Chemical studies on dissolved carbohydrates in the water samples collected from the North Pacific and Bering Sea

Dissolved carbohydrate
Chemical study
GC-MS
North Pacific
Bering Sea
Glucides dissous
Études chimiques
GC-MS
Pacifique Nord
Mer de Béring

Hiroshi Sakugawa, Nobuhiko Handa
Water Research Institute, Nagoya University, Chikusa-ku, Nagoya 464, Japan.

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ABSTRACT

Structural characterization of dissolved carbohydrates in the North Pacific and Bering Sea was conducted. Dissolved carbohydrate was concentrated from seawater samples collected from 10 and 2,500 m, and 1 and 2,000 m depths of the North Pacific and Bering Sea respectively, and separated into mono-, oligo- and polysaccharide fractions by a combined charcoal column chromatography and dialysis system. Mono-, oligo- and polysaccharides in these water samples were measured with the ranges of 4.2-100, 6.5-11 and 11-33 $\mu g\ l^{-1}$ respectively. The following results were obtained by chemical studies of these sugar fractions.

1) The monosaccharide fraction consisted of glucose with small amounts of galactose, mannose, xylose, arabinose, ribose and rhamnose. In these water samples, however, considerable regional variabilities of monosaccharide composition were observed.

2) Oligosaccharides were analyzed by gas chromatography and combined gas chromatography and mass spectrometry after conversion to permethylated derivatives. 1-0-β-D-galactosylglycerol, sucrose, trehalose and melibiose were identified in these water samples, while a glycosylglycerol and four disaccharides consisting of hexose were tentatively characterized.

3) The polysaccharide fraction was treated by Sephadex G-25 column chromatography to differentiate polysaccharides with molecular weight less than 4,000 ($MW < 4{,}000$) and greater than 4,000 ($MW > 4{,}000$). Further chemical characterization was conducted for the polysaccharide with $MW > 4{,}000$ which accounted for 58-70% of total polysaccharide dissolved in seawater.

4) Uronic acid content of the polysaccharide with $MW > 4{,}000$ was 11-30%. Thus, the polysaccharide was treated with Cetavlon to isolate acidic polysaccharide which accounted for 11-43% of the polysaccharide. The acidic polysaccharide was abundant in the surface water of the Bering Sea, while lower values were obtained in the deep water of this oceanic area. However, no vertical change in the percentage of the acidic polysaccharide was observed in the North Pacific.

5) The polysaccharide with $MW > 4{,}000$ was treated with Sephadex G-150 column chromatography to separate high molecular weight polysaccharide ($MW > 1 \times 10^5$) consisting mainly of glucose with some monosaccharides in the surface water of the North Pacific; such predominance of glucose was not found in the polysaccharide of the surface water sample from the Bering Sea. Methylation study indicated that a glucan with 1.4 and 1.6-linkages occurred in the high molecular weight polysaccharide from the North Pacific, while monosaccharide analysis suggested that the high molecular weight polysaccharide from the Bering Sea was a heteropolysaccharide which was most likely derived from diatoms growing in the surface water.

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RÉSUMÉ

Études chimiques des glucides dissous dans des échantillons d'eau de mer prélevés dans l'Océan Pacifique Nord et la Mer de Béring

On a caractérisé la structure des glucides dissous provenant de l'Océan Pacifique Nord et de la Mer de Béring. Les glucides dissous ont été concentrés à partir

d'échantillons d'eau de mer prélevés respectivement à 10 et 2 500 m et à 1 et 2 000 m dans l'Océan Pacifique Nord et dans la Mer de Béring. Ces glucides ont été séparés en trois fractions : mono-, oligo- et polysaccharides, en combinant chromatographie sur colonne de carbone et dialyse. Dans ces eaux, ces fractions représentent respectivement de 4,2 à 100, de 6,5 à 11 et de 11 à 33 µg de glucides par litre. Les résultats suivants ont été obtenus par l'étude chimique de ces fractions glucidiques.

1) Dans ces échantillons, bien que les monomères soient essentiellement représentés par le glucose, d'autres substances telles que le galactose, le mannose, le xylose, l'arabinose, le ribose et le rhamnose, sont aussi présentes. Toutefois, des variations régionales importantes de la composition des monosaccharides ont été observées.

2) Les oligosaccharides ont été analysés en chromatographie gazeuse et en chromatographie gazeuse couplée à la spectrométrie de masse après conversion en dérivés perméthylés. On a identifié dans ces eaux du 1-O-β-D-galactosylglycérol, du sucre, du tréhalose et du mélobiose. La caractérisation du glycosylglycérol et de quatre autres disaccharides, constitués d'hexose, n'a toutefois pas pu aboutir.

3) Par passage sur colonne de Séphadex G-25, les polymères ont été séparés en polysaccharides de poids moléculaire inférieur à 4 000 (MW < 4 000) et supérieur à 4 000 (MW > 4 000). L'étude des caractérisations chimiques a permis de montrer que les polysaccharides de poids moléculaire supérieur à 4 000 représentent de 58 à 70% de la totalité des polysaccharides dissous dans l'eau de mer.

4) Les polysaccharides de poids moléculaire supérieur à 4 000 contiennent de 11 à 30% d'acides uroniques. De plus, les polysaccharides acides isolés par le Cetavlon représentent de 11 à 43% des polysaccharides. Les polysaccharides acides sont plus abondants dans les eaux de surface de la Mer de Béring, et les valeurs plus basses sont observées dans les eaux profondes de cette même aire océanique. Cependant, le taux de polysaccharides acides ne change pas avec la profondeur dans les eaux de l'Océan Pacifique Nord.

5) Les polysaccharides de poids moléculaire élevé ($MW > 10^5$) ont été isolés à partir de la fraction de poids moléculaire supérieur à 4 000 par passage sur colonne de Séphadex G-150. Ces polysaccharides sont essentiellement constitués de glucose auquel sont associés quelques autres monomères dans les eaux de surface de l'Océan Pacifique Nord. Toutefois, cette prédominance du glucose n'a pas été observée dans les polysaccharides isolés des eaux de surface de la Mer de Béring. Les études par méthylation montrent qu'un glucan présentant des liaisons 1,4- et 1,6- est présent dans la fraction de poids moléculaire élevé de l'Océan Pacifique Nord; toutefois, l'analyse des monosaccharides suggère que les polysaccharides de poids moléculaire élevé de la Mer de Béring sont des hétéropolysaccharides, qui ont très probablement pour origine les diatomées des eaux de surface.

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INTRODUCTION

Little information is currently available on the chemical composition of dissolved carbohydrate in seawater. Only free monosaccharide composition has been determined by high performance liquid chromatography after desalting of dissolved carbohydrate by electrodialysis (Josefsson, 1970; Mopper, 1977; Mopper *et al.*, 1980; Liebezeit *et al.*, 1980) or by gas chromatography (GC) (Eklund *et al.*, 1977) and gas chromatography—mass spectrometry (GC-MS) after separation of carbohydrate dissolved in seawater by charcoal column chromatography (Sakugawa, Handa, 1983). It is of importance to determine the chemical nature of carbohydrate dissolved in seawater in order to clarify the mechanism of its production and decomposition in marine environments, to elucidate its significance in the formation of marine humic substance, and to calculate its trace metal complexing capability.

Our studies in Mikawa Bay (Japan) indicated that dissolved carbohydrate consisted of glucose, laminaribiose, laminaritriose, sucrose, raffinose, 2-O-α-D-glucopyranosylglycerol, 1-O-β-D-galactopyranosylglycerol, 6 - O - α - D - galactopyranosyl - 1 - O - β - D - galactopyranosylglycerol, β-1,3 glucan and heteropolysaccharide with the range of 0.5-70 µg l^{-1} (Sakugawa, Handa, 1983; Sakugawa *et al.*, in prep.; Sakugawa, Handa, in prep.). GC, GC-MS, proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), infrared spectroscopy (IR) and methylation analysis were applied to determine their chemical nature after concentration and separation by a combined charcoal column chromatography and dialysis system. Comparative results on the chemical characteristics of carbohydrates from particulate matter and seawater filtered through glass fiber filters (Whatman GF/C) strongly suggested that the sugars dissolved in seawater might be derived from the phytoplankton population of *Prorocentrum minimum* with some diatoms occurring in the surface

water of Mikawa Bay as extracellular products. Thus, it is of great interest to determine the abundance and chemical nature of dissolved carbohydrates not only in coastal but also open oceanic waters because of the considerable significance of carbohydrates dissolved in seawater in the dynamics of organic matter cycling in the marine environments.

In this study the aim was to clarify the chemical nature of mono-, oligo- and polysaccharides of dissolved carbohydrate in the North Pacific and Bering Sea by several analytical methods.

MATERIALS AND METHODS

Water samples

Water samples were collected from the depths of 10 and 2,500 m at station 3 in the northern North Pacific (47°51′N, 176°21′E) and from the depths of 1 and 2,000 m at station 4 in the Bering Sea (53°30′N, 177°15′E) during the Hakuho-Maru cruise (KH78-3) of the Ocean Research Institute, University of Tokyo, from June 5 to Aug. 22, 1978 (Fig. 1). Immediately after collection by Niskin sampler (23 l size), water samples were gently filtered through a glass fiber filter (Whatman GF/C) by gravity using a specifically designed filtration device (filter area, 285 × 420 mm). The filtrates were kept frozen at −20°C until analysis. Phytoplankton mainly consisted of dinoflagellates in the surface water at Stn. 3 in the North Pacific, but diatoms at Stn. 4 in the Bering Sea when the water samples were collected.

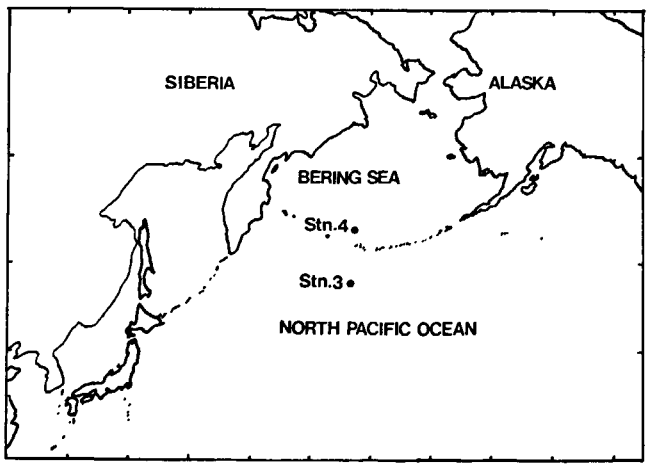


Figure 1
Stations for the collection of the water samples during the cruise of Hakuho-Maru (KH78-3) from June to August, 1978.

Chemicals

Charcoal granules (80-100 mesh, Nishio Industries) were pretreated by baking at 350°C for 4 hrs and then washed with 100 and 50% ethanol, and distilled water successively to remove impurities. The purified charcoal preparation of 4.5 and 45 g suspended in water was packed into glass columns I (column I, 10 mm in diameter × 30 cm in height) and II (column II, 25 mm in diameter × 30 cm in height). Sephadex G-25 and G-150 (particle size 20-80 μm, Pharmacia Fine Chemicals) were packed into glass columns (16 mm I.D. × 100 cm height). Calibration of the molecular weight distribution of these columns was conducted using glucose (MW: 180), raffinose (MW: 504), laminaran (MW: 3,200) and standard dextrans (T-70, MW: 70,000) as standard sugars. A Visking tube for dialysis of seawater samples to collect highly polymerized organic materials was boiled three times with distilled water to remove impurities.

1-O-β-D-galactopyranosylglycerol was prepared from monogalactosyldiglyceride (PL Co.) by deacylation with CH_3ONa. Authentic samples of sucrose, maltose, cellobiose, trehalose, melibiose and gentiobiose were purchased from Waco Pure Chemical. Laminaribiose was prepared by partial acid hydrolysis of pachyman from *Poria cocos*.

Mono-, di-, tri- and tetra-O-methyl sugars used for methylation study of polysaccharide were prepared from α-methyl-monosaccharides by the method of Handa and Montgomery (1969).

Concentration and separation of dissolved carbohydrates

Concentration and separation of dissolved carbohydrates in seawater samples was conducted by the combined charcoal column chromatography and dialysis system described previously (Sakugawa, Handa, 1983). Briefly, 2 l of the water sample were evaporated to a small volume (200 ml) by a rotary evaporator followed by dialysis against distilled water for 20 hrs. After dialysis, polysaccharide remaining in the dialyzed solution was further fractionated into polysaccharides with MW $<4{,}000$ and $>4{,}000$ by Sephadex G-25 column chromatography. The dialysate containing mono- and oligosaccharides was passed through charcoal column system I and II which were connected directly. Oligosaccharide was easily retained in column I, while monosaccharide was trapped in column II without adsorption onto column I. After desalting with 1 l of distilled water, the two columns were disconnected. Oligo- and monosaccharides adsorbed were eluted with 50 and 10% ethanol from columns I and II, respectively. The recoveries of mono- and oligosaccharides from the charcoal columns, and of polysaccharides from dialysis procedures were 46-74, 61-89 and 70%, respectively (Sakugawa, Handa, 1983).

Determination of monosaccharide composition

Sugars of the monosaccharide fraction were converted into corresponding alditol acetates through reduction with $NaBH_4$ followed by acetylation with acetic anhydride in the presence of sodium acetate and then analyzed by GC and GC-MS. Inositol was used as internal standard. Fructose, if present, however, can only be determined as glucitol and mannitol by this method because of its reduction with $NaBH_4$.

Oligosaccharides and polysaccharides with MW <4000 and $>4{,}000$ were hydrolyzed to monomeric constituents with 1 N H_2SO_4 at 100°C for 10 hrs. After neutralization with $Ba(OH)_2$ and removal of insoluble material by filtration through glass fiber filter (Whatman GF/C), the filtrate was evaporated to dry-

ness. Monosaccharides from these procedures were converted into alditol acetates through reduction and acetylation reactions. The alditol acetates were analyzed by GC and GC-MS.

For quantitative determination, these mono-, oligo- and polysaccharides were corrected for recoveries of the sugars through the charcoal columns and from the dialysis procedure as previously reported (Sakugawa, Handa, 1983).

Separation and identification of oligosaccharides

10 l of the water sample were passed through column I at a flow rate of 4 ml min^{-1} by a peristaltic pump. After the column was washed with 100 ml of distilled water to remove inorganic salts, oligosaccharides were eluted with 750 ml of 50% ethanol. The eluate was evaporated to a small volume (5 ml) by a rotary evaporator at 40°C. After reduction of the oligosaccharides with $NaBH_4$, reduced sugars were methylated as follows:

To the dried sample was added 2 ml of dimethylsulfoxide under N_2. The reduced oligosaccharides were allowed to react with methylsulfinyl carbanion to produce sugar alkoxides, which were methylated with CH_3I to convert to permethylated derivatives of the oligosaccharide-alditols.

For clean-up of the reaction product, methylated sample was passed through a silica gel column to remove dimethylsulfoxide. The reaction product was dissolved into 2 ml of chloroform, which was transferred to the top of the column. Permethylated oligosaccharide was eluted with 20 ml of each of the following organic solvent mixtures; *n*-hexane:ethyl ether (1:1), ethyl ether: ethyl acetate (1:1), ethyl acetate: methanol (1:1) and then methanol. The permethylated sugars were eluted in the ethyl acetate: methanol fraction. The fraction was collected and evaporated to dryness to commence GC and GC-MS analysis.

Determination of acidic polysaccharide and uronic acid

Acidic polysaccharide was separated from neutral polysaccharide by the method of Teller *et al.* (1962). The polysaccharide with MW > 4,000 (*ca.* 50 µg) was dissolved into 1.2 ml of distilled water. 60 µl of 5% Cetavlon was added to the solution. After cooling at 2°C for 24 hrs., the precipitate was separated from the supernatant by centrifugation at 3,000 rpm for 15 min. The supernatant was decanted and the precipitate was then washed three times with 1.5 ml of 95% ethanol saturated with NaCl. The material insoluble in ethanol was washed successively with acetone, ethyl acetate and ethyl ether and then dried in a desiccator over P_2O_5. Acidic polysaccharide was determined by the phenol sulfuric acid method (Handa, 1966*b*).

Uronic acid content of the polysaccharide with MW > 4,000 was determined by the method of Blumenkrantz and Asboe-Hansen (1973), which was standardized with glucuronic acid.

Sephadex G-150 column chromatography

The polysaccharide with MW > 4,000 separated from 5 l of seawater as mentioned above was further tested by Sephadex G-150 column chromatography to determine molecular weight distribution. The polysaccharide with MW > 4,000 was dissolved in 2 ml of distilled water. The solution was transferred to the top of the gel and then eluted with 0.05 M NaCl + 0.05 M Na_2HPO_4 at a flow rate of 25 ml hr^{-1}. Each of the 5 ml eluates was collected in test tubes with the aid of a fraction collector. The carbohydrate content of each of the fractions was determined by the phenol sulfuric acid method.

Isolation of high molecular weight polysaccharide

Eluate of the elution volume of 50-80 ml in Sephadex G-150 column chromatography was collected and evaporated to a small volume (5 ml) by a rotary evaporator. The concentrate was transferred into a Visking tube and then dialyzed against distilled water for 24 hrs. to remove inorganic salts. After dialysis, one volume of ethanol was added to the dialyzed solution to precipitate polysaccharide. After cooling at 2°C for 24 hrs., the precipitate was separated by centrifugation. The resulting precipitate was washed successively with ethanol, ethyl acetate and ethyl ether. The material washed was dried in a desiccator over P_2O_5. 50 µg of the polysaccharides was obtained. This procedure was repeated several times to obtain sufficient sample of the high molecular weight polysaccharide.

Monosaccharide composition and methylation study of high molecular weight polysaccharide

The high molecular weight polysaccharide (50 µg) was hydrolyzed with 1 N H_2So_4 at 100°C for 10 hrs. Monosaccharide composition of the hydrolysate was determined by GC after conversion of the monomeric constituents to the corresponding alditol acetates.

The polysaccharide (50 µg) was reduced with $NaBH_4$ and then methylated three times with methylsulfinyl carbanion. Permethylated polysaccharide was hydrolyzed with 1 N H_2SO_4 at 100°C for 10 hrs. Partially methylated monomeric sugars of the hydrolysates were converted into the corresponding alditol acetates through reduction with $NaBH_4$ followed by acetylation. Partially methylated alditol acetates were analyzed by GC and GC-MS.

GC and GC-MS analysis of alditol acetates, partially methylated alditol acetates and permethylated oligosaccharides

Alditol acetates and partially methylated alditol acetates were separated by a glass column (2 m × 2.5 mm I.D.) packed with 3% ECNSS-M on Gas Chrom Q (80-100 mesh) in a chromatograph (Yanaco G-180) equipped with a flame ionization detector. Analytical conditions for these derivatives were the same as those described elsewhere (Sakugawa, Handa, 1983).

Permethylated oligosaccharides were separated by glass column (2 m × 2.5 mm I.D.) packed with 3% OV-17 on Chromosorb W (80-100 mesh) and by fused silica wall coated open tubular (FS-WCOT) columns (25 m × 0.35 mm I.D.) coated with OV-1701 and SE-52 in a GC-9A chromatograph (Shimadzu) equipped with a flame ionization detector. The following analytical conditions were applied in GC analysis of permethylated samples; flow rates of nitrogen as a carrier gas 30, 0.66 and 0.55 ml min^{-1}, over temperature programmes 120-280°C at 5°C min^{-1}, 140-280°C at 2°C min^{-1} and 140-275°C at 2°C min^{-1} for the column of OV-17, OV-1701 and SE-52, respectively. The injection port and detector were heated to 250°C and 300°C, respectively. Quantification and identification of these permethylated oligosaccharides were conducted by Chromatopac C-R1A (Shimadzu). Solvent cut injection system (Shimadzu) in the injection port was used for the analysis of microgram order of magnitude of sugar derivatives.

GC-MS analysis was performed with a JMS-D300 (JEOL) gas chromatograph-mass spectrometer with an integrated JMA-2000 data analysis system. Gas chromatographic conditions in GC-MS analysis were the same as those of GC, except that He served as carrier gas. Mass spectra of alditol acetates, partially methylated alditol acetates and permethylated oligosaccharides were obtained by both chemical ionization (CI) and electron impact (EI) modes of mass spectrometry. Ammonia was used as a reagent gas in CI mode. Analytical conditions in GC-MS were the same as those described elsewhere (Sakugawa, Handa, 1983).

RESULTS

Monosaccharide

Glucose, galactose, mannose, xylose, arabinose, ribose and rhamnose were identified in the water samples from stations 3 and 4. These monosaccharides exhibited a wide range of concentrations (Tab. 1). Total concentration of the monosaccharides was 4.2 to 100 μg l^{-1} in the water samples. Glucose was predominant in all of the water samples and accounted for 69.0-78.3% of total monosaccharides.

Table 1
Compositions of the free monosaccharide fractions separated from v water samples of northern North Pacific (station 3) and Bering Sea (station 4).

Site	Station 3		Station 4	
Depth (m)	10	2,500	1	2,000
	μg l^{-1}			
Compound				
Glucose	69.3	2.9	5.2	14.4
Galactose	Tr.	Tr.	0.7	1.0
Mannose	25.9	1.3	0.9	2.4
Xylose	4.2	Tr.	Tr.	0.6
Arabinose	0.7	ND	Tr.	Tr.
Ribose	ND	ND	ND	Tr.
Fucose	ND	ND	ND	ND
Rhamnose	ND	ND	Tr.	ND
TOTAL	100	4.2	6.8	18.4

Tr.: trace, ND: not detected.

Table 2
Monosaccharide compositions of the oligosaccharide fractions separated from water samples of northern North Pacific (station 3) and Bering Sea (station 4).

Site	Station 3		Station 4	
Depth (m)	10	2,500	1	2,000
	μg l^{-1}			
Compound				
Glucose	4.4	5.8	3.5	4.7
Galactose	1.9	0.8	1.3	Tr.
Mannose	1.4	1.0	1.6	1.8
Xylose	0.8	0.8	0.9	Tr.
Arabinose	0.3	0.4	1.1	Tr.
Ribose	Tr.	ND	0.8	Tr.
Fucose	0.4	ND	0.9	ND
Rhamnose	0.5	0.5	0.9	ND
TOTAL	9.7	9.3	11.0	6.5

Tr.: trace, ND: not detected.

Oligosaccharide

Eight monosaccharides were identified as monomeric constituents of the oligosaccharides (Tab. 2). Total concentration of the oligosaccharides was 6.5-11.0 μg l^{-1} in the water samples from the surface and deep water layers of stations 3 and 4. Glucose was a dominant monomeric constituent of the oligosaccharide in all of the water samples, indicating abundant occurrence of oligosaccharides consisting of glucose. Further attempts were thus made to characterize the oligosaccharides.

A mass chromatogram of the permethylated oligosaccharide-alditols from the depth of 2,000 m at station 4 is shown in Figure 2. The ions were monitored at *m/z* 187, which is one of the major fragment ions of permethylated glycosides. The compounds 1-9 were detected as permethylated glycosides. These compounds were analyzed by CI and EI mass spectrometry. Permethylated oligosaccharide-alditols were identified

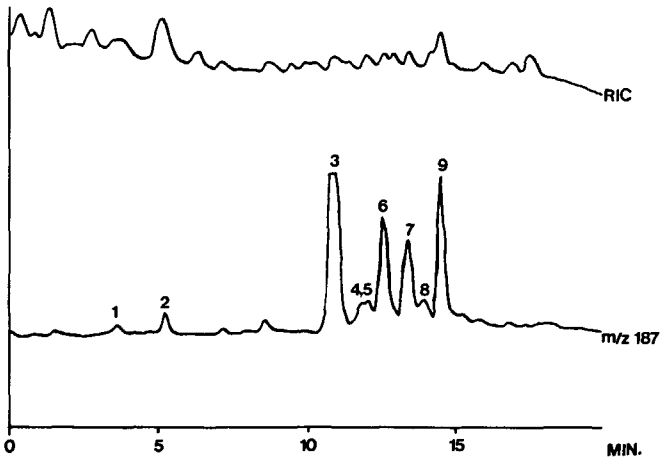


Figure 2
Mass chromatogram of permethylated oligosaccharide-alditols in the oligosaccharide fraction which was separated from the depth of 2,000 m at station 4 in the Bering Sea. The ion at m/z 187 is a characteristic fragment ion of permethylated derivatives of 1) glycosylglycerol, 2) 1-O-β-D-galactosylglycerol, 3) sucrose, 5) trehalose, 8) melibiose, and 4), 6), 7), and 9) hexosyl disaccharides, respectively. RIC is reconstructed ion chromatogram. Glass column packed with 3% OV-17 on Chromosorb W was used for the gas chromatographic separation of permethylated oligosaccharide-alditols.

Table 3
GC and GC-MS data of the compounds 1-9. These compounds are permethylated derivatives of dissolved low molecular weight carbohydrates separated from the water sample of the depth of 2000 at station 4 in the Bering Sea.

Compound No.	Structure assigned	Retention time[a] Column I[b]	Column II[b]	Column III[b]	Molecular ion, m/z
1	Glycosylglycerol A	0.43	—	—	338
2	1-O-β-D-galactosylglycerol	0.54	0.63	0.63	338
3	Sucrose	1.00	1.00	1.00	454
4	Disaccharide A	1.08	—	—	470
5	Trehalose	1.09	1.06	1.06	454
6	Disaccharide B	1.13	—	—	470
7	Disaccharide C	1.20	—	—	470
8	Melibiose	1.28	1.20	1.16	470
9	Disaccharide D	1.35	—	—	470

[a]:Retention times relative to permethylated sucrose (1.00).
[b]:3% OV-17, OV-1701 (FS-WCOT) and SE-52 (FS-WCOT) were used as liquid phases for column I, II and III, respectively.
[c]: Molecular ions of permethylated low molecular weight carbohydrates were determined by CI (NH_4) mass spectrometry.

by their retention times in GC using the co-injection technique with authentic standards. CI and EI modes of mass spectra greatly facilitated identification of these permethylated sugars.

Both of the compounds 1 and 2 gave molecular ion (M^+) of m/z 338 in CI mode of mass spectrometry (Tab. 3). Fragment ions of 103 and 163 characteristic to glycosylglycerols and of 45, 75, 88, 101, 187 and 219 characteristic to permethylated glycosides were found in EI mass spectrometry of these compounds (Sakugawa *et al.*, in prep.; Fig. 3*a*). Thus, the compounds 1 and 2 were assigned as permethylated derivatives of glycosylglycerols. In addition, the retention time of the compound 2 in GC corresponded well with the permethylated 1-O-β-D-galactopyranosylglycerol.

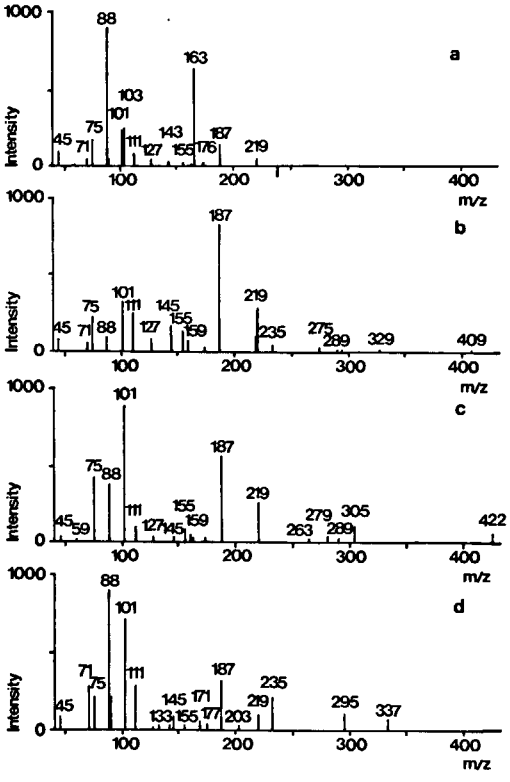
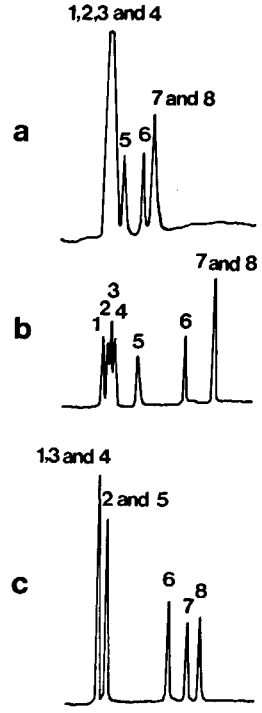


Figure 3
EI mass spectra of the compounds 2 (a), 3 (b), 5 (c) and 8 (d) (see Fig. 2).

Figure 4
Separation of permethylated disaccharide-alditols by gas chromatography using three different liquid phases such as OV-17 (3%, 2 m) (a), OV-1701 (FS-WCOT, 25 m) (b) and SE-52 (FS-WCOT, 25 m) (c). The compounds 1-8 were permethylated derivatives of 1) trehalose, 2) cellobitol, 3) laminaribitol, 4) maltitol, 5) lactitol, 6) isomaltitol, 7) melibitol and 8) gentiobitol. Analytical conditions of GC are described in the "Materials and Methods".



Separation of permethylated preparations of authentic oligosaccharides was undertaken by GC using three different columns. None of the columns served to separate the 8 disaccharides into its single components (Fig. 4). However, chromatograms *b* and *c* indicated that identification of each of the disaccharides could be achieved only by separate GC using capillary columns of OV-1701 and SE-52 as liquid phase. Thus, permethylated derivatives of oligosaccharide-alditols separated from the water samples of stations 3 and 4 were analyzed by these columns.

The compound 3 gave the same retention time and mass spectrum as permethylated sucrose in GC and GC-MS, respectively (Tab. 3 and Fig. 3*b*). Thus, this compound was assigned as the permethylated derivative of sucrose. The compound 5 gave a fragment ion of m/z 101 as base peak (Fig. 3*c*), which is an exclusive characteristic of permethylated trehalose (1.1-linked glucosyl disaccharide) in mass spectrometry apart from permethylated disaccharides consisting of only glucose

with 1.2-, 1.3-, 1.4-, 1.5- or 1.6-linkage giving an ion of m/z 88 as base peak (de Jong *et al.*, 1979). The retention time of the compound corresponded well to permethylated derivative of trehalose. Thus, this compound was assigned as the permethylated derivative of trehalose. The compound 8 gave the same retention time with permethylated reduced preparation of authentic melibiose. The fact that the compound 8 gave fragment ions, m/z 145, 177 and 337 (Fig. 3*d*) characteristic of permethylated reduced disaccharide of hexose with 1.6-linkage (Kärkkäinen, 1970) also supports the identification of the compound 8 to be melibiose.

The compounds 4, 6, 7 and 9 also gave m/z 470 of M^+ in Cl mass spectrometry indicating that these compounds are permethylated derivatives of reducing disaccharides consisting of hexose. However, the identifications of the compounds were incomplete because retention times of these compound in capillary GC were uncertain.

The concentration of the oligosaccharides found in the water samples was very low, less than several hundred nanograms per liter, so the compounds were tentatively determined semi-quantitatively from their peak height in GC (Tab. 4). Large amount of sucrose and trehalose were found in most all of the water samples.

Table 4
Dissolved glycosylglycerols and disaccharides in northern North Pacific (station 3) and Bering Sea (station 4).

Site	Station 3		Station 4	
Depth (m)	10	2,500	1	2,000
Compound				
Glycosylglycerol A	+	+	+	+
1-O-β-D-galactosyl, glycerol	++	+	+	+
Sucrose	++	+++	++	+++
Disaccharide A	+	—	+	+
Trehalose	+++	+++	++	+
Disaccharide B	—	++	++	++
Disaccharide C	—	++	++	++
Melibiose	—	+	—	+
Disaccharide D	++	++	+	++

Peak intensity, +:small, ++: medium, +++: large.

Polysaccharide with MW < 4,000

Eight monosaccharides were determined in the polysaccharide with MW < 4,000 from the water samples collected at stations 3 and 4 (Tab. 5). Glucose was a dominant constituent in all of the water samples, accounting for 72.4% of this polysaccharide in the water sample from the depth of 2,500 m at station 3. This finding suggests that glucose occurs as glucan in the deep waters, but further chemical studies of this polysaccharide remain to be made.

Table 5
Monosaccharide compositions of the polysaccharide with MW < 4,000 fractions separated from the water samples of northern North Pacific (station 3) and Bering Sea (station 4).

Site	Station 3		Station 4	
Depth (m)	10	2,500	1	2,000
	μg l^{-1}			
Compound				
Glucose	5.8	4.2	1.6	1.5
Galactose	1.0	0.5	1.0	0.4
Mannose	1.1	0.5	0.7	0.3
Xylose	1.1	Tr.	0.5	0.4
Arabinose	0.6	Tr.	0.4	0.3
Ribose	ND	ND	Tr.	0.3
Fucose	0.6	Tr.	0.4	0.4
Rhamnose	1.3	0.6	0.5	0.4
TOTAL	11.5	5.8	5.1	4.0

Tr.: trace, ND: not detected.

Polysaccharide with MW > 4,000

Table 6 shows the monosaccharide composition of the polysaccharide with MW > 4,000, which was determined with a range from 6.7 to 23.5 μg l^{-1} in the water samples from the surface and deep water layers at stations 3 and 4. Further detailed chemical studies of the polysaccharide fraction were conducted as mentioned below.

Uronic acid and acidic polysaccharide

Contents of uronic acid and acidic polysaccharide in the polysaccharide with MW > 4,000 were determined

Table 6
Monosaccharides compositions of the polysaccharide with MW > 4,000 fractions (total), and the polysaccharide with MW > 1 × 10⁵ which were separated from the water samples from northern North Pacific (station 3) and Bering Sea (station 4).

	Station 3			Station 4		
Site	10		2,500	1		2,000
Depth						
Polysaccharide	TOTAL	$>1\times10^5$	TOTAL	TOTAL	$>1\times10^5$	TOTAL
	μg l^{-1}					
Compound						
Glucose	8.4	5.1	4.0	4.0	0.6	1.9
Galactose	2.3	0.3	0.7	1.8	0.8	1.2
Mannose	1.9	0.1	0.8	1.6	0.3	0.9
Xylose	2.8	0.2	0.5	1.3	0.4	0.8
Arabinose	2.1	0.1	0.4	0.9	0.2	0.4
Ribose	0.7	ND	Tr.	0.4	ND	Tr.
Fucose	2.4	0.2	0.7	1.0	0.4	0.9
Rhamnose	2.9	0.3	0.8	0.8	0.4	0.6
TOTAL	23.5	6.3	7.9	11.8	3.1	6.7

Tr: trace, ND: not detected.

Table 7

Contents of uronic acid and acidic polysaccharide (5% Cetavlon precipitate) in the polysaccharide with MW>4,000 fraction (total) separated from the water samples of northern North Pacific (station 3) and Bering Sea (station 4).

Site	Station 3		Station 4	
Depth (m)	10	2,500	1	2,000
(1) Total ($\mu g\ l^{-1}$)[a]	67.5	18.0	57.9	19.1
(2) Uronic acid ($\mu g\ l^{-1}$)[b]	7.1	5.4	8.6	5.7
(3) Acidic polysaccharide ($\mu g\ l^{-1}$)[a]	14.2	3.4	24.2	2.2
(2) /(1) × 100 (%)	10.5	30.0	15.0	30.1
(3)/(1) × 100 (%)	21.0	19.1	42.5	11.4

[a]: Carbohydrate contents were determined by the phenol sulfuric acid method.
[b]: Uronic acid contents were determined by the method of Blumenkrantz and Asboe-Hansen (1973).

separately (Tab. 7). Uronic acid and acidic polysaccharide accounted for 10.5-30.1 and 11.4-42.5% of the polysaccharide with MW>4,000, respectively in the water samples.

Molecular weight distribution of polysaccharide with MW>4,000

The polysaccharide with MW>4,000 was fractionated by Sephadex G-150 column chromatography according to molecular size. The gel chromatograms of the polysaccharide with MW>4,000 separated from the water samples are shown in Figure 5. Two peaks were observed in the elution volumes ranging from 50 to 80 ml and from 140 to 180 ml in the surface waters of both sampling stations, while no peaks were detected in the polysaccharides from the deep waters. The fraction with low elution volumes ($MW>1\times10^5$) accounted for 24 and 33% of total polysaccharide with MW>4,000 in the surface waters of stations 3 and 4, respectively, while this fraction of the deep waters accounted for only 6.4 and 8.6%, respectively. This indicates that high molecular weight polysaccharide was abundant in the surface waters rather than in the deep waters.

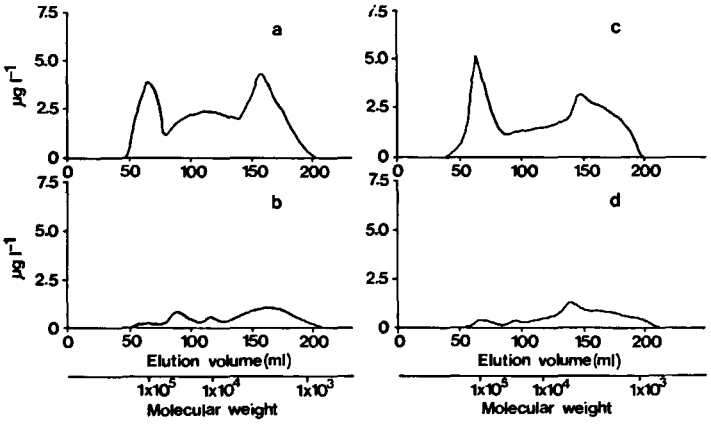


Figure 5

Sephadex G-150 column chromatograms of polysaccharides with MW>4,000 which were separated from the water samples. Carbohydrate content of the eluate was determined by the phenol sulfuric acid method. The chromatograms of polysaccharides a *and* b *are from the depths of* 10 *and* 2,500 *m at station* 3 *in the North Pacific, respectively, and* c *and* d *are from the depths of* 1 *and* 2,000 *m at station* 4 *in the Bering Sea, respectively.*

High molecular weight polysaccharide ($MW>1\times10^5$)

Monosaccharide composition of high molecular weight polysaccharide separated from the surface waters was determined (Tab. 6). Glucose accounted for 81% of total monomeric constituents of the polysaccharide isolated from the depth of 10 m at station 3, while large quantities of diverse monomeric constituents were found in the polysaccharide from the water sample of the depth of 1 m at station 4. These results strongly suggested that the high molecular weight polysaccharide found in the surface water of station 3 mainly consists of a glucan, whereas that of station 4 was a heteropolysaccharide.

Methylation study of the high molecular weight polysaccharide isolated from the depth of 10 m at station 3 was performed in order to determine the type of glycosidic linkages. The partially methylated monosaccharides derived from acid hydrolysis of permethylated polysaccharide were converted to corresponding alditol acetates, which were analyzed by GC (Tab. 8). 2,3,4,6-tetra-O-, 2,3,6-tri-O- and 2,3,4-tri-O-methylglucoses were identified as major compounds, while several tetra-, tri-, di- and mono-O-methyldeoxyhexoses, pentoses and galactose were found as minor compounds. These results indicated that the high molecular weight polysaccharide mainly consisted of glucan having 1.4- and 1.6-linkages, although a small amount of a highly branched heteropolysaccharide co-existed.

Table 8

Approximate percentages of methylated sugars from the methylated high molecular weight polysaccharide which was isolated from the depth of 10 *m at station* 3 *in the North Pacific.*

Methyl sugars	Retention time[a]	Percentage (%)
2,3,4,-tri-O-methylrhamnose	0.45	3.2
2,3,4-tri-O-methylfucose	0.63	2.1
2,3,4-tri-O-methylxylose	0.64	1.5
Di-O-methyldeoxyhexose	0.98	2.5
2,3,4,6-tetra-O-methylglucose	1.00	16.9
2,3,4,6-tetra-O-methylgalactose	1.26	2.1
Di-O-methylpentose	1.38	2.1
2,4,6-tri-O-methylglucose	2.06	Tr.
3-mono-O-methyldeoxyhexose	2.07	Tr.
2,3,6-tri-O-methylglucose	2.46	58.3
2,3,4-tri-O-methylglucose	2.56	10.3
Mono-O-methylpentose	2.89	Tr.
2,3-di-O-methylgalactose	3.64	Tr.

[a];Retention times relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol (1.00).
Tr.: trace.
The percentages of methylated sugars were calculated on the basis of the peak areas to total peak areas in GC.

DISCUSSION

Monosaccharide

Glucose accounted for 69.0-78.3% of total carbohydrate in the monosaccharide fraction separated from the stations 3 and 4 (Tab. 1). These values were slightly higher than the 59-69% of the water samples from Mikawa Bay (Sakugawa, Handa, 1983). It has been observed that glucose is the most abundant monosaccharide in the free sugar fraction dissolved in seawa-

ter (Josefsson, 1970; Mopper, 1977; Mopper *et al.*, 1980; Liebezeit *et al.*, 1980). Mopper *et al.* (1980) reported that the concentration of glucose was 5.7-121 $\mu g\ l^{-1}$ in water samples collected from various coastal and open ocean sites. This glucose accounted for 40-59% of total free monosaccharides. Phytoplankton is reportedly the most probable source of glucose which is one of the important metabolites in organisms (Craigie, 1974). It was also found that glucose was released from blue green lichen symbionts (Richardson *et al.*, 1968) and from zoochlorellae (Muscatine, 1965) as an extracellular product.

Fructose is another of the most abundant monosaccharides other than glucose, as reported by other workers (Mopper, 1977; Mopper *et al.*, 1980; Liebezeit *et al.*, 1980; Ittekkot *et al.*, 1981). Mopper *et al.* (1980) reported that fructose occurred at about the same concentration as glucose in the free monosaccharide fraction. Unfortunately, our analytical method of monosaccharides does not permit identification of this sugar because its reduction with $NaBH_4$ produces equimolar amounts of glucitol and mannitol. Thus, no information is obtained about the occurrence of fructose in seawater samples. However, higher concentration of mannitol (as mannose) together with glucitol (as glucose) found in all the water samples suggests that some portion of these sugars may be derived from fructose due to its reduction in the analytical process. Further derivatization of free monosaccharides is now under way in order to detect fructose.

Total monosaccharides were determined with a wide range of values (4.2-100 $\mu g\ l^{-1}$) in the water samples from stations 3 and 4 in the North Pacific and Bering Sea, whereas oligo- and polysaccharides did not evidence much change in their concentrations (Tab. 1, 2, 5, 6). Regional and vertical variabilities in the concentration of dissolved monosaccharides have been observed at various sites along coasts and in open oceans (Burney *et al.*, 1979; Mopper *et al.*, 1980; Liebezeit *et al.*, 1980; Sakugawa, Handa, 1983). This variability may be attributed to several biological processes occurring in seawater (*i.e.*, extracellular production of sugars by phytoplankton and decomposition by bacteria). The increase in dissolved carbohydrate concentration was frequently accompanied by phytoplankton bloom (Ittekkot, 1982), which indicates that algal extracellular production of sugars largely affects the concentration of dissolved carbohydrate. Inversely, monosaccharide seems more likely to be easily uptaken by bacteria. Turnover rate of ^{14}C-labelled glucose was estimated to be high (Joint, Morris, 1982). Thus, it is highly conceivable that the concentration of monosaccharides dissolved in seawater may greatly depend on *in situ* activities of phytoplankton and bacteria living in seawater.

Oligosaccharide

Two glycosylglycerols were found in the water samples from both stations 3 and 4 (Tab. 4). One of them is identified as 1-O-β-D-galactosylglycerol which was also found in the water samples from Mikawa Bay as well as 2-O-α-D-glucosylglycerol (Sakugawa *et al.*, in prep.). The authors concluded that these glycosylglycerols found in Mikawa Bay were derived from phytoplankton living in the surface water.

Monogalactosyldiglyceride is one of the major photosynthetic products of algae as well as terrestrial higher plants (Wood, 1974). Galactosyldiglycerides seem to be easily hydrolyzed by enzymes in plant tissues (Sastry, Kates, 1964), which results in the formation of the free form of galactosylglycerols. Diatoms and flagellates which were dominant phytoplankton species in the surface waters of stations 3 and 4 also have monogalactosyldiglyceride as a photosynthetic product (Harrington *et al.*, 1970; Holdsworth, Colbeck, 1976; Anderson *et al.*, 1978). Shaw (1975) reported the occurrence of monoglycosyldiglycerides in bacteria, but the anomeric configuration and monosaccharide species of their glycosyl moiety were different from 1-O-β-D-galactosylglycerol. Thus, it is most likely that 1-O-β-D-galactosylglycerol found in the water samples from stations 3 and 4 was derived from monogalactosyldiglyceride occurring in phytoplankton, but not from bacteria.

Occurrence of 1-O-β-D-galactosylglycerol in the surface and deep waters of stations 3 and 4 (Tab. 4) indicated that the compound, which originated from monogalactosyldiglyceride of phytoplankton through enzymic hydrolysis, has to be surprisingly resistant to bacterial degradation. Hence, 1-O-β-D-galactosylglycerol may be useful as a marker substance for algae in marine environments while its input from land still remains to be considered in coastal regions because terrestrial higher plants also have monogalactosyldiglyceride as mentioned above.

Sucrose, trehalose and melibiose were positively identified in the water samples, while the chemical nature of several hexosyl disaccharides still remains to be elucidated. Trehalose was firstly identified in seawater, while sucrose was reported to occur in seawater (Schaefer, 1965; Josefsson, 1970). Melibiose was also found in the particulate matter from Mikawa Bay (Sakugawa *et al.*, in prep.). Possible source of these disaccharides in marine environment is algae or heterotrophic microorganisms.

Sucrose is one of the most important low molecular weight reserve carbohydrates in phytoplankton (Craigie, 1974). Trehalose was reportedly found to exist in several algae, euglena and blue green algae (Craigie, 1974) and shown to be released from blue green algae in brackish marsh as an extracellular product using ^{14}C-labelling techniques (Hall, Fisher, 1983). Phytoplankton is, therefore, a possible source of these disaccharides occurring in the water samples.

Another identifiable source of disaccharides is the heterotrophic organisms found in seawater. Trehalose commonly occurs in fungi and yeast as a reserve carbohydrate and is found in bacteria (Elbein, 1974). Identification of melibiose and relatively large concentrations of sucrose and trehalose in deep waters (Tab. 4) suggested that microorganisms may play an important role in producing melibiose, sucrose and trehalose in deep-ocean environment. However, it is still difficult to evaluate the role of microorganisms as a producer of these oligosaccharides dissolved in seawater because of the

limited information available on the production of oligosaccharide by marine fungi and bacteria.

Polysaccharide

The occurrence of acidic polysaccharides in seawater is also a little-known subject, although combined forms of uronic acid have been determined by ion-exchange chromatography after acid hydrolysis (Mopper, 1977) and decarboxylation with 12% HCl at 130°C followed by measurement of released CO_2 (Williams, Craigie, 1970). Analysis of uronic acid in the polysaccharide with MW>4,000 in the water samples indicated that the uronic acid occurred in polyuronides or complex polysaccharides as one of the monomeric components. It is well known that acidic polysaccharide (*e.g.*, polyuronide, sulfated polysaccharide and acidic mucopolysaccharide) commonly exist in various marine algae (Percival, McDowell, 1967), microorganisms (Stacey, Barker, 1960) and animals (Jeanloz, 1970). Extracellular release of acidic polysaccharide containing sulfated sugars or uronic acids from algae was observed in culture experiments (Hellebust, 1974). Thus, it seems likely that the acidic polysaccharide found in the water samples is derived from phytoplankton and other marine organisms through excretion and/or cell lysis processes.

The phenol sulfuric acid method was used to determine "total sugars", which include neutral sugar and uronic acid, in the polysaccharide with MW>4,000 and acidic polysaccharide (Tab. 7). This method is simple and sensitive for determination of μg order of sugars compared with GC, although overestimation of sugars dissolved in natural water were often observed due to its reaction with humus (Stabel, 1977) and some organics other than sugars. Higher values of total amounts of the polysaccharide with MW>4,000 by the phenol sulfuric acid method compared with those in GC (Tab. 6) may partly result from overestimation of sugars by the former method.

The present experimental results showed that acidic polysaccharide appeared to be a minor portion of the polysaccharide with MW>4,000 in the water samples, accounting for <21% of the polysaccharide with MW>4,000, except for the 42% in the surface water of the Bering Sea (Tab. 7). Despite its small presence in the water samples, acidic polysaccharide may play a specific role in marine environments as a high molecular weight organic ligand of trace metals found in seawater because acidic polysaccharide such as alginic acid was found to have a similar ability to complex with metals (Tanaka *et al.*, 1972) as humic substance (Mantoura *et al.*, 1978).

Uronic acid contents were equivalent with 50 and 35% of acidic polysaccharides in the surface waters of stations 3 and 4, respectively, while the former was two or three times higher than the latter in the deep waters (Tab. 7). The results suggest that the structures of acidic polysaccharides were different between the surface and deep waters. The higher concentration of uronic acid than acidic polysaccharide in deep waters suggests that there were polysaccharides containing uronic acids which were not precipitated with 5% Cetavlon. The non precipitated polysaccharides may be a complex polysaccharide which is difficult to precipitate quantitatively with Cetavlon. Whether these polysaccharides consist of only uronic acids or both uronic acids and neutral sugars remains uncertain because structural studies of these polysaccharides were unsuccessful due to lack of sufficient material for analysis.

A high molecular weight glucan was found to occur in the surface water at station 3 in the North Pacific. Methylation study indicated that this glucan had 1.4- and 1.6-linkages. 2.3-di-O-methylglucose, however, was missing in the acid hydrolysate of the permethylated derivative of this glucan. Thus, this glucan is assumed to be a mixture of 1.4- and 1.6-glucans. It would be extremely interesting to know whether the glucans are α- or β-polysaccharide, because of the great significance of α-glucans for marine organisms as the substrate for their energy metabolism relative to that of β-glucans. However, sufficient quantities of these glucans could not be obtained to determine their steric configuration.

It was striking to find heteropolysaccharide consisting of glucose, galactose, mannose, xylose, arabinose, fucose and rhamnose in the surface water of the Bering Sea (Tab. 6), whereas almost no such polysaccharide was observed in the deep waters (Fig. 5). According to the chemical studies of polysaccharides dissolved in Mikawa Bay, mucilaginous heteropolysaccharide consisting of seven monosaccharides, having highly branched structure and half ester of sulfate with molecular size of less than 5×10^6, appeared to be derived from phytoplankton occurring in the surface water of Mikawa Bay (dinoflagellates and diatoms), because of the fact that almost identical heteropolysaccharide was also found in the particulate matter collected from the surface water. These findings indicate that the heteropolysaccharide found in the surface water of the Bering Sea is most likely derived from the diatoms living abundantly in the surface water of the Sea through extracellular release or cell lysis.

Total concentration of dissolved carbohydrate in the water samples which was determined by GC ranged from 27.2 to 144.7 μg l^{-1}. These values accounted for 1-6% of dissolved organic carbon (DOC) which was reported elsewhere (Handa *et al.*, 1979). These percentage of DOC seem to be less than those determined by colorimetric methods. Handa (1966*a*) reported that dissolved carbohydrate accounted for 10-20% of DOC in the water samples collected from the Indian Ocean. Burney *et al.* (1979) also reported that it accounted for 8.0-24.5% of DOC in the water samples from the North Atlantic, which was determined by a colorimetric method using MBTH (3-methyl-2-benzatolinone hydrazone hydrochloride) reagent. Reported higher values by colorimetric methods may partly result from overestimation of sugars due to their reaction with some organics such as humus in addition to sugars as described above.

Large differences in the concentration and chemical nature of dissolved carbohydrate between the surface waters of stations 3 and 4 were observed—*e.g.*, large occurrence of free monosaccharide (especially glucose)

and high molecular weight glucan in station 3 while small occurrence of free monosaccharides and existence of high molecular weight heteropolysaccharide in station 4. This difference may be attributed to several biological processes occurring in the surface waters of these stations, especially such as *in situ* phytoplankton activity. A variety of carbohydrates were found to be released from algae, of which chemical natures seem to be different according to algal species (Hellebust, 1974). Dinoflagellates were dominant at station 3, but diatoms at station 4. These biological environments might greatly affect the variability in the chemical nature of dissolved carbohydrates at these stations. On the other hand, it is unlikely that regional biological processes largely affect in the concentration and chemical nature of dissolved carbohydrate in deep waters, because our results showed that their concentration and chemical nature in deep waters were similar at both sampling stations.

Several mono-, oligo- and polysaccharides were identified in the North Pacific and Bering Sea. Especially glucose, and oligo- and polysaccharides consisting of only glucose (gluco-oligosaccharide and glucan) largely existed in all the water samples, indicating significance of glucose and its combined forms in marine environment. In these fractions, mono- and oligo-saccharides may have an important role as substrates for bacteria occurring in the surface through deep waters, because low molecular weight organics such as amino acid, sugar and organic acid were found to be easily uptaken by bacteria, their turnover rates in seawater being very high (Joint, Morris, 1982).

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