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Analysis of anomeric configurations in glyceroglycolipids and glycosphingolipids by chromium trioxide oxidation

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Abstract Acetylation and CrO_3 oxidation in acetic acid (Angyal and James, *Aust. J. Chem.* **23:** 1209–1221, 1970) was applied to 18 different glyceroglycolipids and glycosphingolipids of known structure. The lipids studied contained from one to five pyranosic monosaccharide units including α- and β-linked glucose, galactose, mannose, and *N*-acetylgalactosamine and β-linked *N*-acetylglucosamine. Monosaccharides bound to the lipids through β-glycosidic linkages reacted to the extent of 80–97%, but in the case of α-glycosidic linkages the oxidation proceeded only to the extent of 0–6%. A partial reaction was observed in lipids in which a given monosaccharide unit was present in both anomeric forms. Therefore, oxidation with CrO_3 allows the determination of anomeric configurations in simple glycolipids. Samples of only 100–300 μg are required.

Treatment with CrO_3 in acetic acid oxidizes acetylated hexopyranosides, in which the aglycone occupies an equatorial position, giving acetylated 5-hexulosonates (1) (**Fig. 1**). The corresponding anomers in which the aglycone occupies an axial position are oxidized only very slowly. Thus, the β-glycosidic hexopyranoside units of natural glycolipids should be oxidized, whereas the corresponding α-glycosidic units should not. The possibility of using this difference in the analysis of anomeric configurations of natural lipids has been studied by Hoffman, Lindberg, and Svensson (2), who analyzed a bacterial lipopolysaccharide, and by Laine and Renkonen (3), who studied some mannose-containing sphingolipids. The present report shows that the CrO_3 treatment oxidizes almost completely all β-glycosidically linked glucose, galactose, mannose, *N*-acetylglucosamine, and *N*-acetylgalactosamine units in simple glycero- and sphingolipids, whereas the α-glycosidically linked monosaccharide units appear to resist the oxidation.

MATERIALS AND METHODS

The pure glycolipids studied are listed in **Table 1.** CrO_3 oxidation of acetylated glyceroglycolipids was carried out essentially as described by Hoffman et al. (2), but the sample sizes were scaled down about 10 times. The glycerolipids (100–300 μg), together with myoinositol as an internal standard, were acetylated with 0.1 ml of pyridine–acetic anhydride 1:1 (v/v) at 100°C for 15 min. An aliquot (50%) of the acetylated samples was used for the oxidation as follows. The samples were evaporated to dryness, 0.50 ml of acetic acid and 50 mg of CrO_3 were added, and the mixture was treated for 15 min at 40°C in an ultrasonic cleaning bath. The reaction mixture was diluted with 3 ml of water and extracted three times with 3 ml of chloroform. The monosaccharides surviving the oxidation were analyzed from the combined chloroform extracts, which contained 95% of the internal standard added and which appeared to contain all glyceroglycolipids present. The monosaccharide composition of the original acetylation mixture was analyzed using another aliquot.

The sphingolipids were acetylated and oxidized in the same way as the glycerolipids, but with the sphingolipids it was necessary to use chloroform–methanol for a quantitative extraction of the reaction mixtures. Therefore, the oxidation mixtures, diluted with 1 ml of water, were extracted once with 6 ml of chloroform–methanol 2:1 (v/v) and then twice with 2 ml of chloroform. This treatment extracted more than 94% of all sphingolipids from the acetic acid–water phase.

The compositions of neutral monosaccharides in the original and in the oxidized samples were analyzed by acetolysis followed by hydrolysis (8). The glycolipid was dissolved in 0.3 ml of 0.5 N H_2SO_4 in 90% acetic acid and heated in a Teflon-lined, screw-capped tube for 16 hr at 80°C. The mixture was heated for another 5 hr after the addition of 0.3 ml of water. The hydrolysate was filtered through 200 mg of dried Dowex 1-X8 (acetate form)

Abbreviations: Cer, ceramide; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose.

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in a small column and washed with 2 ml of methanol. The filtrate and the washings were combined and evaporated under nitrogen. The liberated monosaccharides were then reduced with $NaBH_4$, acetylated, and analyzed by gas–liquid chromatography (9). The yield of hexoses as measured by this technique was 98% for ceramide monohexoside of brain and 102% for the globoside of red blood cells. The assay of amino sugars of the original and the oxidized glycolipids was performed with the Elson-Morgan reaction (10); the hydrolysis was carried out with 0.25 ml of 2 N HCl at 100°C for 16 hr. The amino sugars of the original and the oxidized globoside (XVI, Table 1) were also analyzed by ion exchange chromatography (11). For this, the hydrolysate was evaporated to dryness, dissolved in 0.067 M sodium citrate buffer, pH 2.2, applied to a column (0.9 × 14 cm) of Beckman custom spherical resin type PA-35, and analyzed in a Beckman amino acid analyzer 120C using 0.117 M sodium citrate buffer, pH 5.25, for elution.

RESULTS

The recoveries of monosaccharides from the oxidized lipids, based on the amounts recovered from the glycolipids without prior oxidation, are shown in **Table 2.** In the case of the glycerolipids, which contained only one or two pyranosic monosaccharide units, the results were clear-cut.

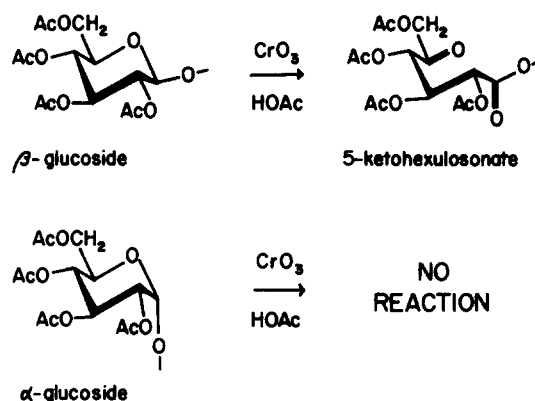

Fig. 1. Fate of acetylated glucosides under the conditions of CrO_3 oxidation.

Glucose and galactose units linked β-glycosidically were almost completely oxidized in all samples. The small fractions (5–8%) of unreactive glucose and galactose of these lipids probably resulted from a too short reaction time. In contrast to the β-glycosides, the α-glycosidically linked glucose, galactose, and mannose units of the glycerolipids resisted the oxidation almost completely.

The sphingolipids of short pyranosic carbohydrate chains also gave distinct results. β-Glycosidic glucose, galactose, and mannose and sulfated galactose units of ceramide monosaccharides and ceramide disaccharides were almost completely oxidized. Even in all of the more com-

TABLE 1. Structure and origin of pure glycolipids studied

	Structure	Origin	Reference
I	3-*O*-β-D-Glucopyranosyl-1,2-*sn*-diacylglycerol	*Pseudomonas rubescens*	13
II	3-*O*-β-D-Galactopyranosyl-1,2-*sn*-diacylglycerol	*Phaseolus multiflorus*	14
II	3-*O*-β-D-Galactopyranosyl-1,2-*sn*-diacylglycerol	*Arthrobacter*	12
III	3-*O*-[β-D-Glucopyranosyl-(1′ → 6′)-*O*-β-D-glucopyranosyl]-1,2-*sn*-diacylglycerol	*Staphylococcus*	12
IV	3-*O*-α-D-Glucopyranosyl-1,2-*sn*-diacylglycerol	*Mycoplasma laidlawii*	12
V	3-*O*-[α-D-Glucopyranosyl-(1′ → 2′)-*O*-α-D-glucopyranosyl]-1,2-*sn*-diacylglycerol	*Streptococcus faecalis*	12
VI	3-*O*-[α-D-Galactopyranosyl-(1′ → 2′)-*O*-α-D-glycopyranosyl]-1,2-*sn*-diacylglycerol	*Lactobacillus*	12
VII	3-*O*-[α-D-Mannopyranosyl-(1′ → 3′)-*O*-α-D-mannopyranosyl]-1,2-*sn*-diacylglycerol	*Micrococcus lysodeikticus*	12
VIII	3-*O*-[α-D-Galactopyranosyl-(1′ → 6′)-*O*-β-D-galactopyranosyl]-1,2-*sn*-diacylglycerol	*Phaseolus multiflorus*	14
IX	*O*-β-D-Glucopyranosyl-(1-1′)-ceramide	Wheat flour	3
X	*O*-β-D-Galactopyranosyl-(1-1′)-ceramide	Ox brain[a]	
XI	*O*-β-D-Galactopyranosyl-(3-sulfate)-(1-1′)-ceramide	Ox brain[a]	
XII	*O*-β-D-Mannopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1-1′)-ceramide	Wheat flour	3
XIII	*O*-β-D-Mannopyranosyl-(1 → 4)-*O*-β-D-mannopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1-1′)-ceramide	Wheat flour	3
XIV	*O*-β-D-Galactopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1-1′)-ceramide	Human RBC membranes[a]	
XV	*O*-α-D-Galactopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1-1′)-ceramide	Rabbit RBC membranes[a]	6
XVI	*O*-β-D-2-Acetamido-2-deoxygalactopyranosyl-(1 → 3)-*O*-α-D-galactopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1-1′)-ceramide (globoside)	Human RBC membranes[a]	5
XVII	*O*-α-D-2-Acetamido-2-deoxygalactopyranosyl-(1 → 3)-*O*-β-D-2-acetamido-2-deoxygalactopyranosyl-(1 → 3)-*O*-α-D-galactopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1-1′)-ceramide (Forssman hapten)	Goat RBC membranes	4
XVIII	*O*-α-D-Galactopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-2-acetamido-2-deoxyglucopyranosyl-(1 → 3)-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1-1′)-ceramide	Rabbit RBC membranes[a]	7

[a] Isolated in this laboratory.

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TABLE 2. CrO_3 oxidation of acetylated glycolipids[a]

	Lipids	Recoveries (%) of Monosaccharides after Oxidation			
		Glucose	Galactose	Mannose	N-Acetyl-hexosamine
Glycerolipids					
I	β-Glc-diglyceride	7.7 ± 0.3 (2)			
II	β-Gal-diglyceride		5.5 ± 1.2 (4)		
III	β-Glc-(1 → 6)-β-Glc-diglyceride	6.7 ± 0.8 (2)			
IV	α-Glc-diglyceride	105 ± 3.0 (2)			
V	α-Glc-(1 → 2)-α-Glc-diglyceride	100 ± 14 (2)			
VI	α-Gal-(1 → 2)-α-Glc-diglyceride	94 ± 9.0 (2)	100 ± 11 (2)		
VII	α-Man-(1 → 3)-α-Man-diglyceride			94 ± 2.0 (2)	
VIII	α-Gal-(1 → 6)-β-Gal-diglyceride		63 ± 14 (4)		
Sphingolipids					
IX	β-Glc-Cer	8.1 ± 6.2 (2)			
X	β-Gal-Cer		5.9 ± 1.5 (3)		
XI	3-OSO_3-β-Gal-Cer		3.1 ± 1.8 (2)		
XII	β-Man-(1 → 4)-β-Glc-Cer	7.5 (1)		3.0 (1)[b]	
XIII	β-Man-(1 → 4)-β-Man-(1 → 4)-β-Glc-Cer	10 ± 0.5 (2)		6.5 ± 3.5 (2)	
XIV	β-Gal-(1 → 4)-β-Glc-Cer	6.7 ± 2.1 (2)	20 ± 0.6 (2)		
XV	α-Gal-(1 → 4)-β-Gal-(1 → 4)-β-Glc-Cer	10 ± 6.3 (4)	60 ± 7.8 (4)		
XVI	β-GalNAc-(1 → 3)-α-Gal-(1 → 4)-β-Gal-(1 → 4)-β-Glc-Cer	8.9 ± 5.6 (5)	30 ± 2.9 (5)		0 (4)
XVII	α-GalNAc-(1 → 3)-β-GalNAc-(1 ↔ 3)-α-Gal-(1 → 4)-β-Gal-(1 → 4)-β-Glc-Cer				51 ± 20 (3)
XVIII	α-Gal-(1 → 3)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 3)-β-Gal-(1 → 4)-β-Glc-Cer	11 ± 2.5 (2)	40 ± 3.0 (2)		0 (2)

[a] Mean ± SD if more than three analyses were made, and mean ± half of the range when fewer analyses were performed; the number in parentheses is the number of experiments.
[b] Oxidation time, 60 min.

plex sphingolipids, the oxidation of β-linked mannosyl and glucosyl residues proceeded to near completion.

The galactose units were bound through both β-glycosidic and α-glycosidic linkages to some of the more complex glycolipids. In all these cases the pyranosic galactose units were oxidized only partially, suggesting that again only the β-glycosidically linked molecules were reactive. In the *O*-α-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1-1′)-ceramide (XV), 60% of the galactose resisted the oxidation, and the corresponding amount for *O*-α-D-galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-2-acetamido-2-deoxyglucopyranosyl-(1→3)-*O*-β-D-galactopyranosyl - (1→4)-*O*-β-D-glucopyranosyl-(1-1′)-ceramide (XVIII) was 40%. Both values were a little higher than expected for an ideally selective oxidation, which should have given 50% and 33%, respectively. This suggests that some of the β-linked galactose units had resisted the reaction. Analogous results were obtained also with the 3-*O*-[α-D-galactopyranosyl-(1′→6′)-*O*-β-D-galactopyranosyl]-1,2-*sn*-diacylglycerol (VIII) of plants. Also globoside (XVI), which contains one α-galactose and one β-galactose unit, lost its galactose units only partially in the oxidation, but the amount of the stable galactose was in this case clearly lower than expected, 30% instead of 50–60%. We do not have a good explanation for the different behavior of XV and XVI. However, the possibility remains that our globoside was not a pure sample of the structure XVI; e.g., it may have contained molecules with a β-linked pyranosic galactose, or with a reactive galactofuranose unit (1), instead of the unreactive α-linked galactopyranose.

The pyranosic *N*-acetylhexosamine units of the sphingolipids appeared to behave like the neutral monosaccharides in the oxidation. The globoside (XVI), which contains a β-linked *N*-acetylgalactosamine unit, lost this residue completely in the oxidation. Galactosamine was present in the chromatogram after hydrolysis of the original sample, but it could not be detected from the oxidized sample; furthermore, the original sample gave a positive Elson-Morgan reaction, but the oxidized sample gave a negative reaction (Table 2). The Forssman hapten (XVII) of goat, which contains one α- and one β-linked *N*-acetylgalactosamine unit (4), lost 49% of its Elson-Morgan reactivity in the oxidation. This finding is compatible with an α-linked *N*-acetylhexosamine unit stable towards the oxidation. The ceramide pentasaccharide (XVIII) of rabbit erythrocytes, which contains a β-linked *N*-acetylglucosamine (7), completely lost its Elson-Morgan reactivity in the oxidation. It appears, therefore, that the β-linked *N*-acetylglucosamine was also oxidized quantitatively. The

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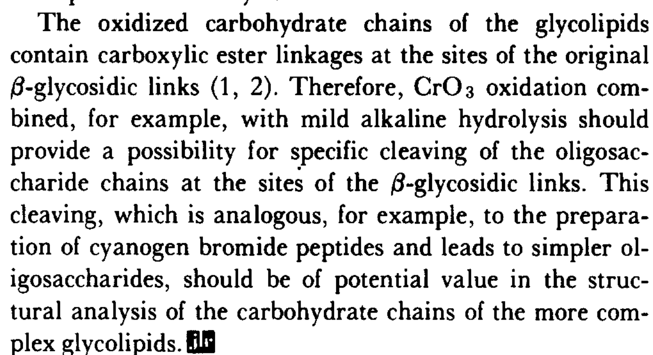
data of Hoffman et al. (2) confirm our findings. These authors studied the methyl glycosides of *N*-acetylglucosamine and found that the α-glycoside was stable towards the oxidation whereas the β-glycoside reacted rapidly.

DISCUSSION

The present report shows that CrO_3 oxidation in acetic acid attacks β-glycosidically bound pyranosic glucose, galactose, mannose, *N*-acetylgalactosamine, and *N*-acetylglucosamine units in acetylated glyceroglycolipids and glycosphingolipids. On the other hand, the same monosaccharides react much more slowly when linked α-glycosidically. Therefore, CrO_3 oxidation can be used for the determination of anomeric structures of these lipids. The oxidation allows distinct separation of an α-glycoside from the corresponding β-glycoside. However, when the sample contains a given monosaccharide bound both in α- and in β-glycosidic linkages, the relative numbers of these units cannot yet be adequately obtained.

Other methods available for anomeric analysis include measurement of optical rotation, IR spectroscopy, NMR spectroscopy, enzymic hydrolysis, and, in special cases, the use of antibodies and lectins. CrO_3 oxidation has some advantages over these methods. The sample size needed is smaller than that normally required in the physical methods, and pure reference substances are not necessary for comparison. In contrast to the enzymic method, only one reagent is required in the chemical procedure; furthermore, the reagent is easy to obtain, it is stable, and it does not require the use of detergents to disperse the substrates. Sometimes the enzymes appear to be specific for other structural features of the substrate in addition to the identity of the distal monosaccharide and its anomeric configuration. For instance, the mixture of IX, XII, and XIII does not react in the presence of purified β-mannosidase or α-mannosidase.[1] Another example is the nonreactivity of XV in the presence of α-galactosidase of *Mortierella vinacea* (5). Such limitations are so far not apparent for the CrO_3 method. For example, our samples contained α-galactose units linked to positions 2, 3, 4, and 6 of pyranosic galactose or glucose, and all these units resisted the oxidation.

However, in its present form, even the CrO_3 oxidation method has some limitations. For instance, complete acetylation of the sample is necessary because any unprotected hydroxyl groups will become oxidized and the keto groups formed may render the monosaccharide unstable during the subsequent acid hydrolysis. The same complication may result also if sialic acid or other acid-labile groups become detached from the carbohydrate chain during the oxidation, which is carried out in acetic acid solution. To prevent this, the gangliosides should probably be desialylated prior to the analysis.



The oxidized carbohydrate chains of the glycolipids contain carboxylic ester linkages at the sites of the original β-glycosidic links (1, 2). Therefore, CrO_3 oxidation combined, for example, with mild alkaline hydrolysis should provide a possibility for specific cleaving of the oligosaccharide chains at the sites of the β-glycosidic links. This cleaving, which is analogous, for example, to the preparation of cyanogen bromide peptides and leads to simpler oligosaccharides, should be of potential value in the structural analysis of the carbohydrate chains of the more complex glycolipids.

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[1] Li, Y-T. Personal communication.

REFERENCES

1. Angyal, S. J., and K. James. 1970. Oxidation of carbohydrates with chromium trioxide in acetic acid. *Aust. J. Chem.* **23:** 1209–1221.
2. Hoffman, J., B. Lindberg, and S. Svensson. 1972. Determination of the anomeric configuration of sugar residues in acetylated oligo- and polysaccharides by oxidation with chromium trioxide in acetic acid. *Acta Chem. Scand.* **26:** 661–666.
3. Laine, R. A., and O. Renkonen. Ceramide di- and trihexosides of wheat flour. *Biochemistry.* **13:** 2837–2843.
4. Taketomi, T., A. Hara, N. Kawamura, and M. Hayashi. 1974. Further investigations on the chemical structure of Forssman globoside obtained from caprine erythrocyte stroma. *J. Biochem.* (Tokyo). **75:** 197–199.
5. Hakomori, S-I., B. Siddiqui, Y-T. Li, S-C. Li, and C-G. Hellerqvist. 1971. Anomeric structures of globoside and ceramide trihexoside of human erythrocytes and hamster fibroblasts. *J. Biol. Chem.* **246:** 2271–2277.
6. Eto, T., Y. Ichikawa, K. Nishimura, S. Ando, and T. Yamakawa. 1968. Chemistry of lipid of the posthemolytic residue or stroma of erythrocytes. XVI. Occurrence of ceramide pentasaccharide in the membrane of erythrocytes and reticulocytes of rabbit. *J. Biochem.* (Tokyo). **64:** 205–213.
7. Stellner, K., H. Saito, and S-I. Hakomori. 1973. Determination of aminosugar linkages in glycolipids by methylation. Aminosugar linkages of ceramide pentasaccharides of rabbit erythrocytes and of Forssman antigen. *Arch. Biochem. Biophys.* **155:** 464–472.

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8. Yang, H-J., and S-I. Hakomori. 1971. A sphingolipid having a novel type of ceramide and lacto-N-fucopentaose III. *J. Biol. Chem.* **246:** 1192–1200.
9. Sawardeker, J. S., J. H. Sloneker, and A. R. Jeanes. 1965. Quantitative determination of monosaccharides as their alditol acetates by gas–liquid chromatography. *Anal. Chem.* **37:** 1602–1604.
10. Gatt, R., and E. R. Berman. 1966. A rapid procedure for the estimation of amino sugars on a micro scale. *Anal. Biochem.* **15:** 167–171.
11. Laine, R. A., H. Söderlund, and O. Renkonen. 1973. Chemical composition of Semliki Forest virus. *Intervirology.* **1:** 110–118.
12. Shaw, N. 1970. Bacterial glycolipids. *Bacteriol. Rev.* **34:** 365–377.
13. Wilkinson, S. G. 1968. Glycosyl diglycerides from *Pseudomonas rubescens. Biochim. Biophys. Acta.* **164:** 148–156.
14. Sastry, P. S., and M. Kates. 1964. Lipid components of leaves. V. Galactolipids, cerebrosides and lecithin of runner-bean leaves. *Biochemistry.* **3:** 1271–1279.

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