Determination of the Relative Nutritive Value of Proteins

Factors Affecting Precision and Validity

D. M. Hegsted, Raymond Neff, and Jane Worcester

The method for the estimation of the relative nutritive value (RNV) of protein utilizing the slope-ratio assay with young rats has been described in detail. A computer program has been written for the easy evaluation of the validity and precision of the results obtained. Comparisons of the results obtained with weight gain, body water, and body nitrogen as measures of response demonstrate generally similar results. In view of the ease with which body water may be determined, it is probably the metameter of choice. The relative precision which may be expected when six proteins are assayed utilizing different numbers of animals per test protein has been determined. As few as nine animals per test protein (three animals per group, three levels of each protein) yield reasonably satisfactory results.

EARLIER papers of this series (Hegsted and Chang, 1965, a and b; Hegsted and Worcester, 1967) have described a slope-ratio assay for the assessment of the relative nutritive value (RNV) of proteins using young rats. The validity, precision, and cost of an assay will depend primarily upon the number of animals used and the measure of response to the dietary protein that is selected. Three measures of response to dietary protein have been commonly employed. Gain in weight has been most widely used but will be unsatisfactory if the percentage of protein in the body is influenced by the diet fed. Measurement of body protein is usually presumed to be the measure of choice but this is expensive and relatively difficult to determine. Several authors (Bender and Miller, 1953; Dreyer, 1957, 1962; Henry and Toothill, 1962) have concluded that body water is so closely correlated with body protein that it may be used to predict body protein and, since it is relatively simple to determine, may be the measure of choice.

Precision will fall as fewer animals are used. It is useful to have estimates of the precision that will be achieved when varying numbers of animals are employed. Data are presented here indicating the relative precision of the assay when various numbers of animals are used as well as estimates of the suitability of body weight gain, body water, and body nitrogen as the metameters of response to dietary protein.

EXPERIMENTAL

The procedure used in these assays was as follows: for the assessment of five unknown proteins, 114 weanling rats were used. These were divided into 19 groups of similar weight containing six rats each. One group received the diet containing no protein (blank). Three groups received diets containing approximately 4, 7, and 10% lactalbumin (General Biochemicals, Chagrin Falls, Ohio). These levels supplied approximately 3, 5.4, and 7.7% of protein (N × 6.25) and served as the standard. The amount of protein supplied by this and all proteins assayed was determined by prior Kjeldahl analysis. Each unknown protein was fed to three groups of rats at three different levels. The levels were selected to cover the widest range possible but with the highest level below that which allowed maximum growth. Food consumption was measured for each animal. The animals were weighed twice weekly although only the final weights have been utilized in the analysis. Total protein consumption was calculated for each animal from the protein content of the diet and food consumed.

The protein-free diet contained corn starch, 84.3%; hydrogenated vegetable oil, 9.5%; cod liver oil, 0.5%; salt mixture (Hegsted *et al.*, 1941), 5%; choline chloride, 0.2%; and vitamin mixture (Chang and Hegsted, 1964), 0.5%. Usually, the basal dietary mixture was made of all of these constituents except for some of the corn starch. The final diets were then made by combining appropriate amounts of the basal mixture, the protein source, and sufficient starch to complete the diet. None of the nonprotein constituents in the diet are believed to be at critical levels. Any diet which supports good growth with an adequate protein supply should be usable since in each assay the response of the unknown proteins is compared with that of the standard, lactalbumin.

The animals were killed with ether on the 21st day. They were weighed, the stomach and cecum removed and discarded, and the remaining carcass reweighed. The carcasses were placed in plastic sandwich bags and frozen until analyzed. The decision to utilize a 21-day test period and to discard the stomach and cecum with their contents was made arbitrarily.

The frozen carcasses were chopped or sliced into relatively small pieces, placed in tared beakers, weighed, and dried at 95° C. until constant weight was obtained. For larger animals this required approximately 3 days.

Departments of Nutrition and Biostatistics, Harvard School of Public Health, Boston, Mass. 02115

Approximately 6*N* HCl or H_2SO_4 was placed in the beakers which were heated on a hot plate until dissolution began. The contents were then transferred to covered quart jars utilizing approximately 250 ml. of acid in all, and the jars were autoclaved at 20 pounds of pressure for 3 hours. After cooling, the contents were transferred to liter volumetric flasks with water. They were made to volume with minimal mixing, and the fat and insoluble material which floats in the neck was removed with suction. A few milliliters of the toluene added to the flasks before they were made to volume helped remove the fat. If care was taken, there was little mixing, and the insoluble material could be removed with little loss of soluble nitrogen. The contents of the flask were then mixed, and a small portion was filtered and preserved for nitrogen analysis. The automated Kjeldahl analysis with the AutoAnalyzer (Technicon Instruments Corp., Chauncey, N.Y.) was utilized.

The automated procedure gave consistently low results when compared with results obtained with manually performed micro-Kjeldahl analysis. While this did not affect the precision of the assay or the results obtained, the authors have preferred to utilize as standards for the automated procedure a series of hydrolyzates which were analyzed by the manual procedure. Comparable results were then obtained. It should be stressed that the validity of the assay and the accuracy obtained are not dependent upon the absolute levels of nitrogen obtained. The assay demands only consistent results regardless of the procedure followed.

The slope-ratio analysis described by Finney (1964) includes the calculation of the linear regression line, $y = a + bx$; for each protein; y is the response and x is the amount of protein consumed by each rat. All lines go through the same intercept, a. The ratio of the slope, b, for each unknown protein to that of the standard is the measure of potency. The standard error of the ratio which, of course, is influenced by the consistency of the data obtained with both the standard and unknown proteins is a measure of the precision of the assay. The complete analysis of variance also yields tests for curvature (whether the regression lines depart significantly from linearity), intersection (whether the several regression lines depart significantly from a common intersection), and blanks (whether the intersection of the regression lines with the y axis departs significantly from the value obtained with the blank, the value obtained with the protein-free diet). Statistically significant values for any of these tests indicate a less than ideal assay. However, the more extensive the data and the more animals utilized per protein tested, the more likely it is that statistically significant deviations from the ideal assay can be shown.

As previously explained (Hegsted and Worcester, 1967), the statistical treatment differs somewhat from that described by Finney (1964) since the dosage of protein (the amount of protein consumed) is not constant for animals fed the same diet. Rather, there is a significant regression between protein consumed and response within each group. Summations must therefore be taken over the individuals rather than groups. The analysis (details of the computer program are available on request) differs from Finney's in that the curvature includes only quadratic terms; the error term, instead of coming from the within group sum of squares, is computed from the sum of squares of the residuals from the blank mean and from the separate regressions with the quadratic terms included.

RESULTS

The estimated potency obtained from the slope ratios by the method described is defined as the relative nutritive value (RNV) to distinguish it from other methods of evaluating the nutritive value of proteins. The results have been expressed as decimals although it may sometimes be preferable to multiply by 100 and express them as percentages. The results of three typical assays which were evaluated utilizing body water, body nitrogen, and weight gain as the measure of response are shown in Table I. Generally speaking, similar results were obtained regardless of the measure of response, although the use of body nitrogen often resulted in somewhat lower estimates of nutritive value than either body water or weight gain. The reasons for this are not clear since body water, body nitrogen, and weight gain are highly correlated within any particular experiment. In seven experiments previously evaluated (Hegsted and Worcester, 1967), the correlation coefficients between weight gain and body nitrogen ranged from 0.98 to 0.91 (average 0.956), and the correlations between body water and body nitrogen ranged from 0.99 to 0.95 (average 0.981). Over-all correlation coefficients obscure minor differences within groups or within animals fed different proteins. However, only occasionally are the results based upon body nitrogen much different from those based upon body water or weight gain.

The standard error of the relative potency with this schedule usually falls between 1.5 and 3.0% yielding confidence limits which are usually about ± 3 or 4%. The standard errors of the results based upon weight gains were the smallest, those upon body water intermediate, and those based upon body nitrogen the largest in practically every instance. The reason for the smaller errors when weight gain is utilized is probably because the calculation of weight gain automatically takes into account the variation in size of the animals at the start of the experiment whereas the values used for body water and body nitrogen do not. The precision of the assay using body nitrogen or water presumably could be improved by analyzing a group of animals at the start of the experiment, using these values to calculate the original body nitrogen or water of each animal, and then calculating the gain in body water or nitrogen of each animal during the experiment. Whether the results justify the extra calculation is a matter of judgment and would be more important when there is substantial variation in the starting weights.

Complete analyses of variance of the same three experiments are shown in Table II. None of the assays based upon body nitrogen departed significantly from linearity, intersection, or blanks indicating that they adequately fulfill the criteria of a good assay. The data obtained using body water and weight gain as the criteria of response were slightly less satisfactory. Departure from blanks was commonly found with these measures of response. It can be shown, however, that this does not affect the assay value obtained in any important degree by calculating the slope ratios without the blank included. Furthermore, assuming

Table I. A Comparison of Body Water, Body Nitrogen, and Weight Gain as Measures of Response in the Assay of Several Proteins

Expt.	Protein	Protein, %	Water RNV[a]	Water Standard error	Water 95% Fiducial limits	Nitrogen RNV[a]	Nitrogen Standard error	Nitrogen 95% Fiducial limits	Weight Gain RNV[a]	Weight Gain Standard error	Weight Gain 95% Fiducial limits
6	Lactalbumin	76.96	1.000	...	...	1.000	...	...	1.000	...	...
	Cottonseed flour No. 1	51.62	0.485	0.013	0.459–0.512	0.423	0.016	0.392–0.454	0.490	0.014	0.462–0.518
	Cottonseed flour No. 2	37.72	0.652	0.014	0.624–0.680	0.570	0.017	0.537–0.602	0.661	0.015	0.631–0.691
	Cottonseed flour No. 2 (cooked, high temp.)	37.65	0.659	0.014	0.631–0.687	0.625	0.017	0.592–0.657	0.654	0.015	0.625–0.683
	Cottonseed flour No. 2 (low temp.)	36.87	0.609	0.015	0.580–0.638	0.543	0.017	0.510–0.577	0.615	0.015	0.584–0.645
	Peanut meal	48.36	0.540	0.011	0.518–0.562	0.486	0.013	0.460–0.511	0.555	0.012	0.531–0.578
7	Lactalbumin	76.96	1.000	...	...	1.000	...	...	1.000	...	...
	Full fat soya flour	39.37	0.579	0.016	0.548–0.610	0.587	0.018	0.551–0.623	0.582	0.013	0.555–0.608
	Cottonseed, corn, sorghum mix	22.81	0.513	0.019	0.476–0.549	0.480	0.021	0.438–0.521	0.529	0.016	0.498–0.561
	Fish flour	71.93	0.829	0.025	0.800–0.879	0.811	0.029	0.755–0.868	0.796	0.021	0.754–0.837
	High protein rice	19.09	0.442	0.023	0.397–0.487	0.448	0.026	0.396–0.500	0.447	0.019	0.408–0.485
	Lactalbumin +10% cellulose	76.96	0.935	0.027	0.883–0.988	0.979	0.032	0.916–1.042	0.919	0.023	0.875–0.964
8	Lactalbumin	76.96	1.000	...	...	1.000	...	...	1.000	...	...
	Coconut flour	19.13	0.639	0.027	0.584–0.693	0.482	0.030	0.423–0.541	0.615	0.023	0.569–0.660
	Coconut protein concentrate	39.29	0.551	0.032	0.489–0.613	0.455	0.035	0.387–0.523	0.523	0.027	0.471–0.575
	Full fat soya flour	39.37	0.640	0.028	0.584–0.696	0.588	0.031	0.528–0.649	0.648	0.024	0.601–0.695
	Full fat soya flour +10% cellulose	39.37	0.603	0.026	0.553–0.654	0.575	0.028	0.520–0.629	0.595	0.021	0.552–0.637
	Blood albumin	86.00	0.112	0.010	0.092–0.131	0.108	0.011	0.087–0.130	0.105	0.008	0.099–0.121

[a] Relative nutritive value (slope ratio or slope of the regression of the unknown compared to lactalbumin).

a protein-free diet produces a nontypical response, an assay might be devised without the use of blanks. This would require that the intersections of the various regression lines are similar, and fortunately this is usually found. However, weight gain seems clearly to yield data less satisfactory in terms of an ideal assay. Curvature was noted in one assay and significant differences in intersection seen in two of the three assays. Clear explanations for these differences in the usefulness of the various criteria of response are not easily derived in view of the previously mentioned correlations between them and the fact that they yield similar assay results.

Table III shows the results of three assays utilizing different numbers of animals in each assay group. Each protein is customarily assayed with three groups, each containing six animals, or 18 animals per protein. The first columns show the results of the standard assay based upon body nitrogen together with the standard error of the RNV. One animal was removed from each group on a random basis, yielding five animals per group or 15 animals per protein assayed, and the results based on the remaining animals are presented in the next three columns. The standard error is expressed as a percentage of that obtained when six animals per group were used. An additional animal was removed to obtain the results shown in the next three columns, and finally another animal was removed to yield the results shown when there were three animals per group, or nine animals per protein.

The RNV is remarkably constant and seldom varied more than 2 or 3%. The estimated potency is not, of

Table II. Analysis of Variance of Assays Based on Body Water, Body Nitrogen, and Weight Gain

Expt.	Source	Degrees of Freedom	Water		Nitrogen		Weight Gain	
			Mean squares	F	Mean squares	F	Mean squares	F
6	Curvature	6	5.49	0.849	234.31	1.796	52.67	2.480[a]
	Intersection	5	14.19	2.196	67.75	0.519	71.31	3.358[b]
	Blanks	1	33.05	5.116[a]	6.94	0.053	90.02	4.240[a]
	Linear regression	6	5,487.37	84.948[b]	70,525.80	54.080[b]	16,569.29	780.465[b]
	Error	106	6.46		130.41		21.23	
	Total	113	...	...	...	...	...	...
7	Curvature	6	2.61	0.241	158.71	0.876	20.88	1.009
	Intersection	5	6.29	0.581	114.34	0.631	32.00	1.546
	Blanks	1	44.62	4.123[a]	21.06	0.116	146.87	7.098[b]
	Linear regression	6	6,166.21	569.890[b]	78,196.19	432.047[b]	16,035.94	775.012[b]
	Error	105	10.82		180.99		20.69	
	Total	112	...	...	...	...	...	...
8	Curvature	6	1.56	0.151	32.87	0.210	38.60	1.959
	Intersection	5	21.39	2.078	176.34	1.131	139.33	7.072[b]
	Blanks	1	16.95	1.647	141.65	0.908	0.64	0.032
	Linear regression	6	4,184.33	406.640[b]	52,147.52	334.622[b]	11,589.22	588.285[b]
	Error	111	10.29		155.84		19.70	
	Total	118	...	...	...	...	...	...

[a] Significant at 0.05 level.
[b] Significant at 0.01 level.

Table III. The Influence of the Number of Animals in Each Group upon the Accuracy of the Assay

Expt.	Protein	Number of Animals Per Group									
		Six		Five			Four			Three		
		RNV[a]	Standard error	RNV[a]	Standard error	Per cent[b]	RNV[a]	Standard error	Per cent[b]	RNV[a]	Standard error	Per cent[b]
1	Lactalbumin	1.0000	...	1.0000	...	...	1.0000	...	...	1.0000	...	...
	Zein	0.0246	0.0108	0.0224	0.0111	102.7	0.0160	0.0128	118.5	0.0121	0.0133	123.1
	Casein	0.7403	0.0287	0.7640	0.0298	103.8	0.7563	0.0335	116.7	0.7530	0.0362	126.1
	Casein–gelatin (1 to 2)	0.3054	0.0191	0.3107	0.0194	101.5	0.3019	0.0209	109.4	0.2985	0.0227	118.8
	Casein–gelatin (1 to 1)	0.4106	0.0185	0.4196	0.0192	103.7	0.4143	0.0218	117.8	0.4131	0.0237	128.1
3	Lactalbumin	1.0000	...	1.0000	...	...	1.0000	...	...	1.0000	...	...
	Soya flour, low heat	0.4780	0.0111	0.4767	0.0129	116.2	0.4644	0.0130	117.1	0.4530	0.0151	136.0
	Soya flour, cooked	0.5733	0.0142	0.5766	0.0166	116.9	0.5632	0.0166	116.9	0.5530	0.0186	130.9
	Soya flour, toasted	0.5757	0.0152	0.5811	0.0176	115.8	0.5697	0.0178	117.1	0.5647	0.0203	133.6
	Lactalbumin +10% fat	0.9822	0.0195	0.9790	0.0227	116.4	0.9683	0.0227	116.4	0.9650	0.0258	132.3
	Soya flour, low heat +10% fat	0.5027	0.0118	0.5013	0.0135	114.4	0.4956	0.0139	117.8	0.4937	0.0157	133.0
7	Lactalbumin	1.0000	...	1.0000	...	...	1.0000	...	...	1.0000	...	...
	Full fat soya flour	0.5870	0.0183	0.5757	0.0199	108.7	0.5896	0.0202	110.3	0.5946	0.0231	126.2
	Cottonseed, corn, sorghum mix	0.4798	0.0213	0.4762	0.0232	108.9	0.4862	0.0231	108.4	0.4744	0.0260	122.0
	Fish flour	0.8115	0.0289	0.7979	0.0321	111.0	0.8108	0.0327	113.1	0.8153	0.0375	129.7
	High protein rice	0.4479	0.0266	0.4304	0.0301	113.2	0.4841	0.0307	115.4	0.4497	0.0335	125.9
	Lactalbumin +10% cellulose	0.9789	0.0320	0.9813	0.0353	110.3	0.9742	0.0353	110.3	0.9414	0.0398	124.3

[a] Relative nutritive value (slope ratio or slope of the regression of the unknown compared to lactalbumin).
[b] Standard error as per cent of the standard error obtained with six animals.

course, a function of the number of animals utilized. As expected, the reliability of the estimated potency falls, as the number of animals decreases, by a factor which is approximately equal to $\sqrt{\frac{\sigma}{n}}$ where n is the number of animals per group. Reasonably reliable estimates are obtained with as few as three animals per group (nine animals per protein) provided the total number of proteins assayed at one time was five as in the experiments presented.

DISCUSSION

Slope-ratio assays have been used widely in the microbiological assay of vitamins and amino acids. Presumably because the statistical treatment of the assays is complex and tedious when done by hand, few investigators have applied the statistical evaluation recommended by Finney (1964). Rather, most workers have simply plotted the values obtained from the standard material, drawn the standard curve by inspection, and estimated the value for each dose of unknown from the standard curve. There is no doubt that the general suitability of an assay can be estimated in this way. The primary criterion of an adequate assay is that several doses of the unknown yield comparable values—i.e., that there is no drift in values obtained at several doses. This criterion will be met when the regression lines for the standard and unknown are linear and have a common intersection.

The authors wish to stress that in many assays completed to date, significant curvature is rarely seen in spite of the fact that the highest levels of protein fed gave weight gains of 80 to 100 grams in the 21-day test period. Thus, with weight gains up to 4 to 5 grams per day which are near maximal for the animals used, one must conclude that nitrogen deposition per unit protein eaten is practically constant. This is important since Miller and Payne (1961, a and b) have based their method of evaluating proteins upon the assumption that nitrogen utilization decreases linearly with intake at all levels above those required for maintenance. While nitrogen utilization will fall at levels approaching those which produce maximum gain, the data presented in this and other papers show that the assumption of Miller and Payne is no longer tenable. Miller and Payne (1961a) failed to test sufficient levels of proteins to evaluate the assumption they made. Also, Morrison *et al.* (1963) tested high levels of intake and conclude the assumption of Miller and Payne made as to the rate of fall in nitrogen utilization is also apparently in error. Other limitations of the Miller and Payne assumptions have been discussed by Njaa (1962).

The data presented in this paper do not clearly demonstrate advantages or disadvantages of weight gain, body water, and body nitrogen as measures of response to dietary proteins. This would be expected on the basis of the very high correlations between these metameters of response (Hegsted and Worcester, 1967). For most proteins, at least, simple weight gain will give as accurate an assessment of nutritive value as more complex estimates of nitrogen retention. The possibility does always exist, however, that some proteins may give body weights with significantly greater fat content and, if so, weight gain would overestimate the nutritive value. Although measurement of body nitrogen is the presumed measure of choice, the estimate of total body nitrogen may be less accurate than that of body water because of the greater number of manipulations involved. It is relatively expensive and time-consuming. Thus, as various authors have concluded (Bender and Miller, 1953; Dreyer, 1957, 1962; Henry and Toothill, 1962), the measurement of body water appears to be a reasonable compromise. There is the possibility that variations in the body water to body nitrogen ratio may occur with certain diets, and this will be obscured when large numbers of animals fed a variety of diets are utilized to demonstrate the general high correlations between body water and body nitrogen. The fact that measurement of body nitrogen usually produces estimates of nutritive value that are slightly lower than those derived from body water is being further investigated.

The variations in the design which might be introduced into the assay, such as the number of animals per group, the number of levels of protein tested, the length of time allowed for the assay, etc., are numerous and the effects of only a limited number of these have been studied. A prime consideration in the assay is that the regression lines between response and protein consumed must be linear. A reasonable range of protein intakes is desirable to demonstrate clearly departure from linearity should this occur. Since departure from blanks is the most common deviation from the ideal assay that has been found (indicating that animals fed a protein-free diet may respond in an atypical manner), the authors have preferred to utilize at least three levels of each protein under test. This also allows elimination of the highest level fed if one should underestimate the nutritive value and select a level that is too high to fall within a satisfactory range of the assay.

The fact that departure from blanks is quite often found (indicating that there is not always a linear response from the zero dose to higher doses) also suggests that some caution should be exercised in relying upon the usual measure of net protein utilization (NPU) in which tacit assumption is made that the relationship is linear. Animals fed a protein-free diet may conserve or waste body protein to a greater or lesser extent than expected. The data presented by Bender (1961), in which diets completely lacking in certain essential amino acids, yielded NPU's above those expected and indicate inadequacies in NPU determinations. The possible effect of the length of time the assay is run has not yet been investigated thoroughly and is a possible source of error or discrepancies in the various approaches suggested for assessing the nutritive value of proteins.

As few as three animals per group (nine animals per protein) yield reasonably reliable estimates of nutritive value. Since the error of the assay depends upon the total number of animals used per experiment as well as the number per group, the data refer to assays in which at least five unknowns are tested. Regardless of the number of animals used, the assay will be most satisfactory when the diets are selected to give a wide range of protein intake between the zero level and that which allows substantial weight gain. This will be particularly important when few animals are utilized since this will stabilize the regression lines.

Some of the measures for estimating the nutritive value

of proteins which have been proposed should yield essentially the same results as the assay proposed since in essence they constitute a slope-ratio assay based upon a blank and one experimental point. These include the measurement of net protein retention of Bender and Doell (1957) which is based upon weight gain differences of animals fed the test diet and protein-free diet and net protein utilization of Miller and Bender (1955) which is based upon body nitrogen or body water measurements of similar animals. However, in these assays data are not obtained which can provide for statistical validation of the assay. Also, replicate groups of four animals housed together are suggested (Miller, 1963). Only limited evidence of reproducibility can be obtained under these conditions.

As has been indicated previously, lactalbumin is nearly quantitatively converted into body protein in the young rat. Any other similar high quality protein might be utilized as the standard in assays of the kind described. To date, five different lactalbumin preparations have been utilized. All have yielded comparable results.

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Received for review November 6, 1967. Accepted January 8, 1968. This work was supported in part by grants-in-aid from the National Institutes of Health (AM-09520 and 5-K6-AM-18,455) and the Fund for Research and Teaching, Department of Nutrition, Harvard School of Public Health, Boston, Mass.