Vol. 159, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1989 Pages 79-86

MASS DETERMINATION OF RECEPTOR-MEDIATED ACCUMULATION OF PHOSPHATIDATE AND DIGLYCERIDES IN HUMAN NEUTROPHILS MEASURED BY COOMASSIE BLUE STAINING AND DENSITOMETRY

David E. Agwu, Linda C. McPhail, Robert L. Wykle, and Charles E. McCall

Departments of Medicine and Biochemistry
Wake Forest University Medical Center
Winston-Salem, NC 27103

Received December 7, 1988

SUMMARY: Quantitation of 1,2-diacylglycerol (AAG), 1-O-alkyl-2-acylglycerol (EAG) and phosphatidic acid (PA) was conducted in polymorphonuclear leukocytes (PMN) labeled with 1-O-[^{3}H]alkyl-2-acyl-GPC following stimulation with 1 uM fMLP using Coomassie blue staining and densitometry. At 5s AAG and PA increased by 80% and 107%, respectively, over controls. The accumulation of PA, which reached a maximum by 30s, was higher than AAG by 302% at 5s, and 550% at 30s. EAG accumulation was delayed by 15s following stimulation of PMN. These results show that AAG accumulates before EAG and support the role of AAG in cellular activation, perhaps, via the stimulation of protein kinase C (PKC). EAG may serve to counter the effects of AAG or may itself elicit responses. The high concentrations of PA which accumulate early suggest that PA may be generated by the activation of phospholipase D in PMN stimulated with fMLP.

The biological responses of human neutrophils (PMN) stimulated with the chemotactic tripeptide fMLP include chemotaxis, lysosomal enzyme release, phagocytosis, and the respiratory burst. The intracellular mechanisms which trigger these responses may involve the remodeling of membrane phospholipids. Inositol phospholipids of PMN are hydrolyzed upon stimulation with fMLP to form 1,2-diacylglycerol (AAG) and 1,2-diacyl-phosphatidic acid, AAPA(1-3). Choline-containing phosphoglycerides (PC) may also contribute to the formation of AAG and AAPA in rat hepatocytes stimulated with vasopressin (4) and in phorbol ester stimulated MDCK cells (5). In addition,

ABBREVIATIONS: fMLP, N-formyl-methionyl-leucyl-phenylalanine; CB, cytochalasin B; AAG, 1,2-diacylglycerol; EAG, 1-O-alkyl-2-acylglycerol; DG, diradylglycerol; PA, diradyl-phosphatidic acid; AAPA, 1,2-diacyl-PA; EAPA, 1-O-alkyl-2-acyl-PA; PKC, protein kinase C; diradyl-, (1-O-alkyl-2-acyl-, and 1,2-diacyl-); CBB R-250, Coomassie brilliant blue R-250; GPC, glycero-3-phosphocholine; PMN (neutrophils), polymorphonuclear leukocytes; TLC, thin-layer chromatography; TG, triglycerides; MG, monoglyceride; FFA, free fatty acids.

0006-291X/89 $1.50

1-O-alkyl-2-acyl-GPC is directly implicated in the generation of 1-O-alkyl-2-acylglycerol (EAG) and 1-O-alky-2-acyl-PA (EAPA) in MDCK cells (5), in fMLP-stimulated HL-60 granulocytes (6) and in human PMN (7).

AAG is an endogenous activator of protein kinase C (PKC) (8) which has been associated with the activation of numerous cellular responses including the respiratory burst (9). EAG causes differentiation of HL-60 cells (10), inhibits (11) or weakly activates PKC (12,13) and may modulate the activation of the respiratory burst in PMN (14). PA induces a transient rise in cytoplasmic free Ca^{2+} (15,16), induces cell proliferation (16), and may be a second-messenger in the activation of a phospholipase C which hydrolyzes phosphoinositides (17). Elucidation of the mechanism(s) involved in the generation of diradylglycerol (DG) and PA and kinetic analysis of the quantitative accumulations of these metabolites may provide insights to their significance in stimulus response coupling.

Mass measurements of AAG in HL-60 phagocytes (18), and human neutrophils (19) stimulated with fMLP have been performed using E. coli diacylglycerol kinase conversion of AAG to [^{32}P]-labeled PA. This method showed no accumulation of AAG prior to 30s and required selective alkaline hydrolysis to demonstrate formation of EAG. Quantitation of DG has also been conducted by HPLC, a method which requires a high number of cells (>10^8) and uses a time-consuming derivatization procedure (7,20). Mass measurements of PA have been rare. Serhan et al. (21) determined the mass of PA in fMLP-stimulated neutrophils, with no indications as to possible accumulations of EAPA, by measuring total lipid phosphorous.

As an alternative to the above methods, we have utilized 1-O-[^{3}H]alkyl-2-acyl-GPC labeling of cells and a simple yet sensitive method involving densitometric scanning of TLC-separated cellular and standard lipids stained with Coomassie brilliant blue (CBB R-250) to analyze the time-dependent accumulations of AAG, EAG, and PA in fMLP-stimulated neutrophils. Our results demonstrate a rapid accumulation of PA and, for the first time, mass accumulation of AAG as early as 5s, preceding the accumulation of EAG.

MATERIALS AND METHODS

Materials. FMLP, cytochalasin B (CB), 1,2-dipalmitin and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, MO. CBB R-250 was

obtained from Bio-Rad Laboratories, Richmond, CA. Silica Gel 60 plates were purchased from E. Merck, Darmstadt, W. Germany. The following lipid standards for thin-layer chromatography were synthesized by Jeffrey Schmitt (Wake Forest University Medical Center, Winston-Salem, NC) 1-O-alkyl-2-acyl-GPC, 1-O-alkyl-2-acyl-glycerol, 1-O-alkyl-2-acyl-PA, 1-O-alkyl-acylglycerol and 1-O-alkyl-2,3-diacylglycerol. 1,2-Diacylglycerol, 1,2-diacyl-PA and beef heart PC were purchased from Serdary Research Laboratories, London, Ont., Canada. All solvents were either reagent or HPLC grade obtained from Fisher Chemicals, Pittsburg, PA. 1-O-[9',10'-$^3H_2$]hexadecyl-2-lyso-GPC (56 Ci/mmol) of greater than 95% purity was synthesized chemically as previously described (7).

Isolation and radiolabeling of human PMN. Human PMN were prepared to >95% purity from heparinized venous blood after an initial removal of erythrocytes by dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) sedimentation at unit gravity, followed by centrifugation over Isolymph (Gallard-Schlesinger Chemical Manufacturing Corporation , Carle Place, NY), and hypotonic lysis of contaminating erythrocytes (7). Isolated PMNs were resuspended in Hank's balanced salt solution, HBSS (Gibco Laboratories, Grand Island, NY), containing 10 mM Hepes, and 4.2 mM sodium bicarbonate at pH 7.4. Cells were counted in a Coulter Counter (Model ZB1). 1-O-[^{3}H]hexadecyl-2-lyso-GPC (10^{-8}M) was incorporated into PMN 1-O-[^{3}H]alkyl-2-acyl-GPC during a 30 min incubation period as previously described (7). The cells were washed two times and resuspended in HBSS at a final concentration of 3.5 x 10^7/ml prior to stimulation.

Stimulation of PMN. The stimulation of PMN prewarmed for 5 min at 37°C with 10^{-6} fMLP was conducted in the presence or absence of 10^{-5}M CB added to the cell suspension before warming. DMSO, at a final concentration of $\leq$ 0.1%, which was added in place of fMLP, did not stimulate lipid metabolism in control cells. At the indicated times (see figures), reactions were terminated by withdrawing samples of 1.5 ml aliquots, into 6 ml of chloroform:methanol:2% formic acid (1:2:0.2, v/v) and mixing thoroughly.

Analyses of individual lipids. Total lipids were extracted by the methods of Bligh and Dyer (22), dried under a stream of N_2 and resuspended in chloroform. Masses of diradylglycerols and phosphatidic acid were analyzed using 3.5 and 1.75 x 10^7 cell equivalents per determination, respectively. Separation of neutral lipids including diradylglycerols was achieved as described (23), in a solvent system (I), made up of chloroform:methanol:glacial acetic acid (98:2:1, v/v) while phospholipids including phosphatidic acid were separated in solvent system II containing chloroform:methanol:glacial acetic acid:H_2O (75:48:12.5:3.5, v/v). Both neutral lipids and phospholipids were identified by comparing their migration with those of appropriate lipid standards after staining of TLC plates with CBB R-250. The [^{3}H]-labeled lipid fractions were located by scanning individual lanes with a Bioscanner (Imaging Scanner 200-IBM, Washington, D.C.) and were compared with the migration of lipid standards.

Determination of mass of DG and PA in fMLP-stimulated PMN. Both neutral lipids and phospholipids, including their standard lipids, separated in systems I and II, respectively, were stained with CBB R-250 by the methods of Nakamura and Handa (4,24). Briefly, TLC plates were immersed in 0.03% CBB R-250 in 30% MeOH containing 100 mM NaCl for 30 min and destained in 30% MeOH also containing 100 mM NaCl for 5 min before being dried. The bands on plates were scanned with an LKB 2400 Ultroscan XL laser densitometer (LKB-Produkter AB, Bromma, Sweden) at a wavelength of 633nm and peaks were analyzed by the Gelscan XL TM software. Standard curve for the determination of mass of PA was constructed with 1-palmitoyl-2-oleoyl-PA (Avanti Polar Lipids, Birmingham, AL) at 0.25, 0.5, 0.75, and 1.0 ug which correlated with the standard curve for 1,2-diacyl-PA from Serdary Research Laboratories. Masses of diradylglycerols were determined from standard curves of 1,2-dipalmitin (for AAG) and 1-O-alkyl-2-acylglycerol (for EAG) at 0.25, 0.50, 0.75, and 1.0 ug. In order to determine possible losses in PA and DG resulting from the procedures used during CBB R-250 staining, lipid extract obtained after a 30s stimulation with fMLP was divided into two equal volume containing identical [^{3}H]-counts (dpm). One sample was subjected to CBB R-250 staining after separation of lipids in system (I) or (II), the other was not. Following scraping of plates and scintillation counting percentage of counts in PA or EAG was determined in both

samples. In these control experiments, greater than 95% of [^{3}H]-radiolabel was recovered in PA or EAG on plates stained with CBB R-250 compared to unstained plates.

RESULTS

Experiments were conducted to separate AAG from EAG in solvent system I in fMLP-stimulated cells labeled with 1-O-[^{3}H]alkyl-2-acyl-GPC. At 120s, a time-point at which the formation of [^{3}H]EAG was previously demonstrated (7), EAG identified by the migration of lipid standard and location of radiolabel ([^{3}H]EAG) was successfully separated from AAG (Fig. 1). Neither EAG nor AAG was formed at 120s in unstimulated cells.

Based on the above TLC-separation and identification, kinetic analyses of fMLP-induced accumulations of AAG and EAG were undertaken. Fig. 2 shows that AAG accumulated as early as 5s and continued to increase up to 120s as compared with controls. A delay in the accumulation of EAG of 15s was observed (Fig. 3). However, after 15s, EAG accumulated and did not plateau up to 120s. AAG accumu-

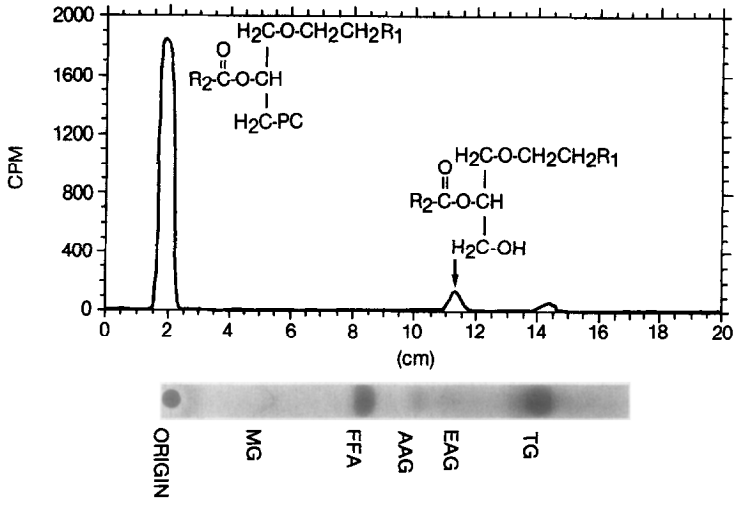


Figure 1 Comparison of representative radiochromatogram and CBB R-250 staining of neutral lipids on TLC plate separated in solvent system I. Cells (3.5 x 10^7 cell equivalents) labeled with 1-O-[^{3}H]alkyl-2-acyl-GPC were incubated at 37^{o}C with 1 uM fMLP, in the presence of 10^{-5}M CB, for 120s. Following termination of reactions, extraction of lipids, separation of individual lipids, and staining of lipid with CBB R-250 as described in "Materials and Methods", the lane at 120s was scanned for radioactivity. The major peaks on the radiochromatogram and the CBBR-250-stained bands on TLC plate were identified by the migration of appropriate standards. Unstimulated controls at 120s did not show radioactivity or stain in the region of EAG.

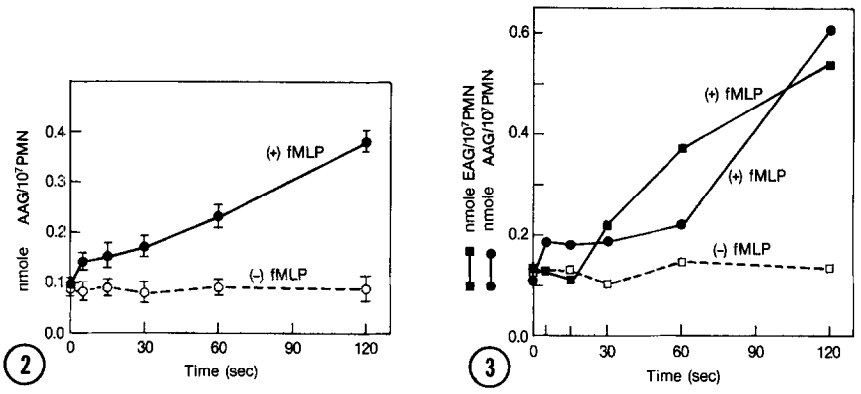


Figure 2 Time-course for accumulation of AAG in fMLP-stimulated human neutrophils. Cells (3.5 x 10^7/ml) were pretreated with 10^{-5}M CB and incubated for 5 min at 37°C prior to stimulation with (-●——●-) or without (-○----○-) 1 uM fMLP. Following termination of reactions and lipid extraction, lipids were analyzed and masses determined as described in "Materials and Methods". Data represent the mean ± SEM for five experiments and were conducted with 3.5 x 10^7 cell equivalents at each time point.

Figure 3 Time-course for comparison of accumulation of AAG and EAG in human neutrophils. Following termination of reactions and extraction of lipids, accumulations of both AAG (-●——●-) and EAG (-■——■-) (nmole/10^7 PMN) were determined on samples containing 3.5 x 10^7 cell equivalents from PMN stimulated with 1 uM fMLP in the presence of 10^{-5}M CB for indicated time points (see "Materials and Methods"). AAG and EAG (-□-□-) did not accumulate in unstimulated controls up to 120s. Curves from one representative experiment are shown.

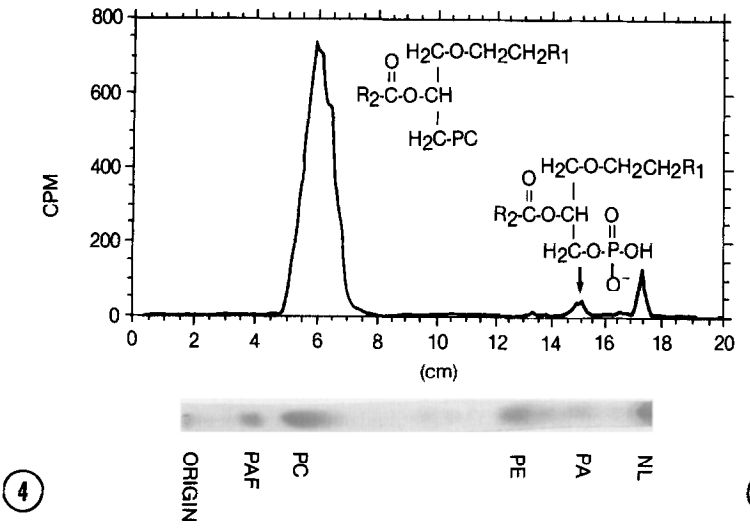
lation preceeded that of EAG (Fig. 3). In the absence of 10^{-5}M CB, fMLP-stimulation did not result in the accumulation of AAG or EAG (data not shown).

Phospholipids, including PA, were separated in system II. Fig. 4 illustrates the identification of PA in cells stimulated with fMLP for 120s by a combined comparison of the migration of standard PA and the position of [^{3}H]EAPA. Mass measurements of PA showed accumulation as early as 5s, maximum accumulation at 30s, and a decline after 60s (Fig. 5).

At any time point, up to 120s, the mass (nmole/10^7 cells) of PA, compared with EAG or AAG, was consistently higher (Fig. 5 vs 3).

DISCUSSION

We (7), and others (5,6), have previously demonstrated the formation [^{3}H]EAG and [^{3}H]EAPA in stimulated cells prelabeled with 1-O-[^{3}H]alkyl-2-acyl-GPC. Cabot et al. (25) have reported on the generation [^{3}H]AAG and [^{3}H]AAPA from [^{3}H]PC in REF52 cells labeled with [^{3}H]myristic acid and stimulated with phorbol diesters. Based on



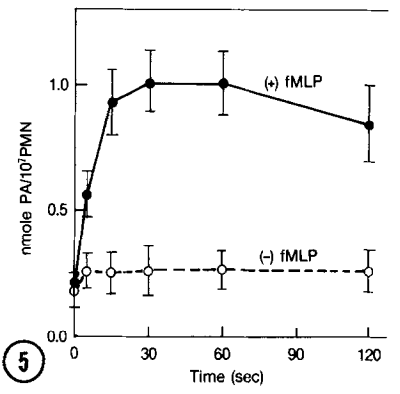


Figure 4 Comparison of representative Bioscan imaging system radiochromatogram and CBB R-250 staining of phospholipids, including phosphatidic acid, on a TLC plate separated in solvent system II. Cells (1.75 x 10^7 cell equivalents) labeled with 1-O-[^{3}H]alkyl-2-acyl-GPC, were treated and analyzed as described in "Materials and Methods" (also see legend to Fig. 1). Stimulation with 1 uM fMLP plus 10^{-5}M CB was for 120s. Unstimulated controls at 120s did not show any radioactivity or stain in the region of PA.

Figure 5 Time-course for accumulation of PA in fMLP-stimulated human neutrophils. Cells (3.5 x 10^7/ml) were pretreated with 10^{-5}M CB and incubated for 5 min at 37°C prior to stimulation with (-●——●-) or without (-○---○-) 1 uM fMLP. At the indicated times, reactions were terminated in samples withdrawn from control and stimulated cells. Analyses of individual lipids and determination of mass of PA were conducted as described in "Materials and Methods". Data represent the mean ± SEM for four experiments determined with 1.75 x 10^7 cell equivalents at each time point.

radiolabeling studies, the hydrolysis of PC and the formation of these bioactive metabolites (DG and PA) have been attriubted to phospholipase D (6,7,25) or C (5). However, the mechanism(s) involved in PC breakdown and the biological significance of the intermediary products formed may become clearer with quantitation of DG and PA. We have used a combination of methods - prelabeling of cells, TLC separations, staining of lipids with CBB R-250 and densitometry-to quantitate AAG, EAG, and PA in fMLP-stimulated PMN. Our results demonstrate an early accumulation of AAG (by 5s), a 15s delay in the accumulation of EAG, and a rapid accumulation of PA which was maximal by 30s. At 5s, the accumulation of PA (0.58 nmole/10^7PMN) was 3.9-fold higher than that of AAG (0.15 nmole/10^7PM).

Mass of diradyl-DG has been determined, without separation into AAG and EAG, by its conversion to [^{32}P]-labeled PA utilizing γ-[^{32}P] ATP and E. coli DG kinase in PMN stimulated with fMLP (19). This assay did not detect these products earlier than

30s following stimulation. The method used in this study is a simple, sensitive assay which makes possible quantitative and kinetic analyses of AAG and EAG in 3.5 x 10^7 PMN without enzymatic conversion of DG. It has advantages over normal phase HPLC, which requires derivatization and a higher number of PMN (7). Our results are in general agreement with earlier reports (7,19) on mass measurements of DG following stimulation of PMN with fMLP and, for the first time, provide mass measurements of AAG at time points less than 30 sec.

CBB R-250 staining and densitometry have recently been used in the quantitation of PA in stimulated rat hepatocytes (4). Our data on accumulations of PA agree with the only other mass measurement of PA in fMLP-stimulated PMN (21). However, not only is our method convenient and sensitive, it also permits detection of the accumulation of EAPA, since the cells were prelabeled. Experiments which will separate and quantitate both EAPA and AAPA are in progress.

Since AAG, an endogenous activator of PKC (9), accumulated before EAG, which inhibits PKC (11,14) and the respiratory burst in human PMN (14), our results agree with current thinking that formation of AAG and activation of PKC are early events in the transduction process in PMN. EAG may serve to counter the effects of AAG or may act itself to elicit responses. The rapid accumulation of high concentrations of PA support observations which suggest PA may be generated before AAG through activation of a phospholipase D (4,6,7) and that PA may function as a second messenger (17) in stimulus-response coupling.

REFERENCES

1. Takenawa, T., Ishitoya, J., Homma, Y., Kato, M., and Nagai, Y. (1985) Biochem. Pharmacol. 34,1931-1935

2. Cockcroft, S., and Allan, D. (1984) Biochem. J. 222,557-559.

3. Volpi, M., Yassin, R., Naccache, P.H., and Sha'afi, R.I. (1983) Biochem. Biophys. Res. Commun. 112,957-964.

4. Bocckino, S.B., Blackmore, P.F., Wilson, P.B., and Exton, J.H. (1987) J. Biol. Chem. 262,15309-15315.

5. Daniel, L.W., Waite, M., and Wykle, R.L. (1986) J. Biol. Chem. 261,9128-9132.

6. Pai, J-K., Siegel, M.I., Egan, R.W., and Billah, M. (1988) Biochem. Biophys. Res. Commun. 150,355-364.

7. Agwu, D.E., McPhail, L.C., Chabot, M.C., Daniel, L.W., Wykle, R.L., and McCall, C.E. (1988) J. Biol. Chem. 264, (in press).

8. Nishizuka, Y. (1984) Nature (Lond.) 308,693-698.

9. Fujita, I., Irita, K., Takeshiga, K., and Minakami, S. (1984) Biochem. Biophys. Res. Commun. 120,318-324.

10. McNamara, M.J.C., Schmitt, J.D., Wykle, R.L., and Daniel, L.W. (1984) Biochem. Biophys. Res. Commun. 122,824-830.

11. Daniel, L.W., Small, G.W., and Schmitt, J.D. (1988) Biochem. Biophys. Res. Commun. 151,291-297.

12. Cabot, M.C., and Jaken, S. (1984) Biochem. Biophys. Res. Commun. 125,163-169.

13. Heymans, F., Da Silva, C., Marrec, N., Godfroid, J-J., and Castagna, M. (1987) FEBS Lett. 218,35-40.

14. Bass, D.A., McPhail, L.C., Schmitt, J.D., Morris-Natschke, S., McCall, C.E., and Wykle, R.L. (1988) J. Biol. Chem., in press.

15. Putney, J.W., Jr., Weiss, S.J., van de Walle, C.M., and Haddas, R.A. (1980) Nature (Lond.) 284,345-347.

16. Moolnaar, W.H., Kruijer, W., Tilly, B.C., Verlaan, I., Bierman, A.J., and deLaat, S.W. (1986) Nature (Lond.) 323,171-173.

17. Murayama, T., and Ui, M. (1987) J. Biol. Chem. 262,5522-5529.

18. Preiss, J.E., Bell, R.M., and Niedel, J.E. (1987) J. Immunol. 138,1542-1545.

19. Rider, L.G., and Niedel, J.E. (1987) J. Biol. Chem. 262,563-5608.

20. Ford, D.A., and Gross, R.W. (1988) J. Biol. Chem. 263,2644-2650.

21. Serhan, C.N., Broekman, M.J., Korchak, H.M., Marcus, A.J., and Weissman, G. (1982) 107,951-958.

22. Bligh, E.G., and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37,911-917.

23. Snyder, F. (1973) J. Chromatogr. 82,7-14.

24. Nakamura, K., and Handa, S. (1984) Anal. Biochem. 142,406-410.

25. Cabot, M.C., Welsh, C.J., Cao, H. and Chabbott, H. (1988) Biohem. Biophys. Res. Commun. 233,153-157.