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Purified membrane and soluble folate binding proteins from cultured KB cells have similar amino acid compositions and molecular weights but differ in fatty acid acylation

(membrane proteins/folate/protein purification/amino acid sequencing)

CAROL A. LUHRS*†, PRASAN PITIRANGGON*†, MARIA DA COSTA*†, SHELDON P. ROTHENBERG*†, BRONISLAW L. SLOMIANY‡, LARRY BRINK§, GUILLERMO I. TOUS¶, AND STANLEY STEIN¶

*Division of Hematology and Oncology, Department of Medicine, Brooklyn Veterans Administration Medical Center, Brooklyn, NY 11209; †State University of New York–Health Science Center at Brooklyn, Brooklyn, NY 11203; ‡New York Medical College, Valhalla, NY 10595; §Roche Institute of Molecular Biology, Nutley, NJ 07110; and ¶New Jersey Center for Advanced Biotechnology and Medicine, Piscataway, NJ 08854

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ABSTRACT **A membrane-associated folate binding protein (FBP) and a soluble FBP, which is released into the culture medium, have been purified from human KB cells using affinity chromatography. By NaDodSO$_4$/PAGE, both proteins have an apparent M_r of ≈42,000. However, in the presence of Triton X-100, the soluble FBP eluted from a Sephadex G-150 column with an apparent M_r of ≈40,000 (similar to NaDodSO$_4$/PAGE) but the membrane-associated FBP eluted with an apparent M_r of ≈160,000, indicating that this species contains a hydrophobic domain that interacts with the detergent micelles. The amino acid compositions of both forms of FBP were similar, especially with respect to the apolar amino acids. In addition, the 18 amino acids at the amino termini of both proteins were identical. The membrane FBP, following delipidation with chloroform/methanol, contained 7.1 mol of fatty acid per mol of protein, of which 4.7 mol was amide-linked and 2.4 mol was ester-linked. The soluble FBP contained only 0.05 mol of fatty acid per mol of protein. These studies indicate that the membrane FBP of KB cells contains covalently bound fatty acids that may serve to anchor the protein in the cell membrane.**

Two forms of folate binding proteins (FBPs) are present in mammalian tissues and biological fluids: soluble and membrane-associated. Soluble FBPs have been identified in milk (1, 2), serum (3), leukocytes (4), and spleen (5). Membrane-associated (membrane) FBPs have been described in choroid plexus (6), placenta (7), intestinal mucosa (8), renal cortex (9), human milk (10), and several cultured human cell lines, such as Molt, HeLa, and KB cells (11). KB cells, which are derived from an epidermoid carcinoma, have an unusually high level of membrane FBP (11, 12) and release a soluble FBP into the medium in which they are cultured (13, 14). In this report we describe the purification by affinity chromatography of the soluble FBP, which is released into the medium, and the integral membrane FBP of these cultured KB cells. Although the purified membrane FBP had hydrophobic properties (i.e., it bound Triton X-100), it had the same molecular weight by NaDodSO$_4$/PAGE, similar amino acid composition, and an identical 18 amino acid sequence at the amino terminus as the purified soluble FBP, suggesting that the hydrophobic property of the membrane protein may be due to a component other than a hydrophobic sequence of amino acids. Fatty acid analysis of the FBPs showed that the membrane FBP contains 150-fold more covalently bound fatty acids than the soluble FBP and this may account for its hydrophobic property.

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MATERIALS AND METHODS

[^{3}H]Pteroylglutamic acid (PteGlu) (37–40 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham Searle; PteGlu, phenylmethylsulfonyl fluoride, and Norit-A charcoal were purchased from Sigma. Ultrapure Triton X-100 was purchased from International Biotechnologies (New Haven, CT). Dithiothreitol was purchased from Calbiochem–Behring. Hydroxylamine hydrochloride was obtained from Aldrich. RPMI 1640 medium, L-glutamine, penicillin, streptomycin, Fungizone, and minimal essential medium (MEM) prepared without folic acid were purchased from GIBCO. Fetal calf serum was purchased from HyClone (Logan, UT). Trasylol was obtained from Mobay Chemical (New York). Complete scintillation cocktail (3a70) was purchased from Research Projects International (Mt. Prospect, IL). Extracti-Gel D and bicinchoninic acid (BCA) protein assay reagent were purchased from Pierce. All other chemicals used were of reagent grade. NaDodSO$_4$, low molecular weight standards for NaDodSO$_4$/PAGE, Bio-Rad silver-staining kit, and Bio-Rad protein assay were obtained from Bio-Rad. The KB cell line, derived from an epidermoid carcinoma, was purchased from the American Type Culture Collection.

Cell Culture. Normal, folate-replete KB cells were maintained as adherent cell monolayers in RPMI 1640 medium that contained 2.26 μM PteGlu (N medium). For the purification of the FBP from the culture medium, KB cells were grown in folate-deficient MEM to which was added 9 pmol of PteGlu per liter (D medium). For the purification of the FBP from the KB cell membrane, KB cells grown in both N and D media were used. All cultures were supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and Fungizone (1 μg/ml).

Preparation of Samples for Purification. *KB cell membrane.* The Triton X-100-solubilized membrane was prepared as described (15). Samples prepared from cells grown in N medium were dialyzed overnight against 0.01 M acetic acid containing 1% Triton X-100 to remove endogenous folates and then neutralized by dialysis against 0.01 M KPO$_4$ (pH 7.5) containing 1% Triton X-100 for 8 hr. The Triton X-100-solubilized membranes from cells grown in N and D media were then pooled and the folate binding capacity (FBC) was determined as described (15).

Culture medium. Every 72 hr, the D medium in which KB cells had been cultured was changed. The spent medium was pooled and centrifuged at 40,000 × *g* for 1 hr to remove particulate material, and the FBC was determined.

Abbreviations: FBP, folate binding protein; PteGlu, pteroylglutamic acid; FBC, folate binding capacity; BCA, bicinchoninic acid.

Purification of FBPs. The FBP in the culture medium was purified as follows. Thirty-nine hundred milliliters of medium was centrifuged at 40,000 × *g* for 1 hr and the supernate was applied to a 20-ml column of epoxy-activated Sepharose 6B coupled to PteGlu using hexane diamine as the linker (E. Sadasivan, unpublished). The column was washed and eluted as described (15). The first 10 fractions, which contained the FBP, were pooled and concentrated using a Diaflo ultrafiltration membrane (10,000 M_r cutoff) (Amicon). The FBP of the Triton X-100-solubilized membranes was similarly purified except that all solutions contained 1% Triton X-100. The mol of [^{3}H]PteGlu bound per mol of protein, which was measured by the Bio-Rad protein assay for the medium FBP and the BCA detergent-compatible protein assay (16) for the membrane FBP, were 1.0 and 0.9, respectively.

Gel Filtration Chromatography. An aliquot of the purified membrane FBP was saturated with [^{3}H]PteGlu and eluted through a Sephadex G-150 column (2.6 cm × 70 cm) previously equilibrated with 0.01 M KPO_4/0.15 M NaCl containing 1% Triton X-100 at a flow rate of 10 ml/hr. All samples were applied to the column in a total volume of 2 ml containing 0.1% ovalbumin and 2-ml fractions were collected.

The purified membrane FBP was then treated with hydroxylamine hydrochloride. An aliquot of the purified protein (0.64 μg) in 0.01 M KPO_4 (pH 7.4) containing 1% Triton X-100 was added to an equal volume of 2 M hydroxylamine hydrochloride containing 1% Triton X-100 at pH 9.8 and incubated for 90 min at 22°C. The sample was then saturated with [^{3}H]PteGlu and eluted through Sephadex G-150 in the presence of 1% Triton X-100.

NaDodSO$_4$/PAGE. The purified FBPs were subjected to NaDodSO$_4$/PAGE by the method of Laemmli (17) on a 15% polyacrylamide slab gel, 1.5-mm thick. Standards and samples were prepared for electrophoresis by adding an equal volume of 0.0625 M Tris·HCl buffer (pH 6.8) containing 15% glycerol (vol/vol), 100 mM dithiothreitol, 0.002% bromophenol blue, and 20% NaDodSO$_4$ and then heating this mixture for 5 min in a boiling water bath. Following electrophoresis, the gels were stained using the Bio-Rad silver-staining kit.

Covalently Bound Fatty Acid Analysis. The lyophilized sample (76 μg) was dissolved in 2 ml of acidified (with acetic acid to pH 4.0) 0.15 M NaCl and vigorously agitated by spinning in a Vortex with equal volumes of chloroform/methanol, 2:1 (vol/vol). The mixtures were centrifuged at 2000 rpm for 5 min and the lipids contained in the lower organic phase were aspirated. The procedure was repeated two more times, and the delipidated protein material contained in the aqueous phase was dialyzed, lyophilized, and subjected to analysis of covalently bound fatty acids. For the analysis of ester-bound fatty acids, the delipidated sample was incubated for 30 min at 37°C with 0.3 M methanolic KOH (18). Acid methanolysis in 1.0 M methanolic HCl at 80°C for 24 hr was used to release the amide- and ester-bound fatty acids (18). The released fatty acid methyl esters were recovered from the reaction mixtures by three consecutive extractions with hexane and analyzed by gas/liquid chromatography using nonadecanoic acid as the internal standard. The content of amide-bound fatty acids was estimated from the difference between the total and ester-bound fatty acids. Gas/liquid chromatography was performed with a Perkin–Elmer 8310 instrument equipped with glass columns (180 × 0.2 cm) packed with 3% SE-30 on Gas-Chrom Q, programed at 4°C/min from 160°C to 250°C.

Amino Acid Analysis. The Triton X-100 was removed from the purified membrane FBP by two passages through a 1-ml column of Extracti-Gel D. The total recovery of the protein following the second elution was 35% of the original binding capacity. Samples of the detritonized membrane FBP and the soluble FBP of the culture medium were hydrolyzed in 6 M HCl containing 4% thioglycolic acid for 24 hr at 110°C and individual amino acids were detected fluorometrically after coupling with fluorescamine (19).

Amino-Terminal Sequence Determination. The procedure used to remove Triton X-100 from the samples for microsequencing was as follows. A Centricon tube (Amicon) (10,000 M_r cutoff) was rinsed with 0.1% NaDodSO$_4$. The protein pellet was dissolved in 2 ml of 0.1% NaDodSO$_4$ and brought down to 0.2 ml by centrifugation. The volume was brought to 2 ml by addition of 1.8 ml of 0.1% NaDodSO$_4$ and recentrifuged in the same Centricon filter. This procedure was repeated an additional three times. An aliquot of the final 0.2-ml sample was applied to the sequencer. Phenylthiohydantoin-amino acids were determined on an Applied Biosystems model 120A analyzer (20).

RESULTS

The silver stain of the NaDodSO$_4$/PAGE of the FBPs eluted from the affinity matrix is shown in Fig. 1. The apparent molecular weights of the FBP from the medium (lane 1) and from the membrane (lane 2) were essentially the same (M_r ≈42,000), whether the sample was prepared in the presence or the absence of reducing agents (data not shown).

The apparent M_r of the FBP purified from the culture medium and subjected to gel filtration through Sephadex G-150 with buffer containing Triton X-100 was ≈40,000, quite similar to the value obtained by NaDodSO$_4$/PAGE (Fig. 2). In contrast, the FBP purified from the membrane and similarly chromatographed through Sephadex G-150 with buffer containing Triton X-100 had an apparent M_r of ≈160,000. A minor component of this preparation eluted with an apparent M_r of ≈40,000. Both of these FBPs have been

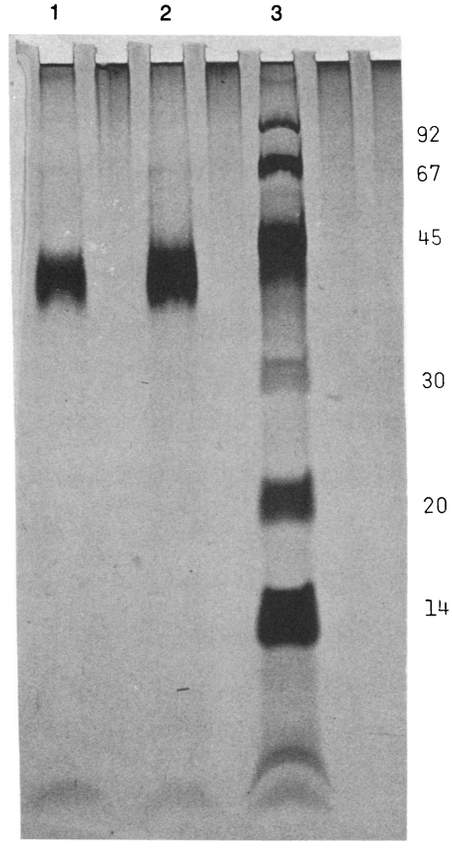

FIG. 1. NaDodSO$_4$/PAGE of the purified preparations of FBP. Lane 1, FBP purified from the culture medium (1.2 μg); lane 2, FBP purified from the Triton X-100-solubilized membrane (1.3 μg); lane 3, marker proteins (shown as $M_r \times 10^{-3}$). The gel was stained by using the Bio-Rad silver-staining kit.

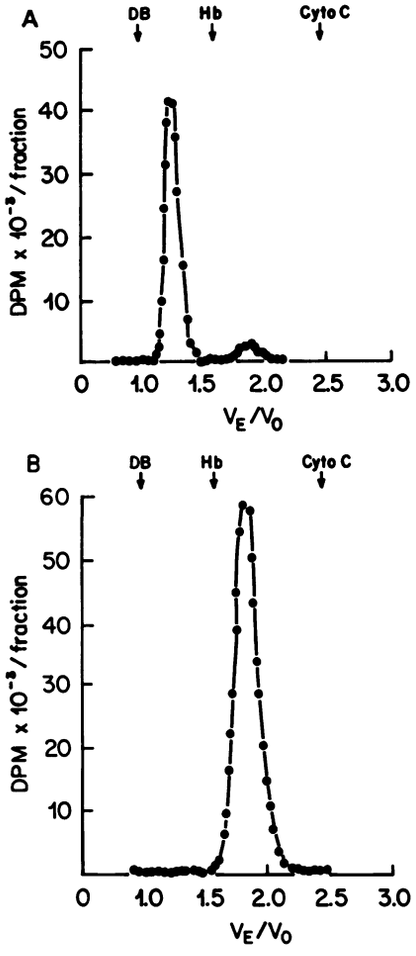

FIG. 2. Gel filtration through Sephadex G-150 of the purified FBPs saturated with [^{3}H]PteGlu. In *A* and *B*, the column buffer was 0.01 M KPO_4 (pH 7.5) containing 0.15 M NaCl and 1% Triton X-100 (vol/vol). The column was calibrated with blue dextran (DB), bovine hemoglobin (Hb), cytochrome *c* (Cyto C), and [^{3}H]PteGlu. (*A*) Purified FBP from the Triton X-100-solubilized membrane. (*B*) FBP purified from the medium in which KB cells had been cultured.

identified in the solubilized crude membrane preparation in approximately the same proportion (15). Treatment of the purified membrane FBP with 1 M hydroxylamine (pH 9.8) at 22°C did not alter its gel filtration profile in the presence of Triton X-100 (data not shown).

The amino acid compositions of the two purified FBPs are listed in Table 1. The percentage of nonpolar amino acid residues (≈30%) is similar for both soluble and membrane FBPs.

The sequences of the 18 amino acids at the amino-terminal end of both the soluble and the membrane FBP are identical (Fig. 3).

The fatty acid contents of both FBPs are listed in Table 2. The purified membrane FBP, after extraction with chloroform/methanol, contained 7.1 mol of fatty acid per mol of protein and the purified soluble FBP of the medium contained 0.05 mol of fatty acid per mol of protein. Of the total fatty acid content of the membrane FBP, 49.3% was palmitic acid and 48.3% was stearic acid. Of the total 7.1 mol of fatty acid per mol of protein, 2.4 mol were ester bonds and 4.7 mol were amide.

DISCUSSION

Two classes of FBPs—soluble and membrane—have been identified in several human tissues, including placenta, milk,

Table 1. Amino acid compositions of purified FBPs

Amino acid	mol%	
	Membrane	Soluble
Aspartic acid	13.0	14.0
Threonine	4.9	5.2
Serine	8.5	7.2
Glutamic acid	14.6	15.2
Proline	ND	ND
Glycine	9.7	6.0
Alanine*	9.3	9.6
Valine*	4.9	5.6
Methionine*	1.7	2.1
Isoleucine*	2.6	2.6
Leucine*	5.7	5.2
Tyrosine	4.4	4.4
Phenylalanine*	4.0	4.4
Histidine	4.0	4.4
Lysine	6.1	7.2
Arginine	6.5	6.8
Cysteine	ND	ND
Tryptophan	ND	ND

ND, not determined.
*These hydrophobic amino acids represent ≈30% of the total amino acids of both soluble and membrane FBPs from KB cells.

and cultured KB cells. The soluble and membrane FBPs of milk (10) have apparent M_rs of 43,000 and 45,000 by NaDodSO$_4$/PAGE, although the apparent M_rs of these two proteins by gel filtration in the presence of Triton X-100 are 40,000 and 160,000, respectively. Antony *et al.* (10) have demonstrated that the higher molecular weight of the membrane FBP on gel filtration was due to the binding of Triton X-100 micelles and these workers presumed this to be a consequence of an interaction of the detergent with the hydrophobic amino acid domain of the protein.

The results presented here for the FBPs of the KB cell are similar to those reported for the FBPs of milk (10). On NaDodSO$_4$/PAGE, each purified protein has an apparent M_r of 42,000, although, by gel filtration, the FBPs of the culture medium and membrane have apparent M_rs of 40,000 and 160,000 respectively.

The similar molecular weights of these two purified FBPs, one soluble and the other an integral membrane protein, suggested to us, initially, that the hydrophobic portion of this protein must be unusually small. However, since most integral proteins are anchored by a transmembrane segment of at least 20–30 hydrophobic residues (21), the difference in molecular weight between the soluble and membrane forms of FBP should have been at least 2000, a value detectable by NaDodSO$_4$/PAGE. In addition, the amino acid composition of each purified protein was similar and the 18 amino acids at the amino termini of the two proteins were identical.

In this study, we have been able to establish that the membrane FBP contains covalently bound fatty acids (i.e., fatty acids not removed by treatment with chloroform/methanol) and this domain on the protein could provide the same property of micellar complexing with Triton X-100 as occurs with a hydrophobic peptide sequence of an integral membrane protein. Covalently bound fatty acids have been

Soluble FBP:
1 5 10 15 18
Ile-Ala-Trp-Ala-Arg-Thr-Glu-Leu-Leu-Asn-Val-?-Met-Asn-Ala-Lys-His-His

Membrane-associated FBP:
1 5 10 15 18
Ile-Ala-Trp-Ala-Arg-Thr-Glu-Leu-Leu-Asn-Val-?-Met-Asn-Ala-Lys-His-His

FIG. 3. Amino-terminal sequencing.

Table 2. Fatty acid content

FBP	mol/mol of protein		
	Total	Ester	Amide*
Membrane	7.10	2.40	4.70
Soluble	0.05		

*The content of amide-bound fatty acids was estimated from the difference between the total and ester-bound fatty acids.

identified in several viral glycoproteins (22, 23), a glycoprotein in the outer membrane of *Escherichia coli* (24), human transferrin receptor (25), brain myelin proteolipoprotein (26), and mucous glycoproteins of gastric and salivary gland mucosa (27, 28). Per mol of protein, there are usually 1–6 mol of fatty acid attached more commonly by means of an ester linkage to serine or threonine and less commonly by means of an amide linkage to lysine or arginine. The difference in fatty acid content following acid methanolysis (which cleaves ester and amide linkages) and alkaline methanolysis (which cleaves ester linkages) established that there were 4.7 mol of amide-linked and 2.4 mol of ester-linked fatty acid per mol of protein (18). That the micellar aggregation of the membrane FBP with Triton X-100 (which was demonstrated by gel filtration) was still observed following mild treatment with hydroxylamine (pH 9.8 at 22°C) is consistent with the greater fraction of amide-linked fatty acid since these bonds are not cleaved by hydoxylamine under these conditions (29).

The report of Elwood *et al.* (30), that the membrane FBP of KB cells has a greater molecular weight ($M_r \approx 50{,}000$) than the soluble FBP ($M_r \approx 40{,}000$) and contains a greater amount of apolar amino acids, is at variance with our findings. Their studies, however, did not include an analysis for fatty acid, and the hydrophobicity of the membrane FBP, similarly demonstrated by aggregation with Triton X-100, was presumed to be a property of the hydrophobic polypeptide domain.

The role of covalently bound fatty acid to membrane proteins is unclear. Schlesinger has suggested that the fatty acid may help to direct or anchor proteins to the cell membrane and that proteolipids in the cell membrane may provide unique regions that facilitate transmembrane signaling and membrane fusions that occur during endocytosis and exocytosis (31).

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1. Ghitis, J. (1967) *Am. J. Clin. Nutr.* **20,** 1–4.
2. Rubinoff, M., Schreiber, C. & Waxman, S. (1977) *FEBS Lett.* **75,** 244–248.
3. da Costa, M., Rothenberg, S. P., Fischer, C. & Rosenberg, Z. (1978) *J. Lab. Clin. Med.* **91,** 901–910.
4. Rothenberg, S. P. (1970) *Proc. Soc. Exp. Biol. Med.* **133,** 428–432.
5. da Costa, M. & Fischer, C. (1981) *J. Lab. Clin. Med.* **98,** 956–964.
6. Suleiman, S. A., Spector, R. & Cancilla, P. (1981) *Neurochem. Res.* **6,** 333–341.
7. Antony, A. C., Utley, C., Van Horne, K. C. & Kolhouse, J. F. (1981) *J. Biol. Chem.* **256,** 9684–9692.
8. Leslie, G. I. & Rowe, P. B. (1972) *Biochemistry* **11,** 1696–1701.
9. Selhub, J. & Franklin, W. A. (1984) *J. Biol. Chem.* **259,** 6601–6606.
10. Antony, A. C., Utley, C. S., Marcel, P. D. & Kolhouse, J. F. (1982) *J. Biol. Chem.* **257,** 10081–10089.
11. McHugh, M. & Cheng, Y. (1979) *J. Biol. Chem.* **254,** 11312–11318.
12. Antony, A. C., Kane, M. A. & Kolhouse, J. F. (1983) *Blood* **62,** 35 (abstr.).
13. Kane, M. A., Antony, A. C. & Kolhouse, J. F. (1984) *Clin. Res.* **32,** 310 (abstr.).
14. Luhrs, C., da Costa, M., Sequeira, J. & Rothenberg, S. P. (1985) *Clin. Res.* **33,** 548 (abstr.).
15. Luhrs, C., Sadasivan, E., da Costa, M. & Rothenberg, S. P. (1986) *Arch. Biochem. Biophys.* **250,** 94–105.
16. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150,** 76–85.
17. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
18. Slomiany, B. L., Murty, V. L. N., Slomiany, A., Zielenski, J. & Mandel, I. D. (1986) *Biochim. Biophys. Acta* **882,** 18–28.
19. Stein, S. & Brink, L. (1981) *Methods Enzymol.* **79,** 20–25.
20. Hewick, R. M., Hunkapiller, M. W., Hood, L. E. & Dreyer, W. J. (1981) *J. Biol. Chem.* **256,** 7990–7997.
21. Gething, M. J. (1985) in *Protein Transport and Secretion*, ed. Gething, M. J. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 1–20.
22. Schmidt, M. F. G., Bracha, M. & Schlesinger, M. J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1687–1691.
23. Schmidt, M. F. G & Schlesinger, M. J. (1979) *Cell* **17,** 813–819.
24. Braun, V. & Rehn, K. (1969) *Eur. J. Biochem.* **10,** 426–438.
25. Omary, M. B. & Trowbridge, I. S. (1981) *J. Biol. Chem.* **256,** 4715–4718.
26. Agrawal, H. C., Randle, C. L. & Agrawal, D. (1982) *J. Biol. Chem.* **257,** 4588–4592.
27. Slomiany, B. L., Takagi, A., Liau, Y. H., Jozwiak, Z. & Slomiany, A. (1984) *J. Biol. Chem.* **259,** 11997–12000.
28. Slomiany, B. L., Murty, V. L. N., Tagaki, A., Tsukada, H., Kosmala, M., Slomiany, A. (1985) *Arch. Biochem. Biophys.* **242,** 402–410.
29. Changchien, L.-M. & Craven, G. R. (1986) *Nucleic Acids Res.* **14,** 1957–1966.
30. Elwood, P. C., Kane, M. A., Portillo, R. M. & Kolhouse, J. F. (1986) *J. Biol. Chem.* **261,** 15416–15423.
31. Schlesinger, M. J. (1981) *Annu. Rev. Biochem.* **50,** 193–206.