PREPARATION AND PROPERTIES OF CELL WALLS OF THE AGENT OF MENINGOPNEUMONITIS[1, 2]

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It is recognized that microorganisms of the psittacosis group are contained within a rigid envelope (Hamre, Rake, and Rake, 1947) which is highly resistant to enzymes (Brown, Itatani, and Moulder, 1952) and mechanical disintegration (Ross and Gogolak, 1957). With the demonstration that penicillin inhibits the growth of bacteria by interfering with cell wall synthesis (Park and Strominger, 1957; Hahn and Ciak, 1957; Lederberg, 1957), the susceptibility of members of the psittacosis group to penicillin (Hurst, 1953) suggested that their outer envelopes might be analogous to the cell walls of bacteria. Since these structures have properties not encountered in other kinds of cells (Cummins and Harris, 1956; Salton, 1956; Work, 1957; Mitchell, 1959), the association of a reasonable number of these unique properties with the outer envelopes of members of the psittacosis group would be of decisive weight in establishing their evolutionary origin.

This report describes the isolation and chemical characterization of the rigid envelopes of the agent of meningopneumonitis, a typical member of the psittacosis group. These structures will from now on be called cell walls as a matter of convenience and without any immediate implication of analogy or lack of analogy with bacterial cell walls. Their serological properties will be reported elsewhere (Jenkin, Ross, and Moulder, *unpublished data*)

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[2] A preliminary account of this work has already been published (Jenkin, 1959).

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METHODS

Preparation of purified particles. The Cal 10 strain of meningopneumonitis virus was passed 2 times at limiting dilution in chick embryo yolk sac and then 8 to 10 times in the allantoic cavity before being used as an inoculum. Purified intact particles were separated from infected allantoic fluid by four cycles of high and low speed centrifugation at 0 C (Colón and Moulder, 1958). In each cycle, the particles were sedimented at 5,000 × *g* for 30 min, resuspended in 0.1 M sodium phosphate buffer, pH 7.4, and clarified at 800 × *g* for 10 min. The low speed pellet was discarded and the particles sedimented at high speed to begin the next cycle. Immediately after the final centrifugation, the chick embryo LD_{50} was measured by yolk sac titration (Moulder and Weiss, 1951), the total particle count was determined by Sharp's (1949) sedimentation method as modified by Litwin (1959), and bacteriological sterility was established by inoculation of thioglycolate broth.

Preparation of cell walls. Freshly prepared purified particles were suspended in 0.01 M phosphate pH 7.4, containing 1 per cent sodium deoxycholate (The Matheson Company, Inc.) and shaken for 4 hr at 45 C (Schaechter et al., 1957). The preparation was cooled to 0 C, centrifuged for 20 min at 8,000 × *g*, and the pellet resuspended in 0.1 M phosphate, pH 7.4, containing 1 mg per ml trypsin (two times recrystallized, in 50 per cent $MgSO_4$. Nutritional Biochemicals Corporation). The tryptic digest was shaken 1 hr at 37 C, cooled again to 0 C, and sedimented for 20 min at 8,000 × *g*. The sediment was then washed three times in distilled water to yield the final cell wall preparation which was cultured to test for bacteriological sterility and counted in the same manner as intact particles.

Electron microscopy. The electron microscope was used to count particles and to follow the course of purification of intact particles and cell walls. Both types of particles were suspended in

TABLE 1

Relations between dry weight, total nitrogen, particle count, and LD_{50}

	Dry Weight per Particle	Total Nitrogen per Particle	Total Nitrogen per Chick Embryo LD_{50}	Particles per Chick Embryo LD_{50}
	$\times 10^{-14}$ g			
No. of preparations	11	7	8	8
Intact particles	1.87 ± 0.13[a]	0.14 ± 0.01	10.0 ± 1.0	66 ± 11[b]
Cell walls	0.85 ± 0.07	0.08 ± 0.02		

[a] Mean ± standard error.

[b] Similar values have been reported by Crocker (1954) and Smith and Manire (1959).

distilled water or the volatile salt ammonium acetate. They were deposited on formvar-coated copper grids directly or by sedimenting or spraying. The samples were dried in air and examined in an RCA electron microscope, with and without chromium shadowing at an angle of 16°.

Analytical methods. Dry weights were determined by drying to constant weight at 60 C in vacuo.

Total nitrogen was determined according to Groman (1951); α-amino nitrogen according to Sobel, Hirschman, and Besman, (1945) and Speck (1949).

Phosphorus partitions were performed by Schneider's (1945) method as modified by Zahler (1953). Bartlett's (1959) conditions were used for phosphate determination.

Two-dimensional chromatography of amino acids was carried out according to Cummins and Harris (1956). Samples were hydrolyzed in 6 N HCl for 16 hr at 100 C. The solvents were phenol:water (80:20) over 1 per cent NH_4OH and NaCN followed by 2,6-lutidine:ethanol: water:diethylamine (55:25:18:2).

Total nucleic acid was measured in terms of the 260 mμ absorbancy of hot perchloric acid extracts (Ogur and Rosen, 1950). Deoxyribonucleic acid was determined by the method of Webb and Levy (1955); ribonucleic acid by that of Webb (1956).

Reducing sugars were measured according to Park and Johnson (1949) before and after hydrolysis in 2 N HCl for 2 hr at 100 C. Hexosamines were released only after 8 hr in 6 N HCl at 100 C. They were determined by the method of Dische (1955).

Qualitative lipid analyses were made by the chromatographic procedure of Hack (1953).

RESULTS

It is difficult to apply the concepts of purity and homogeneity to populations of members of the psittacosis group which are made up of particles of widely different size and internal organization (Litwin, 1959). However, the intact particle preparations were almost completely free of extraneous particles when viewed in the electron microscope and were of relatively constant composition as judged by the reproducibility of the dry weight per particle, total nitrogen per particle, total nitrogen per chick embryo LD_{50}, and particles per LD_{50} (table 1).

Disruption of particles by mechanical forces. Mechanical means of disruption are generally preferred for preparing bacterial cell walls because they minimize extraction of cell wall components during the course of purification. However, the meningopneumonitis particles were disrupted only partially or not at all in the Mickle apparatus and the French pressure cell or by alternate freezing and thawing. Sonic disintegration was not satisfactory because the particles resisted 9 kc for 3 hr and then fragmented into small pieces (Ross and Gogolak, 1957).

Effect of deoxycholate on intact particles. Since mechanical methods for particle disruption were unsuccessful, the method of Schaechter et al. (1957) for preparing cell walls of rickettsiae was next tried. As observed by these workers for *Rickettsiae mooseri*, maximal loss of optical density occurred at 45 C in the presence of 1 per cent sodium deoxycholate (figures 1 and 2). Almost all nucleic acid, as measured by the 260 mμ absorbency of perchloric acid extracts, was also lost under these conditions, and infectivity was rapidly destroyed. However, electron micrographs of deoxycholate-treated particles revealed only slight loss of electron-dense internal mate-

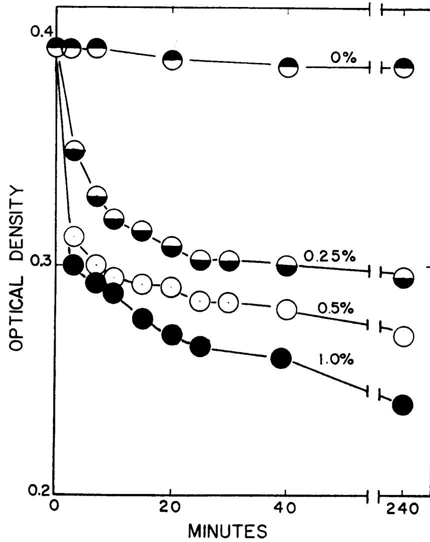

Figure 1. Effect of different concentrations of deoxycholate on the agent of meningopneumonitis. Various concentrations of deoxycholate were added to intact particles in 0.01 M phosphate buffer, pH 7.4. The mixtures were incubated at 45 C, samples removed at different times, and the optical density measured at 460 mμ. Each point represents the mean of two independent experiments.

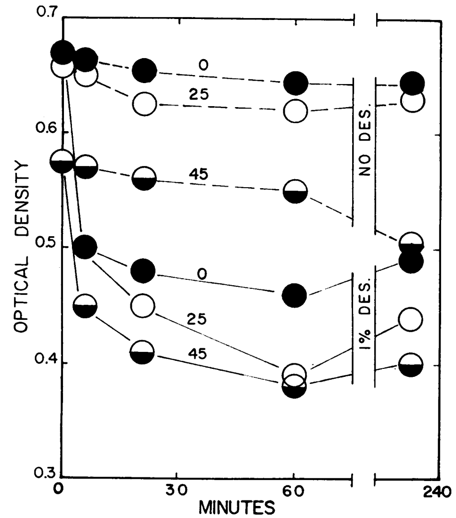

Figure 2. Effect of temperature on the agent of meningopneumonitis in the presence of 1 per cent deoxycholate. Conditions were as given in figure 1.

rial (figure 3*C*). These results are difficult to compare with those of Crocker (1956) who treated the agent of meningopneumonitis with deoxycholate under a different set of conditions and studied the effect of this reagent in different ways.

Effect of trypsin on deoxycholate-treated particles. Ribonuclease and deoxyribonuclease had no effect on deoxycholate-treated particles. However, trypsin destroyed the electron-dense internal material of these particles to yield the structures shown in figure 3*D* which are called cell walls because (1) they are rigid enough to determine the size and shape of the intact particles from which they were derived, (2) they are almost completely free of electron-dense internal material, and (3) they contain only traces of nucleic acid.

In marked contrast, untreated intact particles were unaffected by trypsin (Brown et al., 1952) (figure 3*B*). This sharp change from trypsin resistance to trypsin sensitivity suggests a chemical alteration of the cell wall by deoxycholate.

Morphology of cell walls. Purified preparations of intact particles contained two morphological types, a dense-centered particle 250 to 300 mμ in diameter and a larger, flatter particle of 350 to 1000 mμ diameter. This particle showed scattered areas of electron-dense material instead of a single central mass (figures 3*A* and 4*A*). These two kinds of particles appear to be present in mature populations of all members of the psittacosis group (see Litwin (1959) for discussion). The cell walls were greatly altered in appearance, although it was readily apparent which walls were derived from dense-centered particles and which came from flat ones (figures 3*D* and 4*B*). In forming cell walls, the dense-centered particles lost their central mass, and the residual structure resembled a collapsed and empty envelope. The flat particles became even flatter, lost their electron-dense granules, and acquired a rougher surface texture. These visible surface differences are paralleled by differences in surface charge of the two kinds of intact particles as revealed by starch electrophoresis and cellulose-anion chromatography (Jenkin and Moulder, *unpublished data*).

Relation of cell wall mass to that of the intact particles. About 30 per cent of the dry weight of intact particles was recovered in the form of cell walls, roughly the same recovery generally reported for the cell walls of bacteria. However, total particle counts showed a significant loss of

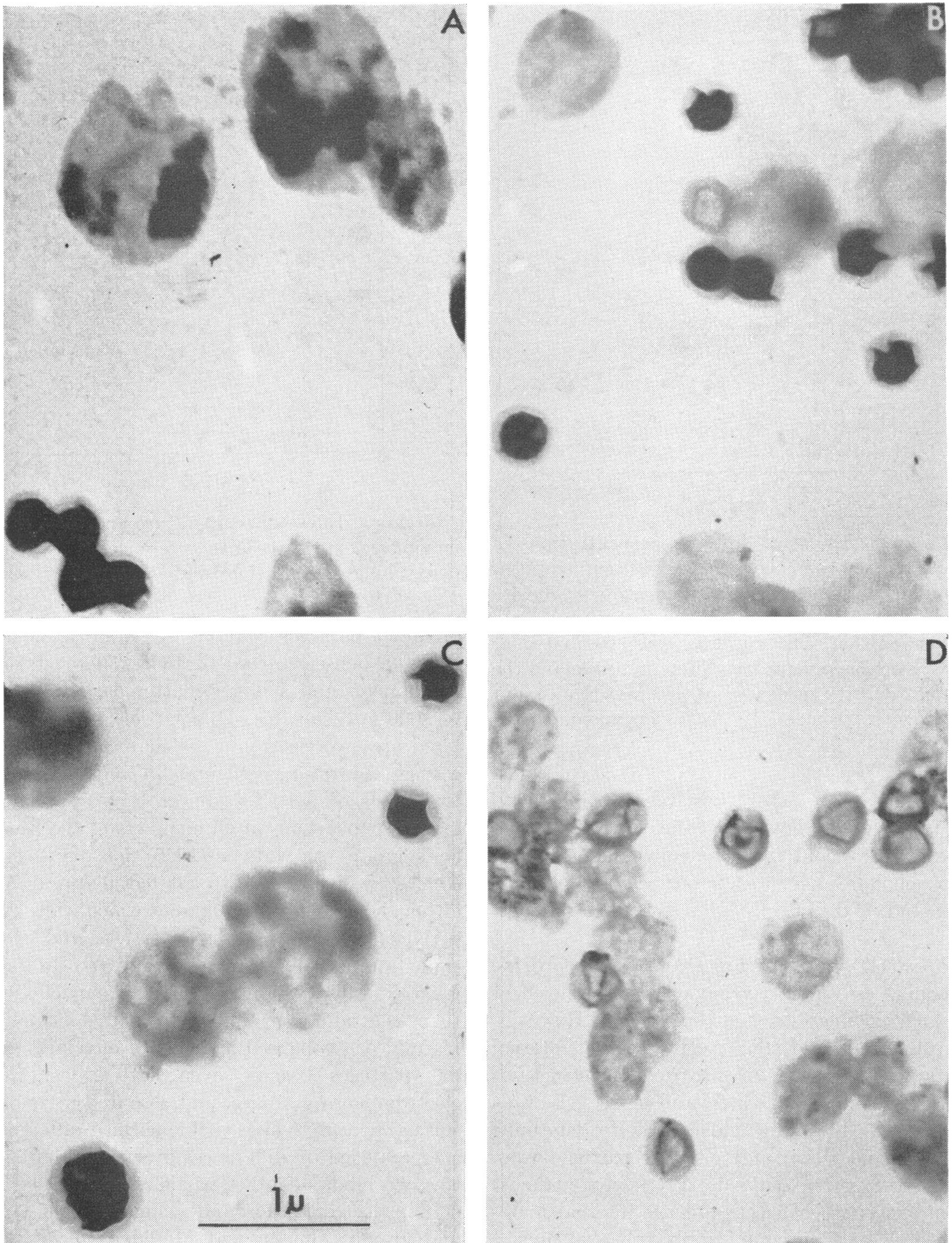

Figure 3. Electron micrographs showing the effects of trypsin and deoxycholate in the preparation of meningopneumonitis cell walls. Unshadowed air-dried preparations (×13,500). *A.* untreated intact particles; *B.* trypsin-treated particles; *C.* deoxycholate-treated particles; *D.* deoxycholate-trypsin-treated particles—the final cell wall preparation

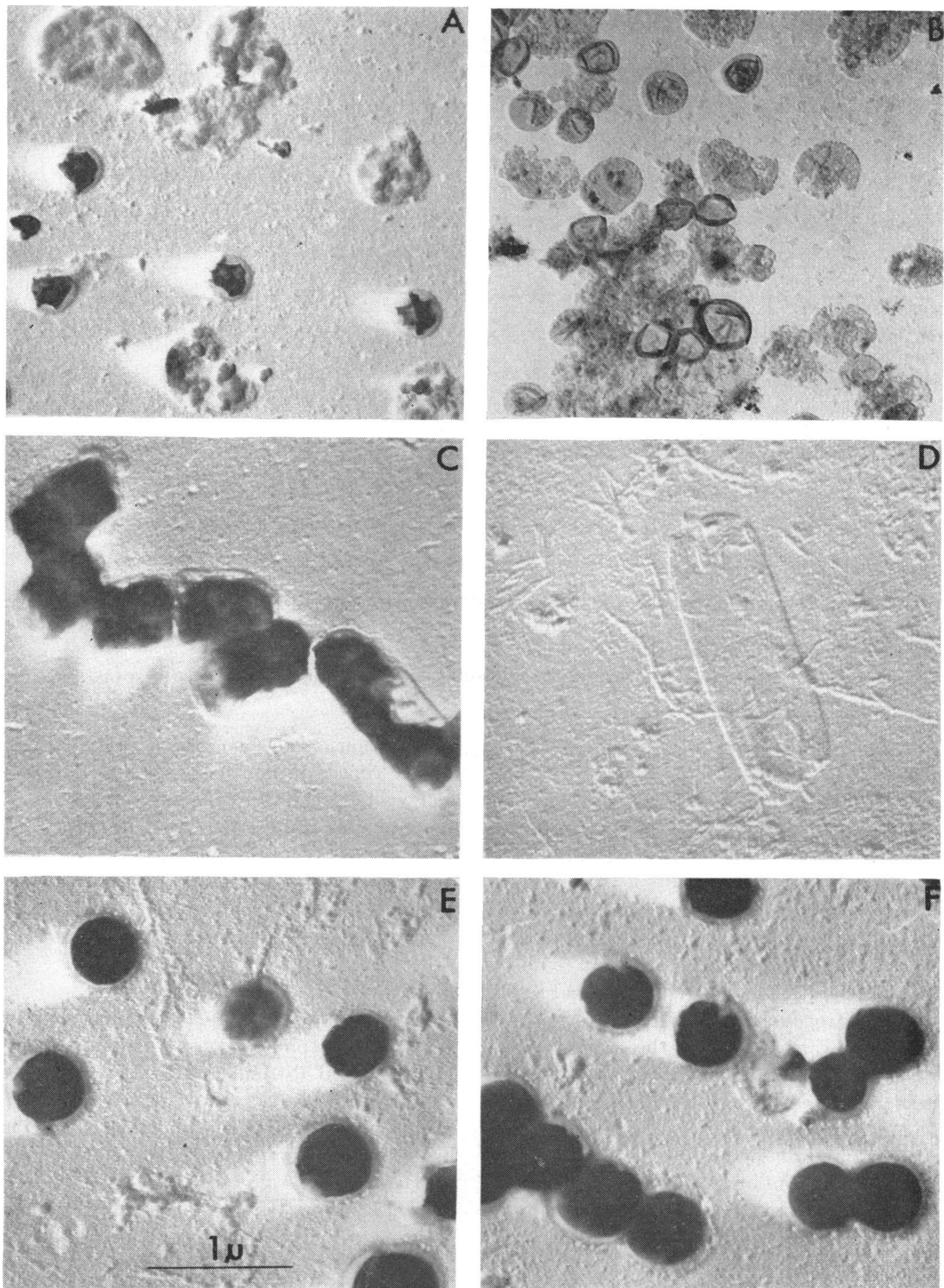

Figure 4. Electron micrographs comparing deoxycholate-trypsin-treated meningopneumonitis agent, *Escherichia coli* and *Staphylococcus aureus*. Chromium shadowed, air-dried preparations (×12,500). *A:* meningopneumonitis—intact particles; *B:* meningopneumonitis—deoxycholate-trypsin-treated particles (cell walls); *C: E. coli*—intact cells; *D: E. coli*—deoxycholate-trypsin-treated cells; *E: S. aureus*—intact cells; *F: S. aureus*—deoxycholate-trypsin-treated cells.

TABLE 2

Chemical composition of intact particles and cell walls

Constituent	No. of Preparations	Dry Weight	
		Intact particles	Cell walls
		%	%
α-Amino acid nitrogen	3	5.30 ± 0.15[a]	5.20 ± 0.31
Total nitrogen	3	6.50 ± 0.40	7.20 ± 0.30
Protein[b]		33.1	32.5
Hexosamine	3	0.62 ± 0.04	0.44 ± 0.05
Reducing sugar (hydrolyzed)	4	1.05 ± 0.05	1.14 ± 0.02
Muramic acid	2	Trace	Trace
Carbohydrate[c]		1.67	1.58
Ribonucleic acid	3	2.50 ± 0.08	Trace
Deoxyribonucleic acid	3	3.40 ± 0.06	Trace
Total nucleic acid	3	5.62 ± 0.30	0.40 ± 0.10
Phospholipid[d]	3	7.5	1.5

[a] Mean ± standard error.

[b] Calculated by multiplying the α-amino acid nitrogen by a factor of 6.5.

[c] Calculated from the sum of the hexosamine and reducing sugar; hydrolytic conditions for reducing sugar were insufficient to release hexosamine.

[d] Calculated from the alcohol-ether soluble phosphorus fraction by multiplying by a factor of 25.

particles during cell wall preparation. When the recovered cell wall weight was corrected for this loss, the yield was increased to 45 per cent of the dry weight of intact particles (table 1). The corrected recovery of total nitrogen was also about 45 per cent.

Chemical properties of intact particles and cell walls. Table 2 compares analytical results on intact particles and cell walls. The two kinds of particle preparation gave almost identical results for total nitrogen and α-amino nitrogen. The total protein calculated from the α-amino nitrogen was lower than comparable values for bacteria.

The amino acid composition of cell walls is of particular interest because gram-positive bacterial cell walls contain as few as 3 amino acids, most frequently alanine, lysine (or diaminopimelic acid), and glutamic acid, whereas gram-negative walls yield most of the amino acids commonly found in proteins (Salton, 1956; Work, 1957). Twelve to 14 amino acids were positively identified in hydrolyzates of intact particles and only 9 to 10 in cell walls. Aspartic acid, alanine, glutamic acid, glycine, and methionine/valine were present in highest concentrations in both preparations. Isoleucine/leucine was found in higher concentration in intact particles than in cell walls. Small amounts of threonine, serine, and phenylalanine appeared in both intact particles and cell walls, whereas traces of tyrosine and proline were found only in intact particles. A small amount of lysine, but no diaminopimelic acid, was detected in both intact particles and cell walls.

Hexosamines and reducing sugars were present in low and approximately equal concentrations in both preparations. A sensitive test for muramic acid (Strominger, J. L., *personal communication*) revealed a trace of this amino sugar in both intact particles and cell walls. This is of interest since muramic acid has been found only in bacterial cell walls.

Table 3 shows the results of a phosphorus partition according to Schneider (1945). The acid-soluble phosphorus fraction was markedly variable, despite repeated washing of the particles in distilled water to remove inorganic phosphate. This probably reflects the lability of the acid-soluble fraction demonstrated by Zahler and Moulder (1953) in experiments with radioactive phosphate. If the acid-soluble fraction is disre-

garded, then relatively constant phosphorus values were obtained for the other three fractions. The total nucleic acid phosphorus content of intact particles was 0.37 per cent, equivalent to 4 per cent nucleic acid. This is a little lower than the total nucleic acid value of 5.8 per cent given in table 2, probably because ribonucleic acid was lost during the extra washing in distilled water (Rappaport et al., 1960). The cell walls contained only 0.04 per cent nucleic acid phosphorus. Thus, this determination, measurement of 260 mμ absorbancy and estimation of ribonucleic acid and deoxyribonucleic acid by specific color tests, all indicated the almost complete absence of nucleic acid from the cell wall preparations.

The lecithin equivalent of the phospholipid phosphorus was 7.5 per cent for intact particles and 1.5 per cent for cell walls (table 3). The loss of phospholipid during cell wall preparation probably occurred during the deoxycholate treatment and was associated with the loss of group-specific complement-fixing antigen because the phospholipid containing group antigen (Ross and Gogolak, 1957) was quantitatively recovered in the deoxycholate extract (Jenkin, Ross and Moulder, *unpublished data*). Present data do not allow a definite decision as to whether the phospholipid and the group antigen were extracted from the endoplasm of the particles or from their cell walls, but the change in sensitivity to trypsin after deoxycholate treatment suggests that the phospholipid came from the cell walls.

Filter paper chromatography (Hack, 1953) showed the absence of detectable amounts of cholesterol and the presence of other nonphosphorus lipids such as neutral fat in both cell walls and intact particles. Attempts to estimate total lipid by direct extraction with lipid solvents gave erratic results, probably because of the very small amounts of material available for study. However, there is no doubt that both intact particles and cell walls contain large amounts of lipid. Only about 50 per cent of the dry weight of either type of preparation is accounted for by the analyses reported in table 2; most of the remainder is probably lipid of as yet unknown composition.

TABLE 3

Phosphorus partition in intact particles and cell walls

Fraction	Dry Weight	
	Intact particles	Cell walls
	%	%
Acid-soluble P	1.44 ± 0.25[a]	0.08 ± 0.02
Alcohol-ether soluble P	0.30 ± 0.02	0.06 ± 0.02
Nucleic acid P	0.37 ± 0.03	0.04 ± 0.01
Phosphoprotein P	0.12 ± 0.02	0.04 ± 0.00
Total P (sum of fractions)	2.23 ± 0.12	0.22 ± 0.01
Total P (direct determination)	2.05 ± 0.07	0.20 ± 0.00

[a] Mean ± standard error of 3 independent preparations.

Effect of deoxycholate on meningopneumonitis virus and bacteria. With the finding that cell walls can be obtained from rickettsiae (Schaechter et al., 1957) and from the psittacosis group by treatment with deoxycholate, it became of interest to see what effect this reagent would have on typical bacteria. Therefore, gram-positive *Staphylococcus aureus* (Oxford strain) and gram-negative *Escherichia coli* (strain B) were inoculated into veal infusion broth, incubated 18 hr at 37 C, washed three times with 0.1 M phosphate buffer, pH 7.4, and treated with deoxycholate and trypsin as in the preparation of meningopneumonitis cell walls. Extremely thin and fragile cell walls were obtained from *E. coli* (figures 4*C* and 4*D*), but *S. aureus* was not visibly affected by the treatment (figures 4*E* and 4*F*).

DISCUSSION

The isolation of cell walls from the agent of meningopneumonitis has provided a new approach to the study of the structure, chemistry, and serology of the psittacosis group. These results and those reported elsewhere (Jenkin et al., *unpublished data*) on the serology of intact particles and cell walls should be repeated as soon as possible on cell wall preparations obtained by purely physical means, so that the question of chemical alteration of the cell wall during preparation will not arise. Such a method for obtaining cell walls would also yield the endoplasmic contents of the particles in a form suitable for chemical examination. A search for an effective method for physically disrupting meningopneumonitis particles is now in progress.

The discovery of appreciable quantities of diaminopimelic acid, muramic acid, or teichoic acid (Armstrong et al., 1958) in meningopneu-

monitis cell walls would have been definite evidence of a relation to bacteria. The trace of muramic acid found in intact particles and cell walls is most interesting, but further study is required to assess its significance. In the absence of such clear-cut findings, the relationship becomes more difficult to discern. It is at once obvious that the meningopneumonitis cell walls are far too complex in chemical composition to be compared with the cell walls of gram-positive bacteria. They more closely resemble the cell walls of rickettsiae and gram-negative bacteria. However, they contain less carbohydrate and more lipid than the gram-negative walls so far analyzed, and their chemical composition is also clearly different from the cell walls of *R. mooseri* (Schaechter et al., 1957).

These results, while not conclusive in themselves, add to the growing weight of morphological (Litwin, 1959), enzymological (Colón, 1960), and genetic (Greenland, 1959) evidence that the members of the psittacosis group resemble the bacteria in most of their fundamental properties.

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SUMMARY

Treatment of purified preparations of the agent of meningopneumonitis with deoxycholate and trypsin yielded structures which are called cell walls because they retained the size and shape of the intact particles, contained only traces of nucleic acid, and were almost completely free of electron-dense internal material. The morphological and chemical properties of the cell walls were compared with those of the intact particles. Morphologically, the cell walls appeared as collapsed envelopes devoid of the electron-dense masses characteristic of the intact particles. Chemically, they were similar to intact particles, but differed from them in having almost no nucleic acid, a different amino acid composition, and less phospholipid. In some chemical properties and in reaction to deoxycholate, meningopneumonitis cell walls resembled those of rickettsiae and gram-negative bacteria.

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