tran from this same strain.[16,52] Our subsequent preparations from this strain have contained 2 distinct structural types of dextran, one of which (fraction L or L-R) apparently was identical with previous preparations and the other (fraction C or S-R)[26] was an entirely new type having a high content of 1,3-like links. Characterization of dextran products from 12 different colonies picked from a plated culture of B-742 failed to reveal evidence of variation or mutation in the culture. These products had somewhat different proportions of the 2 structural types, 1,6-links from 72–75% and intrinsic viscosities near 0.20. The preparation reported in Table I (heterogeneous group), which was obtained from a large-scale fermentation of the original culture, had a higher content of the anomalous fraction and, therefore, showed lower 1,6- and higher 1,3-like linkage contents and higher viscosity and rotation.

The strains B-742 and B-1142 (class C) had a common origin but different subsequent histories. Our B-1142 dextran consisted almost exclusively of the anomalous fraction. Apparently these cultures have been changing, B-1142 more than B-742.

(52) Published information[11] was the basis for our identifying strain NRRL B-742[16] ("number 5" of Hucker[17] and "culture 4" of Tarr and Hibbert[4]) with the strain whose dextran was subjected to methylation-structure analysis by Levi, *et al.*[11] Another source (Ph.D. thesis of I. Levi, McGill University, 1942) now has disclosed that this methylation study was made on dextran from either "culture 1" or "culture 2."[4] Fowler, *et al.*,[5] and later T. H. Evans (Ph.D. thesis, McGill University, 1941), carried out methylation-structure analysis on dextran from "culture 4" but obtained results almost identical with those obtained by Levi, *et al.*, on the other dextran.[11]

A similar change in another strain is indicated by published data[13,53] and by our results on dextran from this strain (B-1375, Table I, class C).

Bacterial Classification and Dextran Type.—The fact that methylation analysis gave no evidence of branching in dextrans from 2 strains of *Leuconostoc dextranicum*[7,8,13] has led to the apparent expectation that all such strains would produce essentially a straight-chain type of dextran.[54] Our observations indicate that although several of our strains of *L. dextranicum* produced dextrans with low percentages of non-1,6-links and long gums (B-640, -1145 and -1146), others produced dextrans with high percentages of non-1,6-links and short gums (B-1420, -1141 and -1375). Dextran B-1193 had 95% 1,6-links, but short gum.

Acknowledgment.—We should like to express our gratitude to the numerous individuals and organizations named in Table I who gave us cultures utilized in this investigation. We are deeply indebted to Dr. E. J. Hehre for advice and encouragement and to members of this Laboratory, as follows: Dr. R. T. Milner for coördination of this project; C. H. vanEtten, T. A. McGuire and Mary Weile for the nitrogen, phosphorus and ash analyses; and Lenora J. Rhodes and Geraldine Bryant for assisting with production of the cultures and of the media, respectively.

(53) S. A. Barker, E. J. Bourne, G. T. Bruce and M. Stacey, *Chemistry and Industry*, 1156 (1952).

(54) S. A. Barker and E. J. Bourne, *Quart. Revs. (London)*, **7**, 56 (1953).

PEORIA, ILLINOIS

[CONTRIBUTION FROM HARRIS RESEARCH LABORATORY AND NATIONAL RESEARCH COUNCIL]

Biosynthesis of C^{14}-Specifically Labeled Cellulose by *Acetobacter xylinum*. II. From D-Mannitol-1-C^{14} with and without Ethanol[1]

BY FRANCIS W. MINOR, GLENN A. GREATHOUSE,[2] HAROLD G. SHIRK,[2] ANTHONY M. SCHWARTZ AND MILTON HARRIS

RECEIVED MAY 10, 1954

Cellulose-C^{14} was biosynthesized by *Acetobacter xylinum* employing D-mannitol-1-C^{14} as the sole labeled nutrient. Label distribution in the D-glucose from the bacterial cellulose showed 84–96% of the activity was located at positions 1 and 6, with the residual activity being at positions 2 to 5. These data indicate that some of the scission products from the original D-Mannitol became oriented in the cellulose. The labeled cellulose had lower specific radioactivity than the D-mannitol-1-C^{14} supplied. The presence of ethanol in the culture media, although it increased the yield of cellulose as well as its C^{14} content, did not affect the distribution of the label among the carbons of the glucose making up the cellulose.

Introduction

This paper is the second in a series concerned with the ability of *Acetobacter xylinum* to produce cellulose-C^{14} from various substrates, possibly providing some information regarding the mechanisms involved in cellulose formation. Presented are results of experiments in which D-mannitol-1-C^{14} was the sole labeled additive to a medium suitable for the organism to produce cellulosic pellicles. Analyses reported include the distribution of the C^{14}-label among the various culture products as well as the spread of radioactivity in the purified glucose from the hydrolyzed C^{14}-cellulose.

(1) This series of papers is based on work supported by the Atomic Energy Commission under contract AT(30-1)-915 with the Harris Research Laboratories.

(2) National Research Council, Washington 25, D. C.

Experimental

Culture Conditions.—The cultures were grown in media containing 0.3% of KH_2PO_4 plus other ingredients, as shown in Table I. Each culture was inoculated from actively growing stock. Culture 5 was incubated at 30° and harvested 7 days following inoculation; while the others were grown at room temperature (20 to 25°) and harvested 14 days after inoculation. The culture vessel, including accessory apparatus for collecting CO_2, was the same as that previously described.[3]

Analyses.—The methods for purifying the cellulose and for determining the C^{14}-content of culture fractions were identical with those described in the preceding paper of this series.[3] Except for the procedure involving lactic acid

(3) F. W. Minor, G. A. Greathouse, H. G. Shirk, A. M. Schwartz and M. Harris, THIS JOURNAL, **76**, 1558 (1954).

TABLE I

CULTURE MEDIA

Culture	Yeast extract, %	Ethanol, %	D-Mannitol, %
1	1	None	2
2	1	0.76	2
3	0.5	None	1
4	0.5	0.76	2
5	0.5	0.76	1

fermentation[4] applied in part to the cellulose from culture 4, the methodology for determining C^{14}-distribution in the glucose units making up the cellulose was similar to that as previously described.[3]

Results and Discussion

The yields of culture products and the quantities of C^{14} found in the carbon dioxide and cellulose are given in Table II. In view of prior evidence[5] that other possible carbon sources in the media do not significantly contribute to cellulose formation, cellulose yields are calculated as the percentage of the weight of mannitol supplied. Experiments recently reported with labeled ethanol[6] established conclusively that none of the carbon of the alcohol nor any carbon in fragments thereof appears in the final cellulose. Carbon dioxide yields, on the other hand, are expressed as percentages of the combined carbon contents of the mannitol and ethanol whenever the latter was added. The C^{14}-yields are expressed as percentages of the total radioactivity added as D-mannitol-1-C^{14}.

TABLE II

YIELDS OF CULTURE PRODUCTS AND DISTRIBUTION OF C^{14}

Culture no.	Total radioactivity added, μc.	Cellulose Wt. yield, %	Cellulose C^{14} yield, %	Carbon dioxide Wt. yield, %	Carbon dioxide C^{14} yield, %
1	2.48	21.2	16.1	62.7	60.9
2 (Ethanolic)	2.48	34.0	28.0	69.0	56.1
3	25.6	17.2	8.6	61.8	60.1
4 (Ethanolic)	9.85	18.5	11.9	52.0	48.8
5 (Ethanolic)	89.4	4.6	2.1	80.3	75.0

Cultures 1 and 2 (Tables I and II), differing only in ethanol content, were grown to ascertain what influence on C^{14}-distribution is exercised by the presence of ethanol in the media. These were purposely inoculated from the same stock culture and incubated simultaneously so as to minimize all possible variables. Comparison of the cellulose weight data from these two cultures demonstrates the customary increase due to the presence of the ethanol[5] and in addition shows that a significantly larger proportion of the C^{14}-label appears in the cellulose. Although the weight yield of CO_2 is increased by the addition of ethanol, it is to be noted that the quantity of C^{14} resulting in this respiratory product is lowered. Since the percentage recovery of C^{14} in the CO_2 from the ethanolic cultures 2 and 4 was significantly lower than that found in the non-ethanolic cultures 1 and 3, it may be concluded that the presence of ethanol spared D-mannitol from oxidation permitting a greater proportion to be utilized for cellulose biosynthesis. As was generally found with the D-glucose-1-C^{14} culture work already reported,[3] whenever oxidation of the substrate was of a high order, indicated by the level of CO_2 produced, the concomitant yield of cellulose was usually of a small magnitude.

From the specific radioactivity values (C^{14}-content per milligram of carbon) presented in Table III, it can be seen that the activity of the cellulose is lower than that of the original D-mannitol. Also, the presence of ethanol in the medium increased the specific radioactivity of the cellulose. The specific activity of CO_2 in the non-ethanolic cultures 1 and 3 is approximately equal to the activity supplied by the D-mannitol-1-C^{14}, whereas the addition of ethanol to the medium lowered the specific activity of the CO_2 confirming this observation already noted in Table II.

TABLE III

SPECIFIC RADIOACTIVITIES

Culture no.	D-Mannitol-1-C^{14} supplied, μc./mg. C	Cellulose produced, μc./mg. C	CO_2 produced, μg./mg. C	Ratio cellulose carbon sp. act. to mannitol carbon sp. act.
1	0.0312	0.010	0.030	0.32
2 (Ethanolic)	.0312	.025	.017	.80
3	.64	.320	.630	.50
4 (Ethanolic)	.121	.074	.063	.61
5 (Ethanolic)	.38	.170	.180	.46

In Table IV are presented the distribution patterns of C^{14} in the D-glucose obtained by hydrolysis of the radioactive cellulose from cultures 3, 4 and 5.

TABLE IV

LABEL DISTRIBUTION IN D-GLUCOSE FROM RADIOACTIVE BACTERIAL CELLULOSE

Carbon position	Culture supplied, %: D-Mannitol-1-C^{14} (No. 3)	D-Mannitol-1-C^{14} and ethanol (No. 4)	D-Mannitol-1-C^{14} and ethanol (No. 5)
1	34.9	34.2	49.0
2 to 5	15.4	17.2	..
6	49.4 (50.2)[a]	47.5 (49.9)[a]	47.0

[a] Calculated from result of direct determination of C^{14} at positions 1 to 5 (lead tetraacetate oxidation of D-glucose).

The major portion of the label was found at positions 1 and 6, as might be expected in view of the symmetry of the D-mannitol molecule and its presumed oxidation to D-fructose-1- and/or -6-C^{14}. Frush and Isbell[7] took advantage of this oxidative characteristic as found in another species, *Acetobacter suboxydans*, and prepared D-fructose-1- and/or -6-C^{14} from mannitol-1-C^{14}. As shown in Table IV, significant quantities of label in the glucose are to be found in the intermediate carbons 2 to 5, inclusive, indicating that scission products became somewhat involved in the biosynthesis. It is interesting to note the significant lack of C^{14} in carbon positions 2 to 5, inclusive, for the cellulose from culture 5 which was cultured for only a 7-day period. It is equally interesting that cultures 3 and 4, differing in absence or presence of ethanol in

(4) H. G. Wood, N. Lifson and V. Lorber, *J. Biol. Chem.*, **159**, 475 (1945).

(5) H. L. A. Tarr and H. Hibbert, *Can. J. Research*, **4**, 372 (1930).

(6) G. A. Greathouse, H. G. Shirk and F. W. Minor, THIS JOURNAL, **76**, 5157 (1954).

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

the medium, invoked no significant effect on the C^{14} distribution in glucose.

Although sufficient supporting evidence is lacking at this time, the distribution pattern data for the glucose resulting from the hydrolysis of the cellulose from culture 5 provides some indication for the hypothesis that cellulose formation even *via* the involved pathways inherent in bacteria is primarily a biosynthesis by direct polymerization.

HARRIS RESEARCH LABORATORIES AND
NATIONAL RESEARCH COUNCIL
WASHINGTON, D. C.

[CONTRIBUTION FROM RADIATION LABORATORY AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

Isolation of Mannoheptulose and Identification of its Phosphate in Avocado Leaves[1]

BY A. NORDAL AND A. A. BENSON

RECEIVED APRIL 2, 1954

Mannoheptulose accumulates in avocado leaves and occasionally in the fruit. Isolation of this sugar and perseitol in good yield from avocado leaves is described. Brief photosynthesis in $C^{14}O_2$ produced labeled mannoheptulose monophosphate as well as sedoheptulose monophosphate and intermediates of carbohydrate synthesis. It was hydrolyzed to free mannoheptulose which was identified chromatographically. Paper chromatographic separation of borate complexes of these heptuloses and the hexoses has been accomplished.

The similarity of mannoheptulose to sedoheptulose suggested that it too might be involved in the regeneration of the carbon dioxide acceptor of photosynthesis and in the phytosynthesis of carbohydrate and aromatic compounds. The stereostructures of these heptuloses are related as are those of galactose and glucose. The nature of the interconversions of these isomeric pairs is not yet clearly understood.

Mannoheptulose had been previously isolated from one variety of avocado (*Persea gratissima*, var. Trapp) fruit[2,3] and we have found that it does not accumulate in several others. It has been identified in the dried root of *Primula elatior*[4] but may not occur widely in the plant kingdom. Avocado leaves appear to be a more reliable source of this sugar. They contain 0.5 to 1% wet weight of mannoheptulose. It was isolated from deionized leaf extract by crystallization from alcoholic solution after removal of hexoses by yeast fermentation.

As sedoheptulose is apparently derived from its 7-phosphate in sedum leaves it was suspected that mannoheptulose monophosphate should be found in avocado leaves. The labeled phosphorylated products of photosynthesis in $C^{14}O_2$ were fractionated by paper chromatography. The area containing sedoheptulose-7- and glucose-6-phosphates gave 12% radioactive mannoheptulose upon chromatography of the phosphatase hydrolysate.

Glucose and mannoheptulose cochromatographed in all the standard solvents used for sugar separations on paper. Glucose, mannoheptulose and sedoheptulose were therefore separated as borate complexes in a butanol–ethanol–borate buffered solvent, and the relative R_f values were 1.0, 0.74 and 0.54, respectively.

Experimental Part

Isolation of Mannoheptulose.—Fresh avocado (seedling tree) leaves (18 kg.) were macerated in Waring Blendors with three parts of cold water. The tissue was removed by filtration through cheesecloth and the yellow-brown aqueous solution was heated to 80–90° for one hour to precipitate proteins. After clarification by centrifuging, the solution was evaporated under reduced pressure to 10 l. Much color and the ionic constituents were removed by passing through columns of Dowex-50 and Duolite A3 ion-exchange resins (80–100 mesh). The pale yellow effluent solution was concentrated (< 40°) to a thin sirup which was mixed with two volumes of ethanol at 70°. The voluminous precipitate of slimy and pectic substances was filtered off and extracted twice with 1.5 l. of boiling 60% ethanol to extract any coprecipitated perseitol. The combined alcoholic filtrates were concentrated to a thick sirup (*ca.* 400 g.) at reduced pressure. This was taken up in 800 ml. of boiling methanol and stored at 10° for crystallization of perseitol[5] which continued several days.

The crude perseitol was dissolved in a minimum volume of hot 80% methanol, decolorized with charcoal, and allowed to crystallize. The yield after one further crystallization was 55 g., m.p. 187–188°.

Paper chromatographic examination of the mother liquor from the crude perseitol showed that the solution contained mannoheptulose and copious amounts of fructose, glucose and sucrose. To remove these, the solution was freed of methanol by evaporation, diluted with water to 2 l. and fermented with baker's yeast for 24 hours at 37°. The yeast was filtered off and the alcohol and some water removed by evaporation at reduced pressure. The residue was again diluted with water and the fermentation repeated. After three such fermentations, the solution contained very little hexose. The residue was concentrated *in vacuo* to a thick sirup and diluted with four volumes of hot methanol. Almost pure mannoheptulose separated on cooling and was recrystallized from 85% methanol. The yield of pure mannoheptulose was 160 g., m.p. 150–152°, with an appreciable amount of recoverable sugar remaining in the mother liquors.

Identification of Mannoheptulose Phosphate in Avocado Leaves.—The concentration of sedoheptulose-7-phosphate in many plants is near 10^{-4} *M* and it was suspected that mannoheptulose monophosphate concentration may be even lower. Therefore, labeled intermediates of $C^{14}O_2$ reduction were prepared in the manner generally used in this Laboratory.[6] After five minutes, photosynthesis in $C^{14}O_2$ was stopped suddenly by plunging the leaf into liquid nitrogen. The frozen leaf was ground and the powder dumped into boiling 80% ethanol for extraction. Two-dimensional (phenol–water, butanol–propionic acid–water) chromatograms were prepared from such extracts. Glucose-6-phosphate and sedoheptulose-7-phosphate are not generally separable in these solvents. Identification of the sugars in this monophosphate area is accomplished by dephosphoryl-

(1) The work described in this paper was sponsored by the U.S. Atomic Energy Commission.

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