*Onderstepoort J. vet. Res.* 38 (2), 83-92 (1971)

# IMMUNOLOGICAL AND CHEMICAL CHARACTERISTICS OF *CORYNEBACTERIUM PSEUDOTUBERCULOSIS* CELL WALLS AND PROTOPLASM

C. M. CAMERON and MARY R. PURDOM, Veterinary Research Institute, Onderstepoort

ABSTRACT

CAMERON, C. M. & PURDOM, MARY, R. Immunological and chemical characteristics of *Corynebacterium pseudotuberculosis* cell walls and ptotoplasm. *Onderstepoort J. vet. Res.* 38 (2), 83-92 (1971).

Successive extraction of purified *Corynebacterium pseudotuberculosis* cell walls with ether: ethanol, cold and hot trichloroacetic acid and crystalline trypsin did not destroy their immunizing potency.

Cell walls were not susceptible to the action of lysozyme unless pre-treated with trichloroacetic acid and NaOH. Treatment with NaOH, however, destroyed the immunizing properties.

Protoplasm also contains immunizing antigen but because of the insolubility of the antigen it could not be purified and characterized.

By gel diffusion it was found that cell walls and a protoplasm fraction contain a common antigen. It could not, however, be proved that this antigen is exclusively responsible for inducing a protective immunity.

## INTRODUCTION

The cell wall of Gram positive bacteria is composed of a mucopeptide mesh which consists of layers of alternating units of N-acetyl muramic acid and N-acetyl glucosamine. These layers are bound together by tetrapeptide bridges and other peptide cross linkages. The latter may occur in a variety of forms (Perkins, 1963; McCarty & Morse, 1964; Martin, 1966; Strominger & Ghuysen, 1967, Ghuysen, 1968).

Other components are intimately associated with this basic structure or may occur more superficially. Many of these substances are responsible for certain antigenic and serological properties of the organisms concerned and some of them have been well characterized and even isolated in pure form. They may be polysaccharides such as the C polysaccharides of group A and other streptococci (Heyman, Manniello & Barkulis, 1963; Montague & Knox, 1968), or teichoic acids that have been found in staphylococci (Haukenes, Elwood, Baddiley & Oeding, 1961; Morse, 1962) as well as in streptococci (Wicken, Elliot & Baddiley, 1963) and other bacteria (Armstrong, Baddiley & Buchanan, 1960). The cell walls may also contain uronic acids (Haskell & Hanessian, 1963; Morse, 1965; Cummings, 1965), proteins (Lancefield, 1962; Grov, Myklestad & Oeding, 1964; Fox & Wittner, 1965; Forsgren & Sjöquist, 1969) as well as lipids (Stöss & Herrmann, 1965).

Much is known about the antigenic and serological properties of many of these antigens and the mucopeptide itself is also antigenic (Kalf & White, 1963; Karakawa & Krause, 1966; Karakawa, Lackland & Krause, 1966; Rolicka & Park, 1969) but with few exceptions little is known about their biological activity in respect to their role in pathogenesis or ability to induce a protective immunity. In certain instances, however, particular roles have been ascribed to certain antigens. Thus it is widely recognised that the M proteins of streptococci protect this organism from phagocytosis (Lancefield, 1962) and teichoic acids in staphylococci may have a similar role (Mudd, Yoshida, Li & Lenhart, 1963; Cameron, 1969). A protein has been isolated from *Listeria monocytogenes* (Murray, Webb & Swann, 1926) with which animals can be immunized (Klasky & Picket, 1968). The immunological properties of mycobacterial antigens have also been studied intensively (Ribi, Anacker, Barclay, Brehmer, Middlebrook, Milner & Tarmina, 1968) and considerable information has been accumulated on the immunological properties of *Bordetella pertussis* (Holland, 1920; Munoz, 1963) and *Salmonella* spp. (Lüderitz, Staub & Westphal, 1966). The latter two organisms are, however, both Gram negative and differ in certain essential aspects from Gram positive bacteria (Bisset & Vickerstaff, 1967).

In a previous paper it was shown that the cell walls of *Corynebacterium pseudotuberculosis* (Buchanan, 1911) possess an antigen which will stimulate immunity in mice. Furthermore, preliminary experiments indicated that, as the antigen could not be extracted with ether: ethanol (E:E), cold trichloroacetic acid (CTCA) or formamide, it is probably an integral part of the cell wall (Cameron, Minnaar & Purdom, 1969). An attempt was therefore made to prepare soluble products from the cell walls by a variety of methods and to assay their biological activity.

Essentially our approach was sequential exposure of purified cell walls to a series of chemical extraction procedures which would not affect their antigenicity, followed by subjection to the effect of mucolytic enzymes. The yield of soluble material was expressed on a dry weight basis and the soluble material and residual insoluble material assayed for its ability to induce immunity in mice.

Apart from E:E, cold TCA and formamide, which were employed in our previous study, other standard methods for extraction of antigens were used. Acids have been widely used for extracting polysaccharides and proteins, particularly from streptococci (Lancefield, 1962; Fox & Wittner, 1965), while wide use has been made of enzymes for studies on the antigenic composition and serological activity of mucopeptides (Allsop & Work, 1963; Karakawa, Lackland & Krause, 1966; Karakawa *et al.*, 1967; Hughes, 1968 and Montague & Knox, 1968). Mucopeptides may be digested by a great variety of enzymes but lysozyme and enzymes derived from *Streptomyces albus* (Rossi-Doria, 1891, *emend.* Krainsky, 1914) are most widely used (Ghuysen, 1968). Not all bacteria are directly susceptible to the action of these enzymes and have to be pre-treated with lipophilic agents, detergents or alkali (Strominger & Ghuysen, 1967; Klasky & Picket, 1968; Azuma & Yamamura, 1969).

Crystalline trypsin has been used to prepare active antigens from mycobacteria (Crowle, 1969) and has also been used to remove proteins from mucopeptides (Park & Hancock, 1960).

Received 4 March 1971 - Editor.

## Materials and Methods

### *Preparation of cell walls*

Purified cell walls (PCW) were prepared exactly as described previously (Cameron, Minnaar & Purdom, 1969). The cell walls obtained from 25g wet whole cells constituted a batch.

### *Chemical extraction of cell walls*

Extractions of purified cell walls with E:E, cold TCA and formamide were done as described by Cameron, Minnaar & Purdom (1969) while extraction with butanol was done as described by Rega, Weed, Reed, Berg & Rothstein (1967) and phenol:acetic acid:water (PAW) extraction as described by Rottem & Razin (1967).

Acid extraction with $H_2SO_4$ was done as described by Montague & Knox (1968) for the extraction of *Streptococcus salivarius* polysaccharide. A batch of purified cell walls previously extracted with E:E and cold TCA was extracted six times in 20 ml of 0,1 N $H_2SO_4$ at 60°C. The combined supernatants were dialyzed against distilled water, pervaporated and lyophilized.

Treatment of cell walls with alkali was done by suspending batches of cell walls pre-treated in different ways in 20 ml of either 0,001, 0,01, 0,1 or 1,0 N NaOH. The suspensions were either heated at 100°C for 10 min or kept at 37°C for 18 h. The residual cell walls were removed by centrifugation at 10 000 *g* for 20 min., washed twice with distilled water and lyophilized. The supernatant fluid was dialyzed against distilled water, pervaporated and lyophilized.

Cell walls were treated with sodium dodecyl sulphate (SDS) by suspending them in 5 ml of a 2 per cent solution of SDS in distilled water. The suspension was stirred for 18h at 37°C, centrifuged and dried as for the NaOH extracts.

Extractions with hot trichloroacetic acid and with crystalline trypsin were done according to the methods outlined by Park & Hancock (1960).

### *Treatment of cell walls with mucolytic enzymes*

(a) *Lysozyme:* The method used for treating cell walls with lysozyme was based on the procedure described by Allsop & Work (1963). A hundred mg dry cell walls or a batch of wet cell walls were suspended in 5 ml distilled water pH 6,5. The pH of the water was adjusted with either 0,01 N HCl or 0,01 N $NH_4OH$ as required. To this suspension was added 1,0 ml of a solution containing 1 mg/ml lysozyme chloride (Seravac)*. It was incubated at 37°C for 24 to 48 h.

(b) *Streptomyces albus enzyme* (SA): Cell walls were treated with SA enzyme (Difco)** essentially as described by Montague & Knox (1968). A batch of cell walls or 30 mg dry cell walls was suspended in 5,0 ml 0,05 M tris-HCl buffer pH 7,8. The enzyme as supplied by the manufacturers was diluted $\frac{1}{10}$ and 0,05 ml of this solution added to the cell wall suspension to give a final concentration of 0,05 per cent v/v.

### *Chemical analysis of cell walls and extracts*

Total protein, hexoses, N-acetyl-hexosamine and phosphorus were determined as described previously (Cameron, Minnaar & Purdom, 1969).

Pentose was determined by means of the Bial reaction as described by Kabat & Mayer (1964). One mg of dry material was hydrolyzed in 2,0 ml of a 0,1 N $H_2SO_4$ at 60°C for 1 h. For preparation of standards, solutions of ribose containing 100$\mu$g, 80$\mu$g, 60$\mu$g, 40$\mu$g and 20$\mu$g/ml were prepared in distilled water. One ml of each of these solutions was mixed with 1,0 ml 0,2 N $H_2SO_4$ and hydrolyzed as in the case of the test material. The hydrolyzed samples and standards were mixed with 2 ml concentrated HCl containing 0,5 ml of a 10 per cent ferric chloride solution per 100 ml. After the addition of 0,2 ml of a 10 per cent alcoholic orcinol solution to each tube, they were heated in boiling water for 30 min.

The tubes were then immediately cooled and kept in an ice bath. The absorbancy was read at 670 $\mu$m and the concentration of the pentose read from a graph compiled from the standards. When the green colour was too intense, in a repeat test, the hydrolyzed material was diluted before addition of the reagents.

Muramic acid was determined by an adaptation of the method described by Stewart - Tull (1968). Twenty or 25 mg of freezedried material was hydrolyzed in sealed Pyrex tubes with 1 ml of 4N hydrochloric acid for 4 h at 105°C. The hydrolyzates were filtered to remove insoluble humins, evaporated to dryness in a beaker of boiling water, and finally suspended in 2 ml of distilled water, to give a concentration of 10 or 12,5 mg of the original sample per ml.

For standards, 5 mg of muramic acids (Sigma)* was dissolved in 25 ml of distilled water. This was diluted to give concentrations of 0,15; 0,1 and 0,05 mg/ml. All four concentrations were used as standards.

Acetylacetone reagent was prepared by dissolving 1 ml of redistilled acetylacetone in 100 ml bicarbonate buffer, pH 9,8. The buffer contained 23,02 g $Na_2CO_3$, 2,76 g $NaHCO_3$ and 5,84 g NaCl per litre. The pH was checked after the addition of the acetylacetone and readjusted to 9,8. The reagent was always prepared just before use.

The p-dimethylaminobenzaldehyde (DMAB) reagent was prepared by dissolving 0,8 g DMAB in 30 ml of ethanol and adding 30 ml of concentrated HCl (A.R.). This reagent was stored for up to 14 days in a refrigerator at 4°C.

Two ml of the standard or hydrolysate was mixed with 5,5 ml of acetylacetone reagent, in a 25 ml flask. The flask was closed with a plastic stopper, and sealed with adhesive tape. It was heated in a beaker of boiling water for 20 minutes. The solution was cooled to room temperature, and transferred to another 25 ml volumetric flask. The original flask was washed three times with 2 ml of distilled water and the washings were added to the solution to give a final volume of 13,5 ml.

The solution was evaporated to 9 ml, cooled to room temperature, and 1 ml of DMAB reagent was added. The solution was mixed with a glass rod, and the absorbancy read at 510$\mu$m 24 hours later. Muramic acid gives a pink colour.

### *Gel chromatography*

Exclusion chromatography of streptomycin treated protoplasm was done essentially as described by Cameron & Smit (1970). Six comma five ml of a fourfold concentrated sample was applied to a 2,5 × 45 cm Sephadex G200 (Pharmacia Fine Chemicals)** column and eluted with 0,05 M phosphate buffer pH 8,0 containing 0,2 M NaCl. Protein in the fractions (2 ml) was determined by measuring the absorbancy at 280$\mu$m. The tubes comprising a particular peak were pooled,

*Seravac Laboratories (Pty) Ltd., Epping Industria, Cape Town
**Difco Laboratories, Detroit, Michigan, U.S.A.

*Sigma Chemical Co. Ltd., St. Louis, Mo., U.S.A.
**Pharmacia Fine Chemicals, Uppsala, Sweden

dialyzed against distilled water, pervaporated and lyophilyzed.

*Preparation of cell wall antiserum*

Dry purified cell walls were suspended in 0,85 per cent NaCl containing 0,5 per cent formalin to give a reading of 48 on the linear scale of an Eel Unigalvo nephelometer*. The nephelometer was set to give a reading of 80 with standard *Brucella abortus* antigen. A portion of the suspension was emulsified with an equal volume of Freund's complete adjuvant (Difco)**

Six rabbits were given eight simultaneous injections of 1,0 ml each subcutaneously at different sites and rested for 3 weeks. A series of intravenous injections of antigens without adjuvant was then given according to the following schedule:

Day 1 — 0,1 ml
Day 2 — 0,3 ml
Day 3 — 0,6 ml

Additional injections at this stage proved to be toxic.

The rabbits were given a further 0,5 ml antigen intravenously six weeks later and bled after 10 days.

The sera were all tested against 10, 20 and 40 mg protoplasm by gel diffusion and the one giving the most numerous and clear lines was used in this study.

*Precipitin tests*

Double diffusion tests were done on microscope slides as described by Cameron (1969).

*Immunity tests*

The immunizing potency of cell walls and extracts was assayed as described previously (Cameron & Minnaar, 1969). Briefly, the antigens were emulsified with Freund's complete adjuvant and 0,1 ml administered subcutaneously to 4-6 week old female albino mice. Each mouse was given two injections of 0,05 ml each at different sites and booster injections were given 4 weeks later. The total quantity of antigen given was either 0,2 mg or 1,0 mg depending on the expected immunizing properties of the antigens in question.

The mice were challenged intravenously with $2 \times 10^5$ live bacteria and cumulative deaths were recorded for 14 days.

*Evans Electroselenium Ltd., Halstead, Essex, England
**Difco Laboratories, Detroit, Michigan, U.S.A.

## Results

Extraction of batches of PCW with E:E, cold TCA, formamide, $H_2SO_4$, butanol or PAW did not yield soluble material of any consequence. PCW extracted with E:E and cold TCA were not susceptible to the action of SA enzyme and only yielded approximately 4 per cent soluble material after treatment with lysozyme (Table 1).

From these results it was evident that PCW of *C. pseudotuberculosis* are exceptionally resistant to chemical procedures as well as to the action of mucolytic enzymes.

In an attempt to render them susceptible to enzymatic degradation, PCW were pretreated with either SDS or NaOH. SDS treatment had virtually no effect but after exposure to 1 N NaOH under severe conditions (100°C, 10 min) the cell walls were readily digested by both SA enzyme and lysozyme. Similar results were obtained by a milder treatment with NaOH (37°C, 18 h) but then SA enzyme was no longer effective (Table 1).

In order to preserve the original natural configuration of the antigens in the cell wall, experiments were done to determine whether extraction with E:E and cold TCA could be omitted before the PCW were exposed to 1 N NaOH. It was found that when the cold TCA step is omitted, the effect of NaOH is markedly reduced. Only approximately 5 per cent soluble material is obtained directly and subsequent treatment with lysozyme is ineffective. However, if the E:E step is omitted, about 23 per cent of the cell wall is dissolved by 1 N NaOH and roughly 45 per cent of the remaining cell wall is digested by lysozyme (Table 2).

When the PCW were extracted with both E:E and cold TCA, the yield was slightly lower and can probably be accounted for by the fact that surface lipid and other constituents have already been removed by the E:E. This latter procedure was, however, preferred because it is known that the material extracted with E:E has no immunizing capacity and that loss of this material would not affect the potency of the final soluble material but would improve its purity. Removal of lipid does, however, destroy the immunogenicity of *L. monocytogenes* although it is not the antigen responsible for inducing immunity (Klasky & Picket, 1968).

Treatment with 1 N NaOH was still considered rather severe and it was thought that it may result in hydrolysis and destruction of the antigens. Conse-

TABLE 1 *Yield of soluble material from cell walls by various extraction procedures*

| Extraction procedure | Yield of soluble material % |
|---|---|
| Ether; Ethanol (E:E) | not tested |
| Cold trichloroacetic acid (CTCA) | trace |
| E:E + CTCA | trace |
| E:E; CTCA; Formamide | trace |
| $H_2SO_4$ | trace |
| Butanol | nil |
| Phenol: acetic acid: water | nil |
| E:E; CTCA; *Streptomyces albus* (SA) enzyme | trace |
| E:E; CTCA; Lysozyme | *c* 4,0 |
| E:E; CTCA; $H_2SO_4$; SA enzyme | trace |
| E:E; CTCA; $H_2SO_4$; Lysozyme | trace |
| E:E; Sodium dodecyl sulphate (SDS); SA enzyme | nil |
| E:E; SDS; Lysozyme | *c* 0,4 |
| E:E; CTCA: SDS; SA enzyme | nil |
| E:E; CTCA; SDS; Lysozyme | trace |
| E:E; CTCA; 1N NaOH, 100°C, 10 min; SA enzyme | *c* 90 |
| E:E; CTCA; 1N NaOH, 100°C, 10 min; Lysozyme | *c* 90 |
| E:E; CTCA; 1N NaOH, 37°C, 18 h; SA enzyme | nil |
| E:E; CTCA; 1N NaOH, 37°C, 18 h; Lysozyme | *c* 40 |

TABLE 2 *Effect of sequence of extraction procedure on yield of soluble material from cell walls*

| Extraction procedure | Yield of soluble material % |
|---|---|
| 1N NaOH, 18h, 37°C | nil |
| E:E; 1N NaOH, 37°C, 18 h | c 5,0 |
| E:E; 1N NaOH, 37°C, 18 h; Lysozyme | nil |
| CTCA; 1N NaOH, 37°C, 18h | c 23 |
| CTCA; 1N NaOH, 37°C, 18 h; Lysozyme | c 45 |
| E:E; CTCA; 1N NaOH, 37°C; 18 h; Lysozyme | c 40 |

TABLE 3 *Effect of concentration of NaOH on yield of soluble material from cell walls*

| Extraction procedure | Yield of soluble material % |
|---|---|
| E:E; CTCA; 0,001 N NaOH, 37°C, 18 h | trace |
| E:E; CTCA; 0,01 N NaOH, 37°C, 18 h | c 15 |
| E:E; CTCA; 0,1 N NaOH, 37°C, 18 h | c 18 |
| E:E; CTCA; 1,0 N NaOH, 37°C, 18 h | c 40 |
| E:E; CTCA; 0,01 N NaOH, 37°C, 18 h; lysozyme | nil |
| E:E; CTCA; 0,1 N NaOH, 37°C, 18 h; lysozyme | nil |
| E:E; CTCA; 1,0 N NaOH, 37°C, 18 h; lysozyme | c 40 |

TABLE 4 *Chemical composition of soluble extracts tested for their immunogenicity*

| Extraction Procedure | Yield | Chemical composition | | | | |
|---|---|---|---|---|---|---|
| | | Protein | N-acetyl-hexosamine | Phosphorus | Hexose | Pentose |
| | % | % | % | % | % | % |
| E:E; CTCA; 1N NaOH, 37°C, 18h | c 40 | 32,0 | 0,4 | 0,13 | 33,0 | 29,0 |
| E:E; CTCA; 1N NaOH, 37°C, 18h; Lysozyme | c 40 | 16,0 | 2,7 | 0,05 | 35,0 | 33,0 |
| CTCA; 1N NaOH, 37°C, 18 h | c 23 | 33,0 | 0,5 | 0,23 | 24,0 | 45,0 |
| CTCA; 1N NaOH, 37°C, 18h; Lysozyme | 45 | 13,0 | 4,0 | 0,08 | 32,0 | 29,0 |

TABLE 5 *Chemical composition of residual cell walls after extraction by various procedures*

| Extraction procedure | Residual cell walls after extraction | Chemical composition | | | | |
|---|---|---|---|---|---|---|
| | | Protein | N-acetyl-hexosamine | Phosphorus | Hexose | Pentose |
| | % | % | % | % | % | % |
| Original cell walls | — | 54,0 | 7,3 | 1,40 | 16,7 | 19,7 |
| Ether: Ethanol (E:E) | c 99 | 19,0 | 5,2 | 0,04 | 18,0 | 40,0 |
| Cold Trichloracetic acid (CTCA) | c 98 | 20,0 | 1,2 | 0,16 | 23,0 | 38,0 |
| E:E; CTCA | c 95 | 20,0 | 2,9 | 0,06 | 19,0 | 12,0 |
| E:E; CTCA; 1N NaOH, 37°C, 18h | c 60 | 10,5 | 1,4 | 0,03 | 30,0 | 45,0 |
| — ; CTCA: 1N NaOH, 37°C, 18h | c 80 | 13,1 | 2,0 | 0,04 | 13,0 | 27,2 |
| — ; — ; 1N NaOH, 37°C, 18h | c 99 | 10,0 | 1,5 | 0,05 | 31,0 | 45,0 |

quently the effectiveness of lower concentrations of NaOH was also tested. The results obtained revealed that by using lower concentrations of NaOH the amount of soluble material which was extracted directly was markedly reduced. Moreover, when concentrations of NaOH below 1 N were used, the cell walls were no longer lysed by lysozyme (Table 3).

In all, four soluble preparations were obtained and their chemical composition investigated. Alkali soluble material had a relatively higher concentration of protein and phosphorus, while the lysozyme lysates had a higher concentration of N-acetylhexosamine. Prior extraction of PCW with E:E did not materially influence the composition of either the NaOH extract or the lysozyme lysate (Table 4).

The ability of these four soluble preparations to induce immunity in mice was determined but none of them exhibited any activity.

In order to find out at which stage of the extraction process the biological activity was lost, the chemical composition and immunizing activity of the residual cell walls were determined. There was an appreciable drop in the protein content after extraction with both E:E and cold TCA but it was higher than reported previously (Cameron, Minnaar & Purdom, 1969).

The protein content decreased further after treatment with 1 N NaOH and the changes in the concentration of the other components were variable throughout (Table 5).

The results of the immunity experiments are shown in Fig. 2a and 2b.

The cell walls which were extracted with either E:E, cold TCA, or both, retained their biological activity which is in accordance with previous results (Cameron, Minnaar & Purdom, 1969) (Fig. 2a). However, as soon as the cell walls were treated with NaOH, they lost all immunizing activity irrespective of whether they were previously treated with E:E, cold TCA, or both (Fig. 2b). It was consequently impossible to obtain an active soluble preparation by this procedure.

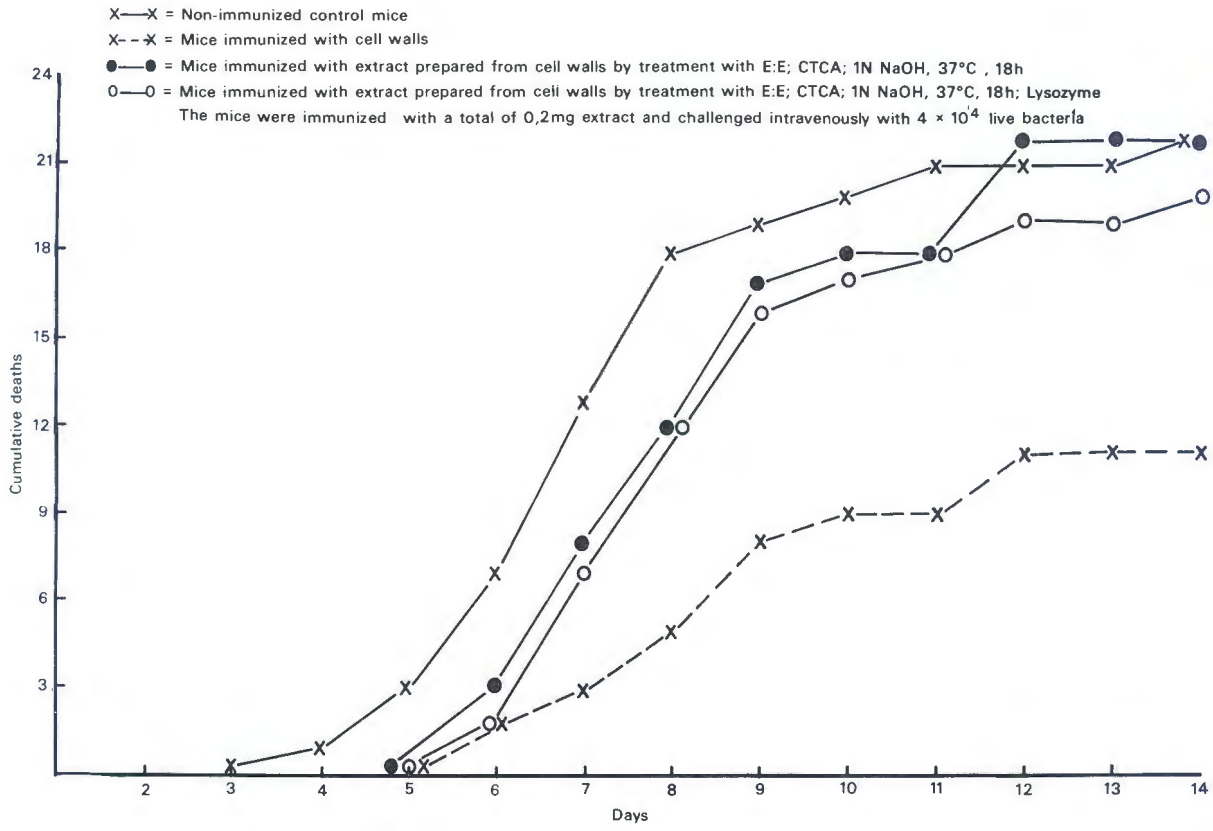


FIG. 1a Immunogenicity of soluble cell wall extracts

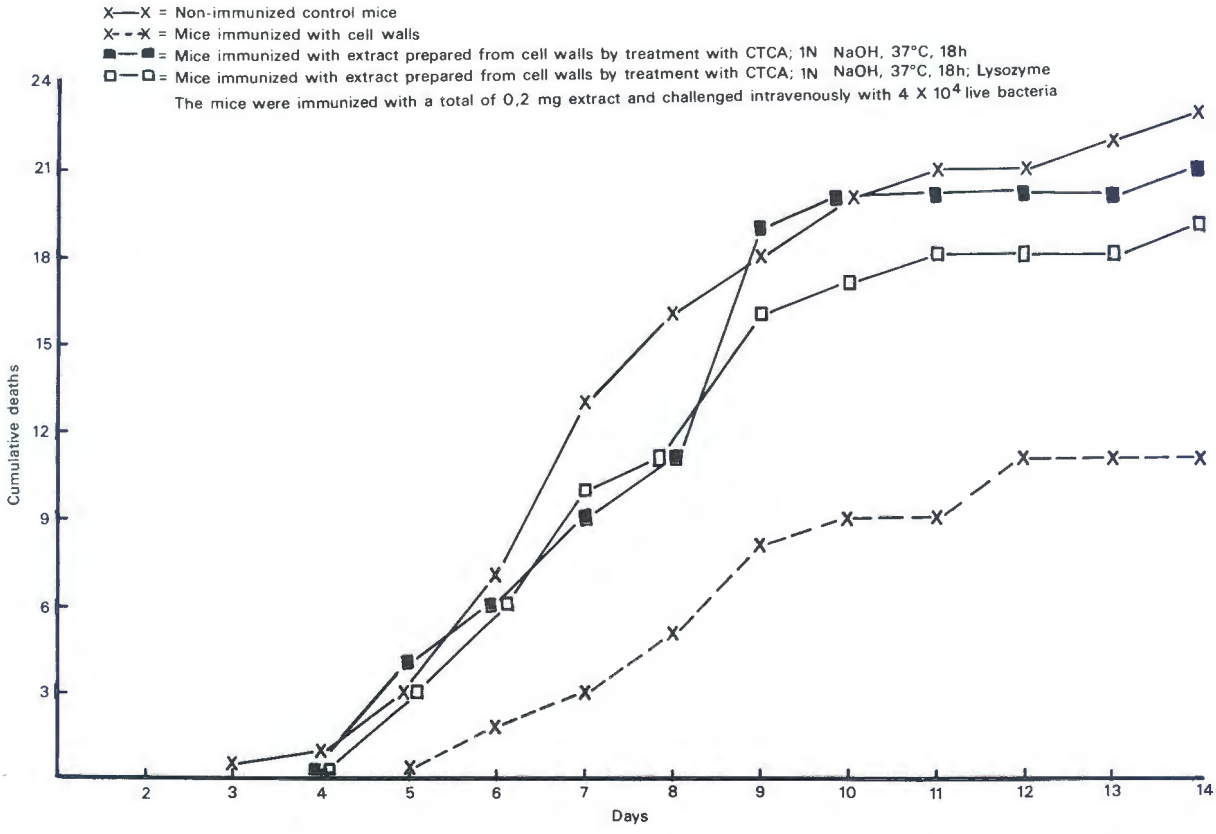


FIG. 1b Immunogenicity of soluble cell wall extracts

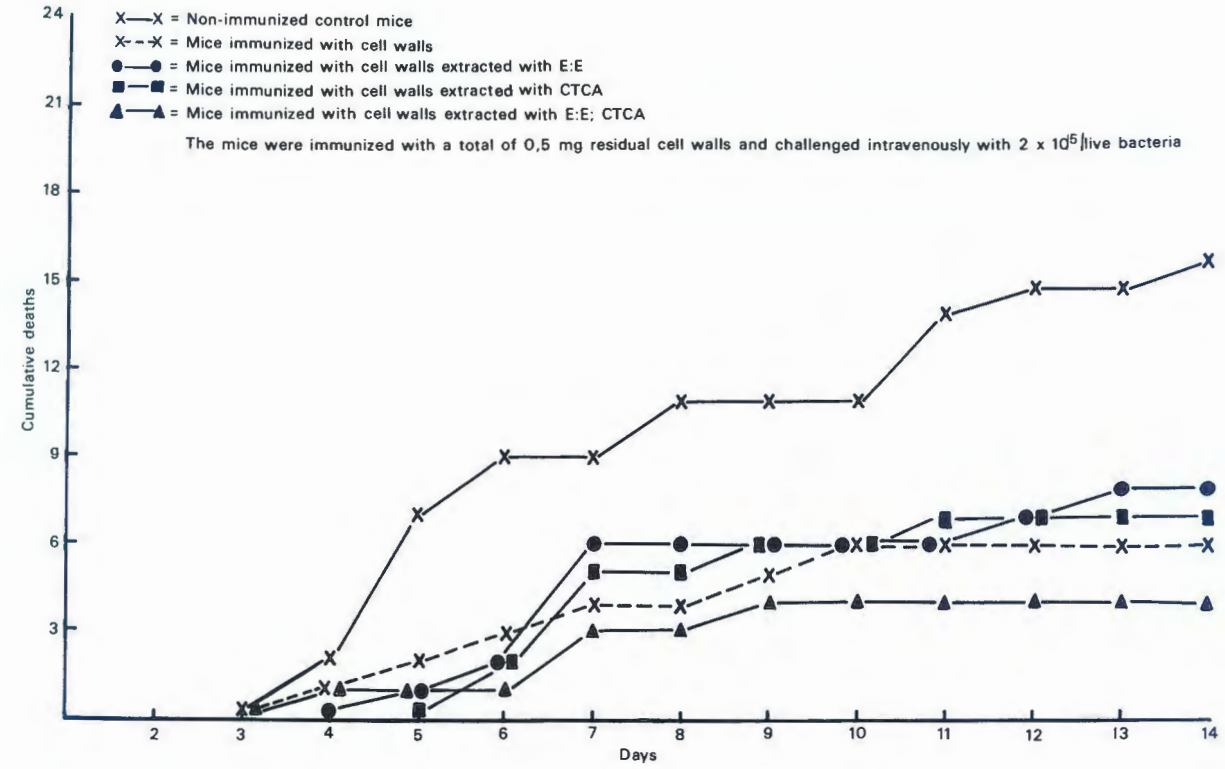


FIG. 2a Immunogenicity of residual cell walls

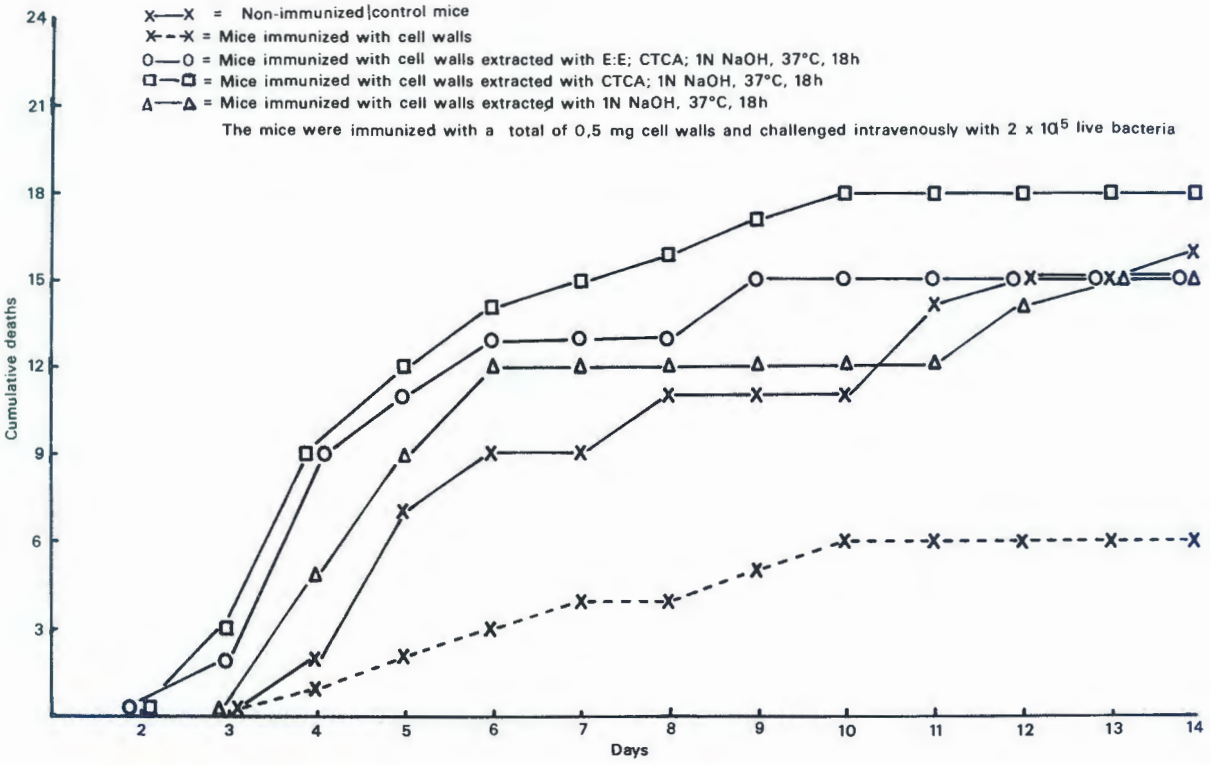


FIG. 2b Immunogenicity of residual cell walls

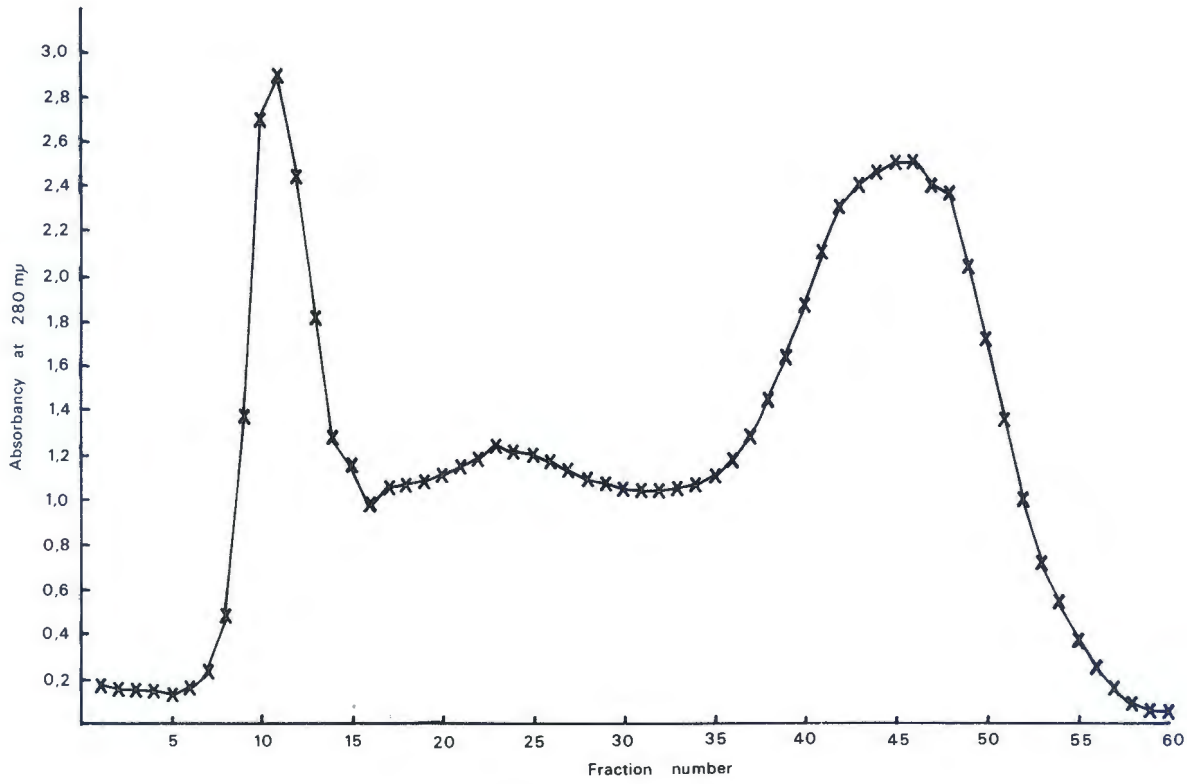


FIG. 3 Chromatography of streptomycin-treated protoplasm on Sephadex G200

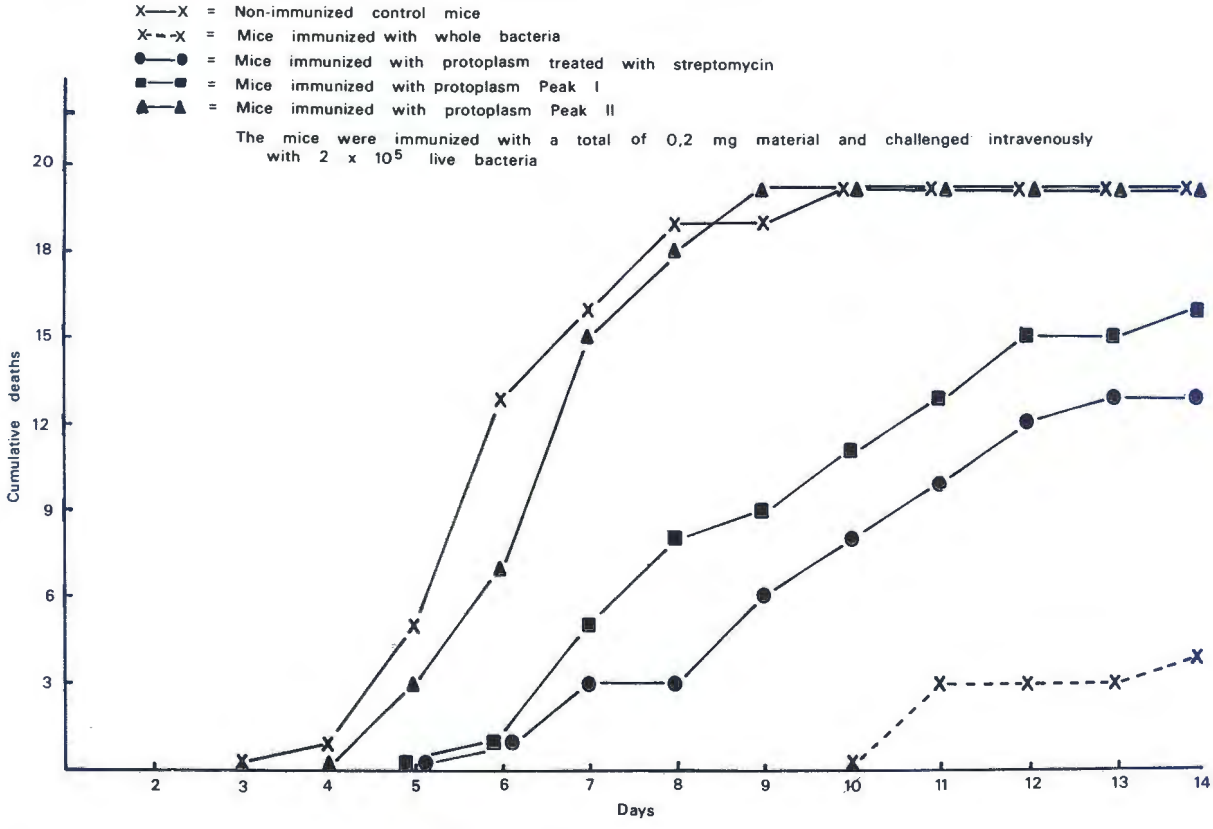


FIG. 4 Immunogenicity of protoplasm fractions

It was previously found that although poor, there is some immunizing potency in the protoplasm of *C. pseudotuberculosis* (Cameron, Minnaar & Purdom, 1969) and an attempt was made to isolate a soluble antigen from this source.

Sephadex G200 chromatography of streptomycin-treated protoplasm yielded two major protein peaks. Tubes 8 to 15 and 37 to 51 were pooled and designated Peak I and Peak II respectively (Fig. 3).

Biological assay of the two peaks revealed that Peak I had satisfactory immunizing properties while Peak II had none whatsoever (Fig. 4).

The material constituting Peak I was very cloudy after gel filtration and exhibited a marked tendency to aggregate. The degree of insolubility was so marked as to preclude further fractionation and assay. An attempt was nevertheless made to examine the relationship between soluble cell wall material and protoplasm Peak I by gel diffusion.

Soluble cell wall material prepared by lysozyme digestion of NaOH treated cell walls would not precipitate with any antisera and consequently the only possibility was to determine whether Peak I possessed any antigens which would react with antibodies present in antiserum prepared from purified cell walls. This was indeed found to be the case. When Peak I was used at a concentration of 20 mg/ml a common strong precipitin line was formed with both protoplasm antiserum and cell wall antiserum, thus indirectly demonstrating that cell walls and protoplasm Peak I possess a common antigen (Fig. 5). At a concentration of 40 mg/ml the common line was still visible, but lines peculiar to the respective antisera were more evident (Fig. 6).

These results proved that Peak I and cell walls share at least one common antigen but it does not necessarily mean that this particular antigen is responsible for immunity.

In order to elucidate this problem further, the protein and muramic acid concentrations of Peak I and cell walls were determined.

Peak I consisted mostly of protein and contained only traces of muramic acid while the cell walls contained muramic acid and between 10 to 20 per cent protein. This concentration of protein is rather high for cell walls and cannot be accounted for by the peptide bonds as they do not contain tyrosine or tryptophane which are necessary for a positive Folin-Ciocalteu reaction. Furthermore the fact that Peak I contained virtually no muramic acid but was nevertheless able to induce immunity suggested that the active component in the cell walls may be a protein rather than the mucopeptide.

TABLE 6 *Comparison of chemical composition of cell walls and Protoplasm Peak I*

| | Protein % | Muramic acid % |
|---|---|---|
| Purified cell walls extracted with E:E & CTCA . . . . | 14,0 | 5,3 |
| Protoplasm Peak I . . . . . | 55,6 | 5,4 |

Cell walls were therefore processed according to the procedure outlined by Park & Hancock (1960). The essential feature of this procedure was the treatment of the cell walls with crystalline trypsin in order to remove all protein from them. Chemical analysis of the final product revealed that this was indeed the case (Table 7). The process also included a step in which the cell walls were treated with hot TCA. This rendered the cell walls extremely insoluble, and this probably accounted for the overall low figures obtained for the different chemical components.

FIG. 5 Immunodiffusion of protoplasm Peak I antigen against protoplasm antiserum and cell wall antiserum showing the existence of antibody common to both antisera.
Left well = Cell wall antiserum
Right well = Protoplasm antiserum
Bottom well = Sephadex Peak I antigen 20 mg/ml in 0,85 per cent NaCl

FIG. 6 Immunodiffusion of protplasm Peak I antigen against protoplasm antiserum and cell wall antiserum showing the existence of specific precipitins.
Left well = Cell wall antiserum
Right well = Protoplasm antiserum
Bottom well = Sephadex Peak I antigen 40 mg/ml in 0,85 per cent NaCl

TABLE 7 *Chemical composition of cell walls at different stages of treatment according to the procedure of Park & Hancock (1960)*

| | Protein % | Phoshporus % | Hexose % | Pentose % | N-acetyl % | Muramic % | Total % |
|---|---|---|---|---|---|---|---|
| Cold TCA . . . . . | 14 | 0,04 | 45 | 21 | 1,4 | 5,3 | 81,74 |
| Hot TCA . . . . . | 12 | 0,02 | 15 | 15,6 | 0,62 | 3,6 | 46,84 |
| Cold TCA + crystalline trypsin . . . | Trace | 0 | 44,5 | 19,6 | 2,2 | 2,8 | 59,1 |
| Hot TCA + crystalline trypsin . . . | Trace | 0,05 | 36 | 18 | 2,64 | 3,2 | 59,89 |

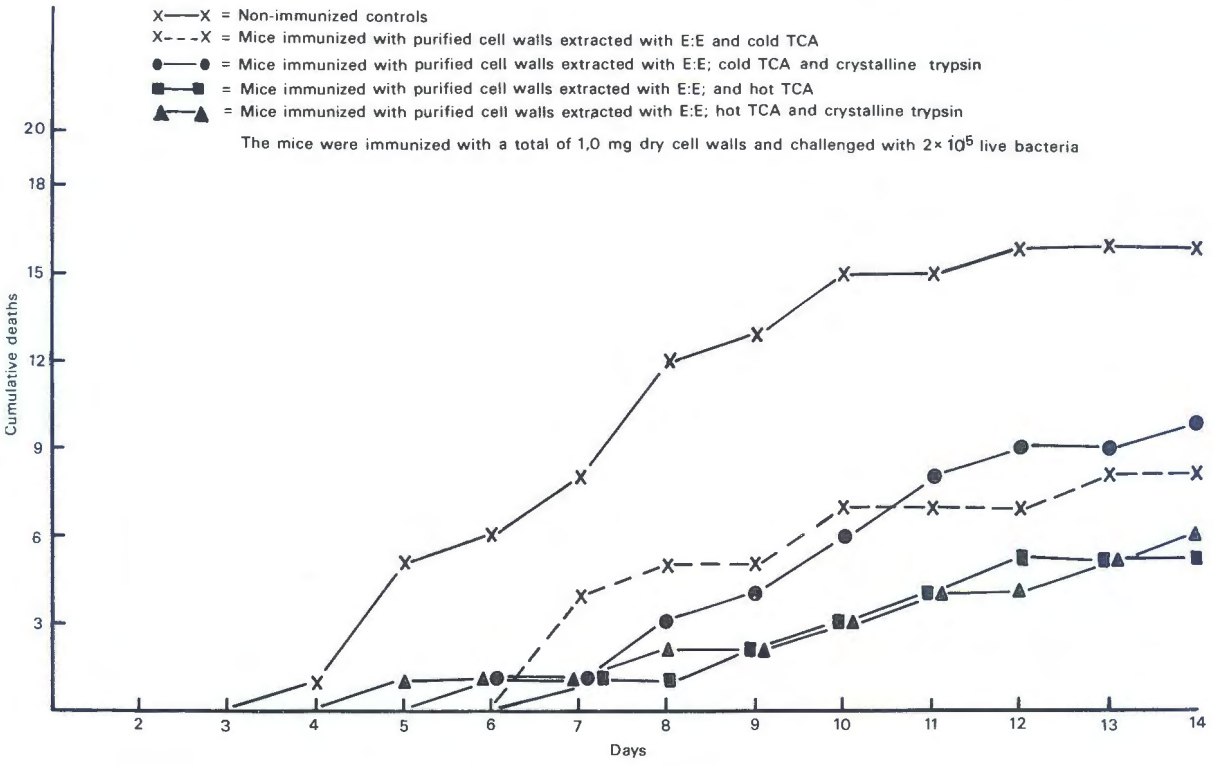


FIG. 7 Immunogenicity of cell walls treated with hot TCA and crystalline trypsin

Evaluation of the immunizing potency of cell walls treated with hot TCA and crystalline trypsin showed that although the immunizing potency was considerably reduced, it was not totally abolished (Fig. 7).

## DISCUSSION

The object of this investigation was to determine which component of the cell wall of *C. pseudotuberculosis* is responsible for inducing a protective immunity. This necessitated either the extraction of a biologically active substance from the cell walls or the demonstration of specific loss of immunizing activity of the residual cell walls after a particular extraction process. Another approach was to digest the cell wall and attempt to isolate a specific substance with immunological activity.

Cell walls of *C. pseudotuberculosis* are, however, extremely resistant to numerous extraction methods (Cameron, Minnaar & Purdom, 1969) and this was confirmed in this investigation. Furthermore, they were found to be resistant to mucolytic enzymes. Strominger & Ghuysen (1967) noted that, among other substances, detergents may render cell walls susceptible to enzymatic activity, but pre-treatment with SDS had no such effect on *C. pseudotuberculosis* cell walls.

Salton & Horne (1951) and Azuma & Yamamura (1969) have observed that alkali partially degrades cell walls and enhances their susceptibility to mucolytic enzymes. This was also found to be the case with *C. pseudotuberculosis* cell walls. However, the concentration of NaOH required to do this completely destroyed the immunizing activity of the cell walls.

It was also found that if the TCA step was omitted the cell walls did not become sensitive to lysozyme. A similar finding has been recorded by Morse (1962) in the case of *Staphylococcus aureus* (Rosenbach, 1884) cell walls.

Treatment of cell walls with crystalline trypsin subsequent to extraction with either hot or cold TCA removed all the residual protein, leaving what would be virtually pure mucopeptide. The immunizing activity of this material was decreased but not totally abolished.

It therefore appears that immunizing activity is not dependent on any particular single substance or antigen component but that effective biological activity is optimal when the different cell wall components are retained in their natural complex configuration. Any disruption of this integrated structure by harsh chemical, physical or enzymatic action would then lead to deterioration, degradation and loss of biological activity.

The protoplasm also possesses antigens which are capable of inducing a protective immunity. The greatest activity is to be found in the first protein peak obtained by gel chromatography. This material is toxic, but is unrelated to the major toxic component as well as to the exotoxin (Cameron & Smit, 1970). It is eluted with the void volume in Sephadex G200 columns and thus appears to have a high molecular weight. However, it tends to become insoluble even after limited storage at 4°C and is therefore difficult to handle. It was nevertheless possible to show that it will produce precipitin lines when tested against cell wall antiserum. This proves that cell walls and protoplasm have common antigens and it is possible that the material isolated from the protoplasm originated from the cell wall or *vice versa*. It could also have been liberated from the cell wall during the process of preparation.

In contrast to the cell walls which, after treatment with crystalline trypsin, contain much muramic acid but no protein, the protoplasmic material consists almost exclusively of protein and is virtually free of muramic acid. These findings preclude any speculation on the chemical composition of the immunity-inducing antigen or antigens.

## Summary

Purified cell walls of *C. pseudotuberculosis* were subjected to extraction with ether:ethanol, cold or hot trichloroacetic acid, butanol: phenol:acetic acid: water and crystalline trypsin. None of these procedures yielded material with any biological activity nor was the activity of the residual cell walls materially diminished.

Treatment of cell walls with 1,0 N NaOH removed much material and also rendered them susceptible to the action of lysozyme. This procedure, however, totally destroyed all biological activity and the soluble cell wall material would also not precipitate with cell wall or protoplasm antiserum.

Gel chromatography of protoplasm yielded two major protein peaks, one of which had considerable immunizing activity. This material was shown to possess antigens in common with cell walls and corresponds to the previously described Toxin A (Cameron & Smit, 1970).

It is concluded that no single cell wall or other antigen is solely responsible for inducing a protective immunity, but that the intact complex configuration of the cell wall is necessary for optimal immunizing activity or that more than one antigen is required in order to induce an effective immunity.



## Acknowledgements

We wish to thank Mrss. M. Engelbrecht and L. v. Reenen for drawing the figures and the section of photography for the reproductions.


## References


Allsop, J. & Work, E., 1963. Cell walls of *Propionibacterium* species: Fractionation and composition. *Biochem. J.*, 87, 512-519.

Armstrong, J. J., Baddiley, J. & Buchanan, J. G., 1960. Structure of the ribitol teichoic acid from the walls of *Bacillus subtilis*. *Biochem. J.*, 76, 610-621.

Azuma, I. & Yamamura, Y., 1969. Chemical and immunological properties of mycobacterial polysaccharides. *Z. ImmunForsch. exp. Ther.* 137, 228-235.

Bisset, K. A. & Vickerstaff, J., 1967. Significance of the characteristic chemical pattern of Gram positive and Gram negative bacterial cell walls. *Nature* (*Lond.*), 215, 1286-1287.

Cameron, C. M., 1969. Antiphagocytic activity of *Staphylococcus aureus* antigens. *Onderstepoort J. vet. Res.*, 36, 199-206.

Cameron, C. M. & Minnaar, J. L., 1969. Immunization of mice against *Corynebacterium pseudotuberculosis* infection. *Onderstepoort J. vet. Res.*, 36, 207-210.

Cameron, C. M., Minnaar, J. L. & Purdom, Mary, R., 1969. Immunizing properties of *Corynebacterium pseudotuberculosis* cell walls. *Onderstepoort J. vet. Res.*, 36, 211-216.

Cameron, C. M. & Smit, M., 1970. Relationship of *C. pseudotuberculosis* protoplasmic toxins to the exotoxin. *Onderstepoort J. vet. Res.*, In Press.

Crowle, A. J., 1969. Immunogen extracted from Tubercle bacilli with trypsin. *Z. ImmunForsch. exp. Ther.*, 137, 71-79.

Cummings, C. S., 1965. Chemical and antigenic studies on cell walls of mycobacteria, corynebacteria and nocardias. *Am. Rev. resp. Dis.*, 92, 63-72.

Forsgren, A. & Sjöquist, J., 1969. Protein A from *Staphylococcus aureus*, VII. Physicochemical and immunological characterization. *Acta path. microbiol. scand.*, 75, 466-480.

Fox, E. N. & Wittner, M. K., 1965. The multiple molecular structure of the M proteins of Group A streptococci. *Proc. natn. Acad. Sci.*, 54, 1118-1125.

Ghuysen, Jean-marie, 1968. Use of bacteriolytic enzymes in determination of wall structure and their role in cell metabolism. *Bact. Rev.*, 32, 425-464.

Grov, A., Myklestad, B. & Oeding, P., 1964. Immunochemical studies on antigenic preparations from *Staphylococcus aureus*. 1. Isolation and chemical characterization of antigen A. *Acta path. microbiol. scand.*, 61, 588-595.

Haskell, T. M. & Hanessian, S., 1963. 2-amino-2 deoxy-D-glucuronic acid, the main constituent of SPA (Staphylococcal polysaccharide antigen). *Nature*, (*Lond.*), 199, 1075.

Haukenes, G., Elwood, D. C., Baddiley, J. & Oeding, P., 1961. Serological cross-reactivity between polysaccharide A and teichoic acid of *Staphylococcus aureus*. *Biochim. biophys. Acta.*, 53, 425-426.

Heyman, H., Manniello, J. M. & Barkulis, S. S., 1963. Structure of streptococcal cell walls. I. Methylation study of C-polysaccharide. *J. biol. Chem.*, 238, 502-509.

Hughes, R. C., 1968. The cell wall of *Bacillus licheniformis* NCTC 6346. Composition of the mucopeptide component. *Biochem. J.*, 106, 41-47.

Kabat, E. A. & Mayer, M. M., 1964. Experimental Immunochemistry. 2nd Ed. Charles C. Thomas, Springfield, III.

Kalf, G. F. & White, T. G., 1963. The antigenic components of *Erysipelothrix rhusiopathiae*. II. Purification and chemical characterization of a type-specific antigen. *Archs Biochem. Biophys.*, 102, 39-47.

Karakawa, W. W. & Krause, R. M. J., 1966. Studies on the immunochemistry of streptococcal mucopeptide. *J. exp. Med.*, 124, 155-171.

Karakawa, W. W., Lackland, H. & Krause, R. M., 1966. An immunological analysis of bacterial mucopeptides. *J. Immun.*, 97 797-804.

Karakawa, W. W., Lackland, H. & Krause, R. M., 1967. Antigenic properties of the hexosamine polymer of streptococcal mucopeptide. *J. Immun.*, 99, 1178-1182.

Klasky, S. & Picket, M. J., 1968. Immunogenicity and specificity of *Listeria monocytogenes* cell walls. *J. infect. Dis.*, 118, 65-75.

Lancefield, Rebecca C., 1962. Current knowledge of type specific M antigens of group A streptococci. *J. Immun.*, 89, 307-313.

Lüderitz, O., Staub, A. M. & Westphal, O., 1966. Immunochemistry of O and R antigens of *Salmonella* and related Enterobacteriaceae. *Bact. Rev.*, 30, 192-255.

Martin, H. H., 1966. Biochemistry of bacterial cell walls. *A. Rev. Biochem.*, 35, 457-484.

McCarty, M. & Morse, S. I., 1964. Cell wall antigens of Gram positive bacteria. *Adv. Immun.*, 4, 249-282.

Montague, Elizabeth, A. & Knox, K. W., 1968. Antigenic components of the cell wall of *Streptococcus salivarius*. *J. gen. Microbiol.*, 54, 237-246.

Morse, S. I., 1962. Studies on the chemistry and immunochemistry of cell walls of *Staphylococcus aureus*. *J. exp. Med.*, 116, 229-245.

Morse, S. I., 1965. Biological attributes of staphylococcal cell walls. *Ann. N.Y. Acad. Sci.*, 128, 191-213.

Mudd, S., Yoshida, A., Li, I.W. & Lenhart, N. A., 1963. Identification of a somatic antigen of *Staphylococcus aureus* critical for phagocytosis by human blood leucocytes. *Nature*, (*Lond.*), 199, 1200-1201.

Munoz, J. J., 1963. Symposium on relationship of structure of micro-organisms to their immunological properties. I. Immunological and other biological activities of *Bordetella pertussis* antigens. *Bact. Rev.*, 27, 325-340.

Park, J. T. & Hancock, R., 1960. A fractionation procedure for studies of the synthesis of cell wall mucopeptide and other polymers in cells of *Staphylococcus aureus*. *J. gen. Microbiol.*, 22, 249-258.

Perkins, H. R., 1963. The chemical structure and biosynthesis of bacterial cell walls. *Bact. Rev.*, 27, 18-55.

Rega, A. F., Weed, R. I., Reed, C. F., Berg, G. G. & Rothstein, A., 1967. Changes in the properties of human erythrocyte membrane protein after solubilization by butanol extraction. *Biochim. biophys. Acta.*, 147, 297-312.

Ribi, E., Anacker, R. L., Barclay, W. R., Brehmer, W., Middlebrook, G., Milner, K. C. & Tarmina, D. F., 1968. Structure and biological functions of Mycobacteria. *Ann. N.Y. Acad. Sci.*, 154, 41-57.

Rolicka, Mary, & Park, J. T., 1969. Antimucopeptide antibodies and their specificity. *J. Immun.*, 103, 196-203.

Rottem, S. & Razin, S., 1967. Electrophoretic patterns of membrane proteins of Mycoplasma. *J. Bact.*, 94, 359-364.

Salton, M. R. J. & Horne, R. W., 1951. Studies on the bacterial cell wall. II. Methods of preparation and some properties of cell walls. *Biochim. biophys. Acta*, 7, 177-197.

Stewart-tull, D. E. S., 1968. Determination of amino sugars in mixtures containing glucosamine, galactosamine and muramic acid. *Biochim. J.*, 109, 13-18.

Stöss, B. & Herrmann, R., 1965. Immunogenic activity of lipids of *M. tuberculosis*. *Nature* (*Lond.*), 1224.

Strominger, J. L. & Ghuysen, Jean-Marie, 1967. Mechanisms of enzymatic bacteriolysis. *Science*, 156, 213-221.

Wicken, A. J., Elliot, S. D. & Baddiley, J., 1963. The identity of streptococcal group D antigen with teichoic acid. *J. gen. Microbiol.*, 31, 231-239.


Printed in the Republic of South Africa by Heer Printing Company (Pty) Ltd., for the Government Printer, Pretoria