77

BARD

FINAL REPORT

PROJECT No. I-174-80

A Comparison of the Mechanism of Cellulose Synthesis in Bacteria and Higher Plants

M. Benziman, D.P. Delmer

1983

December 28, 1982

BARD

P.O.Box 6

Bet Dagan, Israel

BARD No. of Proposal: #I-174-80

Title of Proposed Research:

"A Comparison of the Mechanism of Cellulose Synthesis in Bacteria
and Higher Plants"

Name of Investigators

Principal Investigator: Moshe Benziman

Cooperating Investigator: Deborah P. Delmer

Name and Address of Affiliated Institutions

Principal Institution: The Hebrew University of Jerusalem
Department of Biological Chemistry

Cooperating Institution: Michigan State University
East Lansing, Michigan

Project's Starting Date: September 1980

Type of Report: Final

Signature

Principal Investigator

Institution's Authorizing Official

TABLE OF CONTENTS

Abstract	2
Detailed Report.	3
1. Requirement for a membrane potential for cellulose synthesis in intact cells of <u>Acetobacter xylinum</u>	3
2. The biochemistry of the polymerization process.	4
3. Tracing the path of carbon to cellulose	8
Evaluation of the Research Achievements with Regard to the Original Research Proposal.	11
Description and Results of Cooperation	12
References	13
List of Publications Derived from This Project	15

ABSTRACT

We have applied some preliminary findings with higher plant systems to a study of the properties of the Acetobacter xylinum UDP-glucose: β -1,4 glucan (cellulose) synthase. This study revealed a complex GTP- Ca^{++} -protein factor-mediated regulatory system for the enzyme. This discovery has led to the ability to achieve in vitro rates of 1,4- β -D-glucan synthesis which are 200 times higher than any previously reported. Such rates exceed 40% the in vivo rate of cellulose synthesis from glucose. The enzyme has been effectively solubilized from the membranes with digitonin. The solubilized enzyme is even more active than the membrane-bound form and is also activated by GTP in association with the protein factor.

The factors responsible for loss of cellulose synthesizing activity upon cellular disruption have been investigated. Results of studies with A. xylinum in which we altered transmembrane electrical potential ($\Delta\psi$), ΔpH or both, demonstrated a requirement for a threshold $\Delta\psi$ in order to maintain active cellulose synthesis in intact cells of this organism and raise the possibility that modulation of the $\Delta\psi$ could be one means of regulating the rate of cellulose synthesis in vivo.

An exhaustive characterization survey of possible "cellulose precursor" materials in chloroform/methanol-, water- and urea-soluble cellular fractions has been carried out. The results obtained led us to conclude that no positive evidence exists for any intermediates beyond the level of UDP-glucose on the pathway to cellulose in A. xylinum.

The results of these studies have been incorporated in five publications: two in Proc. Natl. Acad. Sci. USA; two in J. Applied Polymer Sci; and one in J. Biol. Chem.

DETAILED REPORT

As we pointed out in our proposal, cellulose is the world's most abundant organic compound, yet the underlying mechanism of its synthesis remains poorly understood. For obscure reasons, the enzyme(s) involved in the polymerization process are apparently quite labile, and thus it has been difficult to study the reaction in cell-free preparations. One of our goals has been to understand the reasons for this lability and to find conditions which would stabilize the enzyme system for study in vitro. In addition, previous work with Acetobacter indicated the probable existence of high molecular weight intermediates on the pathway, and we hoped to characterize these compounds. As indicated in the summary below, we believe that we accomplished a substantial part of these goals.

1. Requirement for a membrane potential for cellulose synthesis in intact cells of *Acetobacter xylinum*

The marked lability in cell-free preparations of the enzyme system involved in cellulose biosynthesis in most organisms studied, led us to investigate the factors responsible for loss of activity upon cellular disruption. Previous studies with cotton fibers (1, 2) led to the suggestion that the existence of a transmembrane electrical potential ($\Delta\psi$) may be one factor responsible for maintaining an active system in intact cells. As a continuation of these studies, we sought to assess the specific effects of alterations in $\Delta\psi$ and ΔpH on the synthesis of cellulose in living cells of *Acetobacter xylinum*. We have been able to demonstrate that dissipation of the $\Delta\psi$ in metabolizing cells of *A. xylinum* results in severe inhibition of cellulose synthesis. The effect is reversible upon restoration of the $\Delta\psi$. Inhibition of cellulose biosynthesis by dissipation of the $\Delta\psi$ can be observed under conditions where no substantial impairment of energy metabolism occurs, i.e., under conditions where a transmem-

brane pH gradient is of sufficient magnitude to maintain an adequate overall proton motive force across the membrane. The inhibition of cellulose biosynthesis is specifically related to changes in the $\Delta\psi$, since the process can proceed normally in the absence of the pH gradient. More recently, we have observed that there appears to be a critical threshold value of $\Delta\psi$ (between -50 and -70 mV) below which cellulose synthesis ceases. These results support the suggestion that loss of the $\Delta\psi$ upon cellular disruption may be one factor responsible for the low capacity and apparent lability of the cellulose synthesizing system in isolated membrane preparations, and also raise the possibility that modulation of the $\Delta\psi$ could be one means of regulating the rate of cellulose synthesis in vivo.

These results also point to an interesting analogy with the process of chitin synthesis in insects, where even slight damage to cellular integrity leads to loss of activity (3). The existence of a sharp threshold $\Delta\psi$ also has a number of parallels with the so-called "gating effects" observed for Na^+ channels of excitable membranes (4) and for transport processes (5). Indeed, recent studies indicate that a number of apparently unrelated phenomena (reviewed in ref. 7) respond to changes in the $\Delta\psi$, suggesting some fundamental regulatory phenomenon which has to date not been satisfactorily understood at the molecular level.

For a full report of these findings, see ref. 7.

2. The biochemistry of the polymerization process

Until quite recently, attempts to demonstrate convincing in vitro synthesis of cellulose have met with only very limited success. With higher plants, it is not clear that such success has ever been achieved (8), and even in the case of A. xylinum, where in vitro synthesis of 1,4- β -D-glucan from UDP-glucose was first

reported in 1958 by Glaser (9), the rates of in vitro synthesis have traditionally been only about 0.2% of those observed in vivo (10). For this reason, it has been difficult even to be certain of what form(s) of activated glucose serves as a precursor to cellulose. In spite of these difficulties, it is now generally accepted that UDP-glucose is a primary precursor in all organisms capable of cellulose synthesis. Evidence for this in A. xylinum comes from a number of reports indicating that UDP-glucose can serve as a substrate for in vitro synthesis of 1,4- β -D-glucan (11) and from in vivo tracer studies by Swissa et al. (12). In higher plants, a recent comprehensive quantitative analysis by Carpita and Delmer (13) of carbon flow in the developing cotton fiber offered strong support for the concept that UDP-glucose is a precursor to secondary wall cellulose. A number of reports exist of some limited in vitro synthesis of 1,4- β -D-glucan using cell-free preparations derived from a variety of plant tissues (reviewed in refs. 8, 14-16). However, many of these reports should be viewed with caution, as it was not always possible to conclude that the product synthesized in vitro was really a cellulosic 1,4- β -glucan; in some instances, the product could have been a backbone structure for xyloglucan, a portion of a mixed linked β -1,3-1,4-glucan or even 1,3- β -D-glucan which is always synthesized in abundance in vitro by a highly active UDP-glucose: 1,3- β -D-glucan synthase and which is also plasma-membrane localized (8, 14-16).

Some success in enhancing in vitro 1,4- β -D-glucan synthesis in vitro in cotton fibers has been achieved by Delmer's group by imposition of a transmembrane electrical potential across isolated membrane vesicles (2); this group also found a marked and preferential stimulation of this activity by isolating and assaying membranes in the presence of high molecular weight polyethylene-glycols (17). Low substrate concentrations and the presence of Mg^{++} have also been found by a number of workers to enhance 1,4- β -D-glucan synthesis from

UDP-glucose in plant extracts (8, 14-16). Studies by MacLachlan's group with peas (18) and Delmer's group with cultured soybean cells (17) indicated that dissociable activating and/or inhibitory factors may be present in plant extracts which can influence the capacity for β -glucan synthesis in vitro. No one of these factors has led to a dramatic improvement in the rate of in vitro synthesis of cellulose in cell-free preparations derived from higher plants. However, the influence of polyethyleneglycols and possible existence of dissociable factors in these systems have very interesting analogies to the recently discovered regulatory properties of the A. xylinum UDP-glucose: β -1,4-glucan synthase, as described below.

As for the cotton fiber system, polyethyleneglycol (PEG-4000) was found to enhance about 3- to 10-fold UDP-glucose: β -1,4-glucan synthase activity in membranes derived from A. xylinum. In contrast, the activity of a UDP-glucose: β -1,2-glucan synthase also present in the A. xylinum preparations was not stimulated by PEG-4000 (17). Discovery of the PEG effect in A. xylinum led to a series of experiments which revealed a complex regulatory system for the A. xylinum UDP-glucose: β -1,4-glucan synthase. Thus, we have shown that the enzyme present in membrane fractions isolated in PEG-4000 (called PEG-MF) show a marked and specific activation by GTP. However, enzyme in membrane fractions prepared in the absence of PEG-4000 (MF), responded only poorly to addition of GTP. This observation led to the finding that PEG-4000 promotes the association of a protein factor with the enzyme; the factor itself has no enzyme activity nor does it stimulate enzyme activity in the absence of GTP. However, the factor is absolutely required for GTP activation of the enzyme. Factor can be dissociated from the enzyme by washing PEG-MF with a buffer lacking PEG-4000. Re-addition of the supernatant from the washed membranes restores the capacity for GTP activation. Evidence was obtained to show that the factor is a protein which is

heat-labile, destroyed by protease treatment, and has a molecular weight >30,000, as judged by gel filtration chromatography.

We have recently discovered that Ca^{++} can replace PEG-4000 in promoting factor-enzyme association and thus also GTP activation of the enzyme.

The A. xylinum enzyme can be effectively solubilized from the membranes with digitonin. The solubilized enzyme is even more active than the membrane-bound form and is also activated by GTP in association with the protein factor.

The discovery of these regulatory properties as well as our ability to solubilize the enzyme, have opened up a number of exciting possibilities for future research on cellulose biosynthesis. We should re-emphasize that the alkali-insoluble product of this enzyme reaction has been definitively characterized as exclusively 1,4- β -D-glucan. The product is digested by highly purified cellobiohydrolase or endo- β -1,4-glucanase; methylation analysis of the radioactive product yields exclusively the derivative expected for a 1,4-linked glucan. Preliminary analyses indicate that the chain length of the product is >100 glucose residues. Determination of the crystallinity and/or microfibril size awaits future investigation.

Under optimal conditions (saturating levels of substrate, Mg^{++} , factor, GTP, and PEG-4000 or Ca^{++}), we can now achieve initial rates of 1,4- β -D-glucan synthesis in vitro which are 200 times greater than those ever previously reported in the literature. Such rates are approximately 40% of the in vivo rate of cellulose synthesis in A. xylinum. Using the digitonin-solubilized enzyme, even higher rates can be achieved. Thus, using A. xylinum, we now have in hand a model in vitro system which is highly promising for further exploration of the mechanism and regulation of cellulose biosynthesis. Additional knowledge of the details of the mechanism and regulation of cellulose synthesis in this bacterium will undoubtedly provide us with more background information necessary

for future studies with higher plants. Moreover, we believe that A. xylinum may well emerge as an organism of some importance for the commercial production of purified forms of cellulose and, therefore, merits serious study in its own right.

For a full report of these findings, see refs. 19 and 21.

3. Tracing the path of carbon to cellulose

For a cell type in which a major proportion of the carbon is converted into cellulose, it should be possible to trace the flow of carbon from supplied radioactive glucose into cellulose and thus to determine the intermediates on the pathway to cellulose. Resting cells of A. xylinum convert more than half of the carbon from supplied glucose into cellulose (10), making them ideal organisms for such in vivo studies. Similarly, we have studied, as a representative of the plants, the developing cotton fiber which also converts a high proportion of the supplied reduced carbon to cellulose (13).

Benziman's group (10, 12) monitored the flow of carbon from [^{14}C]glucose through sugar phosphates, UDP-glucose, water- and alkali-soluble polymers, and into cellulose. From pulse and pulse-chase experiments, they concluded that glucose was converted through glucose-phosphates to UDP-glucose; the pattern of labeling for UDP-glucose was consistent with it being a precursor to cellulose. However, they observed that labeled carbon also appeared in other water- and alkali- or urea-soluble materials which, in a subsequent chase, moved into the cellulosic fraction. They thus proposed that UDP-glucose serves as a precursor for some soluble polymer(s) which, in turn, donates carbon to cellulose.

We have recently begun to characterize the components present in these water- and alkali- (or urea-) soluble fractions. In sequential extractions

we have shown that the majority of the "precursor material" is present in the water-soluble fraction (WS). We have not yet analyzed the urea-soluble fraction in detail. WS was obtained by first extracting pulse-labeled cells five times with 80% ethanol; these extractions are discarded and are followed by extraction with water to obtain the "precursor" material.

Separation on DEAE cellulose of radioactive components in WS, obtained from cells pulsed with [^{14}C]glucose, repeatedly yielded three peaks which showed substantial reduction in radioactivity in a chase with unlabeled glucose. We have succeeded in elucidating the structure of the components present in two of the three "precursor" fractions: one peak has been identified as fructose-1,6-diphosphate (FDP) and the second peak was found to be a mixture of glucose-6-P and fructose-6-P. We have not yet positively identified the structure of the compounds present in the third peak but this fraction appears to be a mixture of other sugar-phosphates and/or nucleotide sugars. We also now know that the water-soluble and alkali-soluble fractions analyzed by Swissa et al. (12) and Aloni et al. (10) contained, in addition to the compounds described above, very fine fibrils of cellulose which were not pelleted by the centrifugation procedure used in preparing these extracts, but which require either filtration or high-speed centrifugation for removal. In short-term pulses, these small fibrils are abundant, whereas they are much less abundant in extracts derived from cells that had been chased for long intervals in high concentrations of unlabeled glucose, presumably because such fine fibrils become trapped in the large aggregates of cellulose produced in long incubations. Thus, we now believe that the unfiltered dialyzed water- and alkali-soluble fractions analyzed previously contained a mixture of FDP, fructose-6-P, glucose-6-P, and fine fibrils of cellulose. During a chase, carbon from the sugar-phosphates chases rapidly into cellulose, concomitant with a much slower

apparent "chase" of the fine cellulosic fibrils, into the mat of larger aggregates of cellulose. The apparent partial digestion of these "precursor" fractions by protease (12) can be explained as being due to the digestion of random proteins in the extract, which apparently interact with and retard removal of the sugar-phosphates during dialysis.

There has also been much speculation in the literature concerning the possible existence of lipid-linked intermediates in A. xylinum and plants (8). Garcia et al. (22) and Kjosbakken and Colvin (23) reported the synthesis from UDP-glucose of glycosyl-lipids in A. xylinum, one of which was suggested to be lipid-PP-cellobiose. Dankert's group has extended this finding by showing that frozen-thawed, EDTA-treated A. xylinum cells synthesize from UDP-glucose not only lipid-PP-cellobiose but also lipid-PP-mannosyl cellobiose (24) and lipid-PP-oligosaccharides (Dankert, personal communication) in the presence of additional nucleotide sugar substrates. The structure of these oligosaccharides resembles portions of a polysaccharide unrelated to cellulose, which is also produced by their strain of A. xylinum. Thus, they suggest that synthesis of lipid-PP-cellobiose may be related to synthesis of this polysaccharide rather than to cellulose synthesis.

We have carried out extensive searches, both in cotton fibers and in A. xylinum, for the presence of glycosyl-lipids which could serve as precursors to cellulose. To date, in neither system have we found convincing evidence of such compounds. Experiments were performed both in vivo by pulse-labeling cotton fiber or A. xylinum cells with [^{14}C]glucose and in vitro by supplying extracts (or freeze-thawed, EDTA-treated A. xylinum cells) with UDP- ^{14}C glucose. Experiments were also performed in the presence or absence of inhibitors of cellulose synthesis in hopes of causing accumulation or a decrease in the

levels of possible precursors. Radioactive compounds, extracted by a variety of procedures with varying mixtures of organic solvents, were analyzed; in no case did we find lipid-PP-cellobiose nor did we find any other acid-labile lipids with properties consistent with being a precursor to cellulose. As we did not find lipid-PP-cellobiose, even under identical conditions employed by Dankert's group, we can only conjecture that our strain does not produce the non-cellulosic polysaccharide described by them. Although negative findings never provide absolute proof for lack of existence of these intermediates, at present we remain skeptical about their involvement in cellulose biosynthesis.

In summary, we thus conclude from an exhaustive survey of the chloroform/methanol-soluble, water-soluble, and urea-soluble fractions, that no positive evidence exists for any intermediates beyond the level of UDP-glucose in this strain of A. xylinum.

For a full report of these studies, see ref. 17.

EVALUATION OF THE RESEARCH ACHIEVEMENTS WITH REGARD TO THE ORIGINAL RESEARCH PROPOSAL

As is obvious from the above description of results obtained in this work, we began our collaboration by testing a variety of experimental approaches, as outlined in our original proposal, hoping that one or more would develop into interesting projects. To our pleasure, all of these approaches have yielded exciting and interesting results. The highly active cellulose synthase system described here with its interesting regulatory properties should be of great value in the future for dissecting the details of the mechanism and regulation of bacterial cellulose biosynthesis. This, in turn, will undoubtedly provide us with more background information necessary for future studies with higher

plants. The successful solubilization of an active system which synthesizes exclusively β -1,4-glucan, could serve as a useful tool for reconstituting, in the future, the purified components in defined lipid bilayers. This would open the possibility of studying such issues as the vectorial deposition of glucan products across the bilayer as well as the nature of the unusual requirement for a transmembrane electrical potential for cellulose synthesis in vivo discovered in the course of this work.

We have dedicated much effort in the characterization of the possible precursor compounds beyond the level of UDP-glucose on the route to cellulose in A. xylinum. Although the results obtained were negative and, therefore, to a certain extent disappointing, we feel that our conclusion, namely, that no such intermediates are involved in the process, would bring a long-debated issue to rest.

DESCRIPTION AND RESULTS OF COOPERATION

For the projects summarized in this report, Dr. Delmer has been conducting cooperative research on cellulose synthesis in the laboratory of Dr. Ben-ziman during her sabbatical leave in Israel for the first year, and a leave of absence for the second year. Dr. A. Bacic, a post-doctoral trainee in the laboratory of Dr. Delmer, has joined our group in Jerusalem for 6 months, working mainly on characterization of the postulated "intermediates." Samples of these intermediates have been sent for structural analysis in Dr. Delmer's laboratory in Michigan.

We feel that our collaboration has been extremely successful, and we anticipate extending it in the future so as to reap the full benefits of the results accomplished from our present collaborative venture.

REFERENCES

1. Carpita, N.C. and Delmer, D.P. *Plant Physiol.* 66, 911 (1980).
2. Basic, A. and Delmer, D.P. *Planta* 152, (1981).
3. Vardonis, B. *Biochim. Biophys. Acta* 588, 142 (1979).
4. Abood, L.G. *in* "Basic Neurochemistry" (Siegel, G.J., Albers, R.W., Katzman, R. and Agranoff, B.W., eds.) Little, Brown & Co., pp. 103-124 (1976).
5. Lanyi, J.K. and Silverman, M.P. *J. Biol. Chem.* 254, 47 (1979).
6. Delmer, D.P., Basic, A. and Benziman, M. *in* "Cell Walls 1981" (Robinson, D.G. and Quader, H., eds.) Stuttgart Wiss. Verlagsgesellschaft, pp. 94-98 (1981).
7. Delmer, D.P., Benziman, M. and Padan, E. *Proc. Natl. Acad. Sci. USA* 79, 5282 (1982).
8. Delmer, D.P. *in* "Advances in Carbohydrate Chemistry and Biochemistry" Vol. 41 (Horton, D., ed.) Academic Press, N.Y., in press.
9. Glaser, L. *J. Biol. Chem.* 232, 627 (1958).
10. Aloni, Y. and Benziman, M. *in* "Cellulose and Other Natural Polymer Systems: Biogenesis, Structure and Degradation" (Brown, R.M., Jr., ed.) Plenum Press, N.Y., pp. 341-359 (1982).
11. Colvin, J.R. *in* "The Biochemistry of Plants", Vol. III (Preiss, J., ed.) Academic Press, N.Y., pp. 543-570 (1980).
12. Swissa, M., Aloni, Y., Weinhouse, H. and Benziman, M. *J. Bacteriol.* 143, 1142 (1980).
13. Carpita, N.C. and Delmer, D.P. *J. Biol. Chem.* 256, 308 (1980).
14. Franz, G. and Heiniger, U. "Encycl. Plant Physiol. New Series", Vol. 13, pp. 47-67 (1981).

15. Robinson, D.G. Adv. Botan. Res. 5, 89 (1977).
16. Delmer, D.P. Rec. Adv. Phytochem. 11, 45 (1977).
17. Delmer, D.P., Benziman, M., Klein, A., Bacic, A., Mitchell, B., Weinhouse, H., Aloni, Y. and Callaghan, T. J. Appl. Polymer Sci., in press (1983).
18. Chao, H.Y. and MacLachlan, G.A. Plant Physiol. 61, 943 (1978).
19. Aloni, Y., Delmer, D.P. and Benziman, M. Proc. Natl. Acad. Sci. USA 79, 6448 (1982).
20. Benziman, M., Aloni, Y. and Delmer, D.P. J. Appl. Polymer Sci., in press (1983).
21. Aloni, Y., Cohen, R., Benziman, M. and Delmer, D.P. J. Biol. Chem., in press (1983).
22. Garcia, R., Recondo, E. and Dankert, M. Eur. J. Biochem. 43, 93 (1974).
23. Kjosbakken, J. and Colvin, J.R. in "Biogenesis of Plant Cell Wall Polysaccharides" (Leowus, F., ed.) Academic Press, N.Y., pp. 361-371 (1975).
24. Couso, R.O., Ielpi, L., Garcia, R.C. and Dankert, M. Arch. Biochem. Biophys. 204, 434 (1980).

LIST OF PUBLICATIONS DERIVED FROM THIS PROJECT

1. Delmer, D.P., Bacic, A. and Benziman, M. in "Cell Walls 1981"
(Robinson, D.G. and Quader, H., eds.) Stuttgart Wiss. Verlags-
gesellschaft, pp. 94-98 (1981).
- ✓ 2. Delmer, D.P., Benziman, M. and Padan, E. Proc. Natl. Acad. Sci. USA
79, 5282 (1982).
- ✓ 3. Delmer, D.P., Benziman, M., Klein, A., Bacic, A., Mitchell, B.,
Weinhouse, H., Aloni, Y. and Callaghan, T. J. Appl. Polymer
Sci., in press (1983).
- ✓ 4. Aloni, Y., Delmer, D.P. and Benziman, M. Proc. Natl. Acad. Sci. USA
79, 6448 (1982).
- ✓ 5. Benziman, M., Aloni, Y. and Delmer, D.P. J. Appl. Polymer Sci., in
press (1983).
- ✓ 6. Aloni, Y., Cohen, R., Benziman, M. and Delmer, D.P. J. Biol. Chem.,
in press (1983).