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Isolation of a Cell-free Enzyme System from *Acetobacter xylinum* Capable of Cellulose Synthesis

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A cell-free enzyme system was isolated from the cellulose forming bacterium, *Acetobacter xylinum*. Cellulose-C^{14} was produced by this enzyme system from D-glucose-1-C^{14} and the position of the C^{14}-label determined in the D-glucose resulting from the hydrolysis of the cellulose formed. Ninety-six per cent. of the label was found to be in position one of the cellulose molecule.

Introduction

Previous publications[1-5] have been concerned with the biosynthesis of cellulose-C^{14} by *Acetobacter xylinum* from specifically C^{14}-labeled hexoses and trioses. We now report studies on the isolation of a cell-free enzyme or enzymes from the bacterium capable of synthesizing radioactive cellulose from D-glucose-1-C^{14}. Analyses reported include the distribution of the C^{14}-label in the purified D-glucose from the hydrolyzed enzyme cellulose-C^{14}.

Muhlethaler[6] by means of electron microscopic studies of *A. xylinum* presents evidence that the formation of cellulose by this bacterium probably does not take place within the cell or from its surface, but rather at some distance from the organism. This suggests that cellulose results from a possible enzymatically induced polymerization of the embedding slime that can accordingly be thought of as a kind of cellulose-precursor.

Schramm, Gromet and Hestrin[7,8] state that glucose, gluconate, dihydroxyacetone and glycerol in the presence of oxygen were rapidly converted to cellulose by the dry cell preparation of *A. xylinum*. They also state since aldolase was present in the cell extract, elongation and cleavage of the sugar carbon chain could have occurred by an action of this enzyme.

As far as we know this report describes the first experimental proof that a cell-free enzyme system capable of polymerization of hexose to cellulose has been studied.

Experimental

Culture Conditions.—The bacterium, *Acetobacter xylinum*, was grown on the following medium: 20 g. of yeast extract (Difco),[9] 10 g. of D-glucose, 1 g. of KH_2PO_4 and 10 ml. of ethyl alcohol per liter. This medium had a *p*H of 5.88. The inoculum was 1 ml. of a 24-hour liquid culture. The cultures were incubated for 48 hours at 30°. By this time membrane formation was evident.

Methods of Preparing Cell-free Enzymes from *A. xylinum*.—Muhlethaler's[6] study indicated that this organism produced cellulose by means of extracellular enzymes. It is realized that bacteria cells are particularly difficult to disrupt as well as produce cell-free enzyme systems. Thus, the degree of disruption was assessed by high power microscopic examination of stained preparations and making viable count to determine whether the enzyme product was cell-free. In addition, controls were made for each experiment and were left to incubate for 7 days and then examined microscopically for whole or living cells. In no case were cells found using the adopted method described. Hugo[8] discusses methods of preparing cell-free enzymes from microörganisms. The suggested methods of Hugo were evaluated and the one producing satisfactory results as to enzyme activity and being cell-free was selected.

The first experiments were performed utilizing a combination of standard methods for extracellular enzyme isolation. Later experiments used methods of disruption with abrasive particles, crushing without the use of abrasives, disintegration by agitation with small particles, disintegration by alternate freezing and thawing and by chemicals (surface active, dehydrating, etc.). An activity capable of producing cellulose in 15 to 18% yields from D-glucose was obtained with each of the methods studied at an optimum *p*H of 8.5. Consequently, the following method was adopted for this study. The contents (250 ml.) of ten flasks were poured into a chilled Waring Blendor vessel, and the blender was run for 15–30 seconds in order to break up completely the existing membranes. The crushed material (0.5 to 1 g. solids) was centrifuged at 3000 r.p.m. for 5 minutes to remove large particles and any abrasive material. The supernatant fluid was lyophilized. The resulting material was a light brown, hygroscopic powder. The freeze-drying required between 4 and 6 hours. The total solids were ether extracted to remove fats, etc., and then ground with 200-mesh powdered glass. The crude enzyme material was kept cold (−5 to 0°) until used in the experiments 1–2 hours later.

A 10% "enzyme" solution of the dry extracted material was made. Two and one-half ml. of this solution was added to 25 ml. of previously sterilized liquid medium consisting of 1% glucose, 2% yeast extract, 0.1% KH_2PO_4, and 0.5% ethyl alcohol plus 0.1% adenosine triphosphate (ATP). The enzyme system was incubated at 30°. The enzymatically synthesized cellulose was formed within 2–4 hours and no contamination occurred. A small quantity of ether was added to the surface of the cultures to delay or prevent possible contamination.

Preliminary experiments were conducted toward purification of the crude enzyme system. Of the purification methods tried, the glycerol (75%) extraction procedure gave the most promising results. Further research is required on this phase of the study.

Results and Discussion

The effect of varying *p*H values on the extent of cellulose produced by the cell-free enzyme systems is shown in Table I.

These data indicate the optimum *p*H for the enzyme systems is 8.5 to 9.0. Consequently all enzyme systems were adjusted to a *p*H of 8.5 for further studies.

Preliminary experiments indicated that the addition of adenosine triphosphate (ATP) to the other medium ingredients increased the cellulose yield. Thus experiments were designed to evaluate this factor. These data are presented in Table II.

(1) F. W. Minor, G. A. Greathouse, H. G. Shirk, A. M. Schwartz and M. Harris, THIS JOURNAL, **76**, 1658 (1954).

(2) F. W. Minor, G. A. Greathouse, H. G. Shirk, A. M. Schwartz and M. Harris, *ibid.*, **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) K. Muhlethaler, *Biochim. et Biophys. Acta*, **3**, 527 (1949).

(7) M. Schramm, Z. Gromet and S. Hestrin, *Bull. Res. Council of Israel*, **5A**, No. 1, 99 (1955).

(8) W. B. Hugo, *Bacteriol. Rev.*, **18**, 87 (1954).

(9) No satisfactory synthetic medium has been developed for the production of cellulose by *A. xylinum*, therefore, the utilization of yeast extract in the culture and enzyme medium.

TABLE I

OPTIMUM *p*H OF ENZYME SYSTEMS CAPABLE OF CELLULOSE FORMATION

*p*H	Cellulose yield, mg.	*p*H	Cellulose yield, mg.
5.0	0.40	8.0	34.30
5.5	0.82	8.5	48.04
6.0	4.71	9.0	47.35
6.5	12.34	9.5	32.50
7.0	19.85	10.0	0.25
7.5	25.22		

TABLE II

THE EFFECT OF ADDING ADENOSINE TRIPHOSPHATE TO THE MEDIUM ON CELLULOSE YIELD (30°, *p*H 8.5)

Amount of ATP added, g.	Cellulose yield, mg.	Amount of ATP added, g.	Cellulose yield, mg.
0	0.2	0.10	43.4
0.02	10.5	.20	45.1
.04	18.7	.40	44.8

Practically no cellulose was produced by this enzyme system without the addition of ATP. The yield was increased to 40 to 45 mg. by the addition of 0.1 to 0.4 g. of ATP. The energy for this possible direct polymerization of the glucose or glucose phosphate to cellulose by the cell-free enzyme system is likely phosphorylation, high energy phosphate bonds of ATP.

Production of Cellulose-C^{14} by the Isolated Cell-free Enzyme System.—The crude enzyme preparation was added to the medium containing D-glucose-1-C^{14} (50 microcuries) and 0.1% ATP along with the standard D-glucose, and other medium ingredients after adjusting to a *p*H of 8.5. Cellulose was produced as before, purified and the radioactivity determined. The yield based on the D-glucose-1-C^{14} was approximately 15%.

The cellulose was hydrolyzed and the resultant labeled D-glucose purified and crystallized. Paper chromatographic analysis of the cellulose hydrolyzate showed only one radioactive spot, being an R_f value identical with that of a D-glucose reference shot. An aniline hydrogen phthalate spray revealed only the same spot. The hydrolyzates were mixed with unlabeled D-glucose and purified by crystallization, to give D-glucose-C^{14} with the specific activity of 1.665 mc./m*M*. A 50-mg. sample of this radioactive glucose was oxidized by oxygen in aqueous potassium hydroxide to remove carbon 1.[10] The resulting potassium D-arabonate was separated and recrystallized and the percentage of C^{14}-activity determined. Calculations showed that 96.7% of the label was in the original position 1. Consequently, no other carbon positions were analyzed.

The radioactivity of the D-glucose was obtained with an α-β-γ proportional counter Model PC-1 and checked 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 of $BaCO_3$ and D-glucose 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. equalled one disintegration per second.

These data are most interesting, since they constitute strong evidence that glucose-1-C^{14} was synthesized to cellulose directly by the cell-free enzyme system.

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