Monosaccharides Constituents of the Walls of *Rhodopseudomonas spheroides*

By

Zensaku Yosizawa, Eiaki Tsutsumi and Tokutaro Sato

*From the Department of Biochemistry, School of Medicine
Tohoku University, Sendai*

(Received for publication, February 14, 1962)

Rhodopseudomonas spheroides produced large amounts of porphyrin when it was grown under light-anaerobic conditions. In contrast, the dark-aerobically grown cells have only trace amounts of bacteriochlorophyll.¹⁾

In an attempt to learn what differences exist between these cells grown in the different environments, the present authors studied on the monosaccharides constituents of the wall preparations of *R. spheroides*.

The results showed that the light-anaerobic walls contained glucose (1.5%), ribose (0.8%), methylpentoses (rhamnose and fucose) (5.0%, as rhamnose) and aminosugar (as glucosamine) (2.3% by Dische-Borenfreund reaction; 3.4%, by Elson-Morgan reaction). On the other hand, glucose (0.6%), ribose (0.7%), methylpentoses (rhamnose and fucose) (5.3%, as rhamnose) and aminosugar (as glucosamine) (2.1%, by Dische-Borenfreund reaction; 4.2%, by Elson-Morgan reaction) were also found in the dark-aerobic walls. Although there were some divergencies in monosaccharides contents between the two different wall preparations, it seemed not to be of significance.

Since Aaronson and Lessie²⁾ reported the presence of nonulosaminic acid (sialic acid) in this bacteria, the present authors examined the wall preparations by Direct-Ehrlich reaction, but our wall preparations could not be found to contain this substance. In addition, uronic acid, heptose, 2-deoxyaldose, 3-deoxyaldose were not detected in the walls, either.

MATERIALS AND METHODS

Materials—*R. spheroides*, originally obtained from Dr. Van Niel, was grown light-anaerobically or dark-aerobically in medium S of Lascelles,³⁾ usually for 72 hours at 30°C. Harvested cells were washed twice with 0.02 M phosphate buffer (pH 6.8), then suspended in 20 times the volumes of 0.04 M phosphate buffer (pH 6.8). The suspension was sonicated for 4 minutes with 10Kc. Kubota

吉沢善作, 堤 栄 昭, 佐藤徳太郎

Sonic oscillator. The sonicates were centrifuged at $10,000\times g$ for 30 minutes. To the resulting precipitates were added 10 times the volumes of water, and the suspension was centrifuged at 8,000 r.p.m. for 40 minutes. The precipitates thus obtained were washed several times with water, then with cold acetone until no more color was removed by acetone. The residue was washed with ether, then dried over anhydrous calcium chloride *in vacuo*. The dried wall debris was pulverized and used as the wall preparations.

Methods of Analysis—Glucose and ribose were estimated by the PHR_2 method of Masamune and Sakamoto⁴⁾ with thionalide-sulfuric acid reagent. Methylpentoses were determined by the method of Dische and Shettles⁵⁾ with cysteine-sulfuric acid reagent. Aminosugar was analyzed by the procedures of Yosizawa *et al.* with Dische-Borenfreund reaction⁶⁾ and with Elson-Morgan reaction.⁷⁾ Sialic acid (nonulosaminic acid) was examined by the Direct-Ehrlich reaction of Werner and Odin.⁸⁾ Uronic acid and heptose were checked by the procedures of Dische with carbazole-sulfuric acid reagent⁹⁾ and with cysteine-sulfuric acid reagent,¹⁰⁾ respectively. 2-Deoxyaldose and 3-deoxyaldose were examined by the methods of Fromme *et al.*¹¹⁾ using Webb's reaction.

Paper Chromatography — Paper chromatography was carried out on Toyo Roshi filter paper No. 3, in the descending way by the procedure of Masamune and Yosizawa¹²⁾ using the irrigation solvents (a) *n*-butylacetate-acetic acid-butanol-methanol-water (3:2:2:1:1, by volume),¹²⁾ (b) ethylacetate-pyridine-water (2:1:2, by volume, upper layer)¹³⁾ for non-aminosugars and (c) *n*-butanol-pyridine-dilute hydrochloric acid (5:3:2, by volume) (pH 5.0)¹⁴⁾ for aminosugar. The sugars on the papers were detected with aniline-hydrogen-phthalate reagent.¹⁵⁾

RESULTS

Paper Chromatograms — An amount (80~100 mg.) of the wall preparation was hydrolyzed with *N* sulfuric acid (2 ml.) in a boiling water bath for 5 hours. From the hydrolyzate, sulfuric acid was removed as barium sulfate. The resultant filtrate was passed through a column (2.2 $\times$ 15 cm.) of Amberlite IR 120 (H^+) and the column was washed with water (300 ml.). The effluent and washing were mixed together and concentrated to a small volume under reduced pressure. Non-aminosugars in the concentrate were examined by paper chromatography using the solvents (a) and (b) as described in METHODS. On the other hand, aminosugar absorbed on the Amberlite IR 120 resin was eluted with 5% cold aqueous ammonia (300 ml.). The eluant was concentrated to a small volume under reduced pressure below 35°C., then the condensate was concentrated several times from its methanol solution. The final residue was dissolved in 0.5 ml. water and the aminosugar in the solution was examined by paper chromatography using the solvent (c) as described in METHODS. The

Fig. 1. Paper chromatograms of non-aminosugars (A, B) and aminosugar (C) in the light-anaerobic walls (II) and in the dark-aerobic walls (III) of *R. spheroides*.

A: Developed with n-butylacetate-acetic acid-butanol-methanol-water (3:2:2:1:1, by volume) at 27°C for 24 hours. B: Developed with ethylacetate-pyridine-water (2:1:2, by volume, upper layer) at 27°C for 13 hours. C: Developed with n-butanol-pyridine-dilute hydrochloric acid (5:3:2, by volume) (pH 5.0) at 27°C for 40 hours. Coloration: Aniline-hydrogen-phthalate. Authentic sugars (I): 1, rhamnose; 2, ribose; 3, L-fucose; 4, glucose; 5, galactose; 6, glucosamine hydrochloride; 7, galactosamine hydrochloride.

paper chromatograms obtained are shown in Fig. 1.

As can be seen in Fig. 1, both wall preparations contained glucose, ribose, rhamnose, fucose and glucosamine.

Spectrophotometric Determinations *Glucose and Ribose*—An amount (8~12 mg.) of each wall preparation was hydrolyzed in a stoppered glass tube with 10 ml. *N* sulfuric acid in a boiling water bath for a certain periods of time as shown in TABLE I. The hydrolyzate was centrifuged and the resulting supernatant solution was subjected to the PHR_2 reaction of Masamune and Sakamoto.⁴¹ The spectra of PHR_2 reaction products of the light-anaerobic walls (hydrolyzate for 1 hour) and of the dark-aerobic walls (hydrolyzate for 12 hours) are shown in Fig. 2.

The amounts of glucose were calculated on the basis of the differences of the optical densities at 425 $m\mu$ and 465 $m\mu$ (D_{425} - D_{465}) as compared with that of

Fig. 2. Spectra of the PHR_2 reaction products of glucose, ribose and of the walls of *R. spheroides*.

1: Glucose (6 mg. % soln.). 2: Ribose (2 mg. % soln.). 3: Light-anaerobic walls (hydrolyzate with *N* sulfuric acid for 1 hour). 4: Dark-aerobic walls (hydrolyzate with *N* sulfuric acid for 12 hours).

authentic glucose. The contents of ribose were estimated on the differences of the optical densities at $393\text{ m}\mu$ and $438\text{ m}\mu$ ($D_{393}-D_{438}$) as compared with that of authentic sample. The values obtained are listed in TABLE I.

The analytical data showed that the light-anaerobic walls contained approximately 2.5 times as much as glucose than the dark-aerobic walls. On the other hand, the contents of ribose were similar in both wall preparations.

Methylpentose — Rhamnose and fucose were estimated on the hydrolyzates, prepared in the above glucose and ribose analyses, by the method of Dische and Shettles,⁵⁾ in which the differences of the optical densities at $400\text{ m}\mu$ and $430\text{ m}\mu$ ($D_{400}-D_{430}$) were read against the blank. The spectra of the reaction products of both wall preparations (hydrolyzates for 2 hours) are given in Fig. 3. The amounts of rhamnose and fucose (as rhamnose) in the walls are listed in TABLE II.

When the light-anaerobic walls were hydrolyzed with *N* sulfuric acid for 2 hours, maximal value of methylpentoses was obtained. No considerable difference

TABLE I. Amounts of Glucose and Ribose in the Walls of *R. spheroides*

Time of hydrolysis (hours)	Light-anaerobic walls		Dark-aerobic walls*	
	Amounts of glucose (%)	Amounts of ribose (%)	Amounts of glucose (%)	Amounts of ribose (%)
1	—	0.52	—	—
2	0.92	0.60	—	—
4	1.03	0.61	—	—
8	1.47	0.78	—	0.73
12	1.51	0.69	0.62	—
16	1.01	—	—	—
24	0.91	—	—	—

* Because of the shortage of the sample, analyses were carried out at the conditions giving the maximal values with the light-anaerobic walls.

Fig. 3. Spectra of the Dische-Shettles reaction products of rhamnose and of the walls of *R. spheroides*.

1: Rhamnose (2 mg. % soln.). 2: Light-anaerobic walls (hydrolyzate with *N* sulfuric acid for 2 hours). 3: Dark-aerobic walls (hydrolyzate with *N* sulfuric acid for 2 hours).

TABLE II. Amounts of Methylpentoses (Rhamnose and Fucose) in the Walls of *R. spheroides*.

Time of hydrolysis (hours)	Amounts of methylpentoses (as rhamnose) (%)	
	Light-anaerobic walls	Dark-aerobic walls*
1	4.69	—
2	4.98	5.26
4	4.39	—
8	4.29	—
12	4.19	—

* Signification, the same as in TABLE I.

was noticed in the methylpentose contents between the light-anaerobic walls and the dark-aerobic walls.

Aminosugar — 10 mg. of each wall preparation were hydrolyzed in a stoppered glass tube with 2 ml. of 1~6 *N* hydrochloric acid for 1~12 hours as shown in TABLE III. The hydrolyzate was neutralized with 4~0.5 *N* sodium hydroxide to about pH 6.4 under cooling in an ice-bath. Then the solution was diluted to 10 ml. with water. The contents of aminosugar(s) in the hydrolyzates were determined by Dische-Borenfreund reaction and Elson-Morgan reaction.

Aminosugar (as glucosamine) determined by Dische-Borenfreund reaction — The contents of aminosugar in the above hydrolyzates were analyzed by the procedure of Yosizawa⁶⁾ with Dische-Borenfreund reaction, in which the differences of the optical densities at 492 m μ and 425m μ (D_{492} - D_{425}) were read against the blank. The values obtained are listed in TABLE III. The spectra of the Dische-Borenfreund reaction products of the light-anaerobic walls (hydrolyzates with *N* hydrochloric acid for 1~12 hours) and of the dark-aerobic walls (hydrolyzate with 4 *N* hydrochloric acid for 2 hours) are shown in Fig. 4.

As shown in Fig. 4, the spectra of the reaction products of the hydrolyzates

Fig. 4. Spectra of the Dische-Borenfreund reaction products of glucosamine and of the walls of *R. spheroides*.

g: Glucosamine hydrochloride (2 mg. % soln.). 1, 2, 4, 8 and 12 are the hydrolyzates of the light-anaerobic walls with *N* hydrochloric acid for 1, 2, 4, 8 and 12 hours, respectively. 4' is the hydrolyzate of the dark-aerobic walls with 4 *N* hydrochloric acid for 2 hours.

of the light-anaerobic walls with *N* hydrochloric acid for 1, 2 and 4 hours had the absorption maxima at 530 $m\mu$, besides at 492 $m\mu$, but others showed single absorption peak at 492 $m\mu$. None of the spectra of the Dische-Borenfreund reaction products of the hydrolyzates of the walls with 2 *N*, 4 *N* and 6 *N* hydrochloric acid for 1~12 hours had the absorption maximum at 530 $m\mu$.

TABLE III. Amounts of Aminosugar (as Glucosamine) Estimated by Dische-Borenfreund Reaction in the Walls of *R. spheroides*.

Time of hydrolysis (hours)	Amounts of aminosugar (as glucosamine) (%)				
	Light-anaerobic walls				Dark-aerobic walls*
	Concentration of hydrochloric acid (<i>N</i>)				Concentration of hydrochloric acid (<i>N</i>)
	1	2	4	6	4
1	1.45	1.66	2.26	2.00	—
2	1.72	2.14	2.33	1.97	2.07
4	1.81	2.32	2.30	1.93	—
8	1.77	1.93	2.13	1.74	—
12	1.71	1.89	1.95	1.54	—

* Signification, the same as in TABLE I.

As can be seen in TABLE III, the contents of aminosugar (as glucosamine) determined by the Dische-Borenfreund reaction in both wall preparations were approximately the same.

Aminosugar (as glucosamine) determined by Elson-Morgan reaction — In case of aminosugar determination with Elson-Morgan reaction by the procedure of Masamune and Yosizawa,⁷⁾ the differences of the optical densities at 530 $m\mu$ and 450 $m\mu$ ($D_{530}-D_{450}$) were read against the blank. The values obtained are listed in TABLE IV. The spectra of the Elson-Morgan reaction products of the light-anaerobic walls (hydrolyzates with 1~6 *N* hydrochloric acid for 1~12 hours) and of the dark-aerobic walls (hydrolyzate with 4 *N* hydrochloric acid for 2 hours) are shown in Fig. 5.

As shown in Fig. 5, most of the spectra of the Elson-Morgan reaction products of the hydrolyzates of the walls had absorption maxima at 570 $m\mu$, besides 530 $m\mu$, whereas that of authentic glucosamine showed single absorption maximum at 535 $m\mu$. The hydrolyzates obtained by milder conditions were found to contain much more substances giving the absorption maximum at 570 $m\mu$ than those obtained by more drastic conditions.

The content of aminosugar (as glucosamine) estimated by Elson-Morgan reaction in the dark-aerobic walls was slightly much more than that in the light-anaerobic walls. The values obtained by Elson-Morgan reaction were much more than those obtained by Dische-Borenfreund reaction. The former values

Fig. 5-I.

Fig. 5-II.

Fig. 5-IV.

Fig. 5-VI.

Fig. 5. Spectra of the Elson-Morgan reaction products of glucosamine and of the walls of *R. spheroides*.

g: Glucosamine hydrochloride (2 mg. % soln.). I, II, IV and VI are represent the hydrolyzates of the light-anaerobic walls with 1 *N*, 2 *N*, 4 *N* and 6 *N* hydrochloric acid, respectively. 1, 4 and 12 show the hydrolyzates for 1, 4 and 12 hours, respectively. IV-2' is the hydrolyzate of the dark-aerobic walls with 4 *N* hydrochloric acid for 2 hours.

TABLE IV. Amounts of Aminosugar (as Glucosamine) Estimated by Elson-Morgan Reaction in the Walls of *R. spheroides*.

Time of hydrolysis (hours)	Amounts of aminosugar (as glucosamine) (%)				
	Light-anaerobic walls				Dark-aerobic walls
	Concentration of hydrochloric acid (N)				Concentration of hydrochloric acid (N)
	1	2	4	6	4
1	1.29	1.84	2.87	2.63	—
2	1.69	2.08	3.43	2.76	4.15
4	2.16	2.81	3.28	2.28	—
8	1.99	2.74	2.60	1.85	—
12	1.73	1.88	2.10	1.80	—

* Signification, the same as in TABLE I.

might be enlarged by the presence of the substances giving color with Elson-Morgan reagent.

Sialic acid (Nonulosaminic acid) — Sialic acid in the wall preparations was checked by Direct-Ehrlich reaction of Werner and Odin.⁸⁾ The spectra of the Direct-Ehrlich reaction products of the walls, shown in Fig. 6, had two absorption maxima at 550 m μ and 585 m μ , whereas that of authentic sialic acid showed two

Fig. 6. Spectra of the Direct-Ehrlich reaction products of sialic acid and of the walls of *R. spheroides*.

1: Sialic acid. 2: Light-anaerobic walls. 3: Dark-aerobic walls.

absorption peaks at $530\text{ m}\mu$ and $565\text{ m}\mu$. Moreover, the absorption maxima at $550\text{ m}\mu$ and $585\text{ m}\mu$ of the spectra were observed when the walls were treated directly with Ehrlich reagent without heating. Of the substances giving the reaction, one was identified as tryptophan,¹⁷⁾ but another was still unknown as yet. These findings indicate that no sialic acid is present in the wall preparations.

Uronic acid and Heptose — Uronic acid and heptose were checked by the procedures of Dische with carbazole-sulfuric acid reagent⁹⁾ and cysteine-sulfuric acid reagent,¹⁰⁾ respectively, on the hydrolyzate with *N* sulfuric acid for 2 hours at 100°C of the light-anaerobic walls. The spectra of these reaction products are shown in Figs. 7 and 8, respectively. These spectra of the reaction products did not show any specific absorption at $535\text{ m}\mu$ for uronic acid in Fig. 7, nor at $510\text{ m}\mu$ for heptose in Fig. 8, either.

2-Deoxyaldose and 3-Deoxyaldose — 2-Deoxyaldose and 3-deoxyaldose were examined by the method of Fromme *et al.*¹¹⁾ using Webb's reaction on the hydrolyzate of the light-anaerobic walls with *N* hydrochloric acid at 100°C for 2 hours. In the spectra of the Webb's reaction products, as shown in Fig. 9, no specific absorption at $560\text{ m}\mu$ for 2-deoxyaldose or 3-deoxyaldose was found.

Fig. 7. Spectra of the Dische's carbazole-sulfuric acid reaction products of glucuronic acid, glucose and of the walls of *R. spheroides*.

1: Glucuronic acid (10 mg. % soln.). 2: Glucose (6 mg. % soln.). 3: Light-anaerobic walls (hydrolyzate with *N* sulfuric acid for 2 hours).

Fig. 8. Spectra of the products of Dische's cysteine-sulfuric acid reaction for heptose of the light-anaerobic walls of *R. spheroides*.

1: Immediately after the reaction. 2: Standing for 2 hours after the reaction. 3: Standing for 20 hours after the reaction.

Fig. 9. Spectra of the products of Webb's reaction for 2-deoxyaldose (1) and for 3-deoxyaldose (2) of the light-anaerobic walls of *R. spheroides*.

DISCUSSION

As can be seen in RESULTS, the light-anaerobic walls contained glucose (1.5%), ribose (0.8%), methylpentoses (rhamnose and fucose) (5.0%, as rhamnose) and aminosugar (as glucosamine) (2.3%, by Dische-Borenfreund reaction; 3.4%, by Elson-Morgan reaction). On the other hand, glucose (0.6%), ribose (0.7%), methylpentoses (rhamnose and fucose) (5.3%, as rhamnose) and aminosugar (as glucosamine) (2.1%, by Dische-Borenfreund reaction; 4.2%, by Elson-Morgan reaction) were also found in the dark-aerobic walls. Although there were some divergencies in monosaccharides contents between the two different wall preparations, it seemed not to be of significance.

Most of the spectra of the Elson-Morgan reaction products of the hydrolyzates of the walls had the absorption maxima at 530 $m\mu$ and 570 $m\mu$, whereas that of authentic glucosamine showed single absorption peak at 535 $m\mu$. That is, the absorption maximum at 530 $m\mu$ of the Elson-Morgan reaction product of the aminosugar(s) in the walls shifted slightly from that of authentic glucosamine. Since the spectra of the Elson-Morgan reaction product of the C₃-substituted aminosugar is known to have the absorption maximum at 510 $m\mu$,¹⁶ there might be a possibility that a very small amount of the C₃-substituted aminosugar(s) such as muramic acid is also contained in the wall preparations, though muramic acid was not detected on the paper chromatograms. As to the substance(s) giving the absorption maximum at 570 $m\mu$, further study has been continued. On the other hand, it was found that both wall preparations contained the substances giving the color, after treatment directly with Ehrlich reagent without heating, which spectra had the absorption maxima at 550 $m\mu$ and 585 $m\mu$. One of the substances giving the reaction was identified as tryptophan,¹⁷ but another was still unknown as yet.

SUMMARY

The monosaccharides constituents of the walls of *Rhodopseudomonas spheroides* grown under light-anaerobic and dark-aerobic conditions were studied.

The light-anaerobic walls contained glucose (1.5%), ribose (0.8%), methylpentoses (rhamnose and fucose) (5.0%, as rhamnose) and aminosugar (as glucosamine) (2.3%, by Dische-Borenfreund reaction; 3.4%, by Elson-Morgan reaction). On the other hand, glucose (0.6%), ribose (0.7%), methylpentoses (rhamnose and fucose) (5.3%, as rhamnose) and aminosugar (as glucosamine) (2.1%, by Dische-Borenfreund reaction; 4.2%, by Elson-Morgan reaction) were also found in the dark-aerobic walls.

Sialic acid, uronic acid, heptose, 2-deoxyaldose and 3-deoxyaldose were not detected in the walls.

Acknowledgement The present authors were indebted to Prof. G.

Kikuchi for supply of the walls of *R. spheroides*, for which and for his encouragement throughout this work, they wish to express their acknowledgement.

References

- 1) Cohen-Bazire, G., Sistrom, W.R. & Stanier, R.Y., J. Cell. Comp. Physiol., 1957, **49**, 25.
 - 2) Aaronson, S. & Lessie, T., Nature, 1960, **186**, 719.
 - 3) Lascelles, J., Biochem. J., 1956, **62**, 78.
 - 4) Masamune, H. & Sakamoto, M., Tohoku J. Exper. Med., 1956, **63**, 345.
 - 5) Dische, Z. & Shettles, L.B., J. Biol. Chem., 1948, **175**, 595.
 - 6) Yosizawa, Z., Tohoku J. Exper. Med., 1961, **74**, 69.
 - 7) Masamune, H. & Yosizawa, Z., Tohoku J. Exper. Med., 1957, **65**, 169.
 - 8) Werner, I. & Odin, L., Acta Soc. Med. Upsaliensis, 1952, **57**, 230.
 - 9) Dische, Z., J. Biol. Chem., 1947, **167**, 189.
 - 10) Dische, Z., J. Biol. Chem., 1953, **204**, 983.
 - 11) Fromme, I., Lüderitz, O., Stierlein, H. & Westphal, O., Biochem. Zeitschr., 1958, **330**, 53.
 - 12) Masamune, H. & Yosizawa, Z., Tohoku. J. Exper. Med., 1953, **59**, 1.
 - 13) Kuhn, R., Gauhe, A. & Baer, H.H., Chem. Ber., 1954, **87**, 289.
 - 14) Masamune, H. & Yosizawa, Z., Tohoku J. Exper. Med., 1956, **64**, 53.
 - 15) Partridge, S.M., Nature, 1949, **164**, 443.
 - 16) Cifonelli, J.A. & Dorfman, A., J. Biol. Chem., 1958, **231**, 11.
 - 17) Yosizawa, Z. & Tsutsumi, E., Unpublished observation.
-