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Protein Assay of Rat Liver Mitochondria and Mitochondrial Membrane Preparations

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Various methods of protein assay, including modification of the Lowry and biuret procedures, as well as measurements of absorbance of detergent-solubilized proteins, have been applied to rat liver mitochondria and mitochondrial membrane preparations. Total and protein nitrogen have been determined in the same preparations by the Kjeldahl procedure. The different methods are examined critically and conversion factors are given in order to obtain (real) Kjeldahl values from any of the methods tested.

Protein determination is essential as a preliminary step in most studies concerning biological membranes, since a great deal of structural and functional membrane parameters, e.g. the specific activity of enzymes, are expressed in relation to a known protein amount.

Preliminary experiments carried out in our laboratory revealed remarkable differences in protein contents of mitochondrial preparations as determined by the LOWRY (18) or biuret (19) methods. The former gave values that were about 40 % below those obtained when biuret methods were used. BERNATH and SINGER (1) had already reported a substantial overestimation of mitochondrial succinate dehydrogenase protein by the biuret method. In agreement with their report, DAVIS and HATEFI (2) obtained biuret values of the same protein preparation 22 % higher than those obtained by the LOWRY method (18).

The KJELDAHL of protein nitrogen determination (28) is usually considered as the most reliable and accurate among the various technique of protein determination. However, the procedure is lengthy and tedious, and this prevents its use as

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a routine laboratory assay. In the present study, we have attempted to examine three frequently used methods of protein assay as applied to rat liver mitochondria and rat liver inner mitochondrial membranes, namely LOWRY *et al.* (18), biuret (19) or absorbance at 280 nm. For this purpose, results from these three methods have been compared to those obtained on the same mitochondrial preparations following the KJELDAHL method (28).

Materials and Methods

Rat liver mitochondria and inner mitochondrial membranes were prepared as described previously (10, 11), and resuspended in 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.4.

The biuret reaction was performed essentially as described (19), either in the absence («Biuret I») or in the presence («Biuret II») of the detergent Triton X-100 (0.1 %, w/v). The LOWRY method was carried out either as originally described (18) («Lowry A») or following the modification of WANG and SMITH (30), that includes the addition of 0.1 % Triton X-100 («Lowry C»). The reaction time of LOWRY's «reagent C» was changed from 15 to 30 min in the original procedure («Lowry B») and in the above mentioned modification («Lowry D»). Bovine serum albumin was used as standard.

Absorbance measurements at 280 nm were carried out in a double-beam DB-GT Beckman spectrophotometer. Membrane suspensions were diluted 50-500 times in 1 % sodium dodecylsulphate (final volume in the cuvette: 1 ml), and their absorbance read against a blank containing the same detergent solution and a volume of buffer equivalent to the added volume of membrane suspension. Light scattering was negligible under these conditions.

Total nitrogen was determined by a micro-modification of the KJELDAHL method. NH_3 produced in the distillation process was collected on a saturated boric acid solution, and the resulting borate was titrated with 0.1 N HCl in the presence of a mixture of methyl red and methylene blue as indicator (28). The appropriate blanks were run in the same way. Calibration with acetanylide gave an accuracy better than 98 %.

Protein standards were of the highest available purity from Sigma (London). All other reagents were analytical grade.

Results

Differences found in protein contents of mitochondrial preparations according to the LOWRY and biuret procedures prompted us to use the KJELDAHL method in order to estimate membrane protein from total membrane nitrogen. For this purpose, data of protein nitrogen contents and membrane non-protein nitrogen contents are required.

Protein nitrogen contents were determined experimentally by subjecting known amounts of pure proteins (bovine seroalbumin, haemoglobin and casein) to the KJELDAHL procedure. A mean value ($\pm$ S.E.M.) of 14.93 ± 0.584 g N/100 g protein was found. This is in good agreement with a mean value (15.04 ± 1.25 %) calculated from the aminoacid composition of 21 proteins (24, 25). On the basis of these experiments and calculations, a factor of 6.66 can be safely established for the conversion of g nitrogen into g protein.

Non-protein nitrogen was estimated from bibliographic data as 1.3×10^{-2} g N/g protein, in the case of rat liver mitochondria, and as 4.7×10^{-3} g N/g protein, for mitochondrial inner membranes. This corresponds to 8.0 % and 3.0 % of the total nitrogen, respectively (table I) (3, 4, 6, 7, 13, 14, 16, 17, 20, 22-27, 29). The conversion factor for g nitrogen into g protein can thus be modified as 6.13 and 6.46, for whole mitochondria and inner membranes, respectively.

Tables II and III show the protein con-

Table I. *Non-protein nitrogen contents of rat liver mitochondria and inner mitochondrial membranes.*

Origin of non-protein N	Mitochondria		Inner membranes	
	g N/g protein	Ref.	g N/g protein	Ref.
N-containing phospholipids	9.2×10^{-3}	a	3.9×10^{-3}	f
Cytochrome chromophores	2.1×10^{-4}	b	6.6×10^{-5}	g
Purines and pyrimidines	8.1×10^{-4}	c	—	—
Nucleotidic coenzymes	7.6×10^{-4}	d	—	—
Mitochondrial DNA	2.1×10^{-3}	e	—	—
Total flavins	1.3×10^{-5}	h	6.8×10^{-4}	h
Total	1.3×10^{-2}		4.7×10^{-3}	

a (3, 6, 16, 23, 24, 26, 27, 29); b (24); c (16); d (16); e (4, 16, 27); f (7, 14, 17, 22); g (7, 13, 17, 20); h (7, 13, 30).

Table II. *Protein contents of four different preparations of rat liver mitochondria estimated by different methods.*
Figures represent mean values (mg protein/ml) ± S.E.M. The number of analysis performed on each mitochondrial preparation is expressed in parenthesis. (See text for details).

Method	Protein contents (mg/ml)			
	1	2	3	4
Kjeldahl	19.0	17.5	23.4	18.3
Lowry A	10.3 ± 0.75 (5)	9.6 ± 0.60 (5)	16.8 ± 0.36 (5)	10.7 ± 0.65 (5)
Lowry B	10.4 ± 0.32 (5)	7.6 ± 0.15 (5)	11.4 ± 0.58 (5)	—
Lowry C	10.6 ± 0.83 (5)	9.5 ± 0.40 (5)	12.4 ± 0.58 (5)	10.0 ± 0.45 (5)
Lowry D	11.3 ± 1.32 (5)	8.1 ± 0.37 (5)	12.4 ± 0.59 (5)	—
Biuret I	17.4 ± 1.17 (4)	15.0 ± 0.12 (4)	16.0 ± 0.14 (4)	15.6 ± 0.44 (4)
Biuret II	19.9 ± 0.24 (4)	17.5 ± 0.39 (4)	18.4 ± 0.92 (4)	14.7 ± 0.63 (4)

Table III. *Protein contents of four different preparations of rat liver inner mitochondrial membranes estimated by different methods.*
Figures represent mean values (mg protein/ml) ± S.E.M. The number of analysis performed on each mitochondrial preparation is expressed in parenthesis. (See text for details).

Method	Protein contents (mg/ml)			
	1	2	3	4
Kjeldahl	12.0	14.7	11.7	10.1
Lowry A	7.5 ± 0.34 (4)	11.6 ± 0.28 (5)	7.7 ± 0.20 (5)	6.5 ± 0.12 (5)
Lowry B	6.7 ± 0.27 (4)	10.0 ± 0.22 (4)	7.2 ± 0.19 (4)	6.2 ± 0.15 (5)
Lowry C	9.2 ± 0.25 (5)	11.0 ± 0.40 (5)	7.6 ± 0.03 (4)	5.3 ± 0.17 (5)
Lowry D	7.9 ± 0.22 (5)	10.1 ± 0.32 (4)	7.0 ± 0.27 (4)	5.8 ± 0.32 (4)
Biuret I	7.4 ± 0.84 (4)	10.0 ± 0.13 (4)	6.8 ± 0.03 (4)	5.6 ± 0.17 (4)
Biuret II	8.3 ± 0.75 (4)	10.4 ± 0.11 (4)	7.5 ± 0.08 (4)	6.3 ± 0.23 (4)

Table IV. *Protein contents expressed as percentage of the corresponding Kjeldahl value, and conversion factors.*
Figures represent mean values ± S.E.M. Number of experiments is indicated in parenthesis. See text for details.

Method	Mitochondria		Inner membrane	
	% protein detected	Conversion factor	% protein detected	Conversion factor
Kjeldahl	100	1.00	100	1.00
Lowry A	59.8 ± 3.55 (4)	1.67	66.9 ± 2.45 (4)	1.49
Lowry B	48.9 ± 2.66 (3)	2.04	61.6 ± 2.14 (4)	1.62
Lowry C	54.4 ± 0.50 (4)	1.83	67.3 ± 4.84 (4)	1.48
Lowry D	52.9 ± 3.11 (3)	1.89	63.0 ± 2.31 (4)	1.59
Biuret I	82.7 ± 4.99 (4)	1.21	60.8 ± 1.16 (4)	1.64
Biuret II	90.9 ± 5.79 (4)	1.10	66.7 ± 1.78 (4)	1.50

Table V. *Protein assay of rat liver mitochondria and inner mitochondrial membranes by a spectrophotometric method.*
Absorbance at 280 nm was measured as described under «Methods». A factor is included for converting the reading into proteins (mg/ml in the cuvette) according to Kjeldahl. Data are shown for 3 or 4 measurements of 4 independent preparations, as mean values ± S.E.M.

Sample No.	mg protein/ml	A_{280}	Conversion factor
Mitochondria			
1	0.47	0.274±0.0074 (3)	1.71
2	0.43	0.238±0.0062 (3)	1.80
3	0.58	0.286±0.0086 (3)	2.03
4	0.45	0.261±0.0013 (3)	1.72
		Mean conversion factor	1.81±0.064
Inner mitochondrial membranes			
1	0.30	0.235±0.0004 (3)	1.27
2	0.37	0.262±0.0047 (3)	1.41
3	0.29	0.190±0.0049 (4)	1.52
4	0.25	0.164±0.0048 (4)	1.52
		Mean conversion factor	1.43±0.051

tents, in mg/ml, of four different preparations of rat liver mitochondria and inner mitochondrial membranes. For each preparation, one KJELDAHL and a number of LOWRY and biuret assays were performed, as indicated in each case. The latter methods were also applied in their modified versions, as indicated in the Methods section. Assuming that the KJELDAHL value corresponds to the total (100 %) proteins present, a conversion factor can be calculated for each of the six simpler procedures, in order to convert the LOWRY or biuret protein values into (real) «KJELDAHL proteins». The conversion factors calculated for each case are shown in table IV.

The same four preparations of whole mitochondria and inner mitochondrial membranes, whose protein contents had been determined according to KJELDAHL, were examined by the spectrophotometric method in the presence of sodium dodecylsulphate, as described above. The corresponding values of A_{280}, together with the conversion factors into mg protein, are given in table V.

Discussion

The method of LOWRY *et al.* (18) is the most frequently used for protein determinations in biomembrane systems, because of its simplicity and sensitivity. It allows the determination of 5-10 μg protein, thus, this method is about 100 times more sensitive than the biuret or cupro-protein reaction. Nevertheless, there are some

drawbacks, such as its lack of specificity, long reaction times, instability of some reagents and non-linearity of the standard curve beyond narrow limits. Its application to biomembrane systems has also the inherent difficulty of the complex architecture and composition of these structures. For these and other reasons, many modifications of this method have been proposed (21).

The use of detergents in the analysis of membrane components is based on the fact that they interact with the membrane lipids and proteins, producing effects that go from mild changes in membrane properties to more drastic effects of lysis and solubilization, according to the nature and concentration of the detergent (12). Detergents can also induce corformational changes in membrane proteins, thus exposing certain residues or groups originally unaccessible to the reagent. Triton X-100, that has been used in the modifications «Lowry C», «Lowry D» and «Biuret II» (see Methods) is a non-ionic detergent that has found wide application in studies concerning mitochondrial and other biomembranes (5, 8-11).

The comparison of the various methods, as applied to the same membrane preparations, allows us to establish the advantages and disadvantages of each one, especially with reference to the KJELDAHL procedure. The data for comparison are especially those in tables IV and V. The method of absorbance measurements at 280 nm (15, 31) was found to be useful in a concentration range of 0.1-0.8 mg protein/ml in the spectrophotometric cuvette. This is the simplest method for membrane protein determination and, although subjected to certain variability, it should be recommended for routine protein assays with the conversion factors given in table V, respectively 1.81 and 1.43 for whole mitochondria and inner mitochondrial membranes.

The biuret method, although less sensitive than the LOWRY procedure, is more accurate for the determination of mitochondrial proteins (table IV) although no difference can be appreciated when applied to inner mitochondrial membranes.

With respect to the use of Triton X-100, the biuret method benefits by an increase of 6-8 % in the amount of protein determined in the presence of the detergent (table IV), whereas no difference is seen with the LOWRY method. In this procedure, doubling the time of reaction of reagent C (see Methods) does not appreciably alter the results.

The results summarized in this paper show the necessity of calibrating the more commonly used methods of protein assay with respect to the less convenient, but more accurate, KJELDAHL nitrogen determination. Also these and other results show that the calibration should be performed for each biomembrane system under investigation.

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Resumen

A unas mismas preparaciones de mitocondrias de hígado de rata y de membranas internas mitocondriales se han aplicado una serie de métodos de determinación de proteínas, entre ellos varias modificaciones de los métodos de Lowry y Biuret, y un procedimiento de medida de absorbancia de proteínas solubilizadas por detergentes. También se ha determinado el contenido en nitrógeno total y nitrógeno proteico de dichas preparaciones según Kjeldahl. Se han evaluado críticamente los resultados obtenidos por los distintos métodos y se han calculado factores de conversión que permiten obtener valores «reales» (Kjeldahl) de concentración de proteínas a partir de cualquiera de los otros métodos.

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