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# Genetical and structural analyses of cell-wall formation in *Chlamydomonas reinhardi*

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SUMMARY

Fifteen mutant strains of *Chlamydomonas reinhardi* were isolated which showed defects in some aspect of the process of cell-wall formation. Genetic analyses indicated that most of the mutations were due to single gene changes; two were anomalous in that non-Mendelian segregations were obtained on crossing with other genotypes, and on selfing they frequently gave rise to wild-type phenotypes.

Occasional somatic revertants were also obtained from these two strains. On the basis of these analyses it is suggested that there are two levels of control operating in the process of cell wall biogenesis – one concerned with subunit production at the nuclear level and another, possibly concerned with three-dimensional organization, at another level. Electron-microscope analyses of the different mutants showed the mutants to be divided into three main categories: those in which the wall was formed but was not attached to the plasma membrane, those in which the wall was attached to the membrane, and those in which very little wall was formed. In the last class in particular, vesicles containing wall precursors were clearly visible, and were shed through the plasma membrane into the medium.

## 1. INTRODUCTION

Genetic analyses of morphogenetic processes in eukaryotic systems could contribute to an understanding of the biogenesis and organization of many complex cellular structures. In prokaryotic systems the detailed studies of phage morphogenesis (Levine, 1969) have clearly demonstrated the resolving power of such techniques. The analyses of flagellar structure in *Chlamydomonas reinhardi* (Randall, 1969) constitute a similar attempt to resolve by genetic means the structure of an organelle in a eukaryotic system. Such studies necessarily presuppose the availability of mutants which show defects in the structure being investigated. In an attempt to resolve some aspects of the process of cell-wall formation we have initiated a programme to accumulate mutants of *C. reinhardi* which show defects in their cell walls. In the present report we describe the results we have obtained from electron-microscope studies of the comparative structure of the wild-type and mutant cells, and the genetic analyses undertaken with the mutants.

## 2. MATERIALS AND METHODS

The wild-type (WT) strains of *C. reinhardi* were initially obtained from Professor R. P. Levine. The media used were: MIN, minimal medium (Sueoka, 1960); Y, MIN supplemented with 0·4% Difco yeast extract; YA, Y supplemented with 0·2% sodium acetate; YAP, YA supplemented with 0·5% peptone. Both liquid and agar media were used. WT stocks were maintained on YA slopes, and mutants defective in some aspect of cell-wall biogenesis (CW) on YAP. Gametes were produced using either the cell synchronization technique of Kates & Jones (1964) or the technique of Sager & Granick (1954). Diploid vegetative cells were produced using the method described by Ebersold (1967). All vegetative cultures were maintained at 25 °C and 800 ft-c light. Zygotes were produced and matured using standard techniques (Lawrence & Davies, 1967).

*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (N.N.G.) was the only mutagen used in the present experiments. This was made up in citrate buffer pH 5, at a concentration of 300 $\mu$g/ml. Fully differentiated gametes from synchronous cultures were treated; these were washed and resuspended in citrate buffer at a concentration of $4 \times 10^6$ cells per ml, prior to adding freshly prepared N.N.G. to give a final concentration of 50 $\mu$g/ml. The suspension was kept at 25 °C in the dark for the 15 min treatment, and the cells were then washed twice with buffer, resuspended in MIN and plated immediately at a low dilution on YA agar. Plates were examined 7 days later for the presence of mutant colonies.

For electron microscopy of sectioned material the cells were fixed for 60 min in 2% glutaraldehyde in 0·025 M phosphate buffer, pH 7. They were then spun down, washed twice in 0·025 M phosphate buffer, and transferred to 0·5% veronal-buffered osmic acid. The samples were dehydrated rapidly through an alcohol series, the 70–90% alcohols containing uranyl acetate, then into a 1:1 alcohol–acetone mixture and finally left overnight in acetone prior to embedding in Epon (Luft, 1961).

Sections were post-stained with lead acetate (Millonig, 1961) or 2% barium permanganate for 1 min and examined in a Siemens Elmiskop 1A electron microscope.

Measurements were made on plates taken at a fixed magnification of nominally 40000 which was calibrated by measuring plates of catalase negatively stained with uranyl acetate at 38500 ± 3% (Luftig, 1967).

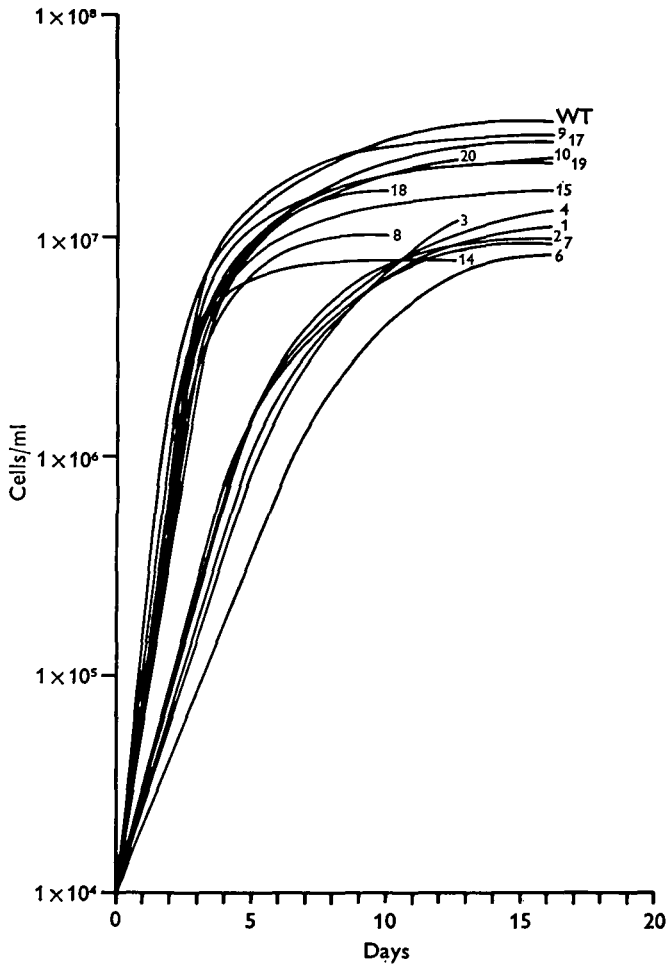
## 3. RESULTS AND DISCUSSION

### (i) *Induction of mutants*

Mutant colonies showing cell-wall defects were recognized by scanning plates under a low-power dissecting microscope. They were readily distinguishable from WT by the fact that the wall defect resulted in a loss of structural strength and the cells, on the outside of colonies in particular, assumed a flat 'amoeboid' configuration (Plate 1). In the centre of colonies, the cells' mutual support made the loss of

structural strength of less significance. In liquid, the wild-type and mutant cells were morphologically indistinguishable at the light-microscope level.

Fifteen mutants were isolated in the initial experiments following N.N.G. treatment, and in later experiments to be described elsewhere (J. S. Hymans & D. R. Davies, unpublished) another 66 have been isolated. The rate of induction in the former experiments was not determined, but in the latter could be as high as 30 per $10^3$ surviving cells. Only the first 15 mutants induced are considered here.




Text-fig 1. Comparative growth rates of WT and mutant lines in liquid YAP.

### (ii) *Growth of mutants*

In YA liquid medium the growth of some CW mutants was poor and in MIN many failed to survive. Various supplements were tried, and YAP was found to give adequate growth in all instances (Text-fig. 1). In agar media also the peptone supplement was necessary, and in addition the agar concentration had to be reduced from the normal 1·5 % to 0·8 %. With the former the plating efficiencies

were very low – the precise value varied with the concentration of cells plated. Though this might indicate a difference in osmotic properties of WT and CW cells, the growth of both forms was similar when tested on a wide range of concentrations of glucose, sucrose or sorbitol. No change occurred in the phenotype of the mutants when grown in these different conditions – i.e. they were not osmotic remedial, neither were they temperature sensitive.

### (iii) *Electron-microscope analyses*

Sections of all the CW mutants were compared with WT. The structure of the walls in the WT cells is not readily resolved. Beyond the plasma membrane there is first a diffuse region, then beyond this a two-partite structure, approximately 19 nm wide (Plate 2); on the outside is the capsular layer, which is continually secreting material into the surrounding medium. This structure is comparable to that described by Sager & Palade (1957). There appeared to be three main categories of mutants: those in which the wall was formed but was not attached to the plasma membrane (category A, Table 1), those in which the wall was attached (B), and those in which very little wall was formed (C). Category A mutants released

Table 1. *Phenotypic characterization of the mutants*

| Mutant | Phenotype | Category |
|---|---|---|
| CW 1, 2, 4, 6, 7, 9, 14, 17, 19 | Walls produced in normal quantities; not attached to plasma membrane | A |
| CW 8, 18, 20 | Walls produced in normal quantities; attached to plasma membrane | B |
| CW 3, 10, 15 | Minute amounts of wall produced; not attached to plasma membrane | C |

large quantities of free cell walls into the medium when grown in liquid (Plate 3). To determine whether more than one wall per cell per generation was being produced, the cells were grown immersed in agar medium; this resulted in any walls shed being trapped in the immediate proximity of the cell and allowed wall lineages to be constructed (Plate 4). Using this technique it was shown that this class of mutants did not produce an excess of wall. The wall differs from that in the WT in that the unit shed is clearly three-partite when seen in cross-section. An electron-dense continuous structure is seen on the inner and outer surfaces, with a discontinuous series of units in between. The whole wall is approximately 32 nm wide (Plate 5). The inner ill-defined layer seen in the WT is not apparent, and in its place there is the well-organized inner continuous layer (cf. Plates 2 and 5). The category A and C mutant cells, with their lack of walls, are thus equivalent to bacterial spheroblasts or protoplasts, and have only a plasma membrane as an outer surface. Nevertheless they are normal in most respects.

Further analyses and comparisons of isolated WT and category A (CW 2) walls have been made using negatively stained preparations. Detailed interpretations of the molecular architecture of the walls, as revealed by optical diffraction techniques, will be given elsewhere (Horne & Davies, 1970); in essence the surface

layers of the wall in both forms appear similar and are constructed of a series of lamellae of parallel fibres. The basis of the defect which leads to non-attachment of walls to the plasma membrane is not apparent to us at present.

Category B mutants have the wall attached (Plate 6), and no obvious difference between them and the WT can be seen in sectioned or negatively stained preparations; the loss of structural strength which leads to the typical CW colony morphology is therefore not yet understood. Again analyses of negatively stained preparations of CW 8 and CW 20 walls indicated that they also had a similar lamellar structure to that seen in the WT.

Category C mutants produce traces of wall, but so little is formed that it is normally undetectable unless the 'trapping' technique is used. These mutants are of particular interest in that large vesicles filled with densely staining material are seen both inside the cell and being secreted through the plasma membrane into the medium (Plate 7). In the WT such vesicles are seen both within the cell and located between the plasma membrane and the cell wall, but by the time the vesicles arrive beyond the plasma membrane they do not contain electron-dense material. It is likely that these vesicles provide the means whereby cell-wall precursors can be transported from the Golgi apparatus or endoplasmic reticulum to the outside of the plasma membrane (cf. Muhlethaler, 1967). In the category-C mutants the electron-dense material present in the vesicles may be modified or aberrant subunits of the wall, or, if some degree of assembly takes place within the vesicles, it may be incorrectly assembled subunits. In the case of the three mutants representative of this class, the end-result of the defect is that the contents of the vesicles are not assembled into cell walls once they have passed outside the plasma membrane.

Further work is being undertaken to determine both the nature and structure of the material within the vesicles, and whether different subunits or wall precursors are present in different mutants. On the basis of such information it may then be possible to determine both the nature of the defect which prevents normal wall formation, and the steps in the assembly of such precursors into wall structures.

### (iv) *Genetic analyses*

Mutants were induced in cells of WT background (mating type +) and subsequently crossed with WT −. Such crosses, with the exception of those involving CW 17 and CW 18, segregated 2:2 for wild and mutant phenotype, indicating single gene control of the character. The two exceptions will be considered separately later. Mutants were intercrossed in all combinations and the results are depicted in Text-fig. 2. The walls of the diploid zygote spores obtained on selfing mutants were normal in all instances, i.e. there is a different genetic system controlling this from that of the vegetative cell wall. In some, though not all, cases the first haploid cells produced from a homozygous mutant spore, following meiosis, were wild-type in phenotype, and this could persist for several cell generations before the mutant phenotype was expressed. Progenies from the various crosses were examined for the presence of WT recombinants. CW 2, 8, 1 and 19 were closely linked and possibly allelic (Table 2); the same was true for CW 10 and 15; CW 1 and 19 gave

occasional recombinants. Thus there are at least 12 loci represented among the 15 mutants analysed.

Complementation tests were attempted using diploid vegetative cells. Wild-type diploids were recovered at a low frequency when known non-allelic CW mutants were combined. The diploid status of these WT cells was confirmed by the production of a low frequency of CW cells after treatment with para-fluoro-phenylalanine (Lhoas, 1961), and also by crossing them with WT haploids; in the latter instance the WT diploids × WT haploids segregated CW cells. As yet the complementation tests are unsatisfactory in our experience. A low and variable frequency

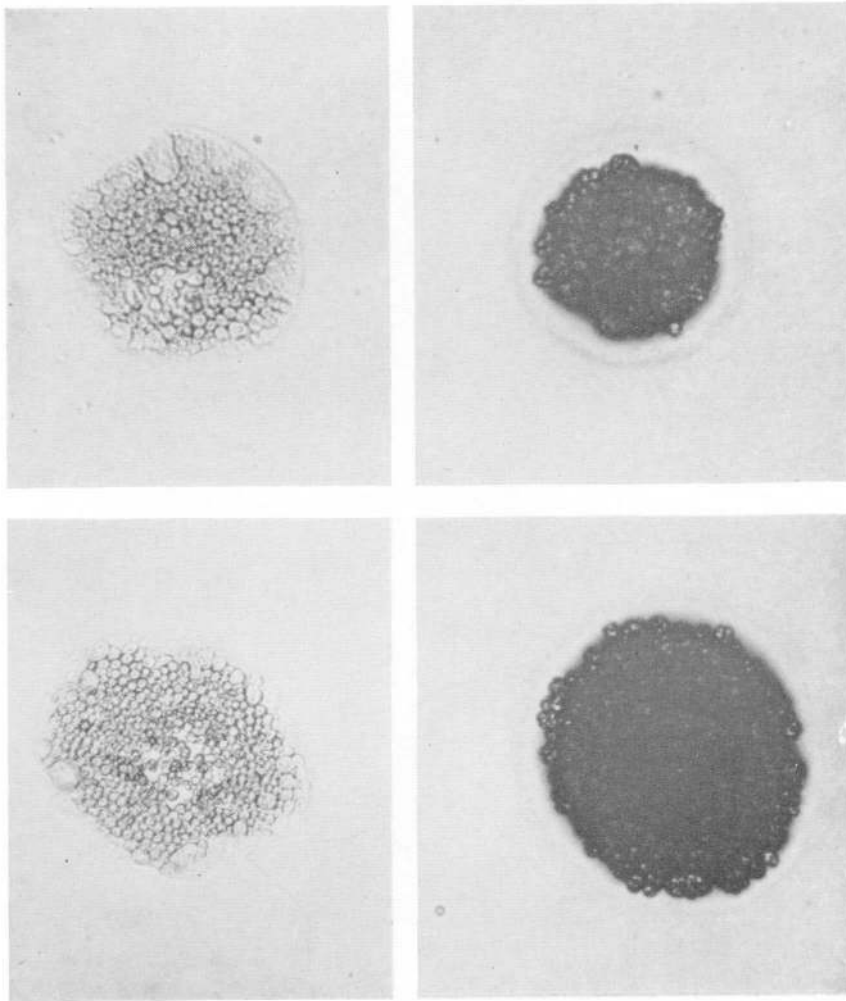
| | 1 | 2 | 3 | 4 | 6 | 7 | 8 | 9 | 10 | 14 | 15 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | × | × | × | × | 0 | × | × | × | × | | | × | × |
| 2 | | 0 | × | × | × | × | 0 | × | × | × | × | | | 0 | × |
| 3 | | | 0 | × | × | × | × | × | × | × | × | | | × | × |
| 4 | | | | 0 | × | × | × | × | × | × | × | | | × | × |
| 6 | | | | | – | × | × | × | × | × | × | | | × | × |
| 7 | | | | | | 0 | × | × | × | × | × | | | × | × |
| 8 | | | | | | | 0 | × | × | × | × | | | 0 | × |
| 9 | | | | | | | | 0 | × | × | × | | | × | × |
| 10 | | | | | | | | | 0 | × | 0 | | | × | × |
| 14 | | | | | | | | | | 0 | × | | | × | × |
| 15 | | | | | | | | | | | 0 | | | × | × |
| 17 | | | | | | | | | | | | | | | |
| 18 | | | | | | | | | | | | | | | |
| 19 | | | | | | | | | | | | | | 0 | × |
| 20 | | | | | | | | | | | | | | | 0 |

Text-fig 2. Results of crosses involving different CW mutants. ×, Presence of WT recombinant progeny in a given cross; 0, absence of such progeny. CW 6 × CW 6 zygotes did not germinate. Progenies of crosses involving CW 17 and CW 18 have been omitted (see text).

Table 2. *Numbers of recombinant zygotes obtained from crosses of different mutants*

| Cross | Zygote progenies | No. of zygotes containing recombinants |
|---|---|---|
| CW 1 × CW 2 | 2510 | 0 |
| CW 1 × CW 8 | 2209 | 0 |
| CW 1 × CW 19 | 1811 | 2 |
| CW 2 × CW 8 | 1129 | 0 |
| CW 2 × CW 19 | 1089 | 0 |
| CW 8 × CW 19 | 1045 | 0 |
| CW 10 × CW 15 | 799 | 0 |

of diploid cells is produced from many crosses and the absence of WT diploids from any given cross cannot yet be taken as an indication of non-complementation. Of the crosses involving different combinations of CW 1, 2, 8 and 19 and CW 10 and 15, only CW 1 × 8 has given WT diploids. Further tests, involving the use of other markers to test for diploidy, will be undertaken.



A tetrad derived from a CW 2 × WT cross showing 2:2 segregation for the CW and WT phenotypes.



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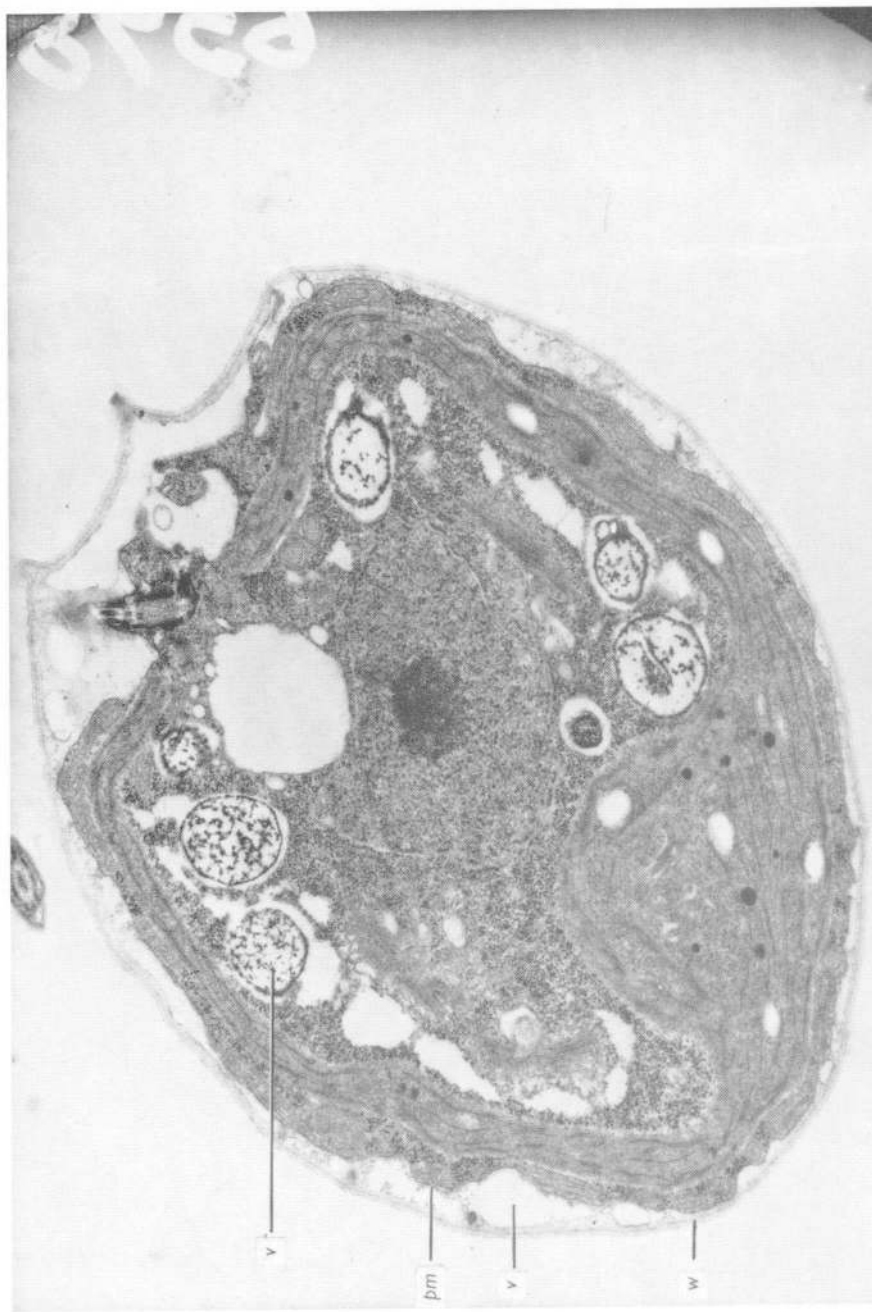


Longitudinal section through a WT cell. Electron micrograph, ×18000. *v*, Vesicle; *pm*, plasma membrane; *w*, wall.



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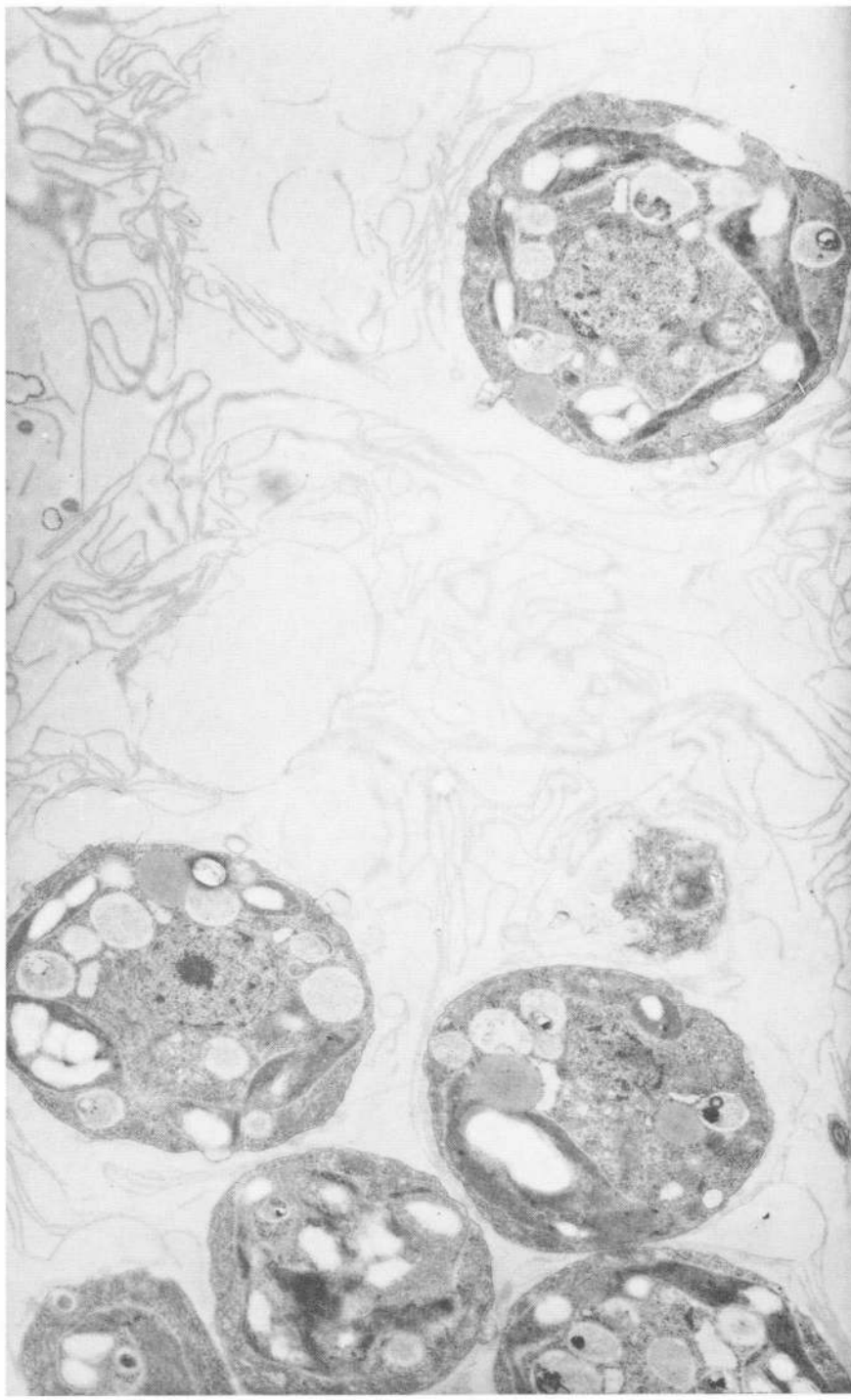

Section through a pellet spun down from a liquid suspension of CW 2 cells. Note the free walls present in the medium and the cells bounded only by a plasma membrane. Electron micrograph, × 9000.



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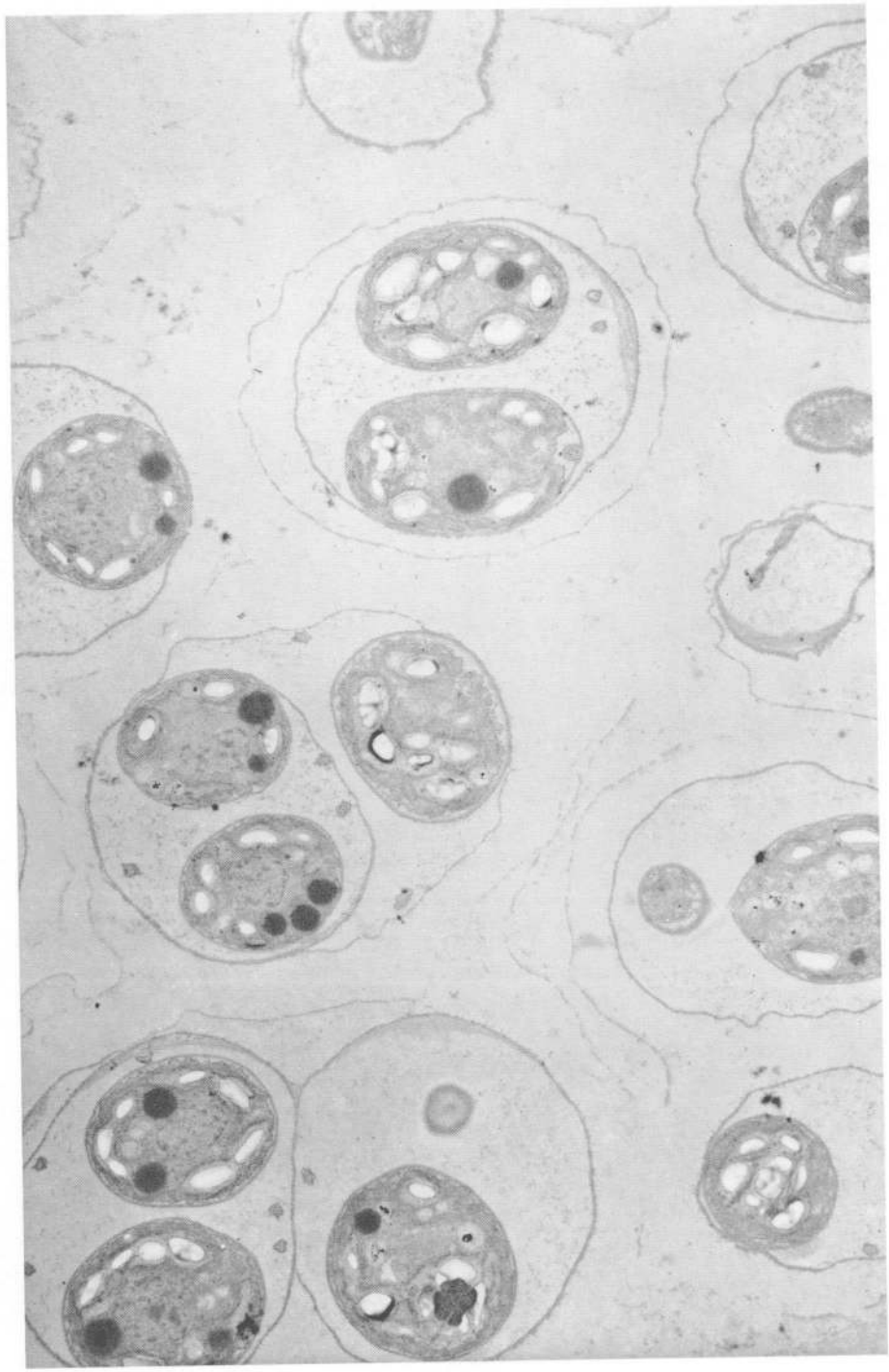

Section through WT cells grown immersed in an agar medium. Note the walls laid down as each cell divides successively within the parent wall. Electron micrograph, × 4500.

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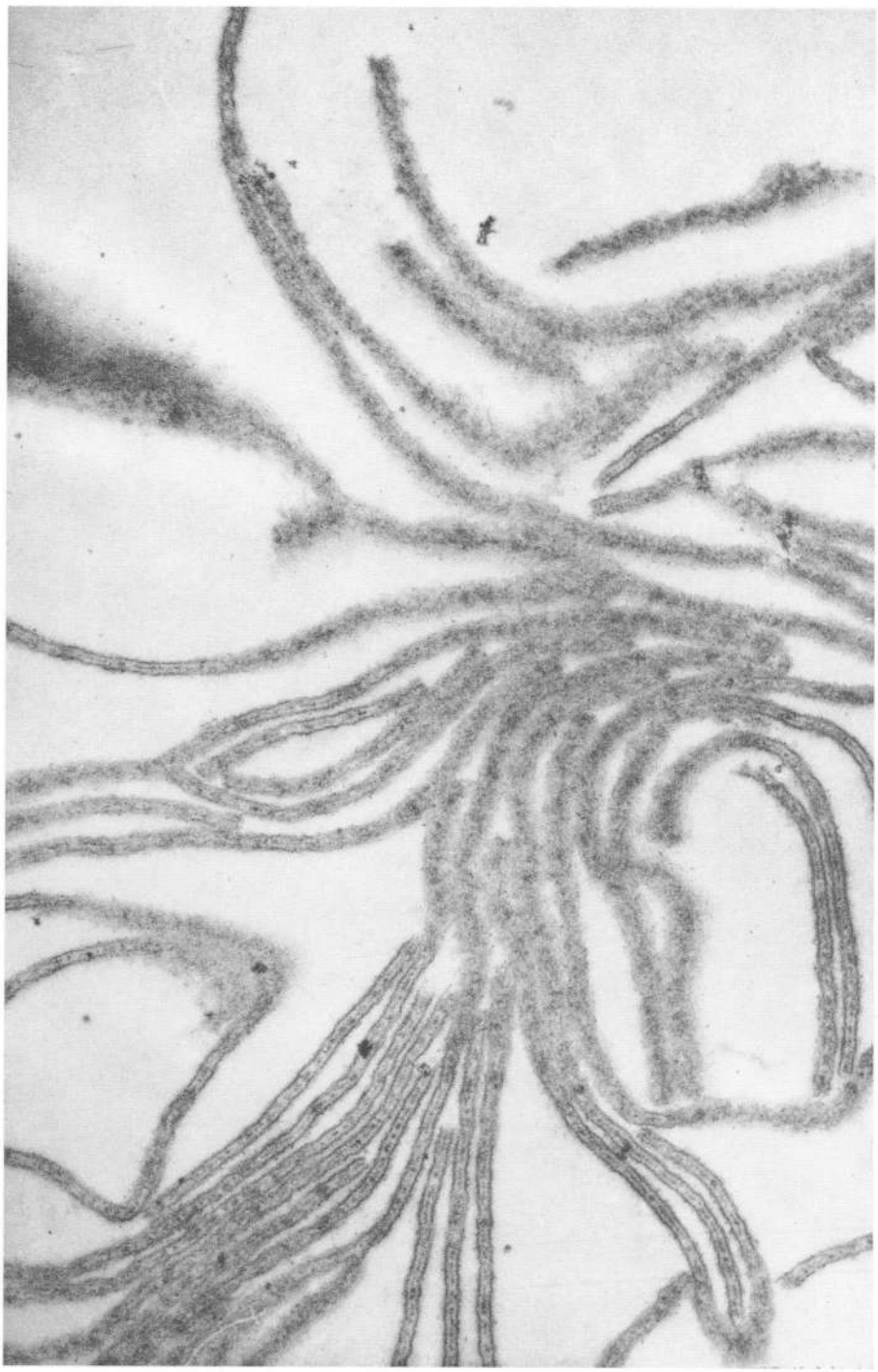

Longitudinal section through the walls of CW2 cells shed into a liquid medium. Note the change, as the plane of cutting changes from a section through the wall into a surface view. Electron micrograph, × 50000.

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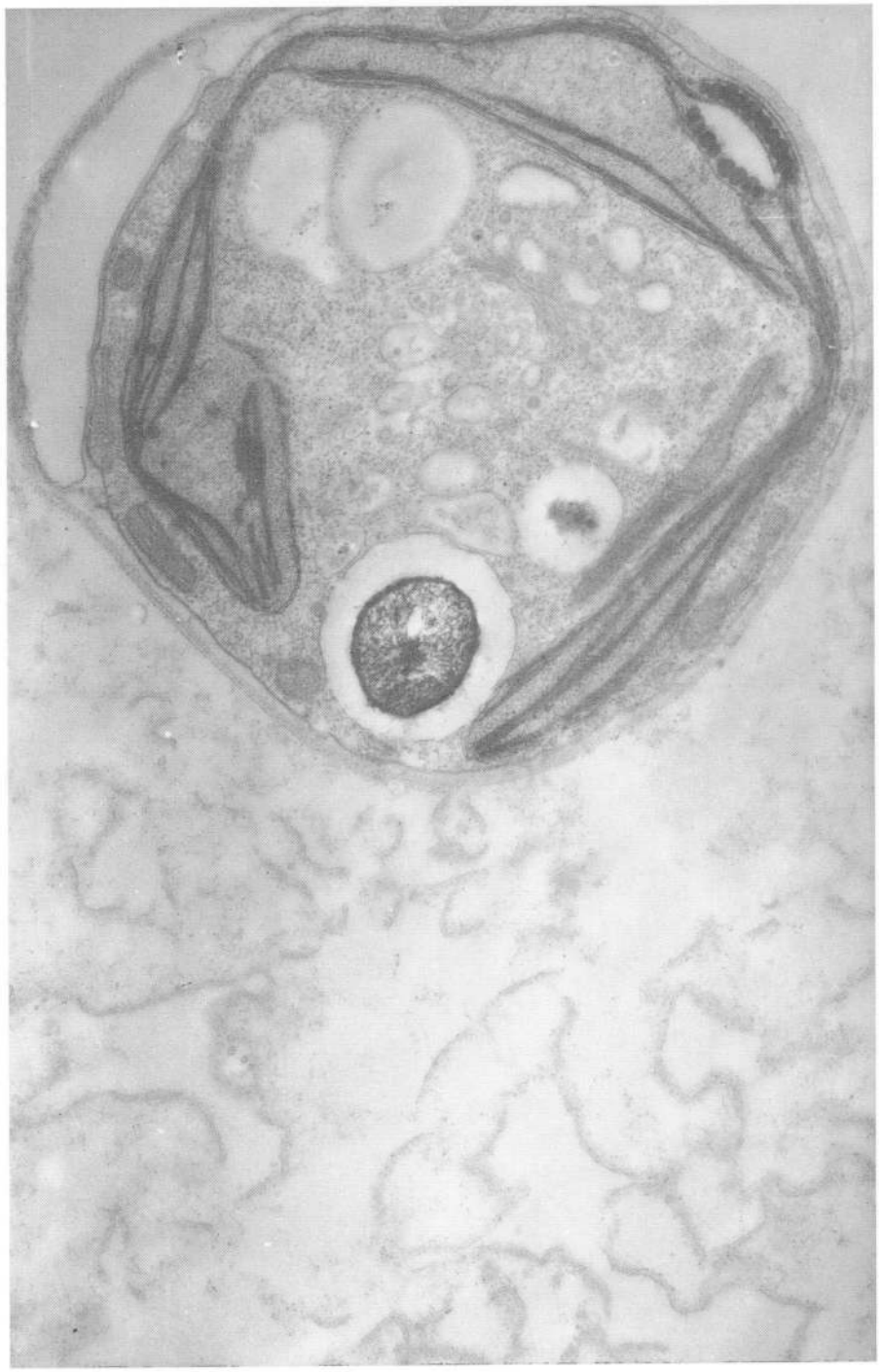

Longitudinal section through a CW 20 cell. Note the free walls and attached wall yet the colony morphology is typical of the CW phenotype. Electron micrograph, × 26 000.

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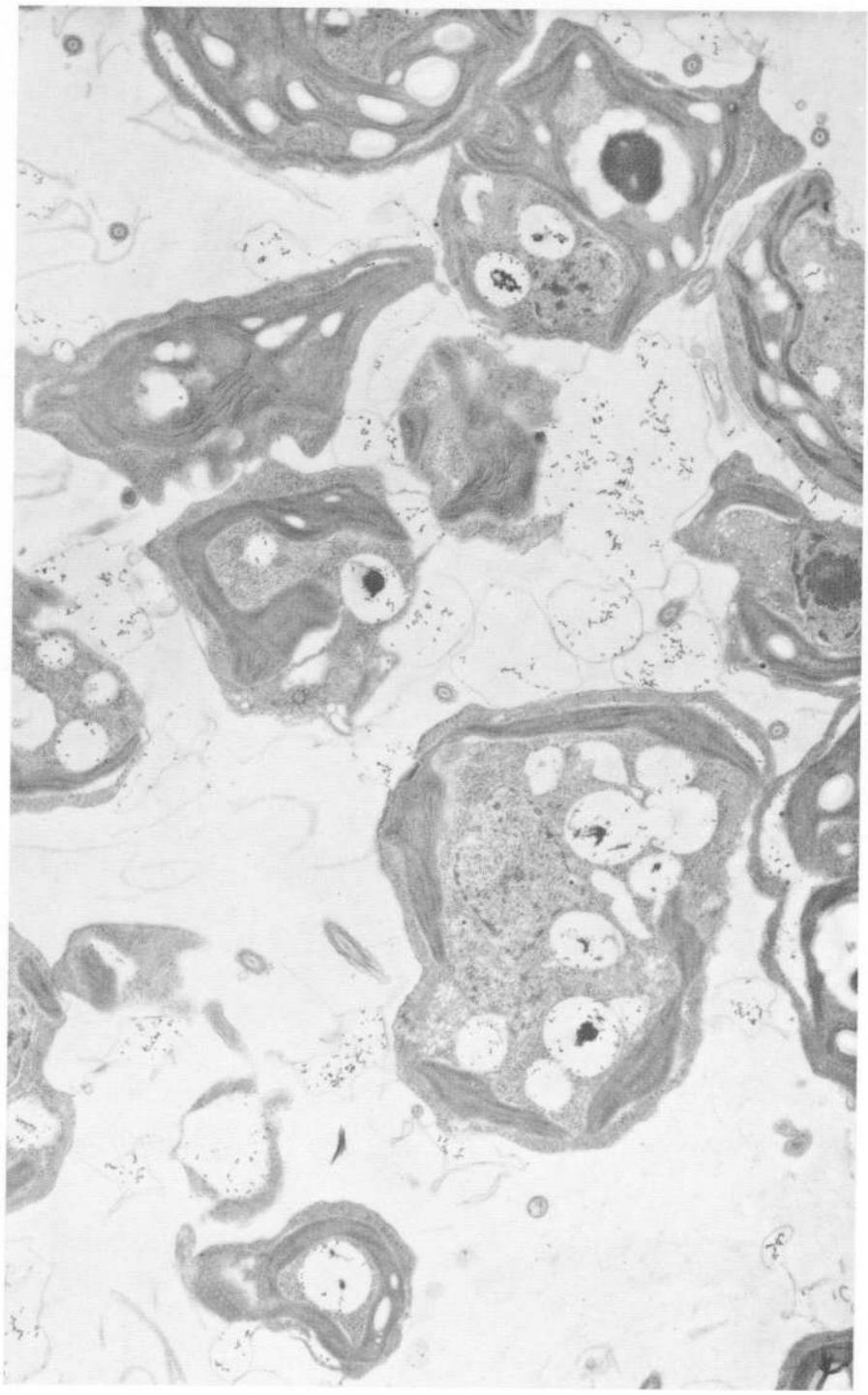

Section through CW 15 cells grown immersed in an agar medium. Note the vesicles containing electron-dense contents being secreted through the plasma membrane. Electron micrograph, × 10000.

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### (v) *CW 17 and CW 18*

Crosses involving these two mutants were eliminated from consideration earlier, because they were exceptional in three respects: (*a*) they frequently segregated wild-types on selfing, (*b*) they gave anomalous ratios in other crosses, and (*c*) on rare occasions somatic reversion to pseudo-wild-type occurred. The genetics of one of these mutants – CW 18 – has been analysed in more detail. The term pseudo-WT has been deliberately used, as they were sometimes, though not always, distinguishable from true WT in colony morphology, when examined under the light microscope. (As seen in Table 1 electron-microscope studies showed that even the original CW 18 had walls attached and was difficult to distinguish from WT.)

Table 3. *Tetrad classes obtained on crossing CW 18 and WT*

| | CW 18 – | | WT – | |
|---|---|---|---|---|
| | Tetrad | No. | Tetrad | No. |
| CW 18 + | 4 CW : 0 WT | 8 | 2 CW : 2 WT | 15 |
| | 3 CW : 1 WT | 23 | . | . |
| WT + | 2 CW : 2 WT | 27 | . | . |
| | 3 CW : 1 WT | 12 | . | . |
| | 4 CW : 0 WT | 2 | . | . |

(*a*) On crossing CW 18 × CW 18, a large number of tetrads gave 3 CW : 1 WT (Table 3) as opposed to the expected 4 CW : 0 WT. Two of these anomalous 3 CW : 1 WT tetrads were analysed further (Table 4). The WT spore (A 3) from tetrad A was lost due to contamination. Again, exceptions were observed to predicted genetic ratios, assuming normal nuclear gene segregations, as seen in the data for A 2 – × WT, A 4 + × WT, B 1 + × CW 18 in Table 4. The anomalous segregation patterns were not correlated with the CW parent being of any particular mating type. The case of the B 3 WT is different in that it behaved as a normal WT in crosses with CW and with WT (Table 4). However, it is difficult to visualize the B 3 as a true back-mutation from CW 18 to WT in view of the high rate of production of such WT forms from CW 18 × CW 18 crosses (Table 3). Equally if we assume that this change is due to a high rate of back mutation, then there is an equally high forward mutation rate observed in other crosses; for example, B 4 – × WT (Table 4) gives an excess of CW forms.

(*b*) On crossing CW 18 + with WT – 2 : 2 ratios were obtained, but CW 18 – × WT gave 3 : 1 and 4 : 0 as well as the expected 2 : 2 ratios. Segregation of mating type was normal in the aberrant tetrads. Occasionally, and sometimes at a late stage – even when a colony had several thousand cells – a 3 CW : 1 WT tetrad would change to a 2 : 2 tetrad, i.e. a mass and uniform change of a CW colony to WT would occur.

There is no evidence to suggest that anomalous chromosome behaviour could explain these switches from expected tetrad ratios in CW 18 × WT crosses – viability and mating-type segregation are both normal, and certainly the production

of WT from the cross CW 18 × CW 18 is not explicable in these terms. It is equally difficult to interpret them in terms of aneuploidy, and conversion-type phenomena cannot be invoked to account for the production of WT forms from the CW 18 × CW 18 crosses (Table 3). An alternative interpretation in terms of an extra-nuclear

Table 4. *Tetrad classes obtained on crossing the progeny of tetrads A and B with CW 18 or WT*

(Tetrads A and B were obtained from a CW 18 × CW 18 cross.)

| | CW 18 (+ or −) | | WT (+ or −) | |
|---|---|---|---|---|
| | Tetrad | No. | Tetrad | No. |
| A 1+ (CW) | 4CW:0WT | 19 | 2CW:2WT | 16 |
| A 2− (CW) | 4CW:0WT | 21 | 2CW:2WT | 17 |
| | | | 3CW:1WT | 9 |
| | | | 4CW:0WT | 5 |
| A 4+ (CW) | 4CW:0WT | 13 | 2CW:2WT | 5 |
| | | | 3CW:1WT | 8 |
| | | | 4CW:0WT | 1 |
| B 1+ (CW) | 4CW:0WT | 14 | 2CW:2WT | 26 |
| | 3CW:1WT | 19 | 4CW:0WT | 2 |
| | 0CW:4WT | 1 | 0CW:4WT | 1 |
| | | | 3CW:1WT | 3 |
| B 2− (CW) | 3CW:1WT | 11 | 2CW:2WT | 31 |
| | | | 3CW:1WT | 2 |
| B 3+ (WT) | 2CW:2WT | 22 | 0CW:4WT | 14 |
| B 4− (CW) | 4CW:0WT | 11 | 2CW:2WT | 14 |
| | | | 3CW:1WT | 28 |

system may be feasible. Jinks (1963) has listed several criteria which can be applied to test for such a system, and many fit the present data. Vegetative persistence, sudden changes in the character, absence of, or non-Mendelian segregation are those which apply in this instance, but at present they do nothing more than indicate that this interpretation is at least possible.

(*c*) Somatic reversion of CW 18 to WT has been observed in two circumstances. As described earlier, a member of a tetrad could show a sudden and uniform change in all the members of a colony from CW to WT. The other instance was among vegetatively propagated cells, when routine cloning of CW 18− showed that a large number of pseudo-WT were present. The latter were isolated and have been extremely stable subsequently, only two spontaneous revertants to CW being found out of several thousand colonies examined. Subsequent attempts to isolate more pseudo-WT from vegetative cultures of CW 18 have proved unsuccessful. Ten subcultures of CW 18− were set up, five have been sampled, and subcultured sequentially for several months, and five allowed to age before being sampled sequentially; no further changes to pseudo-WT were observed. (The somatic stability of the pseudo-WT line was also shown on exposure to two mutagens. Treatment with N.N.G. or u.v. (2537 Å) gave no more CW forms than are normally produced by treatment of WT cells with similar doses.)

On crossing the pseudo-WT somatic revertant of CW 18 –, referred to subsequently for convenience as CW 18 – ($\phi$), to both CW 18 and WT forms, abnormal ratios were once again obtained (Table 5). Another anomalous feature of this series of crosses was that in all those involving CW 18 – ($\phi$) a large proportion of the tetrads were comprised of uniformly small, slow-growing aberrant colonies. Characterization of the phenotype was difficult, and the whole colony frequently died. True CW phenotypes were nevertheless frequently recovered from crosses of WT + × CW 18 – ($\phi$), demonstrating that the CW 18 – ($\phi$) phenotype did not involve a back mutation.

Table 5. *Examples of tetrads obtained from crosses involving CW 18 – ($\phi$)*

| CW 18 – ($\phi$) × WT + | | CW 18 – ($\phi$) × CW 18 + | |
|---|---|---|---|
| Tetrad | No. | Tetrad | No. |
| 0 CW : 4 WT | 6 | 2 WT : 2ab | 4 |
| 3 WT : 1ab | 3 | 3 CW : 1 WT | 8 |
| 2 CW : 2ab | 2 | 2 CW : 2 WT | 5 |
| 2 WT : 2ab | 6 | 4 CW : 0 WT | 6 |
| 1 CW : 3 WT | 1 | 4ab | 15 |
| 3 CW : 1 WT | 3 | | |
| 1 CW : 1 WT : 2ab | 4 | | |
| 1 WT : 3ab | 1 | | |
| 1 CW : 3ab | 1 | | |
| 1 CW : 2 WT : 1ab | 1 | | |
| 2 CW : 2 WT | 6 | | |
| 4 CW : 0 WT | 1 | | |
| 4ab | 5 | | |

The origin of CW 18 – ($\phi$), and its genetic behaviour, are again difficult to explain. An origin by back-mutation does not agree with the presence of a high frequency of CW 18 in the progeny of crosses with WT. Neither does a nuclear gene suppressor of CW 18 account for the behaviour of CW 18 – ($\phi$). If the latter was present then in a cross of CW 18 – ($\phi$) × WT it would be impossible to obtain, for example, tetrads of 3 CW : 1 WT (Table 5). The aberrant, non-Mendelian segregation could, however, be understood if an extra-nuclear system was involved in some aspect of cell-wall biogenesis, or if an extra-nuclear suppressor system was involved (Cox, 1965). The basis of the slow growth and abortion of many tetrad products is not so readily reconcilable with this interpretation, if indeed the two phenomena are in some way linked. If an extra-nuclear system is involved in the change from CW 18 to CW 18 – ($\phi$) then on the basis of the mutation experiments described earlier it must be highly stable, at least in vegetative culture, or possibly does not even involve nucleic acid.

We have to reconcile the following observations with any proposed interpretation of the data. Most of the CW mutants are due to nuclear gene changes. Others, since they give rise to non-Mendelian segregations on crossing, appear to involve changes in another system. A further feature is that wild-type walls can be produced in the first products of a tetrad from a homozygous CW zygote. Such a wild-

type phenotype can be perpetuated for a limited number of generations in some, a considerable number in others, and permanently in yet others. In other tetrad products a switch of colony morphology from CW to WT can occur. The final observation is the rare somatic production of pseudo-wild-type cells; the pattern of inheritance of this latter phenotype is also non-Mendelian.

The mechanism of the origin and perpetuation of complex three-dimensional units within cells is not understood. It is true that work on viruses has demonstrated that three-dimensional structures can be represented by one-dimensional information, but in the case of more complex cellular structure there could be two levels of control involved. If this is so, then in the case of wall biogenesis the first of these would be at the nuclear gene level and would be concerned with specifying basic subunit structure. At another level there would be a second system of control specifying three-dimensional organization. Most of the mutants available from the present experiment would be examples of change at the first level – that is, they involve nuclear gene changes; but CW 18 with its non-Mendelian segregation may be an example of a change at the second level.

If there is a second level of control involved, it could operate in one of two ways; it could involve merely pre-existing structure on which the subunits controlled at the nuclear gene level are assembled, or alternatively it could involve extra-nuclear informational macro-molecules. If the latter is the correct alternative then the origin of CW 18 by mutation in the first place is readily understood, but the basic problem of specifying three-dimensional structures from one-dimensional information then remains.

If the first alternative is correct, then the origin of the CW 18 mutant is more difficult to understand and must have been due to the loss of, or change in, a pre-existing or 'template' structure. Such 'template' structures have been invoked to account for the inheritance and persistence of cortical patterns in *Paramecium* (Beisson & Sonneborn, 1965). It is difficult at present to formulate a quantitative interpretation involving such template structures which will account for both the origin by mutation and the switches between CW 18 and wild type. Template structures could occasionally be renewed from a master and this would account for the sudden switches in both meiotic and mitotic cells. Alternatively there may be a number of templates in a cell and a change in the population structure would account for the switches, but the origin by mutation is more difficult to understand on this interpretation.

Thus while control at a second level can be invoked to account for the delayed expression of the CW phenotype in cells emerging from zygotes and the persistence of the WT phenotype in many tetrad products, and also to account for the production of pseudo-wild-type somatic segregants, any attempt at a more detailed analysis of the mechanism of this control is unjustified at present.

It is hoped that further analyses of the structure and genetics of these and other mutants now available will not only allow an elucidation of the basic steps involved in cell-wall biogenesis, but also confirm our assumptions and extend our understanding of a second level of control. Until this is achieved the concept that three-

dimensional structures arise simply by a process of a self-assembly of subunits must remain a valid alternative.

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