Cells in culture often undergo a “burst” of free sphingosine, sphingosine 1-phosphate, ceramide, and other bioactive lipids upon removal of “conditioned” medium, and at least one lipid signaling pathway (protein kinase C) has been shown to be affected by these changes (Smith, E. R. & Merrill A. H., Jr. (1995) J. Biol. Chem. 270, 18749–18758; Smith, E. R., Jones, P. L., Boss, J. M. & Merrill, A. H., Jr. (1997) J. Biol. Chem. 272, 5640–5646). Whereas increases in sphinganine and dihydroceramide are responses to provision of precursors for sphingolipid biosynthesis de novo in the new medium, the sphingosine burst is due to sphingolipid turnover upon removal of suppressive factor(s) in conditioned medium. This study describes the purification and characterization of these suppressive factors. Conditioned medium from J774 cells was fractionated into two components that suppress the burst as follows: ammonium ion, which reaches 2–3 mM within 48 h of cell culture; and a low molecular weight, cationic compound that has been assigned the structure 2,6-bis(ω-aminobutyl)-3,5-diiminopiperazine (for which we suggest the name “battrachamine” based on its appearance) by 1H and 13C NMR, Fourier transform infrared spectroscopy, and mass spectrometric analyses. The physiological significance of these compounds as suppressors of sphingolipid metabolism is unclear; however, ammonium ion is a by-product of amino acid catabolism and reaches high concentrations in some tissues. Battrachamine is even more intriguing because this is, as far as we are aware, the first report of a naturally occurring compound of this structural type. Considering the many cell functions that are affected by sphingoid bases and their derivatives, the effects of NH4 and battrachamine on sphingolipid metabolism may have important implications for cell regulation.

Identification of Ammonium Ion and 2,6-Bis(ω-aminobutyl)-3,5-diiminopiperazine as Endogenous Factors That Account for the “Burst” of Sphingosine upon Changing the Medium of J774 Cells in Culture* (Received for publication, July 15, 1999)

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Free sphingoid bases (sphinganine and sphingosine), sphingosine 1-phosphate, and ceramides affect numerous cell regulatory pathways when added exogenously or are formed endogenously as lipid mediators (1–4). In most cases, these compounds have been studied as intracellular “lipid second messengers” (1–4) (and in the case of sphingosine 1-phosphate as a “first messenger” (5) for agonists such as tumor necrosis factor-α, interleukin-1β, platelet-derived growth factor, nerve growth factor, cytotoxic agents, and various forms of stress. Nonetheless, a common laboratory procedure, the changing of cells in culture to fresh medium, induces a transient “burst” of sphingosine, sphinganine, and other bioactive lipids (6–10) to levels that are sufficient to affect at least one signaling pathway, the membrane association and activity of protein kinase C (10).

The increase in free sphingosine (as well as sphinganine 1-phosphate and dihydroceramide) of the burst arises from de novo sphingolipid biosynthesis upon provision of new medium (9), which contains serum and other precursors that are known to affect flux through this pathway since serum palmitoyltransferase activity is sensitive to its substrates in vitro and in situ (11, 12). In contrast, the burst of sphingosine (and sphingosine 1-phosphate and ceramide) arises mainly from turnover of complex sphingolipids, apparently involving acidic compartment(s) because NH4Cl and chloroquine are inhibitory (9). As will be shown in this report, the sphingosine burst is due to removal of suppressive factors in conditioned medium. Two of these factors have been purified and characterized and may represent compounds that regulate sphingolipid metabolism, and possibly signaling, in vivo.

EXPERIMENTAL PROCEDURES

Materials—J774A.1 cells (number TIB 67), a murine macrophage-like transformed cell line, were obtained from the American Type Culture Collection (Manassas, VA). The sphingolipid standards for HPLC (13) (D-erythro-sphingosine) were obtained from Sigma or synthesized (C18-sphinganine) (13). All other reagents were of high quality from commercial vendors.

Cell Culture—J774A.1 cells were grown in DMEM and 10% FBS (purchased from Life Technologies, Inc.) and sodium bicarbonate (3.7 g/liter) in a spinner flask (Corning Glass) at 37 °C and an atmosphere of 5% CO2. Cells were passaged every 2–3 days by a 1:4 dilution with fresh medium to a density of approximately 2.5 × 105 cells/ml. Cells were used between passages 3 and 24.

Analysis of the Sphingosine Burst—Unless indicated differently, suspended cells were removed from the spinner flask, collected by gentle centrifugation (in a table top centrifuge), resuspended in new medium, and added to 60-mm tissue culture dishes (Corning) at 5 to 7.5 × 105 cells per ml of medium. Under these conditions, the cells adhere to the dish rather than grow in suspension. The cells were incubated at 37 °C.

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1 The abbreviations used are: HPLC, high pressure liquid chromatography; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; TNBS, 2,4,6-trinitrobenzene sulfonic acid; FAB, fast atom bombardment.
5% CO₂ for 3 days before beginning the experiment by removing the conditioned medium and adding fresh medium to initiate the burst. After incubation of the cells under the particular conditions described in the text, the medium was removed; 0.5 ml of ice-cold methanol was added, and the cells were scraped from the dishes with a rubber spatula. The plates were scraped again with 0.4 ml of deionized water, followed by 0.4 ml of methanol. Cells and washes were pooled in 13 × 100-mm test tubes; 200–300 pmoles of C₂₀ sphinganine was added as an internal standard, and the long chain bases were extracted and analyzed by HPLC (14) with C₆₀ sphinganine as an internal standard. Purification of the Factors Responsible for Suppression of the Burst—Conditioned media were collected over time from the suspension cultures of the HT29 cells and stored frozen at −20 °C until used. The following steps were selected after testing various methods. 1) The media were separated into high and low molecular weight species by ultrafiltration of 5 liters of conditioned media using an Amicon DC 2 device with a Diaflow hollow fiber filtration cartridge (type HFIP3-20-1806 with a molecular mass cut-off of 2000–3000 daltons) (W. R. Grace & Co., Beverly, MA). All of the suppressive activity was recovered in the ultrafiltrate, so this was characterized further. 2) The cationic species in the ultrafiltrate were isolated by ion exchange chromatography using Bio-Rex 70 macroreticular carboxylic acid cation exchange resin (from Bio-Rad). Three liters of the ultrafiltrate were applied (at 4 °C) to a column (6 × 15 cm) containing approximately 100 g of the pre-swollen, 100–200 mesh size resin prepared as described by the manufacturer. After applying the sample, the column was washed with 6 liters of ultrapure H₂O, followed by 0.5 liters of 0.1 M NaCl. The suppressive factor(s) were step-eluted with 0.5 liters of 0.5 M NaCl. 3) The 0.5 M NaCl eluate from the Bio-Rex 70 column was concentrated to approximately 10 ml by lyophilization and then applied to a 2 × 60-cm P2 column (Bio-Rad Bio-Gel Polyacrylamide Gel, Fine, 100–200 mesh). The column was eluted with ultrapure H₂O (sterilized), and 1–2-ml fractions were collected. The fractions containing suppressive activity were identified by bioassay with J774A.1 cells and were stored at −20 °C in pooled fractions of 3–5 ml each (I–IV in Fig. 6).

Chemical Analysis and Structural Characterization of the Isolated Factors—Thinline chromatography (TLC) was conducted using cellulose plates (Eastman Kodak Cellulose 13254) developed with butanol:acetic acid:water (15:5:3, v/v), and silica plates (EM DC Plastikfolien Kiegelgel 60) were developed with methanol. The amino compounds were detected with ninhydrin (ninhydrin was sprayed on the plate as a 0.25% solution in acetone, air-dried, and then heated for 2–3 min) or ortho-phthaldialdehyde (sprayed on the plate as a 0.20% solution in acetone).

Ammonium ion was analyzed by the Clinical Chemistry Laboratory at Emory Hospital. The organic amine content was estimated using the 2,4,6-trinitrobenzene-sulphonic acid (TNBS) method (14). Since the molar extinction coefficient for the new compound is not known, an average of the molar extinction coefficients in Ref. 14 was used (e 20,000 M⁻¹ cm⁻¹ at 420 nm).

¹H NMR spectra were recorded on a Nicolet NT-360 (361.03 MHz), a General Electric QE-300 (300.15 MHz), or a General Electric GN-500 (500.10 MHz) spectrometer; the following abbreviations are used: δ, singlet; d, doublet; t, triplet; q, quartet; g, quintet; m, multiplet; and br, broad. The ¹³C NMR spectra were recorded on a General Electric QE-300 (75.48 MHz) with CDCls (triplet, δ = 77.00 ppm), d₆-methanol (septet, δ = 49.00 ppm), or d₆-Me₂SO (septet, δ = 39.50 ppm) as an internal standard. All ¹³C NMR spectra were obtained using the attached proton test (APT, quaternary carbons only or quaternary and methylene phased up; methyl and methine phased down) pulse sequence. Infrared spectra were obtained using a Perkin-Elmer 1430 ratio recording spectrometer. Mass spectrometry was performed using a JEOL JMS-SX102/SX102A/E, five-sector, tandem (MS1-MS2-MS3) mass spectrometer (19, 20). Full-scan negative ion fast atom bombardment (FAB) mass spectra were acquired using MS1 and frit-FAB in which the solvent was 2:1 CHCl₃/MeOH containing 1% triethanolamine.

RESULTS

The Sphingosine Burst of J774 A.1 Cells—Shown in Fig. 1 is a typical response of J774 A.1 cells upon changing from old to new medium. There is an increase in sphingosine to >100 pmoles per 10⁶ cells within an hour (which we term the sphingosine burst) followed by a return to a basal level (ca 20 pmol) in approximately 8 h. In previous studies (9, 10), increases in sphinganine were also described and attributed to increased de novo sphingolipid biosynthesis. Hence, this study focused only on the factor(s) that account this sphingosine burst.

Reversibility of the Sphingosine Burst—The probable causes of the sphingosine burst are that changing from old to new medium adds factor(s) that trigger sphingolipid turnover or removes factor(s) that are suppressive. The former did not appear likely because the burst could be seen if cells were changed to very minimal media, such as phosphate-buffered saline (data not shown). It was possible, nonetheless, that the procedure of removing and replacing the medium triggered the increases in sphingosine (for example, due to changes in pH or mechanical stimulation of the cells). To test this hypothesis, either new or "conditioned" medium was added to the cells, and sphingosine was measured after varying times (Fig. 2). Conditioned medium completely suppressed the burst (Fig. 2, •), even after the sphingosine burst had been activated by new medium (Fig. 2, ▲ followed by ▲). The suppressive effect of conditioned medium was also reversible (Fig. 2, ◆ followed by ◆). These findings suggest that conditioned medium contains factor(s) that suppress the sphingosine burst.

Appearance of Suppressive Factor(s) in Condition Medium—If J774 cells produce such factor(s), the amounts in conditioned medium should be time-dependent. Medium was collected from cells after various intervals and then added to new cells to determine whether there is an effect on the sphingosine burst. Shown in Fig. 3 are the amounts of sphingosine after 45 min of incubation of the cells with these media. New medium and media that had been conditioned for ≤8 h allowed a robust sphingosine burst, and some increase in sphingosine was seen with media conditioned for up to 18 h. Depending on the selection of the "maximum" increase in sphingosine (i.e., at time 0 or the somewhat higher amount at 4 h) (the latter is shown in the dashed line in Fig. 3), the half-time for 50% suppression is 8–12 h, respectively.

Identification of NH₂ as a Suppressive Factor in Conditioned Medium—A naturally occurring factor that has been shown to

The data in Fig. 6 are given for the chromatographic conditions described here, but subsequent analyses have found that NaCl can be replaced by ammonium formate (0.1 M wash followed by elution with 0.5 M), which is removed by lyophilization and facilitates concentration of the eluate in preparation for the next step.

In this experiment, J774 cells changed to phosphate-buffered saline actually produced more sphingosine in 45 min (111 ± 9 pmol/10⁶ cells) than cells changed to DMEM (53 ± 4 pmol/10⁶ cells).

For the purposes of this study, medium was considered "conditioned" if it had been incubated with J774 cells for at least 3 days.
suppress the sphingosine burst of J774 cells is NH₄⁺, apparently due to neutralization of acidic compartments in the cells (9). Ammonium ion is produced by amino acid catabolism and non-enzymatic deamidation of glutamine; hence, NH₄⁺ was measured in culture medium after varying intervals (Fig. 4A). Over the first 4–8 h, the NH₄⁺ concentration rose to 1.4 mM and continued to increase to 3 mM at 36 h. The results in Fig. 4, A and B, were used to calculate the expected suppression of the sphingosine burst by ammonia, and these estimates (Fig. 5 dashed line) and the actual suppression were compared (Fig. 5). At most time points, the ammonia concentration of the conditioned medium could account for $\sim$50% of the suppression of the sphingosine burst.

**Purification of Suppressive Factors from Conditioned Medium**—Because it did not appear that ammonia accounted for all of the suppression of the sphingosine burst by conditioned medium, the medium was fractionated to determine if there is more than one factor. The first analyses determined whether the factor(s) were of high or low molecular weight. Conditioned medium was dialyzed against water using membranes with a 6,000–8,000 molecular weight cut-off. The dialysate was lyophilized and re-dissolved to the volume of the original conditioned medium. Assay of the same volumes of the retained and low molecular weight fractions found no inhibition by the former and 97% suppression by the latter. Similar results were obtained using dialysis membranes with a 3500 molecular weight cut-off.
Shown in 100%) is shown by the comparison, the magnitude of the burst in DMEM alone (expressed as incubated with aliquots of each fraction as described in the text. For a decrease in the amount of sphingosine that was formed by J774 cells upon chromatography of the pooled 0.5 M NaCl eluate from the membranes with a 2000–3000 dalton cut-off, then applied to a Bio-Rex Bio-Gel P2 gel filtration column. The suppressive factor eluted by 0.5M NaCl.

Figure 6: Elution of suppressive "factors" upon cation exchange (A) and size exclusion (B) chromatography. Low molecular weight species were obtained from conditioned medium by ultrafiltration using membranes with a 2000–3000 dalton cut-off, then applied to a Bio-Rex 70 column (6 x 15 cm) followed by elution with deionized water containing increasing concentrations of NaCl (upper panel, open circles). Shown in A is the elution profile, with suppressive activity reflected as a decrease in the amount of sphingosine that was formed by J774 cells incubated with aliquots of each fraction as described in the text. For comparison, the magnitude of the burst in DMEM alone (expressed as 100%) is shown by the upper dashed line (t = 40 min in DMEM alone) versus the lower dashed line, the amount of sphingosine in cells at time 0 (which was also the same as the amount in cells incubated with unfractio- nated conditioned medium). B, shows the elution of the suppressive activity upon chromatography of the pooled 0.5 M NaCl eluate from the column in A (after concentration by lyophilization) on a 2 x 60-cm Bio-Rad P2 column (the volume of each fraction was 3 ml).

cut-off. Thus, as a first step for purification of the factor(s) from conditioned medium, the medium was passed through a Diaflo hollow fiber filtration system with a 2,000–3,000-dalton cut-off. When the ultrafiltrate was analyzed, it had 80–90% of the potency of original condition medium for suppression of the sphingosine burst (data not shown).

The ultrafiltrate was applied to cation and anion exchange columns, but the suppressive activities did not bind to anion exchange columns (data not shown). Shown in Fig. 6A is the elution profile using the cation exchange resin Bio-Rex 70 with elution by water and 0, 0.1, and 0.5 M NaCl. The column fractions were assayed by adding aliquots to DMEM and incubating the new medium with J774 cells; therefore, fractions that contain suppressive factors show little or no increase in sphingosine after 45 min versus the zero time point. There was some suppressive activity in the column flow-through and initial washes, but the majority remained on the column and was eluted by 0.5 M NaCl.

The 0.5 M NaCl eluate was lyophilized and applied to a Bio-Gel P2 gel filtration column. The suppressive factor eluted from this gel filtration column as a broad band of activity which, when compared with the volumes of elution of several standards, suggested that the molecular masses for fractions III and IV were 240 and 130 daltons, respectively (Fig. 6B) (similar results were obtained using Sephadex G-10 columns, data not shown). Four regions of the eluate (fractions 1–IV in Fig. 6B) were examined by silica and cellulose TLC, and fraction III was the only eluate that contained a single species (see below for $R_f$); therefore, this compound was characterized further. The compounds in the other fractions were also ninhydrin-positive but did not yield the blue-gray product with ortho- 

Phthalaldehyde that was obtained with III (see below).

The organic amine content of fraction III was estimated by reaction with TNBS (15). Although this is only an approximation, it suggested that the amine concentration was ~200 mM.\footnote{Since subsequent structural characterization of this compound revealed that it has two amines that can react with TNBS, this concentration is likely higher.}

Titration of the sphingosine burst with the purified fraction III (Fig. 6B) revealed 50% suppression of the burst with 30 μl, or an apparent concentration of 1–3 mM\footnote{The concentration of the purified factor is likely higher than this, as subsequent structural characterization revealed that it has two amines that can react with TNBS.} (Fig. 7). Based on a volume to volume comparison of the amount of fraction III that was needed to suppress the sphingosine burst comparable to that of conditioned medium, the fraction was >200-fold more concentrated than conditioned medium. Since the purification produced 3 ml of this fraction from 3,000 ml of conditioned culture medium, the yield for the procedure appears to be 22%.

Because the suppressive activities in the other column fractions have not yet been obtained in pure form, they have not yet been characterized.

Characteristics of the Purified Suppressive Factor—On both silica and cellulose TLC plates, the suppressive factor reacted with ninhydrin to give an orange compound that was fluorescent under long wavelength ultraviolet light and with ortho- 

Phthalaldehyde gave a blue gray spot. The $R_f$ (versus the solvent front) on cellulose plates was 0.40 (for comparison, the $R_f$ for tryptamine was 0.7; for tryptophan, 0.5; for glucosamine, 0.24; and for lysine, 0.1); on silica the $R_f$ was 0.56 (plus some color at the origin) (the $R_f$ for tryptamine was 0.12; for trypto- 

Phan, 0.5; for glucosamine, 0.21; and lysine and arginine were slightly above the origin). In addition, the isolated suppressive factor was Ehrlich reagent-negative (therefore is not an indole amine, pyrrole, aromatic amine, sulfonamide, urea, or allanto- 

in), did not react with vanillin (therefore is not proline, a polyhydric sugar, an aldopentose, an aldohexose, inositol, or ornithine), and was not able to be visualized with iodine vapor, which makes it unlikely that it is a lipid, a catecholamine, a sugar mercaptal, an alcohol, a glycoside, or an N-acylamino sugar (a close running, I2-positive contaminant was, however, found in some impure extracts). For further confirmation that
the unknown amine was not a standard amino acid, it was submitted to the Emory Microchemical Center for amino acid analysis, which failed to account for more than 2% of the total amine content as known amino acids; hence, these are minor contaminants but not the major species. The NH$_4^+$ concentration was also low (0–0.04 mM).

**Structural Determination of the Novel Suppressive Factor**—

The chemical identity of the compound in fraction III was elucidated by a combination of methods, and the structure that is consistent with all of the analytical data is 2,6-bis(α-aminobutyl)-3,5-dimino-piperezine, which is shown in Fig. 8.

High resolution mass spectrometry (FAB$^-$ ion mode) gave an m/z for the factor of 255.2295, which provides the molecular composition of C$_{12}$H$_{28}$N$_6$. Electrospray ionization mass spectrometry gave a M + H$^+$ ion at m/z 257, which is also consistent with a protonated species of this composition.

The $^1$H NMR spectrum in D$_2$O revealed five distinct signals integrating in a 1:2:2:2:2 ratio, whereas the $^{13}$C NMR spectrum showed six absorptions with five clustered in the aliphatic region below 55 ppm and one signal at 174.6 ppm. An attached proton test established that the $^{13}$C spectrum was comprised of four methylenes, one methine, and one non-protonated carbon signal. Since the mass spectrum required 12 carbons, these data suggested that the compound was comprised of two identical elements, as shown by the structure in Fig. 8.

The two sites of unsaturation required by the molecular formula could be assigned to the amide groups ($\delta$, 174.6) based on a strong absorption at 1586 cm$^{-1}$ in the IR spectrum. The remaining mass units could be accommodated by two terminal primary amines. The presence of amine and amidine groups was supported by strong absorptions at 3446 cm$^{-1}$ in the IR spectrum, characteristic of exchangeable hydrogens on nitrogen.

COSY and HMQC analyses confirmed that the methine carbon ($\delta$ 54.2, C-6 in Fig. 8) corresponded to the $^1$H NMR signal at $\delta$ 3.61 and was connected to a chain of methylenes as follows: C-7 and C-7': $^1$H NMR, $\delta$ 1.74 (4H); $^{13}$C NMR, 29.9; C-8 and C-8': $^1$H NMR, $\delta$ 1.38 (4H); $^{13}$C NMR, 21.5; C-9 and C-9': $^1$H NMR, $\delta$ 1.58 (4H); $^{13}$C NMR, 26.4; and, C10 and C10': $^1$H NMR, $\delta$ 2.87 (4H); $^{13}$C NMR, 39.2. Analysis of the decoupled one-dimensional spectra also revealed that the aliphatic signal furthest downfield ($\delta$ 3.76 t) assigned to C-2 and C-6 in the parent structure was coupled to C-7 methylene protons ($\delta$ 1.74) which in turn were coupled to the C-8 methylene protons ($\delta$ 1.38). The methylene protons assigned to C-10 and C-10' (α to the amine) were second furthest downfield ($\delta$ 2.87, t) and were coupled only to the C-9 and C-9' protons ($\delta$ 1.58). The C-2 and C-6 protons showed, as expected for the structure in the figure, coupling to C-7 and C-7' methylene protons.6 The splitting and couplings were consistent with a straight chain of methylene units with the terminal methylene unit ($\delta$ 2.87, C-6) connected to a primary amino group.

**DISCUSSION**

Sphingosine bursts have been seen with many types of cells in culture as follows: J774 cells (6, 9, 10), Swiss 3T3 cells (7), NIH-3T3 cells (8), A431 cells (8), NG108–15 cells (8), and primary cultures of rat hepatocytes and mouse peritoneal macrophages.7 Studies with J774 cells have established that the sphingosine arises from sphingomyelin hydrolysis in what appears to be acidic intracellular compartment(s) (such as lysosomes or endosomes) because NH$_4^+$ and chloroquine are inhibitory (9). This led us to hypothesize that media conditioned by J774 cells may contain factor(s) that suppress the production of sphingosine, and two compounds were identified in conditioned medium with this capacity, NH$_4^+$ and 2,6-bis(α-aminobutyl)-3,5-dimino-pipererezine.

The NH$_4^+$ concentration found in conditioned medium after 36 h (3.4 mM) would suppress the sphingosine burst by ~60%; hence, this can account for a substantial portion of the suppressive activity in J774 conditioned medium. It is common for cells in culture to produce (mostly from glutamine) 1–3 mM NH$_4^+$ in the medium (16, 17); thus, NH$_4^+$ might also play a role in the behavior of the many other cell lines where sphingosine bursts have been noted. This disruption of sphingolipid metabolism by NH$_4^+$ may cause an appreciable accumulation of sphingolipid mass (9) and, thus, might have some of the characteristics of a genetic defect in sphingolipid hydrolase(s) of sphingolipid activator proteins (18). The presence of ammonium ion is well known to be growth inhibitory and toxic for many types of cells (16, 19), and given the roles of sphingolipids in cell growth and apoptosis (1–5), interference with sphingolipid metabolism might play a role in this toxicity. Thus far, however, we have only evaluated whether the changes in sphingolipid metabo-

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6 There was no clear evidence for coupling of the two methylene protons to each other, which distinguished 2,6-bis(α-aminobutyl)-3,5-dimino-piperezine as the most likely structure versus 1,10-diamino-5,6-diaminododecan, which is otherwise consistent with all of these analytical data. The isomer 2,5-bis (α-aminobutyl)-3,6-dimino-piperezine might also fit the analytical data but, according to energy minimization calculations, would be most stable with the double bonds in the pyrazine ring and is not as easy to rationalize by a simple biosynthetic pathway.

7 L. A. Warden, E. Wang, and A. H. Merrill, Jr., unpublished results from our laboratory.
Inhibitors of Sphingolipid Turnover in Conditioned Medium

Ammonium ion concentrations in vivo are kept substantially below 1 mM through trapping as amino acids, urea, etc., with a few notable exceptions (19). In the lumen of the colon, digestion of amino acids by microflora produces NH$_4^+$ (plus ammonia) in concentrations ranging from 3 to >50 mM (19, 22–25); these amounts have been shown to cause colonic mucosal cell damage (24). In the latter experiment, rats were perfused in situ with NH$_4^+$; hence, there is a possibility that the cells undergo the equivalent of a sphingosine and/or ceramide burst after the NH$_4^+$ is depleted. If so, the formation of sphingosine and ceramide, which are well known to induce apoptosis in cells in culture (1–4), might participate in the toxicity of NH$_4^+$. Elevations in NH$_4^+$ also occur in liver and kidney disease (23) as well as a number of other metabolic disorders; these concentrations are not of the same magnitude as are found in the colon and probably do not affect sphingolipid metabolism (unless, perhaps, localized regions of the tissues have higher concentrations).

The second suppressive factor in conditioned medium was a compound, we suggest that it be given the common name "batrachamine" after the Greek "batracheios," of or belonging to a frog. The origin of batrachamine is not known; however, it might be biosynthesized by a fairly straightforward series of reactions that proceed through a monomeric intermediate (diamino-hexanamide) that undergoes condensation and cyclization by reactions such as those proposed in Fig. 9. The decarboxylation of amino acids is a common reaction catalyzed by enzymes with reactive aldehyde moieties in the active site (pyridoxal 5'-phosphate or pyruvoyl groups), and the intermediate can be utilized to form carbon-carbon bonds with a co-substrate with a suitable leaving group (for example, in the reactions catalyzed by δ-aminolevulinate synthase and serine palmitoyltransferase). With a guanidinium compound (e.g. arginine) as the co-substrate, this could produce the amidino moiety of batrachamine by reactions I and II. As is also shown in Fig. 9, pyridoxal 5'-phosphate (and pyruvoyl) enzymes are known to effect transformations that produce the types of amine and ketone intermediates shown in II—IV; hence, these might condense (V) and cyclize (V to VI) to form the core batrachamine structure, where only one additional step (reduction) is needed to yield VII. Experiments are underway to explore this pathway.

It is possible that intermediates III and/or IV (in the latter case, as the ketone) account for the lower molecular weight suppressive factor(s) that also elute from the P2 column (fraction IV, Fig. 6B); however, the other suppressive factor(s) have not yet been identified. The mechanism(s) for the suppression of the sphingosine burst by batrachamine (and other factors) is (are) also not known, but the presence of amino groups makes it possible that it is, like NH$_4^+$, acting as a lyso-omotropic factor. Nonetheless, the only other naturally occurring inhibitor of sphingolipid turnover that has been found in mammalian cells is glutathione, which inhibits the neutral sphingomyelinasem (26). Batrachamine may function as an inhibitor of sphingolipid turnover by enzyme(s) that have acidic pH optima. Now that this new class of compound(s) has been structurally elucidated, it is possible to synthesize quantities suitable for more mechanistic studies as well as develop analytical methods to test for the presence of batrachamine in other systems, including ones where as-yet-uncharacterized heat-stable factors that affect signal transduction have surfaced (27).

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