

# Total nitrogen and L-hydroxyproline content of adipose tissue from various sites in rats of different ages

P. B. CURTIS-PRIOR, T. HANLEY, J. TRETHERWEY, and G. A. STEWART

The Department of Medicine, Guy's Hospital,  
London, England; and the Bio-assay Laboratories,  
The Wellcome Foundation, Ltd., London, England

**ABSTRACT** The total nitrogen and L-hydroxyproline concentrations of two intra-abdominal and two subcutaneous sites of adipose tissue were measured in male and female Wistar rats.

At 100 g body weight the nitrogen concentration of intra-abdominal adipose tissue was slightly lower than that of subcutaneous adipose tissue. As age advanced beyond sexual maturity, the nitrogen content of intra-abdominal adipose tissue was steadily reduced, whereas that of subcutaneous tissue did not change.

The L-hydroxyproline concentration of intra-abdominal adipose tissue was also lower than in subcutaneous adipose tissue. In male rats it rose in most adipose tissue sites when sexual maturity was reached, then fell with advancing age. In female rats the pattern of change was less consistent, but the L-hydroxyproline content of intra-abdominal adipose tissue was reduced as age increased.

**SUPPLEMENTARY KEY WORDS**    intra-abdominal  
subcutaneous · age trends

**T**HE RELATIONSHIPS between adipose cell size, cell number, and adipose tissue mass are receiving increasing attention due to their relevance to adipose tissue growth in the normal and obese condition (1–3). DNA concentration has been used as an index of the cellularity of adipose tissue (4), but the validity of this is now questionable since much of the DNA of adipose tissue is present in the collagenous-supporting matrix and not in the adipocytes (3). Tissue nitrogen content has been sug-

gested as an alternative to DNA. However, since stromal collagen makes some contribution to the total tissue nitrogen, we have investigated the extent to which collagen contributes to the total tissue protein of AT from various sites at different times in the life-span of male and female Wistar rats.

## METHODS

### *Animals*

The observations on female rats were made on albino Wistar animals maintained for breeding by the Biological Assay Laboratories of the Wellcome Foundation, Ltd., Dartford, England. Each litter was kept together in a single cage until the animals were weaned at 3 weeks of age. They were then redistributed randomly, 20 to a cage, with other animals of a similar age. The rats were weighed weekly and, on attaining a body weight of about 150 g, were again distributed randomly into cages containing 10–12 animals. All the weaned female animals were fed the Porton Rat and Mouse Diet, obtained from Christopher Hill Ltd., Poole, Dorset, England.

The rats used in the work reported here were taken from the main stock in the following weight groups: 100, 150, 200, and 300 g ( $\pm 10$  g). The facilities for housing animals decreased as their age advanced, hence the smaller number of animals in the older groups.

The observations on male rats were made on a Porton strain of albino Wistar animals, maintained in Guy's Hospital Medical School, London, England, in a way similar to that of the female rats of this experiment. The males were fed the Thompson Rat Cube Diet obtained from Haygate and Son Ltd., Edgbaston, Birmingham, England.

Abbreviation: AT, adipose tissue.

The animals were killed by decapitation, and a piece of adipose tissue was removed from the gonadal, perirenal, groin, and interscapular sites. Samples were taken from both the left and right sides and were measured separately; the results were averaged and reported as one observation.

Care was taken to remove from the interscapular adipose tissue pad all identifiable "brown" AT, and the "white" and "brown" portions were analyzed separately.

### Nitrogen

Nitrogen was measured by a micro-Kjeldahl technique according to Peters and Van Slyke (5) on AT samples weighing from 40 to 500 mg, depending on the size of the fat pad. The adipose tissue was first defatted by homogenization for several minutes in Potter-Elvehjem tubes containing 2 ml of 30% trichloroacetic acid in diethyl ether (w/v); the homogenate was then washed into centrifuge tubes with an additional 3 ml of extraction mixture and was centrifuged for 10 min. The supernatant solution, which contained the lipids, was discarded, and the protein pellet was washed into a 50 ml Kjeldahl flask with diethyl ether, which was then removed by evaporation in a water bath at 60°C. The digestion mixture was 2 ml of 50% sulfuric acid, using the Campbell and Hanna catalysts (5). Digestion was continued until the solutions were clear and either colorless or of a light straw color. Up to 2 hr of digestion were required depending upon the weight of sample and its site of origin.

We investigated both the precision of the analytical procedure itself and the reproducibility of the technique when applied to adipose tissue. The analytical precision was checked by digesting, distilling, and titrating replicate aliquots of a 0.1 M ammonium oxalate solution (nitrogen content 2.802 mg/ml). Nine replicate samples of 0.1 ml gave nitrogen recoveries of  $0.284 \pm 0.018$  mg (coefficient of variation 5.6%; mean recovery, 101.4%). Four samples of 1.0 ml yielded nitrogen values of  $2.812 \pm 0.018$  mg (coefficient of variation, 1.3%; mean recovery, 100.4%). Further studies were then made using human albumin as a convenient, biological standard material. 10 replicate samples of 20 mg of albumin yielded nitrogen values of  $2.902 \pm 0.032$  mg giving a coefficient of variation of 3.5%. After taking into account the presence of sodium caprylate, used as a preservative (1.38% w/w) and of water (5.2% w/w), and after assuming that the Van Slyke factor of 6.25 applied, the mean recovery was 97.7%. Nine samples of 200 mg of albumin yielded  $28.500 \pm 0.287$  mg nitrogen (coefficient of variation, 3.0%; mean recovery, 95.3%). These results showed that the analytical method had an acceptable precision over the range of nitrogen content of the samples (0.28–29.0 mg).

To test the reproducibility of the technique when

TABLE 1 THE REPRODUCIBILITY OF KJELDAHL NITROGEN MEASUREMENTS ON ADIPOSE TISSUE SAMPLES

Tissue	Sample Wet Weight	Nitrogen	Coefficient of Variation
	mg	mg/g $\pm$ SEM	%
Groin AT	100 (7)*	$7.46 \pm 0.51$	18
	200 (7)	$7.96 \pm 0.24$	8
Epididymal AT	50 (8)	$1.78 \pm 0.03$	5
	100 (8)	$1.67 \pm 0.01$	2
	200 (8)	$1.59 \pm 0.04$	8

\*Figures in parentheses are the numbers of observations.

applied to adipose tissue, we pooled and homogenized separately the groin pads and the epididymal fat pads of several animals.

Replicate samples weighing approximately 100 or 200 mg from the groin AT pool, and samples weighing 50, 100, or 200 mg from the epididymal pool were analyzed. The results are shown in Table 1.

The nitrogen content of the whole adipose tissue ranged from approximately 0.1 mg for a 50 mg sample of epididymal tissue, up to 1.6 mg of nitrogen in a 200 mg sample of groin AT, and over this range of nitrogen content, the results were acceptably reproducible.

### L-Hydroxyproline

Woessner's method (6) was used, employing AT samples of 50–500 mg, according to how much tissue was available. The preliminary digestion of the samples was carried out at 130°C in sealed, thick-walled, Pyrex glass ampoules.<sup>1</sup>

Although Woessner's method has been applied to many tissues (6), we were unable to find any previous reports of the application of this method specifically to adipose tissue. We examined, therefore, the recovery of collagen and the reproducibility of the method. 10 samples of collagen (Batch No. 10579; Koch-Light Laboratories, Ltd., Colnbrook, Bucks, England), weighing approximately 10 mg, were analyzed for L-hydroxyproline. The results indicated a mean recovery of  $95.4 \pm 4.2\%$  of the collagen added.

Reproducibility measurements made on 10 samples of a homogenate of groin AT gave a value of  $2.4 \pm 0.03$  mg of hydroxyproline per g of AT, with a coefficient of variation of 3.4%.

### Calculations

Total tissue protein was calculated by multiplying the measured nitrogen value by 6.25. The collagen concen-

<sup>1</sup> *Safety note.* If the digestion is being carried out on large batches of samples, maximum safety precautions to prevent sympathetic detonation of the whole batch by one weak ampoule are essential: a life-endangering explosion may otherwise occur.

TABLE 2 THE TOTAL NITROGEN CONCENTRATION OF DIFFERENT ADIPOSE TISSUE SITES, IN RATS OF DIFFERENT AGES

Adipose Tissue	Weight of Male Rats				Weight of Female Rats			
	100 g	150 g	200 g	300 g	100 g	150 g	200 g	300 g
Gonadal	4.25 ± 0.17* (8)†	3.95 ± 0.32 (9)	3.03 ± 0.22 (8)	2.24 ± 0.10 (7)	5.18 ± 0.26 (11)	4.26 ± 0.29 (6)	1.93 ± 0.12 (15)	2.11 ± 0.20 (5)
Perirenal	4.07 ± 0.38 (11)	5.79 ± 0.69 (7)	3.65 ± 0.41 (6)	2.40 ± 0.18 (7)	5.47 ± 0.20 (11)	4.65 ± 0.61 (6)	2.55 ± 0.24 (15)	2.32 ± 0.23 (5)
Groin	5.15 ± 0.22 (15)	7.96 ± 0.68 (9)	6.20 ± 0.70 (8)	6.57 ± 0.16 (6)	9.88 ± 0.48 (18)	10.88 ± 0.99 (7)	9.89 ± 0.61 (15)	5.84 ± 0.36 (2)
Interscapular (white)	7.03 ± 0.84 (9)	9.61 ± 1.01 (9)	8.23 ± 1.27 (7)	9.03 ± 0.85 (5)	4.37 ± 0.46 (4)	11.75 ± 1.27 (6)	4.32 ± 0.28 (3)	5.65 ± 0.48 (3)
Interscapular (brown)	20.97 ± 0.87 (5)	22.59 ± 1.18 (5)	15.54 ± 2.41 (5)	10.59 ± 0.41 (4)	16.79 ± 1.08 (4)	17.33 ± 1.16 (4)	15.52 ± 0.82 (3)	16.72 ± 1.71 (3)

\* mg/g (wet weight) ± SEM.

† The number of estimations comprising the mean is shown in parentheses.

TABLE 3 THE HYDROXYPROLINE CONTENT OF ADIPOSE TISSUE FROM VARIOUS SITES, IN RATS OF DIFFERENT AGES

Adipose Tissue	Body Weight ±10 g (Male)				Body Weight ±10 g (Female)			
	100 g	150 g	200 g	300 g	100 g	150 g	200 g	300 g
Gonadal	0.88 ± 0.10* (11)†	0.88 ± 0.09 (8)	0.53 ± 0.04 (7)	0.40 ± 0.04 (6)	0.78 ± 0.06 (5)	0.57 ± 0.08 (7)	0.78 ± 0.05 (14)	0.61 ± 0.08 (5)
Perirenal	0.80 ± 0.11 (10)	1.91 ± 0.32 (8)	0.79 ± 0.08 (7)	0.49 ± 0.03 (6)	0.61 ± 0.08 (5)	0.61 ± 0.08 (6)	0.68 ± 0.06 (9)	0.51 ± 0.03 (3)
Groin	2.41 ± 0.13 (12)	4.80 ± 0.53 (8)	3.52 ± 0.34 (7)	3.53 ± 0.34 (5)	4.25 ± 0.40 (6)	4.61 ± 0.51 (7)	3.35 ± 0.29 (11)	3.28 ± 0.52 (2)
Interscapular (white)	2.68 ± 0.32 (10)	4.70 ± 0.78 (6)	3.20 ± 0.28 (9)	3.91 ± 0.78 (9)	1.50 ± 0.30 (4)	3.27 ± 0.54 (10)	2.27 ± 0.66 (3)	2.44 ± 0.56 (3)
Interscapular (brown)	2.71 ± 0.70 (7)	3.20 ± 0.76 (5)	2.67 ± 0.58 (5)	3.44 ± 0.59 (6)	2.20 ± 0.35 (4)	2.27 ± 0.30 (4)	1.73 ± 0.32 (3)	1.44 ± 0.17 (4)

\* mg/g (wet weight) ± SEM.

† The number of estimations comprising the mean is shown in parentheses.

tration was derived by multiplying the L-hydroxyproline value by 7.46 (7). Noncollagen protein was derived from the formula: (Kjeldahl nitrogen × 6.25) - (L-hydroxyproline × 7.46). All analytical values are given as mean ± SEM. The coefficient of variation (%) was calculated

$$\text{from } \frac{\sqrt{\text{variance}}}{\text{arithmetic mean}} \times 100.$$

## RESULTS

The measured values of nitrogen and L-hydroxyproline concentration in AT are given in Tables 2 and 3. Tables 4 and 5, derived by calculation from the data of Tables 2 and 3 (see Methods) show the noncollagen protein and the collagen/total protein ratios respectively.<sup>2</sup>

### Nitrogen

The nitrogen concentration of the intra-abdominal AT sites (gonadal and perirenal) fell progressively and con-

siderably as age advanced beyond sexual maturity, in both males and females (Table 2). The intra-abdominal AT nitrogen levels at all ages were lower than those of the subcutaneous AT sites; this difference was accentuated with advancing age, since the nitrogen concentrations of subcutaneous AT did not change materially with age. Brown AT from the interscapular region had a high nitrogen concentration, which in both males and females exceeded those of intra-abdominal and groin AT at all ages, and which significantly exceeded white interscapular AT in all animals except males weighing 300 g.

### L-Hydroxyproline

The L-hydroxyproline concentration of the two intra-abdominal AT sites was considerably lower than that of either the white or brown subcutaneous AT (Table 3). There was a temporary rise of L-hydroxyproline concentration in all white AT with attainment of sexual maturity (at a body weight of 150–200 g), and then a moderate decline with age. The brown and white elements of interscapular AT showed similar L-hydroxyproline concentrations, in contradistinction to the large differences observed between their total nitrogen concentrations.

<sup>2</sup> Tables showing, in matrix form, the probability levels of differences between the following pairs of variables: (a) the same AT in each sex at different ages, (b) any two AT sites at the same age (within one sex), and (c) the sex differences for any particular AT site at a given age and applying to the data of Tables 2, 3, 4, and 5 can be obtained from P. B. Curtis-Prior upon request.

### Noncollagen Protein

The calculated values of noncollagen protein (see Methods) showed some interesting changes with age (Table 4). In females, in the two intra-abdominal AT sites examined, noncollagen protein fell very sharply as body weight increased from 150 to 200 g. A lesser and more gradual change of the same kind occurred in male animals.

In the two subcutaneous AT sites in males, noncollagen protein showed no significant change with advancing age, whereas in females the level in groin pad AT dropped abruptly after a body weight of 200 g had been attained and showed a temporary, marked elevation of the level in white interscapular AT at 150 g body weight.

Brown AT from the interscapular region showed very high values of noncollagen protein, attaining up to six times the levels in intra-abdominal white AT at some ages.

### Collagen/Total Protein Ratios

Details of the calculated ratios are given in Table 5. In

the male animals, collagen accounted for 20–30% of the total protein of intra-abdominal AT, and for a much larger proportion in groin and white interscapular AT, where it ranged from 50–80%. There were no significant age trends in any AT site examined in the males.

In female animals the results were less satisfactory since it was not considered valid to calculate this ratio unless the collagen and total protein measurements had been made on the same rats. Apart from a striking rise of collagen/total protein ratio of the two intra-abdominal AT sites after sexual maturity was reached at 150 g body weight ( $P < 0.01$ ), there were no significant age trends.

The collagen/total protein ratio of interscapular brown AT was close to the low levels of white intra-abdominal AT. There were no significant age trends in either sex, but at 300 g the level in female AT was ( $P < 0.001$ ) lower than in the males.

### DISCUSSION

The main purpose of this paper was to distinguish non-

TABLE 4 THE NONCOLLAGEN PROTEIN CONCENTRATION OF DIFFERENT AT SITES IN RATS OF DIFFERENT AGES

Adipose Tissue	Male Rats				Female Rats			
	100 g	150 g	200 g	300 g	100 g	150 g	200 g	300 g
Gonadal	19.94 ± 1.33* (8, 11)†	18.14 ± 2.13 (9, 7)	15.02 ± 1.43 (8, 7)	11.05 ± 0.70 (7, 6)	26.54 ± 1.70 (11, 5)	22.42 ± 1.93 (6, 7)	6.26 ± 0.82 (15, 14)	8.61 ± 1.40 (5, 5)
Perirenal	19.46 ± 2.49 (11, 10)	21.95 ± 4.94 (7, 8)	16.92 ± 2.63 (6, 7)	11.40 ± 1.12 (7, 6)	29.58 ± 1.36 (11, 5)	24.50 ± 3.85 (7, 7)	10.87 ± 1.59 (15, 9)	10.70 ± 1.48 (5, 3)
Groin	14.22 ± 1.65 (15, 12)	13.91 ± 5.81 (9, 8)	12.48 ± 5.05 (8, 7)	14.75 ± 2.75 (6, 5)	30.05 ± 4.24 (18, 6)	33.55 ± 7.27 (7, 7)	36.83 ± 4.37 (15, 11)	12.01 ± 4.50 (2, 2)
Interscapular (white)	23.98 ± 5.78 (9, 10)	25.00 ± 8.58 (9, 6)	27.63 ± 8.20 (7, 9)	27.22 ± 7.88 (5, 9)	16.18 ± 3.65 (4, 4)	49.00 ± 8.88 (6, 10)	10.05 ± 5.24 (3, 3)	17.11 ± 5.13 (3, 3)
Interscapular (brown)	110.89 ± 7.57 (5, 7)	117.29 ± 9.30 (5, 5)	77.27 ± 15.67 (5, 5)	40.54 ± 5.07 (4, 6)	88.50 ± 7.22 (4, 4)	91.43 ± 7.57 (4, 4)	84.08 ± 5.64 (3, 3)	93.72 ± 10.77 (3, 4)

Noncollagen protein was calculated on the basis of differences between pairs of mean values i.e.  $\bar{a} - \bar{b}$ , where  $\bar{a}$  is the mean value of total protein, and  $\bar{b}$  is the mean for collagen. The standard error (SE) of this difference was calculated from the formula:

$$SE = \sqrt{[SE \bar{a}]^2 + [SE \bar{b}]^2}$$

\* mg/g (wet weight) ± SEM.

† The figures in parentheses are the number of observations; the left-hand figure applies to total protein, the right hand to collagen.

TABLE 5 THE COLLAGEN PER TOTAL PROTEIN RATIO (%) OF ADIPOSE TISSUE FROM VARIOUS SITES IN RATS OF DIFFERENT AGES

Adipose Tissue	Body Weight ±10 g (Male)				Body Weight ±10 g (Female)			
	100 g	150 g	200 g	300 g	100 g	150 g	200 g	300 g
Gonadal	24.63 ± 4.02* (7)†	27.42 ± 3.58 (7)	21.73 ± 1.87 (7)	21.60 ± 2.24 (6)	‡	16.76 ± 2.70 (6)	51.03 ± 3.68 (14)	34.47 ± 1.76 (5)
Perirenal	25.58 ± 3.72 (8)	43.01 ± 5.81 (5)	26.11 ± 3.41 (6)	25.34 ± 2.08 (6)	‡	14.81 ± 1.45 (5)	39.95 ± 4.80 (9)	‡
Groin	55.29 ± 3.68 (12)	75.52 ± 8.35 (7)	78.64 ± 11.62 (7)	64.98 ± 7.07 (5)	39.68 ± 2.56 (4)	51.02 ± 4.15 (7)	42.51 ± 3.13 (11)	‡
Interscapular (white)	60.63 ± 11.73 (5)	56.42 ± 11.43 (6)	51.78 ± 7.84 (7)	68.39 ± 18.03 (4)	43.43 ± 9.93 (4)	41.24 ± 7.04 (4)	64.25 ± 21.22 (3)	‡
Interscapular (brown)	17.68 ± 5.34 (5)	16.92 ± 4.06 (5)	20.33 ± 3.96 (5)	32.06 ± 2.48 (4)	15.43 ± 1.52 (4)	15.59 ± 1.91 (4)	13.28 ± 2.12 (3)	10.32 ± 0.75 (3)

\* Mean ± SEM.

† The number of estimations comprising the mean is shown in parentheses.

‡ These ratios were not calculated since the collagen and protein values were not measured on the same animals.

collagen protein, which can reasonably be assumed to represent the protein content of the adipose tissue cells, from the collagen of the supporting matrix. Although noncollagen protein comprised about three-quarters of the total protein of intra-abdominal AT, it accounted for only 20–50% of the total protein of the subcutaneous pads examined, and it is clear that total nitrogen of adipose tissue cannot be generally equated with fat cell protein. However, where the intra-abdominal fat pads customarily used for bioassay are concerned, the age trends in total nitrogen and adipocyte protein were very similar. The observed reduction of the nitrogen concentration of intra-abdominal AT with advancing age agrees with the results of Benjamin and Gellhorn (8, 9). These workers postulated that the fall of nitrogen content indicated that adipose cells grow exclusively by enlargement of the central fat globule, while the total cell nitrogen remains constant; in effect, the total nitrogen concentration of the tissue is “diluted” by neutral fat. This view is supported by the work of Knittle and Hirsch (2) and more recently by Hirsch and Han (3). They observed that the total cell number of a particular AT body increased rapidly during early growth and then reached a plateau at adulthood (150–200 g body weight in our rats). Any later increase in the AT body was achieved by hypertrophy of the adipocytes. Constancy of the number of adipocytes does not preclude the possibility of a continuous turnover of cells, i.e. as many old cells die as new ones form.

The only other data on AT hydroxyproline we have been able to find was given for male white rats by Christophe and Wodon (10). They found that collagen comprised 16% of the protein in epididymal AT in 200 g animals, as compared with 22% in our male rats of the same age. However, Christophe and Wodon commented that collagen comprised an increasing proportion of the total protein as the animals aged, and they also observed an increase of collagen in absolute terms (i.e. per g wet weight), whereas our results showed no significant age-related change in the collagen/protein ratio, and a fall in collagen when expressed on a wet weight basis.

#### *Intra-Abdominal Compared with Subcutaneous AT*

In general, both total nitrogen and hydroxyproline con-

centrations were higher in subcutaneous than in the intra-abdominal adipose sites (not observed in the male rats studied by Benjamin and Gellhorn) (8), and the reduction with advancing age was much smaller in subcutaneous AT.

Interscapular brown AT had a higher total nitrogen concentration than any other AT site, at all ages in both sexes, and had a relatively low collagen/protein ratio compared with the other adipose tissues. Although brown adipose tissue contains up to 4% by weight of phospholipids, which contain nitrogen (11), this could not account for more than about 1/40 of the observed nitrogen content of brown AT. This tissue has, therefore, a very high concentration of cell protein, as compared with white AT, a finding compatible with its intense metabolic activity (11, 12).

It is a pleasure to acknowledge the continued encouragement of Professor W. J. H. Butterfield, and valuable statistical help from Mr. F. W. Harpley.

*Manuscript received 4 December 1969; accepted 17 March 1970.*

#### REFERENCES

1. Pawan, G. L. S. 1969. *In Obesity Medical and Scientific Aspects*. I. McLean Baird and A. N. Howard, editors. Livingstone Ltd., (E. & S.), London, England. 75–82.
2. Knittle, J. L., and J. Hirsch. 1968. *J. Clin. Invest.* **47**: 2091.
3. Hirsch, J., and P. W. Han. 1969. *J. Lipid Res.* **10**: 77.
4. Enesco, M., and C. P. Leblond. 1962. *J. Embryol. Exp. Morphol.* **10**: 530.
5. Peters, J. P., and D. D. Van Slyke. 1932. *Quantitative Clinical Chemistry*. The Williams & Wilkins Co., Baltimore, Md. **2**: 516–538.
6. Woessner, J. F., Jr. 1961. *Arch. Biochem. Biophys.* **93**: 440.
7. Neuman, R. E., and M. A. Logan. 1950. *J. Biol. Chem.* **186**: 549.
8. Benjamin, W., A. Gellhorn, M. Wagner, and H. Kundel. 1961. *Amer. J. Physiol.* **201**: 540.
9. Gellhorn, A., and W. Benjamin. 1965. Adipose Tissue. *In Handbook of Physiology*. A. E. Renold and G. F. Cahill, Jr., editors. The American Physiological Society, Washington, D. C. 399–405.
10. Christophe, J., and C. Wodon. 1963. *Arch. Int. Physiol. Biochem.* **71**: 720.
11. Smith, R. E., and B. A. Horwitz. 1969. *Physiol. Rev.* **49**: 330.
12. Johansson, B. 1959. *Metab. Clin. Exp.* **8**: 221.