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A COMPARISON OF ASSAYS FOR THE ANALYSIS OF PROTEIN CONTENT OF LIVER HOMOGENATE SUBFRACTIONS*

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Abstract—Several assay procedures commonly employed to determine protein content of subfractions of liver homogenates were compared. It was found that a microbiuret assay which utilizes optical density measurements in the ultraviolet range (310 mμ) yielded high values for each of the subfractions studied. These values were particularly high in subfractions containing predominantly nuclei, rough-surfaced microsomes, and smooth-surfaced microsomes. A standard biuret assay, which utilizes optical density readings in the visible range (545 mμ) also yielded inordinately high values in fractions containing smooth-surfaced microsomes. The standard biuret method gave significantly lower values in the soluble fraction than the other three assay procedures. Assays performed by the method of Lowry yielded the lowest values in each of the subfractions studied except the soluble fraction. In many cases, however, the observed differences between the Lowry and micro-Kjeldahl methods were not statistically significant. The results indicate the need for accurate descriptions of methodology employed when reporting protein content of tissue homogenates or specific enzymic activity based upon protein content.

THE ENZYMIC activity of subfractions of rat liver homogenates is often expressed in terms of the protein content of such homogenates. A wide variety of assay procedures has been employed for the determination of protein. It is apparent from the literature, however, that the estimation of protein content of various subfractions of liver homogenates varies considerably from one laboratory to another, contributing to considerable confusion with regard to comparisons of enzymic activity when such activity is expressed in terms of protein content. Heretofore it has not been clear whether such large variations are attributable to variations in fractionation procedures or in the nature of the protein estimation *per se*, especially since many authors often fail to state the exact method (or modification thereof) of protein determination.

The purpose of the present investigation was to compare several commonly employed procedures for the estimation of the protein content of several subfractions of liver homogenates. The micro-Kjeldahl, Lowry, and biuret assays and certain variations thereof were compared. Other methods were not studied in this paper because of their relative lack of sensitivity, their specificity for certain types of proteins, or their tendency to be affected by the presence of other substances in liver homogenates. We were particularly interested in studying a microbiuret method developed by Itzhaki and Gill,[1] which was reported to be sensitive, reasonably nonspecific, rapid,

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and unaffected by high concentrations of deoxyribonucleic acid (DNA). It possesses the additional advantage of producing optical density readings which are linearly related to protein concentration. It therefore appeared to be particularly suitable for the measurement of protein concentrations of tissue homogenates and subfractions thereof.

METHODS

Preparation of subcellular fractions. Male Long–Evans rats (60–90 g and 250–300 g) and male Dutch rabbits (700–900 g) were employed in these experiments. Animals were fed lab chow *ad libitum* until 24 hr prior to sacrifice. They were sacrificed by cervical dislocation; their livers were quickly removed and minced in ice-cold 1·15% KCl. The minced livers were then rinsed in a separate volume of 1·15% KCl. This procedure appeared to remove blood from the liver tissue as effectively as perfusion of intact livers. The liver tissue was then blotted, weighed, and homogenized in 5 volumes of 0·25 M sucrose in a Potter homogenizer with a plastic pestle. Nuclei, cell membranes, mitochondria, and lysosomes were separated by methods described by Li *et al.*[2] In certain experiments, microsomes were separated from the 12,000-*g* supernatant; in such experiments EDTA was omitted from the sucrose solutions. Supernatant fractions, rough-surfaced microsomes, and smooth-surfaced microsomes were separated by four separate procedures designated as A, B, C, and D.

In procedure A, the 12,000-*g* supernatant which remained after the sedimentation of lysosomal-rich fractions was reconstituted to the original volume of the whole homogenate with distilled water. Two ml of 1·31 M sucrose was layered under 8·0 ml of the reconstituted 12,000-*g* supernatant in ultracentrifuge tubes. This was then centrifuged at 40,000 rev/min (104,000 *g*) for 8 hr (Spinco No. 40 angle rotor). The layer containing the smooth-surfaced microsomes and the supernatant was separated from the pellet (containing predominantly rough-surfaced microsomes) by aspiration, mixed, and again reconstituted to the original volume. This mixture was centrifuged at 104,000 *g* for 1 hr. The supernatant fraction was aspirated from the pellet of the second centrifugation (this pellet contained predominantly smooth-surfaced microsomes), care being taken not to aspirate the top (fatty) layer nor to aspirate that supernatant within one half inch of the pellet. The pellets containing rough- and smooth-surfaced microsomes were each washed once (to remove residual protein of the soluble fraction) with phosphate buffer (0·5 M, pH 7·4) and resuspended in 0·25 M sucrose such that 1 ml of suspension contained microsomes from 1 g liver tissue (wet weight).

In procedure B, fractions containing rough- and smooth-surfaced microsomes and supernatant fractions were separated according to a method described by Juchau and Fouts.[3] In procedure C, the above-mentioned fractions were separated according to the methods of Dallner *et al.*,[4] and in procedure D, the same fractions were separated according to the method of Fouts.[5] Weanling Long–Evans rats (60–90 g) were used in all experiments designated A or B. In each experiment designated A, protein determinations were made on pooled homogenates of 10 rat livers; in experiments B, pooled homogenates of 3 rat livers were employed for each protein determination. In procedure C, adult Dutch rabbits and adult Long–Evans rats (250–300 *g*) were used; in procedure D, adult Long–Evans rats were employed. In the last two

procedures, each protein concentration was determined from the homogenate of a single liver.

Protein assays. Homogenates were assayed for protein content by the following methods. A microbiuret method described by Itzhaki and Gill,[1] a micro-Kjeldahl method described by Juchau *et al.*[6] the method of Lowry *et al.*,[7] and a slight modification of a biuret method described by Layne.[8] In this modification, color was allowed to develop for 25 min. Five millliliters of an ethanol–ether mixture (0·5 ml of 95% ethanol plus 4·5 ml ether) was then shaken together with the reaction mixture. Layers were separated by centrifugation and the optical density (O.D.) of the bottom layer was read at 545 mμ.

These methods will be referred to as the microbiuret, micro-Kjeldahl, Lowry, and biuret methods, respectively. Microbiuret, micro-Kjeldahl, and Lowry analyses were performed simultaneously on the same homogenate samples for critical comparisons. Simultaneous analyses of identical tissue samples were also performed with the biuret and microbiuret methods. A Beckman DU spectrophotometer equipped with a Gilford attachment was employed in all assays. All protein values were determined from standard curves, with desiccated crystalline bovine serum albumin (BSA) fraction V (Pentex) as the reference standard. The BSA was dried to constant weight (phosphorous pentoxide as the desiccant material). Standard curves (with five different concentrations of the same BSA solution) were run with each assay, including micro-Kjeldahl assays. Micro-Kjeldahl assays of the desiccated BSA (with ammonium chloride as the reference standard and 6·25 as the factor of multiplication) indicated that essentially 100 per cent (95–100 per cent) of the BSA could be accounted for by this assay. In assays of homogenates, dilutions were made such that all O.D. readings were in the linear range of the standard curves.

Statistical analyses. A randomized complete block was employed in the experimental design. The results were evaluated by analysis of variance and Duncan's new multiple-range test. The level of significance chosen was $P<0{\cdot}05$. All statistical procedures are described by Steel and Torrie.[9]

RESULTS

Protein values obtained from the microbiuret analyses were always higher than those estimated by any of the other three protein assay methods. Micro-Kjeldahl values were consistently higher than Lowry values, but in many instances the differences were not statistically significant; significant differences between the Lowry and micro-Kjeldahl methods were observed when the protein content of fractions containing predominantly smooth-surfaced microsomes was analyzed. This was due, apparently, to a lesser degree of variability (in both methods) associated with assays of protein content of this subfraction (Table 1). Micro-Kjeldahl and Lowry assays of whole homogenate, cell membranes, mitochondria, lysosomes, and soluble fraction (Table 2) yielded values which were about 70–80 per cent of values determined by the microbiuret method (Table 3). However, particularly noteworthy was the fact that, in the analysis of the protein content of fractions containing predominantly nuclei and microsomes (rough or smooth), the microbiuret assay yielded values which were 2–3 times as high as those determined from either Lowry or micro-Kjeldahl analyses.

In general, the values obtained from the biuret assay appeared to agree closely with the values from micro-Kjeldahl or Lowry assays. However, in the case of fractions

containing mitochondria and smooth-surfaced microsomes, the biuret values agreed well with microbiuret values (Table 4). With regard to the soluble fraction, biuret values were only about 50 per cent of those determined by microbiuret assays, whereas Lowry and Kjeldahl assays of the soluble fraction yielded values which were 70–80 per cent of the microbiuret values.

TABLE 1. COMPARISONS OF PROTEIN ASSAYS ON ROUGH- AND SMOOTH-SURFACED MICROSOMES ISOLATED FROM RAT LIVER BY VARIOUS FRACTIONATION PROCEDURES*

Assay	Rough-surfaced microsomes					Smooth-surfaced microsomes				
	A	B	C (rats)	C (rabbits)	D	A	B	C (rats)	C (rabbits)	D
Microbuiret	12·6	19·6	13·5	16·3	8·7	15·7	21·4	13·4	15·3	11·6
Micro-Kjeldahl	9·2	8·7	6·4	7·9	4·5	11·7	9·6	10·5	10·7	6·4
Lowry	5·7	7·4	4·5	6·1	4·2	8·9	9·8	9·2	8·9	5·7
CV† (%)	25·5	13·6	26·3	43·7	12·7	7·3	25·6	1·8	9·2	5·9
N‡	3	5	3	4	3	3	5	3	4	3

* Values represent milligrams protein per gram liver tissue (wet weight) in Tables 1, 2, 4. Any values in these three tables that are joined by the same line are not significantly different ($P > 0{\cdot}05$), whereas any values that are not joined by the same line in these tables are significantly different ($P < 0{\cdot}05$).

† Coefficient of variability, Tables 1, 2, 4.

‡ Number of determinations used to compute the means in Tables 1, 2, 4.

TABLE 2. COMPARISONS OF MICRO-KJELDAHL, MICROBIURET, AND LOWRY PROTEIN ASSAYS ON SEVERAL RAT LIVER HOMOGENATE SUBFRACTIONS

Assay	Whole homogenate	Cell membranes	Nuclei	Mito-chondria	Lyso-somes	Soluble (A)	Soluble (B)
Microbiuret	253	73	27	5·7	5·0	37	76
Micro-Kjeldahl	197	58	13	4·7	4·2	30	58
Lowry	198	48	10	3·8	3·6	25	56
CV (%)	18·0	13·8	24·1	19·0	18·8	23·2	15·8
N	9	7	7	8	7	5	3

TABLE 3. COMPARISON OF BIURET, MICRO-KJELDAHL, AND LOWRY METHODS FOR ANALYSIS OF PROTEIN CONTENT OF LIVER HOMOGENATE SUBFRACTIONS*

Assay	Whole homo-genate	Cell mem-branes	Nuclei	Mito-chondria	Lyso-somes	Soluble (B)	Rough (B) micro-somes	Smooth (B) micro-somes
Biuret	69	68	40	94	81	51	43	108
Micro-Kjeldahl	78	79	48	82	84	76	44	45
Lowry	78	66	37	67	72	74	38	46

* Values in the table represent percentages of microbiuret protein. Percentages are based on the values presented in Tables 1, 2, 4.

The protein content of the soluble fraction obtained from procedure B was approximately double the protein content of soluble fraction obtained from procedure A. This was true regardless of the protein assay method used. Rough- or smooth-surfaced microsomal fractions separated according to procedure B also appeared to contain a somewhat higher protein content than the same fractions which were separated by other procedures. This generalization seemed to hold if assays were performed by microbiuret or Lowry methods but did not hold in the case of the micro-Kjeldahl assays. It should be noted that the animals employed in procedures A and B were weanlings, whereas those used in procedures C and D were adults.

TABLE 4. COMPARISON OF MICROBIURET AND BIURET PROTEIN ASSAYS ON SEVERAL RAT LIVER HOMOGENATE SUBFRACTIONS

Fraction	Biuret	Microbiuret	CV (%)	N
Whole homogenate	170	247	18·3	3
Cell membranes	58	85	12·8	3
Nuclei	14·0	34·6	13·0	5
Mitochondria	6·8	7·2 *	15·6	3
Lysosomes	5·2	6·4 *	7·9	3
Soluble (B)	37·7	74·3	10·5	3
Rough microsomes (B)	8·0	18.5	17·7	5
Smooth microsomes (B)	16·7	15·4 *	17·2	5

* These values (joined by line) are not significantly different.

In separate experiments it was found that repeated freezing and thawing of homogenate subfractions did not change the apparent protein content of any subfraction regardless of the assay procedure employed. Treatment of homogenate subfractions with 0·5 per cent sodium deoxycholate also did not appear to affect protein values in any of the four assay procedures. Extraction of the microbiuret assay mixture with ethanol–ether, either before or after color development, lowered the optical density readings of the final solution, but likewise lowered the O.D. readings of the corresponding blanks such that ΔO.D. values remained the same. Correspondingly, additions of dilute lecithin suspensions, both before and after development of color, increased the O.D. readings of the final solution of the microbiuret assay. The O.D.'s of the corresponding blanks, however, were also increased such that the ΔO.D. readings remained essentially the same. Thus the protein values obtained from these procedures were not changed. Standard curves for these assays were run on solutions of BSA treated in the same fashion.

DISCUSSION

The data presented in this paper indicate that the microbiuret method for estimation of protein content of various subfractions of rat or rabbit liver homogenates yields high values compared to those from micro-Kjeldahl or Lowry analyses. Except for fractions containing predominantly mitochondria or smooth-surfaced microsomes, microbiuret values were also higher than biuret values obtained from the same homogenates. The reason for the discrepancies between the microbiuret and other assays is not entirely clear, but did not appear to be due to turbidity differences, as indicated

by experiments with sodium deoxycholate, ethanol–ether extractions, and lecithin. It would appear that nonprotein substances present in the homogenates may be capable of reacting with copper sulfate to produce increases in O.D. readings in the u.v. (310 mμ) spectrum. The fact that fractions containing predominantly nuclei or rough-surfaced microsomes yielded exceptionally high protein values when assayed by the microbiuret method rendered this possibility more likely since these subfractions contain large quantities of nucleic acid derivatives. However, fractions containing predominantly smooth-surfaced microsomes also showed disproportionately high values when assayed by the microbiuret method. (The smooth-surfaced microsomes contain only very small quantities of nucleic acid derivatives.) It should be noted, however, that the biuret assay of smooth-surfaced microsomes yielded values which were very similar to those of the microbiuret analysis.

The fact that the micro-Kjeldahl assays yielded somewhat higher values than corresponding Lowry assays was not surprising, since the micro-Kjeldahl method measures essentially all non-protein nitrogenous material, whereas the Lowry method measures only certain forms of nonprotein material. (Free tyrosine, tryptophane, uric acid, guanine, and xanthine are included in the biological materials which react with the Folin reagent to produce color.)

Analysis of the protein content of the soluble fraction by the biuret method yielded lower values than assays by Lowry or micro-Kjeldahl procedures. This may reflect the greater specificity of the biuret method for protein material. The soluble fraction contains large quantities of free amino and nucleic acids which would give positive color reactions in either Lowry or micro-Kjeldahl assays, but not in biuret assays. In addition, it was found that the protein content of the soluble fraction could vary considerably, depending upon the method used to obtain this subfraction. This is graphically shown in comparisons of soluble fractions obtained by procedures A and B. Furthermore, it was noted that if 104,000-*g* supernatant were simply poured off from microsomal material, subsequent assays of such supernatant material yielded substantially higher values than when the supernatant was aspirated as described above (see Methods).

The results of analysis of mitochondrial protein appeared to be somewhat lower than those obtained by Li *et al.*,[2] who used the same fractionation procedure. Since these authors did not state the method of determination of protein content, rationalization of the apparent discrepancy is difficult but may possibly be explained by the fact that they first precipitated the protein with 10 per cent trichloroacetic acid and washed the precipitate with organic solvents prior to protein analysis. They also used adult rats, whereas our values of mitochondrial proteins are from weanling rats. In our experiments the protein content of microsomal fractions appeared to be somewhat higher in adult animals than in weanlings.

In general, the results obtained indicate the necessity of specifying the protein assay procedure employed. It is evident from the data presented that authors who state that protein was assayed by a biuret analysis, for example, are supplying little helpful information. This is especially true in view of the numerous variations of standard protein assay procedures.

Inchiosa,[10] Parvin *et al.*,[11] and others[1, 8, 12–14] have discussed the advantages and disadvantages of various biuret assay procedures. Theoretically, biuret assays are more specific for protein, i.e. less affected by other biological materials and relatively

independent of amino acid composition of proteins. (However, this may not be the case with the microbiuret assay described here.) Biuret assays in general also possess the advantage of being rapid, simple, and linearly related to protein concentration. The principal disadvantage appears to be due to lack of sensitivity. Advantages and disadvantages of other standard protein assays have been discussed by Layne.[8] No standard protein assay appears to be completely satisfactory for the quantitative evaluation of protein content of liver homogenate subfractions. If a high degree of sensitivity is required, the Lowry method appears to be quite suitable. If sufficient material is available, the biuret assay described here appears to be reasonably suitable, particularly if the nature of the protein being analyzed is unknown. In any case, the limitations of the assay procedure should be recognized and taken into consideration.

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