The Formation of Protoplasts and Quasi-spheroplasts in Normal and Chloramphenicol-pretreated *Bacillus subtilis*

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(Accepted for publication 1 July 1967)

SUMMARY

The lytic action of lysozyme upon *Bacillus subtilis* walls was studied by following the disappearance of bacillary-colony-forming units and the appearance of L-colony-forming-units. The rapidity of cell wall removal by lysozyme fluctuated markedly during growth in a chemically defined medium, presumably because subtle changes in the cell wall were constantly occurring. When lysozyme-sensitive bacilli were grown with chloramphenicol 10 µg./ml. for 3 hr they showed a notable increase in lysozyme resistance; at the same time, their walls almost doubled in thickness. As lysozyme attack proceeded in a given culture, the bacilli passed first through a rod-shaped osmotically sensitive stage, and then a spherical stage characterized by incomplete removal of cell wall before finally reaching the naked protoplast stage. The spherical forms with adherent wall residues formed L colonies on a medium containing the reversion inhibitor D-methionine and bacillary colonies on the same medium without D-methionine. Under the latter conditions, the cell wall residue served as a starting point for rebuilding of complete wall, much as residual wall permits reversion of Gram-negative spheroplasts to the bacillary state. In the presence of D-methionine, the feedback sequence required for wall formation was severed, resulting in heritable propagation of the protoplast state.

INTRODUCTION

The enzyme lysozyme hydrolyses the β -1,4 linkages between the muramic acid residues and the *N*-acetylglucosamine residues of bacterial cell-wall peptido-glycan. Peptido-glycan, a rigid material which holds bacilli to their characteristic shape and helps to protect them from osmotic shock, is found in almost all bacteria and in the blue-green algae. In the Gram-positive bacteria, peptido-glycan is generally a principal cell-wall component, while in Gram-negative bacteria the peptido-glycan or rigid layer is only one of several components which make up the complex wall (Salton, 1964). When Gram-positive bacteria such as *Micrococcus lysodeikticus* or *Bacillus subtilis* are subjected to lysozyme action, the wall is extensively depolymerized so that teichoic acid, the other major wall constituent, is freed and released into the supernatant fluid along with fragments of the peptido-glycan (Miller & Landman, unpublished; Young, 1967). A spherical protoplast results which is bounded only by membrane and is apparently devoid of cell wall (Ryter & Landman, 1964). When Gram-negative *Escherichia coli* organisms are treated with lysozyme, presumably only the rigid (innermost?) layer is attacked. The lipoprotein-containing outermost layer and the lipopolysaccharide-containing middle layer remain (Salton, 1964; Weidel, Frank &

Martin, 1960). Nevertheless, having lost much of their rigid layer, these forms, called spheroplasts, are spherical in shape and sensitive to osmotic shock. Superimposed on this relatively simple and perhaps overgeneralized picture, there are numerous complexities due to differences among bacterial species. Further, as we shall show in this paper, the response to lysozyme of a single strain is clearly different at different times during growth.

Even greater changes in lysozyme sensitivity are produced by incubation of bacteria with antibiotics. It is well known that an important group of antibiotics attacks bacteria principally through damage of the cell envelope. This group includes penicillin, bacitracin, novobiocin, ristocetin, vancomycin, cycloserine, tyrocidin, gramicidin-S, and polymixin (Newton, 1965). Incubation of bacteria in the presence of any one of these substances could plausibly be expected to produce damage to the cell wall. In the present paper, we are concerned with changes in the cell wall produced by incubation with chloramphenicol (CAP), an antibiotic which is primarily an inhibitor of protein synthesis (Newton, 1965). In agreement with previous reports (Hash & Davies, 1962; Shockman, 1965), we have observed that prolonged inhibition of protein synthesis leads to a marked increase in the thickness of the cell wall. A sharp increase in lysozyme resistance was also noted in conjunction with CAP incubation or tryptophan starvation.

In studying the lysozyme-catalysed removal of cell wall from bacteria incubated with CAP it was found that a notable proportion of the bacteria from incompletely treated populations exhibited a plating behaviour intermediate between naked protoplasts and intact bacilli. Like protoplasts, these forms gave rise to heritably stable L colonies on one type of plating medium (Landman & Halle, 1963) and, like bacilli or spheroplasts of Gram-negative bacteria (Landman, Altenbern & Ginoza, 1958), they produced bacillary colonies on a more favourable medium. The intermediate forms are thus on the borderline to commitment to the stable L state. A similar form has been detected in streptococcal populations incompletely treated with a wall-removing enzyme (King & Gooder, 1965). A preliminary report of the present findings has already been published (Miller & Landman, 1965).

METHODS

Organism. *Bacillus subtilis* strain 168 (indole⁻) was used in all experiments.

Media. L forms and bacillary forms were grown on two plating media, DP and SDM. DP medium had the following composition: 0.021 M-K₂HPO₄; 0.011 M-KH₂PO₄; 0.5% acid-hydrolysed casein (Nutritional Biochemicals); 0.2% glucose; 0.002% L-tryptophan; 0.5 M-sodium succinate, pH 7.3; 0.005 M-MgCl₂; 0.5% horse serum or human plasma; 0.9% agar. SDM medium was as DP medium except that 0.1% NH₄NO₃ replaced the casein hydrolysate as nitrogen source and 0.06% D-methionine was added to retard reversion of L forms. For dilutions of bacilli and protoplasts a hypertonic dilution fluid (DFR) was used; it had the following composition: 0.25 M-sucrose; 0.25 M-sodium succinate, pH 7.0; 0.021 M-K₂HPO₄; 0.011 M-KH₂PO₄; 0.01 M-MgCl₂; 0.0005 M-EDTA. The bacilli were grown successively on media SL1 and SL2 (Landman & Halle, 1963) before treatment to form protoplasts. These media were the same as the competence media of Anagnostopoulos & Spizizen (1961) except that medium SL2 contained 0.5 M-sucrose to stabilize protoplasts.

Growth of cultures. The procedure followed to prepare bacilli for treatment to give protoplasts paralleled the procedure used by Anagnostopoulos & Spizizen (1961) to render bacteria competent for transformation: spores of *Bacillus subtilis* 168 were inoculated onto plates of blood agar base medium (DIFCO), germinated, and incubated overnight at 37°. The confluent growth was harvested into a small volume of medium SL1 and adjusted with fresh medium to 45–50 % transmission (at 670 m μ) in the Bausch & Lomb Spectronic 20 Spectrophotometer; this turbidity corresponded to a colony count of about 1.5×10^8 bacilli/ml. This culture was incubated with shaking at 37° for 4 hr, then diluted 1/10 in medium SL2 and incubated with shaking at 37° for a further 90 min. These incubations were usually followed by further incubation in SL2 medium without or with chloramphenicol 10–15 μ g./ml. Samples of these bacilli were treated to give protoplasts at various times during these incubations, by adding lysozyme as indicated to 5 ml. volumes of bacillary suspension in SL2 medium in 125 ml. Erlenmeyer flasks which were kept stationary in a water bath at 32–34°.

Colony counts quoted are averages based on 3–4 plates. Plates inoculated with protoplast-containing suspensions were incubated at 30°; plates from bacillary suspensions were incubated at 30° or 37°.

Arrest of lysozyme action for differential phase microscope counts of spheres and rods. To assess the progress of sphere formation by phase microscopy at intervals during lysozyme treatment, it was important to arrest enzyme action abruptly. Formation of protoplasts in SL2 medium was stopped instantly upon adding an equal volume of 1 % (w/v) OsO₄ in 0.5 M-sucrose. Differential counts in the Petroff-Hausser chamber had to be performed promptly since the spheres and osmotically sensitive bacilli began to lyse about 5 min. after the OsO₄ addition.

Procedure for electron microscopy. Five ml. of suspension of bacilli, protoplasts or 'protoplasting' bacilli were placed into a plastic centrifuge tube at 4° and centrifuged for 20 min. at 8000 g. The pellet was rinsed 3 times with fresh SL2 medium and suspended in 0.1 ml. SL2 medium. Then 0.05 ml. of 4 % (w/v) glutaraldehyde (redistilled) in 0.1 M-cacodylate buffer (pH 6.0) was added and the tube warmed to 45°. Molten 3 % agar (0.2 ml.) containing a 0.5 $\times$ concentration of SL1 salts and 0.5 M-sucrose was then mixed gently into the slurry. After the agar had cooled to room temperature, the plastic tube was cut open and the agar with the suspended organisms cut into 2–3 mm.³ blocks which were fixed overnight in cacodylate-buffered 4 % glutaraldehyde. The following day the agar blocks were washed in 3 changes of veronal buffer (pH 6) for a total of 3 hr, according to the procedure of Kellenberger, Ryter & Séchaud (1958). Overnight fixation with OsO₄, washing, treatment with uranyl acetate and further washing all were now followed according to the prescription of these authors. Finally, the blocks were dehydrated in graded concentrations of ethanol, washed twice for 15 min. in propylene oxide and embedded in Epon (Luft, 1961). Sections, approximately 500 Å in thickness, were cut with the MT 1 Porter-Blum microtome and stained for 1 min. in 0.2 % lead citrate in 0.1 N-NaOH. The sections were examined in the RCA EMU 2D electron microscope.

RESULTS

Changes in lysozyme sensitivity during growth

When lysozyme is added to a suspension of *Bacillus subtilis* in hypertonic medium, the bacilli are gradually converted to protoplasts. These protoplasts give rise quantita-

tively to L colonies when plated on certain soft agar media containing 0.5M-Na succinate (Landman & Halle, 1963). The rate of cell wall removal—and hence the rate of disappearance of bacillary-colony-forming elements and the rate of appearance of L-colony-forming units, ‘commitment’, depends on several factors. (‘Commitment’ was earlier defined as ‘The point in time at which a cell loses its ability to give rise to a bacillary colony and gains its capacity to give rise to an L colony’ (Landman & Halle, 1963). With the demonstration in the present paper that a given cell may persist for some time in a borderline state from which it may give rise to either a bacillary colony or an L colony, this definition of commitment requires further refinement.) For example, lysozyme acts much more rapidly in sucrose-stabilized media than in succinate-containing media. Further, both lysozyme concentration and temperature markedly affect the rate of protoplast formation. Table 1 exhibits the effect of a more subtle variable on lysozyme sensitivity, namely, the previous growth history of the

Table 1. *Bacillus subtilis* 168. Changes in sensitivity to lysozyme during competence incubation

Organisms were grown for 4 hr in medium SL1, diluted 1/10 into medium SL2 and incubated further. At the times shown, samples were taken, subjected to 25 min. treatments with lysozyme 100 µg./ml. and plated on DP medium. The % survival was obtained by comparing the total count of colonies (bacillary+L colonies) appearing on these plates with counts of untreated culture samples. A high % of L colonies on the experimental plates is a reflexion of a high degree of sensitivity to lysozyme.

Time of growth in medium SL 1 (hr)	Experiment 1		Experiment 2	
	Survival (%)	L colonies (%)	Survival (%)	L colonies (%)
0	120	21	93	13
1	68	65	75	98
2	117	58	92	93
3	106	18	84	54
4	99	6	105	43
In medium SL 2				
0.5	.	.	102	16
1	.	.	91	47
1.5	.	.	106	99.8
3	81	96	.	.

bacteria. In the experiments shown, samples of culture were protoplasted at various times during the usual ‘competence incubations’ in SL 1 and SL 2 media (see Methods). Table 1 indicates that in SL 1 medium the susceptibility to lysozyme increased to a maximum after 1–2 hr of growth and then decreased again after 1–2 hr of further incubation. During growth in SL 2 medium, lysozyme sensitivity increased again. After 90 min. of incubation (when the bacilli are highly competent for transformation) lysozyme sensitivity also reached a very high value. The two experiments presented in Table 1 show the same pattern of fluctuation of lysozyme sensitivity, but markedly different absolute values for the % of L colonies formed at different times. Further instances of consistent reproducibility of sensitivity patterns in the face of poor reproducibility of absolute sensitivity values are shown in subsequent Tables. We can offer no certain explanation for this phenomenon.

Effect of incubation in chloramphenicol media upon lysozyme sensitivity

Changes in the lysozyme susceptibility of *Bacillus subtilis* 168 were particularly marked after prolonged incubation in media containing chloramphenicol (CAP). For example, in the experiment shown in Table 2, CAP 10 $\mu\text{g./ml.}$ was added to highly sensitive competent bacilli after the 90 min. incubation in SL2 medium. At the time of CAP addition (zero time) both experimental and control suspensions were attacked equally by lysozyme since, as expected, CAP did not affect the activity of this enzyme. However, as incubation in the presence of CAP proceeded, the bacilli became increasingly resistant to lysozyme. For example, after 3 hr in SL2 medium + CAP, 40 % of the organisms still gave bacillary colonies on DP plates after a 25 min. lysozyme

Table 2. *Bacillus subtilis* 168: development of lysozyme resistance during incubation in chloramphenicol

Organisms were grown for 4 hr in medium SL1 and then for 90 min. in medium SL2. At this time the colony count was $1.1 \times 10^8/\text{ml.}$ The culture was divided and chloramphenicol (10 $\mu\text{g./ml.}$ final concentration) was added to one of the subcultures. Samples from both cultures were taken immediately and at hourly intervals and treated with lysozyme 100 $\mu\text{g./ml.}$ for 25 min. The treated samples and lysozyme-free controls were diluted in DFR and plated on DP and SDM plates. The development of lysozyme resistance during incubation in chloramphenicol medium is reflected in the increasing % bacillary colonies remaining after lysozyme treatment.

Time of incubation (hr)	With chloramphenicol				Without chloramphenicol			
	DP medium		SDM medium		DP medium		SDM medium	
	Survival (%) [*]	B colonies (%) [†]	Survival (%)	B colonies (%)	Survival (%)	B colonies (%)	Survival (%)	B colonies (%)
0	80	2	81	0.7	87	1.1	71	0.8
1	100	7	83	1.3	110	1.7	82	0.8
2	99	26	119	3	108	0.8	96	0.2
3	99	40	104	7	92	0.7	76	< 0.1

* % = Ratio of total counts of bacillary + L colonies after lysozyme action to the counts before lysozyme treatment $\times 100$.

† % B = Ratio of number of bacillary colonies total number of colonies $\times 100$.

Table 3. *Bacillus subtilis* 168: percentage of bacillary-colony formers among survivors of lysozyme treatment

Organisms were pretreated and incubated in the presence or absence of chloramphenicol as shown for the 3 hr samples in Table 2. A chloramphenicol-treated and a control culture were then incubated with lysozyme 200 $\mu\text{g./ml.}$ and samples taken at intervals and plated on DP and SDM media. Survival averaged 93 %. Note the striking difference in lysozyme sensitivity of the two cultures. However, after 1 hr of lysozyme treatment the vast majority of the organisms in the resistant cultures had also become committed.

Plating medium	No treatment with chloramphenicol				Treated with chloramphenicol for 3 hr.			
	Time of lysozyme treatment (min.)				Time of lysozyme treatment (min.)			
	25	30	35	60	25	30	35	60
	bacillary-colony formers (%)							
DP	< 0.1	< 0.1	< 0.3	< 0.1	80	63	52	4
SDM	< 0.2	< 0.2	< 0.1	< 0.2	44	34	19	2

treatment. By contrast, sensitivity to lysozyme continued to increase in the control populations incubated in parallel in SL2 medium without CAP. It should be stressed that the lysozyme resistance which was manifest in the 3 hr CAP-incubated culture was not absolute: when the lysozyme treatment was continued long enough, virtually all of the bacilli even in this population become committed to the L-state. This is shown in Table 3.

The marked increase in lysozyme resistance due to incubation with CAP suggested that it might be possible to observe by electron microscopy correlated changes in the thickness of the cell walls. Accordingly, thin sections of CAP-incubated and of control bacilli were examined in the electron microscope. The results (Pl. 1, fig. 1, 2, Table 4) clearly indicate that a thickening in the cell wall occurred during the 3 hr incubation in SL2 medium+CAP. On the average, the wall of the CAP-incubated bacilli was 1.9 times as thick as that of the controls (Table 4). The electron micrographs of the CAP-incubated organisms show that the thickness of the cell wall had increased fairly uniformly all around the bacilli.

Table 4. *Bacillus subtilis* 168: average wall thickness of chloramphenicol-treated and untreated bacilli

Electronmicrographs $\times 103,000$ of 30 randomly chosen bacilli were used to compile each of the 2 sets of figures. Each number is an average of ten measurements. The deviations shown are standard deviations. The difference between the two sets of measurements is significant ($P < 0.001$).

	Chloramphenicol treated	Untreated
	Wall thickness (Å)	
	268	150
	295	160
	275	142
	305	143
	284	145
	295	140
	292	160
	268	155
	289	150
	289	155
Average thickness	268 ± 47	150 ± 29

Effect of tryptophan deprivation on lysozyme resistance

Blockage of protein synthesis by CAP presumably causes accumulation of unused amino acids (Hancock & Park, 1958) and, possibly, diversion of some of these amino acids into cell-wall synthesis. If this view be correct, starvation for tryptophan, which is not a constituent of *Bacillus subtilis* cell walls (Young, Spizizen & Crawford, 1963) should mimic the effect of incubation with CAP. As shown in Table 5, omission of tryptophan from SL2 medium did cause the development of increased resistance to lysozyme. However, tryptophan starvation was notably less effective than incubation with CAP and, in a combination of the two treatments, the CAP effect predominated. Examination of thin sections of tryptophan-starved and control bacilli in the electron microscope has shown that thickening of the cell wall also occurs as a result of tryptophan starvation (L. J. Archer & G. B. Chapman, unpublished).

Table 5. *Bacillus subtilis* 168: effect of tryptophan deprivation and chloramphenicol on lysozyme sensitivity

Organisms were grown in SL 1 medium for 4 hr and then in SL 2 medium for 90 min. After removal of the 0 time sample, the culture was divided into 4 portions ($\pm$ tryptophan, $\pm$ chloramphenicol) as shown and incubated for another 4 hr. All cultures were treated with lysozyme 200 μ g./ml. for 25 min. immediately after sampling and then plated on DP and SDM media.

Time of sampling (hr)	Treatment		Plating medium	Experiment 1		Experiment 2	
	Trypto-phan	Chloram-phenicol		Survival (%) [*]	B colonies (%) [†]	Survival (%)	B colonies (%)
0	—	—	DP	125	1.60	50	0.30
			SDM	73	0.57	33	0.22
4	+	—	DP	23	< 0.56	30	0.44
			SDM	29	< 0.45	21	0.11
4	—	—	DP	92	14	77	5.2
			SDM	86	12	60	< 0.2
4	+	+	DP	91	80	71	32
			SDM	94	43	62	7.1
4	—	+	DP	95	77	74	19
			SDM	96	43	60	5.8

* % = ratio of counts of bacillary + L colonies after lysozyme action to counts before lysozyme treatment $\times 100$.

† % B = ratio of number of bacillary colonies to total number of colonies $\times 100$.

Transient appearance of a spheroplast-like form during protoplasting of Bacillus subtilis 168

A conspicuous feature of the data in Tables 2, 3 and 5 is the discrepancy between the percentage of bacillary colonies observed on DP medium and those counted on SDM medium. For example, Table 2 shows that after 3 hr incubation in SL 2 medium + CAP, a 25 min. lysozyme treatment produced a suspension which gave 40 % of bacillary colonies on DP medium and 7 % of bacillary colonies on SDM medium. Since survival was complete, it must be concluded that 33 % of the total population scored as 'committed to the L state' on SDM medium and as 'bacillary' on DP medium. Control experiments with media lacking either casein hydrolysate or D-methionine have shown that D-methionine is responsible for the difference in the plating behaviour of the intermediate form on the two media. The D-amino acid, a reversion inhibitor (Landman & Halle, 1963), apparently prevents repairs in the lysozyme-damaged walls which are required for the continued maintenance of the bacillary state (Landman, 1967). The intermediate form is characteristic of suspensions incompletely changed to protoplasts and is not restricted to populations which have been pretreated by incubation with CAP. In more lysozyme-sensitive cultures, a briefer lysozyme treatment can be used to show the phenomenon (Table 6).

Since the intermediate form is obtained when the removal of cell wall is incomplete, it became of interest to know whether such forms appeared as spheres or as rods in the phase microscope. The experiments recorded in Table 7 were made to answer this question. In the first of the experiments, microscopic counts of spherical, spheroidal and rod forms were made for a population which, according to its subsequent plating

behaviour, contained about 16 % intermediate forms and 3 % completely committed forms. The microscopic counts gave 18 % 'true' spheres and 82 % rods + spheroids; clearly, therefore, the intermediate forms appeared to be completely spherical in the phase microscope. In the second experiment the microscopic observations and platings were made after lysozyme action had proceeded much further. However, the results

Table 6. *Bacillus subtilis* 168: appearance of intermediate form during brief exposure of sensitive organisms to lysozyme

Organisms were grown in SL1 medium for 4 hr and then in SL2 medium for 90 min. Lysozyme was then added to 250 $\mu\text{g./ml.}$, samples were taken at times shown, then immediately diluted, and plated on DP and SDM media. Intermediate forms are those organisms which form L colonies on SDM medium and bacillary colonies on DP medium (e.g. 29 % of the organisms were in the intermediate form after 10 min. of incubation.)

Plating medium	Duration of lysozyme treatment (min.)				
	10	15	20	25	30
	bacillary colony formers (%)				
DP	63	38	24	9	4
SDM	34	17	7	4	2

Table 7. *Bacillus subtilis* 168: relationship between plating behaviour and morphology in populations forming protoplasts

The bacilli were subjected to the usual competence procedure, then chloramphenicol was added and incubation continued in SL 2 medium for 3 hr more (2.5 hr in Expt. 2). After 0 time platings, lysozyme was added to a final concentration 200 $\mu\text{g./ml.}$; 20 (or 25) min. later samples were taken for platings. OsO_4 was added to block instantly further changes in morphology and Petroff-Hausser total counts were made immediately.

Expt. no.	Columns							
	1	2	3	4	5	6	7	8
	Plating data				Microscope counts			
	Proto- plasting time (min.)	L colonies on DP medium (%)	L colonies on SDM medium (%)	inter- mediate forms (%)	rods (%)	spheroids (%)	spheres (%)	
1*	0	0	0	0	100	0	0	
	20	3	19	16	77	4	18	
2†	0	0	0	0	100	0	0	
	25	91	99	8	1	3	96	

* The number of perfect spheres seen in the phase microscope (col. 8) far exceeded the number of completely committed protoplasts (col. 3). Further, the % spheres (col. 8) was about the same as the combined % of committed forms and intermediate forms (col. 3 and 5 or col. 4). Hence it is concluded that the intermediate forms were spherical in shape.

† The % intermediate forms (col. 5) considerably exceeded the combined count of rods and spheroids (cols. 6 and 7). Hence it is concluded that the intermediate forms were spherical in shape.

led to the same conclusion, namely, that the intermediate forms were spherical. In Expt. 2 the number of intermediate forms was considerably greater than the sum of remaining rod and spheroid forms counted in the microscope.

Plate 2, figs. 3-5 show electron micrographs of spherical forms from populations

which had been treated with lysozyme for, respectively, 20 and 30 min. and which contained 29 % or 23 % intermediate forms. Considerable portions of the surface of the pictured forms are covered with a thinned residual layer of wall. In an earlier experiment, in which a larger number of organisms was photographed, 33 out of a total of 60 spherical forms retained residual cell-wall material. In this latter experiment, 33 % of the population behaved as intermediate forms, and 46 % as committed forms. In the three electron-microscopic surveys of intermediate populations which were made, the residual wall usually adhered fairly closely to the underlying protoplasts in a manner similar to that shown in Pl. 2, figs. 3-5. However, we also observed separation of cell-wall 'casings' from protoplasts, a phenomenon which had been seen earlier in studies of protoplasting on solid media (Ryter & Landman, 1964). A typical form from an extensively (80 min.) lysozyme-treated population is shown in Pl. 2, fig. 6. Of this population, 90 % behaved as committed forms; no cell-wall residues were observed.

DISCUSSION

The observations described in this paper concern several different topics, namely, changes in the cell wall of *Bacillus subtilis* 168 during normal growth, changes in the cell wall produced by inhibition of protein synthesis by chloramphenicol, and the relationship of cell-wall removal to commitment to the L state. These topics will be discussed in turn.

There are reports that, in a given bacterial species, changes in the cell wall may be produced not only by changing the medium of cultivation (Martin, 1966; Whitney & Grula, 1964; Young, 1965), but differences can occur also in the course of the regular growth cycle (Young, 1965). Further, subtle changes in the cell surface which develop under some conditions during growth are apparently responsible for competence in transformation. In competence development in *Pneumococcus* and *Streptococcus*, chemically and serologically identifiable materials appear quite abruptly in growing cultures, and again disappear, at least partly as a result of enzymic action (Tomasz & Hotchkiss, 1964; Tomasz & Beiser, 1965; Pakula & Walczac, 1963). With *Bacillus subtilis*, wall-surface modifications also have been reported to accompany changes in competence (Charpak & Dedonder, 1965; Jensen & Haas, 1963; Young, 1965; Young, 1966); again these variations occur well within the compass of a few divisions or, indeed, in the absence of division (Archer & Landman, 1967). The changes in lysozyme sensitivity which we have observed sometimes run parallel with changes in competence. In lysozyme sensitivity, as in competence, the changes are quite rapid and substantial variations may occur without division or in 1 or 2 divisions. It is likely that the wall-associated autolytic enzyme acetylmuramyl-L-alanine amidase (Young, 1965), which has been tentatively implicated in competence development, plays a role in effecting these variations. Further, enzymes of this type may also enhance or modify the protoplast-formation action of lysozyme in the various samples (Shockman, 1967). In any case, if the varying rates of commitment of organisms growing in SL 1 and SL 2 media are indeed a reflexion of changes in the structural and enzymic constitution of the cell wall, it must be concluded that the walls of growing *B. subtilis* are in a state of almost continual change.

When the metabolism of *Bacillus subtilis* is altered by inhibition of protein synthesis, cell wall changes become even more pronounced. In the terminology of Shockman

(1965), inhibition of protein synthesis produces a state of unbalanced growth which permits continued increase in cell-wall substance while blocking an increase in protein. The increase in cell-wall substance relative to cytoplasmic substance is clearly indicated by the increase in wall thickness observed in the electron microscope. Analogous increases in wall thickness in *Streptococcus faecalis* during both CAP treatment and starvation for threonine or valine were described by Shockman (1965). Tetracycline, another protein synthesis inhibitor, has been shown to produce thickening of cell walls in *Staphylococcus aureus* (Hash & Davies, 1962). (Incubation in tetracycline media also causes development of lysozyme-resistance in *B. subtilis*, I. L. Miller, unpublished.)

We know little about the comparative chemical make-up of normal cell walls and the abnormal walls produced under conditions of inhibition of protein synthesis. However, it seems to us unlikely that the great differences in lysozyme sensitivity which we have observed could be due exclusively to even a 2- to 3-fold increase in thickness of cell wall; it seems much more probable that changes in cell-wall composition are also involved. For one thing, the formation of wall-associated autolytic enzymes (Shockman, 1965; Young, 1966) is almost certainly blocked by chloramphenicol (CAP). If autolytic enzymes play a complementary role with lysozyme depolymerization of normal wall, the slowed lysis of presumably autolysin-depleted CAP-treated organisms may be more easily understood. The assumed lowering in the concentration of autolytic enzymes may also be invoked to explain the unexpected observation that wall thickening in CAP-incubated bacilli was almost uniform all-round the periphery of the organism. A localized thickening might have been anticipated on the basis of the observations of Chung, Hawirko & Isaac (1964) and of Cole (1965) that cell-wall growth in Gram-positive bacteria occurs in localized regions. The partial absence of localized autolysins required for the loosening of expanding cell walls (Shockman, 1965; Young, 1966) might explain an abnormal uniformity in the deposition of wall during CAP treatment.

In addition to modifying the enzyme content of cell walls, the inhibition of protein synthesis alters the pool concentration (Hancock, 1960) and metabolic flow of amino acids. As a result, the peptide content of the wall, the relationship of peptide content to teichoic acid content, and the cross-linking between wall constituents may well be changed. In *Streptococcus faecalis*, a quantitative difference between the amino acid content of the walls of threonine-deprived and of normal cocci has been described (Toennies, Bakay & Shockman, 1955). However, in *Staphylococcus aureus*, growth in presence of CAP did not appear to modify the composition of the cell wall, although wall synthesis continued throughout the CAP treatment period (Hancock & Park, 1958).

In their discussion of the process of protoplast formation, Landman & Halle (1963) distinguished an intermediate between bacillus form and protoplast, namely a rod-shaped osmotically sensitive form which did not survive in hypotonic media but gave bacillary colonies on hypertonic plating medium. In the present paper a succeeding intermediate form is recognized: a sphere-shaped form which gave a bacillary colony on DP medium and produced an L colony on SDM medium. In the upper portion of Table 8 the characteristics of the various forms are summarized. The lower portion of Table 8 shows the appearance and disappearance of the different forms as observed during a typical experiment on protoplast formation. The gradual progression from the bacillary state through the first and then the second intermediate form to the protoplast

state is clearly evident from the shifting percentage composition of the population undergoing lysozyme treatment (Table 8, lower right).

From the point of view of commitment, the second intermediate forms are very interesting since they are evidently able to re-initiate cell-wall formation on DP medium, but not on SDM medium. The primer mechanism which is responsible for this wall re-initiation (Landman, 1967; Landman & Halle, 1963) is apparently present vestigially in these forms. We suggest that the residues of cell wall which are visible in

Table 8. *Bacillus subtilis* 168

Stages of protoplast formation: schematic

Stage	Osmotic sensitivity	Morphology in phase microscope	Colony form on DP medium	Colony form on SDM medium
Bacillus	Insensitive	Rod	Bacillary	Bacillary
1st intermediate form	Sensitive	Rod	Bacillary	Bacillary
2nd intermediate form	Sensitive	Sphere	Bacillary	L-form
Protoplast: committed form	Sensitive	Sphere	L-form	L-form

Stages of protoplast formation: experimental

Predominant stage	Time of lysozyme treatment*	Colony counts ($\times 10^{-6}/\text{ml.}$)						Among survivors: approximate			
		On low salt†	On DP med.		On SDM med.		B (%)	1st form (%)	2nd form (%)	P (%)	
			B‡	L§	B	L					
Bacillus	0	95	101	—	89	—	100	—	—	—	
1st intermediate form	20	4.2	79	7.5	53	32	5	57	29	9	
1st and 2nd intermediate forms	25	1.2	67	16	33	42	2	40	38	20	
2nd form and protoplast	30	0.8	48	32	15	43	1	20	33	46	
Protoplast	35	1.5	37	38	8	50	2	10	31	57	
Protoplast	40	0.5	25	39	5	50	< 1	8	27	65	
Protoplast	45	0.3	12	42	3	52	< 2	4	17	76	

* Organisms incubated with chloramphenicol (15 $\mu\text{g.}/\text{ml.}$) medium for 3 hr before protoplast formation with lysozyme concentration 200 $\mu\text{g.}/\text{ml.}$

† Hypotonic medium was DP medium without succinate. Colony counts shown are actual counts $\times 10^{-6}$.

‡ Bacillary colonies.

§ L-form colonies.

Pl. 2, figs. 3–5 constitute the vestigial primer. How this vestigial primer accomplishes its priming functions is not known; it might do so directly by serving as a nucleus of attachment for further wall synthesis or wall fragments might indirectly serve as primers by facilitating other priming events in adjacent areas of the cell membrane. In any case, re-initiation of cell-wall formation in these incompletely formed protoplasts, unlike the reversion of protoplasts, takes place promptly and efficiently in ordinary media. The difficult initial event required for the reversion of protoplasts and stable L-forms is avoided. However, it is possible that in later phases of protoplast reversion the reverting forms pass through a stage resembling the second intermediate form (see Landman, 1967; Ryter & Landman, 1967).

The second intermediate form presents an interesting parallel with the spheroplasts

of Gram-negative bacteria. Both forms are osmotically sensitive spheres, both retain important quantities of cell-wall material and both revert promptly to the bacillary form as soon as the removal of cell wall is interrupted (i.e. both retain priming activity). In both forms, further removal of wall leads to loss of primer and thereby to an heritably persistent L-state (Landman & Ginoza, 1961; Landman & Halle, 1963). The second intermediate form of *Bacillus subtilis* 168 differs from spheroplasts of Gram-negative bacteria mainly by its lack of persistence under present experimental conditions.

Dr Masaya Kawakami kindly assisted in making the phase microscope counts of intermediate forms and Dr G. Chapman gave advice and help in making the electron micrographs. This work was supported by grants AI 05972 from the National Institutes of Health (USA), GB 1875 from The National Science Foundation (USA), and by a U.S. Public Health Predoctoral Fellowship, IFI-GM33, 752-01 for Mr R. Zsigray.

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EXPLANATION OF PLATES

PLATE I

Fig. 1. Section of normal *Bacillus subtilis* 168 fixed after competence incubation followed by 3 hr additional incubation in SL2 medium. $\times 93,000$.

Fig. 2. Section of chloramphenicol (CAP)-treated *Bacillus subtilis* 168 fixed after competence incubation followed by a 3 hr treatment in SL2 medium + CAP 15 $\mu\text{g./ml.}$ $\times 93,000$.

PLATE 2

Figs. 3, 4, 5. Sections of organisms from CAP-preincubated bacteria.

Fig. 3. Organisms from a population which had been treated with lysozyme 200 $\mu\text{g./ml.}$ for 20 min. and which contained 29% intermediate forms (see Table 8). $\times 19,000$.

Figs. 4 and 5. Spherical forms from a population which had been treated with lysozyme 200 $\mu\text{g./ml.}$ for 30 min. and which contained 23% intermediate forms. Thin remnants of cell wall cover a large proportion of the surface of the spheres. The strings of small bodies clinging to the cell surface (fig. 5) are probably residues of expelled mesosomes. $\times 46,500$. (See Ryter & Landman, 1967; Fitz-James, 1967.)

Fig. 6. Typical organism from the same suspension as figs. 4 and 5, taken after 80 min. of lysozyme treatment. No wall residues are visible in this protoplast. At the time of fixation, 5% of the population still behaved as intermediate forms. $\times 51,000$.