Sci. U.S.A. 60, 622.
Thomas, J. H., and Wakefield, S. M. (1973), *Biochem. Soc. Trans. 1,* 1179.
Tomizawa, H. H. (1962), *J. Biol. Chem. 237,* 428.
Tomizawa, H. H., and Varandani, P. T. (1965), *J. Biol. Chem. 240,* 3191.
Unger, R. H. (1971), *N. Engl. J. Med. 285,* 443.
Varandani, P. T. (1966a), *Biochim. Biophys. Acta 118,* 198.
Varandani, P. T. (1966b), *Biochim. Biophys. Acta 127,* 246.
Varandani, P. T. (1967), *Biochim. Biophys. Acta 132,* 10.
Varandani, P. T. (1972), *Biochim. Biophys. Acta 286,* 126.
Varandani, P. T. (1973a), *Biochim. Biophys. Acta 295,* 630.
Varandani, P. T. (1973b), *Biochim. Biophys. Acta 320,* 249.
Varandani, P. T. (1973c), *Biochim. Biophys. Acta 304,* 642.
Varandani, P. T. (1973d), *Biochem. Biophys. Res. Commun. 55,* 689.
Varandani, P. T. (1974a), *Diabetes 23,* 117.
Varandani, P. T. (1974b), *Biochim. Biophys. Acta 371,* 577.
Varandani, P. T. (1974c), *Biochem. Biophys. Res. Commun. 60,* 1119.
Varandani, P. T., and Nafz, M. A. (1970), *Arch. Biochem. Biophys. 141,* 533.
Varandani, P. T., Nafz, M. A., and Chandler, M. L. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 276.
Varandani, P. T., Nafz, M. A., and Shroyer, L. A. (1971), *Diabetes 20,* 342.
Varandani, P. T., Shroyer, L. A., and Nafz, M. A. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1681.
Varandani, P. T., and Tomizawa, H. H. (1966), *Biochim. Biophys. Acta 113,* 498.
Yalow, R. S., and Berson, S. A. (1971), *Biochem. Biophys. Res. Commun. 44,* 439.
Yip, C. C. (1972), *Biochem. Biophys. Res. Commun. 46,* 2127.

Characterization by Mass Spectrometry of Blood Group A Active Glycolipids from Human and Dog Small Intestine†

E. L. Smith, J. M. McKibbin, K.-A. Karlsson,* I. Pascher, and B. E. Samuelsson

ABSTRACT: Glycolipids with blood group A activity isolated from human and dog small intestine have been characterized by mass spectrometry of intact lipid in methylated and in methylated and reduced ($LiAlH_4$) form. Without degradative studies the glycolipids were conclusively shown to be hexaglycosylceramides with phytosphingosine as the major long-chain base and hydroxypalmitic acid as the major fatty acid. The exact sugar ratio was hexose–hexosamine–deoxyhexose 3:2:1 and the sequence established as hexosamine–[deoxyhexose–]hexose–hexosamine–hexose–hexose–ceramide. Evidence is presented that mass spectrometry can differentiate between type 1 and type 2 saccharide chains.

A blood group A active hexaglycosylceramide was indicated several years ago in human erythrocytes (Hakomori and Strycharz, 1968), and the glycolipid nature of cellular ABH antigens has been known for a long time (see summary by Hakomori and Strycharz, 1968). Although the structure of the immunological determinants should bear resemblance to those of blood group active secreted glycoproteins (Watkins, 1972; Rovis et al., 1973), no conclusive structure of human A active glycolipids has so far been presented (Hakomori et al., 1972), and an A active glycolipid of hog gastric mucosa was proposed to be a heptaglycosylceramide (Slomiany and Horowitz, 1973; Slomiany et al., 1974). Recently, however, strong evidence for the structure of one of the basic H active structures of human erythrocytes has been presented as L-fucopyranosyl-α-(1→2)-galactopyranosyl-β-(1→4)-*N*-acetylglucosaminosyl-β-(1→3)-galactopyranosyl-β-(1→4)-glucopyranosyl-β-(1→1)-ceramide (Stellner et al., 1973; Kościelak et al., 1973). On the other hand, fucose attachment to glucosamine was reported for A active fucolipids from human erythrocytes (Kościelak et al., 1970).

In the present investigation blood group A active glycolipids isolated from human and dog small intestine have been characterized. By a novel mass spectrometric method for intact glycolipids (Karlsson, 1973) the carbohydrate composition and sequence of the lipids were conclusively established.

Materials and Methods

The methods of isolation and immunological characterization of the glycolipids have been described elsewhere (Vance et al., 1966; McKibbin, 1969; Smith and McKibbin, 1972; Hiramoto et al., 1973; Smith et al., 1973). The human glycolipid was identical with sample Hu-3F-1 of Hiramoto et al. (1973) and Smith et al. (1973), and the dog A active fractions were 15F-1, 20F-1, 26F-1, 29F-1, and

† From the Department of Biochemistry, University of Alabama, Birmingham, Alabama 35294 (J.M.M. and E.L.S.), and the Department of Medical Biochemistry, University of Göteborg, Fack, S-400 33 Göteborg 33, Sweden (K.-A.K., I.P., and B.E.S.). *Received December 2, 1974.* Supported by a grant from the Swedish Medical Research Council (No. 03X-3967), University of Alabama Medical Center Faculty Research Grants 82-6111 and 82-6117, and American Cancer Grant IN 66K.

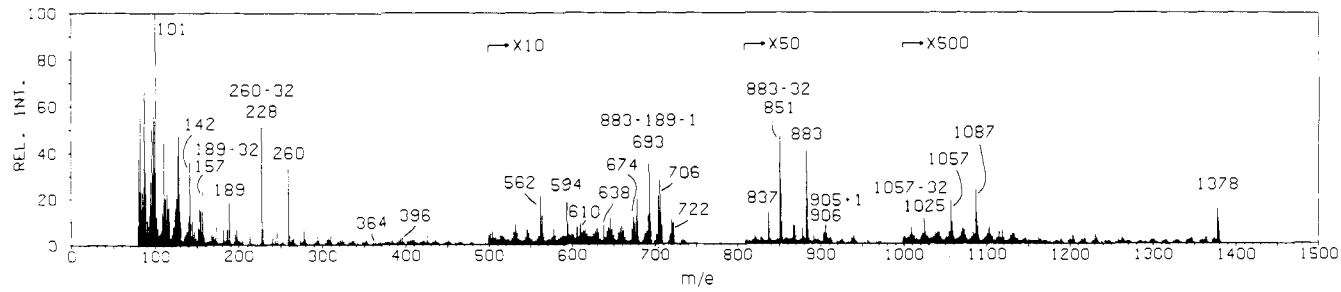

FIGURE 1: Mass spectrum of methylated blood group A active glycolipid of human small intestine. The conditions of analysis were as follows: electron energy 70 eV, trap current 500 μA, acceleration voltage 5 kV, ion source temperature 300°, and probe temperature 330°. A simplified formula for the interpretation of fragments is reproduced in Figure 2.

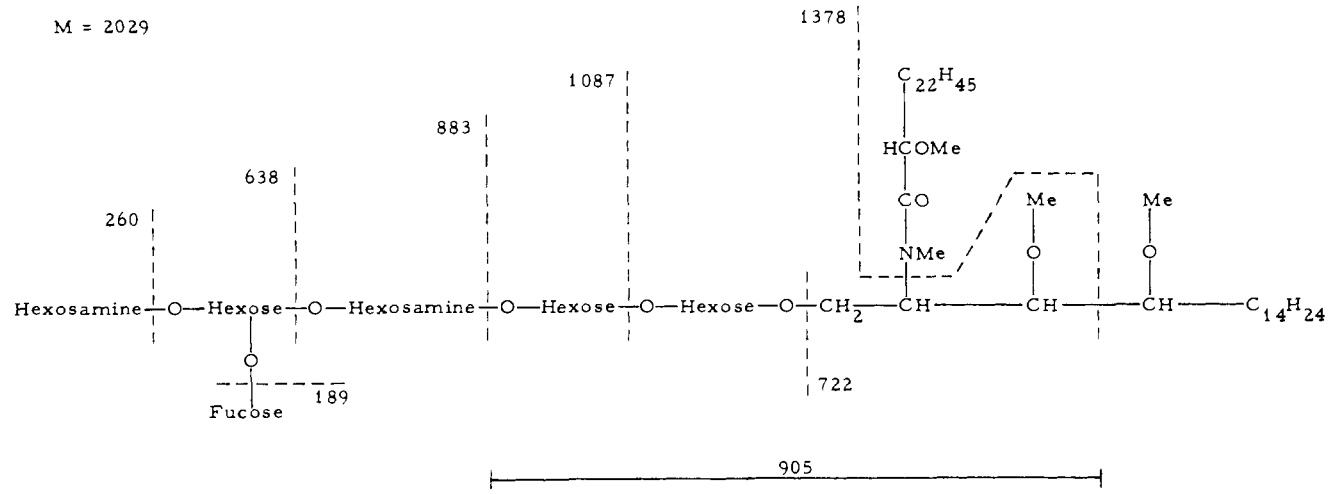

FIGURE 2: Simplified formula for the interpretation of the mass spectrum of Figure 1, showing the methylated derivative of a blood group A active glycolipid containing phytosphingosine and hydroxytetracosanoic acid.

32F-1. Methylation was done according to the procedure of Hakomori (1964), and the reagent conditions for this and the reduction with $LiAlH_4$ have been reported elsewhere (Karlsson, 1974). Mass spectra were recorded on an MS 902 instrument (AEI Ltd., Manchester, England) equipped with a separate probe heater. The conditions of analysis are given in the legends for figures. The spectra were taken at the maximum intensity of the total ion current, and this has been found to give a representative fatty acid profile for samples with known fatty acid composition. The mass numbers were obtained by counting by hand and are therefore nominal masses. The exact masses in the upper region, however, are by one unit higher than those reproduced.

Results

Complete mass spectra are reproduced only for the human sample as the dog species gave almost identical results (see below). The mass spectra of the methylated and methylated and reduced derivatives of the blood group A active glycolipid of human small intestine are reproduced in Figures 1 and 3, respectively, and simplified formulas for the interpretation are given in Figures 2 and 4, respectively. The conclusions concerning structure were based on spectra recorded for the same derivatives of synthetic monoglycosylceramides (Karlsson et al., 1974a), several gangliosides (Karlsson, 1974; Karlsson et al., 1974a,b), the Forssman glycolipid hapten (Karlsson et al., 1974c), and several human and dog fucolipids with H, Le^a, and Le^b blood group activities (unpublished results). The structures of several of the ions in the lower mass regions have been suggested elsewhere (Karlsson et al., 1974a).

The spectrum recorded for the completely methylated glycolipid (Figure 1) has been reproduced up to *m/e* 1378 (the calculated molecular weight for the structure concluded is 2029, see Figure 2). Intense peaks are found for a terminal hexosamine at *m/e* 260 and 228 (260 − 32, which is due to a loss of methanol) and for a terminal deoxyhexose (fucose) at *m/e* 189 and 157. Abundant ions were produced at *m/e* 883 and 851 corresponding to a terminal tetrasaccharide with two hexosamines, one hexose and one fucose. Evidence for the binding of fucose to hexose is the peak at *m/e* 693, corresponding to a loss of 190 mass units. We have found (unpublished) a loss of 190 + 16 (oxygen) mass units if fucose is bound to hexosamine. The peak at *m/e* 638 is further evidence for the sequence given in Figure 2. The significant ions at *m/e* 1087 contain the terminal pentasaccharide. Relatively intense peaks at *m/e* 1057 and 1025 may be due to an admixture of a second glycolipid with an additional fucose at the terminal tetrasaccharide. An heptaglycosylceramide of this structure has been identified in dog small intestine and human pancreas (unpublished results).

Phytosphingosine-containing glycolipids produce a specific ion (unpublished) as indicated at *m/e* 1378, containing all sugars and part of the long-chain base, probably a rearrangement ion of the segment shown in Figure 2. This is evidence for a hexaglycosylceramide containing trihydroxy base. The existence of phytosphingosine in animal tissues has been known for some years (Karlsson, 1964, 1970). Further evidence for phytosphingosine in combination with hydroxytetracosanoic acid are the ceramide peaks at *m/e* 722 and 706 (722 − 16). The corresponding peaks for hydroxyhexadecanoic acid containing ceramides are found at

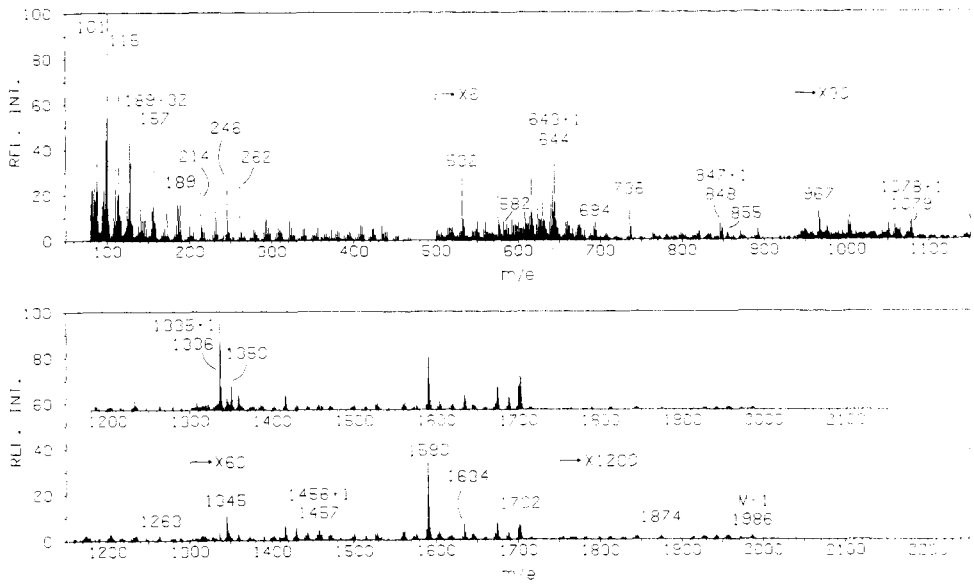

FIGURE 3: Mass spectrum of methylated and reduced blood group A active glycolipid of human small intestine. The conditions of analysis were as follows: electron energy 30 eV, trap current 500 μA, acceleration voltage 3.4 kV, ion source temperature 320°, and probe temperature 350°. The inserted spectrum in the region *m/e* 1200–2100 was recorded at a probe temperature of 320°. A simplified formula for the interpretation of fragments is reproduced in Figure 4.

m/e 610 and 594. A peak for ceramide with sphingosine and hydroxyhexadecanoic acid is seen at *m/e* 562. The two specific ions (Karlsson et al., 1974a) for sphingosine (*m/e* 364) and phytosphingosine (*m/e* 396) are of very low abundance. The rearrangement ions at *m/e* 906 (see explanation below the formula of Figure 2) are evidence for two hexoses in closest position to ceramide with trihydroxy base and hydroxytetracosanoic acid.

The spectrum recorded for the methylated and reduced derivative (Figure 3) is an important supplement to these conclusions. In this case molecular weight ions (at *m/e* 1986) are found for the heaviest molecular species with phytosphingosine and hydroxytetracosanoic acid, which is reproduced schematically in Figure 4. As demonstrated before (Karlsson, 1973, 1974; Karlsson et al., 1974 a–c) for other glycolipids, ions produced by a loss of the long-chain base chain are very abundant (a series of peaks in the interval 1590–1702). This is important evidence for the number and type of sugars (three hexoses, two hexosamines, and one deoxyhexose) and a semiquantitative information on fatty acid composition. The major acid is hydroxyhexadecanoic acid (*m/e* 1590) but other homologs with 22 (at *m/e* 1674), 23 (at *m/e* 1688), and 24 (at *m/e* 1702) carbon atoms are also seen. The peak at *m/e* 1700 is probably due to the species with monounsaturated hydroxytetracosanoic acid (see also below, and the peaks at *m/e* 704, 720, and 904 in Figure 1).

Intense peaks are found for a terminal hexosamine (*m/e* 262, 246, and 214, which is 246 − 32) and a terminal fucose (*m/e* 189 and 157). As for other reduced glycolipids (Karlsson, 1973, 1974; Karlsson et al., 1974 a–c), a series of rearrangement ions is a good indication of carbohydrate sequence of the molecule. These are indicated below the formula of Figure 4, for one sugar at *m/e* 644, two sugars at *m/e* 848, three sugars at *m/e* 1079, and five sugars at *m/e* 1457. The homologs with C_{16} acid are found at *m/e* 532, 736, 967, and 1345, respectively. Evidence for monounsaturated hydroxytetracosanoic acid are the peaks at *m/e* 642, 846, 1077, and 1455. Thus the carbohydrate composition and sequence of the glycolipid as given in the formulas of Figure 2 and Figure 4 are well supported by mass spectra of the two derivatives.

In the lower half of the spectrum of Figure 3, a second spectrum has been inserted, which was recorded at somewhat lower temperature (see legend of figure) than the main spectrum. In this case two additional peaks are seen, at *m/e* 1336 and 1350, of the probable origin as indicated in Figure 4. Their disappearance at higher temperature may be due to pyrolysis. The ions at *m/e* 1634 are due to a loss of the fatty acid as indicated in Figure 4 and this type of ion has been found for the same derivative of other glycolipids with 2-hydroxy fatty acids.

There is additional information in Figure 1 concerning preferential combinations of fatty acid and long-chain base. As noted above, ceramide peaks were seen at *m/e* 562 (sphingosine plus hydroxyhexadecanoic acid), 594 and 610 (phytosphingosine plus hydroxyhexadecanoic acid), and 706 and 722 (phytosphingosine plus hydroxytetracosanoic acid). The peak at *m/e* 706 is relatively more intense than that at 594. However, the molecular species with sphingosine and hydroxytetracosanoic acid is present at *m/e* 674 and is of lower abundance than the C_{16} homolog (*m/e* 562). Several spectra recorded in sequence during the evaporation curve confirm this result. This is evidence for a preferential combination of phytosphingosine with longer chain (C_{24}) fatty acid, which has been found by us before for sphingomyelins of bovine kidney (Karlsson and Steen, 1968) and intestine (Breimer et al., 1975), and for the Forssman glycolipid hapten of horse kidney (Karlsson et al., 1974c). In accordance with this the major fatty acid as indicated by the spectra of the reduced derivative (Figure 3) was hydroxyhexadecanoic acid (*m/e* 1590) and the relative intensities of C_{16} (*m/e* 1590) and C_{24} (*m/e* 1702) was approximately the same as the relation of *m/e* 562 plus 594 (C_{16} acid) to 706 (C_{24} acid) of Figure 1.

The spectra of glycolipids of human and dog origins looked very similar except for two differences. The dog samples all lacked the peaks at *m/e* 1057 and 1025 as seen in Figure 1. As noted above these are evidence for a second glycolipid with two fucose residues, probably lacking in the

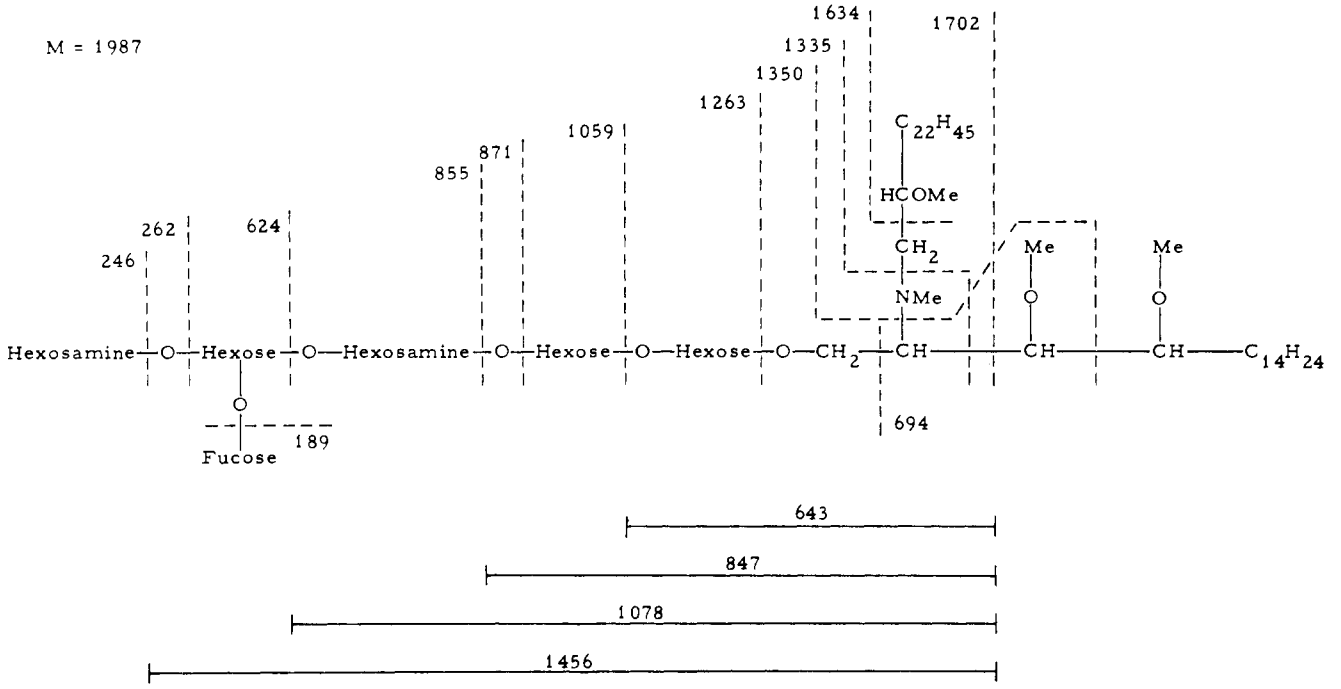

FIGURE 4: Simplified formula for the interpretation of the mass spectrum of Figure 3, showing the methylated and reduced derivative of a blood group A active glycolipid containing phytosphingosine and hydroxytetracosanoic acid. The reduction of amide groups of ceramide and two amino sugars results in a loss of 3 times 14 mass units.

dog samples. The second important difference was the peak at *m/e* 182 of the methylated derivatives, which was very intense for the dog samples (see Figure 5) but was practically absent in the human sample (Figure 1). In several H-like and Le^a-like pentaglycosylceramides of human and dog origins we have found this difference to be related to the binding position of galactose to glucosamine (unpublished results), i.e., a 1 → 3 (type 1 chain) or a 1 → 4 (type 2 chain) binding (Watkins, 1972). As will be reported (J. M. McKibbin and E. L. Smith, in preparation) the human intestinal A active glycolipid has been shown by degradative studies to contain about 60% of the type 1 chain whereas the dog glycolipid exclusively had the type 2 chain. They also differ immunologically (J. M. McKibbin and E. L. Smith, in preparation). The ions at *m/e* 182 produce the base peaks of several dog intestinal fucolipids with type 2 chains and also of human erythrocyte fucolipids (unpublished), which exclusively have type 2 chains (Stellner et al., 1973; Kościelak et al., 1973). High-resolution analysis of *m/e* 182 gave a composition of $C_9H_{12}O_3N$. Our conclusion therefore is that *m/e* 182 originates in a hexosamine (glucosamine) substituted with hexose (galactose) in a 1 → 4 linkage.

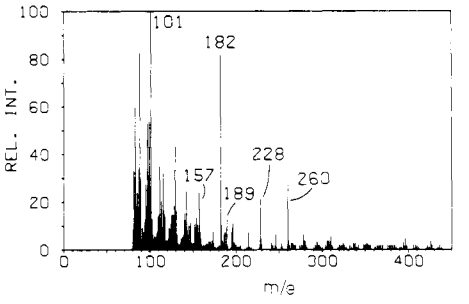

FIGURE 5: Part of the mass spectrum recorded for the methylated derivative of blood group A active glycolipid of dog small intestine. The conditions of analysis were as for the human sample (see legend of Figure 1). The intense peak at *m/e* 182 is practically lacking in the spectrum of the human glycolipid (compare Figure 1), and is probably due to a difference in binding position of sugars (see text).

Discussion

The blood group A active glycolipids isolated from human and dog small intestine are hexaglycosylceramides with a sugar composition and sequence as given in Figure 2 and Figure 4. This conclusion was based on a comparison with earlier published spectra of methylated and reduced glycolipids (Karlsson, 1973, 1974; Karlsson et al., 1974 a–c), and spectra of ABH and Le active glycolipids (unpublished results). Of great importance for the sugar composition data are the sugar and fatty acid composition ions of relatively high intensity in the reduced derivatives, produced by a loss of the long-chain base chain (the interval *m/e* 1590–1702 of Figure 3). The carbohydrate sequence information was derived from the methylated derivative as a series of primary fragments containing a successively increasing number of sugars from the nonreducing end (Figures 1 and 2). This was supported by a series of rearrangement ions in the spectrum of the methylated and reduced derivative (Figures 3 and 4), containing the fatty acid and a varying number of the sugars.

Of the lipophilic components, the fatty acids were semiquantitatively obtained from the reduced derivative in the sugar and fatty acid composition ions. Separate ions for the long-chain bases were of very low abundance. However, knowing the fatty acid spectrum one may get an advanced information on ceramides from the methylated derivatives, even concerning preferential combinations of fatty acid and long-chain base, as shown in the present case for hydroxytetracosanoic acid and phytosphingosine.

The A active glycolipids from human and dog small intestine are hexaglycosylceramides, which composition is expected if a hexosaminyltransferase (Ginsburg, 1972) may

add galactosamine to the H active pentaglycosylceramide as identified in human erythrocytes (Stellner et al., 1973; Kościelak et al., 1973). The composition found is also in agreement with earlier published data on blood group A active glycolipid of human origin (Hakomori and Strycharz, 1968; Hakomori et al., 1972; Ando and Yamakawa, 1973). However, longer carbohydrate chains with the same A-determinant group have been suggested to exist in human erythrocytes (Hakomori et al., 1972), similar to the case in glycoproteins (Watkins, 1972; Rovis et al., 1973), and the major A active glycolipid from hog gastric mucosa was recently proposed to be a heptaglycosylceramide (Slomiany and Horowitz, 1973; Slomiany et al., 1974). On the other hand, a second A active hexaglycosylceramide was identified in hog (Slomiany et al., 1973), with the sugar ratio hexose–hexosamine–fucose 4:1:1, instead of 3:2:1 as found for the glycolipids of human and dog intestine. This difference should be easily recognized by the mass spectrometric method discussed in this paper.

Of interest is the possibility of analyzing for type 1 and type 2 chains of the glycolipids. Glycolipids of human erythrocytes all contained type 2 chains (Stellner et al., 1973; Kościelak et al., 1973), similar to the case for a glycolipid of human adenocarcinoma (Yang and Hakomori, 1971). A pancreas glycolipid with B activity had, however, type 1 and type 2 chains in the approximate ratio of 4:1 (Wherrett and Hakomori, 1973), and the A active glycolipids of hog gastric mucosa also contained both types (Slomiany et al., 1974). In the present case, dog intestinal A active glycolipids were all of type 2, while the human sample had both type 1 and type 2 chains. The meaning of these differences is not known, but evidently there are species, tissue, and possibly individual specificities.

References

Ando, S., and Yamakawa, T. (1973), *J. Biochem. 73,* 387.

Breimer, M. E., Karlsson, K.-A., and Samuelsson, B. E. (1975), *Lipids 10,* 17.

Ginsburg, V. (1972), *Adv. Enzymol. Relat. Areas Mol. Biol. 36,* 131.

Hakomori, S.-i. (1964), *J. Biochem. 55,* 205.

Hakomori, S.-i., Stellner, K., and Watanabe, K. (1972), *Biochem. Biophys. Res. Commun. 49,* 1061.

Hakomori, S.-i., and Strycharz, G. D. (1968), *Biochemistry 7,* 1279.

Hiramoto, R. N., Smith, E. L., Ghanta, V. K., Shaw, J. F., and McKibbin, J. M. (1973), *J. Immunol. 110,* 1037.

Karlsson, K.-A. (1964), *Acta Chem. Scand. 18,* 2397.

Karlsson, K.-A. (1970), *Chem. Phys. Lipids 5,* 6.

Karlsson, K.-A. (1973), *FEBS Lett. 32,* 317.

Karlsson, K.-A. (1974), *Biochemistry 13,* 3643.

Karlsson, K.-A., Leffler, H., and Samuelsson, B. E. (1974c), *J. Biol. Chem. 249,* 4819.

Karlsson, K.-A., Pascher, I., Pimlott, W., and Samuelsson, B. E. (1974a), *Biomed. Mass Spectrom. 1,* 49.

Karlsson, K.-A., Pascher, I., and Samuelsson, B. E. (1974b), *Chem. Phys. Lipids 12,* 271.

Karlsson, K.-A., and Steen, G. O. (1968), *Biochim. Biophys. Acta 152,* 798.

Kościelak, J., Piasek, A., and Górniak, H. (1970), in Blood and Tissue Antigens, Aminoff, D., Ed., New York, N.Y., Academic Press, p 163.

Kościelak, J., Piasek, A., Górniak, H., Gardas, A., and Gregor, A. (1973), *Eur. J. Biochem. 37,* 214.

McKibbin, J. M. (1969), *Biochemistry 8,* 679.

Rovis, L., Anderson, B., Kabat, E. A., Gruezo, F., and Liao, J. (1973), *Biochemistry 12,* 5340.

Slomiany, A., and Horowitz, M. I. (1973), *J. Biol. Chem. 248,* 6232.

Slomiany, B. L., Slomiany, A., and Horowitz, M. I. (1973), *Biochim. Biophys. Acta 326,* 224.

Slomiany, A., Slomiany, B. L., and Horowitz, M. I. (1974), *J. Biol. Chem. 249,* 1225.

Smith, E. L., Bowdler, A. J., Bull, R. W., and McKibbin, J. M. (1973), *Immunology 25,* 621.

Smith, E. L., and McKibbin, J. M. (1972), *Anal. Biochem. 45,* 608.

Stellner, K., Watanabe, K., and Hakomori, S.-i. (1973), *Biochemistry 12,* 656.

Vance, W. R., Shook, C. P., and McKibbin, J. M. (1966), *Biochemistry 5,* 435.

Watkins, W. M. (1972), in Glycoproteins, Gottschalk, A., Ed., Amsterdam, Elsevier, p 830.

Wherrett, J. R., and Hakomori, S.-i. (1973), *J. Biol. Chem. 248,* 3046.

Yang, H.-J., and Hakomori, S.-i. (1971), *J. Biol. Chem. 246,* 1192.