separated almost at once. These were collected and dried, giving 0.30 g. (27%) of colorless needles, m.p. 156–160°.

The crystals (0.30 g.) were recrystallized from 12 ml. of cyclohexane–benzene (5:1), giving 0.25 g. of colorless needle clusters, m.p. 158–159.5°.

For analysis a 150-mg. sample was recrystallized from about 7 ml. of absolute ethanol, m.p. 159–160°.

Anal. Calcd. for $C_{13}H_{13}BrN_2O_6$ (373.17): C, 41.84; H, 3.51; Br, 21.42; N, 7.51. Found: C, 41.83; H, 3.66; Br, 21.50; N, 7.51.

The compound gave a positive Beilstein halogen test, but was inert to alcoholic silver nitrate even after one minute boiling.

The compound was not attacked on prolonged stirring with 15 *M* aqueous ammonia or 4 *M* aqueous sodium hydroxide at 25°. It was attacked by boiling sodium hydroxide, giving a red solution. When heated with strong ethanolic ammonia in a sealed tube for several hours at 100°, the compound gradually dissolved, giving a solid product which we have not yet been able to purify.

DL-*trans*-**Cyclohexanediol-1,2 Bis-3,5-dinitrobenzoate.**—To 893 mg. (2.8 millimoles) of dry, finely pulverized silver 3,5-dinitrobenzoate in 3.6 ml. of dry benzene was added dropwise 254 mg. (1.0 millimole) of iodine in 2.5 ml. of benzene. To the resulting light tan suspension was added dropwise 0.112 ml. (90.3 mg., 1.1 millimoles) of cyclohexene in 1.2 ml. of benzene. After a few minutes stirring, the mixture was boiled under reflux for one hour. The hot mixture was filtered at once.

On cooling, the hot filtrate gave a small amount of crystals, presumably of the complex $AgI(C_6H_5COO)_2$. The solution was again filtered, and evaporated down to an oily residue.

The original residue (consisting mainly of silver iodide) was washed with 20 ml. of chloroform. The chloroform solution was used to dissolve the above oily residue. The resulting solution was concentrated to about 10 ml. and 10 ml. of cyclohexane was added at the b.p. On cooling, 218 mg. of crystals, m.p. 160–188°, was obtained.

The product was recrystallized from acetic acid, giving 52 mg. (10%) of the diol bis-dinitrobenzoate, yellow prisms, m.p. 181–182°, reported[18] m.p. 179°. (The corresponding iodohydrin ester reportedly[9b] melts at 159°.)

DL-*trans*-**Cyclohexanediol-1,2 Dibenzoate.**—The procedure was similar to that for the bis-dinitrobenzoate. From 11 millimoles of cyclohexene, 10 millimoles of iodine and 21 millimoles of silver benzoate (reflux period 3 hours) there was obtained an oily, yellowish crude product. This was recrystallized from absolute ethanol, giving 1.4 g. (44%) of the diol dibenzoate as colorless prisms, m.p. 92.5–93.5° (block), reported[18] m.p. 93°.

Acknowledgment.—We are indebted to Tennyson Campbell and Daniel Glick for assistance in some of the preparative work. Financial support by the National Research Council, the Research Council of Ontario and the Advisory Committee on Scientific Research is gratefully acknowledged.

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[CONTRIBUTION FROM THE HARRIS RESEARCH LABORATORIES]

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

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C^{14}-Specifically labeled cellulose was biosynthesized by *Acetobacter xylinum* from D-glucose-1-C^{14} as the sole labeled carbon source. The presence of ethanol in the medium increased the yield and the quantity of C^{14} found in the cellulose. The distribution of C^{14} in D-glucose from the bacterial cellulose hydrolysate indicated that approximately 82% of the activity was in position 1 for the cellulose produced from D-glucose-1-C^{14} in the ethanol-free medium compared to 70% in that position for the cellulose produced with ethanol present. Positions 3 and 4 contained the remainder of the activity in approximately equal amounts. The cellulose had a lower specific radioactivity than the D-glucose-1-C^{14} that was supplied. This result shows that some of the original hexose units are cleaved prior to cellulose formation. Thus, polymerization of the D-glucose as such, without prior chain cleavage, is not the sole mechanism of cellulose biosynthesis by this bacterium.

Introduction

This study was initiated to determine the ability of *Acetobacter xylinum* to produce labeled cellulose from various C^{14}-specifically labeled carbohydrates and derivatives used as substrates for the growth of the organism, and at the same time to provide some information regarding the involved mechanism in cellulose formation.[2] It is the purpose of this paper to report experiments concerned with the biosynthesis of C^{14}-specifically labeled cellulose by this bacterium when cultured in suitable media containing D-glucose-1-C^{14}. Since the inclusion of ethanol in the culture medium has been observed to increase the yield of cellulose from a given quantity of D-glucose,[3] experiments were performed to determine what advantage, if any, such addition might have with respect to the distribution and specific activity of C^{14} in the cellulose.

The biosynthesis of cellulose by *A. xylinum* at the surface of appropriate media was first noted by Brown[4] in 1886. Corroboration that this membraneous bacterial product, synthesized from either D-glucose or other suitable carbohydrate substrates, is chemically identical to cellulose produced by higher plants was obtained by Hibbert and Barsha[5–7] employing chemical analytical procedures in conjunction with X-ray techniques. Others[8–10] have also compared the X-ray patterns of bacterial cellulose with those of cotton cellulose. More recently electron microscopic techniques

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have been used for structural studies.[11-14] Shirk and Greathouse[15] employing infrared spectral techniques, have established the cellulosic nature of the pellicle produced by *A. xylinum*.

The ease with which cellulose may be produced by *A. xylinum* makes this organism attractive for producing isotopically labeled cellulose for the purpose of investigating the mechanism of cellulose formation. In this paper, the first of a projected series, experiments are reported in which D-glucose-1-C^{14} is the sole labeled source which was supplied to growing cultures of *A. xylinum*. Subsequent papers will report the results of similar experiments with the following substances: D-glucose-2-C^{14}, D-mannitol-1-C^{14}, ethanol-1-C^{14}, ethanol-2-C^{14}, acetic acid-1-C^{14} and acetic acid-2-C^{14}.

Experimental

The D-glucose-1-C^{11}, which was synthesized by Isbell,[16] was incorporated in the medium in which the bacteria were grown. The C^{14} furnished by the D-glucose-1-C^{14} was accounted for in carbon dioxide produced during the life of each culture, the purified cellulose obtained, and the liquid portion of the culture. The cellulose was examined further to determine the distribution of C^{14} over the six positions in the glucose units of the cellulose.

Culture Conditions and Products.—The medium, essentially that used by Tarr and Hibbert,[3] was composed basically of 0.5% Difco yeast extract and 0.3% KH_2PO_4 to which D-glucose was added so that the total content of both labeled and unlabeled glucose was 1%. The labeled component was added in amount so that the 20-ml. quantity used for culturing purposes contained approximately 25 microcuries of radioactivity. These media were sterilized by passing through a sterile UF Pyrex sintered glass filter. Following inoculation, using cells either from an actively growing broth or slant culture, the cultures were incubated for 7 days at 30°. Each culture, consisting of 20 ml. of medium and containing a total D-glucose content of approximately 200 mg., was grown in a 250-ml. erlenmeyer flask which was fitted to an all glass apparatus permitting the flow of CO_2-free air over the culture. The carbon dioxide formed was collected throughout the life of the culture and weighed as $BaCO_3$. At the end of the culture period the resultant pellicles were purified by boiling in 1% NaOH for 1 hour. Successive washes were made with boiling water, then 1% acetic acid, and again with water until free of acid. The purified pellicle was dried to constant weight at 105° and the yield was based on this dry weight. The cellulose yields from five cultures containing only D-glucose and no ethano ranged from 1.4 to 4.0%. The yields from cultures con taining 0.2 ml. 95% ethanol per 20 ml. of media and the customary quantity of D-glucose varied from 4.7 to 8.3%.

Counting Apparatus.—Count rates were calculated from the results of counts obtained by means of a Q-gas flow counter connected to a scaler. Necessary precautions were taken to assure a precision of about 1% of the observed count rate. Counts were made of thin samples, in most cases of $BaCO_3$, and the results were corrected in the standard way for the effects of background count, geometry, and self-absorption. The self-absorption correction for plates of material other than $BaCO_3$ was assumed equal to that of D-glucose plates of the same thickness.

Radioactivity Determinations of the Culture Products.—The activity of the CO_2 was determined from the $BaCO_3$ formed. Two methods were used for determination of the activity in the cellulose depending on the further work planned. When the position of the label in the cellulose structure was to be determined, the C^{14} content of the cellulose was calculated from a count of the hydrolysate prepared by the method of Monier-Williams[17]; otherwise, the cellulose was wet ashed,[18] and the C^{14} was determined from the CO_2 formed. The C^{14} in the remainder of the culture, including the material obtained from the purification of the cellulose, was determined by counts on dried aliquots after adjustment to *p*H 7–8.

Methods Used for Location of C^{14} in Cellulose.—The distribution of C^{14} within the 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 hydrolysate to obtain the desired specific activity. The activity at position 1 was obtained by Sowden's method.[19] The activity at position 3, positions 4 and 5 together, and positions 1 through 5 inclusive, were determined by oxidation with lead tetraacetate according to Abraham.[20] The CO_2 from positions 1 through 5 is determined, and compared with the total activity obtained by wet ashing so that activity at position 6 is determined by the difference. Oxidation of methyl glucoside, prepared by Fischer's method,[21] yielded CO_2 from position 3. It is important to recrystallize until the glucoside is free of glucose, for a small percentage of glucose impurity seriously affects the result. Activity at positions 4 and 5 was determined from the CO_2 produced by lead tetraacetate oxidation of phenylglucosotriazole prepared by the method of Hann and Hudson.[22]

In addition to the indirect determination noted above, direct determination of the activity at position 6 was made by the method of Reeves,[23] 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 50% aqueous pyridine solutions.

From the results of the analyses described, activity at position 2 can be calculated, and activity at positions 1, 3 and 6 is directly determined. The activity at position 6 may also be calculated from the other analyses as a check on the direct determination. Further analyses are required to determine the C^{14} at positions 4 and 5, and the scheme of Wood, Lifson and Lorber[24] was used with some modifications. This scheme involves fermentation to lactic acid, and degradation of it to locate the carbon atoms. This determines the activity in the position pairs 3 and 4, 2 and 5, and 1 and 6 in the original D-glucose. The modifications in the Wood, *et al.*, method were (a) the acetone–bisulfite obtained from decarboxylation of the lactic acid was decomposed with a 10% excess of $NaHCO_3$ which greatly minimized loss; (b) the aqueous acetaldehyde solution was converted to iodoform by adding 1.0 *M* NaOH so that the solution was 0.25 *M* in NaOH, and immediately adding 20% I_2 in KI solution as rapidly as it was decolorized. Persistence of a faint yellow color for 5 minutes was taken as the endpoint of the reaction. The iodoform (from the methyl group in lactic acid derived from positions 1 and 6 of the D-glucose) was washed with water, dissolved in $CHCl_3$ and again washed with water several times. The iodoform was recovered by evaporating the $CHCl_3$ under a blast of air at room temperature. It was then subjected to wet ashing, and from the specific radioactivity of the carbon dioxide (counted as $BaCO_3$), the C^{14} content of the carbon atoms at positions 1 and 6 was calculated.

The aqueous phase of the iodoform reaction mixture contained sodium formate derived from positions 2 and 5 of the glucose. Traces of dissolved iodoform in the aqueous phase of the reaction mixture were removed by extraction with ether until free of iodoform. This step was necessary when the C^{14} content at positions 1 and 6 was relatively large. Formic acid (from position 2 in the lactic acid derived from positions 2 and 5 of the D-glucose) was then distilled from the reaction mixture after acidification and reduction of the

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excess iodine. The distillate was subjected to oxidation with mercuric acetate which selectively oxidized the formic acid to CO_2.

The reliability of the analytical methods and procedures as described above is indicated by the results given in Table I. Analysis of known D-glucose-1-C^{14} showed 99.2% of the label at position 1, within 1% of the theoretical value. The methods selected in this work were further checked by analysis of a sample of D-glucose-C^{14} supplied by Abraham. The results shown in Table I were in good agreement with his values.[25]

TABLE I

TEST OF METHODS FOR LOCATION OF C^{14} IN D-GLUCOSE

Structure position	Randomly labeled D-glucose-C^{14} Reported,[a] %	Found, %	Glucose-1-C^{14} Found, %
1	19	18	99.2
2	20	20	0.8
3	11	11	0.0
4	11	12	−0.2
5	21	22	−0.4
6	18	19	0.21

[a] Abraham, *et al.*, ref. 25.

Results and Discussion

The yields of C^{14} accounted for in the cellulose and other culture products are shown in Table II.

TABLE II

YIELDS OF CULTURE PRODUCTS

	D-Glucose-1-C^{14} only		D-Glucose-1-C^{14} plus ethanol	
	Yield, mg. C.	C^{14} yield, %	Yield,[a] mg. C.	C^{14} yield, %
D-Glucose supplied	80.1		80.8	
Cellulose	2.1	0.55	6.6	3.7
Carbon dioxide	56.8	76.0	119.0[a]	58.4
Liquid products	21.0	23.4	34.5	38.6

[a] The medium contained 79.3 mg. of ethanol carbon, which accounts for much of the yield of carbon dioxide.

Most of the label from the culture containing only D-glucose was found in the CO_2. When ethanol and D-glucose were present, the percentage of label in the CO_2 was decreased, and at the same time the percentage of label in the cellulose was increased about sixfold. These data suggest a sparing action in the presence of ethanol in that less of the D-glucose label was metabolized to CO_2 and relatively more was incorporated in the cellulose.

The specific radioactivities of the CO_2 and the cellulose from the cultures are compared with the specific radioactivity of the substrate D-glucose-1-C^{14} in Table III.

TABLE III

SPECIFIC RADIOACTIVITY VALUES OF CULTURE PRODUCTS

	D-Glucose-1-C^{14} only, microcuries/mg. C.	D-Glucose-1-C^{14} plus ethanol, microcuries/mg. C.
D-Glucose-1-C^{14} supplied	0.34	0.36
D-Cellulose-C^{14} obtained	.071	.16
CO_2	.37	.14[a]

[a] A considerable portion of the CO_2 from this culture was derived from the ethanol in the medium. This accounts for the low specific radioactivity.

The specific radioactivity of the carbon dioxide was essentially the same as that for the substrate D-glucose-1-C^{14} in the non-ethanolic culture, indicating that all six of the D-glucose carbon atoms were equally metabolized to carbon dioxide. In the case of the culture containing ethanol, a large part of the alcohol was metabolized to carbon dioxide, accounting for the reduced specific radioactivity shown.

The specific radioactivity of the cellulose, on the other hand, was lower than that of the substrate D-glucose-1-C^{14} in both cultures, and was appreciably higher in the ethanolic than the non-ethanolic culture.

Table IV shows the distribution of C^{14} in the D-glucose obtained by hydrolysis of the radioactive cellulose. Although the major portion of the label was found at position 1, appreciable quantities were found at positions 3 and 4. This spread of radioactivity in the D-glucose molecule indicates that scission products, presumably 3-carbon intermediates, were involved in the cellulose biosynthesis. These data are in accord with the observed ability of this microörganism to synthesize cellulose with glycerol as the substrate.[3] The results from this study alone do not reveal the mechanism of cellulose biosynthesis. However, the fact that there is no activity in position 6 would indicate that the triose fragments formed by the usual glycolytic process are not involved since such a mechanism would result in activity at the 6-position. Additional biosynthesis studies using D-glucose-2-C^{14}, labeled ethanol and labeled acetate indicate another mechanism is operating and this will be discussed in a later paper.

TABLE IV

LOCATION OF LABEL IN STRUCTURE OF GLUCOSE UNITS IN BACTERIAL CELLULOSE

Structure position	D-Glucose culture Millimicrocuries per mM.[a]	Per cent.	D-Glucose + ethanol culture Millimicrocuries per mM.[a]	Per cent.
1	53.0	82.2	65.7	70.0
2	0.4[b]	0	−0.5[b]	0
3	7.0	10.8	14.1	15.0
4	5.6	8.7	12.9	13.7
5	−0.1[b]	0	1.3	1.4[b]
6	0.6[b]	0	1.5	1.6[b]

[a] Found by analysis after dilution with carrier glucose. [b] Within the realm of experimental error.

From the ratio of the specific radioactivities of cellulose to glucose of 21%, which can be calculated from the data in Table III for the non-ethanolic culture, and the amount of label at position 1 of 82.2%, as given for the same culture in Table IV; it can be calculated that at most 17% of the cellulose could have been formed from the intact D-glucose-1-C^{14} molecule by direct polymerization. On the other hand, in the presence of ethanol, 31% of the cellulose might have been formed in this manner. These data would indicate that in the presence of an easily oxidized substrate such as ethanol, where glucose is not the sole source of carbon along with the carbon of the yeast extract which is present in both cultures, more of the label enters the cellulose molecule. The pathway of formation of cellulose from the intact labeled D-glucose by the bacteria apparently differs appreci-

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ably from that reported for the formation of cellulose in the cotton boll.[2]

The pattern of label distribution in the celluloses from the ethanolic and non-ethanolic cultures was similar; that is, the distribution of label was confined to positions 1, 3 and 4, with the major proportion located at position 1. For this reason the addition of ethanol to the culture medium is clearly advantageous where it is desired to produce optimum yields of isotopically labeled cellulose from D-glucose-1-C^{14} by *Acetobacter xylinum*.

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[CONTRIBUTION FROM THE BEN MAY LABORATORY FOR CANCER RESEARCH, UNIVERSITY OF CHICAGO]

Benzylidene-L-iditols

BY JEAN SICÉ*

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The acid-catalyzed reaction of L-iditol with benzaldehyde gave two acetals, a 2,3,4,5-di-*O*-benzylidene and a tri-*O*-benzylidene derivative.

A number of three-carbon dioses and trioses were needed in this Laboratory to study the steric requirements for the substrates of some enzymes involved in anaerobic glycolysis. It was thought that L-iditol might give access to the substances of the L-series, if suitable acetals or ketals were easily obtainable; this procedure would obviate the laborious preparation of L-mannitol.[1]

A 2,4:3,5-di-*O*-methylene-L-iditol[2] is the only well characterized acetal of iditol. The acid-catalyzed condensation of D-iditol with benzaldehyde has been reported to give a tribenzylidene derivative, $[\alpha]_D$ − 6° in acetone,[3] m.p. 224–228°.[4] Its enantiomorph, melting at 224–228°,[4] 242°[5] or 249°,[6] has been similarly prepared from L-iditol. Some doubt has recently been cast[7] on the possible existence of triacetals of iditol. A dimorphic tribenzylidene-L-iditol, $[\alpha]_D$ + 5.2° in chloroform, can indeed be synthesized. The wide range of melting points recorded in the literature is explained by the unusual melting phenomenon of this substance. It melted at 226–228° or 252–254° depending on the isolation procedure; the melting point of either form would, after a few days, be found in between those extreme values. The favored ring forms of this compound[8] are 1,3:2,5:4,6 as in mannitol[9] or 1,3:2,4:5,6 as in talitol.[7]

A dibenzylidene-L-iditol, melting at 192°[10] or below 190°,[6] has been reported as a by-product in the preparation of the triacetal. This compound (I) can now be prepared in good yield as the major product of the condensation; it was, however, found difficult to separate it by crystallization from the triacetal formed in the reaction. This substance was dimorphic also, m.p. 207–208° or 229–231°; the latter form did not give an appreciable depression of the melting point on admixture with the lower melting form of the tribenzylidene derivative. It was not sufficiently soluble to permit a reading of its rotation.

This dibenzylideneiditol (I) did not reduce periodate. Its ditoluenesulfonate ester was quite resistant to the exchange reaction with sodium iodide or lithium aluminum hydride under usual conditions. A more drastic procedure gave the expected diiodo derivative II which could be reduced to the dimorphic 1,6-dideoxydibenzylidene-L-iditol (III). Replacement of the acetal groups by acetolysis and subsequent catalytic deacetylation gave 1,6-dideoxy-L-iditol identified by conversion to its known[2] dimethylene acetal. By analogy with the latter compound, the dibenzylidene derivative may have the structure 2,4:3,5.

A small amount of triacetal was in every case formed during the preparation of the diacetal. Some diacetal was always isolated from the preparation of the triacetal.

Experimental

The melting points were determined in evacuated capillaries and corrected. The optical rotations were determined in alcohol-free chloroform, *c* being the concentration in g./100 ml. of solution; the tube length was 1 dm. The microanalyses were performed, unless otherwise specified, by Mr. W. Saschek.

2,3,4,5-Di-*O*-benzylidene-L-iditol Diacetate (Ia).—A solution of 1.86 g. of crystalline L-iditol[11] in 4 ml. of 6 *N* hydrochloric acid was shaken for a few hours with 2.20 g. (2.0 mol. eq.) of benzaldehyde. After standing overnight at room temperature the reaction mixture had set to a gel. The cake was broken, transferred to a filter, washed with dilute ammonium hydroxide, water and dried over calcium hydride *in vacuo*. The white mass (3.12 g.) was then dissolved in 10.0 ml. of pyridine and treated with 10.0 ml.

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