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Carbohydrates and Nucleotides in the Red Alga *Porphyra perforata.**

I. Isolation and Identification of Carbohydrates

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An investigation of the marine red alga *Porphyra perforata* showed that taurine, laminitol, *scyllo*-inositol and 1-*O*-α-D-galactopyranosyl-D-glycerol were present in the ethanolic extract of the plant; these were isolated in crystalline form. Although no isolation could be achieved, indirect evidence indicated that 2-*O*-α-D-galactopyranosylglycerol was also present. The algae also contained an insoluble polysaccharide. Hydrolysis of the latter with acid produced D- and L-galactose and 6-*O*-methyl-D-galactose, all of which were isolated in crystalline form. Evidence was also presented for the presence of 3,6-anhydro-L-galactose. The polysaccharide was shown to contain sulfate in the ester form. Analysis of the sodium salt of the polysaccharide indicated that the polysaccharide consists of D-galactose, 6-*O*-methyl-D-galactose, 3,6-anhydro-L-galactose, and sulfate in the molar ratio of approximately 1:1:2:1. The galactose is present in the polysaccharide as a mixture of the D- and L-isomers in the ratio of 1.3:1. A microquantitative method for estimating D-galactose with the specific D-galactose dehydrogenase from *Pseudomonas saccharophila* was developed in connection with this study.

Seaweeds belonging to the order *Bangiales* are known to contain an intercellular polysaccharide consisting chiefly of D- and L-galactosyl residues. *Porphyra crispata*, a member of this order, was shown to contain a DL-galactan in its hot-water extract, while a D-galactoside was present in the ethanolic extract (Su, 1956).

This paper represents the results of an investigation of the various carbohydrates present in the ethanolic extract and of the ethanol-insoluble galactan isolated from this marine alga.

EXPERIMENTAL PROCEDURE

Materials and Methods.—The algae were collected from the east side of the Golden Gate Bridge, San Francisco, California, in October, 1959. After collection, the algae were placed in sea water and brought to the laboratory immediately. A 5.2-kg quantity of the algae (700 g dry weight) was washed with tap water, drained, and dropped into 5.2 liters of boiling ethanol. The mixture was brought to boiling again and kept at that temperature for 2 minutes. The algae were kept in ethanol at −10° until used.

A culture of *Pseudomonas saccharophila* from which the specific D-galactose dehydrogenase was prepared (Doudoroff *et al.*, 1958) was provided by Dr. M. Doudoroff of the Bacteriology Department. Floridoside and its hexa-acetate were provided by Dr. E. W. Putman. *scyllo*-Inositol and its hexa-acetate were furnished by Dr. C. E.

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Ballou. Mono- and di-*N*-methyl taurines were synthesized and purified according to the procedure of Lindberg (1955a).

Infrared absorption spectra of the crystalline materials were recorded with a Baird-Atomic infrared spectrophotometer, Model 4-55, using KBr pellets containing 0.5 or 1% samples. Melting points were determined with a calibrated Kofler hot-stage apparatus.

Reducing sugars were estimated by the method of Nelson (1944). Periodate consumption was measured spectrophotometrically according to the procedure of Dixon and Lipkin (1954), with the following modifications. The concentration of periodate was observed at 260 mμ instead of 220 to 240 mμ; the lower extinction coefficient of periodate at 260 mμ permitted larger amounts of sample to be used. Another modification consisted in buffering the reaction mixture at *p*H 4.0 with 0.1 M acetate. The acetyl content of inositol acetate was determined by a colorimetric method (Snell and Snell, 1953); the principle of this method consists of converting the acetyl ester to the ferric complex of acetylhydroxamate. Pure floridoside hexa-acetate was used as the standard substance. The methoxyl content was determined by the method of Clark (1929). The quantitative elementary analyses, including a *C*-methyl analysis by the method of Kuhn and Roth (1933), were performed by the microanalytical laboratory of the University of California.

Paper chromatography of monosaccharides, galactosylglycerols, and inositols was performed by radial development with propyl alcohol–ethyl acetate–water, 7:1:2 (Albon and Gross, 1952), butyl alcohol–acetic acid–water, 4:1:5 (Partridge, 1948), or ethyl acetate–pyridine–water saturated with boric acid, 12:5:4.[1] Acetone-water, 9:1, was used for the ascending development of taurine and methyltaurines (Lindberg, 1955a).

Reducing sugars were detected with *p*-anisidine phosphate spray reagent (1 g *p*-anisidine phosphate in 140 ml of 70% ethanol) (Feingold *et al.*, 1958) and polyhydric alcohols by the benzidine–periodate dipping technique (Gordon *et al.*, 1956). For detection of 3,6-anhydrogalactose, urea phosphate spray reagent (Wise *et al.*, 1955) was employed. Bromothymol blue indicator spray (Brown, 1950) was used to locate taurine and methyltaurines.

The diethyl dithioacetals of 3,6-anhydro-L-galactose (m.p. 110–112°) and D-glalactose (m.p. 140–141°) were prepared by the mercaptolysis of agar as described by Araki and Hirase (1953). The paper-chromatographic standard of the free anhydrogalactose was prepared by demercaptalating the diethyl dithioacetal with cadmium carbonate and mercuric chloride, as described by Yaphe (1959). The excess mercuric chloride remaining in the demercaptalated solution was removed by extraction with ether.

3,6-Anhydro-L-galactose dimethyl acetal obtained by methanolysis (2.5% HCl in methanol, 75°, 20 hours) of agar served as the chromatographic reference for the anhydro sugar in question obtained from the polysaccharide. For the chromatographic separation of diethyl dithioacetals, butanol–ethanol–water, 3:1:1, was used (Painter, 1960).

The latter compounds were detected by spraying the chromatogram with 1% potassium permanganate and washing off the unreacted permanganate under a tap. The diethyl dithioacetals were revealed as brown spots against a white background (Onodera and Morisawa, 1961).

For estimation of microquantities of D-galactose, an enzymic method using the D-galactose dehydrogenase of *Pseudomonas saccharophila* has been developed. The enzyme preparation was made according to the method of Doudoroff *et al.* (1958). The enzyme proved to be completely inert toward L-galactose, D-glucose, and D-mannose. The following technique has been found to be applicable for the estimation of amounts up to 0.2 μmole of galactose in 1 ml of the assay mixture.

One ml of the assay mixture contained 2.5 units of enzyme, 1 μmole of DPN, 3.3 μmoles of reduced glutathione, and the sugar to be determined in a total of 1 ml of 0.033 M tris(2-amino-2-(hydroxymethyl)-1,3-propanediol) buffer at *p*H 8.6. The reaction was usually initiated by the addition of the enyme with a micropipet. A blank without the sugar was prepared at the same time. After the reaction mixture and the blank in the cuvet had been incubated at 37° for 30 minutes, the optical density at 340 mμ was determined with a Beckman DU spectrophotometer. The difference of the blank reading before and after incubation was usually less than 0.01. The amount of D-galactose was calculated from equation (1), the cuvets having an optical path of 1 cm.

$$\mu\text{mole D-galactose} = \frac{\Delta \text{OD}_{340}}{5.90} \qquad (1)$$

Separation of Taurine and of Carbohydrates from the Ethanolic Extract.—To the ethanol-fixed algae, sufficient ethanol was added to adjust the volume of 95% ethanol in the mixture to 10.5 liters, and the leaves were disintegrated in a large Waring Blendor. The ethanol containing the crushed material was heated to approximately 65° ± 5° as quickly as possible and kept at that temperature for 5 minutes. The residue was removed by filtration and the ethanolic extract concentrated *in vacuo* at 40° to about 2.7 liters. After most of the pigments had been extracted with ether, the resulting yellow aqueous solution was adjusted at 0° to *p*H 2.5 with nitric acid. An equal volume of 95% ethanol was then added and the nucleotides were precipitated with 270 ml of mercuric acetate reagent (Caputto *et al.*, 1950). The optimum amount of the reagent was determined by a series of trial precipitation tests.

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[1] Dr. C. E. Ballou, personal communication.

The precipitate was centrifuged, washed, and saved for a subsequent study of the nucleotides. The supernatant liquid and washings of the precipitate were combined. On standing in the cold, the ethanolic solution yielded a yellowish deposit of unknown nature, which was removed by filtration and discarded. After the *p*H had been adjusted to about 5 with a concentrated solution of sodium hydroxide, the solution was concentrated under diminished pressure to a small volume. The mercuric ion in the concentrate was precipitated as HgS by passage of H_2S gas. The HgS precipitate formed was removed by filtration and the excess H_2S by aeration. The clear solution was evaporated again under diminished pressure to remove acetic acid. After dilution with water, it was deionized by passing through columns of Dowex 50 (H^+) and Duolite A3 (OH^-), and evaporated to a thick sirup, weighing 30 g. A 10-g portion of the sirup was saved for a preliminary examination, while the remainder was kept in a desiccator to induce crystallization. A white material soon crystallized; this was collected by filtration after addition of a small amount of ethanol to reduce the viscosity of the adhering sirup. The yield of the material was 0.7 g. To the filtrate, one volume of 95% ethanol was added, which resulted in the precipitation of the second crop (2.0 g) of the white crystalline material. On sodium fusion, this material was shown to contain nitrogen and sulfur. The material was also shown to consume periodate. While repeated recrystallization could not separate the nitrogen- and sulfur-containing compound from the polyhydric alcohols, two inositols and taurine were isolated from this material according to the following procedure.

On passage of the aqueous solution of the mixture through a Dowex 1 (OH^-) column, taurine was retained while the polyhydric alcohols passed through. The taurine was recovered by eluting the column with 2 N NaOH and the eluate was passed through a Dowex 50 (H^+) column to free the solution from Na^+ ions. On concentration and recrystallization, a glittering crystalline material (0.11 g), later identified as taurine, was obtained. A good separation of the polyhydric alcohols into two components was achieved with an ethyl acetate–pyridine–water solvent on a cellulose-powder column (25 × 4.5 cm). The chromatographic eluate was cut into fractions by means of an automatic fraction-collector and tested for polyhydric alcohols by paper chromatography. Two peaks were obtained with slight overlapping. Only the well-separated fractions were combined and evaporated under diminished pressure to dryness. After recrystallization from dilute ethanol, 171 mg of the fast-moving compound (laminitol) and 36 mg of the slow-moving one (*scyllo*-inositol) were obtained from 0.36 g of the crude material.

TAURINE.—Taurine was characterized by comparison of its melting point (325–328°, decomp.) and infrared spectrum with those of an authentic specimen. Since Lindberg (1955a, 1955b) reported that a small amount of monomethyltaurine was present together with taurine in *P. umbilicalis*, a search for the former compound was made in the crude ethanolic extract and in the mother liquor from which the pure taurine was separated. No monomethyltaurine could be found.

LAMINITOL AND *scyllo*-INOSITOL.—The inositol compounds laminitol and *scyllo*-inositol have been previously reported to occur in *P. umbilicalis* (Lindberg, 1955b), and the structure of the former has been established as 4-*C*-methyl-*myo*-inositol by Lindberg and Wickberg (1959). By comparing the physical constants and analytical data of the two compounds isolated from *Porphyra perforata* with those reported by Lindberg and McPherson (1954), it was concluded that they were laminitol and *scyllo*-inositol.

Analytical data for laminitol:

Reported values (Lindberg and McPherson, 1954) for $C_7H_{14}O_6 \cdot 2H_2O$: H_2O, 15.6; $[\alpha]^{20}_D$ (*c*, 2 in water), −3°; m.p., 266–269°.

Found: H_2O, 15.6; $[\alpha]^{24}_D$ (*c*, 2 in water), −3°; m.p., 266–268°.

Calcd. for $C_7H_{14}O_6$: C, 43.3; H, 7.2; CH_3, 7.3.

Found: C, 43.2; H, 7.2; CH_3, 7.7.

Calcd. for $C_7H_8O_6(COCH_3)_6$: $COCH_3$, 57.9.

Found: $COCH_3$, 57.0.

The compound consumed 5.45 moles of periodate per mole of substance (theoretical, 6 moles).

Analytical data for *scyllo*-inositol:

Calcd. for $C_6H_{12}O_6$: C, 40.0; H, 6.7.

Found: C, 40.0; H, 6.8.

The compound's consumption of periodate was 5.7 moles per mole of substance (theoretical, 6 moles). The melting point of the *scyllo*-inositol was 353°, while that of the hexa-acetate was 300–302°. These values closely agree with those reported by Lindberg and McPherson (1954), 352° for the m.p. of *scyllo*-inositol and 301° for the hexa-acetate.

The complete agreement of the infrared spectra of the algal *scyllo*-inositol and its hexa-acetate with those of the authentic specimens gave additional proof for the identity of the compound.

Galactosyl Glycerols

1-*O*-α-D-GALACTOPYRANOSYL-D-GLYCEROL.—The ethanolic supernatant liquid from which taurine and inositols had been isolated was concentrated to a thick sirup and placed in a desiccator. Crystallization took place in about 2 weeks. After trituration with ice-chilled methanol, 6.8 g of grayish material was collected on a filter. From this material, 2.5 g of a pure galactoside which melted at 150–152° was obtained after three recrystallizations from methanol.

It should be noted here that, besides the above-mentioned crystalline galactoside, one more nonreducing glycoside could be observed on the paper chromatogram; also, no reducing sugar could be detected in the concentrate of the ethanolic extract of the algae by any chemical means.

The fact that only D-galactose [m.p. 166–168°; $[\alpha]^{25}_D$ + 80° (*c*, 1.50, water)] could be isolated and its phenylosazone [m.p. 160–160.5°; $[\alpha]^{25}_{\mathrm{D}}$ −21° (c, 1.19, water)] could be separated directly from the acid hydrolysate (1 N HCl, 100°, 1.5 hours), indicated that only D-galactosides were present in the ethanolic extract of the algae. The crystalline D-galactoside produced only D-galactose and glycerol on hydrolysis. This galactoside was identified as 1-*O*-α-D-galactopyranosyl-D-glycerol by comparing the physical constants (m.p. 150–152°; $[\alpha]^{30}_{\mathrm{D}}$ +154°) and the analytical results (C, 42.37%, H, 7.05%; moles of periodate consumed per $C_9H_{18}O_8$, 3.00) with the theroetical and literature values (Wickberg, 1958) (m.p. 150–152°; $[\alpha]^{20}_{\mathrm{D}}$ + 155°; C, 42.5%; H, 7.08%; moles of periodate consumed per mole, 3). The complete agreement of the infrared spectrum of the compound with that reported for the synthetic 1-*O*-α-D-galactopyranosyl-D-glycerol served as additional proof for the identity of the compound.

2-*O*-α-D-GALACTOPYRANOSYLGLYCEROL.—The second nonreducing galactoside observed on the paper chromatogram was suspected to be 2-*O*-α-D-galactopyranosylglycerol (floridoside), because the crude galactoside crystals showed a slightly higher specific rotation (+156°) and on oxidation consumed less periodate (2.54 moles per mole of substance); floridoside contamination could affect the analytical values in this fashion (Wickberg, 1958; Putman and Hassid, 1954).

The galactoside, 1-*O*-α-D-galactopyranosyl-L-glycerol, shown to occur in *P. umbilicalis* together with 1-*O*-α-D-galactopyranosyl-D-glycerol (Wickberg, 1958), does not appear to exist in *P. perforata*. This is deduced from the observation that an isomorphous mixture of the two isomers cannot be resolved into its components by repeated recrystallization, while we could isolate the 1-*O*-α-D-galactosyl-D-glycerol simply by three recrystallizations from methanol.

Polysaccharide

PREPARATION AND PROPERTIES OF GALACTAN.—The alcohol-extracted residue of the algae from the previous experiments served as the source of material. From 5.2 kg of the fresh algae, 825 g of air-dried residue was obtained. The moisture content of the material was 15.2%.

Two hundred g of this air-dried residue was extracted with 4 liters of water at 80–90°, with occasional stirring, for 2 hours. The viscous extract was first passed through cheesecloth, then filtered with suction through filter paper, while hot, with Celite added as a filter aid. A light-brown, slightly opalescent solution was obtained. Since the polysaccharide could easily be precipitated with ethanol at an alkaline *p*H, 10 ml of saturated NaOH solution and 4 volumes of 95% ethanol were added to the extract with vigorous stirring. The white polysaccharide precipitate was allowed to settle overnight and collected by decantation and centrifugation. The polysaccharide was washed twice with 80% ethanol and thoroughly mixed with 2% HCl in 80% ethanol. It was centrifuged again and washed with 80% ethanol until the washings were free from chloride. After dehydration with 95% ethanol and then with anhydrous ether, the polysaccharide was dried quickly in a vacuum desiccator and then in the open air until it was well equilibrated with the atmospheric moisture. Forty g of slightly gray-colored material was obtained. Considering that the moisture content of this material was 14.3% and that of the starting material 15.2%, the yield of the crude polysaccharide was calculated to be 22% on a dry-weight basis.

Since the polysaccharide contained organic sulfate residues, a sample of the sodium salt of the polysaccharide was prepared for quantitative analyses as follows; 1 g of the polysaccharide was dissolved in 100 ml of water and centrifuged to obtain a clear solution, which was then passed through Dowex 50 (H^+) column. The *p*H of the effluent was about 4. Addition of 12.3 ml of 0.100 N NaOH was required to bring the solution to a phenolphthalein end-point. This figure indicated that the equivalent weight of the anhydrous polysaccharide was approximately 700. The solution was then concentrated to a small volume under diminished pressure, and the polysaccharide was precipitated with 5 volumes of 95% ethanol, washed, and dried in a vacuum oven at 40°; yield 0.55 g

The polysaccharide formed an opalescent solution when dissolved in water. However, a clear solution resulted when 0.5 N NaOH was used as solvent. The specific rotation in 0.5 N NaOH was $[\alpha]^{25}_{\mathrm{D}}$ −61° (*c*, 1.60), which is in close agreement with the value obtained for *P. capensis* polysaccharide (Nunn and von Holdt, 1957; Wu and Ho, 1959). The sodium salt of the polysaccharide showed an almost identical specific rotation (−60°, *c*, 1.50, in water at 25°). The polysaccharide did not reduce hot Fehling solution. After hydrolysis with dilute HCl, it showed a positive Fehling and a sulfate test. A test with ninhydrin was negative. When the polysaccharide was autoclaved with 0.02 to 0.2 N sulfuric acid at 125° for 2 hours, the maximum degree of hydrolysis was reached. The maximal yield of the reducing sugar was 59% when the reducing value obtained by the method of Nelson (1944) was expressed as glucosyl residue. Because of the acidity of the polysaccharide itself, autoclaving in water yielded slightly more than 50% of the amount of reducing sugar obtainable under the most favorable conditions of hydrolysis. The hydrolysate was chromatographed in butanol–acetic acid–water, 4:1:5. A sharp resolution of three spots resulted. The slowest-moving spot was identified as galactose.

The spot that traveled closely behind the solvent front reacted immediately with *p*-anisidine phosphate spray at room temperature to give a yellow color, and an intense blue color developed when it was heated with urea phosphate reagent. The aqueous eluate of the compound obtained from a large chromatogram sheet showed an ultraviolet absorption maximum at 282 mμ, indicating that the compound was probably 5-(hydroxymethyl)-2-furaldehyde (reported $\lambda_{\max}$. of 5-hydroxymethyl)-2-furaldehyde, 285 mμ). This furfural derivative is usually formed from 3,6-anhydrogalactose, which is readily converted to 5-(hydroxymethyl)-2-furaldehyde in an acid medium.

ISOLATION OF MONOSACCHARIDE CONSTITUENTS.—Four g of the polysaccharide was autoclaved with 400 ml of 0.02 N sulfuric acid at 125° for 2 hours. The pale yellow hydrolysate was neutralized with $BaCO_3$ and filtered. The filtrate was concentrated to a small volume, adsorbed on 5 g of well-washed cellulose powder, and the powder put on the top of a cellulose column (4.5 × 35 cm). The dry column was developed with butyl alcohol saturated with water, and the elution of the sugars was followed by spot testing on paper with *p*-anisidine phosphate color reagent. The fractions falling under the same peak were pooled and concentrated under diminished pressure.

6-*O*-METHYL-D-GALACTOSE.—By desiccation of the sirup obtained from the fraction with the intermediate R_F value observed on paper chromatograms, a white crystalline mass was obtained. When this crystalline material was triturated with acetone and collected on a Büchner funnel, a yield of 0.38 g was obtained. On recrystallization from 95% ethanol, 0.15 g of material resulted in the form of colorless plates, m.p. 122–124°. The phenylhydrazone and phenylosazone derivatives prepared from this compound melted at 181.5–182.5° and 200–202°, respectively, after recrystallization from dilute ethanol. The specific rotation of the sugar in water was $[\alpha]^{26}_D$ +78° (*c*, 1.50). These properties agreed well with those described for 6-*O*-methyl-D-galactose (Vogel and Georg, 1931). The identity of this sugar was further substantiated by the periodate consumption data and methoxyl content determinations. The compound consumed 4.02 moles of periodate per mole of the sugar, which is in good agreement with the theoretical value of 4.00. Its methoxyl content was found to be 15.9%; theoretical value, 16%.

D- AND L-GALACTOSE.—The last fraction of the eluate was identified chromatographically as galactose. The whole fraction, after evaporation to dryness, showed a specific rotation $[\alpha]^{25}_D$ +10.9° (*c*, 1.65). The specific rotation of this material, calculated from the reducing sugar content (Nelson, 1944), was $[\alpha]^{25}_D$ +11.9° (*c*, 4.60). From these data, the D-galactose content of the galactose fraction was calculated as 57% and L-galactose, 43%. When part of this fraction was oxidized with nitric acid, galactaric acid (mucic acid) with m.p. 216° (decomp.) was obtained. On recrystallization of the crude galactose from methanol, a crystalline sugar was obtained (0.15 g) from the crude galactose sample, which showed zero rotation (*c*, 2.10) and melted at 143–144°, indicating that it was the racemic compound, DL-galactose (Elsner, 1935). From the mother liquor, a second crop crystallized (0.10 was g) and shown to contain 57% of D-galactose by polarimetry.

Isolation of L-galactose from this second crop of D- and L-galactose mixture was achieved by fermenting the D-isomer with a suspension of D-galactose-adapted *Saccharomyces cerevisiae* cells. About 15 mg of L-galactose with m.p. 163–165°, $[\alpha]^{24}_D$ −79° (*c*, 1.47) was obtained.

Quantitative Analysis of the Sodium Salt of the Polysaccharide

TOTAL SULFATE.—A sample of the polysaccharide was sealed with 100 volumes of 0.2 N HCl and heated in an autoclave at 125° for 2.5 hours. After cooling, the tube was opened and the contents diluted to a definite volume. The sulfate in the hydrolysate was estimated by a turbidimetric method (Treon and Crutchfield, 1942). The percentage of $SO_4^=$ ions was 13.3, which indicated an equivalent weight of 722 for the sulfur-containing polysaccharide. This value is in close agreement with the titration data of 700 described in the previous section.

SULFATE ASH.—A sample of the polysaccharide was ignited to a constant weight after the addition of a drop of concentrated sulfuric acid. If the sulfate in the polysaccharide had been completely neutralized with sodium ion, the ash would be pure sodium sulfate, and half of the sulfate would be lost by the ignition (Smith and Montgomery, 1959). Theoretically, the ash obtained in this manner should be 10.5%. The actual result was 11.2%, which is in close agreement with the theoretical value. This result suggested that the sulfate in the polysaccharide existed in the form of a salt of sulfate ester. The ash in the crucible was extracted with hot 0.1 N HCl and the extract made up to a definite volume. The sulfate in the extract was then determined by the turbidimetric method as before. Expressing the amount of $SO_4^=$ in the ash as the per cent of total $SO_4^=$ in the original sample, the result was 52%, which is in good agreement with theory (50%).

These results indicate that the sulfate in the polysaccharide is organic in nature and that the polysaccharide contains approximately four monosaccharide residues per sulfate residue.

GALACTOSE AND 6-*O*-METHYL-D-GALACTOSE.—A portion of the polysaccharide (0.1 g) was hydrolyzed with 0.2 N H_2SO_4 at 125° for 2 hours in a sealed tube. Galactose and 6-*O*-methyl-D-galactose in the hydrolysate were estimated, after separation on a paper chromatogram, with maltose as reference sugar (Flood *et al.*, 1948), by the anthrone method (Koehler, 1952) and Nelson's method (1944). By these methods, the percentage of the galactosyl residue in the polysaccharide was found to be 26, and that of the 6-*O*-methyl-D-galactosyl residue, 30. These were only approximate values, since the paper blanks obtained by the methods employed were high and variable.

To check the accuracy of the chemical determination of the galactose eluate, the enzymic method, using D-galactose dehydrogenase of *P.*

saccharophila, was applied. From the amount of D-galactose obtained, total galactose was calculated from the polarimetric data of the galactose recovered from the cellulose column. The percentage of the galactosyl residue in the polysaccharide calculated in this way was 22. If this figure is accepted as the true value, it can be concluded that one galactosyl residue is present per one sulfate group in the polysaccharide (theoretical value, 22.2%). The same conclusion was reached, for 6-*O*-methyl-D-galactose, from the methoxyl content of the polysaccharide, which was found to be 4.20% (equivalent to 23.8% of 6-*O*-methyl-D-galactosyl residue). This value is in good agreement with the theoretical value of 4.25%, calculated on the assumption that one methoxyl group was present per equivalent weight of the polysaccharide. 6-*O*-Methyl-D-galactose is known to occur in the *P. capensis* polysaccharide (Nunn and von Holdt, 1957).

3,6-ANHYDRO-L-GALACTOSE.—Attempts to demonstrate directly the presence of 3,6-anhydro-L-galactose, the labile constituent in the hydrolysis products of the polysaccharide, were not successful. However, the presence of this compound could be shown by preparing its diethyl dithioacetal derivative as follows:

One g of the polysaccharide was stirred with 10 ml of concentrated HCl and 2.5 ml of ethanethiol in a closed vessel at 2°. After 2 days, 2.5 ml of ethanethiol was added, and the stirring at 2° was continued for 3 more days. After a total of 5 days, the mixture was diluted with 100 ml of cold methanol and the solution neutralized with lead carbonate to a methyl red end-point. Upon filtration of the solution and evaporation to a small volume in a vacuum desiccator over KOH pellets, a white crystalline material separated out. After filtration and recrystallization of the material from methanol (yield, 0.03 g), the crystals showed a wide melting range, 118–136°. From the mother liquors of the recrystallization, a second (0.01 g) and third (0.01 g) crop were obtained, which melted at 122–135° and 139–140°, respectively. The three fractions produced spots corresponding to galactose diethyl dithioacetal on paper chromatographic separation. The spots obtained from the material with the wide melting range were suspected to be mixtures of the diethyl dithioacetals of D- and L-galactose, and the third crop with a sharp melting point, which did not show a depression of melting point on admixture with an authentic D-galactose diethyl dithioacetal, was considered to be pure D-galactose diethyl dithioacetal.

After collection of the galactose diethyl dithioacetals, the filtrate was concentrated to dryness. This material was dissolved in 20 ml of cold methanol and the small amount of insoluble, inorganic matter removed by filtration. The solution was again evaporated in a vacuum desiccator to a viscous sirup. Chromatographic analysis of the sirup showed three spots, the fastest-moving spot corresponding to 3,6-anhydrogalactose diethyl dithioacetal and the slowest one to galactose diethyl dithioacetal; the one located between the two was assumed to be 6-*O*-methyl-D-galactose diethyl dithioacetal.

The sirup was dissolved in 50 ml of water and extracted five times with 20-ml portions of ether. The combined extract was dried with anhydrous sodium sulfate and evaporated to a small volume. This extract showed the same chromatographic analytical results as the original sirup. The diethyl dithioacetal of the anhydro sugar was then separated on a large chromatogram sheet, which was cut to small strips, and the sugar was eluted with water. The eluate showed a positive optical rotation, indicating that the anhydro sugar is the L-enantiomorph. The separated diethyl dithioacetal was demercaptalated by the method of Yaphe (1959) to the free anhydro sugar, which co-chromatographed with an authentic specimen of 3,6-anhydro-L-galactose in butanol–acetic acid–water system.

Additional proof of the presence of the anhydro sugar was obtained from the methanolysis of the polysaccharide. The methanolysis of the polysaccharide was effected by heating 0.1 g of the polysaccharide at 75° for 20 hours with 2 ml of 2.5% HCl in methanol. After removal of the chloride with silver carbonate and subsequent chromatographic analysis of the methanolic solution, a spot was obtained which gave a ketose test and co-chromatographed with 3,6-anhydro-L-galactose dimethylacetal. Upon hydrolysis with 0.1 M acetic acid at 100° for 2 hours, the free anhydro sugar, together with some 5-(hydroxymethyl)-2-furaldehyde, could be detected.

A direct estimation of the anhydrogalactose in the polysaccharide, based on the acid-catalyzed formation of 5-(hydroxymethyl)-2-furaldehyde (Smith *et al.*, 1955), was attempted. Only about 0.3 residue of the anhydro sugar per one equivalent of the polysaccharide was found. When the method with resorcinol reagent (Yaphe, 1960) was tried, approximately 0.9 residue per equivalent was obtained. Since the titration value and the sulfur content of the polysaccharide indicated the presence of four monosaccharide residues per equivalent of substance, two of these residues must have been anhydrogalactose. The results obtained from the 5-(hydroxymethyl)-2-furaldehyde formation or by the resorcinol method could not be considered correct. It is, therefore, tentatively concluded that an equivalent of the sodium sulfuric ester galactan consists of one sodium sulfate group, one galactosyl residue, one 6-*O*-methyl-D-galactosyl residue, and two 3,6-anhydro-L-galactosyl residues. This unit has an equivalent weight of 729, which closely agrees with the value of 722 obtained from the sulfate determination.

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Carbohydrates and Nucleotides in the Red Alga *Porphyra perforata.**

II. Separation and Identification of Nucleotides

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The nucleotides present in the marine red alga *Porphyra perforata* have been investigated. On chromatographic separation on ion-exchange resin and on paper of compounds present in the ethanolic extract of the alga, AMP, UMP, GMP, IMP, ADP, UDP, IDP, DPN, TPN, UDP-D-glucose, UDP-D-galactose, UDP-glucuronic acid, GDP-D-mannose, and GDP-L-galactose were isolated and characterized. A new nucleotide that appears to be adenosine 3′,5′-pyrophosphate was also isolated. It is suggested that (a) GDP-L-galactose is formed from GDP-D-mannose by a mechanism similar to that involved in the GDP-D-mannose–GDP-L-fucose transformation; (b) GDP-L-galactose serves as the glycosyl donor for the L-galactose component in polysaccharide synthesis; and (c) 6-sulfation of the galactosyl residue is the key intermediate step in the modification of the D- and L-galactosyl residues of the polysaccharide by different modes of etherification.

Nucleoside diphosphate sugars are an important and widely distributed class of compounds in nature. Their importance lies in the fact that the sugar moiety of the nucleotide is activated and can undergo a variety of transformations. A most noteworthy metabolic function of the sugar nucleotides is their role in transglycosylation reactions. These reactions include such diverse processes as the synthesis of sucrose, callose, glycosides, and starch in higher plants; chitin in fungi and cell walls of bacteria; trehalose in yeast; and glycogen and glucuronides in animals (Leloir *et al.,* 1960; Hassid and Neufeld, 1962).

While the metabolic functions of the sugar nucleotides have been studied in animal tissues, yeast, and microorganisms, there is practically no information available regarding these compounds in marine algae.

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