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Biosynthesis of C^{14}-Labeled Cellulose by *Acetobacter xylinum*. IV. From D-Glucose-1-C^{14}, D-Glucose-6-C^{14} and Glycerol-1,3-C^{14}

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The distribution of C^{14} in the D-glucose from the bacterial cellulose hydrolyzate produced from D-glucose-1-C^{14}, added 24 hours after inoculation as compared with that added initially was 10–12% higher in the original position 1. The hydrolyzate of cellulose-C^{14} produced from D-glucose-6-C^{14} indicated that approximately 82% of the label was in the original position 6 and had 9% in position 1, with smaller quantities in positions 2, 3, 4 and 5. The distribution of C^{14} in the D-glucose units of cellulose produced from glycerol-1,3-C^{14} was approximately 12% position 1; 4% position 2; 22% position 3; 29% position 4; 3% position 5; and 30% position 6.

Introduction

Preceding publications[2-5] have been made on the incorporation of labeled sugars into cellulose. Both *Acetobacter xylinum*[2-4] and wheat plants[6] appear to incorporate D-glucose directly. However, some of the original hexose units are cleaved prior to cellulose formation.

It would assist in determining the mechanism of cellulose formation, if the percentage of label could be changed in the original position by adding the labeled hexose after the growth of the bacteria had progressed and enzyme formed. Such experiments utilizing D-glucose-1-C^{14} are reported herein, along with the new data on the incorporation of D-glucose-6-C^{14} and glycerol-1,3-C^{14} into cellulose.

Procedures

Culture Conditions.—The culture conditions were identical with those described in the preceding paper of this series, unless designated as different.[4]

Counting Apparatus.—Counting rates were calculated from the results of counts obtained with an α-β-γ proportional counter Model PC-1 and in certain instances with a Vibrating Reed Electrometer. The proportional counter is a "P-gas (90% argon, 10% methane) windowless type counter." The geometry for small samples is 2π and the efficiency of a thin sample is 50% with this counter. Necessary precautions were taken to assure a precision of about 1% of the observed count rate. Counts were made of thin samples, as $BaCO_3$, glucose, etc. and the results were corrected in the standard way for the effects of background count, geometry and self-absorption.

The samples counted using the vibrating reed electrometer were first combusted by the Van Slyke method and the CO_2 was collected in ionization chambers. The activity of the sample was then determined by the rate at which the condenser was charged or millivolts per second drift rate. The difference between this drift rate and the normal background of the reed gives the measure of the activity of the combusted sample. For this vibrating reed 0.1270 mv./sec. equaled one disintegration per second.

Radioactivity Determinations of the Culture Products.—The procedure for the determination of C^{14} content in the CO_2, the C^{14} remaining in the culture media and the C^{14} activity of the cellulose were the same as those previously described.[2a]

Purification of the Cellulose Hydrolyzate D-Glucose.—The D-glucose-C^{14} resulting from the cellulose-C^{14} was purified by the method of Whistler and Durso[7] and by several recrystallizations before assay for C^{14}.

Methods Used for Location of C^{14} in Cellulose.—The distribution of C^{14} within the D-glucose units in the cellulose was determined by analysis of the D-glucose obtained by hydrolysis of the cellulose-C^{14}. Carrier D-glucose was added to the hydrolyzate to obtain the desired specific activity and increase the amount of C^{14}-glucose.

The activity at carbon 1 was obtained by the Frush and Isbell method.[8] Positions 2 and 3 were obtained by using the potassium D-arabonate from carbon 1 analysis and preparing its benzimidazole according to the method of Moore and Link.[9]

Carbon atoms 2 and 3 of D-glucose were then degraded stepwise by a method described by Roseman[10] by formation of benzimidazole carboxylic acid and carbon atom 2 by the benzimidazole. Position 2 was counted directly and position 3 was determined by difference of 2 and 3.

Activity at positions 4 and 5 were by difference of positions 1, 2, 3 and 6 or as described in previous publication.[2a]

Carbon atom 6 was made by the method of Reeves,[11] which involves oxidation with periodic acid of the C_6-carbon to CH_2O and its conversion to the dimedone derivative. Counts were made of this derivative, plated on aluminum pans from aqueous methanol or pyridine solutions.

Results

Cellulose-C^{14} Produced from D-Glucose-1-C^{14} Added Initially and 24 Hours after Inoculation.—The weight yields, as carbon content and the percentage of C^{14}-label in each of purified cellulose and carbon dioxide, are indicated in Table I. The table shows also, for comparison, the carbon and percentage of label in the D-glucose-1-C^{14} and ethanol that was supplied.

TABLE I

SUBSTRATES, YIELD OF PRODUCTS AND C^{14}-LABEL DISTRIBUTION

	A. When D-glucose-1-C^{14} was added initially		B. D-glucose-1-C^{14} added 24 hr. after inoculation	
	Wt., mg. C	Label C^{14}, %	Wt., mg. C	Label C^{14}, %
Substrates				
D-Glucose-1-C^{14}	80.5	100	80.5	100
Ethanol	79.3	None	79.3	None
Products				
Carbon dioxide	88.2	30.5[a]	92.4[a]	24.5[a]
Cellulose	26.2	28.7[a]	28.4[a]	33.6[a]

[a] These data were secured from three separate experiments and analyses. There was little variation in the three individual sets of data.

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(2) (a) F. W. Minor, G. A. Greathouse, H. G. Shirk, A. M. Schwartz and Milton Harris, THIS JOURNAL, **76**, 1658 (1954); (b) **76**, 5052 (1954).

(3) G. A. Greathouse, H. G. Shirk and F. W. Minor, *ibid.*, **76**, 5157 (1954).

(4) F. W. Minor, G. A. Greathouse and H. G. Shirk, *ibid.*, **77**, 1244 (1955).

(5) G. A. Greathouse, *Science*, **117**, 553 (1953).

(6) S. A. Brown and A. C. Neisch, *Can. J. Biochem. Physiol.*, **32**, 170 (1954).

(7) R. L. Whistler and D. F. Durso, THIS JOURNAL, **72**, 677 (1950).

(8) H. L. Frush and H. S. Isbell, *J. Research Natl. Bur. Standards*, **51**, 167 (1953).

(9) S. Moore and K. P. Link, *J. Biol. Chem.*, **133**, 293 (1940).

(10) S. Roseman, THIS JOURNAL, **75**, 3854 (1953).

(11) R. E. Reeves, *ibid.*, **63**, 1476 (1941).

The original percentage activity per 1 mg. of D-glucose (100/80.5) is 1.24. The percentage activity per 1 mg. for the resultant cellulose when D-glucose-1-C^{14} was added initially (28.7/26.2) is 1.10; when D-glucose-1-C^{14} was added 24 hours after inoculation (33.6/28.4) is 1.18. Thus the activity was slightly lower than the original D-glucose-1-C^{14}.

The label in the cellulose-C^{14} was distributed over the six positions in the glucose units as shown in Table II. These experiments were performed at the same time and under identical conditions except the time of adding the D-glucose-1-C^{14}.

TABLE II

LOCATION OF LABEL IN D-GLUCOSE FROM BACTERIAL CELLULOSE

	D-Glucose-1-C^{14}	
Structure position	Added 0 hr.	Added 24 hr. later
1	62.5	79.1
2	1.6	0.4
3	19.2	11.0
4	15.4	8.0
5	0.9	0.4
6	0.4	1.1
	100	100

Cellulose-C^{14} Produced by D-Glucose-6-C^{14}.—Radioactive cellulose was produced from D-glucose-6-C^{14} by use of *A. xylinum*. The procedure was similar to that described for the other labeled sugar and cellulose formation. The results of these experiments are summarized in Tables III and IV.

TABLE III

SUBSTRATES, YIELD OF PRODUCTS AND C^{14}-LABEL DISTRIBUTION

	Wt., mg. C	Label C^{14}, %
Substrates		
D-Glucose-6-C^{14}	80.5	100
Ethanol	79.3	None
Products		
Carbon dioxide	111.0	28.9
Cellulose	32.6	23.8

TABLE IV

LOCATION OF LABEL IN GLUCOSE FROM BACTERIAL CELLULOSE—FROM D-GLUCOSE-6-C^{14}

Structure position	%	Structure position	%
1	9.3	4	3.5
2	1.7	5	
3	3.9	6	81.6

The major portion (81.6%) of the label was located at position 6, corresponding to its position in the labeled substrate. Position 1 contained 9.3% and the remaining 9% was distributed at positions 3, 4 and 5 and 2 in descending order.

Cellulose-C^{14} Produced by Glycerol-1,3-C^{14}.—Cellulose-C^{14} was produced by *A. xylinum* using glycerol-1,3-C^{14} as the sole carbon label source. The procedures and analyses were the same as for the previously described radioactive sugar studies. These data are presented in Table V and VI.

TABLE V

SUBSTRATES, YIELD OF PRODUCTS AND C^{14} LABEL DISTRIBUTION

	Wt., Mg. C	Label C^{14}, %
Substrates		
Glycerol-1,3-C^{14}	98.0	100
Ethanol	79.3	None
Products		
Carbon dioxide	87.4	6.1
Cellulose	11.8	5.7

TABLE VI

LOCATION OF LABEL IN GLUCOSE FROM BACTERIAL CELLULOSE FROM GLYCEROL-1,3-C^{14}

Structure position	%	Structure position	%
1	12.7	4	28.9
2	3.7	5	3.0
3	21.7	6	30.0

Smaller quantities of C^{14} were found in the CO_2 and cellulose with glycerol-1,3-C^{14} as the label source than with glucose-C^{14} during the 7 day incubation period. Most of the 87.4 mg. carbon as CO_2 must have come from the oxidation of the ethanol. The liquid products of the cultures contained a large portion of the label. *A. xylinum* is required to form cellulose from trioses in this case and it may be the time required for the bacteria or its enzyme system to produce sizable quantities of cellulose intermediate (hexoses, hexose phosphates, etc.) exceeds the 7-day period of the experiments. Experiments (to be reported elsewhere) concerned with the isolation of the cellulose intermediates between glycerol-1,3-C^{14} and cellulose formation bear out this reasoning.

The label in the cellulose was distributed over the six positions in the glucose units, with the smaller quantities being in position 2 and 5.

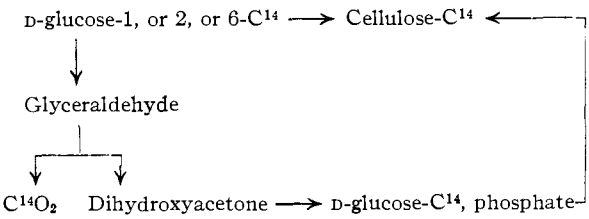
Discussion

This paper presents data for the first time on the biosynthesis of cellulose-C^{14} from D-glucose-1-C^{14} added 24 hours after inoculation as compared with that added initially. The delayed addition of D-glucose-1-C^{14} produced a 10–12% higher concentration of the label in the original position 1. These data indicate that polymerization of the D-glucose, as such, without prior cleavage is favored by adding the radioactive sugar after the bacteria have initiated growth. No previous data have been reported on the biosynthesis of cellulose-C^{14}, and the position of label, utilizing D-glucose-6-C^{14} or glycerol-1,3-C^{14}. The hydrolyzate of cellulose-C^{14} from D-glucose-6-C^{14} showed that approximately 82% of the label was in the original position 6. When glycerol-1,3-C^{14} was the sole carbon source, the label was 12% position 1, 4% position 2, 22% position 3, 29% position 4, 3% position 5 and 30% position 6.

In every case of cellulose biosynthesis studied, some of the original hexose units are cleaved prior to cellulose formation. The concentration of the label in the original position depends on which of

the six carbon atoms is labeled and on the time of adding the radioactive sugar. Thus, the mechanisms of cellulose polymerization is complex. At least two major mechanisms appear from these studies, (1) direct polymerization, possibly involving phosphorylation and (2) cleavage of the hexose and resynthesis of hexose phosphates from trioses such as glycerol. Hexose phosphates[12] have been isolated and identified as intermediates in the biosynthesis of cellulose from D-glucose, thus the reason for suggesting these products as possible energy sources in the major mechanism of polymerization.

To summarize the research, a schematic representation of the processes of cellulose formation may be shown as



A cell-free enzyme system has been isolated from *A. xylinum* capable of producing cellulose-C^{14}.[13] D-Glucose-1-C^{14} was polymerized to cellulose and the hydrolyzate (D-glucose) analyzed. Ninety-six per cent. of the label was found in position one of the cellulose molecule.

(12) Unpublished data.

(13) G. A. Greathouse, THIS JOURNAL, **79**, 4503 (1957).

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[JOINT CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF WAYNE STATE UNIVERSITY AND THE INSTITUTO DE QUIMICA AGRICOLA, MINISTERIO DA AGRICULTURA, RIO DE JANEIRO]

The Chemistry of Rosewood. Isolation and Structure of Anibine and 4-Methoxyparacotoin[1]

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From the wood of the South American rosewood trees (genus *Aniba*) there has been isolated a new alkaloid, $C_{11}H_9NO_3$, which has been named anibine. By various degradations, in particular by alkaline cleavage, it was shown that anibine is 4-methoxy-6-(3′-pyridyl)-α-pyrone (IV). A neutral companion substance of anibine, when subjected to similar reactions, was proved to be 4-methoxy-6-piperonyl-α-pyrone (X) (4-methoxyparacotoin) and attention is called to the structural similarity with the constituents of the closely related *Coto* barks.

The South American rosewood trees belong to the *Lauraceae* family, and the derived essential oil has long been an article of commerce.[4] The botanical identification has been confusing, and the trees were once believed to belong to the genus *Ocotea*.[5] It is now accepted that the *bois de rose* from French Guiana and adjacent areas is *Aniba rosaeodora* Ducke and the Brazilian *páu rosa* from the lower Amazon basin *Aniba Duckei* Kostermans (*A. rosaeodora* var. *amazonica* Ducke).[6,7] The main constituent of the rosewood essential oil is linalool, but a ready differentiation of the two species is even possible on chemical grounds since the essential oil from French Guiana is strongly levorotatory while the Brazilian one is only slightly so or even dextrorotatory.[4]

Aside from the essential oil, no chemical work seems to have been done with these plants, and a detailed examination of the composition of these *Aniba* species was undertaken by one of us (O.R.G.) The Brazilian *Aniba Duckei* Kostermans represented commercial material from Manaus (State of Amazonas) while a sample of *Aniba rosaeodora* Ducke came from an isolated tree from the region of the Amaparí river (Territory of Amapá, Brazil).[8] A number of products were isolated, and the present paper is concerned with the alkaloid and one of the neutral constituents, which were found in both *Aniba* species.

Extraction of the rosewood sawdust with benzene followed by removal of basic material with hydrochloric acid and basification yielded a single, crystalline alkaloid, m.p. 179–180°, in *ca.* 2.6% yield. Its empirical formula, $C_{11}H_9NO_3$, immediately indicated that it must differ considerably from the few alkaloids (isoquinoline types) hitherto encountered among the *Lauraceae*,[5,9] and we have named the substance "anibine." Functional group analysis demonstrated the presence of one methoxyl group and the absence of N-methyl or C-methyl groups. Anibine contained no active hydrogen atom and was optically inactive over the range 700–400 mμ. The alkaloid, though forming a crystalline methiodide, hydrochloride and picrate, was weakly basic and in fact could be removed from hydrochloric acid solution by continuous extraction with chloroform. The nitrogen atom was, therefore, assumed to be part of a heterocyclic ring which was not inconsistent with the ultraviolet

(1) This article should be considered paper XVIII in the Wayne series "Alkaloid Studies"; for preceding article see THIS JOURNAL, **79**, 2203 (1957).

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(3) Instituto de Quimica Agricola, Rio de Janeiro, Brazil.

(4) E. Guenther, "The Essential Oils," Vol. IV, Van Nostrand Co., Inc., New York, N. Y., 1950, p. 191.

(5) See C. Wehmer, "Die Pflanzenstoffe," Vol. I, Gustav Fischer, Jena, 1929, pp. 364–365. References to earlier chemical investigations of the *Lauraceae* can be found on pp. 350–373.

(6) A. Ducke, *Arq. Jard. Bot. Rio de Janeiro*, **5**, 109 (1930).

(7) A. J. G. H. Kostermans, *Rec. trav. bot. neerl.*, **35**, 918 (1938).

(8) The botanical identification was carried out by Dr. Arthur de Miranda Bastos (Botanical Garden, Rio de Janeiro) and confirmed by the rotation ($[\alpha]_D$ −16°) of its essential oil.

(9) See T. A. Henry, "The Plant Alkaloids," Blakiston, Philadelphia, 1949, p. 319.