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## Accelerated Publications

# Action of Monensin, a Monovalent Cationophore, on Cultured Human Fibroblasts: Evidence That It Induces High Cellular Accumulation of Glucosyl- and Lactosylceramide (Gluco- and Lactocerebroside)†

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ABSTRACT: We have exposed cultured human fibroblasts to micromolar concentrations of the ionophore monensin. A salient result was a rapid accumulation in these cells of glucosylceramide (glucocerebroside, GlcCer) and lactosylceramide (lactocerebroside, LacCer). When we incubated these cells with radioactively labeled galactose, GlcCer and LacCer became highly labeled. These results indicate that monensin greatly increases these simplest glycosphingolipids that are the precursor to the major plasma membrane glycosphingolipids. We observed, simultaneously, a decreased incorporation of labeled galactose into some more highly glycosylated neutral glycosphingolipids and sialoglycosphingolipids (gangliosides), and unlike GlcCer and LacCer, the cellular content of these more highly glycosylated compounds remained the same in the presence or absence of monensin. We have found that cultured Gaucher disease fibroblasts, with genetically impaired lysosomal glucocerebrosidase activity, accumulated even more GlcCer and LacCer than normal cells upon exposure to monensin. This finding shows that monensin affects biosynthesis rather than merely disrupting lysosomal degradation that is already deleted with respect to GlcCer in Gaucher disease cells. These results represent the first indication of an apparently remarkable effect of the monovalent ionophore, monensin, on plasma membrane glycosphingolipid biosynthesis. The evidence suggests a regulatory distinction between initial and higher intracellular glycosylation steps. Monensin does not diminish and may augment initial anabolic mono- and diglycosylations and also appears to inhibit higher glycosylations of glycosphingolipids.

Monensin, an ionophore for monovalent cations, inhibits some of the posttranslational modifications of proteins (Tartakoff & Vassalli, 1979; Tartakoff et al., 1981; Tajiri et al., 1980; Nishimoto et al., 1982; Townsend & Benjamins, 1983) and the synthesis of mucopolysaccharides (Goldberg & Toole, 1983), which are believed to occur in the Golgi apparatus. Recently, the monovalent ionophore monensin has been shown to disrupt the Golgi apparatus and slow down intracellular transport of newly synthesized proteoglycans, secretory proteins, and plasma membrane glycoproteins [see review (Tartakoff, 1983)]. It has been postulated that elongation of sugar chains in glycosphingolipids that are important in the plasma membrane occurs through stepwise addition of monosaccharide units to the nonreducing end of the lengthening oligosaccharide chain (Li & Li, 1982; Basu & Basu, 1982), and this process has been shown to occur mainly in the Golgi apparatus (Keenan et al., 1974; Wilkinson et al., 1976; Richardson et al., 1977; Pacuszka et al., 1978). We have investigated the possibility that treatment with monensin may influence glycosphingolipid biosynthesis in the Golgi apparatus and discovered that the effect of monensin on biosynthesis of simple neutral glycosphingolipids in cultured normal and lysosomal β-glucosidase-deficient (Gaucher) human fibroblasts is striking, and sharply different from the effect on higher, more extensively glycosylated glycosphingolipids. The findings uniquely indicate a separation of the intracellular locus of the initial and subsequent glycosylations of glycosphingolipids. Cells exposed to micromolar concentrations of monensin rapidly incorporate labeled galactose into mono- and dihexose glycosphingolipids (gluco- and lactocerebrosides) and accumulate notably high amounts of these compounds. Higher neutral and sialo homologues are not subject to this effect.

## Materials and Methods

*Fibroblast Culture.* Normal cultured human skin fibroblasts (GM3440), obtained from the Institute for Medical Research (Camden, NJ), and type 2 Gaucher disease fibroblasts, sup-

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Table I: Effect of Monensin on Glycosphingolipid Synthesis[a]

| neutral glycosphingolipids | % of control | | gangliosides | % of control | |
|---|---|---|---|---|---|
| | normal | Gaucher | | normal | Gaucher |
| GlcCer | 336 ± 35 | 809 ± 299 | $G_{M_3}$ | 131 ± 19 | 97 ± 12 |
| LacCer | 190 ± 50 | 278 ± 89 | $G_{M_2}$ | 93 ± 17 | 93 ± 23 |
| $GbOse_3Cer$ | 99 ± 10 | 99 ± 5 | $G_{M_1}$ | 111 ± 35 | 85 ± 8 |
| $GbOse_4Cer$ | 99 ± 24 | 108 ± 31 | $G_{D_3}$ | 101 ± 9 | 100 ± 21 |
| | | | $G_{D_{1a}}$ | 115 ± 21 | 96 ± 14 |

[a] Normal and Gaucher fibroblasts (10–13 days after seeding) were incubated for 20 h in the presence or absence of 10 μM monensin in the growth medium. The cells were harvested, and their glycosphingolipids were extracted and analyzed as described under Materials and Methods. The amounts of glycosphingolipids of monensin-treated cells are listed as percent of control. The values represent means ± SD from three experiments. Glycosphingolipid content is given in Table II.

plied by Dr. D. Wenger, University of Colorado Medical Center, were cultured in 10 mL of growth medium consisting of 83% Dulbecco's modified Eagle's medium (Gibco), 15% fetal calf serum (Gibco), and 2% penicillin–streptomycin (5000 IU and 5 mg/mL, respectively, Flow Laboratories) in 75-$cm^2$ plastic tissue culture bottles (Falcon) at 37 °C in a 5% $CO_2$ atmosphere in air. To observe the effects of monensin (Calbiochem), confluent fibroblasts (10–13 days after seeding) were incubated with 0.1–10 μM monensin in the culture medium for 6–24 h.

*Isolation and Identification of Glycosphingolipids.* Fibroblasts in 75-$cm^2$ plastic tissue culture bottles were washed 3 times with 2 mL of 0.15 M NaCl–0.01 M sodium phosphate buffer, pH 7.4 (PBS), scraped with rubber policemen, and centrifuged at 600*g* for 5 min. Total lipids were extracted from the pellet 3 times with 1 mL of chloroform–methanol (2:1 v/v), 1 mL of chloroform–methanol (1:2 v/v), and 1 mL of chloroform–methanol (2:1 v/v). The isolation of glycosphingolipids from total lipids was done according to a modified method (Irwin & Irwin, 1979) as described previously (Saito & Rosenberg, 1982). The gangliosides were separated by high-performance silica gel G thin-layer chromatography (HPTLC) plates (E. Merck) with chloroform–methanol–0.25% $CaCl_2$ in water (65:35:8 v/v/v) and visualized by resorcinol reagent. Neutral glycosphingolipids were developed in chloroform–methanol–water (65:25:4 v/v/v) and visualized by anthrone reagent. Glucosylceramide and galactosylceramide were separated on HPTLC plates presprayed with 1.5% sodium borate and developed in chloroform–methanol–water–15 M $NH_4OH$ (280:70:6:1 v/v/v/v). For the determination of each ganglioside and neutral glycosphingolipid, direct densitometric measurement was performed in a Kratos (Schoeffel) densitometer after TLC development. The isolation of glycosphingolipids from cells labeled by [$^3$H]Gal[1] (11.5 Ci/mmol, New England Nuclear) was done as described above. The patterns of radioactivity were obtained by scraping each 2 mm wide strip from TLC plates, transferring the samples into scintillation vials, adding 5 mL of Aquasol (New England Nuclear), sonicating, and counting in a Beckman scintillation spectrometer. Authentic reference glucosylceramide was purified from a total lipid extract of splenic tissue from a subject with the adult form of Gaucher disease (Kuske & Rosenberg, 1972). Galactosylceramide, lactosylceramide, trihexosylceramide, and globoside were purchased from Supelco, Inc., Bellefonte, PA. $G_{M1}$ and $G_{D1a}$ were prepared from bovine brain, and $G_{M3}$, $G_{M2}$, and $G_{D3}$ were purified from chick liver, Tay-Sachs diseased brain, and a methanol extract of butter-

[1] Abbreviations: Gal, galactose; GlcCer, glucosylceramide; LacCer, lactosylceramide; $GbOse_3Cer$, globotriaosylceramide; $GbOse_4Cer$, globotetraosylceramide; $G_{M3}$, sialosyllactosylceramide; $G_{M2}$, sialosylgangliotriaosylceramide; $G_{M1}$, sialosylgangliotetraosylceramide; $G_{D3}$, disialosyllactosylceramide; $G_{D1a}$, disialosylgangliotetraosylceramide.

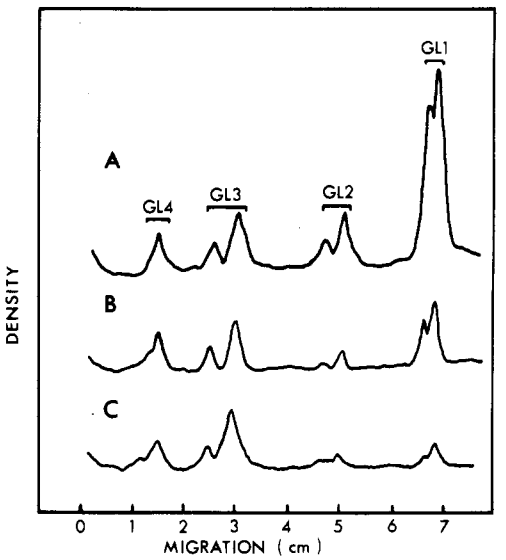


FIGURE 1: Densitometric pattern of neutral glycosphingolipids of monensin-treated fibroblasts. Gaucher fibroblasts were incubated in the presence of 10 μM monensin for 24 (A), 6 (B), and 0 h (C). The neutral glycosphingolipids were extracted from cell samples containing (A) 485, (B) 513, and (C) 430 μg of protein. The glycosphingolipids were separated on HPTLC and visualized by anthrone reagent as described under Materials and Methods. GL1, GL2, GL3, and GL4 indicate GlcCer, LacCer, $GbOse_3Cer$, and $GbOse_4Cer$, respectively.

milk, respectively, by the method of Momoi et al. (1976).

## Results

*Morphology of Fibroblast Cultures Treated with Monensin.* Fibroblasts cultured in the presence of 10 μM monensin became extensively vacuolated after 24 h as described previously (Goldberg & Toole, 1983). The vacuolation decreased after monensin was removed from the medium.

*Accumulation of GlcCer[1] and LacCer.* Figure 1 shows the densitometric pattern on HPTLC of neutral glycosphingolipids from infantile Gaucher (type 2, neuronopathic) fibroblasts. GlcCer, LacCer, $GbOse_3Cer$, and $GbOse_4Cer$ were the major neutral glycosphingolipids (Dawson et al., 1972). GlcCer was markedly increased in fibroblasts treated with monensin. Increase of LacCer was also observed upon monensin treatment. Table I shows the amount of glycosphingolipids upon treatment with 10 μM monensin for 24 h compared to the amount without monensin treatment. GlcCer and LacCer increased similarly after monensin treatment in both normal and lysosomal β-glucosidase-deficient Gaucher fibroblasts, suggesting that the monensin effect is not due to disruption of lysosomal glycosphingolipid catabolism for the most part.

Other neutral glycosphingolipids remained unchanged in both cell types. Major gangliosides of these cells were $G_{M3}$, $G_{M2}$, $G_{M1}$, $G_{D3}$, and $G_{D1a}$, and the amounts of gangliosides

Table II: Effect of Concentration of Monensin on Glycosphingolipid Content[a]

| monensin concn ($\mu$M) | glycosphingolipids (nmol/mg of protein) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | GlcCer | LacCer | $GbOse_3Cer$ | $GbOse_4Cer$ | $G_{M3}$ | $G_{M2}$ | $G_{M1}$ | $G_{D3}$ | $G_{D1a}$ |
| 0 | 1.33 | 0.86 | 1.64 | 1.36 | 4.42 | 2.92 | 0.33 | 0.24 | 1.02 |
| 0.1 | 5.77 | 1.59 | 1.64 | 1.17 | 4.71 | 3.22 | 0.31 | 0.20 | 1.19 |
| 1.0 | 7.47 | 1.55 | 1.77 | 1.56 | 4.01 | 3.18 | 0.38 | 0.23 | 1.08 |

[a] Gaucher fibroblasts (10 days after seeding) were incubated in the presence of monensin in the growth medium for 20 h, and their glycosphingolipids were analyzed as described under Materials and Methods. The values are means of duplicate cultures.

Table III: Effect of Monensin on Radioactivity Incorporated into Glycosphingolipids[a]

| glycosphingolipids | radioactivity (cpm/mg of protein) | | | | | |
|---|---|---|---|---|---|---|
| | normal fibroblasts | | Gaucher fibroblasts | | | |
| | monensin | control | monensin | | control | |
| | expt 1 | expt 1 | expt 1 | expt 2 | expt 1 | expt 2 |
| GlcCer | 33321 | 871 | 20141 | 17468 | 623 | 881 |
| LacCer | 9697 | 185 | 4631 | 4130 | 199 | 121 |
| $GbOse_3Cer$ | 950 | 1653 | 204 | 150 | 841 | 801 |
| $GbOse_4Cer$ | 89 | 316 | 14 | 9 | 155 | 133 |
| $G_{M3}$ | 8952 | 5183 | 2999 | 2367 | 2410 | 2555 |
| $G_{M2}$ | 22 | 240 | 32 | nd[b] | 552 | 588 |
| $G_{M1}$ | 24 | 77 | 14 | nd[b] | 353 | 267 |
| $G_{D3}$ | 17 | 566 | 2 | 1 | 103 | 99 |
| $G_{D1a}$ | nd[b] | 250 | 2 | 7 | 454 | 379 |

[a] Normal and Gaucher fibroblasts (13 days after seeding) were incubated in the presence or absence of 10 $\mu$M monensin in medium containing [$^3$H]Gal (1.5 $\mu$Ci/mL) for 18 h. The glycosphingolipids of these cells were separated, and the radioactivity of each glycosphingolipid was analyzed as described under Materials and Methods. [b] Not detectable.

were also comparable after monensin treatment. Table II shows the effect of different concentrations of monensin. An increase in GlcCer and LacCer was readily detected at a concentration as low as 0.1 $\mu$M monensin.

*Incorporation of [$^3$H]Gal into Glycosphingolipid Fractions.* Fibroblasts were incubated with [$^3$H]Gal (1.5 $\mu$Ci/mL) in the presence or absence of 10 $\mu$M monensin. Gal is readily epimerized and acts as a general hexose donor in these cells. Table III shows the amount of radioactivity incorporated into specific glycosphingolipids. High incorporation was observed in GlcCer and LacCer in the presence of monensin, while inhibition of incorporation actually was observed in all higher glycosphingolipid fractions except $GM_3$. The high radioactivity in cerebroside was due to labeled GlcCer, not GalCer, when analyzed by HPTLC precoated with borate that completely separates these compounds. Fibroblasts contain GlcCer but scantly detectable, if any, GalCer, indicating normal biosynthetic routes for incorporation of labeled Gal. Increase of incorporation of the labeled precursor into GlcCer and inhibition of incorporation into higher glycosylated glycosphingolipids were also observed readily at 0.1 $\mu$M monensin, a reasonably low concentration of ionophore, showing a great sensitivity to monensin.

## Discussion

The results presented in this paper show that, at very low concentration, the monovalent (cat)ionophore monensin specifically affects, in a highly visible manner, the biosynthesis of major glycosphingolipids in human skin fibroblasts. With exposure of the cells to monensin, simple neutral glycosphingolipids accumulated, and the incorporation of labeled precursor ([$^3$H]Gal) into higher glycosylated glycosphingolipids was inhibited. Lysosomal function is supposed to be inhibited in the presence of monensin (Tartakoff, 1983). We actually did observe a relative stability in the amounts of higher glycosylated glycosphingolipids. Nevertheless, our results indicate that, in fact, biosynthesis of these higher glycosylated glycosphingolipids is inhibited. Since the primary site of action of monensin appears to be within the Golgi complex (Ledger et al., 1980; Tartakoff et al., 1977, 1978), and oligosaccharide elongation of glycoproteins occurs in the distal face of Golgi and is inhibited in the presence of monensin (Tartakoff, 1982), we may suggest that GlcCer and LacCer synthesis occurs in monensin-insensitive anabolic membranes (probably in ER or the proximal face of Golgi), while higher glycosylation occurs in negatively monensin-sensitive membranes (probably the distal face of Golgi). Since labeled galactose, as a glycolipid hexose precursor, was readily incorporated into GlcCer, it is possible that monensin stimulates GlcCer synthesis, in addition to a potential accumulation caused by the inhibition of higher glycosphingolipid synthesis, that depends upon further elongation of precursor GlcCer and LacCer. Alternatively, translocation of precursor ceramide or nucleotide glycoses to the initial glycosylation site may be accelerated by the intracellular effects of this monovalent (cat)ionophore while translocation to subsequent glycosylation sites may be inhibited. These possibilities are now under investigation. GlcCer in Gaucher fibroblasts increased more than that in normal fibroblasts upon monensin treatment. The diminished lysosomal glucocerebrosidase activity of the Gaucher fibroblasts (Beutler et al., 1971) might explain this finding. We have found that a similar effect on glycosphingolipid biosynthesis is brought about by $Na^+,K^+$-ATPase inhibition by ouabain treatment (data not shown), indicating the more general possibility that monovalent cation, e.g., $K^+$, flux and pH changes may regulate the anabolism of fibroblast glycosphingolipids. As shown in Table II, $G_{M3}$ is the major fibroblast ganglioside. The pathway for biosynthesis of $G_{M3}$ (sialosyllactosylceramide) begins with the glucosylation of ceramide, to produce GlcCer, and, next, the galactosylation of GlcCer, to produce LacCer, which is then sialosylated to yield this major plasma membrane ganglioside in fibroblasts. These anabolic glycosylations and terminal sialosylation require the activated glycoses UDP-Glc, UDP-Gal, and CMP-*N*-

acetylneuraminic acid and the activity of appropriate transferase enzymes in the smooth endoplasmic reticulum and Golgi membranes (Rosenberg, 1979). Our findings clearly show that the initial glycosylation steps, whether or not they may be definable morphologically, may be considered operationally monensin insensitive, or positively monensin sensitive. We have observed that monensin increases the incorporation of labeled hexose into GlcCer and LacCer and causes a remarkable elevation of the cellular content of these simpler glycosphingolipids in fibroblasts. Experiments with lysosomal β-glucosidase-deficient (Gaucher disease) fibroblasts indicate that the accumulation of GlcCer and LacCer is not due primarily to a disruption of lysosomal glucohydrolase activity since the Gaucher disease cells do not have the capability to deglucosylate or otherwise degrade GlcCer, yet monensin causes an increase of GlcCer in these as well as in normal cells that can lysosomally degrade GlcCer. This effect of monensin is remarkable in view of former studies that indicate that monensin causes an abundance in high-mannose oligosaccharides to occur on the glycoprotein fibronectin, suggesting that monensin does not diminish initial glycosylation, e.g., transfer of mannosyl residues from GDP-mannose, but instead inhibits retailoring of the oligosaccharide moieties to yield the "complex" oligosaccharide compositional form of human fibronectin (Ledger et al., 1983). We find that the level of hexose incorporation into GlcCer and LacCer as well as the cellular amounts of these glycolipids is greatly increased by monensin in human fibroblasts. Sialosylation of LacCer yields $G_{M3}$ ganglioside. Yet, no significant increase in $G_{M3}$ occurs in fibroblasts that have been exposed to monensin and have acquired greatly elevated levels of LacCer. It may be significant in this regard that fibroblasts possess an active plasma membrane $G_{M3}$ sialidase that converts $G_{M3}$ to LacCer and may, therefore, regulate the level of $G_{M3}$ in the cell (Schengrund et al., 1973). Neither do the higher gangliosides nor higher neutral glycosphingolipids accumulate in monensin-treated cells. The values are shown in Table II. It is evident (Table III) that monensin inhibits the incorporation of galactose into these more highly glycosylated sphingolipids, suggesting that intracellular translocation of GlcCer and LacCer may indeed be inhibited by monensin and that, additionally, further glycosylation may take place in another, monensin-sensitive, membrane system. Detailed studies of the effect of monensin on the glycosylation of the glycoprotein fibronectin secreted from cultured human fibroblasts have suggested (Ledger et al., 1983) that monensin impairs translocation of this glycoprotein through the Golgi region of these cells and allows molecules that are incompletely modified posttranslationally, and therefore abnormal in terminal oligosaccharide composition, to reach the cell surface and be excreted. Glucosylceramide and lactosylceramide are the simplest glucosylsphingolipid members of a family of compounds of increasing oligosaccharide complexity that are components of the outer molecular leaflet of the plasma membrane of mammalian cells. Our observation that monensin induces increased labeling and accumulation of these glycolipids in cultured human fibroblasts indicates a hitherto unsuspected effect of monensin on mammalian cells, and implicates monovalent cation flux as a regulatory factor in biosynthesis of glycolipids. It is not unlikely that an effect on monensin is to alter intracellular vesicular pH and anabolic activities of local glycosyltransferases. This possibility is currently under investigation.

**Registry No.** $G_{D1a}$, 12707-58-3; $G_{D3}$, 62010-37-1; $G_{M1}$, 37758-47-7; $G_{M2}$, 19600-01-2; $GbOse_4Cer$, 11034-93-8; $GbOse_3Cer$, 71965-57-6; LacCer, 4682-48-8; GlcCer, 85305-87-9; monensin, 17090-79-8.

References


Basu, S., & Basu, M. (1982) in *The Glycoconjugates* (Horowitz, M., Ed.) Vol. III, pp 265–285, Academic Press, London.

Beutler, E., Kuhl, W., Trinidad, G., Teplitz, R., & Nadler, H. (1971) *Am. J. Hum. Genet. 23*, 62–66.

Dawson, G., Matalon, R., & Dorfman, A. (1972) *J. Biol. Chem. 247*, 5944–5950.

Goldberg, R. L., & Toole, B. P. (1983) *J. Biol. Chem. 258*, 7041–7046.

Irwin, C. C., & Irwin, L. N. (1979) *Anal. Biochem. 99*, 335–339.

Keenan, T. W., Moore, D. J., & Basu, S. (1974) *J. Biol. Chem. 249*, 310–315.

Kuske, T. T., & Rosenberg, A. (1972) *J. Lab. Clin. Med. 80*, 523–529.

Ledger, P. W., Uchida, N., & Tanzer, M. L. (1980) *J. Cell Biol. 87*, 663–671.

Ledger, P. W., Nishimoto, S. K., Hayashi, S., & Tanzer, M. L. (1983) *J. Biol. Chem. 258*, 547–553.

Li, Y.-T., & Li, S.-C. (1982) *Adv. Carbohydr. Chem. Biochem. 40*, 235–286.

Momoi, T., Ando, S., & Nagai, Y. (1976) *Biochim. Biophys. Acta 441*, 479–488.

Nishimoto, S. K., Kajiwara, T., Ledger, P. W., & Tanzer, M. L. (1982) *J. Biol. Chem. 257*, 11712–11716.

Pacuszka, T., Duffard, R. O., Nichimura, R. N., Brady, R. O., & Fishman, P. H. (1978) *J. Biol. Chem. 253*, 5839–5846.

Richardson, C. L., Keenan, T. W., & Morre, D. J. (1977) *Biochim. Biophys. Acta 488*, 88–96.

Rosenberg, A. (1979) in *Complex Carbohydrates of Nervous Tissue* (Margolis, R. U., & Margolis, R. K., Eds.) pp 25–43, Plenum Press, New York.

Saito, M., & Rosenberg, A. (1982) *J. Lipid Res. 23*, 3–8.

Schengrund, C.-L., Lausch, R., & Rosenberg, A. (1973) *J. Biol. Chem. 248*, 4424–4428.

Tajiri, K., Uchida, N., & Tanzer, M. L. (1980) *J. Biol. Chem. 225*, 6036–6039.

Tartakoff, A. (1982) *Philos. Trans. R. Soc. London, Ser. B 300*, 173–184.

Tartakoff, A. (1983) *Cell (Cambridge, Mass.) 32*, 1026–1028.

Tartakoff, A., & Vassalli, P. (1979) *J. Cell Biol. 79*, 284–299.

Tartakoff, A., Vassalli, P., & Detraz, M. (1977) *J. Exp. Med. 146*, 1332–1345.

Tartakoff, A., Vassalli, P., & Detraz, M. (1978) *J. Cell Biol. 79*, 694–707.

Tartakoff, A., Hoessli, D., & Vassalli, P. (1981) *J. Mol. Biol. 150*, 525–535.

Townsend, L. E., & Benjamins, J. A. (1983) *J. Neurochem. 40*, 1333–1339.

Wilkinson, F. E., Morre, D. J., & Basu, S. (1976) *J. Lipid Res. 17*, 146–153.