galactosamine-induced hepatic necrosis. The surviving recipient animals were killed at intervals over 2 weeks. Liver sections were examined by radioautography for the presence of labeled cells. There was no significant uptake of the labeled donor cells by the regenerating livers. Therefore, the transplanted liver cells did not repopulate the recipient injured livers. Moreover, allogeneic (Buffalo and ACI strain) and xenogeneic (porcine) liver cells were as effective as syngeneic cells in prolonging survival after galactosamine administration (*9*). Unless the liver is a privileged site, the rapid destruction of these foreign cells by immune mechanisms argues further against repopulation.

We have shown that either liver or bone marrow cells can prolong the survival of rats with experimental acute hepatic failure. Liver cells can reverse the hepatic failure brought about by toxic or ischemic necrosis. The effect of bone marrow does not appear to be mediated by macrophages, but may be dependent on some other cell type yet to be isolated.

However, the recent evidence indicating that hepatic macrophages (Kupffer fer cells) are of bone marrow origin (*10, 11*) raises interesting questions concerning the role of cells derived from bone marrow. Repopulation of the liver by the transplanted cells does not occur. Whether these cells maintain metabolic support during the period of maximum hepatic failure, or whether a factor derived from these cells increases the rate of regeneration in the recipient liver remains to be determined.

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Calcofluor White ST Alters the in vivo Assembly of Cellulose Microfibrils

Abstract. *The fluorescent brightener, Calcofluor White ST, prevents the in vivo assembly of crystalline cellulose microfibrils and ribbons by* Acetobacter xylinum. *In the presence of more than 0.01 percent Calcofluor,* Acetobacter *continues to synthesize high-molecular-weight β-1,4 glucans. X-ray crystallography shows that the altered product exhibits no detectable crystallinity in the wet state, but upon drying it changes into crystalline cellulose I. Calcofluor alters cellulose crystallization by hydrogen bonding with glucan chains. Synthesis of this altered product is reversible and can be monitored with fluorescence and electron microscopy. Use of Calcofluor has made it possible to separate the processes of polymerization and crystallization leading to the biogenesis of cellulose microfibrils, and has suggested that crystallization occurs by a cell-directed, self-assembly process in* Acetobacter xylinum.

Calcofluor White ST (4,4′-bis[4-anilino-6-bis(2-hydroxyethyl)amino-*s*-triazin-2-ylamino]-2,2′-stilbenedisulfonic acid) is a fluorescent brightener used commercially to whiten cellulosic textiles and paper. Because of its ability to hydrogen bond with β-1,4 and β-1,3 polysaccharides (*1*) as a vital stain (*2*), it has also been used by biologists to localize cellulose and chitin (*3*). In this report we describe results indicating that Calcofluor White ST alters cellulose synthesis in the Gram-negative bacterium *Acetobacter xylinum* by separating the polymerization of high-molecular-weight β-1,4 glucans from their crystallization into cellulose I microfibrils.

Acetobacter xylinum normally produces crystalline 30-Å microfibrils in association with intracellular synthesizing sites and a longitudinal array of extrusion sites in the lipopolysaccharide layer of the bacterium (*4*). The microfibrils hydrogen bond together (*5*) into an extracellular ribbon of cellulose (Fig. 1, a and b); ribbons from many bacteria intertwine into a tough pellicle on the surface of the culture medium.

When actively synthesizing *A. xylinum* cells are incubated in medium or phosphate buffer containing more than 0.01 percent Calcofluor, ribbon assembly is disrupted (Fig. 1, e and f). Instead of twisting ribbons, the bacteria synthesize broad bands of bent fibrils. High-resolution micrographs reveal that the smallest fibrils in the band product measure 15 Å (±4 Å) (Fig. 1i). Larger fibrils appear to arise by fasciation of the smaller 15-Å fibrils. These 15-Å fibrils frequently show pronounced curvature and undulation, which suggests low crystallinity (*6*), while the larger aggregates often bend sharply and appear more rigid.

The altered cellulose synthesized is known to be high-molecular-weight, noncrystalline β-1,4 glucans by the following evidence: (i) the band is rapidly degraded by purified cellulase (*7*); (ii) radioactive glucose is incorporated into an alkali-insoluble product (*8*) and into a band next to the cell, as visualized by autoradiography in the presence of Calcofluor; (iii) viscosity measurements show that the Calcofluor-induced product has a degree of polymerization comparable to that of control samples (*9*); and (iv) x-ray crystallography studies indicate that the Calcofluor-induced product has cellulose I crystallinity after drying although it has no detectable crystallinity in the wet state (*10*). This lack of crystallinity before drying indicates that the Calcofluor-induced product is profoundly different from native cellulose of *A. xylinum*, which has about the same cellulose I crystallinity (*11*) in the wet and dry states, the crystallite size being 65 and 74 Å, respectively. The size of the cellulose I crystallites obtained after drying of the altered cellulose depends on the initial concentration of Calcofluor—69-Å crystallites resulting if a concentration of 0.025 percent is used and 28-Å crystallites if 0.1 percent or 0.5 percent concentration is used to induce the alteration.

The noncrystalline nature of the un-

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dried band cellulose indicates that Calcofluor effectively separates polymerization from crystallization in the wet state by preventing microfibril assembly. On drying, the hydrogen bonds of the nascent crystal lattice must be more favorable than the association between Calcofluor and glucans, so that Calcofluor is displaced and the cellulose I lattice forms. It is exceptional that cellulose I can be produced by drying of noncrystalline cellulose, since the parallel chain cellulose I crystal lattice is thermodynamically less favorable than the antiparallel chain cellulose II crystal lattice (*5*). In order for this to happen, the glucan chains must be synthesized in oriented parallel bundles that do not dissociate after extrusion from the cell. The ordered nature of the wet band cellulose is confirmed by observation with polarized light (Fig. 1c).

The effect of Calcofluor is reversible. In large cultures containing Calcofluor, a soft, filmy mass of altered cellulose is initially produced at the bottom of the culture. After synthesis time, which is proportional to the initial Calcofluor concentration (if inoculum size is constant), normal ribbon assembly and pellicle production resume. Rapid reversion (within seconds) can also be effected by experimental manipulation. When free Calcofluor is removed from the incubation medium of a cell synthesizing band cellulose, ribbon synthesis begins immediately (Fig. 1g); and when it is added to the incubation medium of a cell synthesizing a normal ribbon, band synthesis begins immediately (Fig. 1d). This rapid reversibility, along with the charged nature and large size of the brightener, suggests that Calcofluor exerts its effect extracellularly and does not directly interfere with cell metabolism or cellulose-synthesizing enzymes. Dark-field fluorescence microscopy of whole cells incubated in Calcofluor and thin sections of cells also incubated in this brightener before fixation (*12*) confirm that it is not incorporated or bound to the cells under our experimental conditions. Furthermore, the rapid reversibility described suggests that crystallization occurs near the cell surface.

During the gradual transition from production of band material to production of normal ribbons, aggregates of cellulose are observed that are intermediate between the band cellulose described and

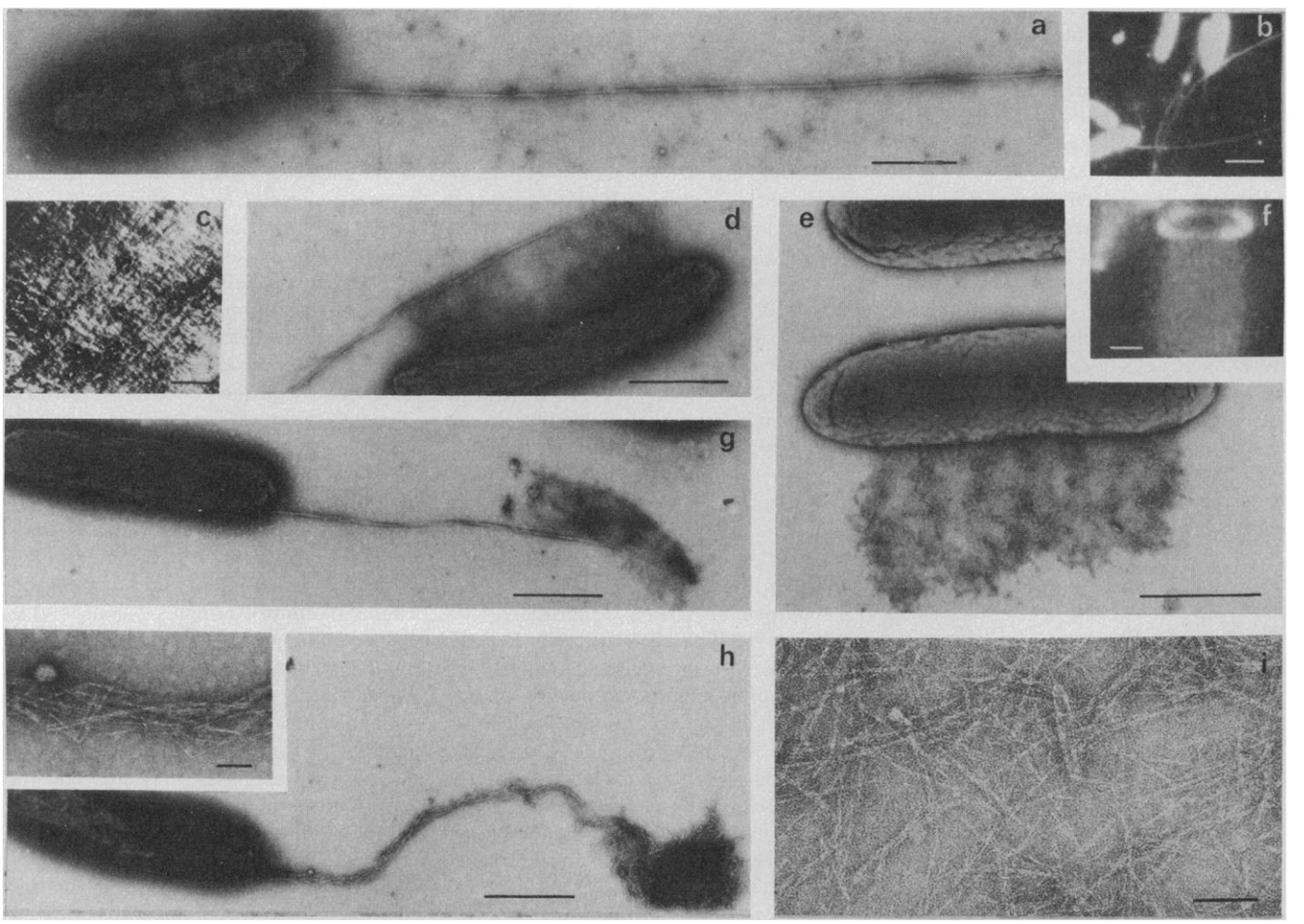

Fig. 1. (a) Electron micrograph of negatively stained dividing *Acetobacter xylinum* showing a newly synthesized, twisting cellulosic ribbon (scale bar, 1 μm). (b) Dark-field light microscopy of *Acetobacter* synthesizing ribbons under normal culture conditions (scale bar, 1 μm). (c) Polarization microscopy with crossed Nicols of band material synthesized in 0.025 percent Calcofluor for 24 hours (scale bar, 25 μm). (d) Electron micrograph of a negatively stained cell that was attached to the grid, exposed to Calcofluor-free medium for 3 minutes, and then to 0.025 percent Calcofluor medium for 3 minutes (scale bar, 1 μm). (e) Electron micrograph of negatively stained *A. xylinum* producing band material in phosphate buffer containing 0.025 percent Calcofluor (scale bar, 1 μm). (f) Dark-field fluorescence microscopy of a cell producing band material (scale bar, 1 μm). (g) Electron micrograph of a negatively stained cell that was attached to the grid, exposed to 0.025 percent Calcofluor medium for 3 minutes, washed in buffered glucose, and then exposed to Calcofluor-free medium for 3 minutes (scale bar, 1 μm). (h) Electron micrograph of a negatively stained cell synthesizing a broad, curving intermediate ribbon. Compare this intermediate form with the two forms in (g) at the same magnification (scale bar, 1 μm). The inset shows the part of this intermediate ribbon near the cell at higher magnification (scale bar, 1 μm). (i) Electron micrograph of negatively stained band material showing the fibrillar subunits in detail. Note the varied size of the fibrils and the frequent bends (scale bar, 0.1 μm).

normal ribbons. The intermediate forms are broad, loose aggregates of continuous fibrillar subunits resembling the microfibrils of the normal ribbon more closely than the bent fibrils of the band (Fig. 1h). These aggregates are probably formed near the threshold concentration, which suggests that concentration of unbound Calcofluor is critical in determining the morphology of the cellulose product. Similarly, the x-ray data indicate that concentration of Calcofluor determines the size of the crystallites obtained after drying. Therefore, we conclude that Calcofluor prohibits ribbon assembly and microfibril crystallization by competing for the hydrogen bonding sites that form the crystalline lattice (*5*). As cellulose synthesis continues, more binding sites become available, the concentration of free Calcofluor decreases, and an increasing degree of normal hydrogen bonding becomes possible.

How does the evidence described here relate to the current understanding of cellulose microfibril and ribbon biogenesis in *A. xylinum*? Zaar (*13*) has interpreted the row of extrusion sites in the lipopolysaccharide layer of *Acetobacter* in light of the multienzyme complex model proposed by Brown and Willison (*4*), suggesting that the extrusion pores facilitate crystallization of bundles of glucans synthesized by multiple enzyme complexes. Since Calcofluor can interfere with the normal process of crystallization only after extrusion of the polymerized product, we conclude that under normal conditions crystallization occurs after the glucan bundles have exited the pores. However, the pores are critical to crystallization because they aggregate parallel glucan chains into nondissociable bundles.

If microfibrils of uniform dimensions and twisting ribbons are formed by the interaction of noncrystalline, oriented glucan bundles at the cell surface, it is probable that the synthesis-extrusion sites are precisely spaced and arranged to coordinate the crystallization process. We propose that biogenesis of cellulose I in *A. xylinum* occurs through a cell-directed process that could occur as follows: (i) the multiple enzyme complexes determine the number and polarity of glucan chains originating at one site, (ii) the extrusion sites facilitate the aggregation of the chains into nondissociable bundles, and (iii) the microfibrils crystallize by self-assembly as glucan bundles from adjacent sites interact. It is possible that bundles from more than one site are involved in the formation of one microfibril. Therefore, we conceive polymerization and crystallization to be consecutive, not simultaneous (*14*), processes. However, the two phases remain closely associated through the formation of ordered bundles of chains and the mediation of the cell surface.

Compatible with the concept of cell-directed crystallization, atypical cases have been observed in which *A. xylinum* synthesizes an abnormal product even without Calcofluor (*15*). This coiled, nonfibrillar product may well result when the cellular organization mediating consecutive polymerization and crystallization into the normal product is disturbed. Giddings *et al.* (*16*) have also suggested that the geometrical arrangement of supposed microfibril-synthesizing complexes, called rosettes, determines the size of fibrils and their ordered pattern of deposition in secondary walls of the green alga *Micrasterias denticulata*. They observed that the size of cellulose fibrils is proportional to the number of aligned rosettes involved in their formation, which is compatible with a mechanism similar to the cell-directed interaction of glucan bundles that we have proposed for *A. xylinum*. Other workers have reported organized microfibril termini in algae and higher plants (*17*), and it is possible that the correlation of highly crystalline, highly organized cellulose I fibrils with such cellular structure will be found to be the most common mechanism of fibril assembly.

How does the Calcofluor-induced alteration of cellulose synthesis that we have described relate to the elementary fibril concept proposed by Frey-Wyssling (*18*)? Giddings *et al.* (*16*) suggest that the elementary fibril in *Micrasterias* could be redefined as the product of one rosette. On the basis of our data, we propose one possible mechanism of fibril formation in *A. xylinum*, namely, that highly crystalline 30-Å fibrils are formed by fasciation of two 15-Å units of synthesis with low crystallinity. The lowest crystallite size of 28 Å obtained from the Calcofluor-altered product corresponds closely to the commonly reported microfibril size of 30 Å, the same size predicted by fasciation of two 15-Å units. The 30-Å microfibril in *A. xylinum* may be the smallest aggregate of chains able to form a highly crystalline cellulose I fibril, or an elementary fibril. A range of larger crystallites could be synthesized in various organisms [for example, 69 Å in *A. xylinum*, 140 Å in *Valonia* (*19*)] by the cell-directed juxtaposition of many terminal complexes that synthesize glucan bundles capable of interaction with their neighbors before final crystallization. Larger crystallites could still be composed of crystalline subunits in the size range of the elementary fibril that would be revealed by mechanical disruption (*20*). The concept of crystalline subunits within larger crystallites would help clarify the long-standing discrepancy between crystallite sizes measured by x-ray diffraction and microfibrils measured in electron microscopy (*5*). It seems possible, then, to define the elementary fibril not only in terms of a specific size but also in relation to its biogenesis from a minimum number of oriented glucan chains that aggregate into a thermodynamically metastable cellulose I fibril.

Thus an experimental probe has been used to alter, in vivo, the molecular dynamics of cellulose microfibril crystallization and ribbon assembly. Our results support the hypothesis advanced before on the basis of morphological evidence (*16, 17*) that the cell-directed coordination of synthesizing sites is essential for the biogenesis of the cellulose I fibrils characteristic of a given organism. The possibility of using Calcofluor White and other probes to experimentally manipulate cellulose assembly in organisms other than *A. xylinum* has great potential for leading to a new understanding of cellulose I biogenesis.

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Micromolar Ca^{2+} Stimulates Fusion of Lipid Vesicles with Planar Bilayers Containing a Calcium-Binding Protein

Abstract. *Fusion of phospholipid vesicles with a planar phospholipid bilayer membrane that contains a calcium-binding protein appears to mimic the essential aspects of cytoplasmic-vesicle fusion with plasma membranes (exocytosis) in that (i) there is a low basal rate of fusion in the absence of Ca^{2+}, (ii) this basal rate is enormously increased by micromolar (~ 10 μM) amounts of Ca^{2+}, and (iii) this rate is not increased by millimolar Mg^{2+}. Essential to this process is an osmotic gradient across the planar membrane, with the side containing the vesicles hyperosmotic to the opposite side. Similar osmotic gradients or their equivalent may be crucial for biological fusion events.*

Exocytosis, the fusion of intracellular vesicles with plasma membranes and the subsequent extracellular discharge of vesicular contents, is fundamental to such diverse biological phenomena as transmitter release at synapses (*1*), secretion by endocrine and exocrine glands (*2*, *3*), and raising of the fertilization membrane after union of sperm and egg (*4*). Recently, we demonstrated that this process is modeled by the interaction of multilamellar phospholipid vesicles with planar phospholipid bilayer membranes (*5*, *6*). In this model system, the existence of an osmotic gradient across the planar membrane is a prerequisite for fusion; the *cis* compartment containing the vesicles must be hyperosmotic with respect to the opposite (*trans*) compartment (*6*). We noted (*6*) that this osmotic condition, or its equivalent, may be required for biological exocytosis, and that there are basic similarities between fusion of phospholipid vesicles with planar phospholipid bilayer membranes, on the one hand, and fusion of cytoplasmic vesicles with plasma membranes, on the other. There is a major difference, however, between these two systems in the Ca^{2+} requirement. Whereas millimolar amounts of any divalent cation (Mg^{2+}, Ca^{2+}, Ba^{2+}) stimulate fusion in the model system (*6*), micromolar amounts specifically of Ca^{2+} are necessary to stimulate biological exocytosis (*7*, *8*). This difference is not surprising, since phospholipids alone have neither the sensitivity nor the specificity for Ca^{2+} manifested in exocytosis (*9*, *10*); Ca^{2+} sensitivity and specificity must arise from other components, presumably a protein or proteins, in the plasma or vesicle membrane. We now report that fusion of multilamellar phospholipid vesicles with planar phospholipid bilayer membranes is catalyzed by 10 μM Ca^{2+}, but not by 1 mM Mg^{2+}, if a calcium-binding protein extracted from synaptic membranes is included in the planar membranes.

Upon addition of vesicles, under appropriate conditions, to one of the two aqueous compartments on either side of a planar membrane, fusion is seen by two independent criteria: (i) the transfer of vesicular contents across the planar membrane into the *trans* aqueous compartment (*5*) and (ii) the incorporation of a vesicular membrane marker into the planar membrane (*6*). The latter criterion is used in the present study. We chose as the membrane marker the intrinsic membrane protein, VDAC (the voltage-dependent anion channel), obtained from outer mitochondrial membranes (*11*, *12*). As reported previously (*6*), fusion events are characterized by sudden jumps in conductance of the planar membrane; because vesicles can contain more than one channel in their outermost lamella, some of these jumps result from "simultaneous" (within 200 μsec) incorporation of several channels into the planar membrane.

Figure 1A demonstrates the effect of Ca^{2+} on the fusion rate of multilamellar vesicles with a planar phospholipid bilayer membrane that contains the calcium-binding protein. This water-insoluble, membrane-associated protein which is partially purified from calf brain, has a molecular weight of $\sim$ 16,000 and a Michaelis constant (K_m) for Ca^{2+} of ~ 15 μM (*13*). In the complete absence of Ca^{2+}, there is a low but finite rate of fusion (not shown in this record) when the *cis* compartment is hyperosmotic with respect to the *trans* side. (The osmotic gradient is here established by 100 mM glucose in the *cis* compartment.) This basal fusion rate is increased by several orders of magnitude on addition to the *cis* compartment of Ca^{2+} to a concentration of 10 μM.

The requirement for an osmotic gradient is shown in Fig. 1B. In the presence of 10 μM Ca^{2+}, no fusion occurs until the *cis* compartment is made hyperosmotic to the other side. An effective osmotic gradient can be established either by adding an osmoticant (solute that creates the osmotic gradient) to the *cis* compartment or by removing one from the *trans* compartment.

When calcium-binding protein is present in the planar membrane, the fusion process also shows Ca^{2+} specificity. Under otherwise appropriate conditions 1 mM Mg^{2+} does not stimulate fusion, but subsequent addition of 100 μM Ca^{2+} initiates fusion events (Fig. 1C). The presence of calcium-binding protein does not alter the effect of Mg^{2+} on fusion; concentrations > 5 mM are still required for Mg^{2+} alone to promote fusion events. Nor does the calcium-binding protein alter any other conditions for fusion of multilamellar vesicles to planar bilayer membranes; only sensitivity and specificity for Ca^{2+} are enhanced. An osmotic gradient across the planar membrane (*cis* side hyperosmotic) is still required; indeed this alone, in the complete absence of divalent cation, causes fusion, albeit at a low rate.

Figure 2 depicts our proposed mechanism for fusion of a phospholipid vesicle with a planar membrane. The figure shows a single-walled vesicle because this is a simpler physical system to analyze than the multilamellar vesicles used in the present study; we have in fact obtained fusion of single-walled vesicles with planar membranes using conditions very similar to those we describe for multilamellar vesicles.

For the sake of clarity, let us assume that both compartments as well as the vesicles initially contain 100 mos$M$ glucose in addition to 200 mos$M$ salt (*14*, *15*). If glucose is now removed from the *trans* compartment, water flows across the planar membrane from the *trans* to

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