Radioactivity in DNA of M-bands isolated from *Chironomus* larvae under different conditions

	Percent total ^{3}H-radioactivity (loaded on the gradient) associated with the M-band (± SD)
M-band isolated from *Chironomus* larvae after 2 h labelling with ^{3}H-thymidine	46 ± 2.6
with ^{3}H-thymidine followed by 8 h chase with cold thymidine (1 mg/ml)	16 ± 2.0

Each value is an average of 3 independent experiments. Total radioactivity of the nuclear lysate loaded on the gradient in these experiments ranged from $5–7 \times 10^4$ cpm.

contained nuclei free from other cellular debris. The nuclei so isolated were suspended in TMK buffer[12] (Tris 0.01 M, maganesium acetate 0.1 M and KCl 0.1 M, pH 7.2) and sonicated in an MSE ultrasonic disintegrator Model 60 W at 1.5 mA for 1 min at ice temperature. The sonicated nuclear lysate (0.5 ml) was overlayered on 0.5 ml of 0.2% sarkosyl (NL-97, Geigy) on a biphasic sucrose gradient in TMK buffer (15 ml of 20% sucrose over 5 ml of 60% sucrose shelf) and centrifuged in an MSE Superspeed 50 ultracentrifuge using an SW 3×23 ml rotor at 15,000 rpm at 4 °C for 30 min. A white layer of membrane-complex bound to magnesium sarkosyl crystals banded at the interphase of the sucrose solutions (M-band). After centrifugation, the contents of the tube were collected by siphoning and trichloroacetic acid-insoluble radioactivity in each fraction was determined[15] and expressed as percent of total radioactivity loaded.

The sedimentation profile of the ^{3}H-thymidine-labelled nuclear lysate of the *Chironomus* larvae in the biphasic sucrose gradient is shown in the figure. It can be seen that most of the labelled DNA is associated to the M-band sedimenting at the interphase of the sucrose solutions. Treatment of the nuclear lysate with deoxyribonuclease I (Worthington Biochemical Corporation) prior to sedimentation resulted in the removal of the radioactivity associated with the M-band indicating that the associated radiactivity was due to labelled DNA. Further it was found that most of the radioactivity (80%) was recovered in the thymidine-5′-monophosphate spot when the M-band material isolated from ^{3}H-thymidine labelled larvae was digested with DNase I and snake venom phosphodiesterase (Sigma Chemical Co., St. Louis, USA) and subjected to paper chromatography using isobutyric acid : liquor ammonia : water (66 : 1 : 33) as solvent mixture. To test whether or not the binding of the DNA with the M-band is due to extraneous factors, ^{3}H-labelled *Escherichia coli* DNA (both heat-denatured and native) was sedimented along with unlabelled nuclear lysate. No radioactivity due to bacterial DNA could be seen associated with the M-band. The radioactivity in the M-band decreased when the larvae were first labelled with ^{3}H-thymidine for 2 h and the label was then chased with cold thymidine for 8 h (table). This suggests that the newly synthesized DNA may be associated with the nuclear membrane. Taken together, the findings provide evidence which implies that in *Chironomus* larvae DNA replication may be associated with the nuclear membrane.

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Isolation and properties of cell walls from *Agrobacterium tumefaciens* B_6[1]

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Summary. Purified cell walls were prepared from *Agrobacterium tumefaciens* B_6 by extraction of intact cells with hot sodium dodecyl sulfate and digestion with proteases. Such preparations contained peptidoglycan that accounted for about 40% of their dry weight. Electron micrographs of the purified walls showed that they conserved their characteristic shape despite the drastic extraction procedure.

Agrobacterium tumefaciens, a gram negative rod, has long been known as the causative agent of crown gall disease in many plant tissues[3]. So far, only crude wall preparations from this organism have been described in the literature[4,5]. In the present study, electron microscopy of whole cells and of partially purified and purified walls of *A. tumefaciens* B_6 are presented along with their amino acid and amino sugar composition.

Materials and methods. Agrobacterium tumefaciens B_6 was obtained through the courtesy of Dr. A. C. Brown, Rockefeller University, New York. Bacterial cultures were grown on liquid medium that contained: casitone (Difco), 2%; yeast extract, 1%; K_2HPO_4, 0.5%, glucose, 0.2% and $MgSO_4 \cdot 7\ H_2O$, 0.01% at 30 °C. When they reached $^1/_4$ to $^1/_3$ log phase, they were quickly cooled and harvested. Crude walls were obtained by mechanical disruption of fresh or frozen cells[6]. Partially purified walls were obtained by extraction of the crude walls for 22h in boiling 4% sodium dodecyl sulfate[7] followed by trypsin digestion (in 0.05 M Tris-HCl pH 8.5 and 0.02 M $CaCl_2$, at 37 °C overnight) of the insoluble residue obtained after heating in SDS, and extensive washing in water. A second extraction cycle with hot 4% SDS and digestion with pronase (0.05 M Tris-HCl, pH 8.0 and 0.02 M $CaCl_2$, at 37 °C overnight) yielded purified cell walls.

For electron microscopy negatively stained samples of untreated cells or cell walls were deposited on copper grids (400 mesh) previously covered with parlodion membrane. After 1 min they were stained with 2% uranyl acetate and 0.2% lead citrate, and after an additional 45 sec a drop of octadecanol was added and the grids were immediately blotted dry and taken for examination in the Philips 300 electron microscope, operated at 80 kV. For thin sectioning, packed cells were mixed with an equal volume of 3% agar solution and the mixture taken up into narrow part of a Pasteur pipette. After solidification, the gel was exhaled and cut into 1 mm^3. The cubes were dehydrated by passing through a graded series of

Analysis of crude and of purified cell walls

	Crude cell walls µmoles/mg	Ratio	Purified cell walls µmoles/mg	Ratio
Amino sugars				
Glucosamine	0.005	0.04	0.974	3.595
Muramic acid	–	–	0.258	0.950
Galactosamine	–	–	0.110	0.450
Total percentage in fraction	0.1%		30.3%	
Amino acids				
Lysine	0.100	0.86		
Histidine	0.083	0.73		
Arginine	0.050	0.44		
Aspartic acid	0.103	0.87		
Threonine	0.053	0.45		
Serine	0.049	0.41		
Glutamic acid	0.118	*1.00*	0.271	*1.00*
Glycine	0.106	0.91	0.026	0.10
Alanine	0.170	1.39	0.413	1.52
Valine	0.053	0.45		
Diaminopimelic acid (+ methionine)*	0.053	0.47	0.342	1.33
Isoleucine	0.039	0.32		
Leucine	0.066	0.56		
Tyrosine	0.020	0.17		
Phenylalanine	0.039	0.32		
Total percentage in fraction	13.8%		15.5%	
Neutral sugars (phenol-H_2SO_4)	**		60%	
Phosphorus	**		0.35%	
Nitrogen	**		6.64%	
OD_{262} of 4 mg (after hydrolysis in 2 N HCl for 2h)	**		0.040	

*In this system diaminopimelic acid and methionine are eluted as one peak; **not tested.

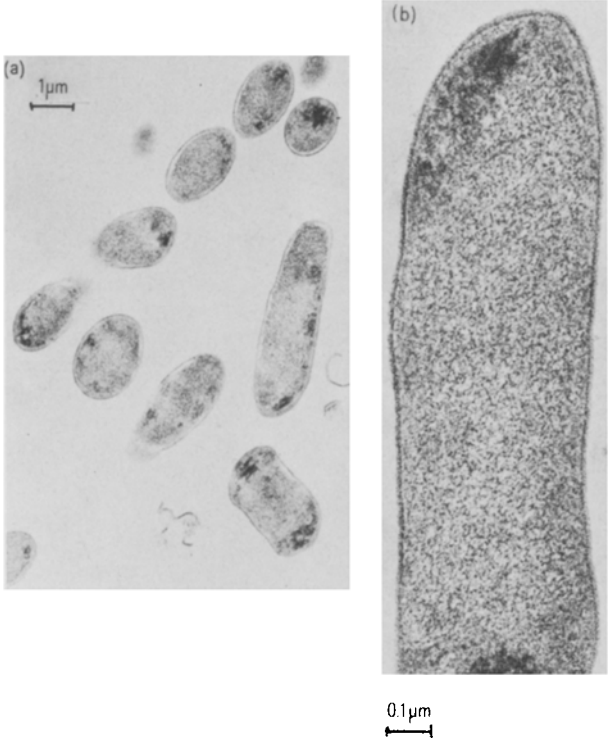

Fig. 1. Thin sections of untreated cells. *a* A general view, in some places the cytoplasm is separated from the wall, the latter can be seen clearly as a distinct layer. *b* A single cell in higher magnification, from which the wall thickness (120–130 Å) was calculated.

increasing concentrations of ethanol and were embedded according to Spurr[8], Sections were cut with glass knives on a Sorvall Porter Blum MT2-B Ultra Microtome and stained with 2% uranyl acetate and 0.2% lead citrate.

Results. Thin sections of *Agrobacterium tumefaciens* B_6 are presented in figure 1. Figure 1, a, shows rod-like cells. In a higher magnification (figure 1, b), it can be seen that the cell is surrounded by a distinct, continuous wall, the thickness of which was found to be 120–130 Å, which is in accordance with published values for bacterial cell walls[9].

1 cycle of SDS extraction and trypsin digestion yielded partially purified walls (table), electron micrographs of which revealed a considerable amount of electron dense material (figure 2, a). The latter was removed by a 2 cycle of SDS extraction and pronase digestion (table; figure 2, b). This suggests that the extracted material consists at least partially of protein. Amino acid analysis of partially purified walls (not presented) shows a significant excess of protein over peptidoglycan components. Braun and Rhen[7] report that trypsin action on the peptidoglycan-lipoprotein complex of *Escherichia coli* releases lipoprotein and results in protein-free peptidoglycan. In our study, the requirement for 2 SDS cycles along with 2 different protease digestions in order to obtain pure peptidoglycan sacculi suggests that the wall

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2 I wish to thank Professor Nathan Sharon for his constant interest and critical discussion during this work.
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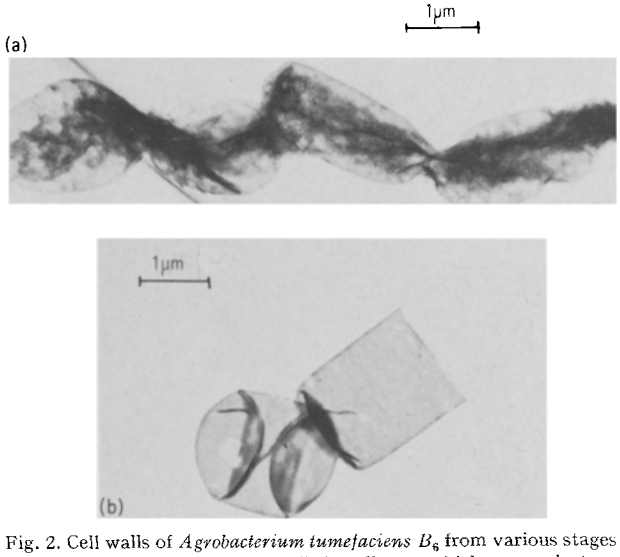

Fig. 2. Cell walls of *Agrobacterium tumefaciens* B_6 from various stages of purification. *a* Partially purified walls, to which some electron dense material adheres. *b* Purified walls, obtained after 2 cycles of hot SDS extractions and proteolytic digestions (for details, see methods). The walls are transparent and devoid of contamination.

protein in *Agrobacterium tumefaciens* B_6 is linked to the peptidoglycan by covalent bonds different from those in *Escherichia coli*.

About 40% of the purified walls is accounted for by typical peptidoglycan components (table). There is a molar excess of glucosamine over muramic acid, which is probably in the form of a polysaccharide. The latter very likely contains also galactosamine and neutral sugars, most of which (80%) was identified as D-galactose (with β-D-galactose dehydrogenase, according to Finch et al.[10]). These may be components of a polysaccharide covalently linked to peptidoglycan. The polysaccharide is different from lipopolysaccharide as the latter is not known to be covalently linked to peptidoglycan[11]. Moreover, lipopolysaccharide prepared from *Agrobacterium tumefaciens* by the hot phenol method[5] does not contain peptidoglycan constituents.

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Cell number and cell doubling times during the development of carrot embryoids in suspension culture

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Summary. A time course for the appearance of different stages in embryogenesis in carrot cell cultures is presented, together with the data on cell doubling times. Removal of auxin appears to dramatically increase the rate of cell division, particularly in early embryogenesis.

There is now good evidence from a number of plant cell cultures that single cells on the surface of small meristematic groups of cells may be induced to form embryoids[2]. Such a phenomenon is usually promoted by decreasing the auxin content of the culture medium, following which a series of rapid cell divisions ensues with the eventual formation of a bipolar embryoid structure. The process of embryoid development encompasses 3 characteristic, morphologically distinct structures usually distinguished as the globular, heart and torpedo stages[3,4].

Table 1. Cell number and time of appearance in the culture of different embryoid stages

Stage (dimensions in mm)	Cell number	Time for appearance (h)
Young globular (diameter 0.15)	70–115 (peripheral cell No. 16–19)	90–100
Globular (diameter 0.2–0.25)	411–607 (peripheral cell No. 29–33)	138–145
Heart (width 0.25) (length 0.3)	900–1300	160–170
Young torpedo (width 0.3) (length 0.5)	2000–3000	190–200

3 separate experiments were performed.

Table 2. Cell growth rate in embryoids at different developmental stages

Interval	Developmental stage	Average doubling time (h)
0– 95	Initial – young globular	14.5*
90–142	Enlargement of globular	21
140–165	Globular – heart	22
165–195	Heart – young torpedo	25

*Assuming single cell origin and no lag period prior to initiation.

During studies into the biochemistry of embryo development in carrot cultures we noted that high rates of cell division were achieved during the early phases of the developmental process. In this communication we report on the time scale of appearance of the different embryoid structures and on the rates of cell division at various points in the process.

Material and methods. Carrot cells were grown in suspension culture in Murashige and Skoog medium supplemented with sucrose (0.073 M), zeatin (10^{-7} M) and 2,4-D[5] (4.52×10^{-7} M) as described previously[3]. Cultures of high embryogenic potential were obtained by inoculating 5 ml of 21-day-old, washed, cells (obtained by filtration through 1 mm diameter glass beads) into 25 ml of Murashige & Skoog medium minus 2,4-D.

The time scale of embryoid development was studied by following the appearance of the different stages in embryogenic cultures initiated as described above. All the embryoids considered were free floating in nature, since cell-group bound embryoids, especially the globular stages, are very difficult to observe. It is known that embryogenic suspensions established from freshly isolated callus and grown in an auxin-containing medium often have slowly developing embryoids which advance rapidly once the auxin level is decreased[6]. To avoid such 'preformed' embryoids the stock embryogenic suspensions used had been subcultured 4 times (every 21 days) and did not contain any microscopically identifiable embryoids. Cultures which had been subcultured considerably more than 4 times showed slower embryoid formation. This could be due to increased sensitivity to auxin carried over from the stock suspension[6] or may represent in part, an increasing lag period during which the cells achieve the metabolic state required for the onset of embryogenesis.

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