold the immediate mobilization and utilization of lipid during this period evidently involves the transient dissolution of the multilocular vacuoles and the concurrent reduction in average cell diameter.

Reconstitution of the multilocular cells coincides with local and general dissolution of white adipose tissue and redistribution of its products. A maximal rate of DNA synthesis in the reticuloendothelial cells at 96 hours in the cold precedes the proliferative activity by about 7 to 9 hours (*cf.* references 2, 3). Thus one can see that cold acclimation is accompanied by hyperplasia of

brown fat tissue which develops maximally during the time period 96 to 192 hours.

The adaptive significance of these changes in the cold is suggested by the coincidence of this hyperplastic activity with the maturation of a new thermogenic steady state, in which shivering no longer represents a major factor in total heat production.

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FIGURES 20 to 23 Autoradiographs from animals that were injected with tritiated thymidine. All sections PAS-hematoxylin stained. Figs. 20 and 21 are from a 96-hour cold-exposed animal injected with isotope one hour prior to killing. Note that labeling appears only over reticuloendothelial cells. Figs. 22 and 23 show sections from the animals cold-exposed for 72 hours but injected with tritiated thymidine 24 hours prior to killing. One now sees labeling over both brown fat and reticuloendothelial cells. $\times 900$.

REFERENCES

1. AUERBACH, M., Das braune Fettgewebe bei schweizerischen und deutschen Nagen und Insectivoren, *Arch. mikr. Anat.*, 1902, **60**, 291.
2. CAMERON, I. L., and GREULICH, R. C., Evidence for an essentially constant duration of DNA synthesis in renewing epithelia of the adult mouse, *J. Cell Biol.*, 1963, **18**, 31.
3. CAMERON, I. L., Is the duration of DNA synthesis in somatic cells of mammals and birds a constant? *J. Cell Biol.*, 1964, **20**, 185.
4. CONINX-GIRARDET, B., Beiträge zur Kenntnis Innersekretorischer Organe des Murmeltiers (*Arctomys Marmota* L.) und Ihrer Beziehungen zum Problem des Winterschlafes, *Acta Zool.*, (*Stockholm*), 1927, **8**, 161.
5. CRAMER, W., On glandular adipose tissue, and its relation to other endocrine glands and to the vitamin problem, *Brit. J. Exp. Path.*, 1920, **1**, 184.
6. FAWCETT, D. W., Adipose tissue, in *Histology*, (R. O. Greep, editor), Philadelphia, The Blakiston Co., 1954, 113.
7. HAMMAR, J. A., Zur Kenntniss des Fettgewebes, *Arch. mikr. Anat.*, 1895, **45**, 512.
8. JOHANSSON, B., Brown fat: A review, *Metabolism*, 1959, **8**, 221.
9. LACHANCE, J. P., and PAGÉ, E., Hormonal factors influencing fat deposition in the interscapular brown adipose tissue of the white rat, *Endocrinology*, 1953, **52**, 57.
10. MESSIER, B., and LE BLOND, C. P., Preparation of coated radioautographs by dipping sections in fluid emulsion, *Proc. Soc. Exp. Biol. and Med.*, 1957, **96**, 7.
11. PAGÉ, E., and BABINEAU, L. M., The effect of cold environment on the hibernating gland of the rat, *Rev. Cancer Biol.*, 1950, **9**, 202.
12. RASMUSSEN, A. T., The so-called hibernating gland, *J. Morphol.*, 1924, **38**, 147.
13. SELYE, H., and TIMIRAS, P. S., Participation of "brown fat" tissue in the alarm reaction, *Nature*, 1949, **164**, 745.
14. SMALLEY, R., and DRYER, R., Brown Fat: thermogenic effect during arousal from hibernation in the bat, *Science*, 1963, **140**, 1333.
15. SMITH, R. E., Thermogenic activity of the hibernating gland in the cold-acclimatized rat, *The Physiologist*, 1961, **4**, 113, abstract.
16. SMITH, R. E., Thermoregulation by brown adipose tissue in cold, *Fed. Proc.*, 1962, **21**, 221.
17. SMITH, R. E., Proceedings of the Second International Symposium on Natural Mammalian Hibernation, Helsinki, in press.
18. SMITH, R. E., Discussion, *Fed. Proc.*, 1963, **22**, 741.
19. SMITH, R. E., and HOCK, R. J., Brown Fat: thermogenic effector of arousal in the hibernator, *Science*, 1963, **14**, 199.
20. SMITH, R. E., and HOIJER, D. J., Metabolism and cellular function in cold acclimation, *Physiol. Rev.*, 1962, **32**, 60.
21. SMITH, R. E., and ROBERTS, J. C., Thermogenesis of brown adipose tissues in cold-acclimated rats, *Am. J. Physiol.*, 1964, **206**, 143.
22. VON HANSEMAN, D., Untersuchungen über das Winterschlaforgan, *Arch. Anat. u. Physiol.*, 1902, 160.