JOURNAL OF BACTERIOLOGY, Sept., 1966
Vol. 92, No. 3

Heat-Labile Antigens of *Salmonella enteritidis*

I. Extraction of Antigens

MARGARET MILNE[1] AND F. M. COLLINS[2]
Department of Microbiology, University of Adelaide, Adelaide, South Australia

Received for publication 13 May 1966

ABSTRACT

MILNE, MARGARET (University of Adelaide, Adelaide, Australia), AND F. M. COLLINS. Heat-labile antigens of *Salmonella enteritidis*. I. Extraction of antigens. J. Bacteriol. **92**:543-548. 1966.—*Salmonella enteritidis* strains of high and low mouse virulence were grown in continuous culture. Cell walls were obtained from both strains by sonic disruption, and the washed walls were extracted at 4 C with sodium dodecyl sulfate or sodium deoxycholate. The extracts were eluted with a saline gradient from a diethylaminoethyl cellulose column, and the separated peaks were tested for precipitin activity against rabbit antisera prepared with heat- or ethyl alcohol-killed vaccines. The separated antigens reacted less intensely with the antiserum to heat-killed cells than they did to the antisera prepared against alcohol-killed vaccine. Little antigenic difference could be detected between the extracts prepared from the virulent and the avirulent organisms.

The pioneer studies by Boivin and Mesrobeanu (4) of the endotoxins of gram-negative bacteria led to numerous detailed studies of the chemistry and biological characteristics of the heat-stable, smooth somatic antigens of the *Enterobacteriaceae* (8, 28). Although of great interest and importance, this work is not directly relevant to the present study, since it has been generally agreed that the heat-stable somatic antigens are not important in determining the virulence of the salmonellae. However, from the work of Felix and his colleagues on the Vi antigen of *Salmonella typhi* (9), the K antigens of *Escherichia coli* (15), and antigen 5 of *S. typhimurium* (17), there arose a great deal of interest in the heat-labile antigens as factors of importance in relation to virulence (1, 3, 14, 16). The destruction of these antigens in heat-inactivated vaccines offered a plausible reason for the well-known failure of such preparations to produce good immunity (12, 14, 20).

Development of milder extraction methods for preparing antigenic material has stimulated interest in the labile-somatic antigens. Sutherland (26) used sodium dodecyl sulfate (SDS) to extract protective antigens from cell walls of *Bordetella pertussis*. Shafa and Salton (25) reported rapid disaggregation of *S. gallinarum* cell walls with this reagent, but later experienced difficulties when they attempted to free the antigenic material from detergent. Jenkin and Rowley (14) overcame this problem by employing alcoholic precipitation to free the endotoxic extract from dodecyl sulfate. Recently, Barta (2) reported that sodium deoxycholate (SDC) caused a rapid disaggregation of *B. pertussis* cell walls. Subsequently, dialysis removed most of the residual bile salts, yielding a protective, nontoxic preparation.

In the present study, cell walls of two strains of *S. enteritidis* differing widely in their mouse pathogenicity were extracted with SDS or SDC. The extracts were fractionated chromatographically, and the antigens from the two strains were compared to determine whether any changes in antigenic makeup could be correlated with the observed difference in the virulence of the two organisms.

MATERIALS AND METHODS

Organism. The strain of *S. enteritidis* was that described in a previous paper (5).

Medium. The synthetic liquid medium described by Collins and Rowley (6) was enriched with 5% heat-inactivated pig serum or with 0.5% casein (Oxo Ltd., London, England). The medium was sterilized by Seitz filtration, incubated at 37 C for 5 days to check for sterility, and stored at 4 C until required. The bacteria were grown in a single-stage 2-liter fermentor under conditions similar to those previously employed (5). The *p*H was maintained at 7.4 in the pig-serum medium, and at 6.6 in the casein-enriched medium.

Preparation of cell walls. These were prepared as

[1] Present address: Microbiology Department, Glaxo Laboratories, Greenford, U.K.

[2] Present address: Department of Experimental Pathology, Trudeau Institute, Saranac Lake, N.Y.

previously described (5), except that sonic disruption was carried out under a continuous stream of nitrogen gas.

Extraction procedures. Cell walls were suspended in 0.1 M phosphate buffer (*p*H 7.5) as a 1.0% (w/v) preparation and were cooled to 4 C. The cell walls were mixed with an equal volume of cold 0.5% (w/v) SDS or SDC made up in 0.1 M phosphate buffer (*p*H 7.5) and were subsequently treated according to the schedule shown in Fig. 1. The final opalescent supernatant fluid was lyophilized and stored as a powder at 4 C.

Chromatography. The samples were dialyzed for 24 hr against cold 0.1 M buffered NaCl (*p*H 7.5), and then a sample, containing approximately 50 mg of protein, was applied to a washed diethylaminoethyl (DEAE) cellulose column. The column was first eluted with 0.1 M buffered NaCl (*p*H 7.5) and then with an increasing linear saline gradient obtained by mixing equal volumes of 0.1 M NaCl (*p*H 7.5) with 2 M buffered NaCl (*p*H 6.0). The optical densities of each fraction were read at 280 mμ, and the protein content of each peak was calculated. The pooled fractions representing each peak were dialyzed against 0.01 M buffered NaCl (*p*H 7.5) and were then freeze-dried.

Analytical methods. Protein, carbohydrate, and total lipid determinations were made by methods previously described (5). Deoxycholate was assayed colorimetrically after thin-layer chromatography (23). Sugars were identified by thin-layer chromatography (23) after acid hydrolysis (5).

Haptenic activity. Haptenic activity of the various fractions (5.0 mg of protein per ml) was checked by the double diffusion technique of Ouchterlony (21). Antisera were prepared in rabbits by use of steamed vaccines (100 C for 90 min) or ethyl alcohol-killed vaccines (70% ethyl alcohol at 4 C overnight). The vaccination program was similar to that recommended by Kauffmann (15).

Virulence tests. Suitable 10-fold dilutions of a logarithmic-phase culture suspended in sterile saline were injected intraperitoneally (ip) into groups of 10 mice. The viability of the suspension was checked immediately after injection. The LD_{50} was estimated from the cumulative mortality data by the method of Reed and Muench (24).

RESULTS

Growth of S. enteritidis in 5% serum medium. The LD_{50} of the inoculum was computed to be between 200 and 500 organisms when given by the ip route of infection. When the initial batch culture was put on flow at a dilution rate of 0.1 hr^{-1}, the yield of cells remained constant at 8 g (dry weight) of cells per liter, but the virulence of the effluent organisms rapidly declined so that, after 100 hr, the LD_{50} was computed at 5×10^5. Serological typing showed that the organism was still a fully smooth strain of *S. enteritidis*. No significant differences could be detected in the in vitro growth rate, gross chemical composition, or "O" agglutination titer of the two strains. The avirulent organisms grown in the pig serum broth were designated strain "Se795 avir" and were stored as a dense, washed suspension at −20 C until required.

S. enteritidis was known to grow well in a simple liquid medium in the fermentor without appreciable effect on the virulence of the organism (5). To find an explanation for the observed decrease in virulence, the cultural conditions and the constitution of the medium were varied extensively. The only measures found to influence this change in virulence were a reduction of the *p*H and the omission of pig serum from the medium.

Growth of S. enteritidis in casein medium. S. enteritidis grew well in the casein-enriched me-

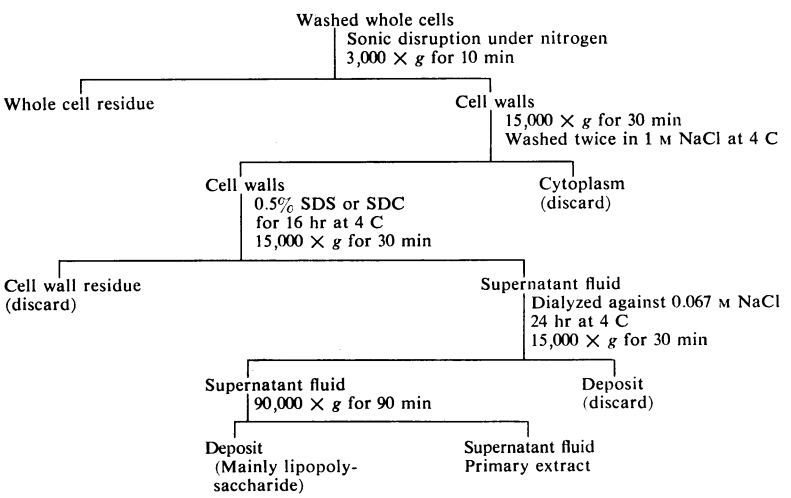

FIG. 1. *Extraction of antigenic material from cell walls of Salmonella enteritidis. Abbreviations: SDS = sodium dodecyl sulfate; SDC = sodium deoxycholate.*

dium, and yields of 8 g (dry weight) per liter were regularly obtained. The *p*H of the medium was adjusted to 6.6, since experiments in the pig-serum medium suggested that loss of virulence was slower in slightly acid media than when the *p*H was kept at 7.4. The LD_{50} of the organisms in the casein medium remained at approximately 500 organisms throughout the 600 hr of cultivation, a period which represented at least 1,000 cell generations. Organisms collected during this period were designated "Se795 vir."

Extraction of antigenic material from washed cell walls. Extraction of 1,100 mg of cell walls of "Se795 vir" with 0.5% SDS yielded 725 mg of primary extract after prolonged dialysis at 4 C (Table 1). This represented a yield of 14.5% of the original whole cells, or 66% of the washed cell walls. Extraction of a similar quantity of "Se795 avir" cell walls yielded 820 mg of primary extract, representing a yield of 16% of the whole cells and 86% of the cell walls.

Alcoholic precipitation of 280 mg of the primary SDS extract from "Se795 vir" cell walls, as recommended by Jenkin and Rowley (14), resulted in the recovery of only 27 mg of soluble material. This represented a recovery of less than 10% of the initially extracted material. Alcohol precipitation thus resulted in such an extensive loss of soluble material that it was decided to omit this step from the purification procedure.

The treatment of 3,040 mg of "Se795 vir" cell walls with SDC resulted in the removal of 660 mg (yield of 22%) of primary extract (Table 1). A second extraction with deoxycholate resulted in the removal of more proteinaceous material, but subsequent tests showed it to have little biological activity; it was therefore discarded. High-speed centrifugation of the initial extract yielded a deposit of heat-stable lipopolysaccharide similar to that obtained by phenol-water extraction (29), both in its sugar content and in its biological properties. It was later shown not to be protective in mice and was discarded (7). The remaining 545 mg of primary extract represented 0.5% of the original whole cells and 18% of washed cell walls.

Extraction of 3,000 mg of "Se795 avir" cell walls with SDC resulted in the recovery of 17.5% of the cell wall material (Table 1). The amount of lipopolysaccharide was slightly lower (99 mg) than in the extract of virulent organisms, but no significance was attached to this. No qualitative difference was found in the sugar content of the two lipopolysaccharides as determined by thin-layer chromatography. The antigenic composition of each extract was checked against rabbit immune sera by gel diffusion; the precipitin patterns, recorded diagrammatically in Fig. 2, show only minor differences, to be discussed later.

Chromatographic separation of antigens in the primary extracts. Gradient elution of antigenic material from DEAE cellulose seemed well suited to the present investigation. The elution patterns for the four extracts are shown in Fig. 3–6. Each extract produced two or three major peaks, as well as several minor ones. The precipitin reactions of the separate fractions obtained from the virulent organisms tested against rabbit immune sera are recorded diagrammatically in Fig. 7 and 8. The corresponding extracts obtained from the avirulent strain behaved in an essentially similar manner. The precipitin lines found when the two primary extracts prepared from the virulent strain were tested against a number of different antisera are recorded in Fig. 9. The reaction of the extracts with anti-*S. gallinarum* serum suggests that the heat-labile antigens were not flagellar in origin.

DISCUSSION

In an earlier study of the growth of *S. enteritidis* in continuous culture, a number of variations in cell wall composition were observed in organisms grown at different rates. These changes were associated with a partial smooth to rough variation which did not, however, result in a detectable change in mouse virulence. Provided that the dilution rate was maintained at 0.1 to 0.2 hr^{-1}, the organisms remained stable in all the characters tested over long periods of time (5). This agreed with the predictions of Herbert et al. (11) that fermentor-grown cells should remain genetically

TABLE 1. *Yield and composition of extracts obtained by treatment of cell walls of Salmonella enteritidis with sodium dodecyl sulphate (SDS) or sodium deoxycholate (SDC)*

Organism	Reagent	Cell wall extracted	Cell wall residue	Spinco deposit	Primary extract	Protein	Carbohydrate	Lipid
		mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Se795 vir	SDS	1,100	274	—[a]	760	290	310	90
Se795 avir	SDS	1,000	220	—[a]	860	344	260	130
Se795 vir	SDC	3,040	2,380	118	545	300	274	153
Se795 avir	SDC	3,100	2,350	99	542	350	325	110

[a] Amount of material in this fraction not determined.

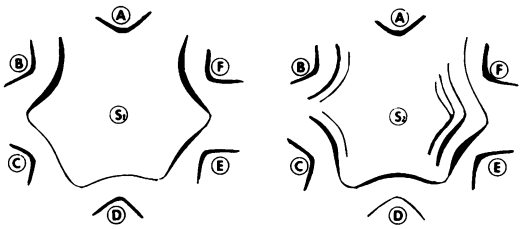

FIG. 2. *Diagrammatic representation of the precipitin lines obtained when rabbit immune sera were tested against cell wall extracts prepared from the virulent and avirulent strains of Salmonella enteritidis. Serum S1 was prepared against boiled S. enteritidis. Serum S2 was prepared against ethyl alcohol-killed S. enteritidis. A = lipopolysaccharide; B = SDS primary extract (virulent); C = SDS primary extract (avirulent); D = sonically disrupted cell wall (virulent strain); E = SDC primary extract (virulent); F = SDC primary extract (avirulent).*

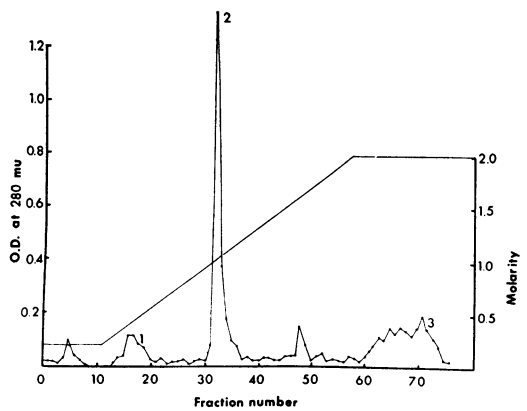

FIG. 3. *Elution pattern for the SDS primary extract (virulent) from a DEAE cellulose column.*

stable for long periods of time. However, when the present organism was grown under similar environmental conditions, but in a highly enriched medium designed to insure good antigen production, a rapid change in the virulence of the organism ensued. The use of enriched culture media was suggested by earlier studies which showed that some somatic antigens were produced in detectable quantities only in highly enriched media (6). Since the present organisms were to be used as a source of heat-labile antigens, it was felt that they should be grown under conditions which afforded the maximal opportunity for them to express their complete antigenic capabilities.

The most obvious change in the cultural conditions used in the present study was the addition to the medium of substantial amounts of serum. Although the presence of the pig serum provided one obvious variation to the cultural conditions

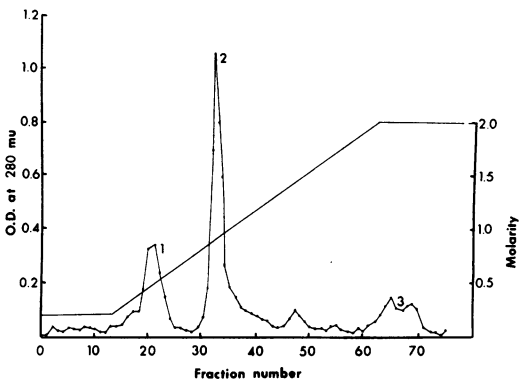

FIG. 4. *Elution pattern for the SDS primary extract (avirulent) from a DEAE cellulose column.*

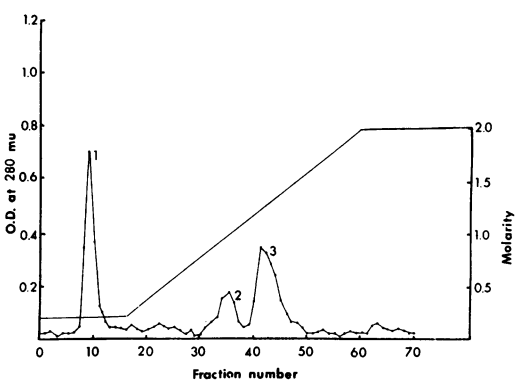

FIG. 5. *Elution pattern for the SDC primary extract (virulent) from a DEAE cellulose column.*

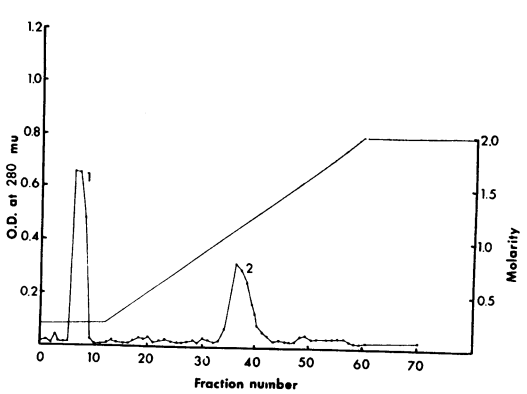

FIG. 6. *Elution pattern from the SDC primary extract (avirulent) from a DEAE cellulose column.*

which may have resulted in the change in virulence, other factors could also have been involved. For instance, Pirt et al. (22) reported that *Pasteurella pestis* grown in continuous culture only expressed its full antigenicity when the *p*H of the

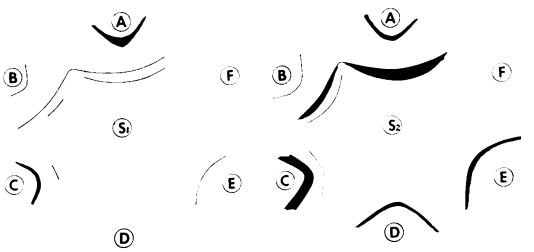

FIG. 7. *Diagrammatic representation of the precipitin lines obtained when the separated fractions obtained from the SDS primary extract (virulent) were tested against rabbit immune sera. Serum S1 was prepared against boiled Salmonella enteritidis. Serum S2 was prepared against ethyl alcohol-killed S. enteritidis. A = SDS primary extract; B = high-speed deposit (lipopolysaccharide); C = SDS primary extract after ethyl alcohol precipitation; D = DEAE cellulose fraction 1; E = DEAE cellulose fraction 2; F = DEAE-cellulose fraction 3.*

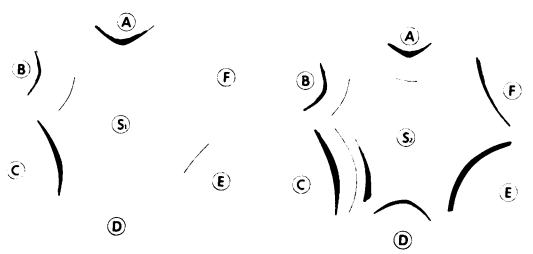

FIG. 8. *Diagrammatic representation of the precipitin lines obtained when the separated fractions obtained from SDC primary extract (virulent) were tested against rabbit immune sera. Serum S1 was prepared against boiled Salmonella enteritidis. Serum S2 was prepared against ethyl alcohol-killed S. enteritidis. A = lipopolysaccharide; B = high-speed deposit (lipopolysaccharide); C = primary extract; D = DEAE cellulose fraction 1; E = DEAE-cellulose fraction 2; F = DEAE cellulose fraction 3.*

medium was kept below 6.9. It was therefore decided to vary the *p*H of the fermentor from the previously used 7.4 to a slightly acid reaction. Adjustment of the *p*H to 6.6 appeared to slow down the emergence of the nonvirulent mutants in the presence of the pig serum, but, even under these conditions, virulence still declined. Replacing the serum protein with an equivalent amount of casein permitted sustained growth of the virulent strain. Thus, it appears that the pig serum was in some way responsible for the change in virulence, but no explanation for this phenomenon has been found.

Both sodium dodecyl sulfate and sodium deoxycholate extracted considerable amounts of material from the cell walls of *S. enteritidis*. The cell walls were never completely dissociated.

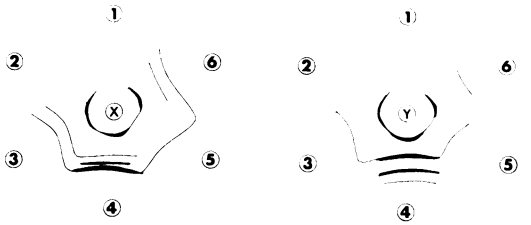

FIG. 9. *Diagrammatic representation of the precipitin lines obtained when the SDS and SDC primary extracts prepared from the virulent strain of Salmonella enteritidis were tested by gel diffusion against a number of different sera. Extract X was the SDS primary extract. Extract Y was the SDC primary extract. Serum 1, normal mouse; serum 2, convalescent mouse; serum 3, rabbit antiserum to boiled Salmonella enteritidis; serum 4, rabbit antiserum to ethyl alcohol-killed S. enteritidis; serum 5, rabbit antiserum to ethyl alcohol-killed S. enteritidis which had been exhaustively absorbed with boiled S. enteritidis; serum 6, rabbit antiserum to S. gallinarum.*

Under the electron microscope, the final residue contained very little electron-dense material and resembled in appearance the walls of *S. gallinarum* after treatment with dodecyl sulfate (27).

The SDS extracts from both the virulent and avirulent organisms produced three main peaks and two or three minor ones from the DEAE cellulose column. The corresponding SDC extracts produced three and two peaks, respectively. The reason for the absence of the third peak from the avirulent SDC preparation was not determined. The differences in the number of peaks obtained with the two extractants may reflect differences in their efficiency. However, Grant and Lawrence (10) reported that SDS treatment of the pure protein legumin resulted in a multiplicity of bands or peaks after electrophoresis or chromatography. Thus, the extra peaks observed in the SDS preparation could have been due to the residual detergent on the cell wall proteins. Despite the extended dialysis, considerable amounts of dodecyl sulfate remained in the cell wall extracts. Although alcohol treatment removed the detergent, it also reduced the number of lines obtained by gel diffusion. On the other hand, after extended dialysis, less than 5% of the deoxycholate remained in the SDC extracts. Antisera prepared by immunization of rabbits with dialyzed SDC extracts gave the same number of precipitin lines against the extract as were observed for rabbit antisera prepared against untreated whole cells (Collins, *unpublished data*). Thus, the smaller percentage yield in the SDC extracts could be set against the greater ease by which the bulk of the residual surface-active material could be removed without seriously affecting the solubility or reactivity of the preparation.

In conclusion, the change in virulence observed in the pig serum-grown cells could not be associated with any differences in the number of heat-labile or heat-stable haptens present. This is in general agreement with the findings of earlier workers (13, 18, 19). In the following report, the immunogenicity of the various extracts will be investigated to see whether the extracts or the separated antigens derived from them varied in their ability to protect mice against subsequent challenge with the virulent organism.

ACKNOWLEDGMENTS

We thank G. Rogers, Biochemistry Department, University of Adelaide, for the electron microscopic examination of the cell wall preparations. Robert Walsh provided excellent technical assistance during the continuous culture experiments.

LITERATURE CITED

1. AUZINS, I., AND D. ROWLEY. 1963. Factors involved in the adherence of *S. typhimurium* C5 and mouse peritoneal macrophages. Australian J. Exptl. Biol. Med. Sci. **41**:539–546.
2. BARTA, G. 1963. Soluble protective antigen from *B. pertussis* prepared with sodium desoxycholate. J. Immunol. **90**:72–80.
3. BAUERJEA, A., AND J. MUNOZ. 1962. Antigens of *Bordetella pertussis*. II. Purification of heat-labile toxin. J. Bacteriol. **84**:269–274.
4. BOIVIN, A., AND L. MESROBEANU. 1935. Recherches sur les antigènes somatiques et sur les endotoxines des bactéries. I. Considérations générales et expose des techniques utilisées. Rev. Immunol. **1**:553–569.
5. COLLINS, F. M. 1964. The effect of the growth rate on the composition of *S. enteritidis* cell walls. Australian J. Exptl. Biol. Med. Sci. **42**:255–262.
6. COLLINS, F. M., AND D. ROWLEY. 1964. The expression of heterophile antigenicity by Salmonellae. Australian J. Exptl. Biol. Med. Sci. **42**:249–254.
7. COLLINS, F. M., AND M. MILNE. 1966. Heat-labile antigens of *Salmonella enteritidis*. II. Mouse-protection studies. J. Bacteriol. **92**: 549–557.
8. DAVIES, D. A. L. 1960. Polysaccharides of Gram-negative bacteria. Advan. Carbohydrate Chem. **15**:271–340.
9. FELIX, A., AND R. M. PITT. 1951. The pathogenic and immunogenic activities of *Salmonella typhi* in relation to its antigenic constituents. J. Hyg. **49**:92–110.
10. GRANT, D. R., AND J. M. LAWRENCE. 1964. Effects of sodium dodecyl sulphate and other dissociating reagents on the globulins of peas. Arch. Biochem. Biophys. **108**:552–561.
11. HERBERT, D., R. ELSWORTH, AND R. C. TELLING. 1956. The continuous culture of bacteria; a theoretical and experimental study. J. Gen. Microbiol. **14**:601–622.
12. HOBSON, D. 1957. Chronic bacterial carriage in survivors of experimental mouse typhoid. J. Pathol. Bacteriol. **73**:399–410.
13. HOBSON, D. 1957. The behaviour of a mutant strain of S. *typhimurium* in experimental mouse typhoid. J. Hyg. **55**:322–332.
14. JENKIN, C. R., AND D. ROWLEY. 1965. Partial purification of the "protective" antigen of *Salmonella typhimurium* and its distribution amongst various strains of bacteria. Australian J. Exptl. Biol. Med. Sci. **43**:65–78.
15. KAUFFMANN, F. 1954. The enterobacteriaceae, 2nd ed. E. Munksgaard, Copenhagen.
16. KAWAKI, M., N. OSAWA, AND S. MITSUHASHI. 1963. Experimental Salmonellosis. III. New toxic fraction (L) obtained from *S. enteritidis* and its immunological properties. J. Bacteriol. **86**:872-879.
17. KOTELKO, K., A. M. STAUB, AND R. TINELLI. 1961. Etude immunochemique sur les Salmonella. VIII. Role des groupements O acetyle dans la specificite du facteur 0:5. Ann. Inst. Pasteur **100**:618–637.
18. MACKENZIE, G. M., H. FITZGERALD, AND R. M. PIKE. 1935. Inter-relationships of antigenic structure, virulence and immunizing properties of smooth and rough cultures of *S. aertryche*. Trans. Assoc. Am. Physicians **50**:242–248.
19. MACKENZIE, G. M., R. M. PIKE, AND R. E. SWINNEY. 1940. Virulence of *S. typhimurium*. II. Studies of the polysaccharide antigens of virulent and avirulent strains. J. Bacteriol. **40**: 197–214.
20. MITSUHASHI, S., M. KAWAKAMI, Y. YAMAGUCHI, AND N. NUGAI. 1958. Studies on the experimental typhoid. I. A comparative study of living and killed vaccines against the infection of mice with *S. enteritidis*. Japan. J. Exptl. Med. **28**:249–258.
21. OUCHTERLONY, O. 1962. Diffusion-in-gel methods for immunological analysis. Progr. Allergy **6**:30–154.
22. PIRT, S. J., E. J. THACKERAY, AND R. HARRIS-SMITH. 1961. The influence of environment on antigen production by *Pasteurella pestis* studied by means of the continuous flow culture technique. J. Gen. Microbiol. **25**:119–130.
23. RANDERATH, K. 1963. Thin layer chromatography. Academic Press, Inc., London.
24. REED, L. J., AND H. MUENCH. 1938. A simple method of estimating fifty per cent end points. Am. J. Hyg. **27**:493–497.
25. SHAFA, F., AND M. R. J. SALTON. 1960. Disaggregation of bacterial cell walls by anionic detergents. J. Gen. Microbiol. **22**:137–141.
26. SUTHERLAND, I. W. 1963. The protective activity of components of *B. pertussis* cell walls. Immunology **6**:246–254.
27. WEIDEL, W., H. FRANK, AND W. LEUTGEB. 1963. Autolytic enzymes as a source of error in the preparation and study of Gram-negative cell walls. J. Gen. Microbiol. **30**:127–130.
28. WESTPHAL, O. 1957. Polysaccharides in biology, *In* G. F. Springer [ed.], Second Macy Conference, New York.
29. WESTPHAL O., O. LÜDERITZ, AND F. BISTER. 1952. Über die Extraction von Bacterien mit Phenolwasser. Z. Naturforsch. **7b**:148–155.