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The nitrogen:water ratios of albino rats and their use in protein-evaluation tests

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In the search for shorter methods for protein evaluation the nitrogen:water ratios of the animals commonly used in these tests have recently become important. Bender & Miller (1953*b*) found that in their black-and-white hooded rats the N:H_2O ratio was of such constancy that it could be used for calculating carcass nitrogen from a knowledge of the water content and the age of the rat. This easy way of estimating carcass nitrogen is an important time-saving factor in the carcass-nitrogen method of protein evaluation proposed by Bender & Miller (1953*a*). These authors, however, doubted whether their calculated regression equation for N:H_2O values, correlated with the age in days, would also be applicable to other rat colonies (Bender & Miller, 1953*b*) and the existence of strain differences was indeed observed later by Forbes & Yohe (1955) when a small number of albino rats was analysed. These authors also concluded that significant errors (up to 20%) can be introduced into determinations of net protein value when the carcass nitrogen content is obtained by relying upon the predetermined N/H_2O × 100 constants—a conclusion that suggests variation in these constants even when the rats are of similar age.

The object of this paper is to present on a larger scale further findings on the stability of the relationship between the N:H_2O ratio and age of our albino rat colony and to report on an observed difference between the N:H_2O ratios of male and female animals.

EXPERIMENTAL

Animals. The animals were of the Wistar strain, bred in this laboratory from rats originally obtained from the Veterinary Research Laboratory, Onderstepoort. The samples of rats analysed were drawn at irregular intervals over a period of about 8 months. A careful record was kept of the age of each rat in every sample. Treatment of the rats after weaning varied a great deal. An occasional rat was kept on the stock diet until it was killed for analysis. The majority of the animals were, however, used in 10-day Bender–Miller protein-evaluation tests before analysis. This routine was followed until results for 300 rats varying in age from 32 to 49 days were collected. The numbers of rats per age group varied from 3 to 76. The sample as a whole was, therefore, of a heterogeneous nature, allowing for seasonal variations as well as the influence of a variety of nutritional conditions.

Carcass analysis. To avoid the practical difficulties involved in cleaning the alimentary canal before analysis, animals were starved for about 18 h before they were killed. Moisture was determined by dehydrating partly dissected carcasses in hot (105°) circulating air for 24 h.

Sampling errors present a major difficulty in the determination of total N in rat carcasses and, to eliminate this source of error, a special procedure for the Kjeldahl digestion of whole carcasses was followed. The dry carcass together with a small piece of N-free filter-paper, used to absorb blood spillings during dissection, was transferred to an 800 ml. Kjeldahl flask. Conc. sulphuric acid (15 ml. + 10 ml./g rat carcass), 30 g anhydrous K_2SO_4 and 4·5 g HgO were used for digestion, roughly according to the method of Perrin (1953). The vigorous frothing which takes place during digestion was kept under control by adding about 1 g N-free paraffin wax (m.p. 50°). The mixture was digested until it became pale yellow, and thereafter for about 30 min. After dilution to 2 l., 50 ml. portions were taken for distillation.

RESULTS

The results obtained for the moisture and N content of 168 female and 131 male rats were used for the calculation of $N:H_2O$ ratios and for further statistical treatment. From inspection it seemed probable that the ratios might be different for male and female animals of the same age. This probability was tested by applying the non-parametric test of Wilcoxon (Wilcoxon, 1945; Mann & Whitney, 1947; Wabeke & van Eeden, 1955) to the different age groups and combining the results (Wallis, 1942; Hemelrijk, 1952). A highly significant difference between the ratios for the two sexes was found.

As in the work of Bender & Miller (1953*b*), the $N:H_2O$ values showed a highly significant positive trend in relation to the age of rats, according to the results of the non-parametric T test of Terpstra (1952, 1953). However, since sex differences were discovered, two separate regression equations had to be calculated to express the above correlation. From the general formula

$$y = a + bx,$$

where $y = N$ (in g)/H_2O (in g) $\times$ 100 and x = age in days, the following equations were obtained:

For males, $$\frac{N}{H_2O} \times 100 = 3{\cdot}3908 + 0{\cdot}0149x \quad \text{(residual S.D.} = 0{\cdot}10) \qquad (1)$$

For females, $$\frac{N}{H_2O} \times 100 = 3{\cdot}4640 + 0{\cdot}0149x \quad \text{(residual S.D.} = 0{\cdot}10) \qquad (2)$$

For males and females together,

$$\frac{N}{H_2O} \times 100 = 3{\cdot}4331 + 0{\cdot}0149x \quad \text{(residual S.D.} = 0{\cdot}13). \qquad (3)$$

To determine the magnitude of the errors which may be introduced into measurements of carcass N content by relying on equations (1) and (2), 267 calculated results were compared with those obtained by chemical analysis. The differences between N calculated and N determined were found to have a standard deviation (σ) of 0·0393 g N, and were normally distributed about zero ($P = 0{\cdot}8$–$0{\cdot}9$ by Pearson's χ^2 test). This finding indicates that calculated results will deviate not more than three times per 1000 observations from the 'true' figures by more than $\pm 0{\cdot}1179$ g N (3σ limits).

As a final check on this conclusion a further sample of forty rats was drawn from our rat population for analysis, followed a month later by another sample of twenty-nine rats. The results obtained by calculation were highly correlated with the figures determined chemically ($r = 0{\cdot}987$) and no variations exceeding the $\pm 3\sigma$ limits were found to occur (Fig. 1).

DISCUSSION

The results reported here confirm the hypothesis that the N:H_2O ratio of albino rats is a constant, varying only within certain close limits. N:H_2O values were again found to increase with the age of the animal, which tends to confirm the findings reported by Bender & Miller (1953*b*). These workers, however, computed the equation N/$H_2O \times 100 = 2{\cdot}92 + 0{\cdot}024 \times$ age in days for black-and-white hooded rats, which obviously differs from the equations reported here for albino rats, so that indiscriminate application of a regression equation formulated for rats of one strain to rats of another is clearly to be discouraged.

In addition to the effect of age and strain on N:H_2O ratios we have now found a third factor, that of sex. The N/$H_2O \times 100$ value of males was approximately 0·073 unit below that of the females over the age range tested. Since this difference was shown to be highly significant for our strain of albino rats, it would be valuable to investigate it further with strains of rats from other colonies.

Equation (3), which was computed from the results for all rats, irrespective of sex, showed a residual S.D. of 0·13, which means that in this computation there was a wider scatter of the observed ratios about the regression line. Carcass-N figures obtained with this equation, would, therefore, be less accurate than figures obtained by relying upon equations (1) and (2). When equations (1) and (2) (discriminating between the sexes) are used for N calculations, results can be obtained that are not likely to differ from the 'chemical' results by more than $\pm 0{\cdot}1179$ g N (99.7% certainty or

3σ limits). It may also be said with 95% certainty that this figure will not be more than $1{\cdot}96\sigma$ or 0·079 g N.

The significance of these figures in determinations of net protein utilization (N.P.U.) by the carcass-N method needs further consideration. Bender & Miller (1953*a*) derived the equation

$$\text{N.P.U.} = \frac{B_f - B_K + I_K}{I_f},$$

(where B_f and I_f = carcass N and N intake, respectively, of animals on the test diet, and B_K and I_K = carcass N and N intake, respectively, on the low-N diet). The error introduced in this equation by using the indirect method for carcass-N determination will therefore have a bearing on $B_f - B_K$. Since $\sigma_{B_K} = \sigma_{B_f} = \sigma$, the standard deviation of the difference of the two variables B_f and B_K is $\sigma\sqrt{(2/N)}$ (where N = number of rats per test group), the standard error in the N.P.U. units is therefore $\sigma/I_f \times \sqrt{(2/N)}$. This error will obviously become less when the number (N) of rats per test group and the N intake (I_f) per animal are increased. Bender & Miller (1953*a*) specified that a group of four rats would suffice, but no mention was made of the amount of the 10% protein (1·6% N) diet to be fed to the test animals. However, a rat weighing about 50 g and receiving daily about 5 g of the diet will consume over a test period of 10 days about $5 \times 10 \times 0{\cdot}016 = 0{\cdot}8$ g N. Assuming this level of N intake, the variability

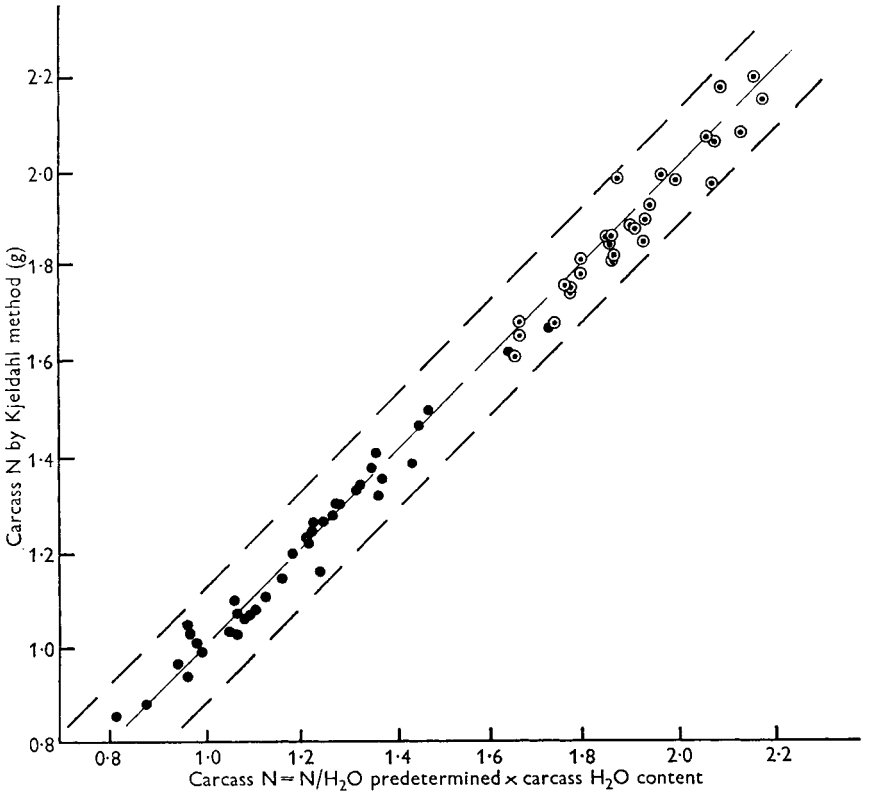

Fig. 1. Correlation of the direct and indirect methods for the determination of rat-carcass N as obtained with two separate samples of rats. The broken lines indicate the $\pm 3\sigma$ limits.

introduced by indirect carcass-N determinations can be calculated according to the above formula. For four rats/group, for example, the standard error is 3·5.

A figure of 0·8 g N for I_f will not be reached under all practical conditions since test diets containing poor-quality proteins are not always readily consumed. As a lower I_f figure in the above formula will increase the limits of the error significantly it seems advisable to check B_K and B_f by direct analysis when such situations arise. To keep I_f at a safe level, it might be wise under all circumstances to select rats of the maximum weight in the range of weights which are regarded as suitable for protein-evaluation tests.

It is doubtful whether the error could be reduced by any means other than the two already mentioned. Forbes & Yohe (1955) suggested that results may be rendered more accurate 'by carefully standardizing the animals by pretreatment with a diet containing an amount of good-quality protein that is barely adequate, thus tending to provide animals of uniform nutritional condition at the start of the test period'. In view of the fact that an inadequate balance of B_f and B_K at the beginning of the test period will introduce an additional source of error, this measure might be a good one. It will, however, not reduce errors introduced during the final stage of the experiment by the inaccuracies of the indirect method of carcass-N determination. Such errors are the direct results of fluctuations in $N:H_2O$ constants, and these constants are assumed to be independent of nutritional status. These errors are, however, significant enough to emphasize that the use of larger numbers of animals per test group is necessary in order to approach the same level of accuracy as that obtainable with the Thomas–Mitchell method (Thomas, 1909; Mitchell, 1923–4). With the further realization of sex and strain differences, the indirect method for carcass-N estimation may certainly find useful applications.

SUMMARY

1. The nitrogen:water ratios of 300 albino rats varying in age from 32 to 49 days were determined by direct analysis of nitrogen and moisture content.

2. The following regression equations for the correlation of these ratios with the age of the rat were calculated:

For males, $$\frac{\text{N (in g)}}{\text{H}_2\text{O (in g)}} \times 100 = 3{\cdot}3908 + 0{\cdot}0149 \times \text{age (in days)}$$

For females, $$\frac{\text{N (in g)}}{\text{H}_2\text{O (in g)}} \times 100 = 3{\cdot}4640 + 0{\cdot}0149 \times \text{age (in days)}.$$

3. The difference between the $N/H_2O \times 100$ figures for the two sexes was about 0·073. Since this difference was found to be highly significant, it is concluded that male and female rats differ in their nitrogen: water ratios.

4. Carcass-nitrogen weights calculated according to the formula:

$$\text{N (in g)} = \frac{\text{N (in g)}}{\text{H}_2\text{O (in g)}} \times 100 \text{ (predetermined)} \times \text{moisture (in g)}$$

were highly correlated with values obtained by Kjeldahl analysis ($r = 0{\cdot}987$); the standard error of the estimate was 0·0393 g N for individual rats.

5. This standard error of estimate was found to be of such a magnitude that it can cause a standard error of about 3·5 N.P.U. units if only four albino rats are being used in Bender–Miller tests.

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Thiamine in the contents of the alimentary tract of sheep

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Interest in the thiamine content of the rumen has been aroused again by the discovery that bracken contains a factor splitting the thiamine molecule into its component pyrimidine and thiazol fractions (Weswig, Freed & Haag, 1946; Carpenter, Phillipson & Thomson, 1950; Evans, Evans & Roberts, 1951). Typical signs of vitamin B_1 deficiency were obtained in rats and in the horse by the feeding of considerable amounts of bracken and were abolished by injections of thiamine. The thiamine content of the rumen digesta in bracken-fed cattle was found to be very small indeed (Phillipson & Reid, 1954), but the syndrome known as bracken poisoning in cattle is not at all like a thiamine deficiency, nor can it be prevented or cured by massive injections of thiamine (Carpenter *et al.* 1950; Evans *et al.* 1951; Naftalin & Cushnie, 1954). From this evidence it might be inferred that the requirements for thiamine in the adult ruminant are small. However, nearly all the existing work on thiamine has been confined to the rumen and little is known about the distribution of thiamine in