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Determination of Monosaccharides in Submarine Sediments Using a Full Automatic Amino-acid Analyzer

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Abstract: This study was performed to determine the optimum condition for extraction of monosaccharides from the submarine sediments and to find the adequate purification method of sediment hydrolysates. The following condition and methods were studied: (1) the concentration of H_2SO_4 and time of heating for hydrolysis, (2) desalting with a set of three columns, and (3) removing acids by evaporation which followed desalting with only one column.

The extraction method, in which the sample was treated with 72% H_2SO_4 in an ample for 4 hours and the solution diluted to concentration of 1N, followed by heating an ample at 110°C for 10 hours, gave the maximum yield of monosaccharides.

The desalting method by a set of three columns is far better than that by only one column. This is because, the monosaccharides were determined more sensitively and more correctly by the sugar solutions obtained from the former than the latter.

Eight of monosaccharides were determined from the submarine sediments studied. As the stability series for monosaccharides in the acid hydrolysis were clarified, they were found different from that in lacustrine environment.

1. Introduction

Carbohydrates are the main framework materials of plants and the most abundant organic constituents in nature. Initially, these carbohydrates are affected qualitatively and quantitatively by source materials and depositional environment, and are supplied to the surface sediments. Secondly, they decompose from high molecular weight polymers to monomers through low molecular weight polymers, on the course of diagenesis. A part of them will disappear, and the others will be concentrated with other organic constituents to be preserved in the sediments. The stratigraphic and diagenetic study on monosaccharides in the sediments will serve to identify source materials (landderived or autochthonous), paleoenvironment and geological history.

The geochemical study on monosaccharides is far behind the studies on amino acids which are the main framework materials of animals in nature. Therefore, analytical studies and introducing papers on monosaccharides are scarce; there are only a few reports such as DEGENS *et al.* (1964), SWAIN (1969) and KOYAMA *et al.* (1970). These works have been made mainly on analytical methods for monosaccharides in water and soil, therefore, these analytical techniques are hardly possible to apply to those in sediments. Because, monosaccharides in sediments condense with kerogen and/or humic substances and become insoluble.

If classified by their occurrences, carbohydrates are devided into free and combined sugars, and by their molecular weight they may be separated into monosaccharides, oligosaccharides and polysaccharides, respectively. And if classified by the affinity for solvent, they may be devided into soluble and insoluble sugars.

On the basis of these facts, monosaccharides, oligosaccharides and low molecular weight polysaccharides are extracted by water or ethyl alcohol. The carbohydrates in humic substances

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are released by alkali extraction (HANDA and MIZUNO, 1973). All carbohydrate polymers in the sample are completely hydrolyzed by strong acid attack, then are extracted as monosaccharides. As most of carbohydrates in the sediments may occur as insoluble polymers, acid extraction of monosaccharides is the best method for the sediment samples.

Chromatographic methods are commonly used for determination of monosaccharides, they are paper-chromatography, gas-chromatography and liquid-chromatography. The paper-chromatography is convenient, but it lacks sufficient accuracy. The gas-chromatography using volatile materials, is superior to the paper-chromatography, as the former can determine monosaccharides more correctly and more rapidly. However, as sugars are not volatile, they must be converted from involatile sugars to volatile sugar derivatives for gas-chromatography. These derivatives are difficult to prepare quantitatively. The last one, liquid-chromatography is much better than the paper- and gas-chromatography for monosaccharides analysis in the sediments, because of much simpler and rapider sample preparation and better yield of monosaccharides. Liquid chromatograph is an apparatus combined with the ion-exchange columns and photometer, and is operated automatically. NEC JLC-5AH amino acid analyzer, a type of automatic liquid chromatograph, was used for monosaccharides analysis in this study.

The main purpose of this study is to determine the optimum condition for extraction of monosaccharides from the submarine sediments. The others are to prepare monosaccharides mixture which are determined more accurately on liquid chromatograph, and to examine the purification method of sediment hydrolysates.

2. Analytical Procedures

2.1 Reagents

All of the reagents in this study are analytical grade, and are as follows;

Sulfuric acid for the hydrolysis: 72%, 18N, 1N and 0.5N H_2SO_4. Columns filled with strongly acidic cation exchange resin: Dowex 50W, X8, 100–200 mesh H^+ form.

Columns filled with weekly basic anion exchange resin: Duolite A-4, 100–200 mesh, OH^- form.

Reagent solution: 0.15% orcin conc. H_2SO_4.

Borate buffer solution: First buffer; pH 7.5, 0.13M H_3BO_3, Second buffer; pH 9.0, 0.25 M H_3BO_3, Third buffer; pH 9.6, 0.35M H_3BO_3.

Sample solvent: pH 8.0, 0.15M H_3BO_3.

Pottassium borate solution for regeneration: 0.5M K_3BO_3.

Monosaccharide standard solution: L-rhamnose 20 mg, D-ribose 10 mg, D-mannose 20 mg, D-fructose 40 mg, L-arabinose 20 mg, D-galactose 40 mg, D-xylose 30 mg, D-glucose 30 mg (in pH 8.0, 0.15M H_3BO_3 100 m*l*).

2.2 Apparatus

A Nippon Electric Company JLC-5AH amino acid analyzer was slightly modified for monosaccharides analysis. Instrumental operat-

Table 1 Instrumental operating conditions for monosaccharides using NEC JLC-SAH amino-acid analyzer.

Stationary phase: JEOL Resin LC-R-3 (Cl^- Type)
Mobile phase: Borate buffer
(1) First buffer pH 7.5 (0.13M)
(2) Second buffer pH 9.0 (0.25M)
(3) Third buffer pH 9.6 (0.35M)
Buffer change time
(1)⟶(2) 100 min
(2)⟶(3) 140 min
Sample size: 1 m*l*
Flow rate:
buffer solution 0.51 m*l*/min
reagent solution 0.92 m*l*/min
(Orcin/H_2SO_4)
Column size: 0.8 ϕ × 15 cm
Temperature: 55°C
Reaction tube length: 1 mmϕ × 15 M
Light pathlength: 2.0 mm
Wave length: 440 mμ
Chart speed: 6.0 cm/hr

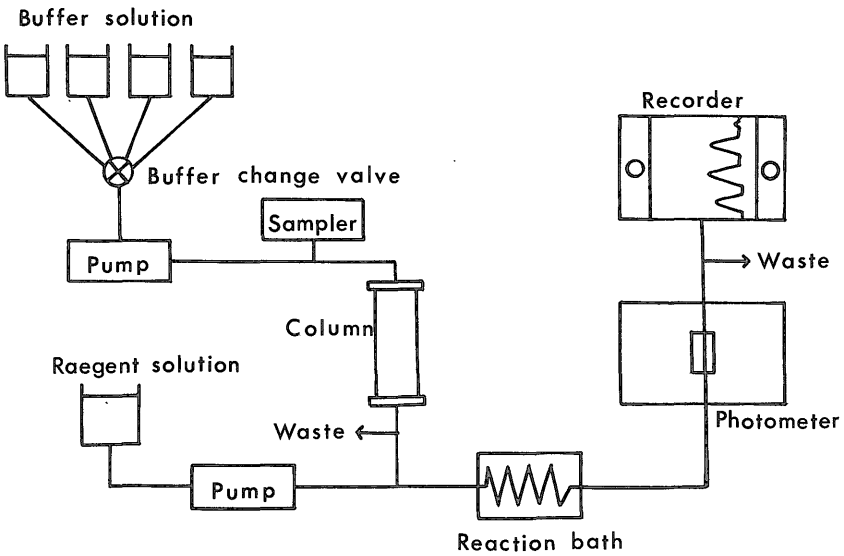


Fig. 1 Flow diagram of Liquid-chromatograph.

ing conditions for monosaccharides and flow diagrams are shown in Table 1 and Fig. 1.

2.3 Samples

Surface sediments from Harima-nada were used for this study. After washing with distilled water, the sediment samples were dried at 70°C for 4 hours and ground by hand tools to 100 mesh powder.

2.4 Analytical techniques

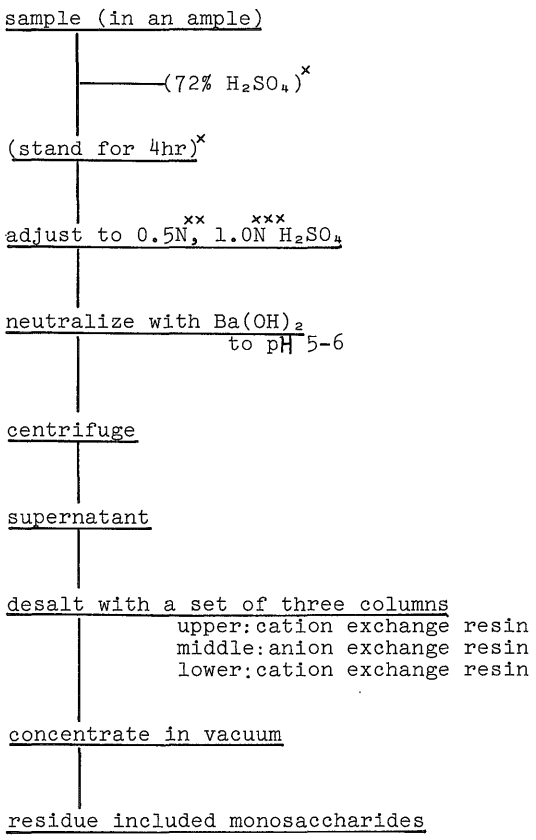
0.3 g of samples were weighed in samples and 0.5 m*l* of concentrated H_2SO_4 were added. After standing for 4 hours, solutions were diluted with distilled water to concentrations 1N (Experiment 1 and 2 do not include this pretreatment with conc. H_2SO_4). Amples containing samples and sulfuric acid were sealed and heated at 110°C for 8, 10, 14, 18 and 24 hours, respectively. Hydrolysates were transferred from amples to beakers and rinsed with distilled water. The hydrolysates and washings were neutralized with $Ba(OH)_2$ until pH 5–6 were reached. The supernatants containing sugars were separated from the precipitation of $BaSO_4$ by centrifuge. The solutions contained with inorganic ions were desalted to pass through one column with strongly acidic cation exchange resin or a set of three columns (the upper and lower columns-strongly acidic cation exchange resin, middle column-weekly basic anion exchange resin). The effluents containing monosaccharides were concentrated with a rotary evaporator. The flow diagrams of sample preparation are shown in Fig. 2 and Fig. 3.

The residues containing monosaccharides were dissolved in sample solvent (pH 8.0, 0.15 M H_3BO_3) to form borate complex. These solutions were analyzed on an automatic amino-acid analyzer according to the instrumental operating conditions shown in Table 1. Namely, borate complex of monosaccharides were absorbed on the column filled with strongly basic anion exchange resin and eluted by changing the concentration and/or pH of boric acid. Each separated monosaccharides reacted with reagent solution (orcin sulfuric acid) and absorbance of coloured compounds at 440 mμ were determined and recorded continuously.

3. Experiment

3.1 Previous works

Oades *et al.* (1970) studied about the condi-



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××: available for II-1 only
×××: available for II-2, 3 only

Fig. 2 Flow diagram of sample preparation for experiment II-1, 2, 3.

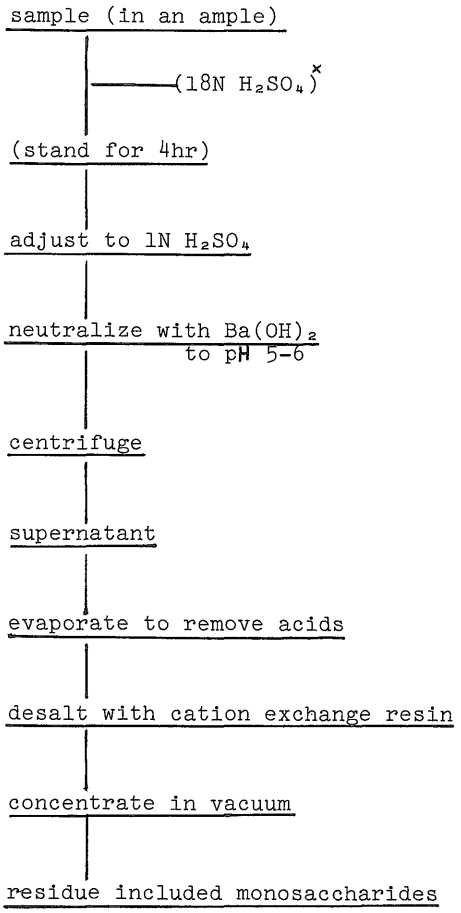

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Fig. 3 Flow diagram of sample preparation for experiment I-2, I-3.

tions of hydrolysis on the extraction of sugars from soil, using gas-liquid chromatography for determination of monosaccharides. He recommended a double hydrolysis procedure: a 20-minute reflux in 5N H_2SO_4 followed by a 16-hours soak in 26N H_2SO_4 and subsequent 5-hours reflux in 1N H_2SO_4. Furthermore, he reported that this procedure gave maximum yield of sugars from the soils, and was efficient to decompose the resistant cellulosic materials. KAGEMORI (1970) determined monosaccharides in the fossil wood, which were extracted to heat amples containing 0.5 g of samples and 10 m*l* of 1N H_2SO_4 at 110°C for 10 hours. SWAIN and BRATT (1971) reported carbohydrate components of core sediments from Broadkill marsh, Delaware Bay. Monosaccharides in their sediments were extracted with cold concentrated sulfuric acid, followed by reflux extraction with 0.5N H_2SO_4 for 8–10 hours, and determined by paper-chromatography. DEGANS *et al.* (1964) studied biochemical compounds in offshore California sediments. Carbohydrates in their sediments were released to stand for one hour with cold concentrated sulfuric acid and to dilute to 1N, followed by reflux extraction at 105°C for 8 hours, and then, the concentrations of monosaccharides were determined by paper-chromatography. They said that this treatment

effectively opened glycoside linkage even in the quite resistant cellulose. KOYAMA *et al.* (1972) reported that monosaccharides in lake sediments were released with 1N H_2SO_4 by reflux extraction for 24 hours, after treating samples with cold 72% H_2SO_4.

3.2 Experiment in this study

The following conditions of hydrolysis and the purification methods were examined in this study. Roman numerals I and II are used for purification methods and arabic one 1, 2 and 3 for hydrolysis.

a. Variation of volumes and concentrations of sulfric acid for hydrolysis.

Experiment 1: at the begining, 5 m*l* of 0.5 N H_2SO_4 were added in 10 m*l* amples containing 0.3 g of samples.

Experiment 2: At the begining, 5 m*l* of 1N H_2SO_4 were added in the 10 m*l* amples containing 0.3 g of samples.

Experiment 3: 0.3 g of samples in 20 m*l* amples were treated with 0.5 m*l* of 72% H_2SO_4 for 4 hours. Solutions were diluted with distilled water to concentration of 1N.

Experiment 3′: 0.3 g of samples in the 20 m*l* amples were treated with 0.5 m*l* of 18N H_2SO_4 for 4 hours. Solutions were diluted with distilled water to concentration of 1N.

b. Variation of time of heating.

8, 10, 14, 18, 24 hours.

c. Variation of purification methods

Experiment I: Sulfuric acid was removed from the solutions by evaporation with a rotary evaporator. The residues dissolved in distilled water were desalted with strongly acidic cation exchange resin.

Experiment II: The solutions were desalted to pass through a set of three columns wherein the upper and lower columns contained strongly acidic cation exchange resin, and the middle was filled with weakly basic anion exchange resin.

The combination of each hydrolysis conditions and purification methods are shown in Table 2.

Table 2 Combination of hydrolysis conditions and purification method.

Experiment	Concentration of H_2SO_4 for pretreatment	Concentration of H_2SO_4 for hydrolysis	Column
I–2	—	1N	one column
I–3′	18N	1N	one column
II–1	—	0.5N	three columns
II–2	—	1N	three columns
II–3	72%	1N	three columns

4. Result and Discussion

4.1 Monosaccharides found from submarine sediments

Monosaccharides found from the submarine sediments were D-glucose, D-galactose, D-mannose, β-D-fructose, D-ribose, D-xylose, L-arabinose and L-rhamnose as shown in Fig. 4. The peaks of D-fructose and L-arabinose overlapped each other and were determined as D-fructose equivalent. The chromatograms of monosaccharides detected from submarine sediments are shown in Fig. 5.

4.2 Effect of different conditions of hydrolysis and of different purification procedures of hydrolysates on total monosaccharides.

The results in Table 3 show the total yield of monosaccharides released by the different conditions of hydrolysis and of different purification procedures of hydrolysates mentioned before.

Total monosaccharides released by the purification method: results of experiment I fluctuate extremely as shown in Table 3. In Fig. 5, No. 1 and No. 3 show the chromatograms of monosaccharides prepared by one column—experiment I, and No. 2 and No. 4 are the ones prepared by a set of three columns—experiment II. The peaks of experiment I- one column are broader than those of experiment II- a set of three columns and the separation degree of formers are worse than the latters as compared

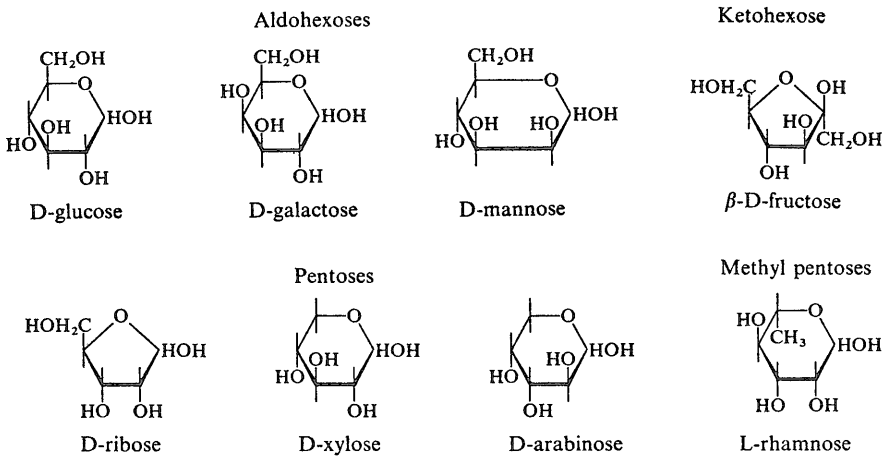

Fig. 4 Monosaccharides from submarine sediments found in this study.

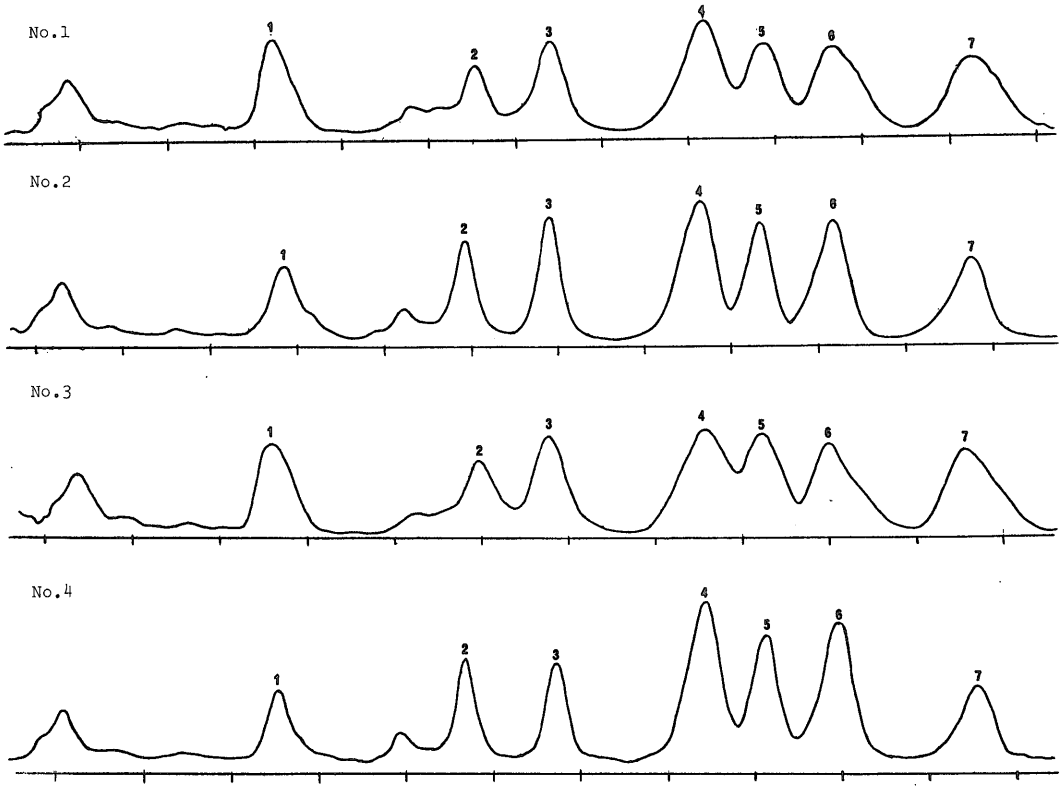

Fig. 5 Chromatograms of monosaccharides.
1, Rhamnose 2, Ribose 3, Mannose 4, Fructose + Arabinose 5, Galactose
6, Xylose 7, Glucose

Table 3 Effect of different conditions of hydrolysis and of different purification procedures of hydrolysates on total monosaccharides (mg/g).

Method	Time of heating				
	8hr	10hr	14hr	18hr	24hr
I-2	1.06	1.51	1.59	1.79	1.62
I-3′	1.86	1.18	1.61	1.52	1.16
II-1	1.54	1.44	1.29	1.18	1.10
II-2	1.57	1.68	1.67	1.57	1.51
II-3	1.57	1.92	1.69	1.63	1.38

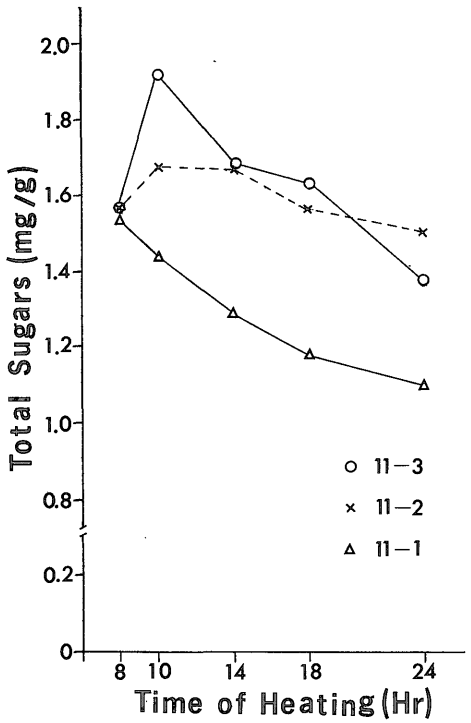

Fig. 6 The effect of different condition of hydrolysis on total monosaccharides, when the desalting method of a set of three columns were used.

in Fig. 5. These facts show that the sediment hydrolysates were not purified fully by experiment I.

The effect of different conditions of hydrolysis on the total monosaccharides are shown in Fig. 6, when the desalting method of a set of three columns were used. In experiment II-1 total monosaccharides become of maximum concentration at 8 hours of hydrolysis, and decrease gradually with time of heating. The total monosaccharides of experiments II-2 and II-3 become of maximum concentration at 10 hours of hydrolysis and show decreasing trend similarly with time of heating. The decreasing trends of total monosaccharides with time in experiment II-1 and II-3 are greater than that in experiment II-2. The total monasaccharides are in the order II-3, II-2, II-1 at for 10 hours of heating, however, after heating for 24 hours they become higher in the order II-1, II-3, II-2. The facts which experiment II-3 for 10 hours heating gave the maximum yield of total monosaccharides, show that the pretreatment with cold concentrated sulfuric acid effectively break up glycocide linkage in highly organized materials (humic acid, kerogen).

If addition of cold concentrated sulfuric acid was not sufficient, the powdered sample will not get wet. On the contrary, if concentrated sulfuric acid is added too excess, the volume of solution becomes larger, when it is diluted to 1N. As it becomes impossible to hydrolyze in an ample, hydrolysis must be done with reflux apparatus in that case. A large volume of hydrolysate makes scales of neutralization, centrifuging, desalting and concentration larger, moreover, it is possible that their larger scales of procedures make the rate of monosaccharide recovery lower. Sasaki (1973) reported 6.1% of average loss of amino acid recovery that was caused by desalting and concentration procedures of amino acid hydrolysates. The scale and procedures of desalting and concentration of sugar hydrolysate are similar to those of amino acid hydrolysate, the average loss of monosaccharide recovery is presumed to be similar value with amino acids. Increasing the volume of cold concentrated surfuric acid unnecessarily will decrease the rate of monosaccharide recovery.

4.3 Effect of different conditions of hydrolysis on the monosaccharides

Figs. 7, 8 and 9 show the effect of different conditions of hydrolysis on the monosaccharides. According to these results, a stability series for

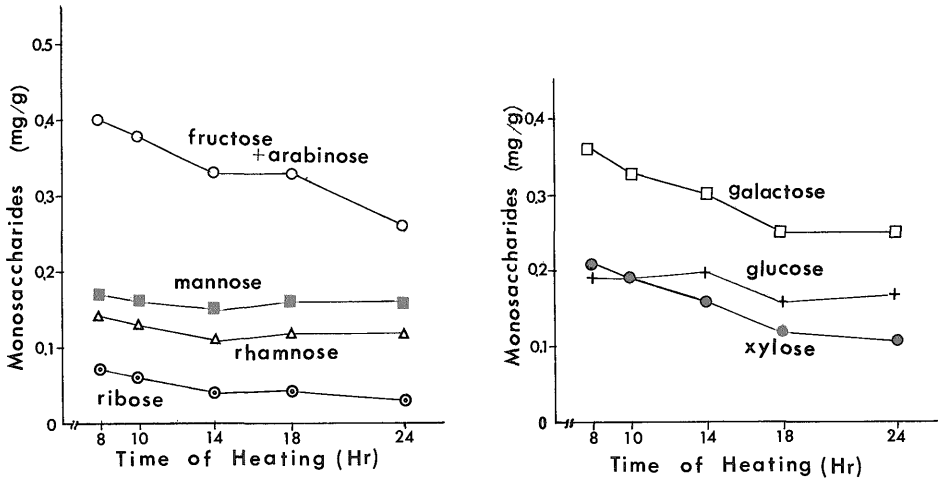

Fig. 7 Effect of different condition of hydrolysis on monosaccharides (Experiment II-1).

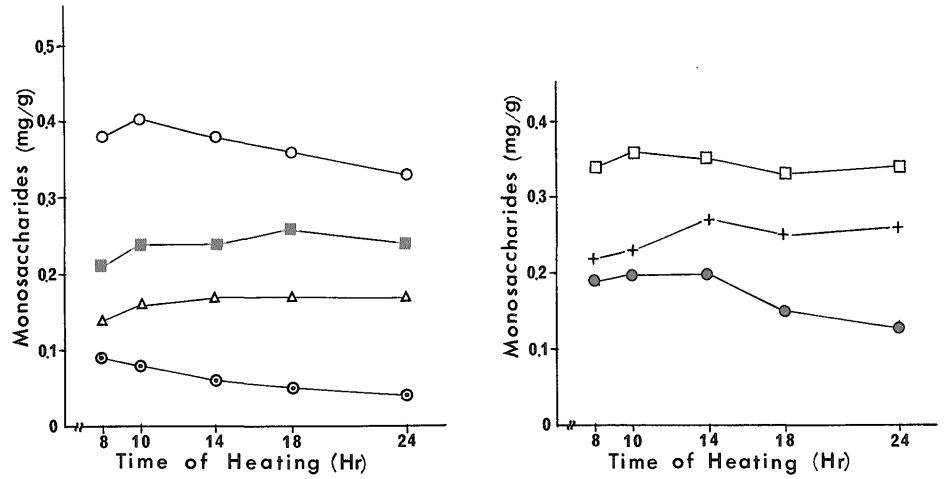

Fig. 8 Effect of different condition of hydrolysis on monosaccharides (Experiment II-2).

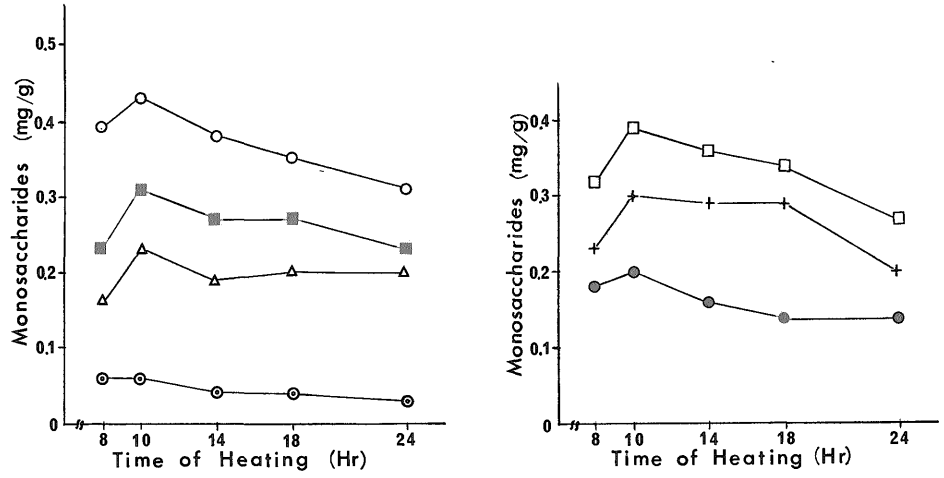

Fig. 9 Effect of different condition of hydrolysis on monosaccharides (Experiment II-3).

monosaccharides in the hydrolysis is: fairly stable: rhamnose, mannose; moderately stable: fructose + arabinose, galactose, xylose; fairy unstable: ribose. Glucose fluctuated relatively. This fluctuation is due to the broad peak of glucose which elutes lastly in the chromatogram. When pH of borate buffer solutions were adjusted higher or lower, the flow rate of glucose gets slower and the width of peak becomes broader.

ROGERS (1965) reported that a natural stability series for carbohydrates in the lacustrine environment was: fairly stable: xylose, glucose, rhamnose, arabinose; moderately stable: ribose, mannose; fairly unstable: galactose; very unstable: glucuronic acid.

Acid hydrolysis process, wherein the various glucosidic linkages having different bond strength are hydrolyzed selectively by strong acid, and monosaccharides released decrease by the effect of acid, are probably different from the natural hydrolysis process. In natural environments, polysaccharide linkages break up biochemically and released monosaccharides become incorporated into the humic acids and the others disappeared with lapse of time. Therefore, it seems that stability series for monosaccharides in both hydrolysis conditions do not coincide.

5. Conclusion

Extraction method of monosaccharides from submarine sediments and purification method of sediment hydrolysate were studied by using an automatic amino acid analyzer to determine monosaccharides. The results are summarized as follows;

1. The hydrolysis procedure; in which the ample containing sample and 1N H_2SO_4 was heated at 110°C for 10 hours, after treatment with 72% cold sulfuric acid, gave maximum yields of monosaccharides from the sediments studied. However, this method gave a fluctuation to the total monosaccharides, compared to the method without treatment of cold sulfuric acid.

2. The desalting method by a set of three columns (the upper and lower-strongly acidic cation exchange resin, middle-weakly basic anion exchange resin) gave better degree of separation and sharper shapes of peaks on the chromatograms of monosaccharides than that by only one column (strongly acidic cation exchange resin).

3. Monosaccharides detected from submarine sediments studied were as follows: D-glucose, L-arabinose, D-galactose, D-mannose, β-D-fructose, D-ribose, D-xylose and L-rhamnose.

4. Monosaccharide stability under the acid hydrolysis were as in the following orders: fairly stable; rhamnose, mannose moderately stable: fructose + arabinose, galactose, xylose; fairly unstable; ribose. These were different from a natural stability series for monosaccharides in the lacustrine environments.

The results suggest that the best method and conditions for extraction of monosaccharides from submarine sediments and for the purification method of acid hydrolysates are as mentiond above. Furthermore, the pretreatments for the determination of monosaccharides by an automatic amino-acid analyzer is simpler than that by a gas-liquid chromatograph, and the rate of monosaccharide recovery in this method is better than in the gas-liquid chromatography.

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全自動アミノ酸分析機による海底堆積物中の単糖類の定量法について

寺島美南子

要　旨

この研究は，海底堆積物から，単糖類を抽出する最適の条件をきめるために，また，堆積物加水分解物の最も良い精製法を求めるために行われた．すなわち，次のような条件と方法；1) 加水分解のための硫酸濃度と加熱時間，2) 3重カラムによる脱塩と，蒸発により酸を除いたのち，ワンカラムによる脱塩が検討された．

アンプル中で試料を72% H_2SO_4 により4時間処理したのち，1Nにうすめ，110℃で10時間加熱する抽出方法が，単糖類の最大の収量を与えた．

3重カラムによる脱塩法がワンカラムによる脱塩法よりすぐれている．なぜならば，単糖類は，後者よりも前者から得られた糖溶液によって，より精度よく，より正確に定量されるからである．

8種類の単糖類が，海底堆積物から検出された．酸加水分解における単糖類の安定順位が明らかにされたが，それらは湖底堆積物における単糖類の安定順位とは異なっていた．

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