VITAMINS AND OTHER NUTRIENTS

Net Protein Ratio Data: AACC–ASTM Collaborative Study

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Seven- and 14-day net protein ratio (NPR) data were obtained from 7 laboratories for 6 protein sources: ANRC casein, lean beef, lactalbumin, textured vegetable protein, and peanut flour were fed as 10% protein (N × 6.25) in the test diet. Wheat flour, casein, and textured vegetable protein were fed as 6% protein (N × 6.25) in the test diet. Weighed dry ingredients for each diet were sent to each collaborator, who mixed the dry ingredients, then added specified amounts of corn oil and water and mixed each complete diet thoroughly. Rats were adapted for 0, 2, or 4 days, and then were fed the test diets for 28 days for protein efficiency ratio (PER) diets. The animal weight gain and feed consumption data obtained after 7 or 14 days of feeding were used to calculate NPR values. Analyses of data were done before [net protein ratio (NPR)] and after (R-NPR [relative-NPR]) adjustment of the data from each laboratory by its results for the reference protein casein. From the analysis of variance for NPR, significant (P <0.05) interactions were observed among laboratories, protein sources, and adaptation times of the animals (0, 2, or 4 days). Inter- and intralaboratory variability were decreased by use of 14-day values compared with 7-day values. Adjustment of the NPR data to R-NPR did not lower the intralaboratory variability but did lower the interlaboratory variability of the data. Increasing adaptation time did not consistently decrease interlaboratory or intralaboratory variability or decrease coefficients of variation (CV) of R-NPR values. The 14-day NPR inter- and intralaboratory variations for the 10% protein diet over all factors (5 protein diets, 3 adaptation periods, and 7 laboratories), as measured by CV values, were 13.2 and 7.7%, respectively. The corresponding R-NPR values were 9.2 and 8.0%, respectively.

Determination of net protein ratio (NPR), a 10-day rat bioassay proposed by Bender and Doell in 1957 (1) for estimation of protein quality, has potential as a standard method. Therefore, NPR with a time modification also was determined during the AACC–ASTM collaborative study on protein efficiency ratio (PER) (2). Hackler et al. (3) have reported the results of the collaborative study on PER. The NPR bioassay is similar to a shortened PER test, except in the NPR bioassay, 1 group of rats is fed a nonprotein diet. The weight loss of the animals fed the nonprotein diet is assumed to be equivalent to the requirements for maintaining rats of the age tested (1, 4–8). This adjustment addresses one of the most serious criticisms of the AOAC PER method (9) for measuring protein quality, which is that the PER assay evaluates proteins for growth (weight gain) only and does not allow for maintenance (10–13).

McLaughlan and co-workers (4, 5, 14, 15) found that the NPR method of Bender and Doell (1) gave essentially the same values as the slope ratio (SR) multiple dose assay of Hegsted and Chang (11, 16, 17) when expressed as a percentage of the values of casein, except for lysine-deficient proteins. The SR assay method produces a relative nutritive value and is considered superior to the PER method (4, 11, 12, 14–16). It was found that the SR procedure may either overestimate [relative nutritive value (RNV)] (18) or underestimate [relative protein value (RPV)] (19) the nutritional quality of lysine-deficient proteins. The NPR procedure yielded higher values than did the SR assay for lysine-deficient proteins (4, 12, 14, 17); the PER assay yielded low values. There is a controversy over which method gives the most appropriate value for lysine-deficient proteins (17, 20).

NPR values have a high correlation with net protein utilization (NPU) values obtained by the method of Bender and Miller (21) for a variety of proteins (22–24). Theoretically, the NPU procedure is probably the most satisfactory of the rat assays because the nitrogen retained in the body is measured directly by nitrogen analyses of the carcass at the termination of the test (22). Henry (23) concluded, however, that for a rapid routine assessment of protein quality, the simpler determination, NPR, is an adequate replacement for NPU.

Lachance et al. (25, 26) reported on studies at Institute of Nutrition of Central America and Panama (INCAP) that indicated a high correlation between NPR and slope ratio data and the PER data obtained by Mertz et al. (27) from 18 samples of grain cereal. He also reported results from similar studies at INCAP, using PER, NPU, and NPR data from 21 legume samples (28). The same approach, comparison of data from PER, NPR, and SR methods, was used for 7 high protein foods under development at INCAP. The correlation coefficients were highly significant, indicating a close relationship between these methods (25, 26).

Two previous collaborative studies on NPR have been reported (10, 17, 29). One, conducted by Samonds and Hegsted (17, 29), compared data for 7 protein sources (lactalbumin, casein, defatted meat, soy flour, a soy protein isolate, wheat gluten, and white flour) from 7 laboratories. The second study, reported by McLaughlan et al. (10), compared data for 6 protein diets (casein plus L-methionine as the reference protein, lactalbumin, egg white, wheat gluten, soybean protein isolate, and wheat gluten and soybean protein isolate) from 6 laboratories.

One of the overall objectives of a third study, the AACC–ASTM collaborative study (2, 3), which is reported here, was to compare NPR values and the precision of the NPR method

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with similar data from the PER method on the same protein sources. The primary objective of the study as reported in the PER section (2, 3) was to better define the specifications of the procedure for determining PER, with the ultimate goal of making some useful changes in the assay. The determination of interlaboratory variation of the data was also of major interest.

Experimental

Seven laboratories collaborated by conducting rat bioassays. The test diets contained either 10 or 6% protein (N × 6.25) (Table 1). The sources and composition of the diets, information on the selection and distribution of the rats, and the environmental conditions of the study were described in an earlier preliminary publication (2) and by Hackler et al. (3).

There were 2 differences between the protocols for determining NPR (Table 1) and PER (3) in this collaborative study: (a) The feeding period for NPR was 7 and 14 days [instead of 7, 14, 21, and 28 days as for PER; 10 days as described by Bender and Doell (1); or 14 days only as used by McLaughlan et al. (10)]. (b) A nonprotein diet was fed to one group of rats for 14 days. NPR calculations were made using the 7- and 14-day weight gain and protein consumption data from each animal fed the test diets and the average weight loss of the 10 animals fed the nonprotein diet, using the following equation (30):

$$\text{NPR} = \frac{\text{wt gain of test animal + av. wt loss of nonprotein group}}{\text{wt protein consumed by test animal}}$$

The above equation was used to calculate the NPR for each rat individually within a trial [instead of for 10 rats collectively (1)], so the intralaboratory variation could be calculated. Thus, it was necessary to use the average weight of 10 animals from the nonprotein diet because an individual animal on the nonprotein diet could not be linked to a specific animal on a test diet.

ANRC casein, the reference standard for the determination of PER by the official method (AOAC) in the United States and Canada, was chosen as the reference protein for calculating relative NPR (R-NPR) and relative PER (R-PER). Casein is readily available worldwide and is standardized for use as the reference protein for PER determinations. The following equations were used to calculate the R-NPR and the R-PER:

$$\text{R-NPR} = \frac{\text{NPR of protein source}}{\text{NPR of ANRC casein (for same week and adaptation period within 1 lab.)}} \times 100$$

Table 1. Protocol for net protein ratio (NPR) study

Variable	10% Protein diets[a]	6% Protein diets[a]
Adaptation period	0, 2, 4 days	2 days
Adaptation diet	10% casein	10% casein
Animals	10 rats/trial	10 rats/trial
Protein sources	ANRC casein	ANRC casein
	freeze-dried lean beef	
	lactalbumin	
	text. veg. protein	text. veg. protein
	peanut flour	wheat flour
	Nonprotein[b]	Nonprotein[b]
Test period	14 days	14 days

[a]Referred to as Test I (10%) and Test II (6%) by Hackler et al. (3).
[b]Nonprotein diet fed for 14 days after 0-, 2-, or 4-day adaptation period on adaptation diet.

$$\text{R-PER} = \frac{\text{PER of protein source}}{\text{PER of ANRC casein (for same week and adaptation period within 1 lab.)}} \times 100$$

Statistical analyses including analysis of variance were carried out, and the intralaboratory variability (repeatability) and interlaboratory variability (reproducibility) were determined by the methods of Youden and Steiner (31) for protein diet (protein sources), adaptation time, for each week individually, and for 7 laboratories. Examination of the data for outlying results yielded no evidence of a consistent bias for any of the 7 laboratories. The intralaboratory variability is a measure of the variability between individual rats, and not between 10-rat NPR replications. Inter- and intralaboratory variability was also determined for each week and adaptation time over all protein diets and for each week over all variables for 7 laboratories.

Results and Discussion

Nonprotein Diet

The data from feeding the nonprotein diet (Figure 1) indicated an increasing mean weight loss of 10 animals over the 7 laboratories with increasing length of the adaptation period of either 0, 2, or 4 days. Mean weight loss standard deviations were similar except for the 2-day-adaptation 14-day-weight-loss data. This is due to a reported mean weight loss of only 45 g per 10 animals from Laboratory 2. The next lowest reported value for this same adaptation–time test period was 106 g. Removing the value of 45 g as an outlier increased the mean for 6 laboratories to 134 g. The SD (standard deviation) value, 17.0 g, and the CV value, 13% (cross-hatched areas on Figure 1), were similar to the data from the other time periods. The CV values for the adaptation periods 0 and 4 days of the second week were generally lower than those of the first week. This was also true of the 2-day adaptation when the 45 g outlier was removed.

Individual Laboratory Data

The NPR mean values for the 10% protein diets for 7 and 14 days (means for 10 rats), intralaboratory SD of the mean, and CV values are given in Table 2 for each laboratory, protein diet, and adaptation period. In general, the 14-day NPR means were lower than those for 7 days. Exceptions (higher 14-day NPR means) can be found in the data reported from Laboratory 2 in the 0-day adaptation group and for Laboratory 6 in the 4-day adaptation group. The CV value is lower for the 14-day mean data for 84% of the values (88 are lower out of 105 comparisons). The 14-day CV values are

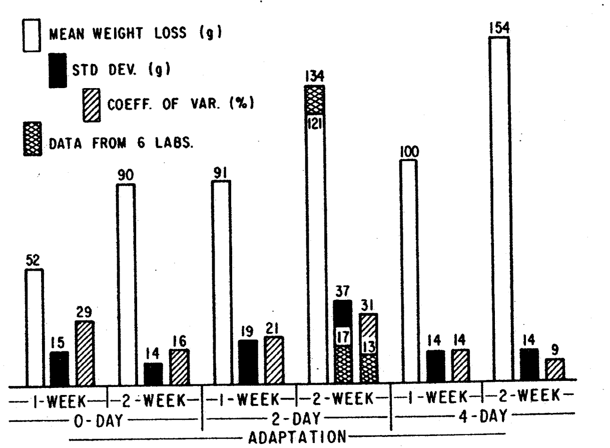

Figure 1. Data from feeding nonprotein diet. Mean weight loss (g) for groups of 10 rats over 7 laboratories.

Table 2. Net protein ratio (NPR) data for 10% protein diets from 7 laboratories[a]

		Casein						Beef					
		7 days			14 days			7 days			14 days		
Adapt. time, days	Lab. No.	Mean	SD[b]	CV,[c] %	Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %
0	1	5.83	0.23	4.0	4.82	0.18	3.7	6.09	0.16	2.7	5.03	0.10	2.1
	2	3.02	2.17	72.0	3.56	0.50	14.0	3.14	2.32	73.8	3.96	0.29	7.3
	3	5.68	0.85	14.9	3.89	0.43	10.9	6.51	0.73	11.2	4.62	0.28	6.1
	4	4.76	0.62	13.0	3.60	0.40	11.1	5.05	1.13	22.3	4.16	0.33	7.9
	5	4.99	0.67	13.5	3.97	0.26	6.5	5.20	0.37	7.0	4.42	0.39	8.8
	6	4.87	0.46	9.4	4.03	0.60	14.9	4.18	2.01	48.2	4.08	0.39	9.6
	7	5.53	0.37	6.7	4.52	0.19	4.1	5.63	0.23	4.1	4.71	0.17	3.6
	Mean	4.9		19.1	4.1		9.3	5.1		24.2	4.4		6.5
2	1	5.75	0.18	3.1	4.47	0.18	4.0	6.19	0.27	4.4	4.88	0.51	10.4
	2	3.90	0.50	12.9	3.22	0.20	6.3	4.35	0.43	10.0	3.67	0.15	4.2
	3	6.48	0.34	5.2	3.84	0.45	11.7	6.94	1.08	15.6	4.72	0.34	7.2
	4	4.50	0.46	10.2	3.81	0.29	7.5	4.90	0.30	6.1	4.02	0.33	8.2
	5	4.72	0.47	10.1	4.02	0.22	5.4	4.53	0.69	15.2	4.18	0.18	4.3
	6	4.06	1.05	25.9	3.79	0.49	12.9	4.37	0.52	11.8	4.07	0.22	5.3
	7	5.67	0.43	7.7	4.55	0.16	3.5	5.69	0.23	4.1	4.48	0.20	4.4
	Mean	5.0		10.7	4.0		7.3	5.3		9.6	4.3		6.3
4	1	5.71	0.31	5.4	4.54	0.18	4.0	6.01	0.20	3.3	4.76	0.16	3.4
	2	4.11	0.66	16.2	3.64	0.23	6.4	4.40	0.50	11.4	4.02	0.15	3.7
	3	5.05	0.70	13.8	3.63	0.30	8.4	5.74	0.43	7.5	4.29	0.15	3.6
	4	4.38	0.49	11.2	3.67	0.13	3.5	5.18	0.36	7.0	4.17	0.22	5.4
	5	4.92	0.27	5.4	3.74	0.23	6.0	4.92	0.45	9.2	3.80	0.35	9.1
	6	4.18	0.70	16.8	3.63	0.34	9.3	3.83	0.77	20.2	4.01	0.40	10.0
	7	5.41	0.23	4.2	4.32	0.09	2.0	5.47	0.24	4.3	4.29	0.14	3.3
	Mean	4.8		10.4	3.9		5.7	5.1		9.0	4.2		5.5
	Overall Mean	4.9		13.4	4.0		7.4	5.2		14.3	4.3		6.1

Table 2. Continued.

Lactalbumin						Peanut flour						Textured vegetable protein					
7 days			14 days			7 days			14 days			7 days			14 days		
Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %	Mean	SD	CV, %
6.01	0.32	5.3	5.27	0.21	4.1	3.38	0.67	19.8	3.00	0.24	8.1	4.84	0.29	6.0	4.23	0.23	5.3
3.56	0.59	16.6	3.98	0.32	8.0	2.38	0.49	20.6	2.33	0.16	6.8	2.85	1.82	63.7	3.25	0.40	12.2
6.19	1.04	16.8	4.68	0.35	7.5	2.67	0.55	20.7	2.27	0.24	10.4	4.88	0.51	10.4	3.76	0.18	4.9
4.03	0.39	9.8	4.04	0.34	8.4	2.96	0.77	26.2	2.21	0.22	9.7	3.31	0.31	9.5	3.11	0.19	6.1
5.05	0.45	8.9	4.49	0.34	7.5	3.09	0.36	11.5	2.53	0.14	5.4	3.95	0.28	7.2	3.50	0.23	6.7
4.19	2.34	55.9	4.39	0.52	11.8	1.96	1.32	67.4	2.24	0.26	11.4	3.58	0.53	14.8	3.46	0.22	6.2
5.56	0.29	5.3	4.95	0.11	2.1	3.06	0.39	12.8	2.95	0.13	4.3	4.35	0.38	8.6	3.93	0.18	4.6
4.9		16.9	4.5		7.1	2.8		25.6	2.5		8.0	4.0		17.2	3.6		6.6
5.96	0.25	4.1	4.98	0.15	3.0	3.62	0.37	10.1	2.83	0.15	5.3	5.00	0.23	4.5	4.00	0.19	4.9
3.72	0.36	9.8	3.49	0.24	7.0	2.34	0.52	22.4	1.77	0.27	15.1	3.58	0.54	15.1	2.98	0.30	9.9
7.12	0.96	13.5	4.71	0.17	3.6	3.25	0.67	20.7	2.49	0.23	9.2	4.99	0.70	14.1	3.79	0.28	7.3
4.43	0.29	6.5	4.28	0.14	3.3	2.40	0.39	16.2	2.03	0.18	9.0	3.24	0.33	10.1	3.06	0.23	7.4
4.44	0.75	16.9	4.40	0.24	5.4	2.92	0.31	10.8	2.47	0.17	7.1	3.81	0.44	11.5	3.47	0.24	6.9
3.56	1.08	30.4	4.01	0.46	11.5	1.92	0.36	18.8	1.99	0.31	15.4	2.90	1.00	34.6	2.92	0.49	16.8
5.75	0.28	4.8	4.90	0.19	3.8	3.29	0.25	7.7	2.87	0.14	4.9	4.59	0.24	5.2	3.74	0.25	6.6
5.0		12.3	4.4		5.4	2.8		15.2	2.3		9.4	4.0		13.6	3.4		8.5
5.93	0.22	3.7	5.06	0.13	2.6	3.54	0.34	9.7	2.89	0.27	9.5	4.81	0.25	5.2	3.96	0.14	3.5
4.40	0.46	10.5	4.17	0.34	8.2	2.59	0.44	16.8	2.41	0.22	9.2	3.80	0.60	15.8	3.37	0.16	4.8
5.88	0.54	9.3	4.41	0.23	5.2	2.82	0.48	16.9	1.98	0.32	16.0	4.62	0.70	15.1	3.25	0.51	15.8
4.84	0.31	6.4	4.12	0.22	5.4	2.47	0.47	19.0	2.24	0.20	8.7	3.57	0.39	11.0	3.06	0.19	6.3
4.87	0.57	11.6	4.01	0.47	11.8	2.89	0.25	8.7	2.19	0.17	8.0	4.10	0.49	11.9	3.12	0.25	8.0
3.69	1.11	30.2	3.86	0.46	12.0	1.66	0.89	53.4	2.35	0.28	11.8	2.51	1.01	40.0	2.89	0.55	19.1
5.46	0.46	8.4	4.61	0.19	4.1	3.27	0.22	6.6	2.72	0.18	6.7	4.35	0.26	6.0	3.49	0.27	7.8
5.0		11.4	4.3		7.0	2.7		18.7	2.4		10.0	4.0		15.0	3.3		9.3
5.0		13.6	4.4		6.5	2.8		19.8	2.4		9.1	4.0		15.2	3.4		8.1

[a]Each NPR mean is the average value for 10 rats.
[b]SD = standard deviation. The SEM (standard error of the mean) can be calculated by the following equation:

$$SEM = \frac{SD}{\sqrt{10}} = \frac{SD}{3.1623}$$

[c]CV = coefficient of variation $= \frac{SD}{mean} \times 100$

frequently lower by a factor of 2 or more for Laboratories 2 and 6, particularly for the 0-day and 4-day adaptations, respectively. Six CV values were about the same for both the 7- and 14-day data. Generally, the data indicated that extending the NPR test to 14 days decreased the SD and CV values, showing an increased precision. The level of variability (precision) is rather consistent in several laboratories for the 10% diets for all protein sources.

Six laboratories (Laboratories 1–4, 6, and 7) exhibited considerably higher variability for the 6% casein diet (Table 3) compared with the 10% casein diet (Table 2, 2-day adaptation, 14-day CV data). However, only 4 laboratories (Laboratories 1–4) showed greater variability for the 6% diet data of textured vegetable protein (Table 2 [2-day adaptation, 14-day CV data] and Table 3).

Analysis of Variance

An analysis of variance (ANOVA) was performed on both the 7- and 14-day NPR and R-NPR data over 5 protein diets, 3 adaptation periods, and 7 laboratories for the 10% diets and over 3 protein diets, one adaptation period (2-day), and 7 laboratories for the 6% diets (Table 4). When the 10% diets were fed, there were significant effects due to laboratories and diets and a significant interaction for laboratory-by-adaptation and laboratory-by-diet for the first week feeding. There was a large increase in the effect of diet in the second week which tended to overshadow (lower) the significance of the laboratory effect and the relatively small effect of adaptation time. Laboratory-by-diet was the most significant interaction. The R-NPR data for 10% protein diets also showed the highly significant effect of diet. The other individual effects and the laboratory-by-diet interaction were lowered by adjustment to the R-NPR.

The analysis of variance for the 6% protein diets showed significant effects due to laboratories and diets for the 7- and 14-day NPR values and a significant laboratory-by-diet interaction. Analysis of the R-NPR data indicated a highly significant diet effect and a significant laboratory-by-diet interaction. The significance of the laboratory-by-diet and laboratory-by-adaptation interaction terms means that the effects of diet and adaptation changed from laboratory to laboratory. This is quite evident from Table 2.

Two-Week Statistical Summary for Each Protein Source and Adaptation Period

Fourteen-day NPR intralaboratory variability data (mean, SD and CV) for each protein source and adaptation period for all laboratories are given in Table 5. Calculating R-NPR did not improve the intralaboratory variability of the NPR for protein source and/or individual adaptation periods as measured by the CV value (Table 5).

Interlaboratory variation or interlaboratory error (reproducibility) is defined by Youden and Steiner: "Reproducibility is determined by the sum of between-laboratory error, interaction, and within-laboratory error" (31). The interlaboratory variability data for each protein diet and adaptation period determined from analysis of data for all laboratories are also shown in Table 5.

The mean values are the same for calculating either intra- or interlaboratory variability. The lowest variability (interlaboratory CV of NPR) was found for the 4-day adaptation groups for casein, beef, and lactalbumin and in the 0-day adaptation groups for peanut flour and textured vegetable protein. Calculating the R-NPR value produced lower interlaboratory CV values for nearly all protein sources, adaptation periods, and the 2 levels of protein. The 2-day adaptation period probably would be the most practical for a minimum time–cost test. The analysis of variance indicated that variability due to laboratory or diet effects had higher significance than those of the adaptation period. The interlaboratory variation (CV) in this study for lactalbumin NPR was higher for 0- and 2-day adaptation data; about same for 4-day adaptation NPR data, but lower for all R-NPR data than that reported in a previous study (10). Lactalbumin was the only protein source common to both studies.

Table 3. Net protein ratio data for 6% protein diets from 7 laboratories[a]

	7 days			14 days		
Lab.	Mean	SD	CV, %	Mean	SD	CV, %
			Casein Diet			
1	5.90	0.39	6.6	4.80	0.66	13.8
2	4.32	0.33	7.6	3.20	0.35	10.9
3	5.79	1.32	22.8	4.49	0.65	14.5
4	3.90	0.48	12.3	3.94	0.45	11.4
5	4.94	0.44	8.9	4.08	0.23	5.6
6	3.04	0.99	32.6	2.93	0.80	27.3
7	5.98	0.50	8.4	4.80	0.34	7.1
Mean	4.8		14.2	4.0		12.9
			Wheat Flour Diet			
1	3.38	0.59	17.4	2.71	0.46	17.0
2	2.37	0.48	20.2	0.69	0.30	43.5
3	3.75	1.19	31.7	2.31	0.39	16.9
4	2.57	0.67	26.1	2.00	0.31	15.5
5	2.73	0.41	15.0	2.27	0.30	13.2
6	2.40	0.74	30.8	2.20	0.47	21.4
7	3.73	0.54	14.5	2.55	0.21	8.2
Mean	3.0		22.2	2.1		19.4
			Textured Vegetable Protein Diet			
1	4.77	0.56	11.7	4.07	0.33	8.1
2	3.84	0.38	9.9	2.69	0.40	14.9
3	3.78	0.98	25.9	3.53	0.41	11.6
4	3.61	0.81	22.4	3.62	0.30	8.3
5	4.47	0.44	9.8	3.95	0.21	5.3
6	3.14	0.57	18.2	3.26	0.39	12.0
7	4.82	0.41	8.5	4.09	0.26	6.4
Mean	4.1		15.2	3.6		9.5

[a]Each NPR mean is the average value for 10 rats.

Table 4. Analysis of variance on NPR and R-NPR data

		NPR						R-NPR					
		7 days			14 days			7 days			14 days		
Source[a]	Error term[b]	Sum of sq.	df	F^c	Sum of sq.	df	F^c	Sum of sq.	df	F^c	Sum of sq.	df	F^c
10% Protein Diet													
A	MS_{AL}	1.91	2	<1	7.98	2	3.1	477.90	2	<1	470.96	2	<1
L	MS_e	543.28	6	175.4**	119.60	6	225.2**	29564.01	6	16.5**	9707.21	6	31.0**
D	MS_{LD}	833.40	4	74.4**	558.65	4	281.2**	303717.77	3	163.6**	349878.60	3	303.8**
A × L	MS_e	54.92	12	8.9**	15.67	12	14.8**	7646.72	12	2.1*	3170.50	12	5.1**
A × D	MS_{ALD}	1.54	8	<1	0.96	8	1.2	911.11	6	<1	456.93	6	1.0
L × D	MS_e	67.24	24	5.4**	11.92	24	5.6**	11137.92	18	2.1**	6909.76	18	7.4**
A × L × D	MS_e	24.83	48	1.0	4.64	48	1.1	8600.89	36	<1	2646.40	36	1.4*
Error		487.80	945		83.63	945		225807.23	756		39440.80	756	
Total		2014.92	1049		803.05	1049		587863.56	839		412681.17	839	
6% Protein Diet													
L	MS_{LD}	99.30	6	8.1**	62.76	6	10.2**	8112.76	6	1.5	17794.33	6	4.1
D	MS_e	120.55	2	128.3**	143.67	2	409.2**	22371.40	1	128.5**	51649.15	1	590.9**
L × D	MS_e	24.58	12	4.4**	12.28	12	5.8**	5533.20	6	5.3**	4325.07	6	8.2**
Error		88.77	189		33.18	189		21933.58	126		11013.98	126	
Total		333.20	209		251.90	209		57950.94	139		84782.53	139	

[a]A = adaptation; L = laboratory; D = diet.
[b]Appropriate mean square (MS = ss/df) to calculate F (e.g., for adaptation, $F = MS_A/MS_{AL}$).
[c]* = ($P < 0.05$); ** = ($P < 0.01$).

Statistical Summary for Each Week and Adaptation Period Over 10 and 6% Diets

Data for each adaptation period and each week over the 5 protein diets fed at the 10% level (table not included) showed that: The NPR mean over all protein sources was lowered by extending the feeding period to 14 days; the 14-day NPR was less variable than the 7-day NPR; the 4-day adaptation 14-day NPR was the least variable and the 0-day adaptation was nearly as precise. The NPR following the 2-day adaptation had the highest variability, but the mean values for all adaptations had a maximum difference of only 0.21 NPR. Calculation of the R-NPR lowered the interlaboratory CV values in all but 1 adaptation period (7-day R-NPR, 0-day adaptation), which remained the same. The differences between the interlaboratory CV values for 14-day NPR and 14-day R-NPR are 4.2, 4.7, or 2.9% for the 0, 2, or 4-day adaptation groups, respectively.

Statistical Summary for Each Week Over All Factors (Adaptation Period, Protein Diets, and Laboratories for 10% and 6% Diets)

The intra- and interlaboratory variability over all factors for the five 10% and three 6% protein diets are given in Table 6. Again the calculation of R-NPR showed a lowering of the

Table 5. 14-day NPR and R-NPR data for 7 laboratories

		Intralaboratory						Interlaboratory			
		NPR		R-NPR		CV,%		SD		CV,%	
Diet	Adapt., days	Mean[a]	SD	Mean[a]	SD	NPR[b]	R-NPR[b]	NPR[b]	R-NPR[b]	NPR[b]	R-NPR[b]
10% Protein											
Casein	0	4.06	0.39	100.00	10.25	9.7	10.3	0.60	10.49	14.8	10.5
	2	3.96	0.31	100.00	8.08	7.8	8.1	0.53	8.37	13.4	8.4
	4	3.88	0.23	100.00	6.18	5.9	6.2	0.44	6.32	11.3	6.3
Beef	0	4.43	0.30	109.52	7.66	6.7	7.0	0.57	9.49	12.9	8.7
	2	4.29	0.30	108.77	7.33	7.0	6.7	0.52	10.49	12.1	9.6
	4	4.19	0.25	108.35	6.57	5.9	6.1	0.39	8.94	9.3	8.3
Lactalbumin	0	4.54	0.33	112.19	8.56	7.3	7.6	0.57	8.94	12.6	8.0
	2	4.39	0.25	111.08	6.58	5.7	5.9	0.58	8.37	13.2	7.5
	4	4.32	0.32	111.50	8.58	7.4	7.7	0.51	10.00	11.8	9.0
Peanut flour	0	2.50	0.20	61.72	5.04	8.1	8.2	0.39	6.32	15.6	10.2
	2	2.35	0.22	59.03	5.77	9.2	9.8	0.48	7.75	20.4	13.1
	4	2.40	0.24	61.71	6.29	10.0	10.2	0.39	7.07	16.2	11.5
Textured vegetable protein	0	3.61	0.24	89.01	6.28	6.7	7.1	0.44	7.07	12.2	7.9
	2	3.42	0.30	86.65	7.78	8.6	9.0	0.52	10.49	15.2	12.1
	4	3.31	0.34	85.27	9.06	10.2	10.6	0.46	10.00	13.9	11.7
6% Protein[c]											
Casein	2	4.03	0.53	100.00	14.50	13.2	14.5	0.90	14.49	22.3	14.5
Wheat flour	2	2.10	0.36	51.98	9.63	17.1	18.5	0.75	18.17	35.7	35.0
Textured vegetable protein	2	3.60	0.34	90.40	9.06	9.3	10.0	0.60	13.78	16.7	15.2

[a]Means of 70 data points, 7 laboratories, 10 rats per laboratory.
[b]The means for intra- and interlaboratory variability of NPR for 1 adaptation period, diet, and 7 laboratories are the same. The R-NPR means are the same. Casein was given the value of 100 for the calculation of R-NPR.
[c]NPR determined following a 2-day adaptation period only.

Table 6. Intra- and interlaboratory variability for NPR data over all factors

Ratio calcd	Mean	SD Intralab.	SD Interlab.	CV,% Intralab.	CV,% Interlab.
		10% Protein Diets[a]			
NPR 7[b]	4.37	0.72	1.11	16.5	25.4
NPR 14[c]	3.71	0.29	0.49	7.7	13.2
R-NPR 7[b]	88.67	17.74	18.62	20.0	21.0
R-NPR 14[c]	93.65	7.46	8.66	8.0	9.2
		6% Protein Diets[d]			
NPR 7[b]	3.96	0.69	1.05	17.4	26.5
NPR 14[c]	3.25	0.42	0.76	12.9	23.4
R-NPR 7[b]	82.26	14.47	16.26	17.6	19.8
R-NPR 14[c]	80.79	11.33	15.44	14.0	19.1

[a]Five protein diets, 7 laboratories, 3-adaptation periods (0, 2, and 4 days), 10 rats/trial (1050 values).
[b]7-day data.
[c]14-day data.
[d]Three protein diets, 7 laboratories, 10 rats/trial, and 2-day adaptation period (210 values).

variability, indicating an increase in the precision of the interlaboratory data, for example, 4.4 and 4.0 percentage units for the 7-day and 14-day data, respectively, for the 10% diets.

The interlaboratory CV values for the 6% diets over the 3 protein sources (casein, wheat flour, and textured vegetable protein), 7 laboratories, and 2-day adaptation period (Table 6) were high. Calculating the R-NPR values for these diets lowered the interlaboratory CV values 4.3%.

Comparison of R-NPR and R-PER Data

R-NPR and R-PER data were compared (Table 7). The R-NPR, PER, and R-PER data from 7 laboratories, 10 and 6% protein diets, and 2-day adaptation period are based on 70 data points. The 7-laboratory R-NPR data over all 3 adaptation periods are based on 210 data points. All data are relative to casein which was given the value of 100. The R-PER data were calculated from the actual PER values obtained for the different test proteins and casein for the feeding and adaptation time periods specified, and not from PER values that were adjusted with the PER value of 2.5 assigned to casein. The latter procedure is commonly used to adjust PER values used for nutritional labeling. The use of either adjusted or unadjusted PER values will give the same R-PER values. The official method for calculating PER, although not used for nutritional labeling, specifies that the unadjusted PER of the test is expressed as a percentage of the unadjusted PER obtained for the reference protein, ANRC casein (9). Thus, the R-PER values shown in Table 7 have been calculated by the official method and the R-NPR values were calculated similarly. There are similarities and differences in the R-NPR and R-PER. The 14-day R-NPR and the 28-day R-PER values are similar for the high quality proteins, lactalbumin, beef, and casein. The data for textured vegetable protein indicate a higher R-NPR than either the 14-day or 28-day R-PER for both the 10 and 6% diets. Peanut flour had the greatest difference among the 10% diets, at least 13 or 14 NPR units higher than the 14- or 28-day R-PER, respectively. Wheat flour exhibited a dramatic difference between R-NPR and R-PER values.

Conclusions

The following conclusions can be drawn from the data in this collaborative study: *(1)* The analysis of variance for the NPR indicated significant ($P < 0.05$) effects due to the factors (laboratories, protein sources, and adaptation times of the animals for 0, 2, or 4 days) and interactions among the factors. The most significant effect identified was between protein diets, indicating that the NPR assay was finding a difference between proteins. The most significant interaction among the factors was laboratory-by-protein diet. *(2)* Inter- and intralaboratory precision were increased by feeding for 14 days compared with 7 days. Therefore, a 14-day NPR test would be advantageous. *(3)* Although results from the 4-day adaptation period were more precise than those from either the 0-

Table 7. Comparison of R-NPR and R-PER data

Diet	R-NPR 14 Days Adapt., days 2	R-NPR 14 Days Overall	PER[a] (2-Day adaptation) 14 Day	PER[a] (2-Day adaptation) 28 Day	R-PER[a] (2-Day adaptation) 14 Day	R-PER[a] (2-Day adaptation) 28 Day
			10% Protein Diet[b]			
Casein	100	100[c]	3.26	2.97	100	100
Beef	108	108[c]	3.66	3.14	112	106
Lactalbumin	111	111[c]	3.73	3.31	114	111
Peanut flour	59	61[c]	1.51	1.35	46	45
Text. veg. protein	86	87[c]	2.68	2.39	82	80
			6% Protein Diets[b]			
Casein		100	2.51	2.25	100	100
Wheat flour		52	−0.15	0.04	−6	2
Text. veg. protein		89	2.09	1.90	83	84

[a]Unadjusted to a PER value of 2.5 assigned to casein.
[b]Based on means of 70 data points (7 labs, 1 adaptation period, and 10 rats/trial).
[c]Based on means of 210 data points (7 labs, 3 adaptation periods, and 10 rats/trial).

or 2-day adaptation period, the differences were small and it would not be of practical significance to recommend a 4-day adaptation period. *(4)* A 2-day adaptation period for the rats to adjust to their environment and to recover from shipping stresses before initiation of the test would be more practical for a minimum time-cost NPR assay. *(5)* Adjustment of the NPR data to R-NPR did not improve the intralaboratory precision, but did increase the interlabortory precision of the data. *(6)* The NPR inter- and intralaboratory variation for the 10% diets over all factors were 13.2 and 7.7%, respectively, as measured by CV values, about the average for that of the 3 adaptation periods. The R-NPR inter- and intralaboratory variation over all factors, as measured by CV values were 9.2 and 8.0%, respectively. *(7)* Fourteen-day R-NPR and 28-day R-PER values agree closely for high quality protein sources, less closely for a medium quality protein source, and show large differences for the lower quality protein sources (peanut flour and wheat flour) in this study. *(8)* The NPR assay can be used as an alternative method to PER for the determination of protein quality. This conclusion is made despite knowledge that the R-NPR may overestimate the quality of lysine-deficient protein sources (4, 12, 15, 17, 18, 20). This is also the case for other rat bioassays (4, 19).

Reference Standard for NPR and R-NPR Determinations

Casein plus L-methionine was used by McLaughlan et al. (10) as the reference protein for NPR assay in a recent collaborative study and by Sarwar and McLaughlan (32) in a study of variables for the NPR method. Casein and purified L-methionine are each readily available to provide a mixture that can be used as a reference standard for the rat, with an NPR approaching that of the highest quality protein sources. High quality protein sources such as lactalbumin and egg white are not readily available as standardized products. Consideration could be given to using casein plus L-methionine as the reference protein in an official method for NPR and R-NPR. However, the greatest disadvantage is that there is little experience in feeding casein plus L-methionine to humans, and it becomes a contrived reference protein for the determination of protein quality or for ranking the quality of protein sources for human consumption and regulatory purposes. The average level of sulfur amino acids (s.a.a.) in the ANRC casein plus L-methionine used in the McLaughlan et al. (10) collaborative study probably was about 46 mg/g protein. Rama Rao et al. (33) found that the rat required about 50 mg s.a.a./g protein for maximum growth. The assumption that a mixture of casein and L-methionine reacts the same nutritionally in humans (infants and children) as in weanling rats is questionable.

The Food and Nutrition Board (FNB) estimated the total s.a.a. content of an ideal protein for humans as 26 mg/g protein (34). Pineda et al. (35) found that 27 mg methionine plus cystine/kg body weight (this translates to 27 mg s.a.a./g protein in a scoring pattern) when fed as milk or milk plus synthetic s.a.a. to children 21–27 months old, was similar to "recommended" or "safe levels" of intake rather than mean requirements. Torun et al. (36) used a level of 27 mg s.a.a./g protein (a value close to that of FNB) in an amino acid scoring pattern for 2-year-old children. The sample of ANRC casein [92.6% protein (N × 6.25)], the reference protein in this AACC–ASTM collaborative study, contained about 38 mg s.a.a./g protein (M. L. Happich (1979) unpublished data). Thus, ANRC casein without added methionine contained adequate s.a.a. and can be considered an appropriate reference protein for the evaluation of protein quality for human consumption, including young children. The use of casein plus L-methionine introduces a bias in relation to the evaluation of proteins for humans. It is appropriate that casein be the reference protein for NPR determinations (and that casein be continued as the reference protein for PER). Continuing with casein allows for direct comparisons with values already in the literature from past research and with values obtained for past production and regulatory control. A protein source that has higher protein quality than casein (or casein plus L-methionine), e.g., egg white, can still be evaluated.

Recommendation

From the results obtained in this study, the net protein ratio (NPR) method, following the general procedure used in this study but with the 5 specifications listed below, is recommended as an alternative method to the PER assay for determination of protein quality. The 5 specifications are as follows: (a) that the test diet contain 1.6% nitrogen originating solely from the test protein source; (b) that a 14-day NPR be determined by feeding the test protein for 14 days; (c) that there be a 2-day adaptation period; (d) that ANRC casein be used as the reference protein for the assay; and (e) that the NPR be expressed as R-NPR by calculating the ratio × 100 of the NPR for each assay group to the NPR for the reference ANRC casein group, and reported as R-NPR.

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