Whole-rat protein content estimation: applicability of the N × 6·25 factor

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The amino acid composition of the protein from three strains of rat (Wistar, Zucker lean and Zucker obese), subjected to reference and high-fat diets has been used to determine the mean empirical formula, molecular weight and N content of whole-rat protein. The combined whole protein of the rat was uniform for the six experimental groups, containing an estimate of 17·3% N and a mean aminoacyl residue molecular weight of 103·7. This suggests that the appropriate protein factor for the calculation of rat protein from its N content should be 5·77 instead of the classical 6·25. In addition, an estimate of the size of the non-protein N mass in the whole rat gave a figure in the range of 5·5% of all N. The combination of the two calculations gives a protein factor of 5·5 for the conversion of total N into rat protein.

Nitrogen conversion factor (6·25): Protein analysis: Protein nitrogen: Rat

The enormous variety of different protein species that can be found in a complex living organism is a severe handicap for their global measurement. The different physical and chemical properties of such a large array of molecules, having in common only their amino acid building blocks and the peptide bonds joining them, lead to serious difficulties in their estimation as a class of compounds.

In N and nutrient energy balance studies the analytical needs are focused on the estimation of 'protein' as a differentiated entity comprising myriads of, often very different, individual protein species at varying concentrations. Most methods used for the undifferentiated estimation of 'protein' are necessarily crude in their approach, which invariably limits their precision and uniformity of application. The most favoured method is the time-honoured measurement of all N contained in the sample by means of a Kjeldahl digestion or using elemental analysers, and multiplying the result obtained by a factor depending on the N content of the protein. The factor of 6·25 for the conversion of N values to protein values has been in use for most of this century in spite of its obscure origins (Hiller *et al.* 1948) and is still commonly applied in such balances despite its known variability (Passmore & Eastwood, 1986). The differences between protein sources as uniform in their composition as dairy or wheat products have urged the application of other N-to-protein factors (Lloyd *et al.* 1978).

The applicability of the 6·25 factor has been discussed (Hiller *et al.* 1948; Lloyd *et al.* 1978; Passmore & Eastwood, 1986), but its use is widespread despite the regular posing of caveats to its indiscriminate application. The practice of estimating protein as a direct

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correlate of N content also incurs the error of assuming that all N in the material being analysed is part of protein amino acids; in addition, N losses are assumed to correspond to protein losses (Food and Agriculture Organization/World Health Organization/United Nations University, 1985). A large number of other compounds containing N are found at low, albeit significant, proportions in animal tissues, which adds a fixed source of error to the already unreliable process of estimation of protein content.

The measurement of total N content in complex samples such as protein implicitly involves systematic error due to other nitrogenous compounds present in the sample. There are insurmountable practical difficulties in the estimation of this N fraction, due both to analytical diversity and small concentration and/or sampling difficulties. Although the existence of these sources of error is known (Oddoye & Margen, 1979; Lin & Huang, 1982; Esteve *et al.* 1992), its quantitative importance is often dismissed or overlooked, because it is commonly appreciated that its magnitude is very probably negligible when compared with that of protein N. We present an estimate of the size of that N fraction, by using the very few global data available and by establishing sequences of assumptions and approximate calculations from the data available for non-protein N compounds related to a whole mammalian organism.

We attempt to determine the percentage N content of the mixed protein of whole adult rats from various strains fed on different diets, as a way of defining the limits of variability in N percentage and the applicability of the commonly used 6·25 factor to studies involving the analysis of whole-rat protein.

MATERIALS AND METHODS

Animals and diets

Female Wistar rats from Iffa-Credo (Saint Germain sur l'Arbresle, France) stock, as well as Zucker lean (Fa/?) and obese (fa/fa) rats from Harlan Olac (Bicester, Oxon.) stock, were bred at the University of Barcelona Animal Service; they were used at 30 d after birth (weaned on day 22). The rats were housed individually in polypropylene-bottomed cages maintained in a light- (on from 08·00 to 20·00 hours), humidity- (70–80 %) and temperature- (21–22°) controlled environment.

Two dietary groups were established for each of the three strains used: the reference groups were fed on a commercial pellet diet (type A04; Panlab, Barcelona, Spain; containing (g/kg) protein 170, digestible carbohydrate 587, and lipid 30 and tap water *ad lib.* The high-fat diet group received a simplified 'cafeteria' diet, consisting of a daily offering of biscuits spread with liver pâté, bacon, banana, chow pellets (as indicated above), tap water and whole milk supplemented with 333 g sucrose/l plus 10 g mineral and vitamin supplement (Gevral; Cyanamid Ibérica, Madrid, Spain)/l, all presented in excess (Rafecas *et al.* 1992). This diet is a simplified version of an earlier high-fat cafeteria diet (Prats *et al.* 1989), scaled down by selecting only the items more likely to be consumed. The rats (n 6) in each group were fed on the allotted diets for 1 month, and on day 60 they were weighed and then killed by decapitation. The rat bodies were dissected and the contents of the stomach and intestines were removed. The remaining carcass was then minced with scissors and ground with a blender, sampled and stored at −20° until processing. The blood shed in the killing process was added to the homologous tissue preparation and mixed before storage. Ten samples (approximately 0·5 g each) were taken from different parts of the ground tissue preparation. These portions were frozen, and then powdered together with a ceramic mortar and pestle under liquid N_2. The finely powdered samples were mixed, and portions were used for analysis. Previous work established the reliability of the sampling procedure and repeatability of the measurements (Esteve *et al.* 1992; Rafecas *et al.* 1992).

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Amino acid and nitrogen analyses

Total N content of the powdered samples was determined by means of a Carlo Erba NA-1500 elemental analyser. Triplicate portions of about 0·2 g powder were mineralized in long pyrex tubes with 20 ml reagent quality HNO_3 (12·7 M) (Merck, Darmstadt, Germany), in a heating block. The tubes were kept for 12 h under reflux at 70° and 1 h at 120°, then concentrated down (150°, no reflux) to about 5 ml. Reagent quality 7 M-perchloric acid (5 ml) was then added and the mixture was incubated for 30 min at 220° under reflux. The tubes were evaporated down to 1 ml (240°, no reflux) and pure (Milli-Q Millipore quality, 15 MΩ cm resistivity) water was added to every tube to bring the final volume up to 10 ml. The S content of the acidic digests was measured with a Polyscan 61E ICAP spectrometer (Thermo Jasell Ash, Franklin, MA, USA). A series of randomly selected samples were also measured following the addition of known amounts of several amino acids and defatted bovine serum albumin, in order to check the linearity of the response in the measurements of N and S.

Portions of the powdered samples were suspended in ten volumes of chilled water and then homogenized with a Politron homogenizer (Kinematica, Littau-Luzern, Switzerland), then their proteins were hydrolysed with 6 M-HCl in sealed glass tubes under N at 105° for 48 h (Allen, 1989). The hydrolysates were cleaned by ultrafiltration and neutralized, and their phenyl-hydantoins were prepared (Heinrikson & Meredith, 1984). All series of determinations included standards of known amino acid mixtures. Before derivatization, an internal standard of norleucine was also added to each sample. Amino acids were determined in an LKB 2150 high-performance liquid chromatograph using Spherisorb ODS-2 (5 μm) columns (150 × 4 mm) and a gradient of ammonium acetate–acetonitrile–methanol as mobile phase at 60° (Heinrikson & Meredith, 1984).

The losses due to the formation of Maillard products (Whistler & Daniel, 1985), as well as acid decomposition, and the efficiency of the derivatization process were corrected by means of internal standards and samples of known amino acid content run with each batch of samples. Since the recoveries of cysteine and methionine were poor, these amino acids, together with tryptophan, which is destroyed in the acid hydrolysis procedure (Kasper, 1970), were not measured.

The amide (glutamine and asparagine)-N in the samples was measured in a second series of portions, suspended in about ten volumes of chilled (−10°) water–acetone (1:1, v/v; Arola *et al.* 1977) and thoroughly blended with a Politron homogenizer. The suspension was left standing for 12 h at −20° and then centrifuged. The protein precipitate was again homogenized with ten volumes of pure acetone, and the delipidated-protein acetone powder was dried under a warm (25°) stream of N_2. Portions of the acetone powder, standards of defatted bovine serum albumin (Sigma), glutamine and asparagine and some samples containing known amounts of crystalline glutamine were hydrolysed in sealed glass ampoules with 100 volumes of 2 M-HCl under N_2 for 3 h at 100° (Chibnall *et al.* 1958). The hydrolysates were neutralized with 1 M-NaOH, and used for the estimation of free ammonium with a standard glutamate dehydrogenase method (Kun & Kearney, 1974). Under the conditions indicated, the release of amino-N as ammonia from either glutamine, asparagine or albumin was quantitative, and the loss of 2-amino-N was negligible.

Evaluation of the extent of contamination of protein by other nitrogenous compounds in the whole-rat samples

It was assumed that, quantitatively, the main sources of error in the ascription of all N in whole-rat samples to protein were the presence of: (a) free amino acids, (b) DNA and RNA, (c) nucleotides, nucleosides and related compounds, (d) creatine and creatine phosphate, (e) the porphyrin ring in proteins, (f) free ammonia, (g) urea, (h) N-containing

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lipids (e.g. sphingosine, choline), (i) other N-containing compounds (e.g. hexosamines, amines). Since the measurement of all of them as classes of molecules was not feasible, they were estimated instead from bibliographic data when available.

Free amino acids constitute about 0·5% of all amino acids in a living mammal (Munro, 1970). A relationship of 70 g in a 70 kg man has also been calculated (Munro, 1976). Assuming a mean molecular weight of 122 this represents 10·5 g N from free amino acids, i.e. 0·16 g/kg body weight. In the rat, assuming a measured mean value for Wistar rats fed on a standard diet of 28·0 g N /kg animal tissue, free amino acids (0·16 g/kg) may represent about 0·57% of all N.

The estimation of whole-body DNA and RNA is more complicated. The relative proportions of RNA and DNA vary in different tissues (Prasad *et al.* 1972). We assumed that their total amounts in the body were roughly equivalent. No reference was found to whole-body DNA levels, but it is commonly accepted that mammals have about 6 g DNA/10^{12} cells (Herd *et al.* 1973). The rat has about 80 million cells/ml (i.e. per 1·1 g) (Nedergaard & Lidberg, 1982), thus 1 kg rat tissue may contain about 0·44 g DNA, i.e. 0·87 g nucleic acids. Assuming a N content of about 22%, this represents 0·19 g, or about 0·68% of all N.

ATP is the most abundant nucleotide, followed by ADP, AMP and others; it was assumed that all nucleotides, nucleosides and related compounds combined would amount to about twice the level of ATP; its concentration is in the range of 2·5 μmol/g tissue (Start & Newsholme, 1968). Since it contains about 14% N, all nucleotides may represent 0·35 g N, or about 1·27% of all N.

Creatine and creatine phosphate are found in large concentrations, essentially in muscle, in the order of 30 μmol/g (Kurahashi & Kuroshima, 1978). Since muscle constitutes about 43% of rat weight (Arola *et al.* 1979), in 1 kg of rat we can expect to find about 0·54 g creatine-N. This represents about 1·93% of all N, which is in agreement with the 120 g creatine contained in an adult man (Best & Taylor, 1964; i.e. 1·71 g/kg or 0·55 g N).

Again it was assumed that the porphyrin ring in haemoglobin would represent about half the porphyrins in the whole body (also found in myoglobin and cytochromes). Most haemoglobin (in which the porphyrin ring is about 6% of the molecule weight) is limited to erythrocytes, which contain 15% haemoglobin (Guyton, 1976). Blood forms about 5% of body weight (Hobbs, 1967) and erythrocytes constitute only 40–45% of blood; thus all blood in 1 kg of rat would contain 1·28 g porphyrin: since it has 9% N, we could calculate that 1 kg of rat contains 0·017 g haemoglobin porphyrin-N, i.e. 0·035 g porphyrin-N, or about 0·12% of all N.

Circulating plasma ammonia concentration is about 20 μM (Zamora *et al.* 1988); even assuming a diffusion space of all the extracellular space of the rat, all this ammonia could not account for even $1/10^5$ of the N in the rat. Since ammonia freely diffuses into all spaces of the body (Kleiner, 1981), and the concentration of ammonia in tissues is in the range of 4 μg/g (Singh & Banister, 1983), we can assume that tissue ammonia can account for about 3·5 mg/kg N, i.e. 0·01% of all N in the rat. For urea (5–10 mM in plasma), assuming a diffusion space in the range of 30–50%, the yield may be about 83 mg/kg, i.e. about 0·29% of all N. Phospholipids may be found in the range of 9 g/kg body weight (computed from organ weights and their phospholipid content from data in Diem (1965)); assuming a N content of about 1·8% (that of the most abundant phospholipid, lecithin), we obtain 0·16 g N, i.e. 0·57% of all N. We have further considered, arbitrarily, that all other N-containing compounds may in all contribute to about 0·1% of all N. Thus (Table 1) we may justify about 5·5% of all N as non-amino-acid-protein sources.

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Table 1. *Estimation of the non-protein-N compounds as sources of error in the measurement of protein through evaluation of total N content**

Source	g N/kg rat	% total N
Free amino acids	0·16	0·57
DNA and RNA	0·19	0·68
ATP and other nucleotides	0·35	1·25
Creatine and creatine-P	0·54	1·93
Porphyrins	0·04	0·12
Free ammonia	0·00	0·01
Urea	0·08	0·29
Lipid N	0·16	0·57
Other sources	—	0·10
Total estimated	1·52	5·52
Total rat N	27·97	100·00

* For the methods used in the calculations and sources of data, see pp. 201–202.

Determination of amino acid composition of rat protein

The estimation of mean amino acid composition of the mixed rat protein involves first a precise measurement of the N content of the sample. We have found that about 5·5% of all N is non-protein; thus the total N content figures should be scaled down by 5·5% to fit the actual protein N value.

Since the analytical method followed does not give reliable data on a series of amino acids (glutamine, asparagine, cysteine, methionine and tryptophan), we devised a series of corrections that limit the difficulties caused by the lack of direct data when establishing a quantitative composition of the protein. The amino acids cysteine and methionine were singled out because they contained S. This was measured independently, and it was assumed that all belonged to either cysteine or methionine. The proportion of each to be computed in the global analysis was established, as an approximation, by taking their mean ratio from a series of 207 eukaryotic protein analyses (Reeck & Fisher, 1973) i.e. 1·74 residues of cysteine per methionine residue. A similar approach was used for tryptophan, taking as calculated percentage tryptophan in the rat protein the value of 1·4 residues per 100 residues found in the compiled protein analyses cited (Reeck & Fisher, 1973). This may result in an overestimation of methionine and tryptophan (and underestimation of cysteine) but since their percentage in most proteins is low it was considered that the overall effect on the empirical formula of the protein would be lower than their non-inclusion.

The extreme lability of the amide groups of asparagine and glutamine has been circumvented by direct measurement, thanks to their marked lability. Thus the value for amide-N has been established. It was decided to give this figure alone and to compute the rest of the molecule of glutamine and asparagine as glutamate and aspartate respectively.

The amide-N and that corresponding to the amino acids already computed (cysteine, methionine and tryptophan) were subtracted from the measured sample N. The remaining protein-N would be that of all other amino acids. The mean values for the different aminograms obtained from the liquid chromatograph were used to compute the percentage of each amino acid with respect to the sum of all those measured in the chromatograms. The distribution, following these percentages, of the protein-N not yet allotted allowed the establishment of a precise distribution of amino acids per unit sample weight. When all the data were combined, the mean protein analyses were obtained.

The computation of the contribution of C, O, H, N and S by each aminoacyl residue corrected by the calculated percentage of that amino acid in the rat protein was used to

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establish the mean empirical formula for that protein. This allowed the estimation of mean molecular weight of the aminoacyl groups, and thence the percentage of N and the protein factor, i.e. that figure which multiplied by the N content would give the protein yield.

Application of the methods to the analysis of a known protein sample

In order to determine the effectiveness of the combined analytical methods used for analysis of rat protein, a standard batch of bovine serum albumin (Cohen's Fraction V; Sigma, St. Louis, MO, USA) was analysed for total N, S, amino acid composition and amide-N content, using the procedures outlined above. This protein was selected because of its ready availability, because it is frequently used as standard for protein analyses, and because it was one of the few for which amide-N data were available. The results were compared with a complete analysis of bovine serum albumin available in the literature (Spahr & Edsall, 1964).

RESULTS AND DISCUSSION

On day 60, Wistar rats fed on the reference diet weighed 198 (SE 5) g and those on the high-fat diet weighed 248 (SE 9) g. The values for lean Zucker rats were 166 (SE 7) g and 209 (SE 12) g respectively, and the values for obese Zucker rats were 279 (SE 8) g and 351 (SE 16) g respectively.

Table 2 shows the mean empirical formulas of the aminoacyl residues of whole-rat protein. Table 2 also includes data on several significant proteins for which complete analyses are available in the literature; these data have been computed into empirical formulas and percentage N content using the procedures outlined in the preceding section. There was considerable uniformity in the mean amino acid empirical formulas of the six strain–diet combinations studied, with uniform N content values around 17·3%, which gives a protein factor of 5·77. When no corrections were introduced for tryptophan or methionine (i.e. it was considered that all S was in cysteine residues), a mean residue molecular weight of 102·8, i.e. 17·26% N, and a protein factor of 5·79 were obtained. The differences between the two approaches are fairly small, implying that even allowing for substantial errors in the appreciation of the levels of these amino acids, the use of either factor may give more precise values than the widely used factor of 6·25.

Many of the individual proteins listed in Table 2 show a lower percentage N content than the rat protein, and are thus closer to a protein factor of 6·25. The fact that a combined sample of about 207 different enzyme proteins yields a factor of 6·30 and 207 non-enzymes yield 6·27 (Reeck & Fisher, 1973), may help understand the prevailing use of the 6·25 factor. However, some proteins such as haemoglobin (Ball *et al.* 1966) and, especially, collagen (Kakiuchi & Kobayashi, 1971) show low or very low values.

The different types of collagen are abundant in connective tissue, skin and skeletal muscle; together they constitute a significant portion of the whole-rat protein, as can be observed in Table 3 where the individual amino acid composition of the whole-rat protein of the different experimental groups is presented along with the composition of bovine serum albumin (Spahr & Edsall, 1964). The rat proteins contain more than 3% hydroxyproline, which is found almost exclusively in collagen (Kakiuchi & Kobayashi, 1971), and albumin, a very common laboratory protein standard, contains none. The contents of proline and glycine in rat protein were also much higher than in albumin; conversely, leucine, lysine, phenylalanine and tyrosine were much lower. The relative abundance of glycine and small amino acids also results in smaller mean residue molecular weights of rat protein and collagen compared with most of the other examples listed. This is an important point, since protein measurement using methods based on the number of peptide bonds (biuret and biuret-based methods like the Lowry *et al.* (1951) method;

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Table 2. *Mean empirical formulas of aminoacyl residues in whole-rat protein and in some selected proteins*

(Breed and diet) Protein source	Mean empirical formula of aminoacyl residues	Mean residue mol. wt.	%N	PF
Experimental data:				
Whole-rat protein:				
Wistar, reference	$C_{4.54}H_{7.09}O_{1.42}N_{1.28}S_{0.06}$	104·0	17·24	5·80
Wistar, high-fat	$C_{4.57}H_{7.13}O_{1.43}N_{1.28}S_{0.06}$	104·6	17·17	5·82
Zucker, reference	$C_{4.57}H_{7.15}O_{1.41}N_{1.29}S_{0.06}$	104·4	17·34	5·77
Zucker, high-fat	$C_{4.51}H_{7.03}O_{1.40}N_{1.29}S_{0.05}$	103·3	17·49	5·72
Zucker obese, reference	$C_{4.49}H_{6.99}O_{1.40}N_{1.28}S_{0.05}$	102·9	17·35	5·76
Zucker obese, high-fat	$C_{4.50}H_{7.02}O_{1.37}N_{1.27}S_{0.06}$	102·8	17·36	5·76
Mean values		103·7	17·33	5·77
Bibliographic data:				
Chicken soluble collagen* (Kakiuchi & Kobayashi, 1971)	$C_{3.71}H_{5.75}O_{1.48}N_{1.21}S_{0.01}$	91·1	18·54	5·39
Bovine muscle myosin* (Bodwell *et al.* 1971)	$C_{5.00}H_{8.19}O_{1.54}N_{1.44}S_{0.03}$	114·0	17·67	5·67
Human haemoglobin A_2 (Ball *et al.* 1966)	$C_{4.91}H_{7.62}O_{1.37}N_{1.33}S_{0.02}$	107·7	17·29	5·78
Rat Glu dehydrogenase* (King & Frieden, 1970)	$C_{4.94}H_{7.70}O_{1.53}N_{1.31}S_{0.03}$	110·7	16·60	6·02
Bovine serum albumin (Spahr & Edsall, 1964)	$C_{5.03}H_{7.90}O_{1.51}N_{1.33}S_{0.07}$	113·4	16·45	6·08
Human fibrogen* (McKee *et al.* 1966)	$C_{4.86}H_{7.45}O_{1.64}N_{1.29}S_{0.05}$	111·7	16·22	6·16
Eukaryotic protein (non-enzyme)* (Reeck & Fisher, 1973)	$C_{4.87}H_{7.54}O_{1.60}N_{1.27}S_{0.05}$	111·0	15·96	6·27
Eukaryotic protein (enzymes)* (Reeck & Fisher, 1973)	$C_{4.89}H_{7.56}O_{1.58}N_{1.25}S_{0.04}$	110·3	15·86	6·30
Beef muscle actin* (Carsten & Katz, 1964)	$C_{4.88}H_{7.61}O_{1.60}N_{1.25}S_{0.04}$	110·5	15·87	6·30
Wool keratin* (Elleman & Dopheide, 1972)	$C_{4.10}H_{6.44}O_{1.53}N_{1.12}S_{0.22}$	102·8	15·28	6·54

PF, protein factor.
* The data for these proteins do not include amide-N.

Peterson, 1979) may yield erroneous estimates of protein weight when used in conjunction with standards of different mean residue molecular weight. The use of bovine serum albumin as weight standard in the measurement of total protein of rat with a biuret method may yield an overestimation of protein of as much as 9·1%.

Other methods for measuring protein are totally or partly based on the colorimetric reaction of a given amino acid residue with a chromogenic reagent. The widely used method of Lowry *et al.* (1951) follows this approach, relying on the reaction of tyrosine residues with the Folin phenol reagent (Peterson, 1979). Again, whole-rat proteins contain about half the tyrosine found in bovine serum albumin, a source of error not directly appreciable but plausibly significant nonetheless.

The analysis of a well-known standard protein, bovine serum albumin (fraction V, Sigma), yielded the results shown in Table 4. The sample contained 35 g water/kg and 956 g protein/kg (determined from N content and amino acid composition); the remainder consisted of other materials (buffer, salts). The N content of the sample was 156·0 (SE 8·8) g N/kg; that of S was 19·6 (SE 2·1) g S/kg. Amide-N content was 0·42 (SE 0·02) mol/kg. The mean measured aminoacyl residue molecular weight was 114·6; the data gave a mean

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Table 3. *Percentage amino acid composition* of reference and whole-rat proteins*

Amino acid‡	Reference†		Rat breed and diet					
	Bovine serum albumin	Chicken leg collagen	Wistar reference	Wistar high-fat	Zucker reference	Zucker high-fat	Zucker obese reference	Zucker obese high-fat
Asx	9·46	5·26	7·52	7·19	7·38	7·46	7·51	6·48
Glx	14·01	8·70	11·20	12·11	11·19	10·50	10·42	9·78
Hyp	0·00	13·83	3·18	2·88	2·89	3·34	3·52	3·49
Ser	4·55	3·08	5·23	5·62	5·44	5·26	5·39	5·02
Gly	2·63	36·54	16·11	15·26	15·47	17·47	17·69	17·43
His	2·98	0·59	1·68	2·12	1·94	1·87	1·65	1·68
Thr	5·60	2·18	3·83	3·88	3·81	3·79	3·26	3·54
Ala	7·71	13·10	7·56	7·55	8·00	7·83	7·92	8·31
Arg	4·03	5·62	4·22	4·19	4·35	4·59	4·54	4·44
Pro	4·90	0·14	8·02	7·33	7·29	7·53	7·60	8·45
Tyr	3·33	0·50	1·46	1·53	1·45	1·59	1·54	1·61
Val	6·13	2·27	4·09	4·38	4·35	3·96	4·46	4·56
Ile	2·45	1·41	3·16	3·32	3·67	3·31	3·41	3·41
Leu	10·16	1·59	6·81	6·69	6·63	6·66	6·43	6·25
Phe	4·55	1·77	2·49	2·61	2·93	2·59	2·57	2·61
Lys	10·16	2·58	6·30	6·22	6·28	5·79	5·37	5·54
Cys	6·30	0·00	3·57	3·52	3·42	3·13	3·28	3·72
Met	0·70	0·86	2·20	2·16	2·11	1·93	2·02	2·30
Trp	0·35	—	1·40	1·40	1·40	1·40	1·40	1·40
Amide-N	4·73	—	4·34	3·94	4·74	4·38	3·85	3·87
Total	104·73	100·00	104·34	103·94	104·74	104·38	103·85	103·87

* The values are given in percentage of aminoacyl residues.

† The data on albumin (Spahr & Edsall, 1964) and collagen (Kakiuchi & Kobayashi, 1972) have been taken from the literature.

‡ Tryptophan, not measured, was estimated from the mean values for 207 proteins (Reeck & Fisher, 1973). Cysteine and methionine values were estimated from the proportions given in the literature for 207 proteins (Reeck & Fisher, 1973) and the direct measurement of sulphur in the samples. No independent glutamine and asparagine data are given; both amino acid skeletons are included, respectively, with the glutamate and aspartate figures (Glx and Asx), and their amide groups are given combined under amide-N, measured separately as indicated on p. 203.

empirical formula of $C_{5\cdot15}H_{7\cdot99}O_{1\cdot49}N_{1\cdot34}S_{0\cdot07}$, a percentage N content of 16·37 and a protein factor of 6·11. The amino acid composition found for this protein was very close to that described in the literature (Spahr & Edsall, 1964). Most amino acids were found in a range of variation lower than 10% of the values given by Spahr & Edsall (1964). The most marked difference was for tryptophan, the calculated value of which was excessively high. In spite of the overestimation of tryptophan, the uniformity of the data obtained with the whole process outlined can be seen in the practical identity of the mean empirical formulas for albumin, using either the data of Spahr & Edsall (1964) or our experimental findings. The molecular weight of the mean amino acid residue was also similar. The global percentage N content found for bovine serum albumin was practically the same as that deducible from the data of Spahr & Edsall (1964), which gave essentially the same protein factor. The tryptophan, methionine and cysteine values incorporated into the calculation for this specific protein are far off the mark, but this does not seem to be the case for a large group of different proteins (Reek & Fisher, 1973). In any case, even if there is a generalized overestimation of tryptophan, the percentage N in the whole protein does not change significantly, both because of the low concentration of tryptophan in proteins and because

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Table 4. *Analysis of bovine serum albumin using the methods for protein evaluation described in the text**

Amino acid	Results found (% published data) Mean	SE
Asx	94·5	4·1
Glx	98·8	1·6
Hyp†	—	
Ser	101·5	2·9
Gly	109·5	3·0
His	107·7	4·3
Thr	94·8	2·3
Ala	94·9	2·5
Arg	100·5	2·7
Pro	106·9	3·5
Tyr	110·2	6·6
Val	113·4	4·2
Ile	97·6	2·0
Leu	90·6	2·8
Phe	105·3	3·3
Lys	99·7	2·9
Cys‡	74·1	
Met‡	382·9	
Trp‡	400·0	
Amide-N	99·6	4·2
Total	101·4	

* The data presented are expressed as percentages of aminoacyl residues divided by the percentages found by Spahr & Edsall (1964) shown in Table 3. For details of analyses, see p. 201.

† Bovine serum albumin does not contain hydroxyproline.

‡ Calculated values.

the percentage N in the tryptophanyl residue is 14·3% (i.e. a protein factor of 7·01). Thus, if the amount of tryptophan in the samples was lower than the value given, the overall N percentage would rise slightly, and the estimated protein factor would, consequently, be even lower than the figures postulated here. On the other hand, since the total amount of S is measured the expected errors in under-evaluation of cysteine may largely correct those incurred in overestimation of methionine; methionyl residues contain 10·7% N (protein factor of 9·37) whilst the values for cysteine are 13·6% N and a protein factor of 7·37. The effect of over- and underestimation of calculated amino acids (tryptophan, cysteine and methionine) in the case of bovine serum albumin results in a deviation in the order of 0·1% in N content, i.e. 0·04 for the protein factor.

The results obtained for a purified and well-known protein help to validate the global calculations deduced from rat protein analyses, and thus reinforce the postulate that the currently used protein factor of 6·25 may not be directly applicable to samples of mixed protein of unknown or undefined amino acid composition.

We have tried to limit the errors induced by the use of the available methodology in our analysis of whole-rat protein. Perhaps extensive analyses of several tissue components would have given a stronger basis for establishing the non-protein sources of error. However, the large number of methodologies applicable, some of which are untried in whole-animal samples, would have made this task impossible. Although the original aims

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of several authors in the literature were different from ours, we have been able to integrate the data from a large number of sources in order to evaluate the extent of the errors incurred when using the protein index as a method for protein quantification. The difference in protein content in a given rat that could be found when applying the protein index we postulate (5·77) or the commonly used 6·25 may amount to 8·3%.

We have found that a significant source of error in earlier estimations of protein N content (and the applicability of the protein factor) lies in the omission of amide-N in the analyses of protein composition. In the case of bovine serum albumin, which contains 0·415 (SE 0·018) mmol amide-N/g protein, the protein factor was found to be 6·12, but when no amide-N was taken into account, the figure rose to 6·34, i.e. a 3·6% increase. The widespread diffusion of protein-amino acid analyses lacking this information on a key component tend to give lower percentages of N for most proteins and may help to explain the, in our view unjustified, continued use of the classical protein factor of 6·25.

The combination of the estimation of non-protein N pool size and the protein factor applicable to whole-rat protein leads to an even lower protein factor: if protein contains only about 94·4% of the rat N, and this N value is converted into a protein value by a factor of 5·77, the protein mass may be calculated from total N by using a corrected protein factor of 5·5. This could result, when using the 6·25 protein factor, in a constant overestimation of protein in whole-rat analyses in the range of 14%, a figure important indeed when measuring protein deposition and body composition changes.

The main conclusions that can be drawn from the experiments presented are the need to estimate protein only after carefully selecting the standards with which to compare the samples, the high N content of rat protein compared with usual standards and the need to bear this in mind when measuring N content and balances, especially in nutritional studies. And last, but not least, the often overlooked importance of non-protein N and of amide-N may profoundly alter the results, and eventually the conclusions, of studies in which no provision for them has been made.

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