STUDIES OF THE BACTERIAL CELL WALL

II. METHODS OF PREPARATION AND SOME PROPERTIES OF CELL WALLS

by

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INTRODUCTION

One of the problems preventing the elucidation of the chemical constitution of the bacterial cell wall has been the absence of suitable procedures for the preparation of pure cell wall material. Many of the early chemical tests on what was assumed to be the cell wall were performed on the residue from alkaline extraction of the cells. This evidence has been reviewed by KNAYSI[1]. However, there appear to be no adequate microscopical observations to support the assumption that only the cell wall remains after extraction of bacteria with alkali.

Cell walls either partially or completely freed of the cytoplasm have been observed by MUDD AND LACKMAN[2], and MUDD, POLEVITSKY, ANDERSON, AND CHAMBERS[3] in sonically vibrated preparations of bacteria. CURRAN AND EVANS[4] found that bacterial spores were destroyed by violent agitation with small inert particles. Following the report by KING AND ALEXANDER[5] of the lethal effects of shaking various organisms with minute glass beads, DAWSON[6] showed that this method could be used to prepare cell wall suspensions of *Staphylococcus aureus*. However, since 7% of the cells remained intact (COOPER, ROWLEY, AND DAWSON[7]), it is evident that this procedure would require modification to avoid chemical estimation of major impurities such as the cytoplasmic components of unbroken cells. More recently WEIDEL[8] has described a method used in the preparation of phage receptor spots. Electron microscope examination of the material prepared by procedures including trypsin and lysozyme digestion showed that it "consists entirely of bacterial cell walls, which are crumpled up and folded to give completely flat, almost circular particles of rather uniform size".

This paper presents the results of an investigation of several methods of preparing pure cell wall material. One of the methods was suggested from our observations (SALTON AND HORNE[9]) on cell wall rupture resulting from heat-treatment of bacteria. The stages in the preparation of bacterial cell walls have been followed by examination of the material in the electron microscope.

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EXPERIMENTAL

Bacteria

The following organisms were used in this investigation: *Escherichia coli* H and *Salmonella pullorum* used in earlier studies (SALTON AND HORNE[9]), and *Streptococcus faecalis* ST (N.C.T.C. No. 6782).

Cultural conditions and harvesting

The organisms were grown on a medium consisting of 3% tryptic digest of casein, 0.1% Marmite, 1% glucose and 2% agar. The surface of 150 ml of the medium in Roux bottles was inoculated with a few ml of overnight cultures of the organisms in liquid medium. The incubation period was for 16 h at 37° C, unless otherwise specified. The cells were harvested by washing from the agar surface with distilled water, centrifugation and washing twice with distilled water on the centrifuge. Cells were finally suspended in distilled water to give suspensions containing approximately 10–20 mg dry weight bacteria/ml.

Electron microscopy

Suspensions of preparations suitably diluted with distilled water were placed on specimen grids covered with nitrocellulose film. Mounted specimens were dried in a desiccator and shadowed with gold-palladium alloy (60%–40%). The shadowing was at an angle of 45 degrees from the plane of the supporting film. Observations were made in the Siemens electron microscope, usually at direct magnifications of 8,000–12,000.

CELL WALL PREPARATION TECHNIQUES

1. *Heat-treatment*

Washed suspensions of *Esch. coli* were heated for 5 min at 75° and 100 C° and *Salmonella pullorum* at 75° C according to the procedure described by SALTON AND HORNE[9].

Suspensions of *Esch. coli* heated at 100° C were centrifuged at 3,000 r.p.m. for 20 min; the supernatant containing soluble cell components was discarded. Fig. 1 shows the ruptured cell walls adhering to the cytoplasmic bodies in the 3,000 r.p.m. deposit. The deposit was resuspended in distilled water and the suspension was gently shaken for 10 min with ⅛ inch glass beads. The suspension was decanted from the glass beads and centrifuged at 3,000 r.p.m. for 20 min. The deposit of cell bodies was discarded, the supernatants collected and centrifuged at 10,000 r.p.m. for 10 min, and the deposit of cell walls washed several times with distilled water on the high speed centrifuge. Figs. 2 and 3 show purified cell wall material.

Esch. coli and *Salmonella pullorum* suspensions heated at 75° C were centrifuged at 3,000 r.p.m. for 20 min. The deposit of cell bodies was discarded, the supernatant fluid collected and the cell walls removed from the cell solutes by centrifugation for 10 min at 10,000 r.p.m. The cell walls thus prepared from *Esch. coli* and *Salmonella pullorum* are shown in Figs. 4 and 5 respectively.

2. MICKLE *disintegration*

The initial stage in the preparation of cell walls was similar to that described by DAWSON[6]. 10 ml of distilled water suspensions containing 10 mg dry weight bacteria/ml were shaken with 10 ml of "ballotini" grade 12 glass beads in the MICKLE disintegrator (described by MICKLE[10]) for 15 min. The glass beads were removed by filtering the suspension through a No. 1 sintered glass filter. Under these conditions more than 90% of the cells of the three organisms studied were ruptured.

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Removal of unbroken cells and debris

The intact cells remaining in the suspension after disintegration were removed by centrifuging for 10 min at 3,000 r.p.m. Fig. 6 shows a typical field of unbroken cells of *Strep. faecalis* deposited by centrifugation at 3,000 r.p.m. Some cell walls are also deposited in this fraction. As shown in Fig. 7 centrifugation of the *Salmonella pullorum* suspensions at 3,000 r.p.m. resulted in the removal of not only unbroken cells but also large clumps of cytoplasmic debris and some cell walls. Similar results were observed with *Esch. coli*.

Removal of cell cytoplasm

The cell walls were then removed from cytoplasmic material by centrifuging for 10 min at 10,000 r.p.m. The separated cell walls of *Strep. faecalis* are shown in Fig. 8. Further washing of cell walls from *Strep. faecalis* with distilled water on the high speed centrifuge resulted in the removal of residual electron-dense cytoplasmic material as shown in Fig. 9.

However, large amounts of cytoplasmic debris were observed in the cell wall fraction deposited by high speed centrifugation of *Esch. coli* and *Salmonella pullorum* suspensions. This cytoplasmic debris adhering to the cell walls could not be removed simply by repeated washing with distilled water as with *Strep. faecalis*. Figs. 10 and 11 show typical fields of cell walls and debris in the 10,000 r.p.m. deposit from *Esch. coli* and *Salmonella pullorum* respectively. To avoid contamination of the cell wall preparations of these two organisms with cytoplasmic debris the following procedures were investigated: (a) disintegration of younger cultures of the organisms (WYCKOFF[11] has observed that the cytoplasm of young cells of colon bacilli is very fluid and sol-like, whereas it appears to be gel-like in old cells); (b) disintegration of the cells in 1% saline instead of distilled water; and (c) washing the separated cell wall material in buffer. Fig. 12 shows that there was a substantial reduction in the contamination with cytoplasmic debris when cell walls were prepared from 9 h cultures of *Esch. coli* (compare with Fig. 10 for cell walls from 16 h culture). The cell walls from the 9 h culture showed no tendency to form the large clumps observed in preparations from 16 h cultures. Although disintegration of cells from 16 h cultures of both organisms in 1% saline prevented the cell walls from clumping, the cytoplasm was more viscous and much of it was deposited with the cell wall fraction on high speed centrifugation. When cells were broken in distilled water and the separated cell wall fraction thoroughly washed several times in *M*/10 phosphate buffer at p_H 7.0, most of the cytoplasmic material was removed. Figs. 13 and 14 show the purer preparations of cell walls obtained in this way from *Esch. coli* and *Salmonella pullorum* respectively.

No attempt was made to make a quantitative recovery of cell walls by either the heat-treatment method or the MICKLE disintegration procedure. Indeed, it appears to be necessary to sacrifice high yields of cell wall material to ensure purity of the final preparation.

EXTRACTION OF CELLS WITH ALKALI

In order to determine whether the alkali-resistant residue is morphologically similar to the cell wall material obtained by the methods described above, washed suspensions of *Esch. coli* and *Salmonella pullorum* were extracted with *N* NaOH for 48 h at 37° C.

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The suspensions were centrifuged for 15 min at 10,000 r.p.m., and the deposit washed three times on the high speed centrifuge and finally suspended in distilled water. Figs. 15 and 16 show that the alkali-resistant residue from *Esch. coli* and *Salmonella pullorum*, respectively, differs markedly in appearance from the cell walls obtained by either the heat-treatment or the MICKLE methods. Although there still appears to be an extremely thin "membrane" enclosing granular material, there is no evidence to support the assumption that alkaline extraction leaves only the cell wall. Indeed, the original cell wall structures were no longer observable in the electron microscope when pure cell wall preparations of *Strep. faecalis*, *Esch. coli* and *Salmonella pullorum* were extracted with *N* NaOH for 48 h at 37° C.

SOME PROPERTIES OF THE CELL WALLS

Pure suspensions of cell walls from the three organisms showed strong light-scattering properties, thus behaving similarly to the material isolated by WEIDEL[8]. The cell wall suspensions prepared by MICKLE disintegration of *Esch. coli* and *Salmonella pullorum* showed strong streaming birefringence. *Strep. faecalis* cell wall suspensions and those prepared by heat-treatment of *Esch. coli* and *Salmonella pullorum* did not show this phenomenon to any marked degree. Freeze-drying preparations of cell wall material from the three organisms yielded a stable white substance of pith-like appearance. Dried cell wall material had the same macroscopic appearance irrespective of the method of preparation. One of the remarkable features also noted by WEIDEL[8] is the insolubility of the cell walls in many organic solvents, dilute acids in the cold and SCHWEIZER's reagent. However, our preparations appear to be partially soluble in *N* NaOH, 10% sodium hypochlorite and 5% phenol in the cold.

Preliminary observations of the pure cell wall material by infra-red spectroscopy suggests that it is of a complex nature. Using the Beckman spectrophotometer the ultra-violet absorption spectra of cell walls from *Strep. faecalis* and *Esch. coli* were measured. The curves for pure cell walls together with the curve for the cytoplasmic fraction from *Strep. faecalis* are given in Fig. 17. It is clear that only traces of nucleic acid or purine-pyrimidine compounds could be present in the pure cell wall preparations, whereas the cytoplasmic fraction shows the characteristic nucleic acid absorption maximum at 260 mμ. This finding is not in agreement with the claim by STACEY[12] that nucleic acid is a component of the cell wall.

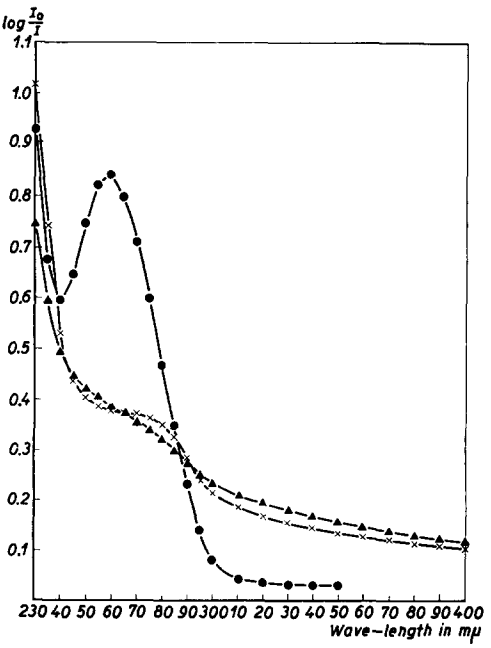

Fig. 17. Ultra-violet absorption spectra
▲——▲ *Strep. faecalis* cell wall suspension;
×——× *Esch. coli* cell wall suspension;
●——● cytoplasmic fraction from *Strep. faecalis*.
Concentrations arbitrary

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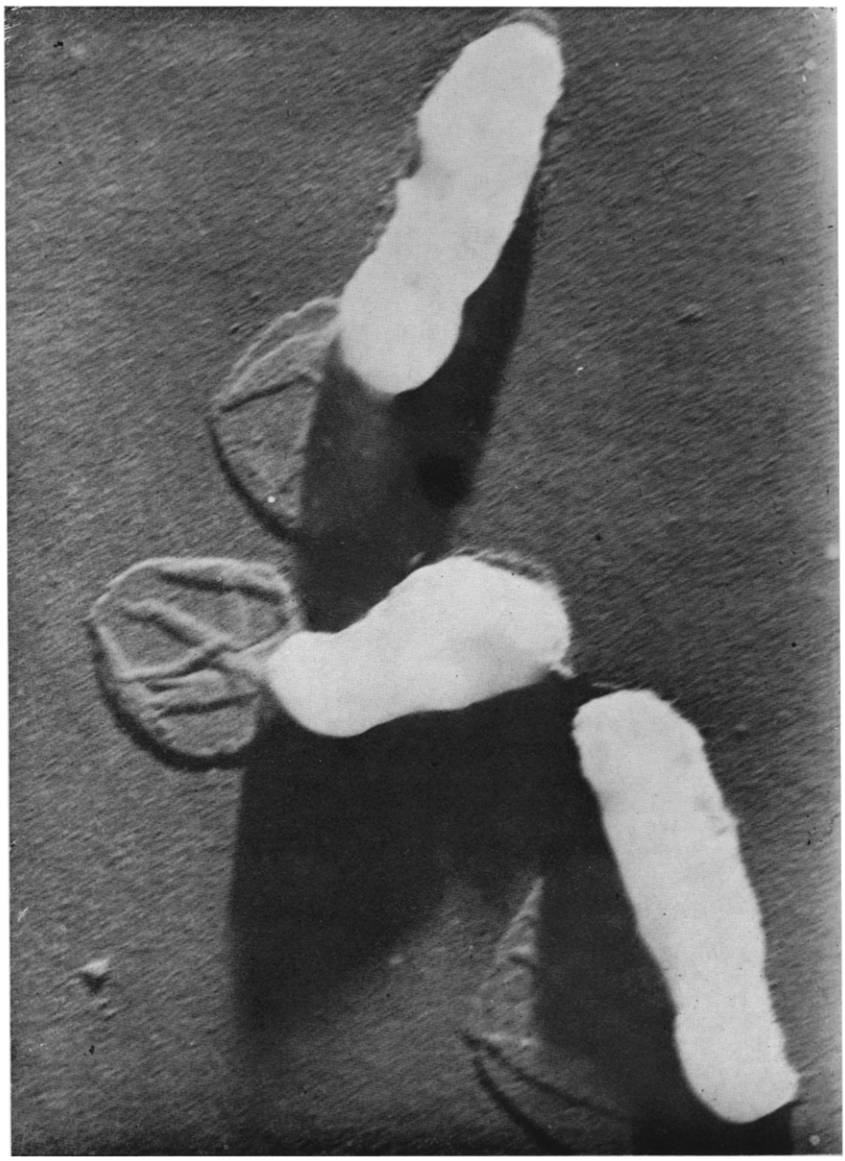

Fig. 1. *Escherichia coli* heated 5 min at 100° C. Shows ruptured cell walls adhering to cytoplasmic bodies in 3,000 r.p.m. deposit. (42,000 ×)

Fig. 2

Figs. 2 and 3. Separated cell walls from *Escherichia coli* heated at 100° C. (Fig. 2: 20,000 ×) (Fig. 3: 41,500 ×)

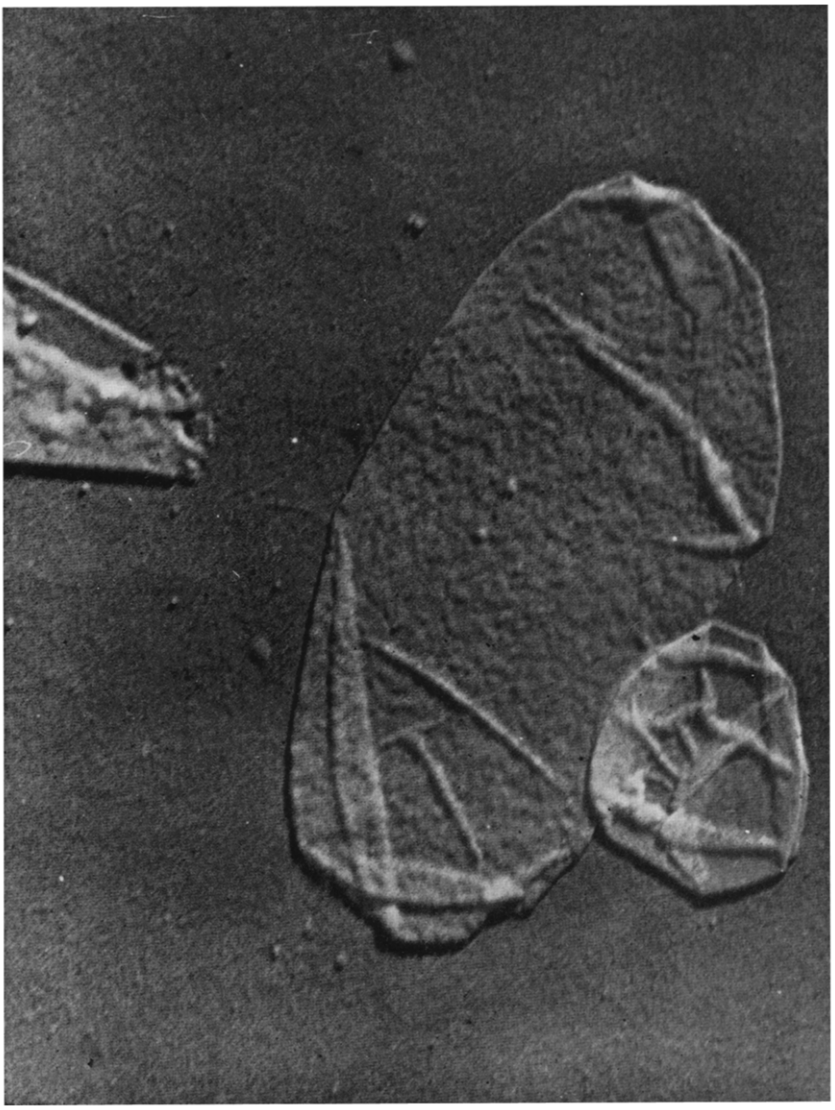

Fig. 3

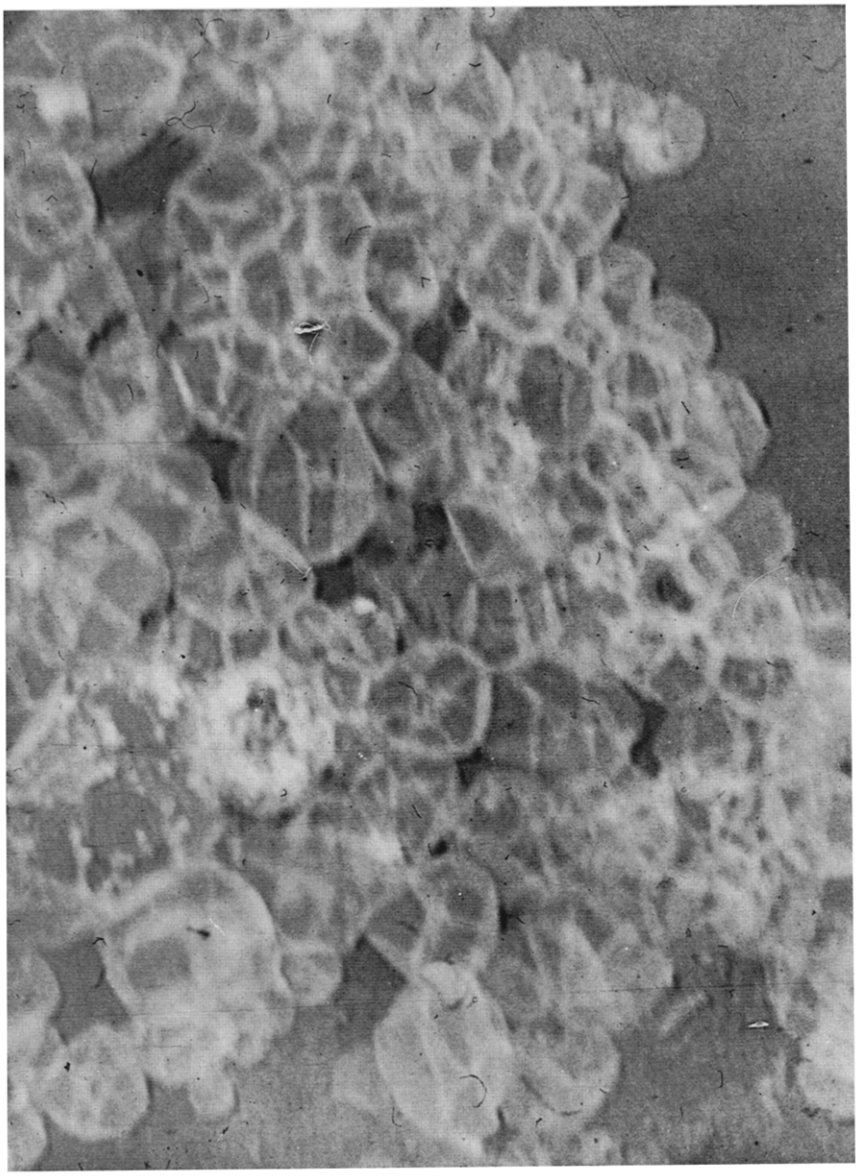

Fig. 4. Cell walls separated from *Escherichia coli* heated at 75° C as described in text. (18,000 ×).

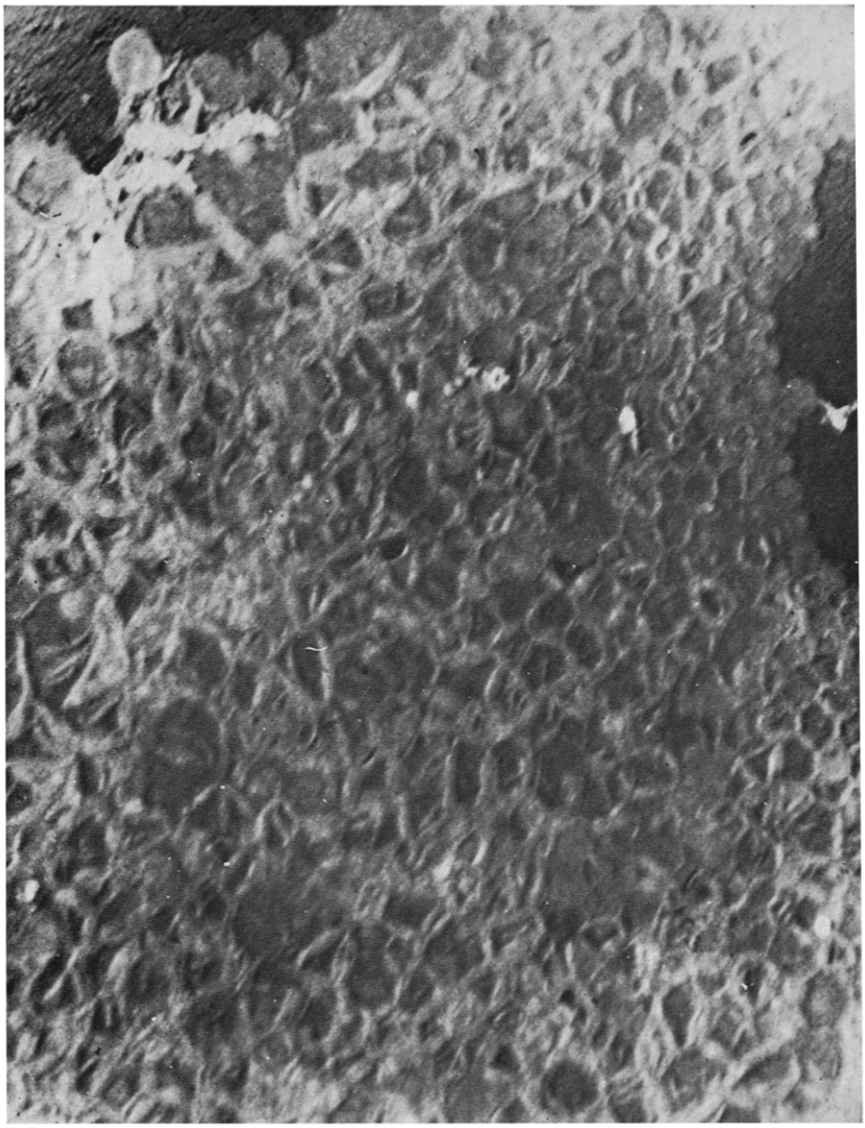

Fig. 5. Cell wall fragments separated from *Salmonella pullorum* heated at 75° C as described in text. (28,500 ×)

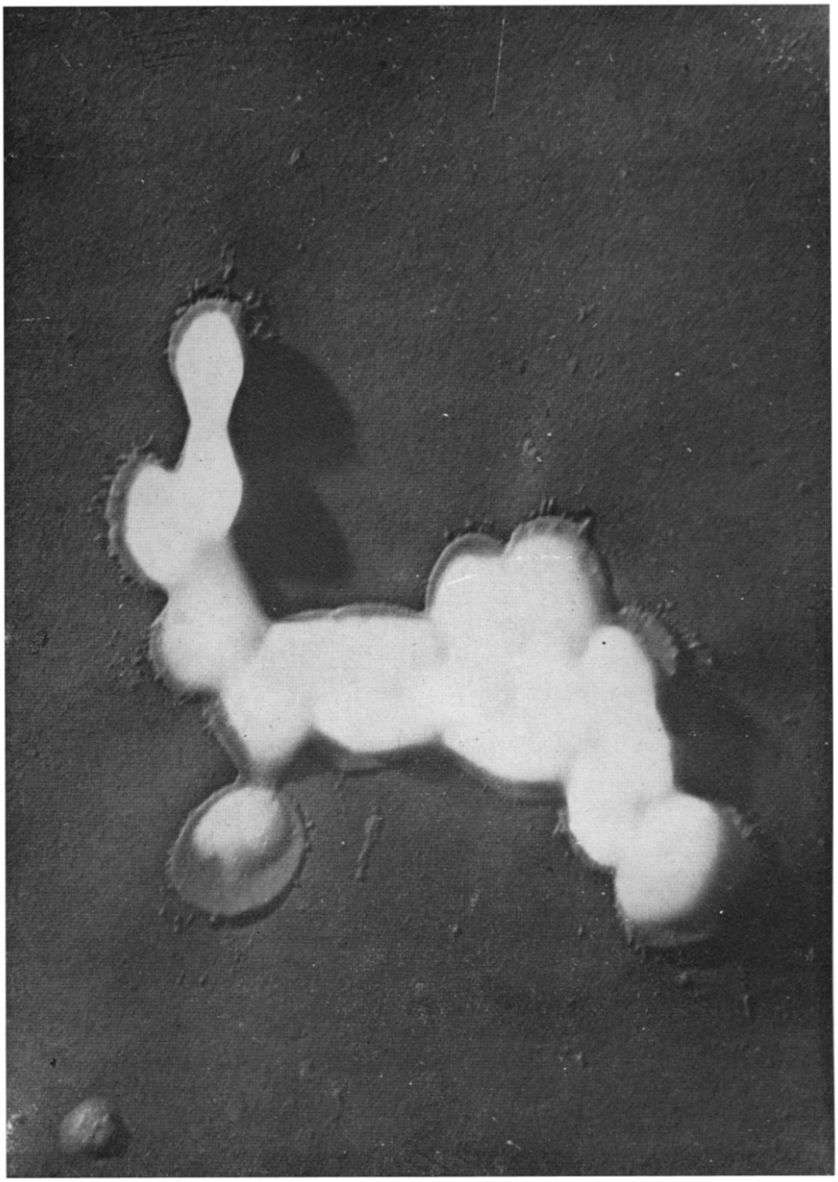

Fig. 6. Intact cells of *Streptococcus faecalis* deposited by centrifugation of MICKLE disintegrated suspension at 3,000 r.p.m. (20,000 ×)

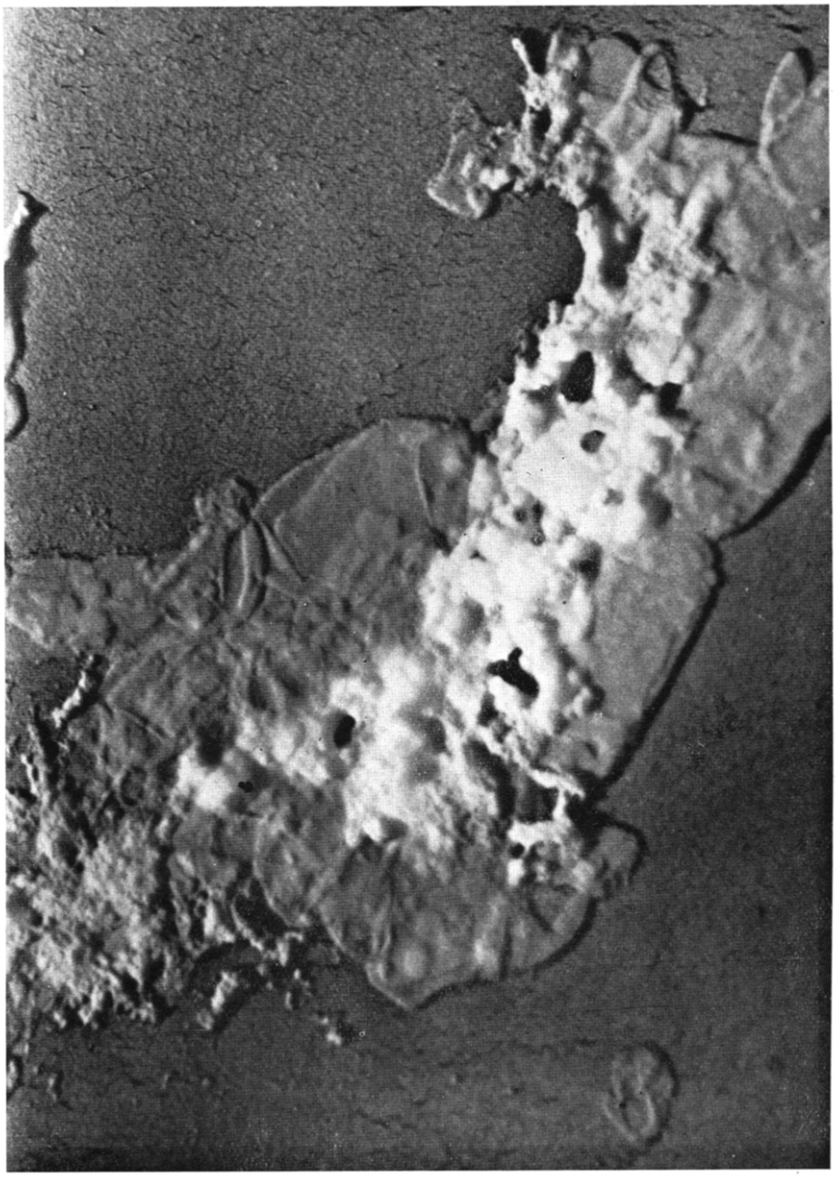

Fig. 7. Material deposited by centrifugation of MICKLE disintegrated suspension of *Salmonella pullorum* at 3,000 r.p.m. (30,000 ×)

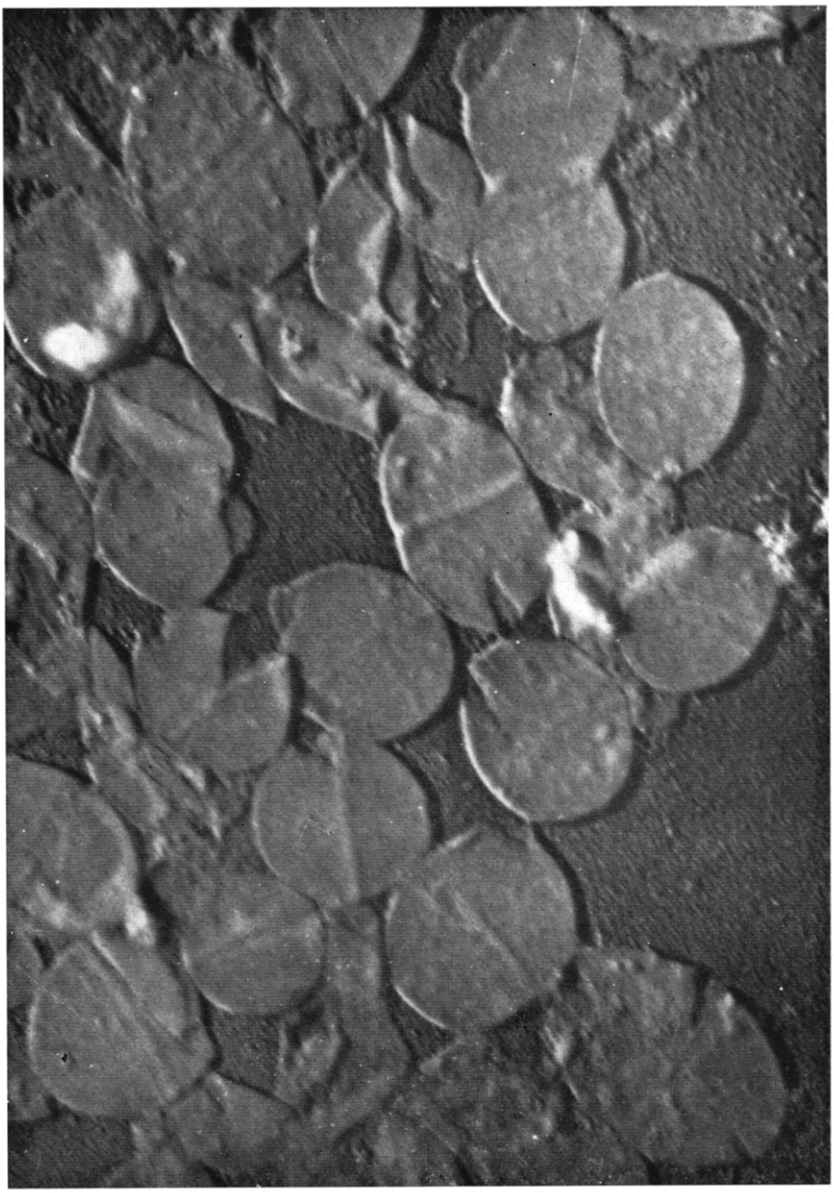

Fig. 8. Cell walls of *Streptococcus faecalis* removed from most of the cytoplasm by deposition at 10,000 r.p.m. (21,000 ×)

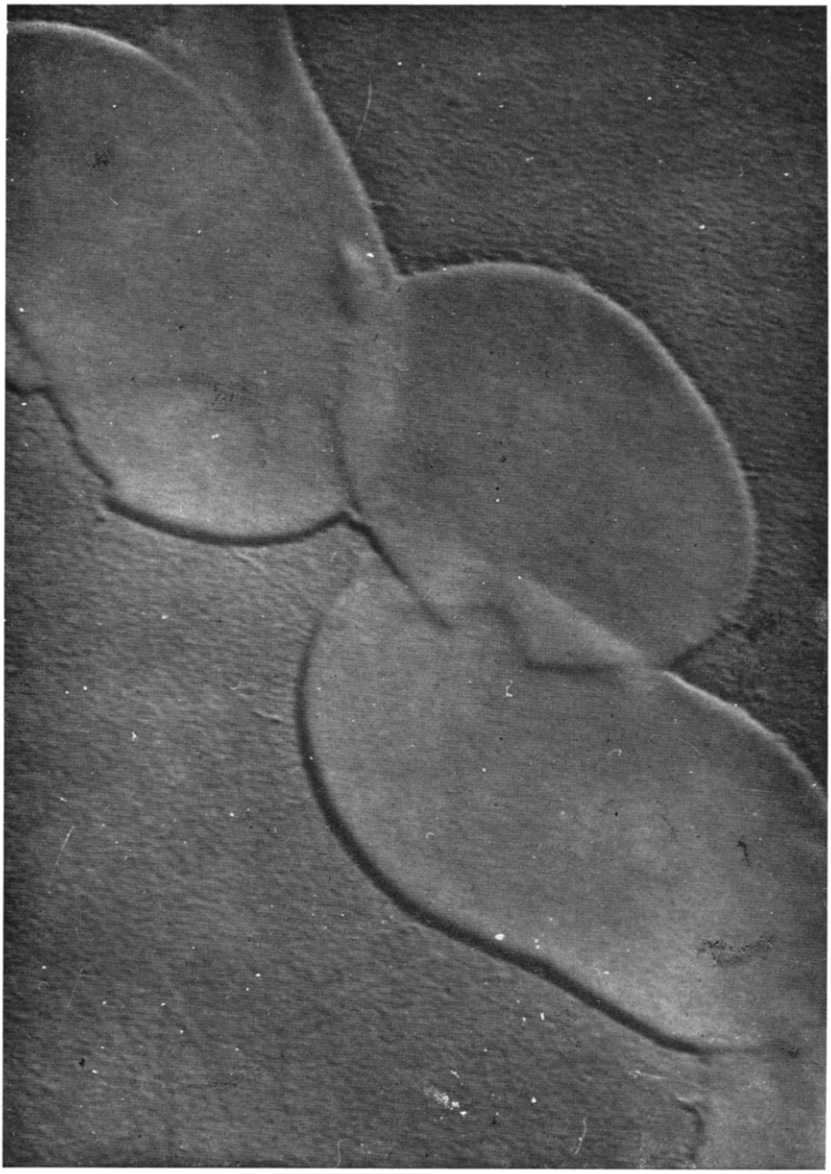

Fig. 9. Cell walls of *Streptococcus faecalis* after washing with distilled water. Electron dense material appears to have been removed from the cell walls. (48,000 ×)

Fig. 10. Cell walls and some debris deposited by high speed centrifugation of MICKLE disintegrated suspension of *Escherichia coli* as described in text. (21,000 ×)

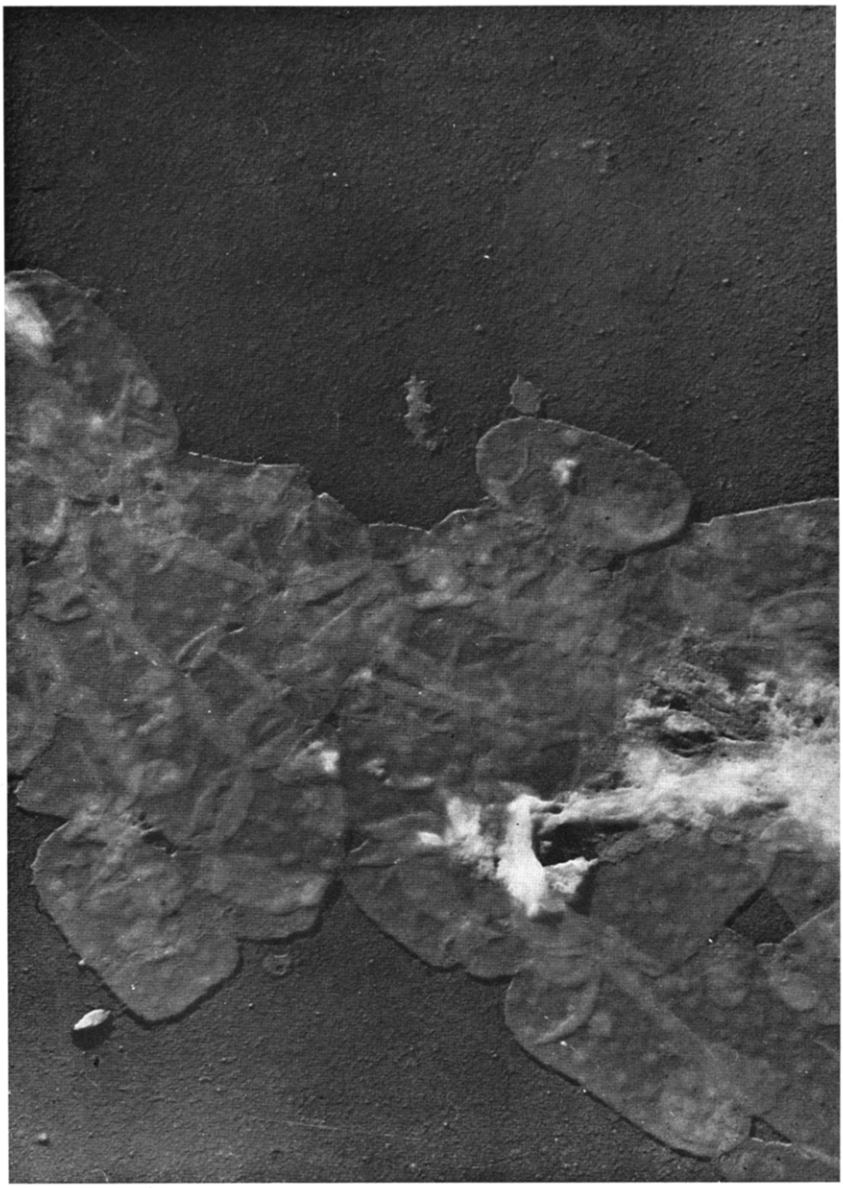

Fig. 11. Cell walls and some debris deposited by high speed centrifugation of MICKLE disintegrated suspension of *Salmonella pullorum* as described in text. (21,000 ×)

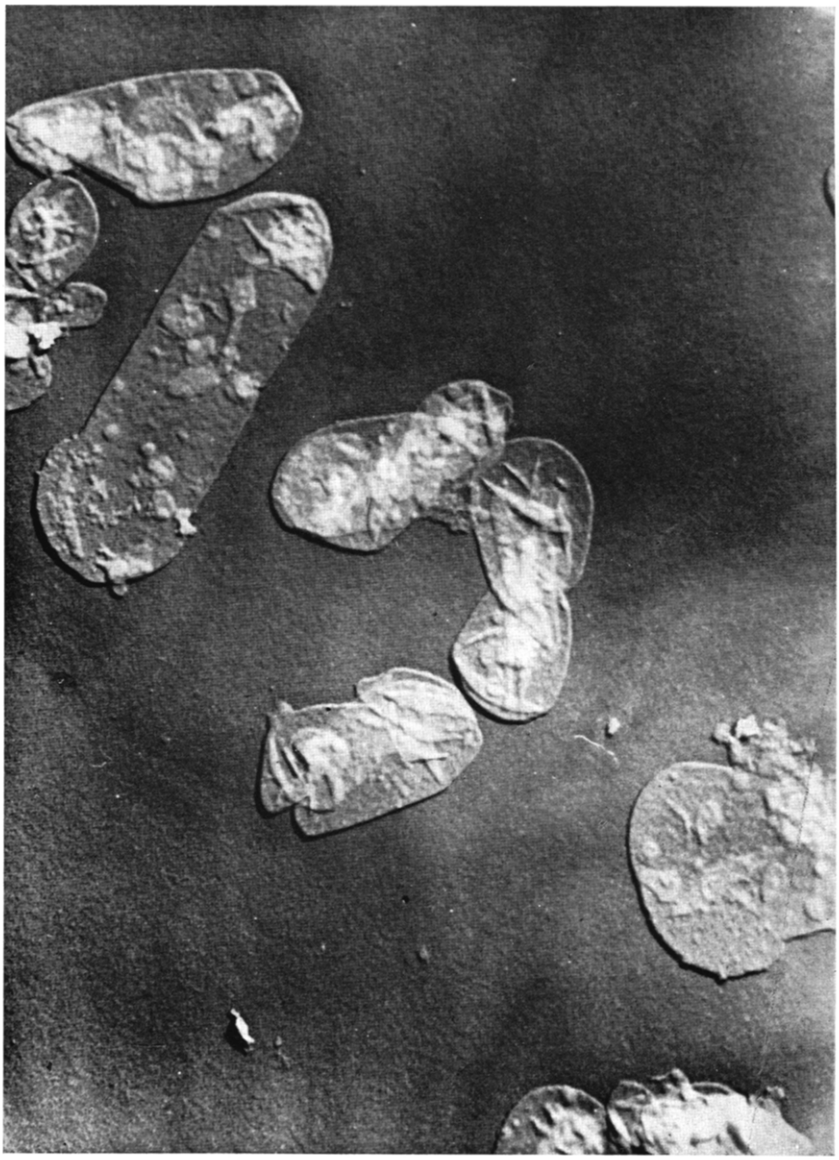

Fig. 12. Cell walls from 9 h culture of *Escherichia coli*. Compare with Fig. 10 for 16 h culture. (21,500 ×)

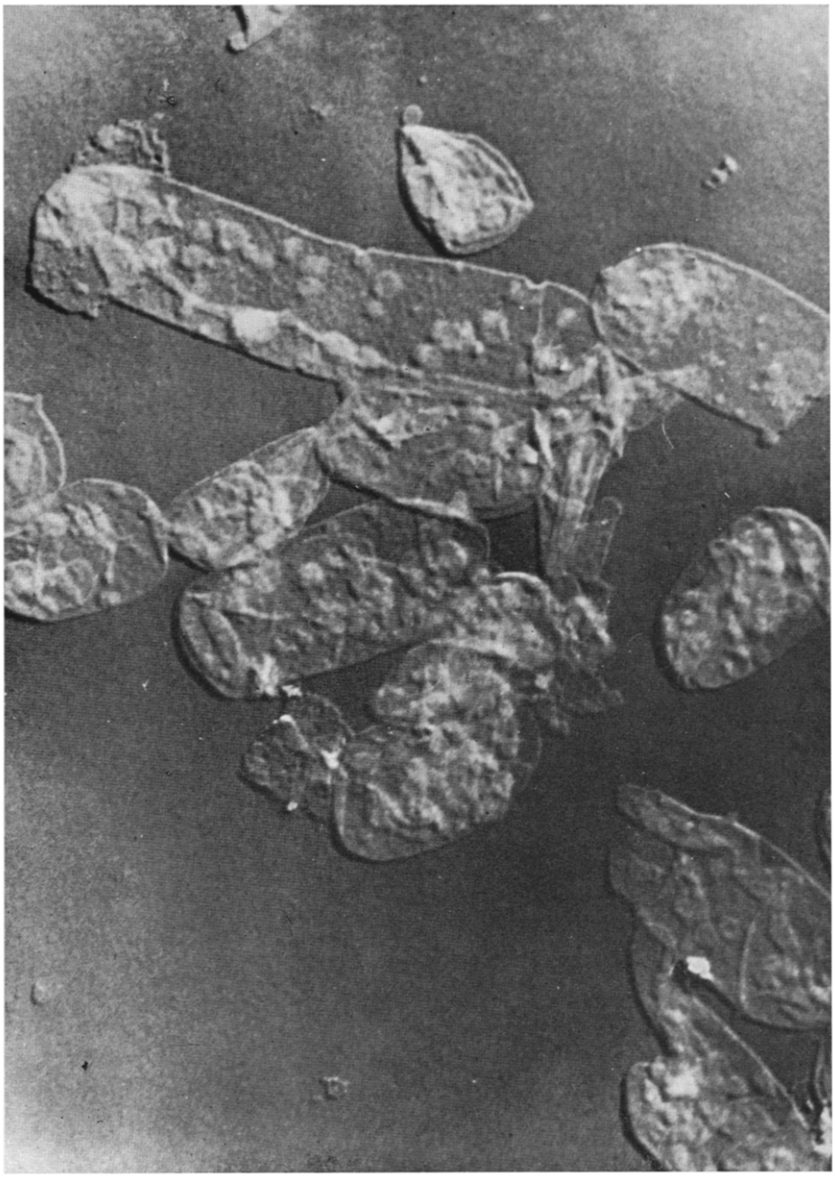

Fig. 13. Cell walls from 16 h culture of *Escherichia coli* washed thoroughly with phosphate buffer to remove adhering cytoplasmic material (compare with Fig. 10). (21,500 ×)

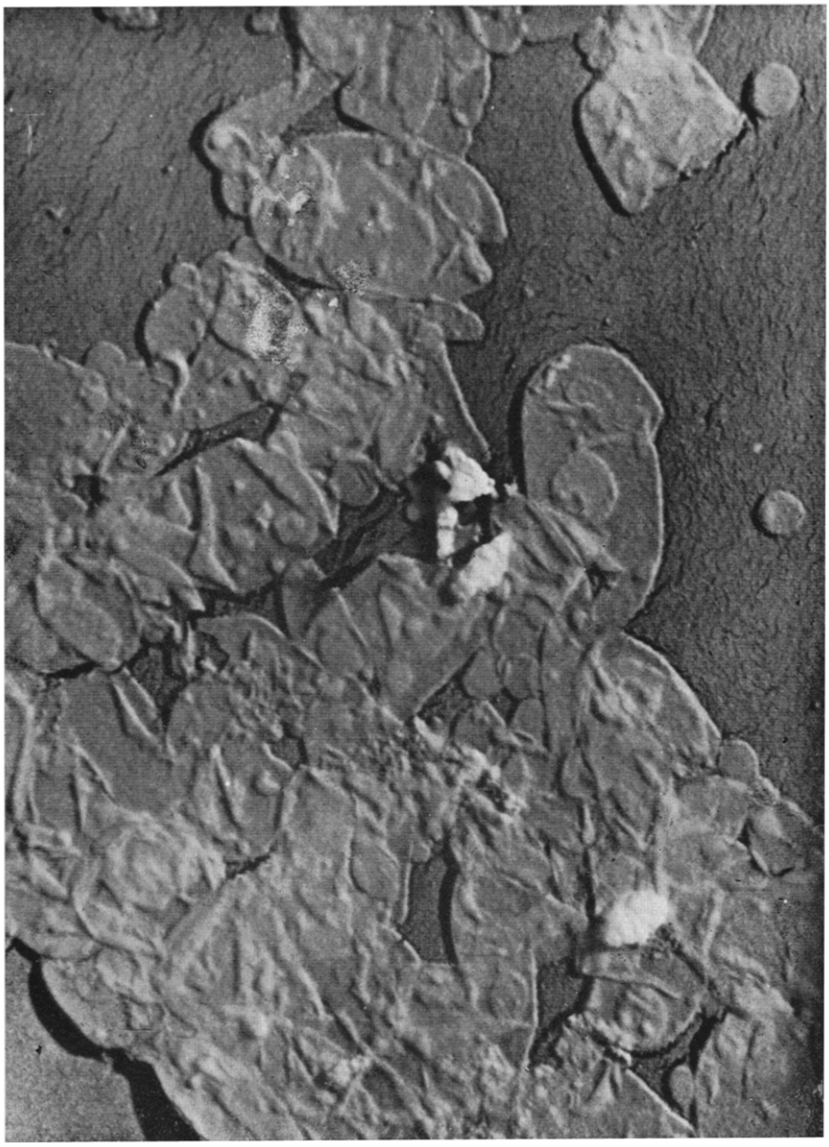

Fig. 14. Cell walls from 16 h culture of *Salmonella pullorum*, washed thoroughly with phosphate buffer to remove adhering cytoplasmic material (compare with Fig. 11). (20,000 ×)

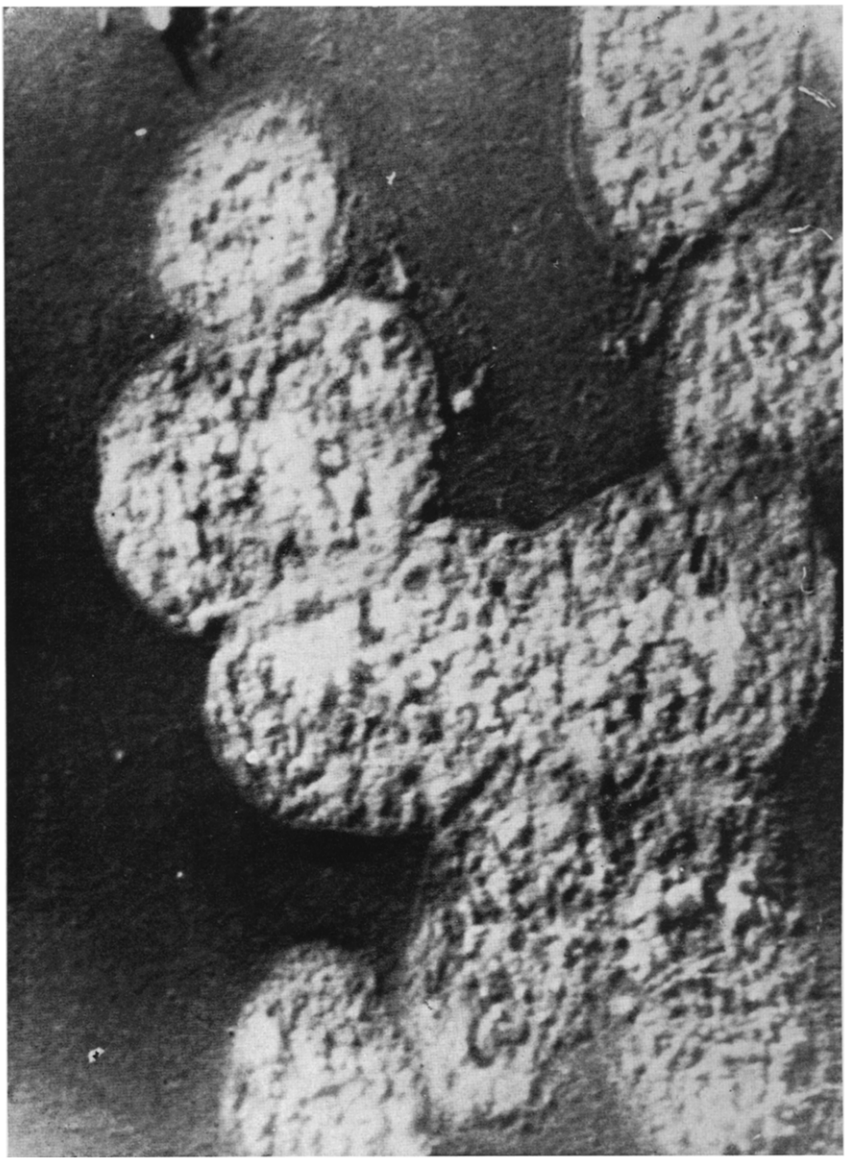

Fig. 15. Residue after extraction of *Escherichia coli* cells with *N* NaOH as described in text. (40,000 ×)

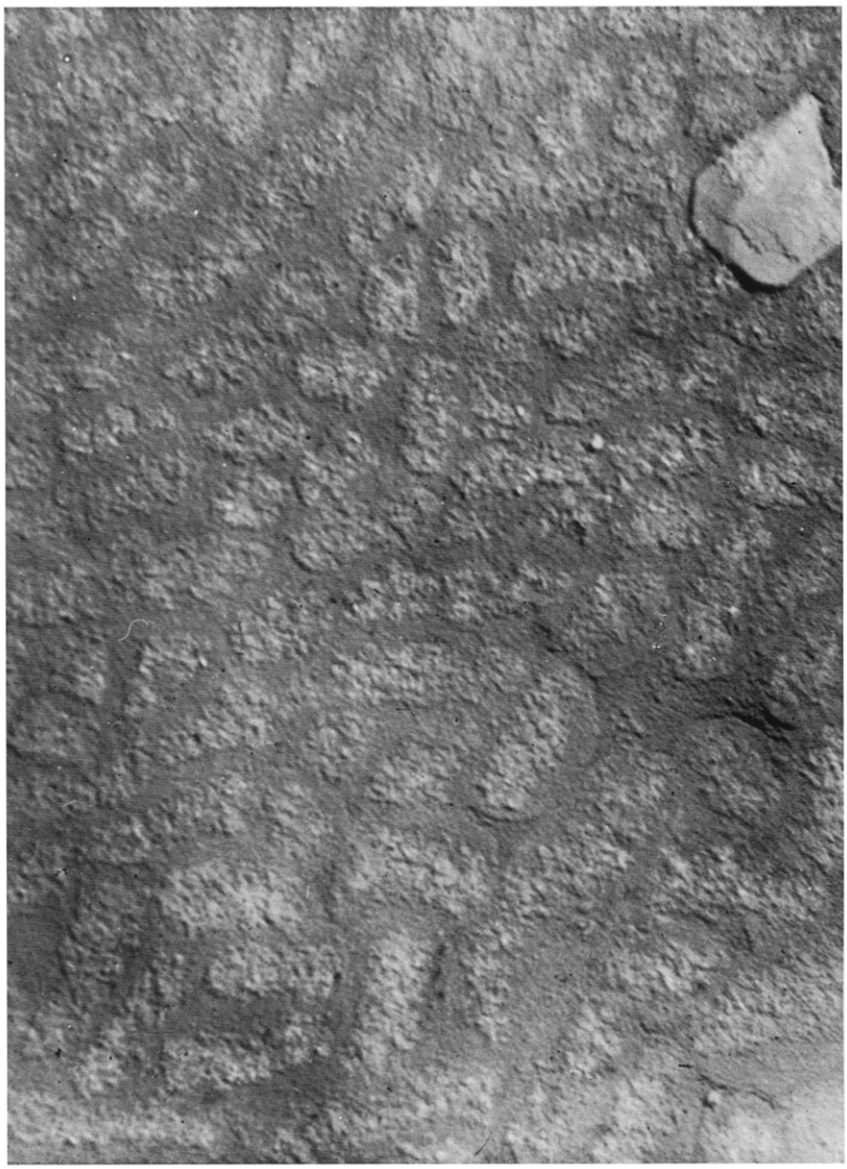

Fig. 16. Residue after extraction of *Salmonella pullorum* cells with *N* NaOH as described in text. (17,500 ×)

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SUMMARY

The preparation of pure cell walls has been followed by examination of material in the electron microscope.

1. A procedure for the preparation of cell walls by heat-treatment of *Esch. coli* and *Salmonella pullorum* has been described.

2. Preparation of pure cell walls from *Strep. faecalis*, *Esch. coli* and *Salmonella pullorum* by disintegration of the cells with minute glass beads has been investigated.

3. The alkaline resistant residue from *Esch. coli* and *Salmonella pullorum* extracted with *N* NaOH has been examined in the electron microscope. The residue differs markedly from the pure cell wall preparations.

4. Some properties of the pure cell wall suspensions and dried preparations have been recorded. Ultra-violet absorption spectra show no maximum corresponding to nucleic acid or purine-pyrimidine compounds.

RÉSUMÉ

Nous avons suivi la préparation de parois cellulaires pures par examen du matériel au microscope électronique.

1. Nous avons décrit un procédé de préparation de parois cellulaires par traitement à la chaleur de *Esch. coli* et *Salmonella pullorum*.

2. Nous avons examiné la préparation de parois cellulaires pures de *Strep. faecalis*, *Esch. coli* et *Salmonella pullorum* par désintégration des cellules par de très petites billes de verre.

3. Nous avons examiné au microscope électronique le résidu résistant à l'alcali de *Esch. coli* et *Salmonella pullorum* extraits au NaOH *N*. Ce résidu diffère considérablement de préparations de paroi cellulaire pure.

4. Nous avons enregistré quelques propriétés des suspensions de paroi cellulaire et de préparations séchées. Des spectres d'absorption u.v. ne montrent pas de maximum correspondant à des composés d'acide nucléique ou de purine-pyrimidine.

ZUSAMMENFASSUNG

Die Darstellung reiner Zellwände wurde durch Prüfung des Materials im Elektronenmikroskop verfolgt.

1. Ein Verfahren zur Darstellung von Zellwänden durch Wärmebehandlung von *Esch. coli* und *Salmonella pullorum* wurde beschrieben.

2. Die Darstellung reiner Zellwände von *Strep. faecalis*, *Esch. coli* und *Salmonella pullorum* durch Zerstörung der Zellen mit kleinen Glasperlen wurde untersucht.

3. Der alkali-resistente Rückstand von *Esch. coli* und *Salmonella pullorum* nach Extraktion mit *N* NaOH wurde im Elektronenmikroskop geprüft. Der Rückstand unterscheidet sich bedeutend von den Präparaten von reiner Zellwand.

4. Einige Eigenschaften der Suspensionen von reiner Zellwand und der getrockneten Präparate wurden verzeichnet. U.v.-Absorptionsspektren zeigen kein Nukleinsäure- oder Purin-Pyrimidin-Verbindungen entsprechendes Maximum.

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