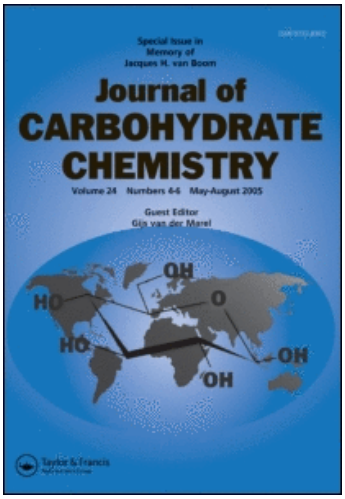

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Biosynthesis of Cellulose From Culture Media Containing ^{13}C-Labeled Glucose as a Carbon Source

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BIOSYNTHESIS OF CELLULOSE FROM CULTURE MEDIA CONTAINING ^{13}C-LABELED GLUCOSE AS A CARBON SOURCE

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ABSTRACT

^{13}C-Labeled celluloses were biosynthesized by *Acetobacter xylinum* (IFO 13693) from culture media containing D-[1-^{13}C]glucose, D-[6-^{13}C]glucose, or D-[2-^{13}C]glucose as a carbon source with or without addition of ethanol, and their structures were analyzed by ^{13}C NMR spectroscopy. The labeling was mainly found in the original position, that is C-1, C-6 or C-2, in cellulose obtained from D-[1-^{13}C]glucose, D-[6-^{13}C]-glucose or D-[2-^{13}C]glucose, respectively, indicating direct polymerization of introduced glucoses, especially with addition of ethanol in culture medium. Furthermore, C-1 carbons in cellulose obtained from D-[6-^{13}C]glucose, and C-1, C-3 and C-5 carbons in cellulose obtained from D-[2-^{13}C]glucose were labeled. From the analysis of labeling, the mechanism of biosynthesis of cellulose was explained by (1) direct synthesis from glucose, (2) isomerization and rearrangement of trioses formed in the Embden-Meyerhof pathway, Entner-Doudoroff pathway, or pentose cycle, followed by neogenesis of glucose and formation of cellulose, (3) the pentose cycle and (4) neogenesis of glucose from fragments produced in various pathways of glycolysis, followed by formation of cellu-

lose. It is noted that ^{13}C-labeling at C-6 and C-2 carbons in the starting glucoses is well preserved in C-6 and C-1 carbons, and C-1 to C-3 carbons, respectively, in celluloses obtained.

INTRODUCTION

Biosynthesis of cellulose using ^{14}C-labeled D-glucose and other low molecular weight compounds as a carbon source have been carried out by many investigators, and the mechanism of biosynthesis of cellulose has been discussed.[1-13] Recently, the biosynthesis of cellulose with deuterium-labeled D-glucose and glycerol was reported.[14,15] However, investigations on the labeling with ^{13}C-carbon are few, with the exception of those of French and coworkers,[16,17] in spite of the fact that the labeling is easier and the analysis of labeling is possible for cellulose itself without further reaction such as hydrolysis. In this paper, labeling of cellulose with D-[1-^{13}C]glucose, D-[6-^{13}C]glucose or D-[2-^{13}C]glucose as a carbon source and the labeling analysis with ^{13}C NMR spectroscopy have been investigated. The mechanism of biosynthesis of cellulose is discussed. The results were compared with those from curdlan.[18]

RESULTS AND DISCUSSION

FIG.1 shows the ^{13}C NMR spectra of celluloses obtained from cultures containing D-[1-^{13}C]- glucose, D-[6-^{13}C]glucose or D-[2-^{13}C]glucose as a carbon source. TABLE 1 shows the ^{13}C intensity ratio (I.R., the ratio of intensity of each carbon to that of C-4 carbon in labeled cellulose) determined from peak areas in FIG.1. TABLE 1 also indicates the labeling ratio (L.R.) of each carbon. L.R. is the ratio of ^{13}C carbon atom introduced into the polymer to the ^{13}C carbon atom intensity of C-1, C-6 or C-2 carbon used for carbon source. Therefore, if the starting glucose directly polymerizes to cellulose, the L.R. of C-1, C-6 or C-2 would be 100%. Total L.R. indicates total ^{13}C carbon atom introduced into the polymer. For calculating L.R., the isotopic purity of D-[1-^{13}C]glucose, D-[6-^{13}C]glucose and D-[2-^{13}C]glucose was assumed to be 99.0%, 98.7% and 99.3%, respectively, as stated in the experimental section.

TABLE 1 shows that in cellulose obtained from D-[1-^{13}C]glucose, ^{13}C carbon atoms introduced are solely found in the original position, that is C-1. L.R. (36.8%) of C-1 carbon of cellulose obtained from a culture containing 1% ethanol is much higher than that(17.3%) of cellulose obtained without addition of ethanol. This result is in accordance with that of Minor et al.,[3] indicating that glycolysis of glucose added decreased due to consumption of ethanol as an energy source.

In our previous paper,[18] in curdlan obtained from D-[1-^{13}C]glucose, a small amount of introduction of labeling to C-3 carbon was observed. Several papers[3,7] on

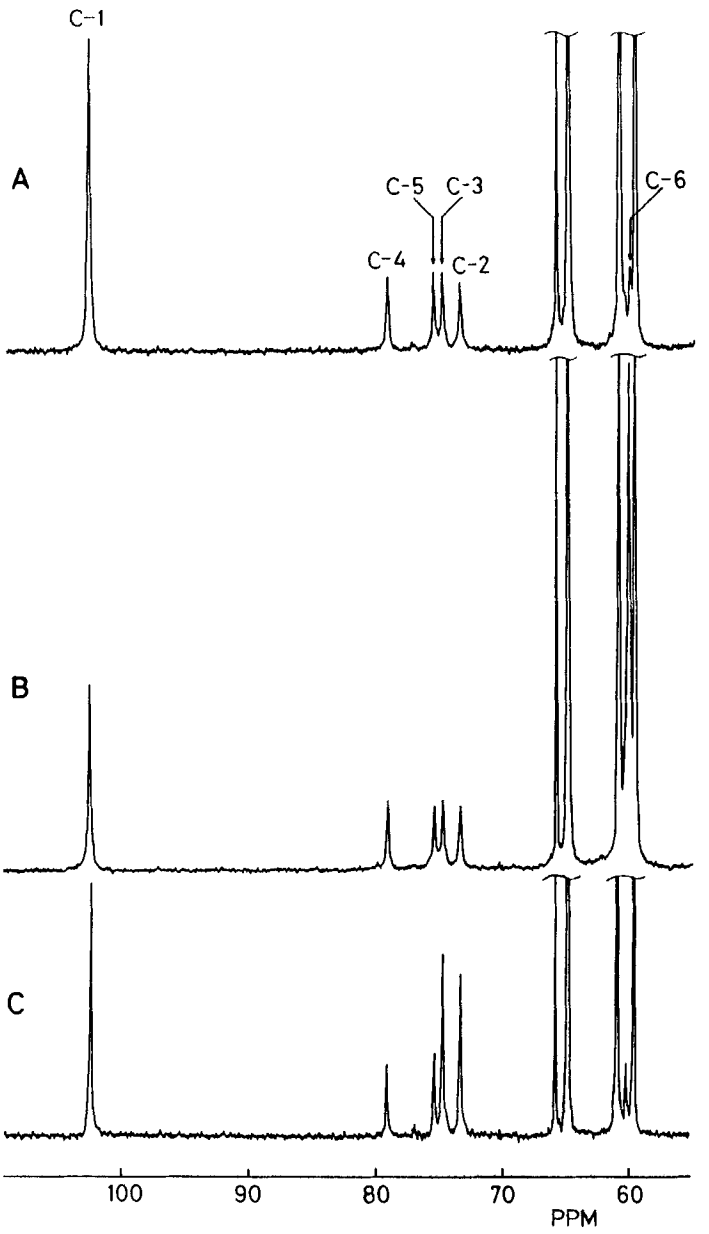

FIG. 1. ^{13}C NMR spectra of celluloses obtained from culture media containing, (A) 10 wt% of D-[1-^{13}C]glucose with addition of 1% ethanol, (B) 10 wt% of D-[6-^{13}C]glucose(no ethanol), and (C) 10 wt% of D-[2-^{13}C]glucose(no ethanol).

TABLE 1. ^{13}C Carbon intensity ratio(I.R.), and labeled ratio(L.R.) for each carbon in celluloses obtained from ^{13}C-labeled glucoses.

Chem. Shift/ppm	C-1 (102.4)	C-2 (73.3)	C-3 (74.7)	C-4 (79.1)	C-5 (75.4)	C-6 (60.2)	Total
Cellulose from D-[1-^{13}C]glucose							
I.R. No EtOH	2.70	1.04	1.01	1.00	1.02	n.d.	
L.R.	17.3%	0.4%	0.1%	0	0.2%	n.d.	18.1%
I.R. 1% EtOH	4.61	1.00	1.05	1.00	1.00	0.97	
L.R.	36.8%	0	0.5%	0	0	-0.3%	37.0%
Cellulose from D-[6-^{13}C]glucose							
I.R. No EtOH	2.98	1.00	1.06	1.00	0.95	9.00	
L.R.	20.2%	0	0.6%	0	-0.5%	81.6%	101.9%
Cellulose from D-[2-^{13}C]glucose							
I.R. 1% EtOH	3.30	3.72	2.40	1.00	1.28	n.d.	
L.R.	23.5%	27.8%	14.2%	0	2.8%	n.d.	68.4%
I.R. No EtOH	3.91	2.56	2.81	1.00	1.27	1.00	
L.R.	29.7%	15.9%	18.5%	0	2.8%	0	66.8%

Purity of D-[1-^{13}C]glucose, D-[6-^{13}C]glucose and D-[2-^{13}C]glucose is 99.0%, 98.7% and 99.3%, respectively. The culture medium contained 10 wt% of one of ^{13}C-labeled glucoses. n.d.; not determined.

the biosynthesis of cellulose from a culture containing D-[1-^{14}C]glucose by *Acetobacter xylinum* also describes transfer of labeling from the C-1 carbon to the C-3 carbon as well as to the C-4 carbon. In this investigation, however, such transfer of labeling from C-1 to C-3 carbon was not observed. This may indicate that rearrangement of [1-^{13}C]dihydroxyacetone 1-phosphate to [3-^{13}C]dihydroxyacetone 1-phosphate in the Embden-Meyerhof pathway may depend on not only species but culture conditions.

The cause of dilution of C-1 carbon labeling to 17-37% is ascribed to isomerization of unlabeled C-6 carbon, pentose cycle,[19] and Entner-Doudoroff pathway, which is an impor-

tant pathway for *Acetobacter xylinum* [20] as well as for *Agrobacterium*[21,22] and results in the loss of C-1 labeling as well as pentose cycle.

In cellulose obtained from D-[6-^{13}C]glucose, labeling is mainly found at the C-6 carbon, as described with curdlan[18] and cellulose,[7,10,13,16] in addition to a considerable amount of labeling at C-1. A high L.R. (82%) at C-6 indicates that the C-6 carbon, namely, the backbone structure of C-4 to C-6 carbons in D-[6-^{13}C]glucose, is well preserved in the biosynthesis of cellulose as already described for curdlan,[18] although the cause of dilution (18%) of C-6 carbon is unknown. A part of dilution (3%) may be explained by isomerization of unlabeled dihydroxyacetone 1-phosphate to D-glyceraldehyde 3-phosphate, followed by neogenesis of glucose, as will be discussed later.

Transfer of labeling from C-6 to C-1 carbon in cellulose obtained from D-[6-^{13}C]-glucose was already reported for cellulose produced by *Acetobacter xylinum*[7,13,16] and that obtained from cotton ball[10] and curdlan.[18] This phenomenon has been explained by reversible isomerization of D-glyceraldehyde 3-phosphate formed in the Embden-Meyerhof pathway,[10] Entner-Doudoroff pathway or pentose cycle[20] to dihydroxyacetone 1-phosphate. A large increase of transfer of labeling from the C-6 carbon to the C-1 carbon under culture condition without ethanol as energy source may account for the increase of glycolysis through various pathways.

An experiment on labeling with D-[2-^{13}C]glucose resulted in a large amount of transfer of labeling from C-2 carbon to C-1 and C-3 carbons, as well as in curdlan.[18] Transfer of labeling from C-2 carbon to C-1 and C-3 carbons in cellulose has been reported,[6] and participation of pentose cycle and Entner-Doudoroff pathway has been suggested.[20] We believe that the transfer can be explained by the pentose cycle,[23] as shown in FIG. 2. Namely, formation of fructose 6-phosphate (**1**) from xylulose 5-phosphate (**3**), which is produced from ribulose 5-phosphate (**2**), and erythrose 4-phosphate (**4**) results in transfer of labeling from the C-2 carbon to the C-1 carbon, and formation of fructose 6-phosphate (**7**) from glyceraldehyde 3-phosphate and sedoheptulose 7-phosphate (**6**), which is produced from ribose 5-phosphate (**5**), results in transfer of labeling from the C-2 carbon to the C-1 and C-3 carbons, followed by formation of cellulose. The percentage of cellulose formed from the latter fructose 6-phosphate (**7**) is 18%, and the percentage from the former (**1**) is 12%. Thus, 30% of polymer is formed by this process in the culture without addition of ethanol. With addition of ethanol, the value decreased to 24%, indicating decrease of glycolysis, while the labeling at C-2 increased from 16% to 28%.

Transfer of labeling from the C-2 carbon to the C-5 carbon was already observed in cotton cellulose[11] and bacterial cellulose[6] labeled with D-[2-^{14}C]glucose. The transfer has been explained by isomerization of dihydroxyacetone 1-phosphate to D-glyceraldehyde 3-phosphate, followed by neogenesis of glucose. The transfer could not be observed in

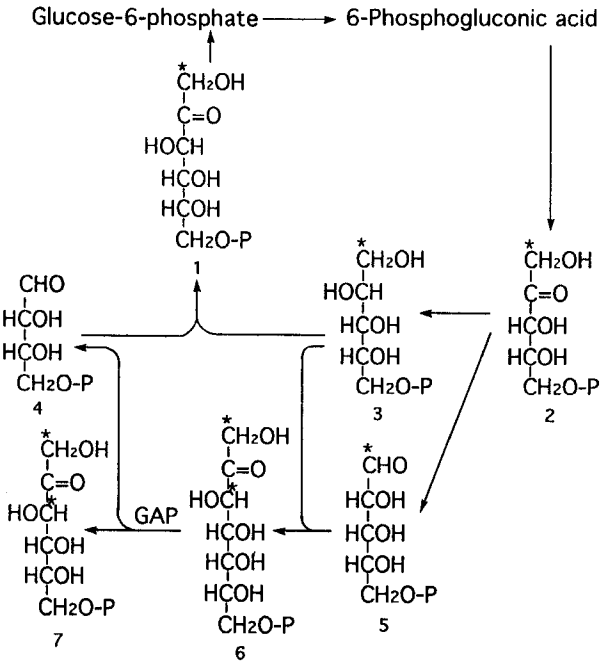

FIG. 2. Transfer of labeling from C-2 carbon to C-1 and C-3 carbons in the pentose cycle. GAP; Glyceraldehyde 3-phosphate.

curdlan labeled with D-[2-^{13}C]glucose, indicating that the extent of isomerization depends on species. It is interesting that the transfer does not change whether ethanol was added to the culture or not.

The mechanism of transfer of labeling from C-2 carbon to C-5 carbon suggests that transfer of labeling from the C-1 carbon to C-6 carbon must occur. It is difficult, however, to definitely prove the transfer of labeling from the C-1 carbon to the C-6 carbon, since determination of the C-6 peak area in ^{13}C NMR spectrum is subject to a large error due to neighboring large solvent absorptions, as described in the experimental section.

It is noted that total L.R. of cellulose obtained from D-[2-^{13}C]glucose is fairly high as with curdlan.[18] Dilution of labeling may be mainly due to isomerization of unlabeled glyceraldehyde 3-phosphate formed in the Entner-Doudoroff pathway, Embden-Meyerhof pathway and pentose cycle to dihydroxyacetone 1-phosphate, followed by neogenesis of glucose.

Dilution of labeling (83% without addition of ethanol) in cellulose obtained from D-[1-^{13}C]glucose can be ascribed to (1) isomerization of unlabeled C-6 carbon, in trioses formed in various pathways, (2) rearrangement of unlabeled C-2 carbon in the pentose

cycle, and (3) neogenesis of unlabeled glucose from fragments formed in various pathways of glycolysis. Since mechanism (1) and (2) can be estimated as 20% and 30%, respectively, from Table 1, mechanism (3) can be calculated as 33% (without addition of ethanol). The details of mechanism (3) are unknown at present.

From the discussions above, the biosynthesis of cellulose is explained by four routes: (1) direct synthesis of cellulose from labeled glucoses, including neogenesis of glucose from cleaved trioses without rearrangement, (2) neogenesis of glucose from isomerized trioses (^{13}C-6 → ^{13}C-1 and ^{13}C-2 → ^{13}C-5) formed in various pathways, (3) from fructose 6-phosphate reconstructed in the pentose cycle(^{13}C-2 → ^{13}C-1 and ^{13}C-3), and (4) neogenesis of glucose and formation of cellulose from fragments produced in various pathways of glycolysis, such as the pentose cycle, Entner-Doudoroff pathway and Embden-Meyerhof pathway. L.R. values (37%, when ethanol was added and 17% without addition of ethanol) at C-1 carbon in cellulose obtained from D-[1-^{13}C]glucose indicate process (1).

These values almost agree with L.R. values (28% and 16%, respectively) of C-2 carbon in cellulose obtained from D-[2-^{13}C]glucose. Thus, biosynthesis of cellulose takes place through routes (1), (2), (3) and (4) in proportions of 17%, 20%, 30% and 33% (without addition of ethanol in culture medium). With addition of ethanol, contribution from route (1) increases to *ca.* 30%, while contributions from mechanism (2) and (3) decrease. These values are comparable with those estimated by statistical treatment of NMR data on cellulose which was obtained from a culture containing statistically ^{13}C-enriched glucose as a carbon source.[16]

In conclusion, the mechanism of biosynthesis of cellulose was elucidated as follows. The direct synthesis of the polymer from the introduced glucose is about one-third when ethanol was added. However, this proportion is considerably decreased without addition of ethanol. Isomerization (^{13}C-6 → ^{13}C-1 and ^{13}C-2 → ^{13}C-5) of trioses formed in various pathways, followed by neogenesis of glucose and formation of cellulose, occurs to some extent. Formation of cellulose from fructose 6-phosphate reconstructed in the pentose cycle takes place to a considerable extent, especially without addition of ethanol. Formation of cellulose from fragments produced in the pentose cycle and Entner-Doudoroff pathway which result in the loss of C-1 carbon also occurs. Percentages of routes described above, through which cellulose is biosynthesized vary according to culture conditions as described.

EXPERIMENTAL

^{13}C-Labeled Glucose. D-[1-^{13}C]Glucose, D-[6-^{13}C]glucose and D-[2-^{13}C]-glucose (Isotec Inc., Ohio, U.S.A.) were used as the labeling sources. The isotopic purity was 99.0%, 98.7% and 99.3%, respectively, as determined with mass spectrometry on di-acetone derivatives of glucose.

Preparation of ^{13}C-Labeled Cellulose. The culture was performed according to the method described in a previous paper.[24] Sixty mL of cell suspension(*Acetobacter xylinum* IFO 13693) were added to 140 mL of Hestrin medium[25] containing 10% of D-[1-^{13}C]glucose, D-[6-^{13}C]glucose, or D-[2-^{13}C]glucose in unlabeled D-glucose, and the culture was incubated at 28.0 °C for 5 days. The product was purified by boiling in 1 wt% aqueous NaOH for 10 h under a nitrogen atmosphere, washed with distilled water, and dried under vacuum. Yield, *ca.* 10% of D-glucose.

Sodium Hydroxide Treatment of Biosynthesized Cellulose. In order to enhance the solubility of biosynthesized cellulose for NMR studies, the cellulose was mercerized in 18% aqueous sodium hydroxide for 1 h at 20 °C. The excess aqueous sodium hydroxide was squeezed from mercerized cellulose, and then alkali cellulose was warmed for 48 h at 30 °C to lower the degree of polymerization. The alkali cellulose was neutralized with 1% aqueous acetic acid and washed with distilled water thoroughly and then dried under vacuum in a desiccator with phosphorus pentoxide as desiccant.

^{13}C NMR Spectroscopy. ^{13}C NMR spectra of cellulose were recorded on cellulose solutions (10 wt%) dissolved in *N*-methyl morpholine oxide/dimethyl sulfoxide-d_6 (1:1, wt ratio) mixed solvent at 90 °C with a JEOL EX 270 spectrometer at 67.8 MHz; repetition time 10.0 sec and 10000 scans with gated decoupling. The reference for chemical shifts was dimethyl sulfoxide-d_6 (39.50 ppm).

Since the resonance of the C-3 carbon slightly overlaps that of C-5 carbon, and that of C-6 carbon severely overlaps the resonances of solvents, the areas from those carbons were determined using a computer program (Gonta) for curve resolving developed by Dr. S. Ando.[26] The accuracy of determined values is ±3% for C-1 to C-5 carbons, but that of C-6 carbon is ±10%.

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