Biochimica et Biophysica Acta, 448 (1976) 589–598

BBA 77465

IDENTIFICATION OF THE MILK FAT GLOBULE MEMBRANE PROTEINS

I. ISOLATION AND PARTIAL CHARACTERIZATION OF GLYCOPROTEIN B

JAY J. BASCH, HAROLD M. FARRELL, Jr. and RAE GREENBERG

*Eastern Regional Research Center**, *Philadelphia, Pa. 19118 (U.S.A.)*

(Received March 23rd, 1976)

SUMMARY

The salt soluble proteins from the fat globule membrane of cow's milk were resolved into three fractions by Sephadex column chromatography in sodium dodecyl sulfate. One of the fractions, termed glycoprotein B, was purified by rechromatography to essentially one band on sodium dodecyl sulfate gel electrophoresis. It was found to contain 14 % carbohydrate including sialic acid, mannose, galactose, glucose, glucosamine and galactosamine. The amino acid composition of glycoprotein B was determined; it has amino terminal serine and carboxyl terminal leucine. The molecular weight of this glycoprotein as estimated by sodium dodecyl sulfate gel electrophoresis is 49 500.

INTRODUCTION

It has long been recognized that the fat globules of milk are encased in a membrane. Furthermore, it is thought that this is a true biological membrane which, according to theory [1, 2], should consist of a lipid bilayer interspersed with proteins of different sizes. The lipid components of the fat globule membrane have been studied extensively [3, 4]. Comparison of the membranes of the milk fat globules with plasma membranes of lactating bovine mammary gland has shown that these two systems are similar in the distribution and the fatty acid composition of their phospholipids [5]. In this work emphasis is being placed on the protein components of the fat globule membrane. Part of the problem encountered in studying membrane proteins has been their insolubility in aqueous media. However, sodium dodecyl sulfate has been reported to be a satisfactory dissociating agent for membrane proteins [1]. Therefore, the use of this reagent provides a good starting point for isolating individual proteins of the milk fat globule membrane.

The chemical composition and physical properties of glycoprotein B, a protein fraction isolated from the fat globule membrane of cow's milk, are reported in this communication.

* Agricultural Research Service, U.S. Department of Agriculture.

MATERIALS AND METHODS

Preparation of proteins of the milk fat globule membrane. Soluble and insoluble fractions of the fat globule membrane proteins were prepared according to the method of Herald and Brunner [6], with the exception that the water-washed cream was churned at room temperature with a Polytron ST-10*.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membrane proteins were examined by sodium dodecyl sulfate-polyacrylamide electrophoresis [7,] using an EC-Vertical Slab Gel Apparatus and pH 9.2 buffer solution (10.1 g/l Tris, 1.3 g/l Na_2 EDTA, and 0.77 g/l H_3BO_3) containing 0.1 % sodium dodecyl sulfate. The gels were prepared from 7 % Cyanogum-41, polymerized with 0.1 % dimethylamino-propionitrile and 0.25 % ammonium persulfate as catalysts, and were prerun for 1 h at 80 mA. Protein samples (2 mg) were solubilized in 90 µl of sucrose solvent system (30 % sucrose, 0.5 % $NaCO_3$, and 2 % sodium dodecyl sulfate) and 10 µl of 2-mercaptoethanol and were heated at 37 °C for 30 min before electrophoresis. The gels were run for 3 h at 80 mA and maintained at 14 °C. The gels were stained for protein with 0.03 % Coomassie blue in 5 % trichloroacetic acid/10 % methanol/7 % acetic acid and destained by washing several times with 10 % methanol/7 % acetic acid. The gels were stained for glycoproteins by the method of Kapitany and Zebrowski [8].

Molecular weights were estimated by sodium dodecyl sulfate gel electrophoresis according to the procedure of Weber and Osborn [9].

Gel chromatography. Sephadex G-200 was equilibrated with 0.083 M Tris · HCl, pH 8.5, containing 0.1 % sodium dodecyl sulfate and 0.05 % 2-mercaptoethanol then poured into a 2.5 · 45 cm column. Chromatography was carried out with a flow rate of 10 ml/h at room temperature and fractions of 5 ml were collected in a refrigerated unit. The effluent fractions were monitored at 280 nm.

Protein analysis. Protein was determined by the method of Lowry et al. [10] using bovine serum albumin as a standard.

Carbohydrate analysis. The total protein-bound hexose, hexosamine, and sialic acid were determined by the methods described by Winzler [11]. Hexose was assayed by the orcinol-sulfuric acid procedure using galactose and mannose as standards, total hexosamine by acetylacetone-Ehrlich's reagent method using glucosamine as a standard, and sialic acid by the diphenylamine reaction method.

Neutral sugars were analyzed by the Technicon system, using automated borate-complex anion exchange chromatography [12]; ribose was used as internal standard. Dilute solutions of glycoprotein (3 mg/ml) were hydrolyzed in 1 M HCl for 6 h in sealed tubes at 100 °C.

Individual hexosamines were determined by the method of Zacharius [13].

Amino acid analyses. The amino acid composition was determined by the method of Piez and Morris [14] with a Phoenix amino acid analyzer. Protein samples (0.3–0.6 mg) were hydrolyzed at 110 °C for 24, 48 and 96 h, respectively, in 1 ml of 6 M HCl in sealed evacuated tubes. Analyses at each time were carried out in duplicate.

Tryptophan analyses were conducted according to Procedure N of Spies and Chambers [15]. The time for reaction I was 6 h and for reaction II, 0.5 h.

---

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

N-Terminal amino acid analysis. Amino end group analysis was carried out by the dansylation technique reported by Woods and Wang [16] and described in detail by Hartley [17].

C-Terminal amino acid analysis. The carboxyl end group of membrane protein was determined by carboxypeptidase digestion as described by Ambler [18]. The mixtures were incubated at 37 °C for time intervals of 0, 1, 4, 7 and 23 h, and the released amino acids were identified and quantitated on an automated amino acid analyzer.

Determination of sodium dodecyl sulfate bound to glycoprotein. The amount of sodium dodecyl sulfate bound to the protein fractions was estimated by the methylene blue method reported by Weil and Stirton [19] using sodium dodecyl sulfate as a standard.

RESULTS

Isolation of glycoprotein B

Milk fat globule membrane proteins were prepared by the method of Herald and Brunner [6]. Table I shows the yield and recovery of protein at several stages in

TABLE I

RECOVERY OF PROTEIN IN FAT GLOBULE MEMBRANE FRACTIONS

Fraction	Total volume or weight	Protein concentration	Total protein (g)	Recovery (%)
Washed cream	1500 ml	1.79 mg/ml[a]	2.69	100.0
Membrane-containing serum	950 ml	3.59 mg/ml[b]	3.41	126.8
Delipidated fat globule membrane proteins	300 ml	4.95 mg/ml[b]	1.49	55.4
Insoluble fraction	1.143 g	0.582 g/g[c]	0.665	24.7
Soluble fraction	0.571 g	0.613 g/g[c]	0.350	13.0
Rechromatographed glycoprotein B	0.171 g	0.482 g/g[c]	0.082	3.0

[a] From Kjeldahl nitrogen.
[b] From Lowry [10] determination of suspension.
[c] Lowry [10] determination from dry weight.

this procedure. 100 mg of the salt soluble fraction were dissolved in a 1.5 ml solution of 4 % sodium dodecyl sulfate and 0.5 % Na_2CO_3 containing 0.15 ml of 2-mercapto-ethanol. The protein solution was heated at 37 °C for 45 min and then stirred at 4 °C for about 5 h. The proteins were then chromatographed on Sephadex G-200 as described above. Fig. 1 shows the elution diagram for the proteins of the soluble fraction. Three major fractions (A, B and C) which are divided by the broken vertical lines in Fig. 1 were obtained .These fractions were dialyzed extensively against water at 4 °C. and then against ion exchange resin (Amberlite MB-1) to remove the sodium dodecyl sulfate. The three fractions were then lyophilized and stored at 4 °C. Fig. 2

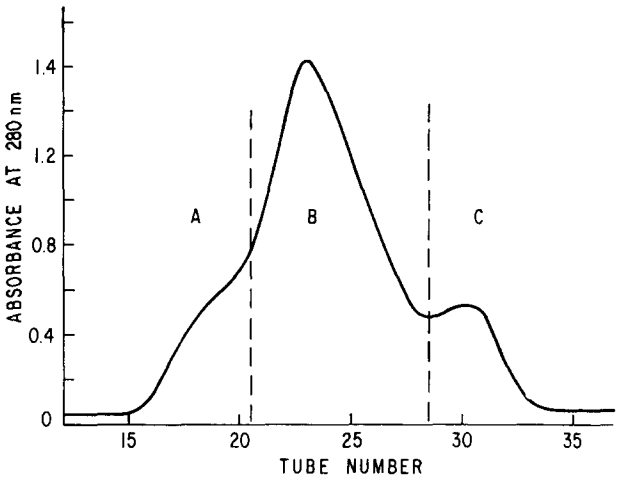

Fig. 1. Fractionation of soluble fraction of milk fat globule membrane proteins on Sephadex G-200 column. A column (2.5×45 cm) was eluted with 0.083 M Tris · HCl, pH 8.5, containing 0.1 % sodium dodecyl sulfate and 0.05 % mercaptoethanol. Dashed lines and letters define separate fractions.

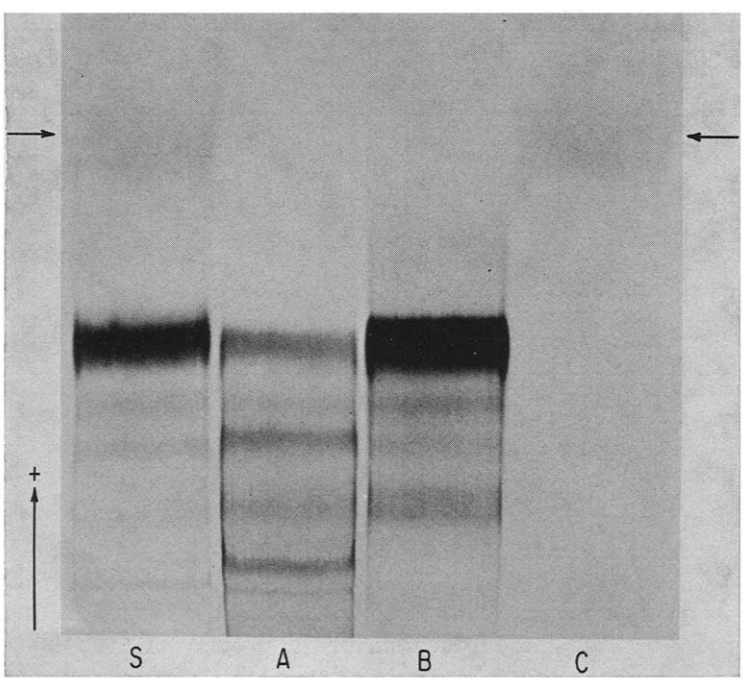

Fig. 2. Polyacrylamide gel electrophoresis in 7 % acrylamide, 0.1 % sodium dodecyl sulfate, at pH 9.2: S, soluble fraction of FGM proteins; A, fraction A; B, fraction B; and C, fraction C. The arrows indicate the locations of faint protein bands in S and C.

shows the electrophoretic patterns of the soluble fraction of fat globule membrane proteins, S, and of fractions, A, B and C. The first peak, fraction A, contained the proteins with the slowest mobilities and yielded 10 mg of the 100 applied to the column. Fraction B accounted for 30 mg and contained typically one major protein, while fraction C accounted for 60 % of sample applied and contained several bands not easily visualized by slab gels. This faint staining property was reported by Kobylka and Carraway [20] when they found that a major glycoprotein did not stain with Coomassie blue due to the high carbohydrate content of the protein.

Fraction B was dispersed in 0.02 M NaCl to a protein concentration of 3.5 % and then centrifuged at 25 000 $\times g$ for 1 h at 5 °C. The precipitate was discarded and the clear supernatant was dialyzed and lyophilized. 100 mg was then rechromatographed as described previously. Fraction B gave an almost symmetrical peak and its rechromatography is shown in Fig. 3. The rechromatographed fraction B was subjected to polyacrylamide slab gel electrophoresis as shown in Fig. 4. The rechromato-

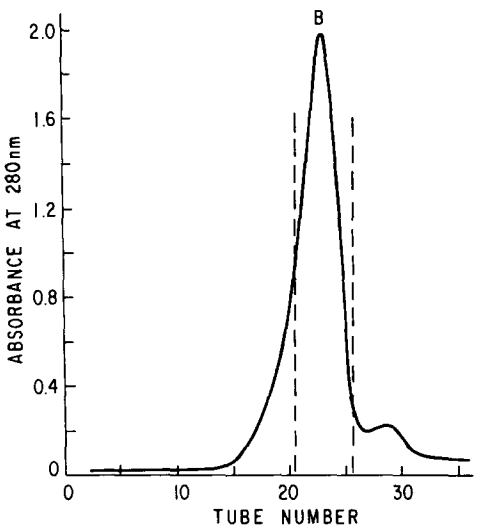

Fig. 3. Rechromatography of fraction B (Fig. 1) on Sephadex G-200. Tubes which were combined to yield glycoprotein B are shown between dashed lines.

graphed fractions A and C and a sodium dodecyl sulfate extract of the whole membrane from cream are shown for comparison. When the gels were stained for either protein or carbohydrate, a single major band was observed for fraction B. Thus, fraction B is hereafter designated as glycoprotein B. A typical yield of glycoprotein B is given in Table I.

Electrophoresis in the presence of sodium dodecyl sulfate on 10 % polyacrylamide slab gels was employed to estimate the molecular weight of glycoprotein B. The standard proteins used to calibrate the gels were lactoperoxidase (93 000), human transferrin (82 000), bovine serum albumin (68 000), ovalbumin (43 000), chymotrypsinogen (25 700), and B-lactoglobulin (18 400). The molecular weight obtained under these conditions was 49 500.

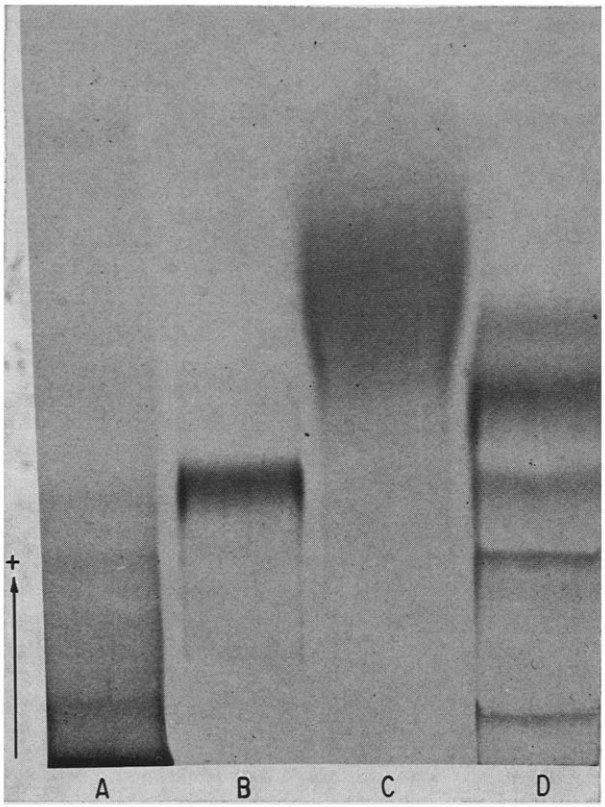

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of fractions A, B, and C (Fig. 1) which were rechromatographed on Sephadex G-200: A, fraction A; B, fraction B or glycoprotein B; C, fraction C; and D, sodium dodecyl sulfate extract of the whole membrane from cream.

Carbohydrate analyses

The carbohydrate analyses of glycoprotein B are presented in Table II. Hexose determination by the orcinol-sulfuric acid reaction [11] gave a value of 2.97 % which agrees well with 2.84 %, obtained by chromatography using the Technicon system. The results showed 1.09 % mannose, 1.04 % galactose, and 0.71 % glucose. In nearest integers, glycoprotein B contained mannose, galactose, and glucose in the ratio 3 : 3 : 2. The total hexosamine content of glycoprotein B, as determined by

TABLE II

CARBOHYDRATE COMPOSITION OF GLYCOPROTEIN B

Carbohydrate	%	Molar ratio
Hexose	2.97	mannose : galactose : glucose (3 : 3 : 2)
Hexosamine	4.02	glucosamine : galactosamine (7 : 2)
Sialic acid	7.35	*N*-acetyl neuraminic acid (13)

the acetylacetone-Ehrlich's reagent method [11], is 4.02 %. Individual hexosamines determined by method of Zacharius [13] gave a glucosamine : galactosamine molar ratio of 7 : 2. No other hexosamines were detected. Sialic acid as determined by the diphenylamine reaction method [11] was 7.35 %. Total carbohydrate as shown in Table II accounted for 14.3 % of glycoprotein B.

Amino acid analyses

The amino acid composition of glycoprotein B is shown in Table III. Molar ratios are calculated with alanine assigned the value of 6. The numbers of amino acid

TABLE III

AMINO ACID COMPOSITION OF GLYCOPROTEIN B

The nearest integers are given in parentheses. Results are the average of six determinations. Values for threonine and serine were calculated by extrapolation to zero time.

Amino acid	Molar ratio (Ala = 6)
Aspartic acid	10.8 (11)
Threonine	7.30 (7)
Serine	6.40 (6)
Glutamic acid	10.0 (10)
Proline	4.62 (5)
Half-cystine	2.60 (3)
Glycine	9.14 (9)
Alanine	6.00 (6)
Valine	5.31 (5)
Methionine	1.08 (1)
Isoleucine	5.24 (5)
Leucine	7.91 (8)
Tyrosine	3.34 (3)
Phenylalanine	4.68 (5)
Histidine	2.67 (3)
Lysine	4.36 (4)
Tryptophan*	2.04 (2)
Arginine	4.22 (4)

* Determined by the method of Spies and Chambers [15].

residues rounded to the nearest integer are also shown in parentheses in Table III. A minimal molecular weight of 10 700 was calculated for glycoprotein B, based on six alanine residues and a single methionine residue and the summation of the appropriate numbers of other amino acid residues derived from the molar ratios.

The tryptophan content of glycoprotein B was determined by the method of Spies and Chambers [15]. This determination gave a value of 2.04 residues of tryptophan, based on the minimal molecular weight of 10 700 as reported in Table III.

Terminal amino acids

Amino terminal analysis of glycoprotein B was performed by the dansylation method and the separation of dansyl-amino acids by polyamide thin-layer chromatography [16, 17]. Glycoprotein B gave one spot corresponding to serine.

Digestions of glycoprotein B with carboxypeptidase A were performed as described by Ambler [18]. Carboxypeptidase A treatment released leucine rapidly while valine and glutamic acid were released slowly, valine slightly faster than glutamic acid. These results suggested the COOH-terminal sequence to be -Glu-Val-Leu.

Determination of sodium dodecyl sulfate bound to glycoprotein B

Sodium dodecyl sulfate effectively dissociated the soluble protein fraction of the milk fat globule membrane. After the column chromatography the glycoprotein B fraction was dialyzed exhaustively in a cylindrical jar of deionized water containing Amberlite MB-1 ion exchange resin. In order to test whether there was complete removal of sodium dodecyl sulfate from glycoprotein B, the methylene blue method of detecting the sodium dodecyl sulfate [19] was applied. A well-characterized protein, α_{s1}-casein, and a glycoprotein, riboflavin binding protein of egg white, were reacted with a known amount of sodium dodecyl sulfate. The results of the methylene blue experiments indicated that the experimental values for the amounts of detergent in the presence of α_{s1}-casein and riboflavin binding protein were 100 and 95 %, respectively. In the absence of added sodium dodecyl sulfate, these proteins gave no reaction with the methylene blue. Analysis of glycoprotein B by this method showed that 16 % of the dry weight of the glycoprotein was attributable to bound sodium dodecyl sulfate.

DISCUSSION

Herald and Brunner [6] reported the preparative procedures for two fat globule membrane protein fractions designated soluble and insoluble on the basis of their ability to dissolve in NaCl. These two fractions were heterogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The soluble protein fraction contains several glycoproteins; this fraction accounts for approximately 23 % of the protein recovered after delipidation (Table I). Employing gel chromatography in sodium dodecyl sulfate and 2-mercaptoethanol, the salt soluble proteins were separated into three fractions, A, B, and C, which contained 10, 30, and 60 %, respectively, of the starting material. When fraction B was rechromatographed, it gave a reproducible peak on Sephadex. Essentially one band was observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with either protein or carbohydrate stain. The appearance of only one N-terminal residue, serine, indicates that the component, designated glycoprotein B, may be a homogeneous protein.

Mather and Keenan [21] reported the extraction of a major glycoprotein fraction from fat globule membranes using $MgCl_2$. This fraction appeared as a doublet on sodium dodecyl sulfate gel electrophoresis. They estimated the molecular weights of these two proteins to be 43 500 and 48 000. In this study the higher molecular weight component is associated primarily with the NaCl insoluble fraction, while the lower molecular weight component is probably identical with glycoprotein B.

Although sodium dodecyl sulfate-polyacrylamide gel electrophoresis provides rather accurate estimates of molecular weights for most proteins, values for glycoproteins may be subject to error [22]. Values reported in the literature for gel bands comparable to glycoprotein B range from 43 500 [21] to 54 600 [22]. Mangino and Brunner [23] estimated the molecular weight of the major glycoprotein of their 1S fraction to be 49 000. This latter value is in agreement with the finding of 49 500 reported here. Side

by side comparisons of glycoprotein B and the 1S fraction of Mangino and Brunner, when carried out on slab gels, showed these proteins to be nearly identical in mobility. The value of 49 500 reported here was calculated from gel electrophoresis experiments which were conducted on polyacrylamide slabs; this enables one to run both the samples and reference proteins simultaneously.

The total carbohydrate content of glycoprotein B is 14.3 %. Glycoprotein B contains sialic acid, mannose, galactose, and glucose. The hexosamines present are glucosamine and galactosamine.

Bigelow [24] has proposed a structural parameter for proteins termed average hydrophobicity which is based on amino acid composition of the molecule and on Tanford's free energies of transfer of amino acid side chains from an organic to an aqueous environment. Glycoprotein B has an average hydrophobicity of 1110 which is lower than other milk proteins, such as α-lactalbumin, α-casein, and β-lactoglobulin, which are reported in the literature [24]. Hydroxy amino acids account for about 15 % of the amino acid content of glycoprotein B.

The minimal molecular weight of glycoprotein B was found to be 10 700 according to the amino acid analysis. Gel electrophoresis showed that this protein has an estimated molecular weight of 49 500. If 14 % carbohydrate is subtracted (6900), then the molecular weight of the protein moiety would be 42 600 which is close to the figure obtained by multiplying the minimal molecular weight by 4. Furthermore, the molecular weight of total carbohydrate, as calculated by the molar ratios of individual sugars, was found to be 6912.

Glycoprotein B was found to consist of 48 % protein by weight (Lowry et al. method) and 14 % carbohydrate. If one takes into account approximately 10 % moisture, the total percentage will fall short of 100 %. Methylene blue analysis for sodium dodecyl sulfate showed that 16 % of the dry weight of the isolated glycoprotein B was due to bound sodium dodecyl sulfate. It appeared that the dialysis failed to remove all of the sodium dodecyl sulfate from glycoprotein B.

Further studies are in progress dealing with the isolation and analysis of the individual proteins of the fat globule membrane as well as attempts to identify those proteins important in the stability of the fat globule in milk.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. C. A. Kiddy for supplying individual milks from the USDA herd at Beltsville, Md. The authors also wish to thank Dr. C. O. Clagett, Pennsylvania State University for performing the Technicon carbohydrate analyses, H. J. Dower for performing amino acid analyses, and D. J. Wolf for his able assistance.

REFERENCES

1 Bretscher, M. S. (1973) Science 181, 622–629
2 Vanderkooi, G. (1974) Biochim. Biophys. Acta 344, 307–345
3 Thompson, M. P., Brunner, J. R., Stine, C. M. and Lindquist, K. (1961) J. Dairy Sci. 44, 1589–1596
4 Brunner, J. R. (1969) in Structural and Functional Aspects of Lipoproteins in Living Systems, (Tria, E. and Scanu, A. M., eds.), pp. 545–578, Academic Press, New York

5 Keenan, T. W., Morré, D. J., Olson, D. E., Yunghans, W. N. and Patton, S. (1970) J. Cell Biol. 44, 80–93
6 Herald, C. T. and Brunner, J. R. (1957) J. Dairy Sci. 40, 948–956
7 Peterson, R. F. (1972) Methods Enzymol. 25, 178–182
8 Kapitany, R. A. and Zebrowski, E. J. (1973) Anal. Biochem. 56, 361–369
9 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
10 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
11 Winzler, R. J. (1955) Methods Biochem. Anal. 2, 290–299
12 Spiro, R. G. (1972) Methods Enzymol. 28, 3–7
13 Zacharius, R. M. (1976) J. Chromat., in the press
14 Piez, K. A. and Morris, L. (1960) Anal. Biochem. 1, 187–201
15 Spies, J. R. and Chambers, D. C. (1949) Anal. Chem. 21, 1249–1266
16 Woods, K. R. and Wang, K.-T. (1967) Biochim. Biophys. Acta 133, 369–370
17 Hartley, B. S. (1970) Biochem. J. 119, 805–822
18 Ambler, R. P. (1972) Methods Enzymol. 25, 143–154
19 Weil, J. K. and Stirton, A. J. (1964) J. Am. Oil Chem. Soc. 41, 355–358
20 Kobylka, D. and Carraway, K. L. (1972) Biochim. Biophys. Acta 288, 282–295
21 Mather, I. H. and Keenan, T. W. (1975) J. Membrane Biol. 21, 65–85
22 Anderson, M., Cawston, T. and Cheesman, G. C. (1974) Biochem. J. 139, 653–660
23 Mangino, M. E. and Brunner, J. R. (1975) J. Dairy Sci. 58, 313–318
24 Bigelow, C. C. (1967) J. Theoret. Biol. 16, 187–211