SYNTHESIS OF CELLULOSE BY *ACETOBACTER XYLINUM**

VIII. On the Formation and Orientation of Bacterial Cellulose Fibrils in the Presence of Acidic Polysaccharides

G. BEN-HAYYIM and I. OHAD, Ph.D.

From the Department of Biological Chemistry, The Hebrew University of Jerusalem, Israel. Dr. Ohad's present address is The Rockefeller Institute

ABSTRACT

The transfer of the glucosyl moiety from uridine diphosphate glucose in the presence of *Acetobacter xylinum* cell-free extracts led to the formation of a mixture of alkali-soluble and -insoluble cellodextrins. Typical cellulose fibrils could not be detected by electron microscopy in this product. Immediately after release into the medium, cellulose formed by whole cells is in a "prefibrous" form which passes through Millipore filters of 0.45 and 0.8 μ pore diameter. Non-filtrable fibrils arise from this material probably by a process of crystallization involving no extracellular enzymes. Fibrils formed in shaken cell suspensions intertwine and form aggregates visible to the naked eye. In quiet suspensions pellicles are formed which float on the surface. Soluble Na-carboxymethylcellulose (CMC) is incorporated into cellulose fibrils formed in its presence, probably by a process of co-crystallization. Aggregation of fibrils containing CMC is delayed because of electrostatic repulsion between carboxylic groups. The aggregation time depends on the amount of CMC incorporated, its degree of substitution, the pH of the medium, and the ionic strength. The amount of CMC incorporated depends on the relative concentration CMC/cellulose and on the similarity of the CMC and the cellulose molecules *i.e.* in molecular weight and the number of carboxyl substitutions. Cellulose pellicles formed in the presence of CMC by unshaken cell suspensions consist of crossed, superimposed layers of parallel oriented cellulose fibrils. The same phenomenon is observed when phosphomannan, but not levan, is substituted for CMC. The biogenesis of oriented cellulose fibrils is envisaged as a process comprising the following steps: polymerization of the monomeric precursor, diffusion of the molecule to crystallization sites, crystallization, and orientation. It is proposed that charged polysaccharides play a role similar to that of CMC in affecting the orientation of cellulose fibrils in the plant cell wall.

INTRODUCTION

The process of biosynthesis and oriented deposition of cellulose fibrils in biological systems may be resolved into four steps: (*a*) polymerization of the activated monomeric precursor to form a cellulose

* The authors wish to dedicate this paper to the memory of Professor Shlomo Hestrin, who initiated and stimulated the research reported here. Professor Hestrin died on February 2, 1962.

TABLE I

Properties and Indexing of CMC Preparations Used in This Work

Source	Code no.*	Mol wt	DS	Mol wt determined by	CPM/μg after tritiation
Prepared in the laboratory	21/2.2	21,000	2.2	Ultracentrifugation	‡
" " "	71/2.2	71,000	2.2	"	‡
I.C.I.	32/0.4	32,000	0.4	Viscosity	450
"	53/0.8	53,000	0.8	"	600
"	83/0.4	83,000	0.4	"	—
"	111/0.8	111,000	0.8	"	—
"	134/0.4	134,000	0.4	"	250
"	129/0.8	129,000	0.8	"	150

* The code is used in this work for shorter description of the molecular weight (first two numbers $\times 10^3$) and degree of substitution (DS) (the number at the right of the stroke).
‡ This CMC was synthesized with chloroacetic acid-C^{14} and contained 180 CPM/μg.

molecule of high molecular weight; (*b*) transport of the molecule from the site of synthesis to that of crystallization; (*c*) crystallization or fibril formation; (*d*) orientation of fibrils during deposition. These steps are not necessarily isolated from one another. Colvin and co-workers consider the polymerization and formation of the fibrils to be a single step in the bacterial cellulose-forming systems (1, 2). Frei and Preston believe that polymerization, fibril formation, and orientation occur simultaneously in plants. They propose that a catalytically active protein moves around the cell membrane and deposits the formed cellulose fibrils in predetermined directions, the whole process being under genetic control (3, 4). Frey-Wyssling has suggested that extrusions of the plasmalemma inside the cell wall are responsible for synthesis and orientation of cellulose fibrils in plants (5). As opposed to this, evidence has been presented that the synthesis, transport, and crystallization may be separate processes in bacterial systems (6) and probably in some plants (7). Thus, incorporation of the glucosyl moiety of uridine diphosphate glucose (UDPG) (8) or guanosine diphosphate glucose (GDPG) (9) into a β-1 $\rightarrow$ 4 alkali-insoluble product, using bacterial or plant cell-free extracts, suggested that a sugar nucleotide might be the glucosyl donor for the polymerization. The enzymes involved in this process in bacteria are probably anchored to the cell membrane (10), whereas cellulose fibrils are randomly deposited as free entities in the surrounding medium and are not physical appendages of the bacterial cell (6, 11). Many workers still regard the process of cellulose fibril orientation in the secondary wall of plant cells as a passive one, rearrangement being the result of mechanical strain or stress (12, 13).

In the present work experiments are described on the synthesis, crystallization, and orientation of cellulose fibrils, using extracts or whole cells of *Acetobacter xylinum*.

EXPERIMENTAL

Acetobacter xylinum cells were prepared as described by Ohad *et al.* (6). Washed cells were suspended in phosphate-citrate buffer, pH 6.4 (2×10^{-2} M phosphate), at 0°C. All the experiments were carried out with fresh cells.

Cell-free extracts used in experiments of cellulose synthesis from UDPG-C^{14} or cellodextrins and UDPG were prepared as described by Glaser (8) and Klungsöyr (14), respectively.

UDPG-C^{14} was synthesized by the method of Munch-Petersen (15) using glucose-6-phosphate-C^{14}, uridine triphosphate (UTP), and an extract of baker's yeast. The UDPG-C^{14} was purified on charcoal and ion exchange columns (16) and finally by paper chromatography. The degree of purity was at least 95 per cent.

Cellodextrins (C^{12} or C^{14}) were prepared from bacterial cellulose (17) by partial hydrolysis with hydrochloric acid (18). After hydrolysis the hydrochloric acid was neutralized with triethylamine, and the cellodextrins were precipitated in 75 per cent ethanol. Water-soluble cellodextrins were extracted from the precipitate with hot water and were separated and analyzed by chromatography on stearic acid–treated charcoal columns (19). They were of an average degree of polymerization (DP)

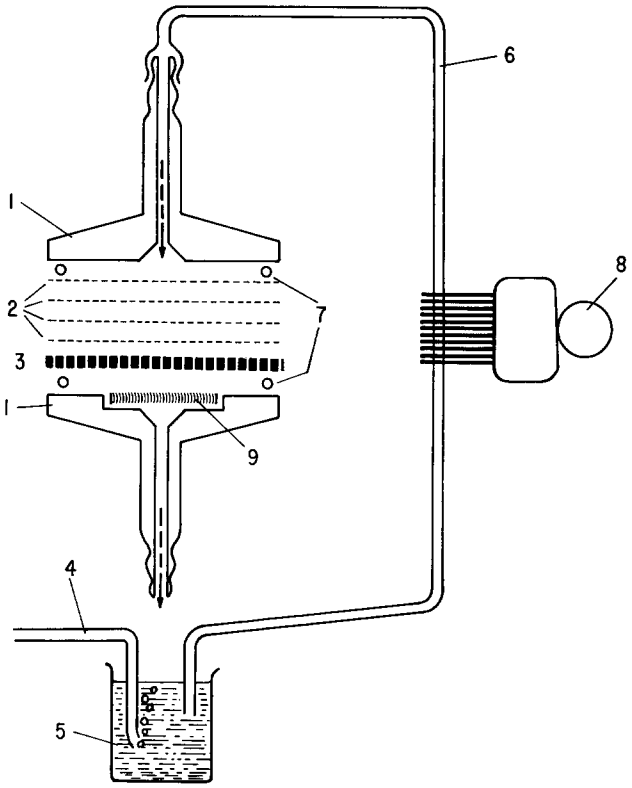


FIGURE 1 Apparatus used for measurement of synthesis of "filtrable cellulose." *1*, filter holder; *2*, Millipore filters; bacterial cells were placed on the second filter; *3*, metallic support for filters; *4*, oxygen supply; *5*, vessel containing buffered glucose-C^{14}; *6*, Tygon tubing; *7*, neoprene ring; *8*, peristaltic pump; *9*, cotton surgical gauze (6 layers) coated with a thin layer of sonicated bacterial cellulose. →, flow direction when the apparatus was operated in "closed circuit"; when it was operated in "open circuit" the buffered glucose solution was collected in a second vessel and not reused.

of 4 and contained glucosides from cellobiose up to hexaose. Soluble Na-carboxymethylcellulose (CMC-C^{14}) was prepared from bacterial cellulose and chloroacetic acid-C^{14} according to McLaughlin and Herbst (20). After dissolution the samples were dialyzed against distilled water to constant radioactivity, and insoluble material was removed by centrifugation at 15,000 *g* for 15 minutes. The molecular weights (mol wt) of the CMC so obtained were calculated from sedimentation and diffusion coefficients.

Sodium CMC-H^3 was prepared according to Wilzbach (21). Five curies of tritium gas were used to tritiate simultaneously, in different compartments of the same vessel, four different CMC samples (10 grams each, obtained from Imperial Chemical Industries (I.C.I.), batches S/E 50, 49, 51, 47). The total volume of the tritiation vessel was 250 ml, and the samples were incubated for 4 weeks at room temperature in the dark. After dissolution, samples were dialyzed until constant radioactivity, and insoluble material was removed as described above. Since these samples were polydisperse as indicated by ultracentrifugation analysis, the average molecular weights were calculated from intrinsic viscosity according to Brown *et al.* (22).

The properties of the CMC preparations used in this work are summarized in Table I.

Bacterial cellulose-C^{12} or -C^{14} was prepared according to Hestrin (17). Before use as a carrier in incorporation experiments, it was sonicated for 30 minutes in a Raytheon 10 kc magnetostriction oscillator.

Standard System for Determination of the Delaying Time of the Aggregation of Cellulose-Synthesizing Cells in Presence of CMC

The standard system contained 10 μmoles/ml glucose, various concentrations of cells, and CMC in 5 ml phosphate-citrate buffer at pH 6.4 (2×10^{-2} M phosphate). Incubation was at 30°C in a 25-ml Erlenmeyer flask with shaking (60 cycles/min.). The aggregation time was determined with an accuracy of ±1 minute, by the appearance of small aggregates visible to the naked eye (23). In control systems without CMC the aggregation time was 1 to 3 minutes.

Incorporation of CMC-H^3 into Cellulose Fibrils

The system was as described above and the cellulose synthesis was stopped with 2.5×10^{-3} M *N*-ethylmaleimide (24). The sediment, after centrifuga-

TABLE II

Incorporation of Glucose-C^{14} from UDPG-C^{14} into Cellodextrins

System	Treatment	Glucose-C^{14} in sediment	C^{14} in neutral disaccharide
		mμ mole	*mμ mole*
A. Active enzyme	1	0.9	36
	2	2.2	
	3	0.9	
B. Active enzyme	1	4.1	41
	2	0.1	
	3	0.1	
C. Heat-inactivated enzyme	1	<0.1	3
	2	<0.1	
	3	<0.1	

The incubation system contained: 0.8 μmole UDPG-C^{14} (specific activity 1.3×10^5 CPM/μmole), 8 mg cellodextrins (average DP ≈ 4), 0.7 ml enzyme (15 mg protein/ml) in a final volume of 1.6 ml Tris buffer, pH 8.2, containing 4×10^{-3} M $MgCl_2$ and 4×10^{-4} M EDTA. The reaction was stopped by boiling for 2 minutes in a water bath. Whatman filter paper powder was added to system A, and sonicated bacterial cellulose fibrils to systems B and C, and the preparations were centrifuged. Bacterial cellulose was added to the supernatants and they were sedimented again; the resulting sediments were boiled in 1 N alkali, washed with water by centrifugation, and counted (A3, B3, C3). The sediments from the first centrifugation were resuspended in 1 N alkali, boiled for 10 minutes, centrifuged again, washed, and counted (A1, B1, C1). Their alkali supernatant was neutralized, and sonicated bacterial cellulose was added, sedimented, washed, and counted (A2, B2, C2).

The neutral disaccharide was analyzed as follows: A small sample was taken from the incubation mixture after the first sedimentation and passed through a Dowex 1 × 8 format column after neutralization with 0.1 N alkali, using brom thymol blue as indicator. Radioactivity of the effluent was measured, and the material was concentrated by evaporation and chromatographed on Whatman filter paper using the following solvent mixtures (by volume): I, methanol, formic acid, water (11.5:0.5:0.5); II, methanol, ethanol, water (4.5:4.5:1); III, ethanol, ammonium acetate 0.1 M (7:3); IV, propanol, ethylacetate, water (7:1:2). The R glucose of both cellobiose and the radioactive spot in the four solvents was 0.66, 1.00, 0.98, and 0.78, respectively, and significantly differed from the R glucose of maltose and trehalose.

tion at 4,000 *g* for 10 minutes, was washed twice with 10 ml 1 N NaOH, then with water to pH 7.0, dried at 100°C, and acetolyzed according to Schramm and Hestrin (25). The tritium content was measured with a Packard Tri-Carb Scintillation Spectrometer with an efficiency of about 9 per cent.

Determination of Rate of Cellulose Synthesis

Glucose-C^{14} was used instead of glucose-C^{12} in an incubation mixture as described above. The product was filtered through a fiber glass filter (H. Reeve Angel and Co., Inc., Clifton, New Jersey), washed three times with 10 ml of 0.5 N NaOH, then with water down to constant radioactivity, and counted in a gas flow counter (Nuclear Chicago Corp., Chicago, Illinois (26)). The amount of cellulose trapped on the filter was not affected by the presence of CMC in the medium.

Total sugar was determined according to Dubois *et al.* (27) using either glucose or CMC as standard.

Synthesis of CMC-Cellulose Complex for X-Ray Diffraction

The rubidium salt of CMC (111/0.8) was used instead of the sodium salt. The system contained 200 mg cells (dry weight), 10 μmoles/ml glucose, and 400 μg/ml CMC in a final volume of 200 ml of buffer phosphate citrate (Rb), pH 6.4 (2×10^{-2} M phosphate). Two minutes after the aggregation time (28 minutes) aggregates were dispersed in a Waring Blendor for 2 minutes and the homogeneous suspension obtained was filtered through a column (4.5 × 9 cm) of 2.5 mm diameter glass beads. The cellulose fibrils remained on the glass beads, whereas the cells passed through the column. In order to get a higher yield of CMC-cellulose complex, the cells were removed by centrifugation and the medium was re-used after addition of fresh cells and glucose. The pooled cellulose synthesized was separated from beads by decantation, freed from enmeshed bacteria by repeated blending and filtration through the glass bead column, and finally washed by centrifugation and dried at 100°C. Seven milligrams of CMC–Rb cellulose complex were obtained containing 0.9 mg of CMC measured as Rb by flame photometry.

Preparation of Hydrocellulose

Preparation of hydrocellulose was carried out as described by Rånby (28). Ten milligrams of cellulose containing 0.43 per cent by weight CMC-H^3 (53/0.8) were hydrolyzed in boiling 2.5 N H_2SO_4 for 4 hours. The degree of hydrolysis was about 11 per cent. The hydrolyzate was centrifuged at 5,000 *g* for 5 minutes and the sediment washed with 10-ml portions of water. Peptization occurred at the sixth washing. The supernatant of this portion contained 3.5 mg hydrocellulose, and the following two super-

natants about 4 mg. Tritium and total sugar of these samples were determined as described above.

Preparation of Specimens for Electron Microscopy

Specimens of cellulose pellicles were prepared, as described by Ohad *et al.* (6), by incubating cells suspended in the "standard system," without shaking, in a Pasteur pipette fitted with a copper grid (200 mesh) in its conical part. Another method was as follows. Copper grids (80 mesh) were pressed onto a sheet of Parafilm paper so that they adhered to the film. Several holes were punctured in the Parafilm below the grid, and a small drop of the incubation mixture was placed on the grid, which was incubated for different periods of time at 30°C in a Petri dish saturated with water vapor. The reaction was stopped by sucking the drops gently through the grid so that the pellicle formed remained on its surface. The pellicles were washed with alkali (2 N NaOH) and water, dried over phosphorus pentoxide, and shadowed with platinum-paladium at an angle of 1:4 to 1:5. Similar results were obtained by both methods of specimen preparation.

The insoluble product formed by incubation of cell-free extracts with UDPG-C^{14} as described by Glaser (8) was examined in the electron microscope by one of the following methods: (*a*) a drop of the incubation mixture after dialysis was dried on a copper grid covered with a collodion film, or (*b*) the agar gel filtration method of Kellenberger and Arber (29) was used. When the latter method was used, extracts were incubated directly on the agar gel covered with a collodion film. No carrier was used in these experiments. Before examination in the microscope the amount of product deposited on the film was estimated by measuring its radioactivity. The specimens were shadowed with Pt-Pd as described above.

All specimens were photographed using an RCA EMU 3B.

Synthesis of "Filtrable Cellulose"

"Filtrable cellulose" was measured using the following device: A Millipore filter of 0.45 μ pore diameter, covered with a layer of bacterial cells ($\approx$1 cell/10 μ^2), was placed on a series of filters in a special filter holder (Fig. 1). An oxygenated buffered solution of glucose-C^{14} was either recirculated through the holder ("closed circuit") or passed through and collected in a separate vessel ("open circuit"). The "filtrable cellulose" formed on the filter coated with bacteria was trapped on the following filters, washed, and counted.

MATERIALS

UTP, UDP, UDPG, and *N*-ethylmaleimide were purchased from the Sigma Chemical Co., St. Louis, Missouri. Glucose-C^{14} and chloroacetic acid-C^{14} were purchased from the Radiochemical Centre, Amersham, England. Tritium 100 per cent pure was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee. Yeast phosphomannan and bacterial levan were kindly supplied by Dr. G. Avigad of this laboratory. All other chemicals used were of an analytical grade.

TABLE III

Synthesis of "Filtrable Cellulose" by Whole Cells as a Function of Time

Pore size and filter sequence	Role	CPM counted after		
		0 min.	30 min.	70 min.
5 μ	Prefilter	0	52	100
0.8 μ	Support for bacteria	38	75	166
0.45 μ	Filter	0	12	10
Cotton gauze (6 layers)	Trap. Coated with a thin layer of sonicated bacterial cellulose	65	550	900

Cells (4×10^7) were spread by filtration on the surface of the second filter before it was inserted in the apparatus. A solution of glucose-C^{14} 3×10^{-3} M (1.5×10^5 CPM/μmole) in phosphate citrate buffer (1.5×10^{-2} M phosphate, pH 6.4) at 30°C was circulated in a "closed (or open) circuit" through the filters, using the device shown in Fig. 1. The flow rate was 2 to 3 ml/cm²/min., at about 1.5 atm.

The zero time system consisted of heat-inactivated bacteria, incubation being for 30 minutes. At the end of the incubation 1 liter glucose-C^{12} solution in the same buffer was circulated and the filters were removed, washed with water in a separate holder, and counted. The radioactivity of the gauze trap was found only on the surface of the first layer, and was not removed by washing with 10 N alkali. Similar results were obtained in an experiment in which the solution was circulated in an open circuit for 30 minutes.

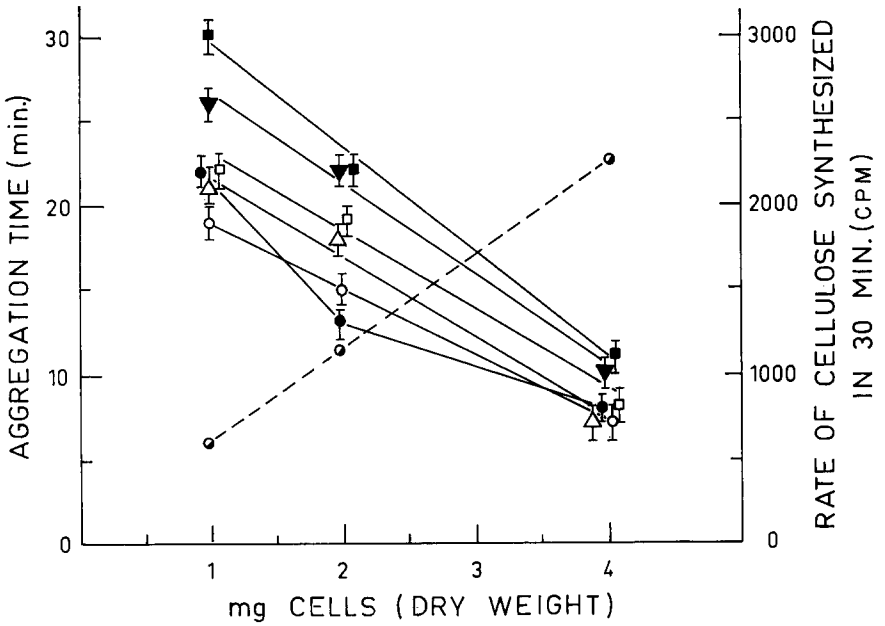


FIGURE 2 Aggregation time of cellulose fibrils formed in presence of CMC, as a function of the quantity of cells (dry weight). The standard system contained 200 μg/ml CMC and increasing quantities of cells. CMC: solid circles, 32/0.4; open circles, 53/0.8; open triangles, 83/0.4; solid triangles, 111/0.8; open squares, 134/0.4; solid squares, 129/0.8. The rate of cellulose synthesis was measured with glucose-C^{14} (6×10^3 CPM/μmole), the incubation time being 30 minutes (dashed line).

RESULTS

Formation of Cellulose by Acetobacter xylinum Extracts

Glucose-C^{14} was incorporated into a water- and alkali-insoluble polymer when UDPG-C^{14} was incubated with extracts of *Acetobacter xylinum*. However, the amount of C^{14}-alkali-insoluble polymer collected was dependent on the nature of the carrier used. Sonicated bacterial cellulose fibrils trapped more counts than Whatman filter paper powder. In the presence of the latter carrier about 50 per cent of the insoluble material synthesized dissolves in hot alkali and is reprecipitated by neutralization in the presence of the carrier.

A neutral disaccharide was also found which had a paper chromatographic mobility of cellobiose (as tested in four different solvents) and contained about 9 times as much glucose-C^{14} as the insoluble product. Glaser (8) also reported the presence of this disaccharide in the system, although it was not possible to identify it with cellobiose. The results of these experiments are represented in Table II. Typical cellulose fibrils could not be detected by electron microscopy in the product of this system. The alkali-insoluble product consisted of aggregates of granules of varying size with no well defined units.

The incorporation of glucose-C^{14} from UDPG-C^{14} into an alkali-insoluble polymer was not dependent upon the presence of cellodextrins in the system. Experiments to detect transfer of glucose from cellodextrins to an insoluble polymer using UDP as cofactor, as described by Klungsöyr (14), failed. Neither net synthesis nor incorporation or C^{14}-labeled cellobiose, or cellotriose up to cellohexaose, into a cellulose-like polymer could be detected.

Synthesis of Filtrable Cellulose by Whole Cell Suspensions

This was tested using the apparatus shown in Fig. 1. The cellulose synthesized passed through the first Millipore filter of pore diameter 0.45 μ and was trapped on a subsequent filter of cotton gauze (Table III).

Synthesis of Cellulose by Whole Cells in Presence of CMC

The rate of cellulose synthesis was slightly increased by addition of CMC to the system (*cf.* Fig. 4), and the time of aggregation of cellulose

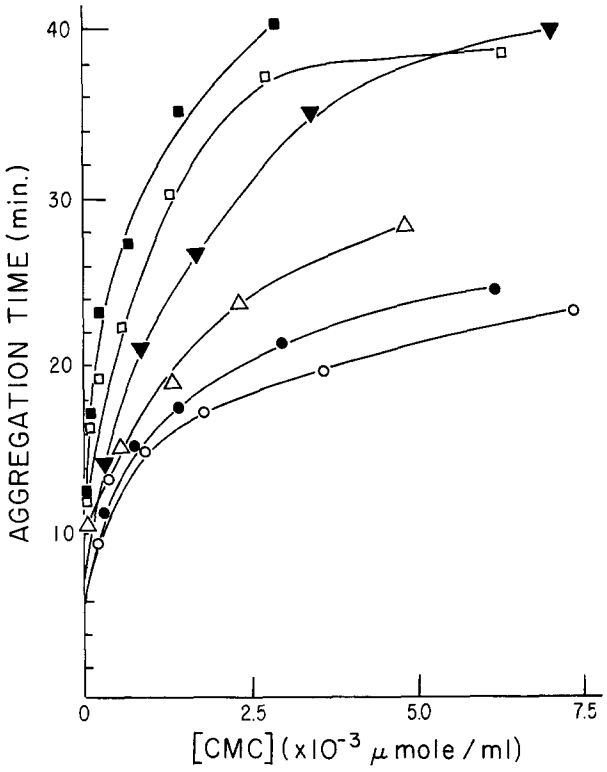


FIGURE 3 Aggregation time as a function of CMC concentration. The standard system contained 1 mg cells per ml (dry weight) and different molar concentrations of CMC. CMC: solid circles, 32/0.4; open circles, 53/0.8; open triangles, 83/0.4; solid triangles, 111/0.8; open squares, 134/0.4; solid squares, 129/0.8.

fibrils was delayed (*cf.* Fig. 3). The aggregation time was inversely proportional to the cell concentration, *i.e.* to the rate of cellulose synthesis (Fig. 2). The amount of cellulose synthesized before aggregation in the presence of a given concentration of a specific preparation of CMC was approximately constant.

For a given cell concentration, the aggregation time was dependent on the CMC concentration (Fig. 3), typical saturation curves being obtained for CMC of varying molecular weight and degree of substitution (DS). CMC of a higher molecular weight was more active on a molar basis in inhibiting the aggregation. CMC of a DS of 0.8 was in general more active than CMC of DS 0.4 (Fig. 3). However, when the DS was 2.2 the activity was significantly less. The influence of molecular weight and DS of CMC on the aggregation time of a standard system is given in Table IV.

The synthesis of cellulose by whole cells is dependent on the pH, with an optimum between pH 6.4 and 7.0, the rate being about half its maximum value at pH 4.5 (Fig. 4; *cf.* also (10)). If at all pH's the aggregation time were dependent on the rate of cellulose synthesis only, one would

TABLE IV

Aggregation Time of Cellulose Fibrils Formed in the presence of CMC as a Function of the Degree of Substitution and Molecular Weight of the CMC Used

CMC used		Onset of aggregation	Morphology of aggregates
DS	mol wt		
		min.	
0.4	32,300	20	Fine
0.4	83,100	23	Fine
0.4	134,500	35	Fine
0.8	53,200	18	Fine
0.8	111,000	30	Fine
0.8	129,500	38	Fine
2.2*	21,500	5	Coarse
2.2*	71,500	6	Coarse

The standard system contained 1 mg cells (dry weight), 2.5 × 10^{-3} μmole/ml CMC. In control system without CMC the onset of aggregation was 2 minutes.

* In these systems the ratio CMC/cellulose in fibrils expressed in monomers and calculated at aggregation time was about 0.02 (compare with Table VI).

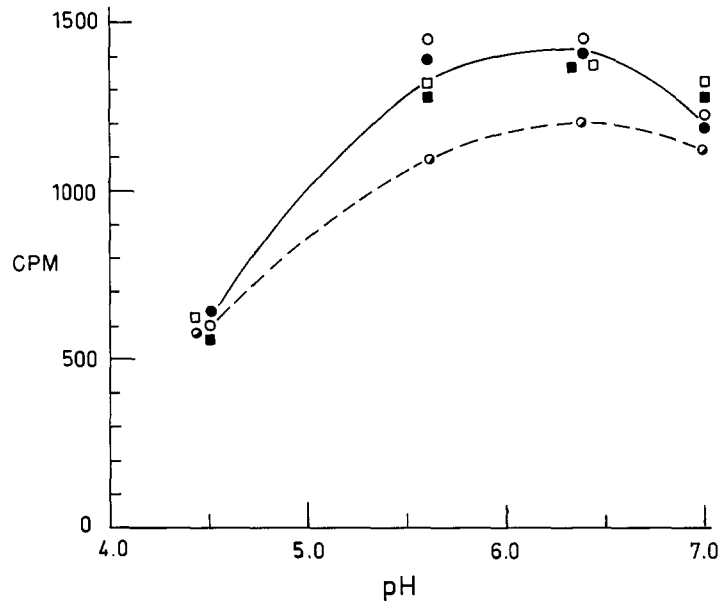


FIGURE 4 Cellulose synthesis in the presence and absence of CMC, as a function of pH. The standard system contained 2 mg cells (dry weight), 200 μg/ml CMC, and glucose-C^{14} (6×10^3 CPM/μmole). The incubation time was 30 minutes; other conditions as in "standard system." CMC: solid circles, 32/0.4; open circles, 53/0.8; open squares, 134/0.4; solid squares, 129/0.8; dashed line, cellulose synthesis without CMC.

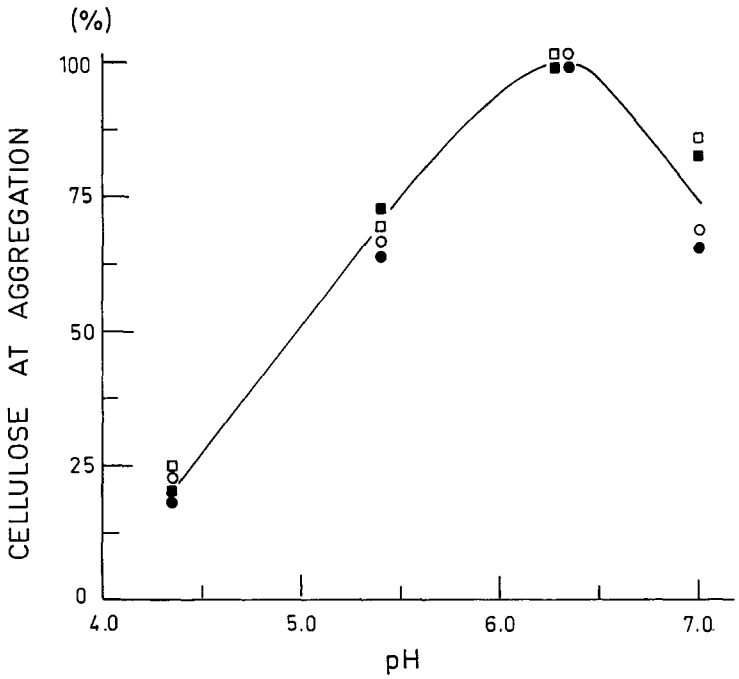


FIGURE 5 Amount of cellulose formed in presence of CMC until onset of aggregation, as a function of pH. The amount at pH 6.4 was taken as 100 per cent. The system was as in Fig. 4. CMC: open circles, 32/0.4; solid circles, 53/0.8; open squares, 134/0.4; solid squares, 129/0.8.

TABLE V

Influence of Ionic Strength on the Amount of Cellulose Synthesized up to Aggregation Time in the Presence of Different Preparations of CMC

Ionic strength	Amount of cellulose at aggregation time (CPM glucose-C^{14} incorporated): CMC					
	32/0.4	53/0.8	83/0.4	111/0.8	134/0.4	129/0.8
0.022	790	645	870	1160	950	960
0.189	435	295	405	540	410	445

The standard system contained 1 mg cells (dry weight), 100 μg/ml CMC, and glucose-C^{14} (7.5×10^3 CPM/μmole).

expect it to increase in proportion to the decrease of the pH. However, if the cellulose fibrils are prevented from aggregation as a result of electrostatic repulsion due to carboxyl groups of CMC bound to fibrils, one would expect a decrease in the time of aggregation at lower pH values as a result of reduction of the charge of carboxyls. This was indeed the result obtained when the amount of cellulose held in suspension was plotted against the pH (Fig. 5). Increasing the ionic strength also reduces the amount of cellulose held in suspension until the aggregation time (Table V).

The rate of incorporation of CMC into cellulose fibrils during synthesis decreases after the onset of aggregation (Fig. 6; *cf.* also (23)). The ratio CMC/cellulose in fibrils after a given period of synthesis would therefore be dependent on the time of aggregation, *i.e.*, on the molecular weight and DS of the CMC preparation used. The ratio CMC/cellulose was calculated for fibrils collected at the aggregation time, on the basis of measurement of the total CMC incorporated, cellulose content, and molecular weight of CMC and of cellulose synthesized in its presence (Table VI).

Hydrocellulose crystallites obtained by acid hydrolysis and peptization of cellulose-CMC complex contained only 0.017 CMC monomer for every glucose monomer as compared with an initial ratio of 0.33 in the complex fibrils and 0.20 in the remaining sediment. Although the rate of cellulose synthesis is slightly increased in the presence of CMC at pH 5.5–6.4, this effect disappears at lower pH (Fig. 4). Similar stimulatory effects were obtained when Rb was substituted for Na or the ionic strength was decreased. It appears that CMC does not act as a primer, since the molar ratio CMC/cellulose in fibrils could be higher than unity (Table VI).

The x-ray diffraction pattern of cellulose fibrils formed in the presence of Rb salt of CMC with a molar ratio CMC/cellulose of 0.4 was similar to that of pure cellulose. The spacings calculated from a powder diagram are given in Table VII.

Orientation of Cellulose Fibrils Formed in the Presence of CMC

Cellulose aggregates formed during shaking or pellicles formed on the quiet surface of suspensions of cells consist of intertwined, randomly oriented fibrils forming a matrix in which bacterial cells are enmeshed (6). As compared with 3 to 5 small round compact bodies formed in a control system, when CMC is added aggregates (about 100

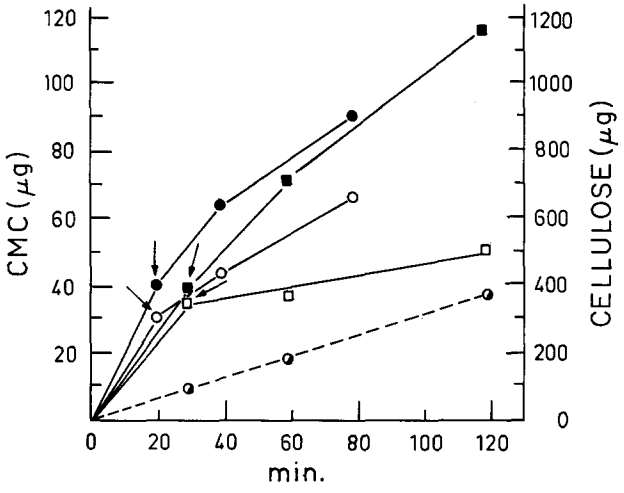


FIGURE 6 Incorporation of CMC-H^3 into cellulose fibrils formed in its presence, as a function of time. The system contained 4 mg cells (dry weight), 200 μg/ml CMC-H^3, in a final volume of 20 ml; other conditions as in the "standard system." CMC: open circles, 32/0.4; solid circles, 53/0.8; open squares, 134/0.4; solid squares, 129/0.8. The synthesis of cellulose was measured with glucose-C^{14} (10^4 CPM/μmole) (dashed line). The reaction was stopped with 2.5×10^{-3} M *N*-ethylmaleimide. Arrows indicate the aggregation time.

TABLE VI

*The Ratio CMC/Cellulose in Fibrils at Aggregation Time**

CMC	Molar ratio CMC/cellulose‡	Ratio in monomers
32/0.4	6.0	0.56
53/0.8	2.7	0.35
134/0.4	0.8	0.33
129/0.8	0.9	0.33

* Calculated from data in Fig. 6.

‡ The mol wt of cellulose synthesized in the presence of CMC under the same experimental conditions was found to be 3×10^5 from viscosity measurements according to Brown *et al.* (22), *i.e.* 1,780 anhydroglucose units.

TABLE VII

Spacings of X-Ray Diffraction Pattern of Cellulose and Cellulose Fibrils Containing CMC

Reflection plane	Spacings in both fibrils containing CMC and native fibrils*	Spacings in native cotton cellulose*
	A	*A*
101	6.08	5.96
$10\bar{1}$	5.31	5.36
021	4.37	4.35
002	3.92	3.92
040	2.60	2.58

* Data from Hall and coworkers (31).

The spacings were calculated from a Debye-Scherer x-ray diffraction pattern with CuK α radiation. Exposure was with a camera of 114.6 mm radius.

The x-ray work was carried out by Dr. W. Traub of the Department of X-Ray Crystallography, The Weizmann Institute of Science, Rehovoth, Israel.

or more) appear as fine filaments several millimeters in length. Pellicles formed in systems containing CMC are thinner and less resistant to stress than those formed in a control system. Both aggregates and pellicles formed in the presence of CMC show a strong birefringence when examined between crossed nicols in a polarizing microscope; birefringence was observed sometimes also in the control systems. When examined in the electron microscope, pellicles and aggregates formed in the presence of CMC were found to contain crossed, superimposed layers of cellulose fibrils oriented in parallel (Figs. 7 and 8). The microfibrils were identical in appearance with those formed in the absence of CMC, but the tendency to form thick composite fibrils and cables was reduced. Orientation was observed at all CMC concentrations tested between 0.3 and 2 mg/ml, when the incubation time was 1 to 3 hours. After longer incubation a decrease in the degree of orientation was observed and the pellicles became too thick to be readily examined in the electron microscope. The degree of orientation was affected by the pH of the system.

The influence of polysaccharides other than CMC on the aggregation time of cellulose synthesized in their presence was previously reported (23). Pellicles formed in systems containing phosphomannan at concentrations which delayed the aggregation time also show the phenomenon of orientation (Fig. 9). Levan, however, tested at concentrations up to 8 mg/ml, did not delay aggregation nor did it cause any significant orientation of cellulose fibrils into pellicles (Figs. 10 and 11).

DISCUSSION

The incorporation of glucose from UDPG into an alkali-insoluble product using cell-free extracts of *Acetobacter xylinum* as reported by Glaser (8) was successfully repeated in this work. Alkali-soluble cellodextrins and appreciable amounts of a neutral disaccharide were also formed. The specific activity of this system was only about 1 per cent of that of the whole cells. Addition of soluble cellodextrins, which was necessary in the experiments carried out by Glaser (8), did not influence the incorporation significantly in our system. The low activity of the cell-free systems described so far could probably be related to lack of a specific acceptor or some structural factor from the cell wall or membrane, which is assumed to be the site where the final polymerization reaction actually occurs (10). The question now arises whether the alkali-insoluble product could be considered to be true cellulose, *i.e.* to consist of typical fibrils built up from high molecular weight molecules. The justification of this question resides in the fact that alkali-insoluble cellodextrins of low molecular weight would also yield glucose, cellobiose, etc. by partial acid or enzymic hydrolysis, which were the only criteria used until now to identify the material synthesized by the above systems (8, 9). In experiments described in

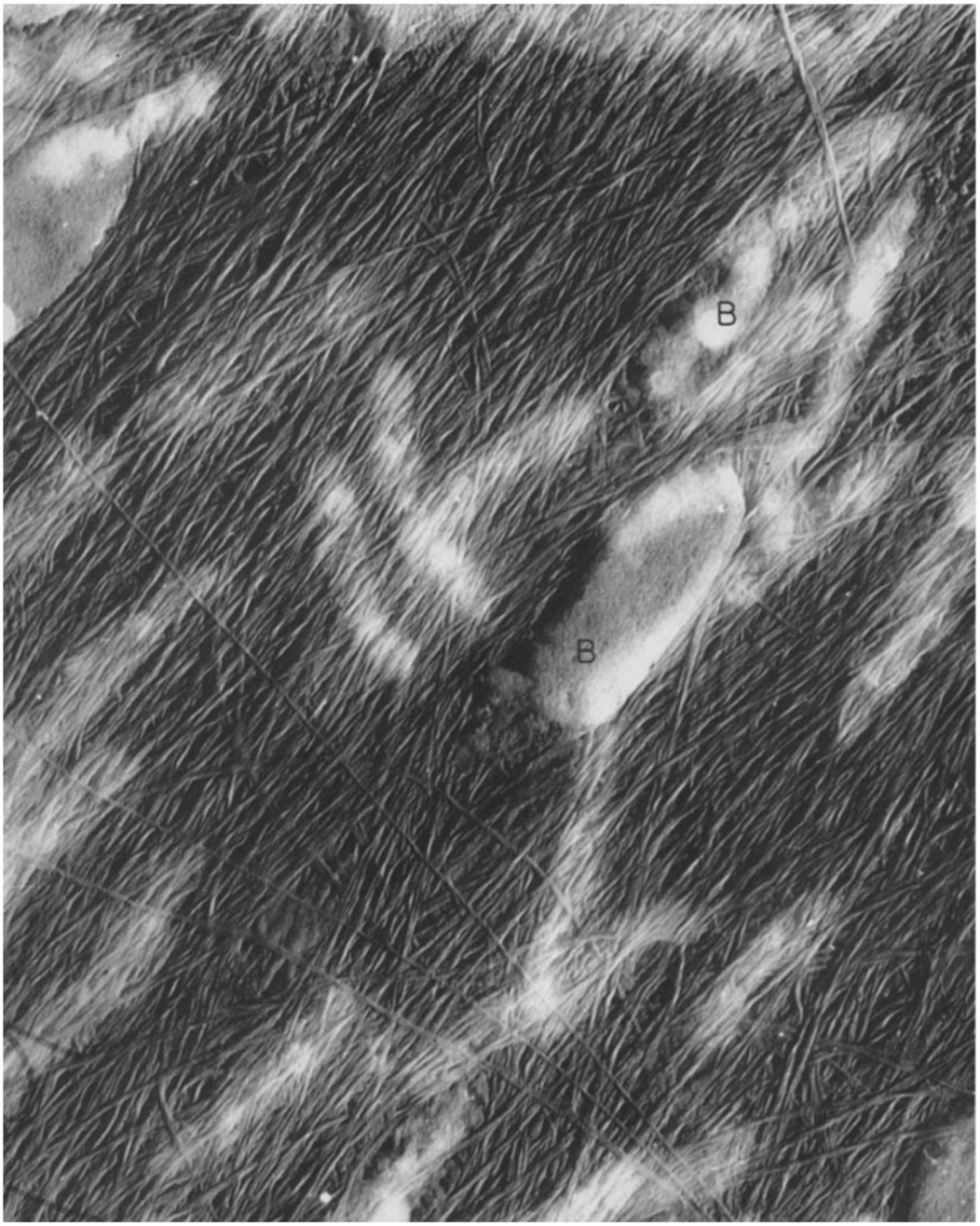


FIGURE 7 Oriented cellulose fibrils in pellicle. The system contained 0.8 mg cells per ml (dry weight), 10^{-2} M glucose in phosphate citrate buffer (1.5×10^{-2} M phosphate, pH 6.4), and 2 mg/ml CMC 53/0.8. The incubation was carried out in Pasteur pipettes. The reaction was stopped after 2 hrs. Pt-Pd shadowed. Ratio of height to shadow 1:4 to 1:5. *B*, bacterial cell wall not removed by alkali treatment. × 16,000.

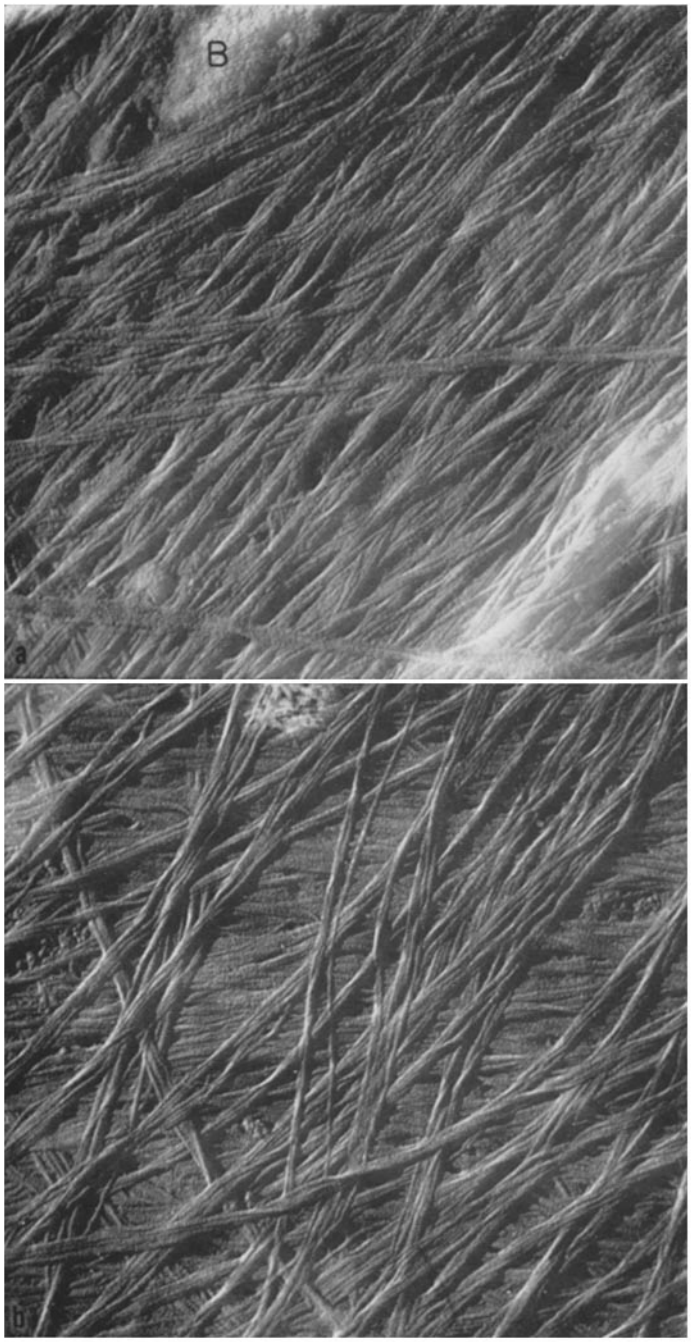


FIGURE 8 Similar to Fig. 7. × 42,000.

this work we were unable to detect typical cellulose fibrils in this material by electron microscopy, even in experiments in which the amount of insoluble glucose-C^{14} present in the specimens allowed detection of 1 per cent of the material as fibrils. The main product of this system is of a low molecular weight, and it is possible also that the alkali-insoluble material is not highly polym-

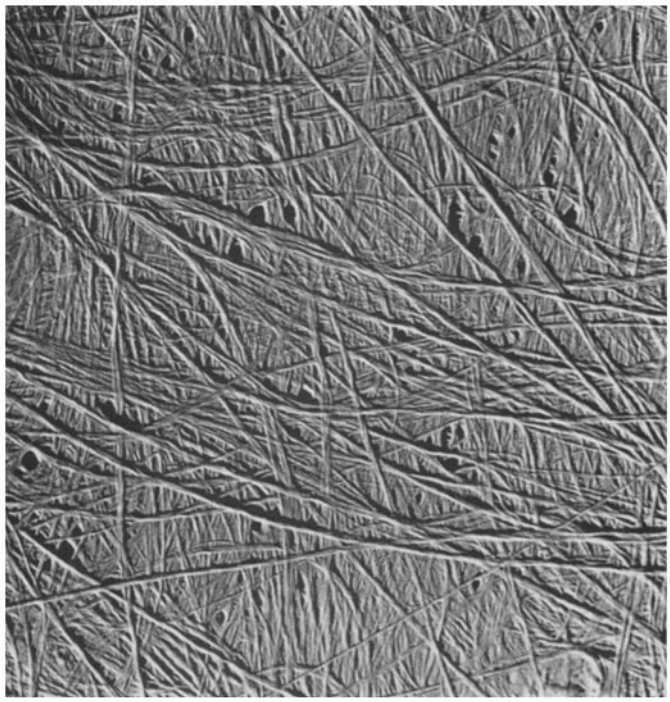


FIGURE 9 Similar to Fig. 7 but with 4 mg/ml phosphomannan. × 16,000.

erized. Although it seems that sugar nucleotides are the donors for cellulose synthesis (8, 9), it is still difficult to characterize the enzymic activity of the cell-free systems now available as true "polymerase" rather than "transferase."

On the basis of kinetic analysis of the morphological appearance of cellulose fibrils using electron microscopy (6, 11), it was suggested that cellulose molecules are released by bacteria into the liquid medium before crystallization and that the transfer of molecules to the crystallization sites on the fibrils could be a process of diffusion. The results presented above provide further support for this view. Cellulose molecules synthesized by bacteria placed on a filter, which acts as a "catalytic surface," pass through pores 0.8 or 0.45 μ in diameter. The radius of a randomly coiled solvated cellulose molecule (DP 2,500) was calculated to be about 0.02 μ (6). The minimal length of cellulose fibrils observed in electron micrographs was in the range of 3 to 5 μ (1, 6). The rigidity of the fibrils is great enough to prevent coiling and filtration through such small pores. Thus a filtrable alkali-insoluble polyglucoside produced by bacteria under conditions in which true cellulose is formed (10) could be cellulose molecules in a pre-fibrillar state.

Hestrin and Schramm (10) have shown that the enzymes involved in the synthesis of cellulose by *A. xylinum* are anchored to the cell so that repeated washings do not reduce the rate of synthesis. Colvin and Khan (30) reported that an extracellular enzyme is involved in the process of fibril formation by bacteria. In the filtration experiments in which the medium was circulated in an "open circuit," the amount of cellulose trapped on the gauze filter was similar to that in "closed circuit" experiments. Thus it seems that no extracellular soluble enzyme is involved in this process.

The solubility of cellulose molecules in water is too low to permit a direct study of their crystallization. It was our hope that some information on this process could be obtained by the use of a soluble cellulose analogue. Thus, addition of a water-soluble substituted cellulose like CMC to a system which generates solvated cellulose molecules could result in the incorporation of the soluble substituent into the fibrils by a process of "cocrystallization" (23). Fibrils containing a polyelectrolyte would show electrostatic repulsion.

This could be the explanation for the inhibition of the aggregation of fibrils formed in the presence of CMC. The factors expected to be involved in this process are the tendency to form interfibrillar bonds, the total charge per fibril unit area, and the fibril length. Cellulose fibrils elongate as a function of synthesis time (2), and could attain 50 μ or more in length. Fibrils so long and thin ($\approx$ 2 mμ thick by 3 mμ wide (32, 33)) would entangle, irrespective of their electrostatic repulsion, when shaken. Through this process, therefore, a critical length would be reached at which aggregation must occur. Before the critical length was attained, the onset of aggregation would depend on the equilibrium between interfibrillar bonds formed and repulsion due to surface charge. If the rate of incorporation of CMC into the fibrils (*i.e.* charge per unit area) is a function of CMC concentration, a saturation curve would be obtained when the aggregation time was plotted *versus* CMC concentration (Fig. 3). The amount of charge per monomer CMC incorporated is a function of the DS, so that in the same mol wt range, CMC of DS 0.8 may be more active in inhibiting the aggregation than CMC of DS 0.4 (Fig. 3). If the incorporation is a process of "cocrystallization," one would expect that the similarity of the analogue molecule to that of the cellulose would influence the incorporation, CMC of mol wt 1.2 $\times$ 10^5 being more active than CMC of mol wt 7 to 8 $\times$ 10^4 or 3 to 5 $\times$ 10^4 (Fig. 3; Table IV). For a given amount of CMC incorporated, the total charge of fibrils in suspension would be a function of the pH and ionic strength of the environment. When the pH decreases or the ionic strength increases, there is a reduction in the total charge of the carboxyl groups so that electrostatic repulsion between fibrils decreases and aggregation occurs earlier (Fig. 5; Table II).

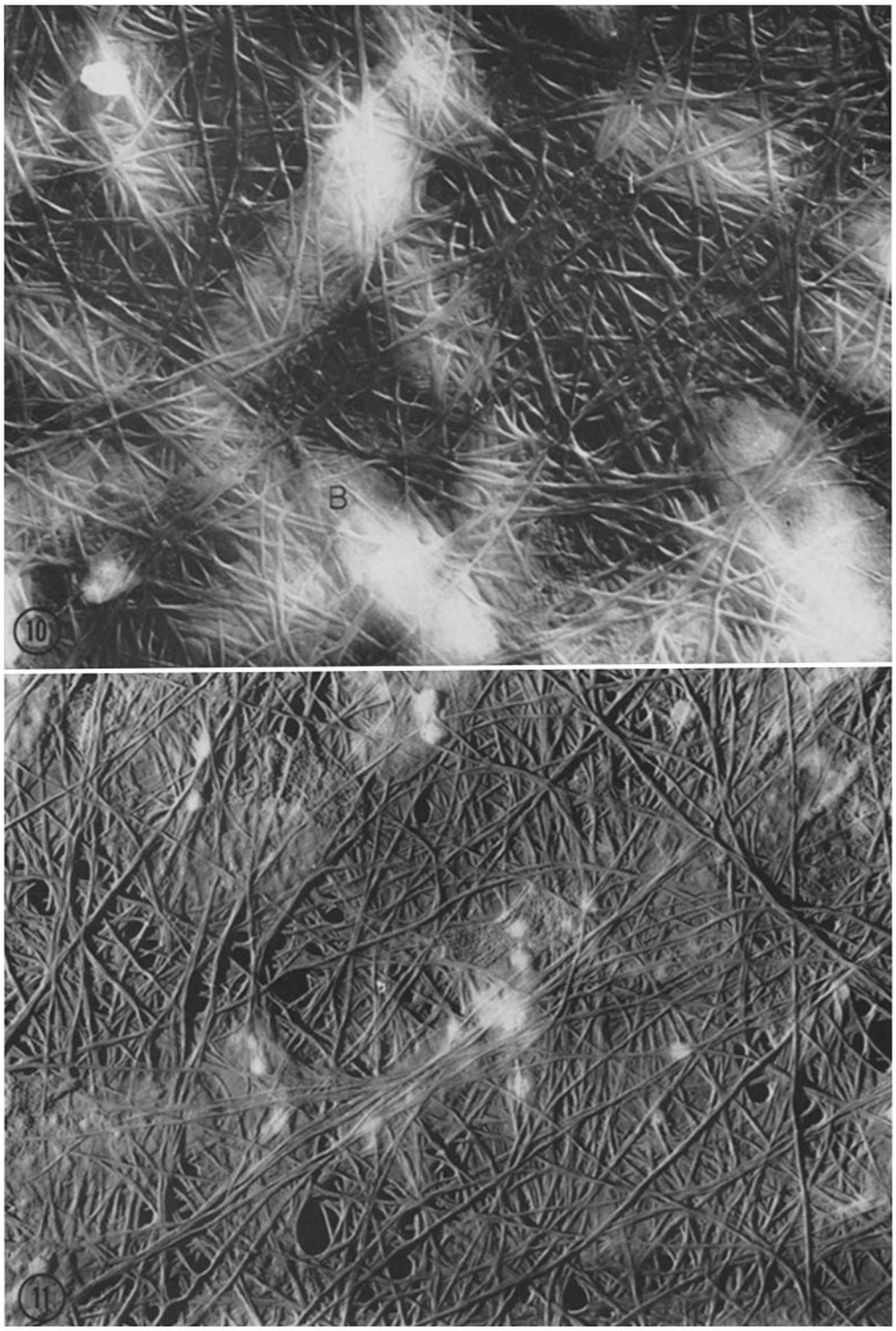
Cells are trapped in aggregates so that 80 to 90 per cent of the synthesizing sites become enmeshed in a matrix of cellulose fibrils (34). It is possible that in this case the relative concentration CMC/cellulose in the internal liquid phase of the aggregate decreases and consequently a decrease in the incorporation rate after aggregation is observed (Fig. 6; (23)).

The maximum amount of CMC incorporated was measured in the complexes formed in systems containing saturative CMC concentration and collected at the aggregation time (Table VI). It appears that the difference between CMC's of mol wt 2 $\times$ 10^4 to 1.5 $\times$ 10^5 having a DS of 0.4 to 0.8 is not enough to affect significantly the degree of incorporation under these conditions. When the DS was about 2, the incorporation was about 10 times less, possibly because the carboxyls of CMC monomers interfere with the hydroxyls of cellulose monomers and prevent hydrogen bond formation between O_6 and O_3'' or O_5 and O_2' (35, 36).

From the ratio CMC/cellulose in complex fibrils (in monomer units (Table VI)) it appears that about half to one-third of the length of every cellulose molecule is linked with CMC molecules. However, the powder x-ray diffraction pattern shows no significant difference between complex fibrils and control, indicating that the crystalline unit cell of cellulose-CMC complex fibrils is not markedly different from that of pure cellulose fibrils (Table VII). Analysis of the specific CMC content of hydrocellulose obtained by acid hydrolysis from a complex showed that the CMC is not uniformly distributed in fibrils, the crystalline parts having a small CMC content as compared with whole fibril. These findings point to the possibility that CMC is found predominantly in the amorphous part of the fibril or that the presence of CMC in fibrils induces amorphousness in its surroundings. These results could be in agreement with the findings of Preston (4) that xylan in cellulose containing up to 50 per cent

FIGURE 10 Randomly oriented cellulose fibrils in pellicle. The incubation mixture contained 0.8 mg cells per ml (dry weight) and 10^{-2} M glucose in buffer phosphate-citrate (1.5×10^{-2} M phosphate, pH 6.4). Other experimental conditions as in Fig. 7. *B*, bacterial cell wall not removed by alkali treatment. $\times$ 16,000.

FIGURE 11 Randomly oriented cellulose fibrils in pellicle. The system contained 0.8 mg cells per ml (dry weight), 10^{-2} M glucose in buffer phosphate-citrate (1.5×10^{-2} M phosphate, pH 6.4), and 8 mg/ml levan. Preparation was on Parafilm sheets. Incubation time 1 hour. Pt-Pd shadowed. Ratio of height to shadow 1:4 to 1:5. $\times$ 16,000.


B
10
11

xylan by weight appears to be located also in the amorphous part of the fibrils. Thus, this system could provide an experimental model for the investigation of the process of association of polysaccharides with cellulose fibrils as it occurs in plants.

The parallel orientation of fibrils containing CMC could be explained, at least partially, in terms of charge interaction and mechanical stress. Non-charged cellulose fibrils crossing each other at any angle would form a random mesh, the rigidity of which would depend on the number of interfibrillar cross-links (van der Waals or H bonds) per unit area. Electrostatic repulsion between fibrils containing CMC can weaken the interfibrillar links occurring in the vicinity of adjacent carboxyls. Stress or strain applied to this kind of mesh could result in an easier deformation of the matrix in the direction of the applied force. The most stable state would be achieved when the fibrils are parallel and the over-all number of links formed between fibrils exceeds the number of weak bonds due to electrostatic repulsion. From similar considerations, it is conceivable that a second layer of fibrils which would be formed above the first would reach a more stable position when oriented at an angle with the first, since then the repulsion between the layers would be minimal. The origin of mechanical forces acting in this system could be multiple, for example Brownian motion, surface tension, streaming due to evaporation, and probably stress during preparation of specimens. The explanation of orientation on the basis of charge interaction is in agreement with the observed facts, *i.e.*, reduction in degree of orientation at lower pH, and orientation of fibrils formed in the presence of other charged polysaccharides like phosphomannan but not in the presence of non-charged polysaccharides like levan. The development of techniques for the quantitation of the degree of orientation in this system is necessary in order to test the above hypothesis, which is not entirely new. In fact, Steward (37) suggested a somewhat similar role for the accompanying polysaccharides, which would serve as a greasing material during a process of passive orientation of cellulose fibrils in some *Valonia* species. Thus a surface absorption or incorporation of charged polysaccharides into cellulose fibrils, as proved experimentally in this work, could be part of the mechanism of orientation of cellulose fibrils in plant cell walls. It is known that the degree of orientation and pattern are specific for different plant cell walls. This could be due to the presence of different polysaccharides which influence the orientation of the cellulose fibrils during their formation and deposition in the cell wall.

For part VII of this work, see *Bull. Research Council Israel*, 1963, **11A**, 279.

Our thanks are due to Dr. W. Traub of the Department of X-Ray Crystallography, The Weizmann Institute of Science, Rehovoth, Israel, for performing the x-ray diffraction experiments and for valuable comments with regard to the interpretation of the results; and to Professor J. Gross and Dr. O. Stein of the Department of Experimental Medicine and Cancer Research of the Hebrew University-Hadassah Medical School for their permission to use facilities of the Rosa and Eliahu Wunch Laboratory of Electron Microscopy. We are also indebted to Dr. G. Avigad and Dr. R. G. Kulka of the Department of Biological Chemistry, The Hebrew University of Jerusalem, for their critical discussion and interest in this work.

Received for publication, July 2, 1964.

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