Cellular Responses of *Bacillus subtilis* and *Escherichia coli* to the Gram Stain

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Exponentially growing cells of *Bacillus subtilis* and *Escherichia coli* were Gram stained with potassium trichloro(1,2-ethylene)platinum(II) (TPt) in place of the usual KI-I₂ mordant. This electron-dense probe allowed the staining mechanism to be followed and compared with cellular perturbations throughout the staining process. A crystal violet (CV)-TPt chemical complex was formed within the cell substance and at the cell surface of *B. subtilis* when the dye and Pt mordant were added. The ethanol decolorization step dissolved the precipitate from the cell surface, but the internal complex was retained by the cell wall and remained within the cell. This was not the case for *E. coli*; the ethanol decolorization step removed both surface-bound and cellular CV-TPt. During its removal, the outer membrane was sloughed off the cells until only the murein sacculus and plasma membrane remained. We suspect that the plasma membrane was also perturbed, but that it was retained within the cell by the murein sacculus. Occasionally, small holes within the murein and plasma membrane could be distinguished through which leaked CV-TPt and some cellular debris. Biochemical identification of distinct envelope markers confirmed the accuracy of these images.

Although the Gram reaction is a technique on which much of the criteria for bacterial taxonomy resides, the actual effect of the stain on cells is imperfectly understood. There is general agreement that the staining response between gram-negative and -positive bacteria is due to a fundamental permeability difference between the two types of cells and that this difference resides within the molecular fabric of their enveloping layers (1, 2, 4, 26, 28, 32). Clearly there are distinct structural differences between the two wall types (4); these unique structural traits are the result of profoundly different chemistries (4, 11, 25). Presumably, it is these features that, in concert, control the end result of the Gram stain.

Recently, we have defined the chemical mechanism of the Gram stain and, using this information, have designed potassium trichloro(1,2-ethylene)platinum(II) (TPt) as an electron-dense chemical mordant to replace the KI-I₂ of Gram's iodine solution (9). Since crystal violet (CV) is a chloride salt, it interacts with iodide in solution by simple metathetical exchange (i.e., the bulky I⁻ replaces the smaller Cl⁻, and a CV-I precipitate results) (9). The TPT anion (TPt⁻) is also a bulky anion and complexes with CV⁺ to produce a CV-TPt precipitate which is completely analogous to CV-I (9). In this paper, we use TPT⁻ as an electron-dense probe to study the cellular responses of *Bacillus subtilis* and *Escherichia coli* to the various steps of the Gram stain.

**MATERIALS AND METHODS**

**Growth of cells,** The Gram staining method, processing for electron microscopy, and conditions of electron microscopy and EDS. *B. subtilis* 168 and *E. coli* K-12 stain AB274 were used for this study. The cells were grown and processed through the Gram reaction as outlined according to Davies et al. (9). LB broth (3) was used with both bacteria for the ³H-labeled phospholipid experiment. All processing for electron microscopy, actual imaging, and energy dispersive X-ray analysis (EDS) was done as previously described (9). All images from thin sections reported in this paper had the contrast increased by uranyl acetate and lead citrate staining, except for the elemental distribution maps which used unstained thin sections.

Platinum distribution maps were produced with the Pt (M₃,0 and L₃,0) lines. Lines slightly removed from the Pt lines (both upstream and downstream) were used to produce maps of the continuum to check that the Pt maps were real. The scanning transmission electron microscope (STEM) was operated at 80 kV and 85 μA with a 50-μm condenser aperture at a magnification of ×37,500. The total scan time was 500 s.

Although some Epon 812 embeddings were attempted, Durcupan (Fluka AG, Buchs SF, Switzerland), which is a water-miscible resin, proved to be the plastic of choice, since it did not leach the CV-TPt from the cells. A time sequence of embeddings was done on *E. coli* during the ethanol decolorization step. In this case, separate batches of CV-TPt cells were
treated with ethanol for 15, 30, 60, 120, and 300 s before equilibration into (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) HEPES buffer and fixation.

Atomic absorption spectroscopy. Both E. coli and B. subtilis were estimated for the TPT content after the Gram reaction by detection of total Pt per cellular dry weight by atomic adsorption. Cells were well washed after the Gram reaction in 0.05 M HEPES (pH 6.8) buffer until the supernatant was free of material which absorbed at 660 nm (a λmax for CV). They were then washed two times in water, hydrolyzed in fuming HNO3, and the residue was dissolved in 0.01 N HNO3 containing 250 μg of CsCl ml⁻¹ as a carrier. Flame atomic absorption was performed on a Perkin-Elmer model 2390 spectrophotometer working in the C2H8N2O flame mode.

Biochemical analyses of the Gram reaction supernatant. (i) Protein. Protein was estimated by the method of Markwell et al. (21), employing lysozyme (Sigma Chemical Co.) as a standard. CV and its various complexes have a λmax at ca. 580 and 660 nm (9) and will interfere with the 600-nm line of the Folin-phosphomolybdic-phosphotungstate complex for protein determination. Therefore, to compensate for this spectral interference, two times the added protein standards in appropriate concentrations; i.e., the CV concentration of each sample was estimated before determinations were begun.

(ii) KDO. The thiobarbituric acid procedure of Weissback and Hurwitz (33) was used, employing purified 2-keto-3-deoxyoctonate (KDO) (Sigma Chemical Co.) as a standard. Since this method forms a chromogen with a λmax at 548 nm, we also had spectral interference with this assay in the presence of CV. Addition of CV to the standard solutions proved unreliable. We therefore were able to estimate KDO only under low CV concentrations in the supernatant (before ethanol decolorization) or in the control samples (no CV) before and after ethanol decolorization. The ethanol control sample was a good estimate of KDO extraction during the decolorization of CV cells.

(iii) Incorporation of [³H]glycerol into the phospholipids of both bacteria and estimation of [³H]phospholipid release during the Gram reaction. [²⁻³H]glycerol (200.0 mCi mmol⁻¹) was obtained from New England Nuclear Corp. For the labeling of B. subtilis, the methods of both Sargent (27) and Smit et al. (29) were used. Both methods incorporated ³H into the plasma membrane fraction of the cells, and since the LB broth (3) of the method of Smit et al. (29) gave a higher cell yield, it was used to label cells for the Gram reaction. The same method was used to label E. coli membranes.

The bacteria were grown overnight in 250 ml of LB broth containing 0.01% of glycerol at 22°C for B. subtilis and 37°C for E. coli. Each culture was pelleted by centrifugation at 10,000 × g for 15 min, suspended in 120 ml of LB broth containing 5 μCi of [²⁻³H]glycerol ml⁻¹ and grown at the appropriate temperature for 4 h. Each culture was then washed twice with 100 ml of 0.05 M HEPES (pH 7.0) buffer, and the cells were checked for the incorporation of the label (which was positive in both cases).

The cells were then put through the Gram regimen by the method of Davies et al. (9), and the supernatants of the various steps of the reaction were checked for radioactivity by liquid scintillation by using a Beckman model LC250 counter. Aquasol (New England Nuclear) was used as the scintillation fluid.

Preparation of potassium TPT. TPT was prepared by the method of Davies et al. (9).

SDS-PAGE. The discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system of Laemmli (20) was used to monitor the protein composition to the Gram reaction supernatant of E. coli. Much of the extracted material was membranous and contained high concentrations of CV that could not be removed by either extensive dialysis (10 × 5 liters of buffer) or treatment with mixed bead resins (e.g., Amberlite MB-3 or ABX; British Drug Houses). This CV contamination affected the electrophoretic properties of the proteins (smearing was common) and the clarity of Coomassie brilliant blue staining. The resulting gels were of poor quality (Fig. 8 is representative), but the major outer membrane proteins could be differentiated above background.

RESULTS

Cellular CV-TPT content of B. subtilis and E. coli. B. subtilis and E. coli were monitored for the CV-TPT complex by atomic absorption detection of Pt during the Gram reaction before and after ethanol decolorization. In B. subtilis, 1.98 and 1.71 mmol of CV-TPT per mg (dry weight) of cells were found before and after treatment, respectively. In E. coli, 2.44 and 1.14 mmol of CV-TPT per mg (dry weight) of cells were found before and after ethanol treatment, respectively. These values were calculated from atomic absorption analysis of total Pt in cells. Atomic absorption analysis also confirmed that the CV-to-TPT ratio was 1:1. After ethanol treatment, B. subtilis lost ca. 14% of its total cellul-associated CV-TPT. The amount of precipitate that remained associated with these cells produced the typical purple color of gram-positive cells (9). E. coli, on the other hand, lost ca. 53% of the CV-TPT precipitate. This must be a low estimate since large numbers of these cells (a total of 0.154 g [dry weight] of cells) tended to aggregate during ethanol treatment to trap CV-TPT within the central mass of bacteria. The brief ethanol treatment (9) was not long enough to completely decolorize all of these cell masses, but light microscopy confirmed that a majority had been washed free of the dye.

Electron microscopy of B. subtilis during the Gram reaction. Previous work had demonstrated that CV-TPT could be detected within cells by electron microscopy (9). The staining of these same sections of cells with uranyl acetate and lead citrate added enough contrast to the cellular structure, without displacing the Pt probe (as monitored by EDS), that ultrastructural detail could be distinguished.

B. subtilis showed a surprising retention of cellular detail when treated with CV (no TPT) and decolorized (Fig. 1a). Ribosomes, plasma membrane, and the wall fabric could be detect-
FIG. 1. (a) Thin section of *Bacillus subtilis* which has been treated with CV alone and then decolorized with ethanol. This and all other sections have been contrasted with uranyl acetate and lead citrate. The dark areas within the cytoplasm are presumed to be ribosomes. In this and all other micrographs, the bar represents 100 nm. (b) This micrograph, from the same preparation as (a), reveals a high magnification of the wall to show that it remains intact after ethanol treatment. (c) This is a CV cell that has been treated with Gram’s iodine to form the CV-I complex and then decolorized. The wall remains intact, as does the PM, but the cytoplasm contains voids (arrows) that once contained CV-I complexes.

ed. The wall remained intact (Fig. 1b) and retained its typical 22- to 25-nm thickness (5). Cells that had been stained by the CV-iodide method resembled the CV cells in every way except that the cytoplasm contained voids (Fig. 1c). These were attributed to areas which had previously contained the CV-I precipitate until the energy of the electron beam sublimed the iodine from the section to leave empty spaces (9).

CV-TPt sections revealed electron-dense deposits scattered throughout the cytoplasm. The plasma membrane was retracted from the wall (Fig. 2a). EDS of the electron-dense deposits confirmed that they contained Pt (9). STEM-EDS platinum maps of bacteria before and after ethanol decolorization failed to reveal differences between the two treatments (Fig. 2b and c). Unlike atomic absorption spectrophotometry, this technique is not sensitive enough to differentiate between untreated and decolorized *B. subtilis* cells.

Plasmolysis was not unique to CV-TPt cells, but was seen in CV and CV-I cells as well (Fig. 2d is representative). This phenomenon was only seen in decolorized cells and was, therefore, a common response to the ethanol treatment. The ethanol had denatured and condensed the cytoplasmic substance away from the plasma membrane (PM). Cells that were not decolor-
FIG. 2. (a) A *B. subtilis* cell that has been treated with CV and TPt and then decolorized. The CV-TPt complexes (arrows) stain dark in this preparation and were not removed by the ethanol treatment. In this and in other CV-TPt images, the dark areas have been identified as containing Pt by EDS. (b) STEM-EDS elemental distribution map of a cell for Pt (i.e., CV-TPt) before ethanol treatment. Each dot represents a high Pt concentration within the section and the highly concentrated area conforms to the shape of a bacterial cell. Maps of the X-ray continuum (i.e., background radiation) for this and other samples demonstrated that the point distribution was due to Pt and was not an artifact of the mass thickness of the specimen. All maps are of...
from protein. Presumably, removed reaction. Gram (12, extracted did which was decolorized iodide tion. Table within analyses the wall occasionally, is evident TPt. This and wall, proved 2e). CV-TPt decolorized decolorized CV-I 0.382 3.2. CV-I decolorized by alcohol 1.319 7.8. CV-TPt decolorized by alcohol 1.104 6.5.

Data were derived from 10 ml of a culture with an optical density at 600 nm of 0.600 with a total dry weight of 32.4 mg. Figures represent the average of three protein estimations for each sample by the Markwell et al. method (21).

ized often contained CV-TPt deposits on the surface of the cell wall (presumably this was external precipitate that had condensed on to the cell surface during chemical metathesis of exogenous CV+ and TPt−) (Fig. 2f). These were not seen after ethanol treatment (the ethanol had dissolved and washed them away) (Fig. 2a), but occasionally, the wall was buckled and contained electron-opaque material within its lumen (Fig. 2e). EDS proved this material to be CV-TPt. This suggested that it was the fabric of the wall, and not the PM, which retained CV-TPt during decolorization.

Biochemical identification of cellular products extracted from B. subtilis during the Gram reaction. Table 1 shows the amount of protein which was extracted from B. subtilis during each step of the Gram reaction. Both the CV-I and CV-TPt methods were employed. In all cases, whether iodide or TPt were used or whether the cells were decolorized or not, the percent protein extracted did not exceed 7.8% of the total cellular protein. Presumably, it was the wall fabric which prevented more substantial loss of protein from cells during the Gram reaction.

Table 2 shows the amount of phospholipid which was removed during each step of the Gram reaction. Since ethanol is a membrane perturant (12, 18) it is expected that lipid would be removed. Of the cellular lipid 30 to 43% was removed by CV, CV-I and CV-TPt without ethanol treatment. This amount, no doubt, was due to the 20% ethanol within the CV staining solution (9). Ethanol treatment did not increase the amount of lipid extracted. Presumably, this treatment further contracted the wall fabric and made it impermeable to the passage of additional lipid. No attempt was made to identify the extracted lipids.

Electron microscopy of E. coli during the Gram reaction. The response of E. coli to the Gram stain was much different than that of B. subtilis. These bacteria tended to agglutinate upon the addition of CV and TPt to form large aggregates of cells and CV-TPt precipitate. The precipitate was found on the cell surface, in the cytoplasm (Fig. 3a) and within the periplasm (Fig. 3b). Alcohol decolorization reduced the amount of cytoplasmic substance, congealed the remaining cellular contents, and produced large regions devoid of electron-scattering power (Fig. 3c). These cells did not produce a strong Pt signal with EDS, and presumably the cytoplasmic voids had initially contained CV-TPt (9). In many instances only the ghosts of cells remained (Fig. 3d).

Close examination of these bacteria during decolorization revealed a disruptive membrane effect (Fig. 4). When a time sequence of embeddings was made during the decolorization process, the membrane perturbation could be more accurately followed (Fig. 5a to f). Initially, the outer membrane (OM) began to buckle and fold until it eventually sloughed off the cell surface. This continued until only small OM fragments were left, or until the murein layer (peptidoglycan [PG]) was laid bare. In some cells, small holes were eventually produced in the remaining layers (PG and PM) through which CV-TPt and

<table>
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<th>% Total cellular protein</th>
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<tr>
<td>CV-I</td>
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<tr>
<td>CV-TPt</td>
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<td>Control decolorized by alcohol</td>
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<tr>
<td>CV-TPt decolorized by alcohol</td>
<td>1.104</td>
<td>6.5</td>
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“ Data were derived from 10 ml of a culture with an optical density at 600 nm of 0.600 with a total dry weight of 32.4 mg. Figures represent the average of three protein estimations for each sample by the Markwell et al. method (21).

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<th>Treatment</th>
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<th>With decolorizing alcohol</th>
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<td>31</td>
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<tr>
<td>CV</td>
<td>43</td>
<td>39</td>
</tr>
<tr>
<td>CV-TPt</td>
<td>42</td>
<td>39</td>
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</table>

All data are expressed as the percent of total counts (lipid) extracted from the cells.
FIG. 3. (a) An *E. coli* cell which has been treated with CV-TPt but not subjected to ethanol decolorization. The darkly stained areas are CV-TPt complexes (arrows). (b) A high-magnification of the envelope from one of the cells of the preparation used in (a). CV-TPt is located within the periplasmic space (arrow). (c) The same preparation after ethanol treatment. Only congealed material remains within the cytoplasmic space and the CV-TPt precipitate can no longer be distinguished. (d) A cell ghost after cellular lysis during ethanol treatment.
cellular debris extruded (Fig. 6). The end result was a lysed ghost that retained the original shape and form of the bacterium (Fig. 3d).

Semi-quantitative STEM-EDS analyses of CV-TPt cells before and after ethanol treatment confirmed that there was a drastic reduction of Pt (i.e., CV-TPt) in bacteria that had undergone decolorization. In fact, elemental maps of the cells revealed that the CV-TPt distribution was more widely dispersed in the treated cells (Fig. 7a and b). Point EDS analyses corroborated the elemental maps (Fig. 7c and d) and this was especially apparent when the spectrum of the treated cells was subtracted from that of the untreated cells (Fig. 7e); a substantial Pt peak remained.

**Biochemical identification of cellular products extracted from E. coli during the Gram reaction.** Table 3 shows the amount of protein which was removed from E. coli during each step of the Gram reaction. The decolorization step had a profound effect on the amount of cellular protein which was leached since up to 36% of the total protein was removed. Likewise, there was a tremendous increase in the [3H]glycerol-labeled phospholipid that was liberated during ethanol treatment (Table 4). The amount of radioactive label which was released from the CV, CV-I, and CV-TPt cells without ethanol treatment, once again, is a reflection of the ethanol content of the CV staining solution (9).

Although electron microscopy clearly revealed that ethanol treatment perturbed the OM, chemical evidence was desirable. KDO is a constituent of the lipopolysaccharide (LPS) which is unique to the OM. Analyses for this component in the Gram supernatant revealed that KDO was extracted by ethanol and CV treatment (CV contains 20% ethanol) (Table 5). In addition, SDS-PAGE of the released products demonstrated that the OmpA, OmpC and possibly OmpF proteins were abundant in the Gram supernatant (Fig. 8). Taken in concert, these biochemical data support the electron microscopic results and suggested that the OM had been disrupted during the Gram stain.

**DISCUSSION**

The results reported in this paper provide an understanding toward the events that occur to bacteria during the Gram stain. Two bacteria were chosen as representatives of the two Gram types, B. subtilis and E. coli, and they were subjected to the Gram reaction using a new electron-opaque probe to replace the iodide of Gram’s iodine (9). Under the conditions employed, these bacteria reacted unequivocally to the stain; B. subtilis stained gram-positive, and E. coli stained gram-negative (9).

With B. subtilis, a metathetical ion exchange product (CV-TPt or CV-I) forms both at the cell surface and within the cell substance during the Gram stain. Ethanol treatment dissolves the surface-bound product and releases small quantities of cellular protein and phospholipid into the external milieu. It does not remove the
FIG. 5. A series of micrographs of CV-TPt E. coli cells depicting the sequence of OM perturbation during the ethanol decolorization step. Exposure times to ethanol were as follows: (a), 15 s; (b), 30 s; (c and d), 60 s; (e), 120 s; and (f), 300 s. After 300 s, little, if any, OM remains on the cells. All images are the same magnification.
product from within the bacteria which is restricted from migration by the molecular fabric of the wall; presumably, the CV-TPt complex is too large to penetrate through the interstices of the PG-teichoic acid matrix. (Under our growth conditions, the walls should be in the teichoic acid form, and teichuronic acid should not be present [6]). The CV$^+$ (1.88 by 0.65 nm; see reference 9) and TPt$^-$ (0.82 by 0.62 nm; see reference 9) ions permeate through the wall fabric and interact and combine with one another inside the cell. Here, a large precipitate of

FIG. 6. A hole in the polar end of a CV-TPt cell after decolorization with ethanol. The debris that is leaking out of the cell (large, left arrow) gave a strong Pt signal by EDS.

FIG. 7. (a) STEM-EDS platinum map of an E. coli cell before ethanol decolorization. (b) Same as (a) but after decolorization. The dots are not as clustered and are more widely disbursed, making them appear smaller. (c) EDS spectrum of the cell in (a). (d) EDS spectrum of the cell in (b). The copper peaks in this and in (c) are derived from the copper EM grids. The copper peak in (c) is larger since the specimen was closer to a grid bar. The chlorine is from the plastic of the section. (e) EDS spectrum after (d) has been subtracted from (c). There is a strong Pt signal left which confirms that there was much less platinum in cells after ethanol decolorization.
CV-TPt is formed which can be detected by electron microscopy. This precipitate is spread throughout the cell substance and occupies a substantial volume of the cell; very little is removed by ethanol treatment (the 14% difference [see Results] is most probably due to surface-bound CV-TPt). Our conception of these cells after ethanol treatment is diagrammed in Fig. 9.

At least three explanations could explain the retention of the complex within *B. subtilis* during ethanol treatment. First, it is possible that the CV-TPt precipitate is not dissolved by the ethanol within the interior of the cell. If this were true, the large dye aggregate would be too large to exit from the cell. Second, it is possible that the CV-TPt aggregate is dissolved but that the dissociated complex (ca. 2.50 by 1.00 nm) is too bulky to penetrate through the wall matrix. As the ethanol is replaced by an aqueous counterstain (e.g., carbol fuchsin) during the next step of the Gram reaction, the CV-TPt would again precipitate out of solution within the contents of the cell. The last possibility is a combination of the first two; in this case, only a small proportion of the CV-TPt complex would be solubilized by the ethanol, and this proportion would be free to migrate from the bacteria.

Of the three possibilities, the second choice is the most attractive. In vitro, CV-TPt is soluble in ethanol, and it is difficult to imagine that the cellular precipitate could be different. The method of decolorization used in our experiments relied on the fact that excess ethanol was used (see reference 9), and this should be sufficient to dissolve all cellular precipitate. Therefore, possibilities 1 and 3 seem untenable. The lattice constant of dried PG is smaller than that of the hydrated form (8, 14). Ethanol should replace or reduce the content of water within the wall fabric, condense the polymeric network (23), and shrink the dimensions of the lattice constant (4, 8, 14). The end result is that the fabric of the wall should become even more impermeable to the dye complex. The lattice constant in dried PG foils suggests that the glycan strands are separated by 0.44 nm and that the dimer repeat along the strand is 0.90 nm. Of course, not all strands of PG are cross-linked by tetrapeptide stems (*B. subtilis* has a cross-linking efficiency of 30%), but the limiting dimension should be 0.44 nm. Indeed, disregarding the secondary polymers, up to 24 separate sheets of PG piled on top of one another could fit into the thickness of the *B. subtilis* wall (4). The planar alignment of these sheets within the wall would also affect

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Data derived from 10 ml of a culture with an optical density of 0.630 with a total dry weight of 11.4 mg. Figures represent the average of three protein estimations for each sample by the Markwell et al. method (21).

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<th>Treatment</th>
<th>nmol</th>
<th>% Cellular KDO</th>
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Data derived from 10 ml of a culture with an optical density of 0.630 with a total dry weight of 11.4 mg. Figures represent the average of three KDO estimations for each sample.

Although alcohol extracted a high proportion of KDO, this amount is only 40% of the total available KDO in the culture. Electron microscopy showed that ethanol produced large aggregates of cells which contained trapped OM. This membrane was not cell-bound but was difficult to remove from the aggregates. Consequently, it did not contribute to the KDO estimations of the extract. We believe the observed discrepancy between the extract and available KDO is due to this nonspecific binding to the cellular aggregates.

ND, Not determinable because of spectral interference from chemically bound crystal violet.

### Table 4

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<tr>
<th>Treatment</th>
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<tr>
<td>CV</td>
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<tr>
<td>CV-TPt</td>
<td>44</td>
<td>92</td>
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</table>

All data are expressed as the percent of total counts (lipid) extracted from the cells.

### Table 5

- **Control**: 0
- **CV**: 0.1
- **CV-I**: 0.15
- **CV-TPt**: 0.12
- **Control decolorized with alcohol**: 0.66
- **CV decolorized with alcohol**: ND
- **CV-I decolorized with alcohol**: ND
- **CV-TPt decolorized with alcohol**: ND

Data derived from 10 ml of a culture with an optical density of 0.630 with a total dry weight of 11.4 mg. Figures represent the average of three KDO estimations for each sample.
the permeability characteristics and further inhibit the dye complex from migrating out of the cells.

The molecular architecture of the *E. coli* wall is different than that of *B. subtilis*. Instead of an amorphous matrix of interwoven peptidoglycan and teichoic (or teichuronic) acid strands, the *E. coli* wall is partitioned into two separate entities, the PG (which is most probably a monolayer [7]) and the OM (an asymmetric LPS-phospholipid bilayer which is interspersed with protein, the majority of which consists of three or four major polypeptide species [4, 11, 15]). The OM is chemically linked to the PG (murine) layer by lipoprotein (through covalent interaction with the diaminopimelic acid of the PG peptide stem) and matrix (or porin) protein (through magnesium salt bridging) [4, 11].

Clearly, the Gram reaction affects these walls in a profoundly different manner than the gram-positive variety. It is the OM which is immediately affected by the ethanol. This is one of the prime differences between the two systems. This membrane eventually disappears from the cell surface to reside permanently in the external milieu as membrane sheets or vesicles after rehydration. The resulting cell becomes leaky at this stage, and exudes the CV-TPt complex and some cellular constituents (protein and nucleic acid can be detected by their UV spectra). Eventually, in some cells, small holes occur in the PG and PM through which particulate cellular debris and CV-TPt are seen to escape.

Two general mechanisms of membrane interaction with ethanol can be assumed. First, it should intercalate into membrane with concomitant disturbance of native membrane structure. Second, the polar hydroxyl group should interact with the hydrophilic head groups of membrane lipids resulting in a disruption of their normal ionic affinity for small ions, such as Mg$^{2+}$ or Ca$^{2+}$, or charged molecules such as proteins or water. The latter possibility would perturb the boundary water of the membrane by the dehydration effect of ethanol. Although membrane fluidity does not seem to be affected, a variety of complex alterations within and without the membrane are possible [12, 16, 17, 18].

Our perceptual view of the OM of *E. coli* is that it is a more rigid membrane than the PM, that it is physically supported by chemical linkage to the PG, and that, consequently, it is more able to withstand environmental stress than the PM [4, 11]. This presents a conundrum; why is the PM not disturbed to a greater degree than the OM? It is our contention that the PM is perturbed; after all, it is not very different from the *B. subtilis* PM, and our *B. subtilis* results indicated that intracellular CV-TPt breached the PM but was trapped by the wall. We believe that the *E. coli* PM is disrupted in a similar manner but that the sheets and vesicles thus formed are too large to penetrate through the PG layer. In fact, PM disruption during the decolorization step...
would leave the PG as the only remaining obstacle to the removal of intracellular CV-TPt. Since this layer is most probably a monolayer (7), since the PG is only 20 to 30% cross-linked (10, 22, 30), and since discontinuities within the monolayer must exist to accommodate lysozyme and autolysin digestion (4, 13), this layer could not present the same sort of barrier to the dye complex as the B. subtilis wall. Once the ethanol-treated bacteria were reequilibrated back to aqueous conditions, the fragments of PM would reanneal to form an encompassing membrane. By electron microscopy, this membrane would resemble the original PM in shape and form.

The observation that some cells of E. coli develop small localized lesions within the PG and PM during ethanol treatment is of interest. There is the possibility that these are distinct sites of autolytic activity within the PG fabric. Our subjective analyses (no statistics were attempted) by electron microscopy suggested that these occurred most frequently at one polar end of the cell (the newly synthesized end?). They did not develop until the OM had been removed (a stringent control?). Ethanol decreases the extent of cross-linkage in the PG causing eventual lysis (16, 17). The reduction of cross linkage is due to a displacement and concomitant folding of the glycopeptide carboxypeptidase and transpeptidase (16) which are membrane bound (19, 24, 31). Since ethanol has disrupted the E. coli membranes during the decolorization step, it is possible that the localized lesions seen in the PG are the end result of carboxypeptidases and transpeptidases displaced from the membrane. If a hole did occur, then the underlying PM could bleb and escape, exposing the cell interior. A model system for the decolorization process in E. coli is presented in Fig. 10.

In summary, using electron microscopy and TPt (which is apparently chemically analogous to the iodide of Gram's iodine [9]), we have followed the stain complex of the Gram reaction and related it to the ultrastructural alterations manifest within one gram-positive and one gram-negative bacterium. These observations have been related to biochemical changes during the staining response. The difference between the two cell types appears to reside solely in the fundamental molecular dissimilarities within their wall fabrics. Milton Salton knew the answer all along (26)!

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FIG. 10. Artist’s rendering of the sequence of events that occur to E. coli during the ethanol decolorization step of the Gram stain. (a) The OM is sloughing off from the cell, whereas the PG and PM remain intact. As explained in the discussion of the text, the intact PM may be an artifact of electron microscopy. The CV-TPt complex is the electron dense precipitate that is scattered throughout the internal cell substance (CS). (b) Most of the OM has been removed (only a few fragments remain) and much of the CV-TPt has been leached from the CS. (c) A small localized hole has developed (usually at one pole) in the PG and PM through which the remaining CV-TPt and some of the CS is passing. The dimensional aspects of these drawings are by no means accurate, and certain structures (e.g., the periplasmic space) have been exaggerated for the clarity of the image. Like all artistic representations and model systems, we have no doubt simplified the series of events. As our tools for deciphering molecular events at the subcellular level become more acute, we expect more definitive explanations to be obtained.
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