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PII: S0144-8617(97)00242-7

Carbohydrate Polymers **35** (1998) 271–278

0144-8617/98/$19.00+0.00

Biosynthesis of ^{13}C-labeled branched polysaccharides by *pestalotiopsis* from ^{13}C-labeled glucoses and the mechanism of formation

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(Received 10 February 1997; revised 5 September 1997; accepted 3 November 1997)

Biosynthesis of branched glucan by *Pestalotiopsis* from media containing D-(1-^{13}C)glucose, D-(2-^{13}C)glucose, D-(4-^{13}C)glucose, D-(6-^{13}C)glucose or a mixture of D-(1-^{13}C)glucose and D-(2-^{13}C)glucose was carried out to elucidate biosynthetic mechanism of branched polysaccharides. ^{13}C NMR spectra of the labeled polysaccharides were determined and assigned. Analysis of ^{13}C NMR spectra of glucitol acetates obtained from hydrolysates of the labeled branched polysaccharides indicated that transfer of labeling from C-1 to C-3 and C-6 carbons, from C-2 to C-1, C-3 and C-5 carbons, and from C-6 to C-1 carbon. From the results the percentages of routes via which the polysaccharide is biosynthesized are estimated. They show that the biosynthesis of the polysaccharide via the Embden–Meyerhof pathway and that from lipids and proteins are more active, and the pentose cycle is less active, than in the biosynthesis of cellulose and curdlan. As for the results, labeling at C-6 carbon in the branched polysaccharide cultured from D-(6-^{13}C)glucose was low, compared to that of cellulose and curdlan.

INTRODUCTION

Biosyntheses of ^{14}C- and ^{13}C-labeled cellulose and starch have been investigated by many researchers in order to elucidate the mechanism of their biosynthesis(Brown & Neish, 1954; Greathouse, 1953; Greathouse, 1957; Greathouse, Shirk & Minor, 1954; Minor, Greathouse, Shirk, Schwartz & Harris, 1954; Minor, Greathouse & Shirk, 1955; Shafizadeh & Wolfrom, 1955; Wolfrom, Webber & Shafizadeh, 1959; Gagnaire & Taravel, 1980; Gagnaire, Mancier & Vincendon, 1980). In our previous papers detailed studies on the biosynthesis of ^{13}C-labeled curdlan and cellulose have been reported, and the mechanism of biosynthesis has been discussed (Arashida et al., 1993; Kai et al., 1993; Kai et al., 1994). In this paper the biosynthesis of ^{13}C-labeled branched polysaccharides having β-(1 → 3)-D-glucan as main chain and (1 → 6)-D-glucosidic residues as side chains is carried out and the biosynthetic mechanism of the branched polysaccharides is discussed comparing with that of curdlan and cellulose. It has been known that such branched polysaccharides are potentiators of antitumor activity (Chihara, 1980; Matsuzaki et al., 1986a; Matsuzaki et al., 1986b) and their sulfated derivatives are anti-HIV agents in vitro (Yoshida et al., 1988; Yamamoto et al., 1990; Kaneko et al., 1990; Osawa et al., 1993). Suda, Ohno, Adachi and Yadomae (1995) reported biosynthesis of branched polysaccharides by *Sclerotinia sclerotiorum* from media containing D-(1-^{13}C)glucose or D-(2-^{13}C)glucose, and

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Table 1. Results of elemental analysis of branched polysaccharides

	Culture	Yield (g/l)	C (%)	H (%)	N (%)
P.crassiuscula	Standard	0.68	39.4	6.15	0.20
	No. 815	3.6	40.0	6.52	0.08
P. microspora	Standard	1.0	38.5	6.56	0.64

determined ^{13}C NMR spectra of the polysaccharides, but did not investigate their biosynthetic mechanism in detail.

EXPERIMENTAL

Preparation of ^{13}C-labeled branched polysaccharides and characterization of the polysaccharides

Pestalotiopsis crassiuscula (IFO 31055) and *Pestalotiopsis microspora* (IFO 31056) were used as microorganism producing branched polysaccharides. The incubated medium (30 ml) was added to fresh medium (100 ml) containing one of ^{13}C-labeled D-glucoses (0.1 g), unlabeled D-glucose (1.9 g), peptone (0.15 g), yeast extract (0.3 g), $MgSO_4 \cdot 7H_2O$ (0.05 g) and KH_2PO_4 (0.1 g). For the medium containing both D-(1-^{13}C)glucose and D-(2-^{13}C)glucose, total weight of labeled D-glucoses was 0.2 g and that of unlabeled D-glucose was 1.8 g. The culture was incubated for 3 days at 28°C with shaking at 140 strokes/min. The culture was filtered and the filterate was concentrated. The polysaccharide was precipitated with adding ethanol and the precipitate was purified by redissolving in distilled water and precipitation with adding ethanol. The results of elemental analysis of polysaccharides are shown in Table 1.

In order to reduce nitrogen contents in branched polysaccharides, a culture (No. 815: D-glucose 3.5 g, peptone 0.05 g, yeast extract 0.05 g, KH_2PO_4 0.1 g and $MgSO_4 \cdot 7H_2O$ 0.05 g) with low contents of nitrogen source was used. As shown in Table 1, it is known that low nitrogen culture is effective to reduce nitrogen content in the branched polysaccharide.

The branched polysaccharide once dried was hardly soluble in water and even in DMSO.

Methylation analysis of the branched polysaccharide by Hakomori method gave the result that the ratio of 2,3,4,6-tetra-O-methyl-D-glucitol acetate:2,4,6-tri-O-methyl-D-glucitol acetate:2,4-di-O-methyl-D-glucitol acetate = 1.4:1:1.8 for *P. crassiuscula* and 1:1:1 for *P.microspora*, indicating that ca. three branch glucosidic residues are at sixth position of every five glucosidic units in the main chain for *P. crassiuscula* and that a branch glucosidic residue at sixth position of every two glucosidic units in the main chain for *P. microspora*.

^{13}C NMR spectra of the branched polysaccharides

The ^{13}C NMR spectra of the branched polysaccharides were recorded for DMSO-d_6 solutions of the polysaccharides at 60°C with a JEOL EX-270 spectrometer under gated-decoupling.

Determination of labeling intensity

It was found that the determination of the labeling intensity of carbons in the branched polysaccharides from their ^{13}C NMR spectra did not give quantitative accuracy, due to scattering of signals, e.g., signal of C-6 carbon appears as C-6A, -6B and -6C, and due to the change of protonation of carbons, e.g., C-6A and -6C carbons are primary and C-6B carbon is secondary.

Therefore, the labeling intensity of the carbons was determined from glucitol acetates derived from the branched polysaccharides. The polysaccharides were hydrolyzed with 90% formic acid for 8 h at 100–110°C followed by the hydrolysis with 2*N* trifluoroacetic acid for 3 h at 100–110°C, reduced with sodium borohydride and then acetylated to glucitol acetate with acetic anhydride in pyridine.

The ^{13}C NMR spectra of glucitol acetates were determined with a JEOL EX-270 spectrometer or with a JEOL GX-400 spectrometer on $CDCl_3$ solutions at 30°C.

RESULTS AND DISCUSSIONS

^{13}C NMR spectra of the branched polysaccharides obtained from media containing ^{13}C-labeled glucoses

Fig. 1(A)–(D) show the ^{13}C NMR spectra determined on DMSO-d_6 solutions of branched polysaccharides (*P. crassiuscula*), which were obtained from unlabeled and C-1, C-4 and C-6 labeled glucoses, respectively. The assignment of the spectra was carried out according to our previous paper (Matsuzaki, Yamamoto, Sato & Enomoto, 1986c). It is clearly observed that the branched polysaccharide obtained from culture containing C-4 labeled glucose showed an apparent increase of peak intensities of 4C and 4AB + 6B, and that from C-6 labeled glucose indicated an increase of peak intensities of 6AC and 4AB + 6B, where A, B and C indicate unbranched main-chain, branched main-chain and side-chain units, respectively, and 4AB means 4A + 4B and so forth, as shown in Fig. 1. The results confirmed the previous assignment.

The determination of peak intensities of unlabeled polysaccharide, however, did not give constant ratios such as

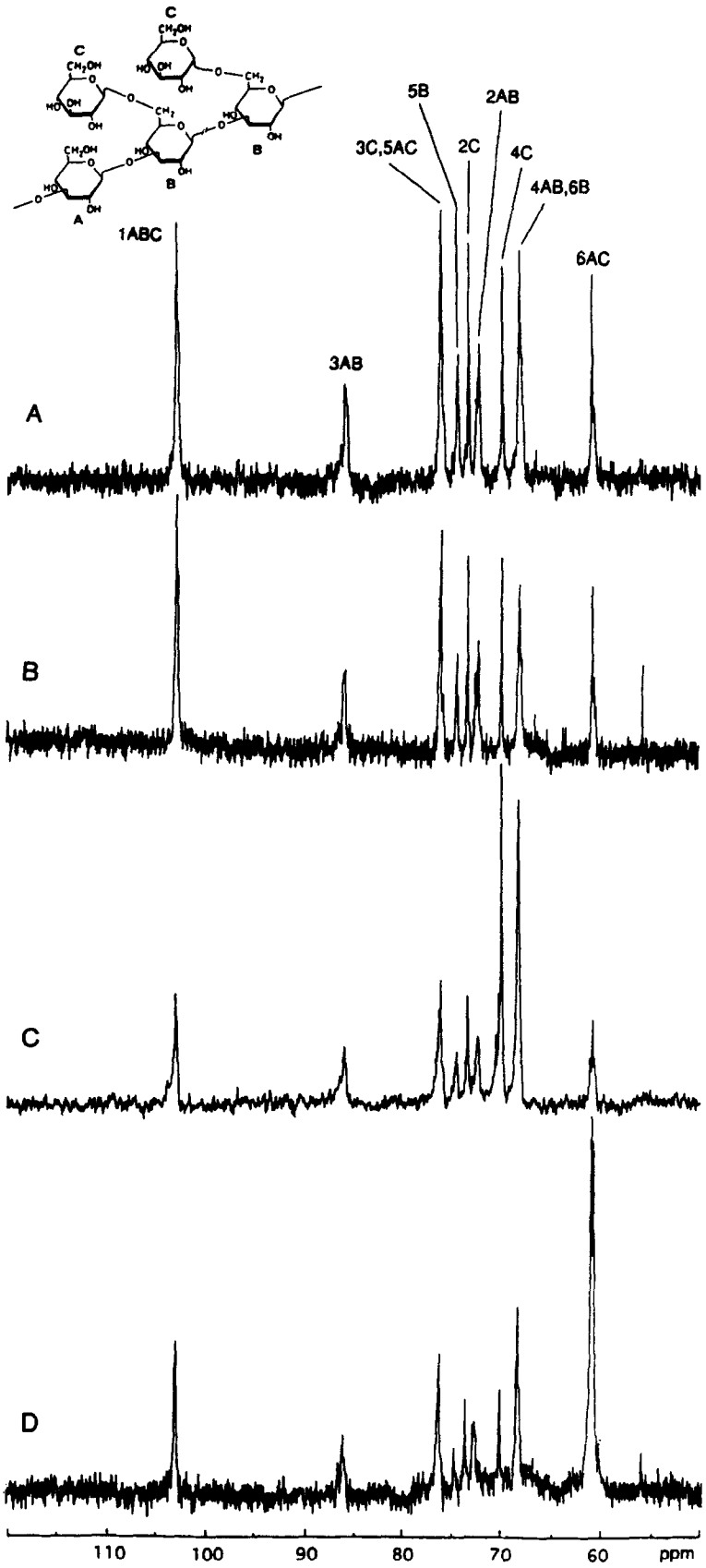

Fig. 1. 67.5 MHz ^{13}C NMR spectra of branched polysaccharides formed by *P.crassiuscula* from culture media containing unlabeled glucose (A), D-(1-^{13}C)glucose (B), D-(4-^{13}C)glucose (C) and D-(6-^{13}C)glucose (D).

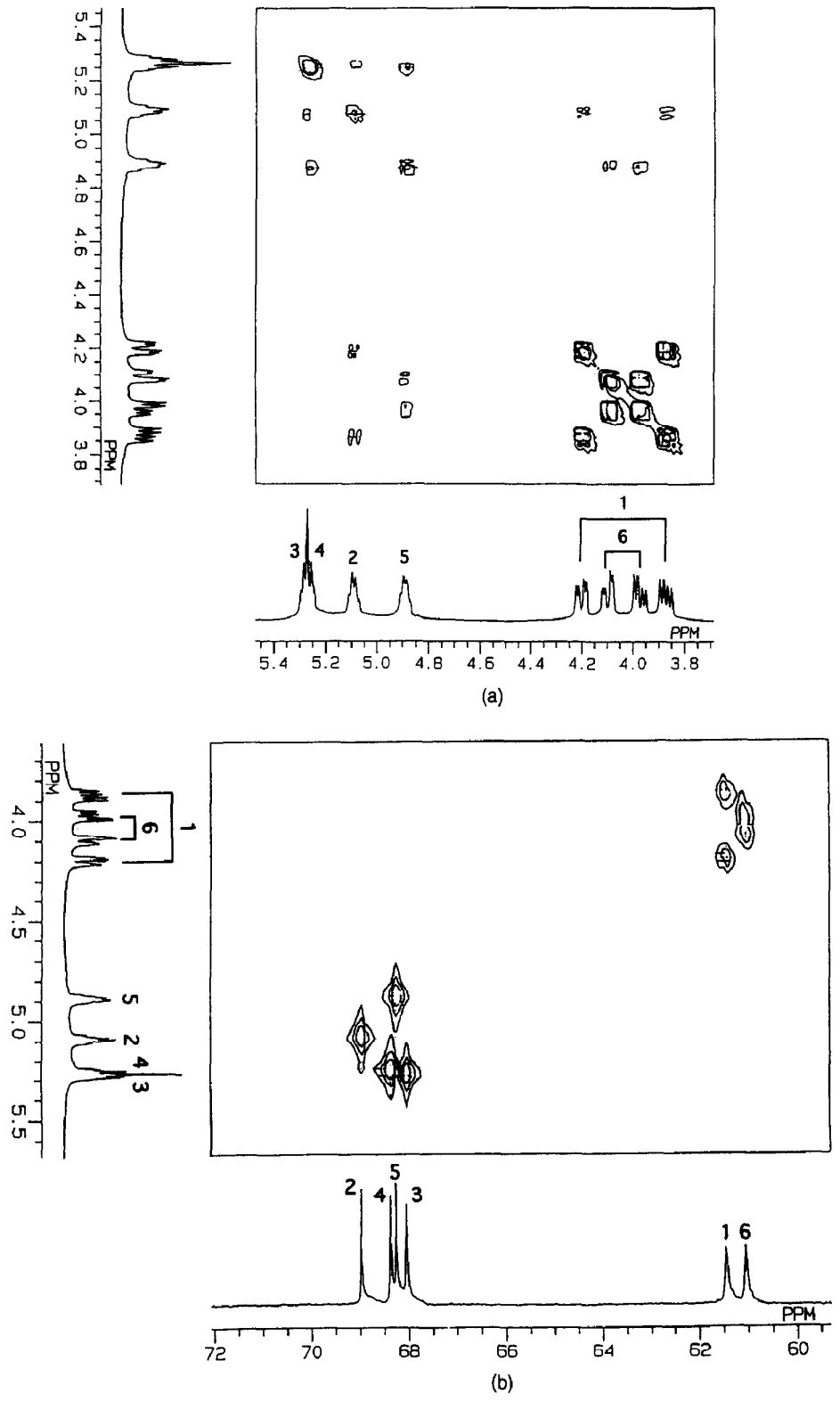

Fig. 2. (A) 400 MHz 2D COSY(^{1}H-^{1}H) spectra of D-sorbitol acetate; (B) 400 MHz 2D COSY(^{1}H-^{13}C) spectra of D-sorbitol acetate.

1ABC:(2AB + 2C):(6AC + 4C + 4AB + 6B) = 1:1:2 within experimental error (±4%), in spite of the fact that the measuring conditions such as delay time were varied. Therefore, direct determination of labeling intensity from ^{13}C NMR spectra of the branched polysaccharides was unsuccessful.

^{13}C NMR spectra of glucitol acetates and the determination of labeling intensity of carbons in the branched polysaccharides

In the ^{13}C NMR spectrum of D-glucitol acetate derived from glucose, that is D-sorbitol acetate, it is necessary to identify

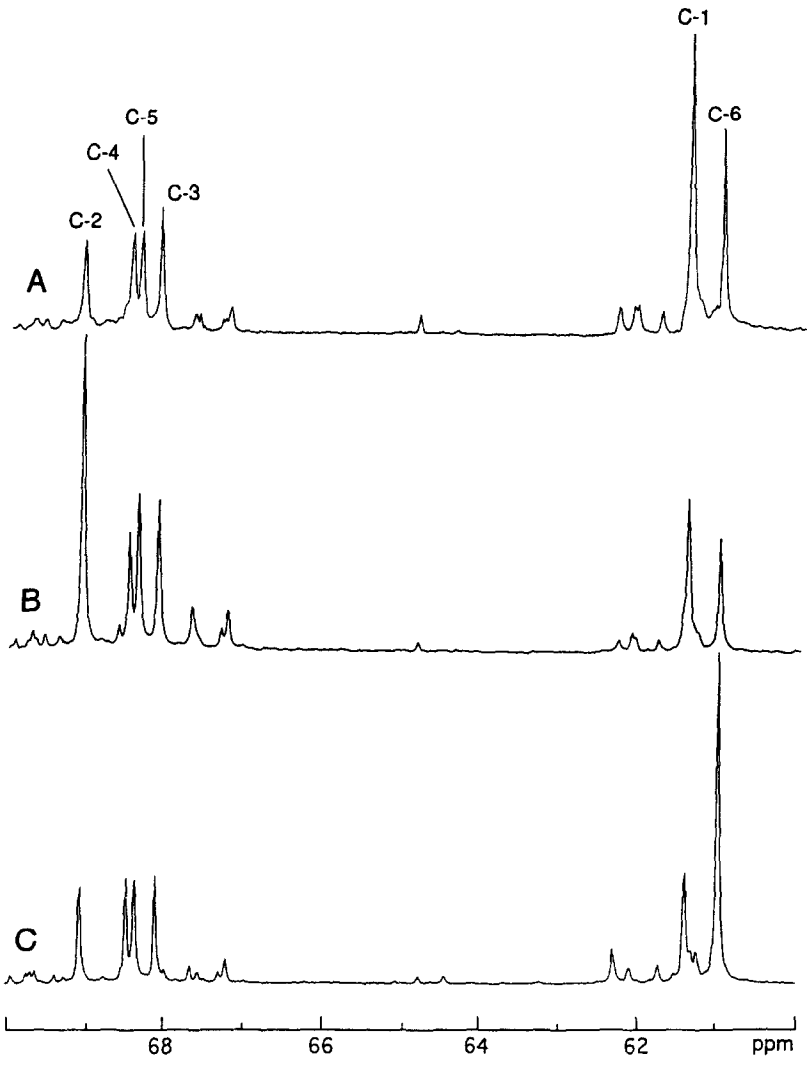

Fig. 3. 67.5 MHz ^{13}C NMR spectra of D-glucitol acetates prepared from branched polysaccharides (*P.microspora*) cultured in media containing (A) 5% of D-(1-^{13}C)glucose, (B) 5% of D-(2-^{13}C)glucose, and (C) 5% of D-(6-^{13}C)glucose (determined in $CDCl_3$ at 30°C).

carbons originated from D-glucose. The assignment was first carried out from 2D-NMR(^1H-^1H and ^1H-^{13}C COSY) spectra, as shown in Fig. 2. In the spectra the assignment for C-1 to C-6 carbons (or protons) can be reversed, that is, C-1 to C-6, C-2 to C-5, and C-3 to C-4. The conclusive assignment was given by a ^{13}C NMR spectrum of glucitol acetate obtained from (1-^{13}C)-labeled polysaccharide, as shown in Fig. 3(A). It was confirmed that glucitol acetate derived from unlabeled polysaccharide gives equal area for each carbon within experimental error (±4%) as shown in Table 2.

The ^{13}C NMR spectra of glucitol acetates obtained from branched polysaccharide (*P. microspora*) produced from culture media containing D-(1-^{13}C)glucose, D-(2-^{13}C)-glucose or D-(6-^{13}C)glucose are shown in Fig. 3(A)–(C), respectively. The peak intensities of ^{13}C carbons were measured and are shown in Table 2 for glucitol acetates obtained from labeled polysaccharides. In the table, IR (intensity ratio) is the relative ratio of ^{13}C carbon intensity in glucitol acetates and LR (labeling ratio) is the ratio of ^{13}C carbon atom intensity introduced into the polymer to the ^{13}C carbon atom intensity of C-1, C-2 or C-6 carbon of labeled glucose used for carbon source, defined by the following equation.

$$LR = \frac{(\text{IR of a carbon derived from labeled polysaccharide}) - 1}{(\text{IR of labeled carbon in glucose used for culture}) - 1}$$

where minus 1 is to eliminate the natural abundance.

The table shows that a large amount of transfer of labeling from D-(1-^{13}C)glucose to C-6 carbon of branched polysaccharide is observed for *Pestalotiopsis*, although both in curdlan and cellulose the transfer from C-1 to C-6 carbon was not observed. This suggests that the Embden–Meyerhof pathway is active in *Pestalotiopsis*, in contrast to organisms giving curdlan and cellulose. Namely, the process of isomerization of dihydroxy acetone 1-phosphate formed in the Embden–Meyerhof pathway to D-glyceraldehyde 3-phosphate, followed by neogenesis of glucose and formation of polysaccharide is active.

A small amount of transfer from C-1 carbon to C-3

Table 2. ^{13}C NMR peak intensity (IR%) and labeling ratio (LR%) of each carbon in branched polysaccharide (*P. microspora*) obtained from media containing ^{13}C-labeled glucoses

Labeled glucose in culture medium		C-1 (61.5)	C-2 (69.0)	C-3 (68.1)	C-4 (68.5)	C-5 (68.4)	C-6 (61.1)
Unlabeled	IR	1.07	1.00	1.00	1.05	1.05	1.01
D-(1-^{13}C)Glc	IR	3.83	1.02	1.29	1.02	1.00	1.97
	LR	57.8	0	5.9	0	0	19.8
D-(2-^{13}C)Glc	IR	1.84	3.05	1.39	1.00	1.48	1.08
	LR	17.1	41.8	7.96	0	9.80	0
D-(6-^{13}C)Glc	IR	1.54	1.02	1.02	1.00	1.05	3.74
	LR	11.0	0	0	0	0	55.9
	IR	1.80	1.00	1.08	1.00	1.08	4.07
	LR	16.3	0	0	0	0	62.7
D-(1-^{13}C)Glc + D-(2-^{13}C)Glc	IR	3.20	2.47	1.48	1.00	1.40	1.58
	LR	44.9	30.0	9.80	0	8.16	11.8

Values in parentheses under carbon number are ^{13}C NMR chemical shifts.

carbon in the branched polysaccharide was observed as in curdlan. This may have resulted from an unknown biosynthetic process.

The labeling in branched polysaccharides obtained from D-(6-^{13}C)glucose shows that there is a considerable amount of transfer of labeling from C-6 carbon to C-1 carbon. This suggests that in the Embden–Meyerhof pathway, isomerization of glyceraldehyde 3-phosphate to dihydroxy acetone 1-phosphate followed by neogenesis of glucose occur considerably. Furthermore, low labeling ratio at C-6 carbon is noted. Both in cellulose and curdlan, obtained from D-(6-^{13}C)-glucose as the labeling source, LR of C-6 carbon is very high (81–85%), indicating that the structure of C-4 to C-6 carbons in the starting glucose are well preserved in the glycolysis such as the pentose cycle and the Entner–Doudoroff pathway. Therefore, low LR of C-6 carbon of the branched polysaccharide is peculiar.

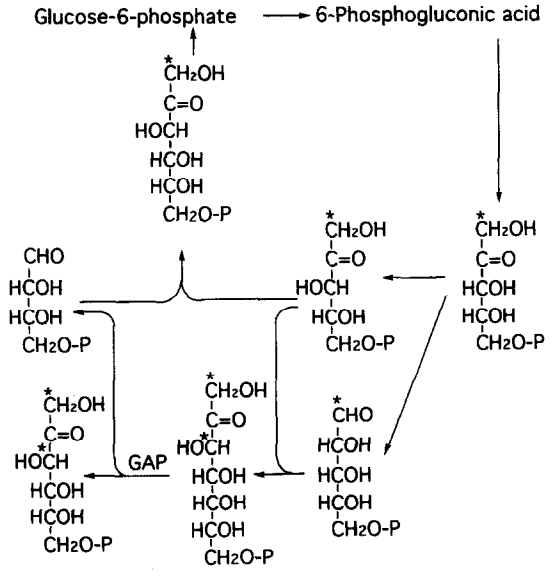

Fig. 4. Transfer of labeling from C-2 carbon to C-1 and C-3 carbons via the pentose cycle.

Use of D-(2-^{13}C)glucose as a labeling source resulted in scattering of labeling. Transfer of labeling from C-2 carbon to C-1 and C-3 carbons can be explained by the pentose cycle as with cellulose and curdlan, as shown in Fig. 4. Transfer of labeling from C-2 to C-5 carbon is explained by isomerization of dihydroxy acetone 1-phosphate to D-glyceraldehyde 3-phosphate in the Embden–Meyerhof pathway, followed by neogenesis of glucose, as with the transfer from C-1 to C-6 carbon.

Mechanism of biosynthetic process

From the above results, it is deduced that the polysaccharides are biosynthesized through the following routes:

(1) direct polymerization of glucose into polysaccharides;
(2) isomerization of dihydroxy acetone 1-phosphate formed in the Embden–Meyerhof pathway into D-glyceraldehyde 3-phosphate, followed by neogenesis of glucose and then polysaccharide;
(3) isomerization of D-glyceraldehyde 3-phosphate to dihydroxy acetone 1-phosphate in the Embden–Meyerhof pathway, followed by neogenesis of glucose and polysaccharide;
(4) neogenesis of glucose or fructose via the pentose cycle, followed by formation of polysaccharide;
(5) neogenesis of glucose from D-glyceraldehyde 3-phosphate formed in glycolysis of introduced glucose;
(6) isomerization of C-1 carbon to C-3 carbon via unknown biosynthetic process; and
(7) neogenesis of glucose from protein and lipids (Stryer, 1988), followed by formation of polysaccharide.

As in the previous paper, it is appropriate to discuss the mechanism of formation of polysaccharides separating glucosi-

Table 3. Percentages of routes via which the branched polysaccharide, cellulose and curdlan are formed

Route:	1	2	3	4	5	6	7
C-1 to C-3							
P. microspora	30–42	–	11–16	15–17	–	3	20–40
Cellulose							
No EtOH	37	–	20	30	–	0	13
1% EtOH	49	–	17	24	–	0	10
Curdlan	60	–	8	20	–	3	9
C-4 to C-6							
P. microspora	30–42	8–12	–	15–17	0–15	–	25–35
Cellulose							
No EtOH	37	3	–	30	15	–	15
1% EtOH	49	3	–	24	12	–	12
Curdlan	60	0	–	20	1		19

dic units into upper (C-1 to C-3) and lower (C-4 to C-6) halves. The upper half is biosynthesized via routes 1, 3, 4, 6 and 7. The lower half is biosynthesized via routes 1, 2, 4, 5 and 7.

Since the proportions of routes via which the branched polysaccharides are synthesized vary according to the species of microorganism and also according to their activity and culture conditions, strict quantitative determination may not be expected. For example, proportion of polysaccharide formed via isomerization of dihydroxy acetone 1-phosphate to D-glyceraldehyde 3-phosphate (route 2) should be the same for polysaccharides cultured from D-(1-^{13}C)glucose and D-(2-^{13}C)glucose. However, the proportions are 20% (LR at C-6) for the former and 10% (LR at C-5) for the latter. The proportion of route 3 obtained from LR of C-1 carbon of polysaccharides cultured from D-(6-^{13}C)glucose also vary from 11 to 16%. The proportion of direct synthesis (route 1) is obtained from LR (58%) of C-1 carbon of the polysaccharide cultured from D-(1-^{13}C)glucose, but it does not agree with LR (42%) of C-2 carbon of the polysaccharide obtained from D-(2-^{13}C)glucose.

Therefore, in order to avoid the effect of fluctuations of experimental conditions, an experiment of culture in an equal mixture of D-(1-^{13}C)glucose and D-(2-^{13}C)glucose as medium was carried out and the result is shown in Table 2. In the table, the proportion of direct synthesis (route 1) indicated by LR of C-2 carbon is 30%, which is near to LR (42%) of polysaccharide obtained from D-(2-^{13}C)glucose and is quite different from that (58%) obtained from D-(1-^{13}C)glucose. The proportions of route 2 are 8% and 12%, as shown by LRs of C-5 and C-6 carbons, respectively, and also they are near to LR (10%) of C-5 carbon of polysaccharide obtained from D-(2-^{13}C) glucose. Therefore, it is deduced that LRs of the polysaccharide obtained from D-(1-^{13}C)glucose are exceptionally high, presumably due to high activity of organism. In the following discussion, therefore, this exceptional case is not considered except for LR of C-3 carbon.

The proportion of polysaccharide produced via the pentose cycle (Fig. 4) is estimated from LR of C-1 carbon of the polysaccharide cultured from D-(2-^{13}C)glucose as 17%, which is near to LR(C-1) − LR(C-2) = 15%, where LR(C-1) and LR(C-2) are LRs of C-1 and C-2 carbons, respectively, of the polysaccharide cultured from a mixture of D-(1-^{13}C)glucose and D-(2-^{13}C)glucose, and indicate direct synthesis + pentose cycle, and direct synthesis, respectively, It is noted that in the pentose cycle, the structure of the lower half of the glucosidic units is maintained. The proportion of route 6 is estimated as half of LR (6%) of C-3 carbon of the polysaccharide obtained from D-(1-^{13}C)glucose taking high activity of organism into consideration. The proportion of route 7 for the upper half of glucosidic units in the polysaccharide is estimated by subtracting the known proportions from 100%.

The proportion of route 5 for the lower half of the glucosidic units in polysaccharide is estimated by subtracting the proportions of direct synthesis (30–42%) and the pentose cycle (15–17%) from LRs (56–63%) of C-6 carbon of the polysaccharides obtained from D-(6-^{13}C)glucose. Since dilution of LR (37–44%) of C-6 carbon in the polysaccharides obtained from D-(6-^{13}C)glucose results from route 2 and route 7, the proportion of route 7 for the lower half of the glucosidic units in the polysaccharide is estimated as 25–35%.

Table 3 lists percentages of the routes via which the branched polysaccharide is biosynthesized, together with those for cellulose and curdlan (Kai et al., 1994). As discussed above, the values are rough numbers and change according to activity of microorganism and culture conditions.

In the table, it is noted that percentages of routes 2 and 7 are high and that of route 4 is low for the branched polysaccharide, compared with those for cellulose and curdlan. This indicates that the Embden–Meyerhof pathway is active and the pentose cycle is not. In contrast to cellulose and curdlan, in which the structure of lower half of the introduced glucose is well preserved (more than 82%), low LR values (56 and 63%) at C-6 carbon in the branched polysaccharides cultured from D-(6-^{13}C)glucose are observed. The low LR value at C-6 carbon in the branched polysaccharides cultured from D-(6-^{13}C)glucose is ascribed to route 2, which results in dilution of labeling at C-6 carbon by the isomerization of unlabeled dihydroxy acetone

1-phosphate to D-glyceraldehyde 3-phosphate, and high values of route 7, which indicates synthesis of polysaccharide from lipids and proteins.

ACKNOWLEDGEMENTS

We wish to thank Prof. N. Ohno of Tokyo College of Pharmacy, Hachioji, Tokyo, for kindly giving us information on culture of microorganisms and purification of polysaccharides.

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