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Analysis of the biosynthetic process of cellulose and curdlan using ^{13}C-labeled glucoses

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In order to elucidate the biosynthetic process of cellulose and curdlan, ^{13}C-labeled polysaccharides were biosynthesized by *Acetobacter xylinum* (IFO 13693) and *Agrobacterium* sp. (ATCC 31749), from culture media containing D-(1-^{13}C)glucose, D-(2-^{13}C)glucose, D-(4-^{13}C)glucose, or D-(6-^{13}C)glucose as the carbon source, and their structures were determined by ^{13}C NMR spectroscopy. The labeling was mainly found in the original position, indicating direct polymerization of introduced glucoses. In addition, the transfer of labeling from C-2 to C-1, C-3 and C-5, from C-4 to C-1, C-2 and C-3, and from C-6 to C-1 was found in celluloses. In curdlan, the transfer of labeling from C-1 to C-3, from C-2 to C-1 and C-3, from C-4 to C-1, C-2 and C-3, and from C-6 to C-1 and C-3 was observed. From analysis of this labeling, the biosynthetic process of cellulose and curdlan was explained as involving six routes. The percentages of each route via which cellulose or curdlan is biosynthesized were estimated for upper (C-1 to C-3) and lower portions (C-4 to C-6) of glucosidic units in the polysaccharides. It is noted that very few polysaccharides are formed via the Embden–Meyerhof pathway. The lower half (C-4 to C-6) structure of introduced glucoses is well preserved in the polysaccharides.

INTRODUCTION

Cellulose is the most important reproducible and biodegradable polymeric material. Recently, bacterial cellulose produced by *Acetobacter xylinum* is attracting interest due to its high modulus and tensile strength. It is now produced in Japan and applied to an acoustic plate in headphones of high quality. Other applications are being sought (Yamanaka & Watanabe, 1992).

Curdlan is also a new material (Harada *et al.*, 1966) when compared with cellulose. It has been used in food additives, and recently its mass production has begun so that it can be applied as an additive for high-performance concrete (Harada *et al.*, 1993). Furthermore, it is the main chain polysaccharide for many antitumor polysaccharides (Chihara, 1980; Matsuzaki *et al.*, 1986*a,b*), and chemically modified curdlan showed strong anti-HIV activity *in vitro* (e.g. Yoshida *et al.*, 1988; Kaneko *et al.*, 1990; Yamamoto *et al.*, 1990; Osawa *et al.*, 1993). Therefore, elucidation of the biosynthetic process of those polysaccharides would lead to their effective production and/or modification.

The biosynthetic process of cellulose from bacteria or cotton boll has been investigated by many researchers using either ^{14}C-labeled glucoses or low molecular

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weight compounds as the carbon source (Greathouse, 1953, 1957; Greathouse *et al.*, 1954; Minor *et al.*, 1954, 1955; Shafizadeh & Wolfrom, 1955; Wolfrom *et al.*, 1959). It has been explained by direct polymerization of introduced glucose and neogenesis of glucose via the Embden–Meyerhof pathway, followed by the formation of cellulose. Investigations using ^{13}C-labeled glucose as the carbon source are limited (Gagnaire & Taravel, 1980; Gagnaire *et al.*, 1980), but pathways other than the Embden–Meyerhof pathway have been considered. However, biosynthesis of labeled curdlan had not been reported, in spite of its industrial and biomedical importance. In our previous papers (Arashida *et al.*, 1993; Kai *et al.*, 1993), biosynthesis of ^{13}C-labeled cellulose and curdlan from culture media containing D-(1-^{13}C)glucose, D-(2-^{13}C)glucose, or D-(6-^{13}C)glucose as the carbon source has been carried out, their structures elucidated by ^{13}C-NMR spectroscopy, and the mechanism of their biosynthesis discussed.

Herein the biosynthesis of cellulose and curdlan from culture media containing D-(4-^{13}C)glucose is reported. In addition, complementary experiments for previous investigations were carried out, and the previously reported biosynthetic process is revised and discussed in more detail.

EXPERIMENTAL

Curdlan and cellulose were biosynthesized with *Agrobacterium* sp. (ATCC 31749) and *Acetobacter xylinum* (IFO 13693), respectively, from culture media containing 5 or 10% of D-(1-^{13}C), D-(2-^{13}C), D-(4-^{13}C), or D-(6-^{13}C)glucose in unlabeled glucose as previously described (Arashida *et al.*, 1993; Kai *et al.*, 1993). The purity of the ^{13}C-labeled glucoses is 99.0, 99.3, 99.0 and 98.7%, respectively. Cellulose synthesis was examined in the presence and absence of ethanol (1%). The addition of ethanol into the culture for curdlan was not effective for the production of polysaccharide.

The ^{13}C NMR spectra of curdlan were recorded for solutions in $(CD_3)_2SO$ at 60°C and those of cellulose for solutions in *N*-methyl-morpholine oxide/$(CD_3)_2SO$ at 90°C with a JEOL EX-270 spectrometer under gated decoupling. Figure 1 shows ^{13}C NMR spectra of cellulose and curdlan obtained from culture media containing D-(4-^{13}C)glucose.

RESULTS AND DISCUSSIONS

Biosynthetic process of cellulose

In this investigation, the biosynthesis of cellulose from culture medium containing D-(4-^{13}C)glucose was carried out. Furthermore, biosynthesis of cellulose from culture medium containing D-(1-^{13}C)glucose was reinvestigated

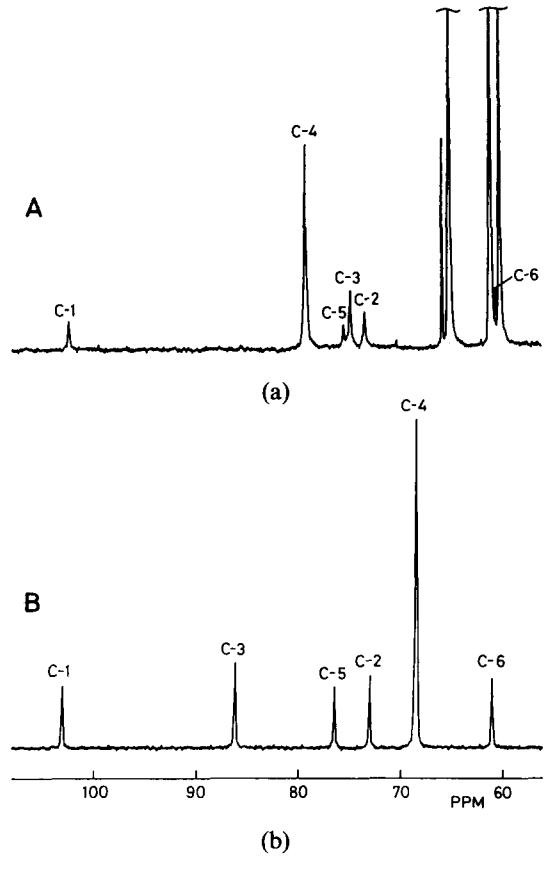

Fig. 1. ^{13}C NMR spectra of (a) cellulose and (b) curdlan obtained from culture media containing 10% and 5% of D-(4-^{13}C)glucose in unlabeled glucose, respectively.

and that from culture medium containing D-(6-^{13}C)glucose with the addition of ethanol was augmented.

^{13}C-labeling ratio (LR, the ratio of introduced ^{13}C intensity, i.e. observed intensity minus the natural abundance of a carbon to that of the labeled carbon in the original labeled glucose) of each carbon in labeled cellulose was determined from the NMR spectra, the results being shown in Table 1. The labeling was found mainly in the original position, indicating direct polymerization of introduced glucoses, especially with the addition of ethanol in the culture media.

It is seen from Table 1 that the LR of C-6 carbon in cellulose obtained from D-(6-^{13}C)glucose agrees well with the LR of C-4 carbon in cellulose obtained from D-(4-^{13}C)glucose (both 85% with the addition of ethanol, and 82% and 77% without the addition of ethanol), indicating that the C-4 to C-6 structure of the introduced glucoses are well preserved. Dilution of labeling (15% with the addition of ethanol, and approx. 20% without the addition of ethanol) may be explained partly (3%) by the isomerization of unlabeled dihydroxyacetone 1-phosphate to D-glyceraldehyde 3-phosphate by the Embden–Meyerhof pathway, followed by neogenesis of glucose and then cellulose. Most of the

Table 1. ^{13}C-labeling ratio (LR, %) for each carbon in cellulose obtained from ^{13}C-labeled glucose

	Labeling[a]	C-1	C-2	C-3	C-4	C-5	C-6	Total
1	No EtOH	37·2	0·7	0	0	−0·6	0·5	37·8
	1% EtOH	48·6	0·9	0·8	0	0	0	50·3
2	No EtOH	29·7	15·9	18·5	0	2·8	0	66·9
	1% EtOH	23·5	27·8	14·2	0	2·8	n.d.	68·3
4	No EtOH	3·5	11·2	22·0	76·9	0	n.d.	113·6
	1% EtOH	3·5	9·2	16·3	84·8	0	n.d.	113·8
6	No EtOH	20·2	0	0·6	0	−0·5	81·6	101·9
	1% EtOH	17·0	1·4	0·5	0	0	84·9	103·8

[a] Labeling carbon in glucose. The LR value of each carbon in celluloses obtained from D-(1-^{13}C), D-(2-^{13}C), or D-(6-^{13}C) glucose was calculated assuming that LR values of C-4 carbon are zero, i.e. only natural abundance, except those in cellulose obtained from D-(4-^{13}C) glucose, in which the LR values of C-5 carbon were taken as zero. Total LR values were obtained assuming that the LR values of C-6 carbon not determined (n.d.) are zero.

dilution (12% with the addition of ethanol and 17% without ethanol), however, is caused by conversion of the organism itself along with organic matters such as yeast extracts and peptone added to the culture to polysaccharide, since lower organisms can easily convert protein and lipids to carbohydrates (Stryer, 1988).

The LR (37% without the addition of ethanol and 49% with the addition of ethanol) of C-1 carbon in cellulose obtained from D-(1-^{13}C), indicates direct polymerization. The values are quite high compared with the previous values (17% and 37%, respectively) (Arashida *et al.*, 1993), indicating that the extent of direct polymerization changes with bacterium activity. It is also noted that with the addition of ethanol, the direct polymerization increased and the percentage of glycolysis decreased (discussed later).

With respect to cellulose obtained from D-(6-^{13}C)glucose, the LR of C-6 carbon is higher and that of C-1 carbon is lower with the addition of ethanol than corresponding LR values without the addition of ethanol, indicating the increase of direct polymerization and decrease of glycolysis. The transfer of labeling from C-6 to C-1 carbon has been explained by isomerization of D-(3-^{13}C)glyceraldehyde 3-phosphate to (1-^{13}C)-dihydroxyacetone 1-phosphate by the Embden–Meyerhof pathway, followed by neogenesis of glucose (Wolfrom *et al.*, 1959). Gromet *et al.* (1957), however, reported that *Acetobacter xylinum* lacks phosphofructokinase to convert fructose 6-phosphate to fructose 1, 6-bisphosphate in the Embden–Meyerhof pathway. In that case, dihydroxyacetone 1-phosphate is produced only from D-glyceraldehyde 3-phosphate formed in various pathways (such as the pentose cycle, the Entner–Doudoroff pathway) and not from glycolysis via the Embden–Meyerhof pathway. On the other hand, transfer of labeling (3%) from C-2 to C-5 carbon in cellulose obtained from D-(2-^{13}C)glucose can be explained by isomerization of (2-^{13}C)-dihydroxyacetone 1-phosphate produced via the Embden–Meyerhof pathway to D-(2-^{13}C)glyceraldehyde 3-phosphate, followed by neogenesis of glucose. Therefore, glycolysis via the Embden–Meyerhof pathway may be present in *A. xylinum* to a small extent, which would also explain the transfer of labeling from C-2 to C-5 carbon being much lower than the transfer of labeling from C-6 to C-1 carbon.

In cellulose obtained from D-(4-^{13}C)glucose, the labeling was found at C-1 to C-3 carbons. Transfer of labeling from C-4 to C-3 carbon is explained by reversible isomerization of D-(1-^{13}C)glyceraldehyde 3-phosphate (formed via the Entner–Doudoroff pathway, the pentose cycle or the Embden–Meyerhof pathway, if any) to (3-^{13}C)-dihydroxyacetone 1-phosphate, as well as the transfer of labeling from C-6 to C-1 carbon. The labeling at C-2 carbon can be explained by the pentose cycle of C-3 labeled glucose (or fructose), as shown in Fig. 2. The labeling at C-1 carbon may be caused by the pentose cycle of C-2 labeled glucose (or fructose) as shown in the previous paper (Arashida *et al.*, 1993). Therefore, without the addition of ethanol, the total LR of C-3 reaches at least 37% (the sum of LR values of C-1 to C-3 carbon; a part of C-1 labeling may be lost in the pentose cycle), thus indicating considerable isomerization.

With the addition of ethanol, the sum of LR (29%) of C-1 to C-3 carbon is lower than that (37%) without the addition of ethanol, indicating a decrease of glycolysis. A similar trend is observed in celluloses obtained from D-(2-^{13}C)glucose with and without the addition of ethanol.

The biosynthetic process of cellulose is interpreted by the following routes:

(1) direct polymerization of introduced glucose;
(2) isomerization of dihydroxyacetone 1-phosphate formed via the Embden–Meyerhof pathway to

D-glyceraldehyde 3-phosphate, followed by neogenesis of glucose and then cellulose;

(3) isomerization of D-glyceraldehyde 3-phosphate formed via various pathways to dihydroxyacetone 1-phosphate, followed by neogenesis of glucose and then cellulose;

(4) neogenesis of glucose or fructose via the pentose cycle, followed by formation of cellulose;

(5) neogenesis of glucose from D-glyceraldehyde 3-phosphate formed in glycolysis of introduced glucose; and

(6) neogenesis of glucose and then cellulose from protein and lipids.

In our previous papers (Arashida *et al.*, 1993; Kai *et al.*, 1993), the percentages of routes via which cellulose are formed were determined for glucosidic units as a whole. However, it now seems reasonable to consider the mechanism of formation of cellulose, separating glucosidic units into the upper (C-1 to C-3) and lower (C-4 to C-6) halves.

The upper half of cellulose is formed via routes (1), (3), (4) and (6). The percentage of routes (1), (3) and (4) can be estimated from LR values of celluloses obtained from D-(1-^{13}C)glucose, D-(6-^{13}C)glucose and D-(2-^{13}C)glucose, respectively, as discussed above and in a previous paper. Therefore, the percentage of route (6) can be estimated as shown in Table 2. It should be mentioned that a portion of cellulose is produced via more than one route such as routes (3) and (4), therefore, the percentage shown is not a strict one and changes with culture conditions.

The lower half portion of glucosidic units in cellulose are formed via routes (1), (2), (5) and (6). The percentage of routes (1), (2) and (6) is estimated from LR values of celluloses obtained from D-(1-^{13}C)glucose, D-(2-^{13}C)glucose and those of celluloses obtained from D-(4-^{13}C)glucose or D-(6-^{13}C)glucose, as already discussed. These results are shown in Table 2. The percentage of route (5) is obtained by subtraction from 100%. It is seen from the table that the dilution of labeling by protein and lipids is a little more for the lower half portion of glucosidic units in cellulose than for the upper half.

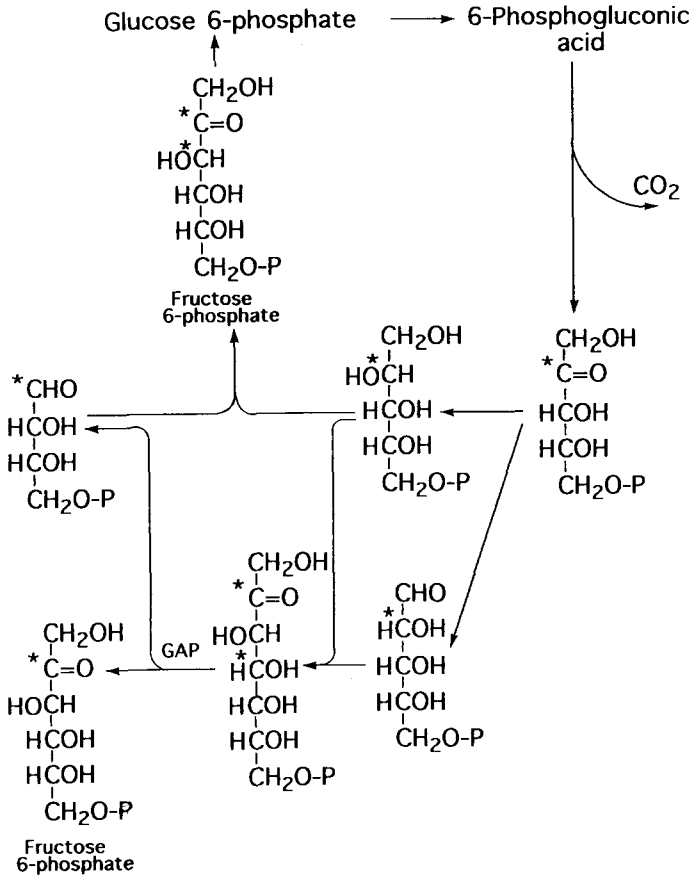

Fig. 2. Transfer of labeling form C-3 to C-2 carbon via the pentose cycle.

Biosynthesis of curdlan

In addition to the biosynthesis of curdlan from culture media containing D-(1-^{13}C)glucose, D-(6-^{13}C)glucose or D-(2-^{13}C)glucose, its biosynthesis was carried out from culture medium containing D-(4-^{13}C)glucose in this investigation. Figure 1(b) shows a ^{13}C NMR spectrum of curdlan obtained from D-(4-^{13}C)glucose.

The labeling ratio of each carbon obtained from ^{13}C NMR spectra is shown in Table 3. The main differences between transfer of labeling in cellulose and that in curdlan are the following: (1) transfer of labeling from C-1 to C-3 carbon in curdlan obtained from D-(1-^{13}C)glucose, and (2) transfer of labeling from C-2 to C-5 carbon in cellulose obtained from D-(2-^{13}C)-glucose.

The transfer of labeling from C-1 to C-3 carbon was ascertained by repeating the NMR measurement. The exact nature of this transfer is not known at present.

The lack of the transfer from C-2 to C-5 carbon and that from C-1 to C-6 carbon in curdlan indicates that the Embden–Meyerhof pathway is not used by *Agro-*

Table 2. Percentages of routes via which cellulose or curdlan is formed

	Route	1	2	3	4	5	6
C-1–C-3							
Cellulose	No EtOH	37	—	20	30	—	13
	1% EtOH	49	—	17	24	—	10
Curdlan[a]		60	—	8	20	—	9
C-4–C-6							
Cellulose	No EtOH	37	3	—	—	43	17
	1% EtOH	49	3	—	—	36	12
Curdlan		60	0	—	—	21	19

[a]For curdlan, 3% of polymer was biosynthesized via ^{13}C-1 to ^{13}C-3 rearrangement.

Table 3. ^{13}C-labeling ratio (LR, %) for each carbon in curdlan obtained from ^{13}C-labeled glucoses

Labeling[a]	C-1	C-2	C-3	C-4	C-5	C-6	Total
1	57·0	1·1	3·3	0	0	0	61·4
2	19·3	61·3	10·8	0	0	0	91·4
4	1·6	1·8	8·1	87·3	0	0	98·8
6	8·4	0	1·6	0	0	81·2	91·2

[a] Labeling carbon in glucose.

bacterium sp. (ATCC 31749). Arthur *et al.* (1975) reported that five out of seven *Agrobacterium* species do not utilize the Embden–Meyerhof pathway for glucose catabolism, and that the pentose cycle and the Entner–Doudoroff pathway are commonly used. It is therefore conceivable that *Agrobacterium* sp. (ATCC 31749) does not utilize the Embden–Meyerhof pathway, and it is noted that lack of Embden–Meyerhof pathway in *Agrobacterium* was proved from the analysis of polysaccharides formed.

As with cellulose, an LR value (87%) of C-4 carbon in curdlan obtained from D-(4-^{13}C)glucose agrees well with an LR value (81%) of C-6 carbon in curdlan obtained from D-(6-^{13}C)glucose, indicating preservation of the lower half structure of the introduced glucoses.

Transfer of labeling from C-4 to C-3 carbon in curdlan obtained from D-(4-^{13}C)glucose is explained by isomerization of D-(1-^{13}C)glyceraldehyde 3-phosphate to (3-^{13}C)-dihydroxyacetone 1-phosphate, followed by neogenesis of glucose and the formation of curdlan, as discussed above. Transfer of labeling to C-1 and C-2 carbons is explained by the pentose cycle, as discussed with cellulose. This transfer (total 12%), however, is much lower than that in cellulose.

It is noted that a small transfer of labeling to C-3 carbon is observed in curdlan obtained from D-(6-^{13}C)glucose. As with cellulose, the percentage of each route via which curdlan was synthesized, was estimated as shown in Table 2. It is noteworthy that for curdlan the percentage of direct polymerization is very high. Therefore, the biosynthesis of curdlan via glycolysis is low compared with that of cellulose.

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