BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **236,** 29–33 (1997)
ARTICLE NO. RC976803

Chromatin Neutral Sphingomyelinase and Its Role in Hepatic Regeneration

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Received April 22, 1997

Ceramide acts as a second messenger and modulates many cellular functions. This molecule can be produced by enzymatic digestion of sphingomyelin, a phospholipid which has been shown to be in high concentration in a chromatin phospholipidic fraction. In order to clarify whether chromatin sphingomyelin has a role in this signal transduction pathway, it is necessary to define the sphingomyelin cycle. Neutral sphingomyelinase represents the first step of the cycle. In this paper we demonstrate that sphingomyelinase activity can be detected also in the chromatin of rat hepatocyte nuclei and it increases in relation to the entrance in S phase of hepatocyte after hepatectomy. The enzyme can be distinguished from that present in the nuclear envelope on the basis of optimum pH and Km. The role of the spingomyelin pathway in relation to liver regeneration is discussed.

Histochemical and biochemical techniques have shown that a small fraction of phospholipids (PLs)[1] is associated with the chromatin in hepatocytes and plant cells (1, 2). This fraction, which represents 10% of the total PLs of isolated hepatocyte nuclei, is particularly rich in sphingomyelin (SM, 35% of that present in the entire nucleus, 3). The metabolites of sphingomyelin are considered to play an important role in many physiological functions (4,5). In fact, stimulating factors, such as 1-25 OH vitamin D_3 (6,7), interferon γ (8), TNFα (9, 10) or Interleukin-1 (11), can induce hydrolysis of SM, with formation of ceramide. The latter is hydrolysed to free sphingosine, which can act as a second messenger by inhibiting protein kinase C (12) localised in liver nuclei (13). Neutral sphingomyelinase (N-SMase) is probably an ubiquitous enzyme. It has been found in the nervous system (14), in the human kidney (15), in rat liver (16), and also *in vitro* in cultured cells like murine glioblastoma and human skin fibroblasts (17) and proximal renal tubular cells (15). The enzyme was isolated from human urine (18) and partially purified from human brain extract (19). In the rat liver the enzyme appears to be associated mainly with the plasma membrane, probably on the external surface (16). Recently, the presence of N-SMase has been also shown in hepatic nuclei (20). The latter enzyme has the same pH optimum as that described for plasma membranes and its activity increases during cell duplication in regenerating rat liver (20). No evidence has been produced so far for a possible localisation of N-SMase inside the nucleus. The aim of this work is to establish i) if N-SMase is present in chromatin and its activity changes in relation to hepatocyte proliferation; ii) if it can be distinguished from the previously described N-SMase, particularly from that of the nuclear membrane.

MATERIALS AND METHODS

Materials. The radioactive SM (choline-methyl ^{14}C, 54.5 Ci/mol) and Atomlight were obtained from NEN (Boston, Massachusetts, USA); SM was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA).

Animals. Thirty-day-old Sprague Dawley rats of either sex were used. They were kept in a normal light-dark period and had free access to pelleted food and water prior to killing. The same animals were subjected to partial hepatectomy between 8 and 10 a.m. and killed at different times post-operation.

Hepatocyte nuclear preparation and chromatin isolation. The hepatic nuclei were isolated according to the procedure of Bresnick *et al.* (21), which yields 95-98% of isolated hepatocyte nuclei. After washing (22), swelling of the nuclei and chromatin extraction were performed according to the method of Shaw and Huang (23) modified by Viola-Magni *et al.* (1).

Liver nuclear preparation and nuclear membrane isolation. The nuclei and the nuclear membranes were isolated according to the procedure of Widnell & Tata (24) and Kay & Johnston (25) respectively.

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Abbreviations used: PL, phospholipid; SM, sphingomyelin, N-SMase, neutral sphingomyelinase; PMSF, phenylmethylsulfonyl fluoride.

0006-291X/97 $25.00

TABLE I

Comparison of DNA, RNA, and Phospholipid Content of Nuclear Membrane and Chromatin

	Nuclear membranes	Chromatin
DNA	87.79 + 31.25	387.94 + 20.64
	(7.05 + 2.58)	(25.56 + 1.4)
RNA	49.18 + 20.99	92.00 + 21.10
	(3.85 + 1.69)	(6.17 + 1.32)
PL	123.63 + 13.32	5.77 + 0.90
	(9.99 + 0.96)	(0.39 + 0.07)

Values are expressed as μg/mg protein and indicate means ± S.D. of 3 experiments for Nuclear Membranes and 4 experiments for Chromatin. In parenthesis, the same values are expressed as per cent of total Nuclear Membranes and Chromatin contents.

Lipid extraction. Lipids were extracted from nuclear membrane (6.2 mg/ml) and chromatin (2.5 mg/ml) with 20 volumes of chloroform/methanol (2:1 vol/vol). The organic phase was washed with 0.2 volumes of 0.5% NaCl according to Folch *et al.* (26).

Biochemical determinations. Protein, DNA and RNA contents were determined according to Lowry *et al.* (27), Burton (28) and Schneider (29) respectively. Since the RNA values were not corrected for the presence of DNA, the amount of RNA is overestimated by about 1.4 fold when the DNA concentration is 5 times that of the RNA, as it is in chromatin (29). The total amount of PLs was determined by measuring inorganic phosphorus (30).

Assay of neutral sphingomyelinase. In the reactions 1.6 nmol of ^{14}C SM was diluted by adding 78.4 nmol cold SM to a final specific activity 1.08 Ci/mol. *Nuclear membranes.* The reaction mixture contained 0.1 M Tris/HCl pH 7.6, 0.8 mM ^{14}CSM, 6 mM $MgCl_2$, 0.1% Triton X-100 and nuclear suspension equivalent to 150 μg protein to a final volume of 0.1 ml. Incubation was performed at 37°C for 10, 30, 60, 90 min. The reaction was stopped by adding 1.5 ml chloroform/methanol (2:1), 0.2 ml of 0.5% NaCl was added to the tubes and mixed with vortex. The tubes were centrifuged at 2000 x g for 10 min and the upper phase was removed and diluted in counting vials with 3 ml Atomlight (NEN). *Chromatin.* Reaction mixtures contained 0.1 M Tris/HCl pH 8.4, 0.04 mM ^{14}C SM, 6 mM $MgCl_2$, 0.1% Triton X-100 and chromatin suspension equivalent to 500 μg protein to a final volume of 2 ml. Incubation was performed at 37°C for 10, 30, 60, 90 min. The reaction was stopped by adding 25 ml chloroform/methanol (2:1), 2 ml of 0.5% NaCl was added to the tubes and mixed with vortex. The tubes were centrifuged at 2000 x g for 10 min, the upper phases were removed, measured and 1 ml was diluted in counting vials with 10 ml Atomlight.

RESULTS AND DISCUSSION

Hepatocyte nuclei contain DNA, RNA and PLs in decreasing concentrations with respect to proteins (Table 1). The nuclear preparation is free of microsomal contamination as shown by enzyme measurement (1) and electrophoretic analysis of RNA (31). The nuclear membranes are characterised by a strong increase (about 7 times) in PL, whereas the DNA is reduced to almost 1/3. On the contrary, chromatin contains more DNA and RNA and only a small amount of PL (Table 1). The composition here described is similar to that reported in previous works (1, 32). In the isolated chromatin the activity of N-SMase can be detected. The radioactive phosphorylcholine, derived by SM hydrolysis, is 886 pmol./90′/mg protein. After hepatectomy the enzyme activity begins to increase slowly during the first 12hr (2.36 nmol /90′/mg protein), then there is sharp increase at 18hr (until 5.39 nmol/90′/mg protein), followed by a decrease at 24 hr (Fig.1). If the behaviour of SMase is compared with the DNA synthesis evaluated by measuring the incorporation of ^{3}H-thymidine, we observe that the peak of N-SMase corresponds to the beginning of hepatocyte S phase (Fig.1). At the same time a decrease in SM in the chromatin PL fraction is observed. In order to ascertain if N-SMase, detected in chromatin, could be ascribed to nuclear membrane contaminations, some physical chemical parameters were analysed. The N-SMase is present both in the nuclear membrane and the chromatin, but at different concentrations. The activity measured at different pHs reaches a maximum at pH 7.6 in the nuclear membranes, whereas in chromatin the maximum activity is found at pH 8.4 (Fig.2). The chromatin N-SMase activity detected at pH 7.2 increases 3 times at pH 7.6, 5 times at pH 8.0 and shows a peak at pH 8.4, followed by a decrease to values lower than those found at pH 7.6. The activity behaviour is thus completely different from that found in the nuclear membranes, thus supporting the hypothesis that the enzyme present in the chromatin is not derived from contamination from nuclear membrane. Also, the activity with respect to the incubation time is different, being linear with time in the nuclear membrane fraction, but increasing sharply after 60 min. of incubation in chromatin fraction (Fig.3). It can be assumed that the first 30 min. are necessary for a better solubilization of chromatin, thus permitting the enzyme to react. The Km values show a greater substrate affinity to the chromatin enzyme as compared to that found in nuclear membranes. The Km is 2.4×10^{-5}M

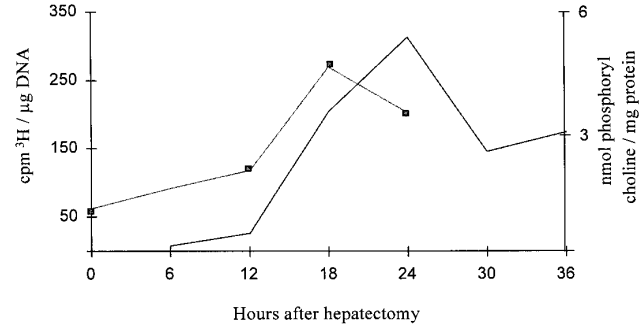

FIG. 1. Chromatin neutral sphingomyelinase activity during hepatic regeneration. Behaviour of ^{3}H-thymidine incorporation and the chromatin neutral sphingomyelinase activity at different intervals after hepatectomy. The data of ^{3}H thymidine incorporation are from Viola Magni *et al.* (39).

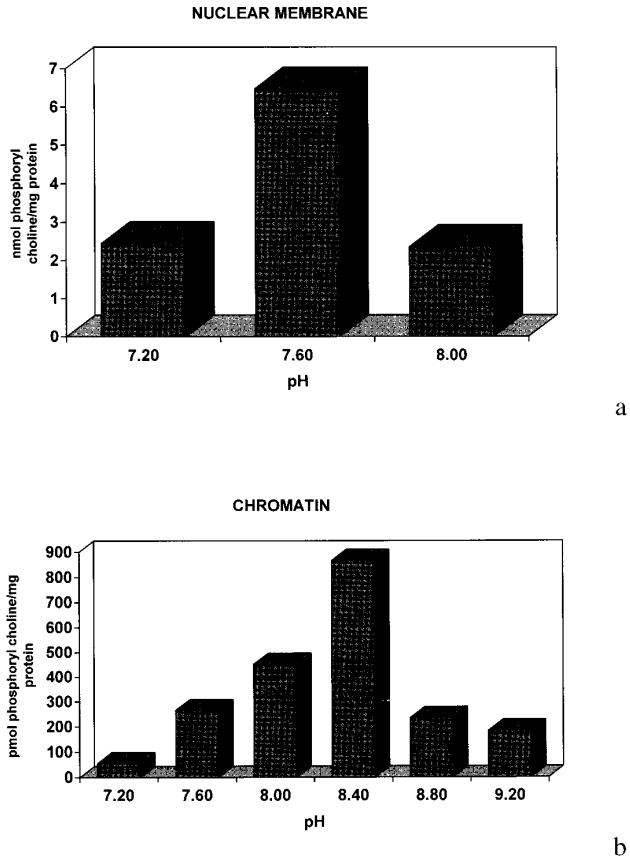

FIG. 2. pH dependence of nuclear membranes (a) and chromatin (b) sphingomyelinase activity. Nuclear membranes were incubated with 0.1 M Tris-HCl, 6 mM MgCl2, 0.1% Triton X-100 and 0.8 mM ^{14}C sphingomyelin at 37°C for 10 min. Chromatin was incubated at 37°C for 90 min. in the same solutions except that 0.04 mM ^{14}C sphingomyelin was used. The mixture, without nuclear membranes or chromatin, was used as blanck.

for the chromatin N-SMase (Fig.4b), and 3.9×10^{-4} M for nuclear membrane N-SMase (Fig.4a), with Vmax 1.39 nmol mg^{-1} prot. 90 $min.^{-1}$ and 9.12 nmol mg^{-1} prot. 10 $min.^{-1}$ respectively. In liver, N-SMase is localised in the plasma membrane and in the microsomes (33). These N-SMase are Mg^{++}-dependent, though a form of Mg^{++}-independent N-SMase has been found in the cytosol (34). Recently, this latter enzyme was found also in hepatocyte nuclei, particularly in the nuclear membranes (20). The Mg^{++}-dependent enzyme localized in the cytoplasm has a pH optimum of 7.2, which is similar to that of the microsomes and plasma membranes. However the Km is different : 6.2×10^{-5} M for microsomes and 1.2×10^{-4} M for plasma membranes (33). On the basis of these Km values, it appears that the nuclear membrane enzyme is more similar to that of the plasma membrane, whereas the chromatin enzyme is similar to that of microsomes. As regards the hepatocyte nuclei, no particular characteristics were reported, the Km was not measured and the pH optimum was around 7.0, but intermediate values were not determined (20). The nuclear hepatocyte enzyme was localized on the nuclear membrane by immuno-cytochemistry (20). All the enzymes described in the liver possess probably the same antigenic characteristic, as shown by the fact that an antibody prepared against N-SMase, extracted from human urine (20), can react with the rat enzyme thus suggesting a similar genomic structure. Therefore, the N-SMase gene appears well preserved also in different species. It is possible that the enzyme is formed by different subunits, which could explain the differences in Km. The activity behaviour of chromatin N-SMase during hepatic regeneration is also different with respect to that of the nuclear membranes, in which there is no evidence of increase at the beginning of S-phase (data not shown). According to the results obtained so far, ceramides, which are the product of enzymatic hydrolysis of SM, have a role in various forms of growth suppression and cell death (35). The increased concentration of ceramides observed in cell treated with

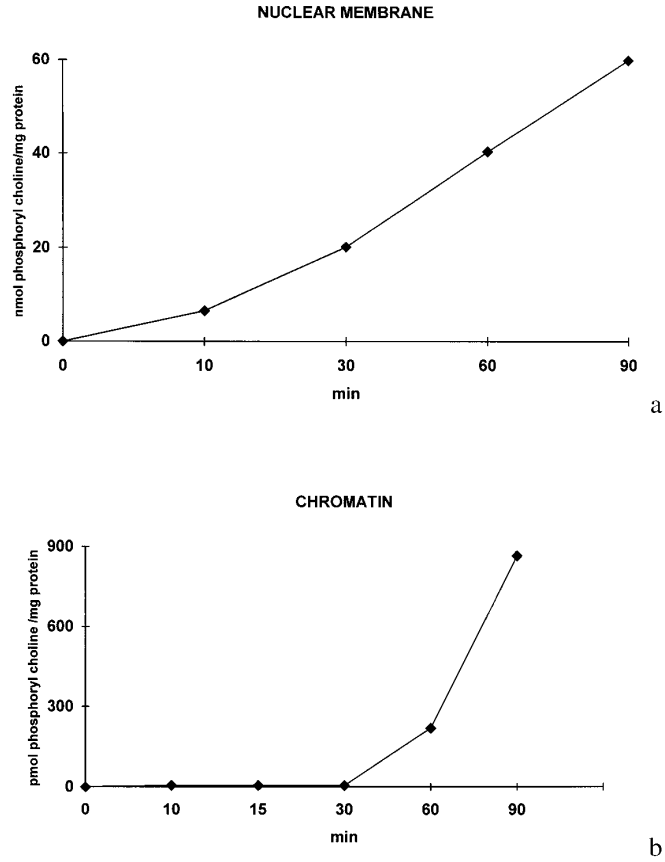

FIG. 3. Neutral sphingomyelinase activity at different incubation times. The activity was measured as described under fig.1 but at different incubation times. The incubations were carried at pH 7.6 and pH 8.4 for nuclear membrane (a) and chromatin (b).

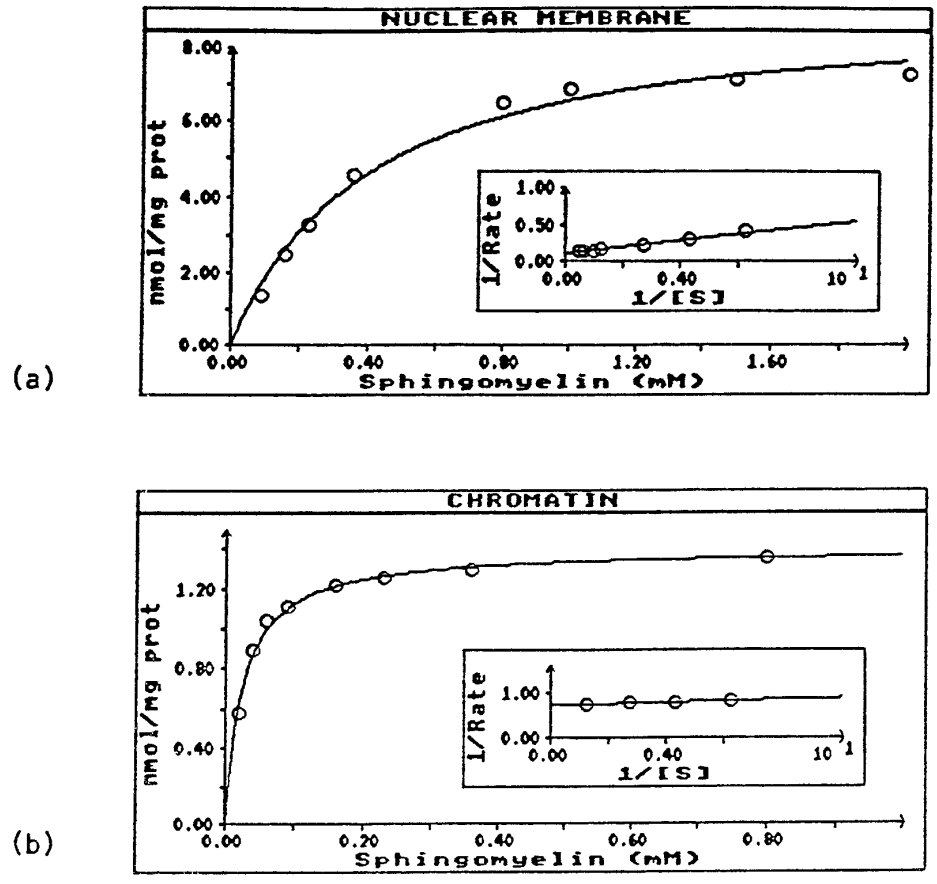

FIG. 4. Effect of sphingomyelin concentration on nuclear membrane sphingomyelinase activity. Incubations were carried at 37°C, pH 7.6 for nuclear membrane (a), pH 8.4 for chromatin (b). The results represent the average of two experiments. In the Lineweaver-Burke plots the data are represented as 1/S (mM) and 1/V (nmol $mg^{-1} \times 10$ min and nmol $mg^{-1} \times 90$ min for nuclear membrane and chromatin respectively).

TNF, dexametasone or deprived of serum is accompanied by apoptosis or cell cycle arrest or both. The cells do not incorporate thymidine, are arrested in G0/G1 phase and there is an early dephosphorilation of the retinoblastoma gene product which seems to have an essential role in the ceramide induced cell cycle block (36, 37). The chromatin N-SMase does not appear involved in the same effect on the cell cycle. In fact, it increases in relation to the transit of cells from G1 to S phase and when the incorporation of thymidine is enhanced. When the cells are in S phase and the incorporation of thymidine increases further as happens 24 hr after hepatectomy, the NSMase decreases. This clearly shows that the variation in enzyme activity is not linked to DNA duplication but to the molecular events which precede DNA synthesis, such as the formation of Pre-Rc complex, by favouring the expression or the link of protein such Cdc6p to the ORC (origin recognition complex, 38). At present there is no proof in favour of this hypothesis; however our results clearly indicate that the increase in chromatin N-SMase activity is not related to cell cycle block in G0/G1 but, on the contrary, to the beginning of S phase. The different role, together with the physico-chemical differences previously described, supports the hypothesis of the presence of a chromatin specific enzyme form.

ACKNOWLEDGMENTS

This work was supported in part by MURST. We thank Dr. L. Corazzi for helpful discussion, Dr. M. Micheli for the collaboration in the hepatectomy experiments, Mr. R. Lazzarini for the technical assistance, and Professor F. Magni for the revision of manuscript.

REFERENCES

1. Viola Magni, M. P., Gahan, P. B., and Pacy, J. (1985) *Cell Biochem. Funct.* **3,** 71–78.
2. Albi, E., Viola Magni, M. P., Lazzarini, R., and Gahan, P. B. (1991) *Cell Biochem. Funct.* **9,** 119–123.
3. Albi, E., Mersel, M., Leray, C., Tomassoni, M. L., and Viola Magni, M. P. (1994) *Lipids* **29,** 715–719.
4. Hannun, Y. A. (1994) *J. Biol. Chem.* **269,** 3125–3128.

5. Spiegel, S., and Milstien, S. (1995) *J. Membrane Biology* **146,** 225–237.
6. Okazaki, T., Bell, R. M., and Hannun, Y. A. (1989) *J. Biol. Chem.* **264,** 19076–19079.
7. Okazaki, T., Bielawska, A., Bell, R. M., and Hannun, Y. A. (1990) *J. Biol. Chem.* **265,** 15823–15831.
8. Kim, M. Y., Linardic, C., Obeid, L., and Hannun, Y (1991) *J. Biol. Chem.* **266,** 484–489.
9. Dressler, K. A., Mathias, S., and Kolesnick, R. N. (1992) *Science* **255,** 1715–1718.
10. Jayadev, S., Linardic, C. M., and Hannun, Y. A. (1994) *J. Biol. Chem.* **269,** 5757–5763.
11. Ballon, L. R., Chao, C. P., Holness, M. A., Barker, S. C., and Raghow, R. (1992) *J. Biol. Chem.* **267,** 20044–20050.
12. Merril, A. H. (1991) *J. Bioenerg. Biomemb.* **23,** 83–104.
13. Masmoudi, A. Labourdette, G., Mersel, M., Freesia, L. H., Huang K. P., Vincendon, G., and Malvija, A. N. (1989) *J. Biol. Chem.* **264,** 1172–1179.
14. Spence, M. W., Burgess, J. K., and Spencer, E. R. (1979) *Brain Res.* **168,** 543–551.
15. Gosh, P., and Chatterjee, S. (1987) *J. Biol. Chem.* **262,** 12550–12556.
16. Slife, C. W., Wang, E., Hunter, R., Wang, S., Burgess, C., Liotta, D. C., and Merill, A. M. (1989) *J. Biol. Chem.* **264,** 10371–10377.
17. Spence, M. W. (1993) *Adv. Lipid Res.* **26,** 3–23.
18. Chatterjee, S., and Ghosh, N. (1989) *J. Biol. Chem.* **264,** 12554–12561.
19. Maruyama, E. N., and Arima, M. (1989) *J. Neurochem.* **52,** 611–618.
20. Alessenko, A., and Chatterjee, S. (1995) *Molecular and Cellular Biochem.* **143,** 169–174.
21. Bresnick, E., Lanclos,K., Sage, J., Schwartz, A., Yawn, D. H., Bush, H. J., and Unuma, T. (1967) *Exptl. Cell Res.* **46,** 396–411.
22. Barnes, D. W. H., Esnonf, M. P., and Stoken, L. A. (1957) *in* Advances in Radiobiology, pp. 211–213, Oliver & Boyd, Edinburg.
23. Shaw, L. M. J., and Huang, R. C. C. (1970) *Biochemistry* **9,** 5430–5441.
24. Widnell, C. C., and Tata, J. R. (1964) *Biochem. J.* **93,** 313–317.
25. Kay, R. R., and Johnston, I. R. (1975) *Methods Cell Biol.* **15,** 277–283.
26. Folch, J., Lees, M., and Sloene-Stanley, G. H. (1957) *J. Biol. Chem.* **226,** 497–509.
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
28. Burton, K. (1956) *Biochem. J.* **62,** 315–323.
29. Schneider, W. C. (1957) *Method Enzymol* **3,** 680–684.
30. Bartlett, G. R. (1959) *J. Biol. Chem.* **234,** 466–468.
31. Albi, E., Micheli, M., and Viola Magni, M. P. (1996) *Cell Biol. Intern.* **20,** 6, 407–412.
32. Gahan, P. B., Albi, E., and Viola-Magni, M. P. (1991) *in* Topics in Aging Research in Europe, Vol. 16, pp. 229–236.
33. Hostetler, K. Y., and Yazaki, P. J. (1979) *J. Lipid Res.* **20,** 456–463.
34. Okazaki, T., Bielawska, A., Domae, N., Bell, R. M., and Hannun, Y. A. (1994) *J. Biol. Chem.* **269,** 4070–4077.
35. Hannun, Y. A. (1996) *Science* **274,** 1885–1888.
36. Riboni, L., Bassi, R., Sonnino, S., and Tettamanti, G. (1992) *FEBS lett.* **300,** 188–192.
37. Gomez-Munoz, A., Waggoner, D. W., O'Brien, L., and Brindley, D. N. (1995) *J. Biol.Chem.* **270,** 26318–26325.
38. Stillman, B. (1996) *Science* **274,** 1659–1662.
39. Viola Magni, M. P., Gahan, P. B., Albi, E., Iapoce, R., and Gentilucci, P. F. (1985) *Bas. Appl. Histochem.* **29,** 253–259.