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The Degradation of Glycosphingolipids by Air

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Exposure of glycosphingolipids to air irreversibly destroys the integrity of these lipids within a few hours. It was established that among the natural constituents of air, ozone, at commonly observed daytime levels, is responsible for the observed degradation. As one product of the reaction of glycosphingolipids with air, an aldehydic fragment containing the carbohydrate moiety was identified. This aldehydic fragment was identical to the one obtained by classical glycosphingolipid ozonolysis. Identical with the latter, the air-induced product is further fragmented by mild alkali treatment with concomitant liberation of the free reducing oligosaccharide. As a consequence of the alteration of glycosphingolipids by air, it was shown that the accuracy of methods of analysis of these glycoconjugates that depend on their physico-chemical integrity, *e.g.*, by tlc-immune overlay, is severely influenced by their prior exposure to the atmosphere.

Key words: air pollution, ganglioside, glycosphingolipid, ozone.

Glycosphingolipids, when exposed to air, were found to be irreversibly altered. Since ozone is one natural constituent of air, it was assumed that this oxidant could be responsible for the observed alteration of the glycolipids. In 1964, it was shown that ozone reacts with glycosphingolipids containing a sphing-4-enine base (*2*). Thereby, the sphingoid C-to-C double bond is cleaved creating products of oxidation. These, in turn, are sensitive to further degradation, whereby carbohydrate, glycosidically linked to the C1-position of the ozonized sphingoid product, is liberated (*3*). We have now observed that the sensitivity of sphingolipids to attack by ozone is such that its commonly present concentration in air during daytime in Germany is sufficient for an effective cleavage of sphingolipids within a few hours of exposure. This, in turn, was found to have consequences for the correctness of methods of quantitative analyses of glycosphingolipids.

MATERIALS AND METHODS

Ozone Concentration—The concentration in air was measured by its absorption at 254 nm using a Dasibi Analyzer, type 1008-RS (Bionics, Wuppertal, Germany), sensitivity minimum 4 $\mu g \cdot m^{-3}$, a government-approved and -supervised instrument.

Glycosphingolipids—All neutral and acidic glycolipids used were products of this laboratory. Gtet1-aldehyde (4-monosialo-gangliotetraosyl-,3-hydroxy,2-*N*-acyl-butyric aldehyde), used as a standard, was produced with a Fischer Laboratories ozone generator as described earlier (*4*).

Antibodies—A murine anti-ganglioside Glac2 monoclonal IgG antibody (R24) was a gift from Professor W. Dippold, Mainz, Germany, while a murine anti-ganglioside Gtri2 monoclonal IgG antibody (704/152/5) was generously provided by Dr. K. Bosslet, Behring-Werke, Marburg, Germany.

Air Treatment on tlc Plates—Glycosphingolipids (GSLs) were applied to silica tlc glass plates (Merck, Darmstadt, Germany, or Baker, Gross-Gerau, Germany) and left exposed to air, nitrogen or oxygen. Serving as controls, equally tlc-applied GSLs were covered for the same periods of time with glass plates or plastic foil. After exposure, tlc plates containing neutral GSLs were developed in a saturated atmosphere with a running solvent of chloroform-methanol-water, 62.5 : 30 : 6 (by volume). For ganglioside and sulfatide containing tlc, a running solvent of chloroform-methanol-0.3% aqueous $CaCl_2$, 45 : 45 : 10 (by volume), was chosen.

Air Treatment on Silica Gel—Ganglioside Gtet1, 50 μg in 200 μl of methanol, was added to 100 mg of dry silica gel (LiChroprep Si 60, Merck, Darmstadt, Germany), and the mixture was carefully brought to dryness. Subsequently, the silica gel was spread on an aluminum foil (2 cm^2) as a thin layer and left exposed to air. Thereafter, the silica gel was recovered from the foil, suspended in 2 ml of chloroform-methanol, 8 : 2 (by volume), applied to a small glass pipette with a glass wool plug, and all glycolipid material was eluted with chloroform-methanol-water, 60 : 35 : 8 (by volume). A subsequent elution with methanol yielded liberated free GSL-oligosaccharide. The filtrates were analyzed by tlc.

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Abbreviations: G, gangliosides of the sialoganglio-series (Gg) are abbreviated as suggested earlier (*1*): an addendum to G designates the neutral carbohydrate portion, *e.g.*, Glac (lactose), Gtri (gangliotriaose, Gg_3), Gtet (gangliotetraose, Gg_4). The number of sialic acid residues is given in Arabic numerals, sialic acid position isomers by a, b, c, *e.g.*, for gangliosides Glac2 (II^3NeuAc_2-LacCer), Gtri2 (II^3NeuAc_2-Gg_3Cer), Gtet1 (II^3NeuAc-Gg_4Cer), Gtet2a (IV^3NeuAc-,II^3NeuAc-Gg_4Cer), Gtet2b ($II^3NeuAc_2Gg_4$Cer), Gtet3b (IV^3NeuAc-,II^3NeuAc_2-Gg_4Cer). GSL, glycosphingolipid; mAb, monoclonal antibody; tlc, thin-layer chromatography.

Air Treatment in Dimethylformamide—Ganglioside Gtet1, 50 μg, was dissolved in 300 μl of dimethylformamide by ultrasonication. Air was passed through the solution for 12 h using a small membrane pump. During this time, two 100-μl portions of dimethylformamide had to be added in order to avoid dryness. Analysis was, as described, by tlc.

Detection of GSL and Ozone Cleavage Products on tlc—Neutral GSLs and sulfatide were visualized with orcinol/sulfuric acid spray-reagent; gangliosides, additionally, with resorcinol/hydrochloric acid spray-reagent. Besides with these reagents, the ganglioside-aldehyde could also be detected with tetrazolium salt by its formazan color formation. To this end, 0.5 g 2,3,5-triphenyltetrazolium chloride was dissolved in 100 ml of methanol. To 5 ml of this solution, an equal volume of NaOH (6 M in water) was added shortly before use. The tlc was sprayed moderately, and heated gently until a pink color appeared.

For immunological detection of gangliosides, murine monoclonal antibodies were used. The secondary antibody was an alkaline phosphatase-labeled anti-mouse-Ig antibody. The procedure was carried out as described before (*5*).

Isolation of the Sialooligosaccharide-Containing Aldehydic Product of Ganglioside Air Treatment—Twenty-five micrograms of ganglioside Gtet1 were applied as an 8-cm-long line, 2 cm off the lower edge of a 10×10 cm tlc plate, leaving 1 cm without application at each side. The plate was left exposed to the open air for 24 h. For detection after chromatography, two strips, 1.5 cm in width, were cut from each side of the tlc-plate with the aid of a glass cutter, and the positions of the ganglioside and its aldehydic degradation product as detected with resorcinol/HCl spray-reagent were marked on the main plate. The silica gel containing the degraded resorcinol-positive material was carefully scraped off the plate with a spatula, suspended in methanol and applied to a glass pipette, prefilled with 100 μl of LiChroprep silica gel (Merck, Darmstadt, Germany). The aldehydic ganglioside degradation product was eluted with 2 ml of methanol, then dried down by rotary vacuum evaporation. The residue was collected at the bottom of the tube by rinsing the tube walls with a small amount of methanol and repeated evaporation to dryness.

Alkaline Degradation of the Sialooligosaccharide-Containing Aldehydic Product of Ganglioside Air Treatment—Ten microliters of 0.1 M aqueous NaOH and 30 μl of methanol were added to either the air-treated ganglioside on silica gel (LiChroprep) or the isolated ganglioside-aldehyde from the tlc plate. The mixture was left for 1 h at room temperature. An aliquot was spotted on a tlc plate. Chromatography was carried out as described above.

RESULTS

GSLs, including sulfatide, gangliosides, and neutral compounds, when applied to silica gel thin-layer chromatograms and left exposed to air, showed altered tlc properties upon subsequent chromatography (Fig. 1).

This air-induced alteration of GSLs was independent of light (data not shown). Comparable exposure of GSLs to nitrogen or oxygen did not change their behavior on subsequent tlc. Shielding the tlc-applied GSL by coverage with a glass plate or plastic foil, completely prevented the degradation (see, Figs. 5 and 6). In addition, degradation of GSLs on tlc by air could be largely avoided by treatment of the GSL-applied plate with polyisobutylmethacrylate (see, Fig. 5D). An identical change in tlc properties to that seen after exposure of tlc-applied GSLs could also be observed when air was passed through a solution of these GSLs, *e.g.*, in dimethylformamide. Furthermore, when coated on a glass plate or polystyrene surface, or adsorbed to silica gel (Lichroprep), GSLs were also degraded by exposure to air. Alteration of GSL by air was dependent on the concentration of ozone (Fig. 2).

The sugar-containing products from air-exposed GSL, *e.g.*, ganglioside Gtet1, migrated on tlc at slower rates than the unaltered GSL (Fig. 3A).

Products with chromatographically identical properties to those obtained by air exposure could be produced by classical treatment of the GSL with ozone. In fact, the identity of the ozone- and the air-produced primary aldehydic GSL degradation products could also be shown by their equal ability to reduce tetrazolium salts with concomitant formazan color formation (Fig. 3B). Furthermore, the air-induced aldehydic GSL fragment was labile to mild basic conditions, whereby free reducing oligosaccharide was liberated (Fig. 4).

In the case of ganglioside Gtet1, this sugar was identical to monosialogangliotetraose as obtained by classical ozonolysis-alkaline fragmentation described earlier (*2*). As expected, the part of the glycosphingolipids containing only saturated sphingoid with no C-to-C double bond at the C4-position was not altered by exposure to air (data not shown). In a comparative experiment, exposure of tlc-applied ganglioside Gtet1, isolated from human brain, to air for 48 h, with a mid-day maximum ozone concentration reaching 120 μg/m^3, cleaved approximately 80% of the total (data not shown). This corresponded to practically all of the glycolipid present with a sphingoid that was unsatu-

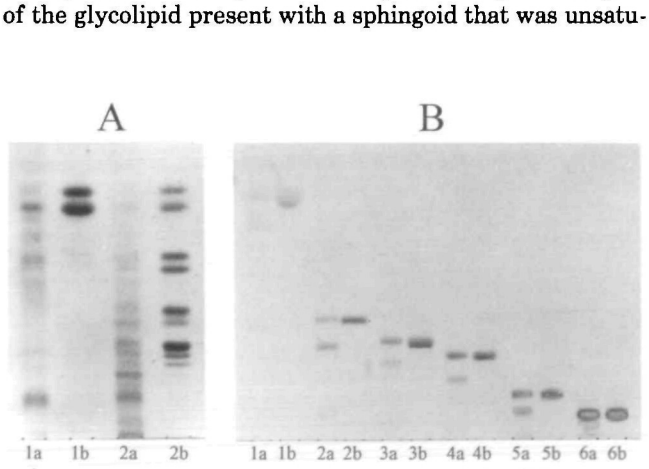

Fig. 1. **Thin-layer chromatogram of neutral and acidic GSLs after exposure to air.** The glycolipids were applied to the tlc plate and left exposed to air, and subsequently chromatographed. Controls were added only shortly before chromatography. A, neutral GSLs: a, exposed to air for 48 h on 2 days with average maximum concentrations of 100 μg O_3 per m^3; b, unexposed glycolipids; 1, galactosylceremide from human brain; 2, neutral mono-, di-, tri-, and tetra-glycosylceramides from human spleen. B, acidic GSLs from human brain: a, exposed to air for 24 h on a day with a maximum concentration of 120 μg O_3 per m^3; b, unexposed glycolipids; 1, sulfatide; 2, Gtet1; 3, Glac2; 4, Gtet2a; 5, Gtet2b; 6, Gtet3b. Visualization: orcinol sulfuric acid spray reagent; running solvents, see "MATERIALS AND METHODS."

rated at the C4-position. Maximal oxidation of the ganglioside by treatment for 2 min on the tlc-plate with ozone, produced from oxygen with an ozone generator, yielded an identical rate of cleavage of the compound.

Because of the sphingolipid degradation induced by air, particularly on tlc, and the altered physico-chemical properties of the ensuing products, it was thought likely that methods of GSL analysis that depended on the integrity of the lipids might also be influenced. As an example, using mAb R24 for the quantitative detection of ganglioside Glac2 on tlc, it was shown that less ganglioside was immunologically detected after exposure to air (Fig. 5C).

Even though the aldehydic Glac2 ozone degradation product showed a positive reaction with the antibody, a lower degree of immuno-detectability was observed, probably due to its partial elution from the plate (Fig. 5A).

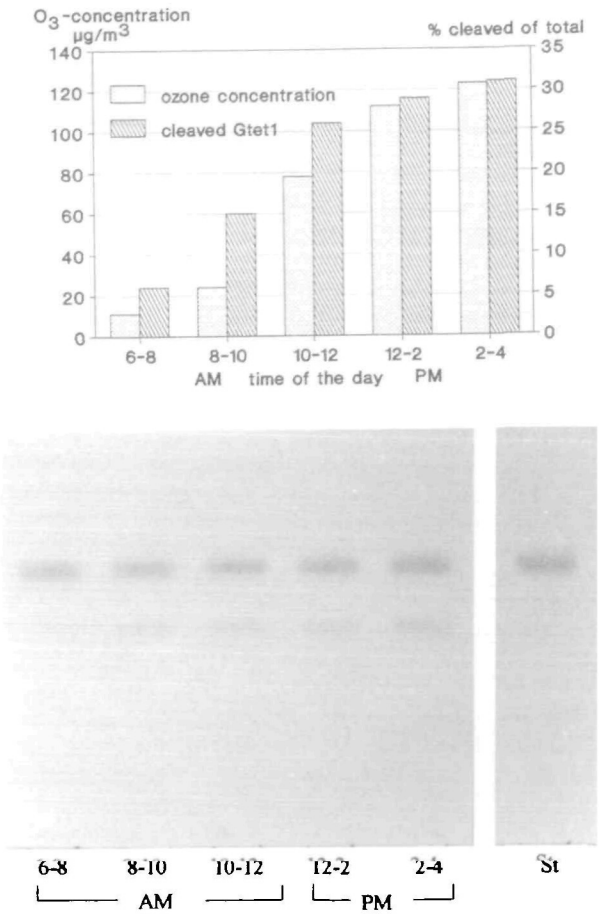

Fig. 2. **Dependence of ganglioside Gtet1 degradation by air on ozone concentration.** Tlc-applied Gtet1 was exposed for 2 h during the rising ozone concentrations of daytime (Marburg, 20 August 1996). St, standard ganglioside Gtet1. Visualization: resorcinol/HCl spray reagent; running solvent, see "MATERIALS AND METHODS."

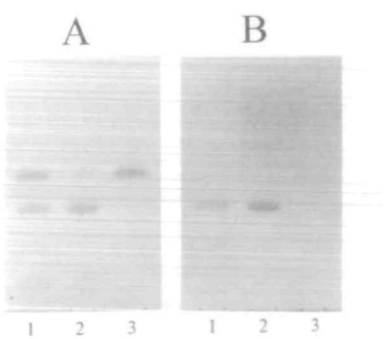

Fig. 3. **Tlc-analysis of the air-induced degradation products of ganglioside Gtet1, at a maximum air O_3-concentration of 120 $\mu g \cdot m^{-3}$.** A, staining with resorcinol/HCl spray reagent; B, color formation with triphenyltetrazoliumchloride: 1, Tlc-applied Gtet1 after exposure to air; 2, tlc-applied Gtet1 after exposure to ozone from a generator; 3, native Gtet1.

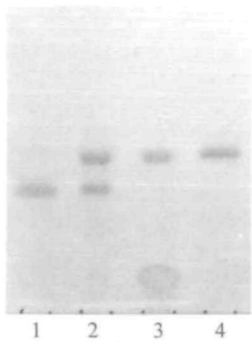

Fig. 4. **Tlc-analysis of the degradation of the air-induced cleavage product of ganglioside Gtet1 by mild alkaline treatment.** The experiment was performed on a day with a maximum air ozone concentration of 90 $\mu g \cdot m^{-3}$. 1, aldehydic sialo-oligosaccharide-containing product of Gtet1 cleavage with ozone from a generator; 2, Gtet1, adsorbed to silica gel, exposed to air for 24 h, and subsequently eluted with chloroform-methanol-water; 3, Gtet1 treated as given under 2, but subsequently treated with alkali; 4, native Gtet1. Visualization: resorcinol/HCl spray reagent.

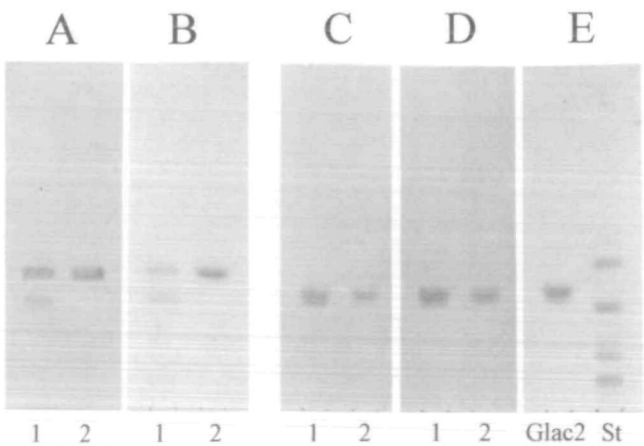

Fig. 5. **Detection of ganglioside Glac2 on tlc by immune-overlay with mAb R24.** A and B: A, 24 h air exposure of tlc plate-applied Glac2, followed by chromatography and immune overlay determination; B, chemical staining with resorcinol/HCl spray-reagent; 1, air-treated Glac2; 2, native Glac2. C and D, tlc of Glac2, followed by 24 h exposure to air and immune overlay determination: C, treatment of tlc with polyisobutylmethacrylate after 24 h air exposure; D, treatment of tlc with polyisobutylmethacrylate before 24 h air exposure; 1, Glac2, protected against air by shielding; 2, Glac2 exposed to air. E: Glac2, native Glac2; St, standard gangliosides from human brain, main components from top to bottom: Gtet1, Gtet2a, Gtet2b, Gtet3b; chemical staining with resorcinol/HCl spray-reagent.

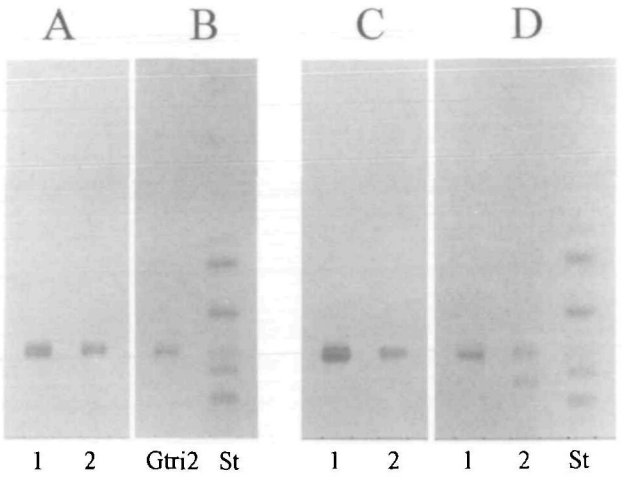

Fig. 6. **Detection of ganglioside Gtri2 on tlc by immune overlay with mAb 704/152/5.** A, tlc of Gtri2, and subsequent exposure to air for 24 h, followed by immune overlay: 1, shielded against air; 2, air-exposed; detection with mAb 704/152/5; B, standard gangliosides: ganglioside Gtri2; standard human brain gangliosides, detection with resorcinol/HCl-spray reagent; C, tlc of Gtri2 after 24 h air exposure: 1, ganglioside Gtri2, shielded; 2, air-exposed; detection with mAb 704/152/5; D: 1, ganglioside Gtri2, shielded; 2, air-exposed; St, standard human brain gangliosides; detection with resorcinol/HCl-spray reagent.

Similarly, detection of ganglioside Gtri2 with monoclonal antibody 704/152/5 by tlc-immune overlay method was also greatly influenced by prior exposure of the tlc-applied glycolipid to air (Fig. 6A).

In this case, the sialo-oligosaccharide-containing aldehydic fragmentation product proved immuno-negative on tlc with the corresponding antibody (Fig. 6C). However, whereas the free sugar could not be chemically detected on the tlc after undergoing the immune overlay procedures, the aldehydic, fatty acid-containing ganglioside ozone cleavage product, though immuno-negative on tlc, could still be visualized on the plate by chemical staining (data not shown).

DISCUSSION

We have observed that GSLs left exposed to air are readily degraded. The active ingredient of air causing the destruction of these glycolipids was established to be ozone. Since the earliest reliable measurements of the air ozone concentration of around 20 to 32 $\mu g \cdot m^{-3}$ were performed in Paris in 1900, these values have continued to increase at a rate of approximately 1 $\mu g \cdot m^{-3}$ per year. Atmospheric ozone levels at the beginning of the century can be regarded as the result of naturally occurring molecular processes induced by solar UV irradiation, and largely uninfluenced by human activity. However, boosted by chemical reactions promoted by emitted nitrogen oxides and hydrocarbons, ozone concentrations now commonly reach daytime levels of up to 120 to 140 $\mu g \cdot m^{-3}$. They are still below the concentrations that are believed to be harmful to human health to the extent that, according to European Community Regulations, the respective governmental agencies (at 180 $\mu g \cdot m^{-3}$) or the population as a whole (at 340 $\mu g \cdot m^{-3}$) would have to be informed. Because of the ease of detection of GSLs by visualization on thin-layer chromatograms, it could be observed that in fact normal air ozone concentrations cause an unexpectedly, and hitherto unrecognized, rapid degradation of these compounds. The speed at which normal air may degrade GSLs depends on their physical state of distribution during exposure, and, possibly, on their purity. Therefore, GSLs spread on silica gel appeared to be particularly vulnerable, and more quickly degraded than, *e.g.*, in solution. The GSL-oligosaccharide-containing product of this degradation, although not of an as yet rigorously proven chemical structure, was believed to be a 4-oligoglycosyl-,3-hydroxy-,2-*N*-acyl-butyric aldehyde. This compound is rather unstable and readily degraded under very mildly basic conditions, with the concomitant liberation of the intact sugar of the parent compound. It was hypothesized that the latter reaction might proceed *via* a Lobry-DeBruyn rearrangement of the 4C-fragment aldehyde and subsequent β-elimination of the sugar. This reaction scheme, though not proven, was supported by the ease of β-elimination of the sugar moiety after introduction of a carbonyl-function at the C3-position of the sphingoid of intact GSL by selective oxidation with dichlorodicyanobenzoquinone (*6, 7*). It was now observed that the aldehydic sugar-containing GSL ozonization product, when distributed on silica gel, is already fragmented in a similar way and to a great extent merely by elution with methanol.

All carbohydrate-containing products of GSL air oxidation, as shown by their altered tlc-migration rates, were considerably more hydrophilic than the parent compounds. Therefore, it was expected that the destruction of GSLs by air *via* ozonization might severely influence methods of GSL quantitation. And indeed, it was shown that after exposure of GSL to air on tlc-plates, values of quantitation by the immune overlay method according to Magnani *et al.* (*8*) were dramatically reduced. As an explanation for this, it is suggested that the aldehydic ozonization product, as well as its liberated oligosaccharide, are more or less soluble in water. Even after stabilization of the plate with polyisobutyl-methacrylate, both products will be desorbed during the immune incubations using aqueous solutions. Thereby, liberated oligosaccharides are completely dissolved from the plate. The aldehydic ozonization products, depending on their hydrophilicity, are more (Gtri2-aldehyde) or less (Glac2-aldehyde) eluted from the silica gel surface of the plate, and thereby become immuno-negative (Gtri2-aldehyde), even though they may in all cases still be detectable by chemical staining from deeper levels of the thin-layer. A similar phenomenon of non-detectability by tlc-immune overlay of an otherwise with the respective specific antibody immuno-positive glycolipid was frequently observed with GSL derivatives missing one of the aliphatic long hydrocarbon chains (lyso-GSL, R. Jennemann, unpublished results).

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