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The Action of Dilute Aqueous *NN*-Dimethylhydrazine on Bacterial Cell Walls

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1. Walls of certain Gram-positive bacteria dissolved on incubation with dilute aqueous *NN*-dimethylhydrazine in the presence of air, by a reaction that probably involves free radicals. 2. Under the conditions described, the soluble products from the peptidoglycan were almost all non-diffusible. After brief incubation of walls of some organisms with reagent, part of the peptidoglycan component was obtained as a high-molecular-weight gel, the viscosity of which was rapidly decreased by incubation with lysozyme. 3. The extent to which peptidoglycan dissolved varied with different organisms, depending possibly on the extent of cross-linking, but the nature of the bonds that were destroyed has not been established. 4. Teichoic acids and polysaccharides were solubilized by this treatment and could be isolated in high overall yield. 5. The procedure is valuable in the examination of the distribution of heteropolymers in walls, and has been used to show that the polysaccharide present in walls of *Lactobacillus arabinosus* 17–5 is phosphorylated and may account for 20% of the total phosphate of the wall.

Walls of Gram-positive bacteria contain teichoic acids and polysaccharides attached to the peptidoglycan component. Chemical extraction of the former polymers from walls has been accomplished in several ways, most of which give complete extraction only after extensive degradation of the various polymers. We have therefore studied further the extraction of teichoic acids by dilute solutions of hydrazines. Hydrazines are known to give rise to free radicals (Scott & Barry, 1968) and the depolymerization of several biological macromolecules by radical oxidation is well documented (Herp, Rickards, Matsumara, Jakosalem & Pigman, 1967). It is likely that such attack accounts for the present observations. The optimum conditions for the dissolution of walls with DMH* have been established with walls of *Micrococcus lysodeikticus* and *Bacillus megaterium*, both of which undergo complete dissolution. Application of these conditions to walls of other bacteria has shown considerable variations in the extent to which they dissolve, but in all cases examined teichoic acids and polysaccharides are extracted in high yield.

MATERIALS AND METHODS

Growth of organisms. M. lysodeikticus N.C.T.C. 2665 was grown at 37° in a medium containing 1% of Bactopeptone, 0·5% of NaCl, 1% of yeast extract and 1% of glucose, the initial pH being adjusted to 7·5. Cells were harvested at the end of the exponential phase of growth. *B. megaterium* N.C.T.C. 7581 was grown for 16hr. at 37° in a medium containing 1% of glucose and 1% of peptone, and *Escherichia coli* K12 was grown for 16hr. at 37° in a medium containing 1% of glucose and 1% of nutrient broth.

Lactobacillus arabinosus 17–5, *Staphylococcus aureus* H and *Bacillus subtilis* (culture provided by Dr M. R. J. Salton) were grown as previously described (Archibald, Baddiley & Buchanan, 1961; Baddiley, Buchanan, RajBhandary & Sanderson, 1962; Armstrong, Baddiley & Buchanan, 1960) and cell walls were prepared as described by Archibald, Baddiley & Shaukat (1968).

Materials. DMH (British Drug Houses Ltd., Poole, Dorset) was distilled immediately before use, the fraction with b.p. 60–63° being used. Anhydrous hydrazine was prepared as described by Archibald *et al.* (1968). Calf intestinal phosphomonoesterase and lysozyme were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

Analytical methods. Phosphate, amino compounds, reducing sugars, hexose and periodate were determined as described by Archibald *et al.* (1968).

Chromatography. Paper chromatography was carried out on Whatman no. 4 paper that had been washed before use with 2M-acetic acid and then water. Thin-layer plates were prepared with silica gel (Kieselgel G; E. Merck A.-G., Darmstadt, Germany). The solvent systems were those described by Archibald *et al.* (1968). Products were detected by the periodate–Schiff reagents for glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956), the molybdate reagents for phosphoric esters (Hanes & Isherwood, 1949), the aniline phthalate reagent for reducing sugars (Partridge, 1949), the alkaline $AgNO_3$ reagent for

* Abbreviations: DMH, *NN*-dimethylhydrazine; DAP, $\alpha\epsilon$-diaminopimelic acid.

reducing compounds (Trevelyan, Procter & Harrison, 1950) and the ninhydrin reagent for amino compounds (Consden & Gordon, 1948).

RESULTS

Experiments with walls of M. lysodeikticus. Suitable conditions for the reaction were established mainly with walls of *M. lysodeikticus*. The actions of DMH, hydrazine and methylamine are given in Table 1, where the superiority of DMH is seen. Conditions were optimum at 80° for 1hr., but comparable results were obtained at 37° for about 20hr. From Table 2, where the effect of concentration of DMH was examined, it was concluded that 0·2% DMH was convenient; the oxygen requirement is shown in Table 3.

The nature of the solubilized wall was studied on a larger scale. Walls (490mg.) in water (400ml.) were heated at 80° for 20min. with a 2% (v/v) solution (100ml.) of DMH adjusted to pH 7 with formic acid; aeration was achieved with a sintered disk. The extinction had decreased from 1·0 to 0·085 during the reaction. The cooled filtered (no. 4 sinter) solution was centrifuged at 30000*g* for 45min. to give a clear gel (13ml.). From an examination of a small sample, this represented 96mg. of dry product. The supernatant solution was dialysed against water and freeze-dried, giving 290mg. of dry product.

The effect of further treatment with DMH was studied on a sample (2ml.) of the gel in 0·2% DMH–formate reagent (90ml.) at 80° for 2hr. with aeration; centrifugation as above gave no gelatinous sediment, whereas a control experiment with water gave the expected gel. Similarly, degradation of the gel was readily achieved with lysozyme (2mg. of enzyme, 1ml. of gel in 20ml. of water for 3hr. at 37°), whereupon no material could be sedimented by centrifugation at 30000*g*. The gel (13mg.) in 0·5M-sodium chloride (15ml.) at 20° had specific viscosity 0·15; after addition of lysozyme (1mg.) in water (0·02ml.) and incubation at 20° for 20min. the viscosity had fallen to 0·02.

The effect of dissolution in aqueous DMH on the composition of walls is shown in Table 4. After 75min. the extinction of the suspension in DMH had fallen by more than 90%, whereas the suspensions in water were only slightly affected. Paper electrophoresis (pH 2·5 and 6·5) of the non-diffusible material obtained by treatment of walls with DMH for 6·5hr. showed that several ninhydrin-positive products were formed.

A measure of the ability of walls and solubilized materials to bind hydrazines was obtained in the following way. Suspensions of walls (0·5mg./ml.) in 0·3% ammonium acetate, pH 7, that was 0·065M with respect to hydrazine were heated at 80° for 3·5hr. under nitrogen and under oxygen. The digest obtained from incubation with oxygen was extensively dialysed against water and 0·1M-sodium

Table 1. *Dissolution of walls with different reagents*

Walls of *M. lysodeikticus* N.C.T.C. 2665 were suspended (0·5mg./ml.) in the reagents and changes in extinction at 550nm. were followed with time. Solutions (2%, v/v) of DMH, hydrazine and methylamine were adjusted to pH 7·0 with formic acid and added to water containing the various salts to give a final reagent concentration of 0·2% and salt concentration of 0·3%. The suspensions were then shaken in open conical flasks at 80°.

	E_{550}		
Reagent	At zero time	After 1hr.	Final pH
DMH–sodium acetate	0·777	0·063	5·5
DMH–$(NH_4)_2CO_3$	0·795	0·110	8·9
DMH–NH_4Cl	0·778	0·252	2·8
DMH–ammonium formate	0·778	0·083	5·4
Methylamine–sodium acetate	0·792	0·779	6·8
Hydrazine–sodium acetate	0·470	0·100	—
Water	0·792	0·768	6·8

Table 2. *Effect of DMH concentration on dissolution of walls*

Walls of *M. lysodeikticus* were suspended (0·5mg./ml.) in solutions containing ammonium acetate (0·3%) and varying amounts of DMH and shaken at 80° in open flasks.

Concn. of DMH (%)	E_{550} after 1hr.	Final pH
0·00	0·883	5·6
0·04	0·237	5·5
0·08	0·100	5·5
0·12	0·060	5·3
0·16	0·058	5·3
0·20	0·057	5·3

Table 3. *Oxygen requirement for reaction between walls and DMH*

Walls of *M. lysodeikticus* were suspended in a solution containing DMH (0·2%) and ammonium acetate (0·3%) and heated at 80° under a gentle stream of oxygen. A duplicate suspension was heated under a stream of nitrogen and samples were removed at intervals for extinction measurements.

	E_{550}	
Time (min.)	Sample under N_2	Sample under O_2
0	0·202	0·294
6	0·207	0·163
25	0·208	0·074
45	0·211	0·047

Table 4. *Analysis of* M. lysodeikticus *walls*

Walls suspended in water (0·5mg./ml.) or in the DMH reagent were heated at 80° for 75 and 155min. Solvent was removed by freeze-drying and the residues were hydrolysed in the absence of air in 6M-HCl for 16hr. Analysis was carried out on a Technicon AutoAnalyzer. Free amino and carboxyl groups were determined as described by Archibald, Baddiley & Shaukat (1968). The non-diffusible fraction was prepared from 200mg. of walls; after 6·5hr. the residue (2mg.) was separated by centrifugation, and the supernatant was dialysed against several changes of distilled water, then freeze-dried (177mg.). Results are expressed as μmoles/g. of wall.

	Untreated walls	Walls in water (75min.)	Walls in DMH (75min.)	Walls in water (155min.)	Walls in DMH (155min.)	Non-diffusible fraction of walls treated with DMH for 6·5hr.
Muramic acid	538	550	422	563	422	461
Glutamic acid	819	793	755	768	781	794
Glycine	909	908	806	832	909	884
Alanine	1562	1536	1561	1421	1485	1575
Glucosamine	640	640	499	589	550	563
NH_3	695	817	1739	797	2427	894
Lysine	755	781	640	755	627	691
NH_2-Alanine	6·9			8·8	11·9	3·8
NH_2-Lysine	446			405	326	320
Bis-NH_2-lysine	2·2			2·1	1·8	0
Glycine-CO_2H	495			475	320	410
Alanine-CO_2H	45			55	55	50

chloride and then freeze-dried. The walls that had been heated under nitrogen were recovered by centrifugation and washed with M-sodium chloride and water and then freeze-dried. Samples were analysed for free and bound hydrazine by the method of Seifter, Gallop, Michaels & Meilman (1960). Walls that had been heated under nitrogen contained 2·05μmoles of free hydrazine/g. and 4·7μmoles of bound hydrazine/g., whereas the non-diffusible fraction of walls incubated with hydrazine and oxygen contained 7·1μmoles of free hydrazine/g. and 34·8μmoles of bound hydrazine/g. These values indicate that no extensive hydrazinolysis of peptide linkages had occurred.

Experiments with walls of B. megaterium 7581. Walls of *B. megaterium* were soluble in the DMH reagent under conditions similar to those described for *M. lysodeikticus*. After 1hr. the extinction had decreased from 0·77 to 0·045 and after 3hr. to 0·03. Analytical results are given in Table 5. After dinitrophenylation of the digest a small amount of DNP-alanine was present before acid hydrolysis; this is referred to as free alanine in Table 5. In the hydrazinolysis experiments, the aqueous washings from the Dowex column were hydrolysed and shown to contain only alanine, derived from alanine hydrazide. This indicated that all of the glutamic acid and DAP residues have at least one carboxyl group free, and is in agreement with independent observations (Bricas, Ghuysen & Dezélée, 1967). This was partly confirmed by examination of the acid eluate; electrophoresis on paper at pH6·5 showed a compound of mobility identical with that of DAP semihydrazide. No glutamic semihydrazide (Ito & Strominger, 1964) was observed, but a compound having the mobility of pyroglutamic acid was present. This presumably arose from glutamyl-γ-hydrazide.

A digest of walls (100mg.) was dialysed and the diffusible and non-diffusible fractions were analysed (Table 6). It was found that less than 10% (w/w) of the soluble peptidoglycan material was diffusible. The non-diffusible fraction was treated for a further 6hr. with fresh DMH reagent, dialysed and the non-diffusible fraction freeze-dried, when 60mg. of material was recovered.

Action of DMH on the peptidoglycan layer of E. coli *K*12. The peptidoglycan layer was prepared from *E. coli* walls as described by Mandelstam (1960). A sample (3mg.) was homogenized in water (2·5ml.) and 3% (w/v) ammonium acetate, pH7 (0·6ml.), and 2% DMH, pH7 (0·6ml.), were added. After heating the mixture at 80° for 5hr. under oxygen the extinction had decreased from 0·74 to 0·33. Centrifugation gave a solid residue (about 0·4mg.), and the supernatant was freeze-dried, hydrolysed and analysed (Table 7).

Action of DMH on DAP. An aqueous solution of DAP (2·5ml. containing 1·2μmoles) was added to 3% (w/v) ammonium acetate solution, pH7·0 (0·6ml.), and 2% (v/v) DMH, pH7 (0·6ml.). The mixture was heated at 80° for 1hr. under oxygen and then evaporated to dryness *in vacuo*; analysis showed 1·1μmoles of DAP, corresponding to 92% recovery.

Action of DMH on the peptidoglycan layer of S. aureus. Walls of *S. aureus* (23mg.) were suspended in 10% (w/v) trichloroacetic acid (20ml.) at 37°

Table 5. *Composition of walls of* B. megaterium *before and after treatment with DMH*

Walls (1 mg./ml.) in a solution containing 0·5% ammonium acetate (added as a 3% solution at pH 7) and 0·3% DMH were heated at 75° in a gentle stream of oxygen for 3 hr. The solution was evaporated to dryness repeatedly *in vacuo*, then hydrolysed and analysed as described in Table 4. *C*-Terminal amino acids were also determined after hydrazinolysis by application to a column of Dowex-1 (OH^- form) resin, washing the column with water and then eluting with 10% (v/v) acetic acid. Results are expressed as μmoles/g. of wall.

	Walls	DMH digest
Muramic acid	510	270
Glutamic acid	690	635
Alanine	1580	1490
Glucosamine	900	570
DAP	690	285
NH_2-DAP	190	34
NH_2-Alanine	36	223+16 free alanine
Alanine-CO_2H	160	190
Glutamic-bis-CO_2H	8	8
DAP-bis-CO_2H	20	9

Table 6. *Diffusible and non-diffusible fraction of* B. megaterium *after 6 hr. incubation with DMH*

Walls (100 mg.) were incubated with DMH as described in Table 1 for 6 hr. and then dialysed against distilled water (4×1 l.). Diffusible and non-diffusible materials were hydrolysed and analysed as described in Table 4.

	Fractions (μmoles/g. of original wall)	
	Diffusible	Non-diffusible
Aspartic acid	10	25
Muramic acid	10	360
Glutamic acid	30	570
Glycine	70	40
Alanine	80	1200
Glucosamine	20	740
DAP	30	360

Table 7. *Action of DMH on the peptidoglycan of* E. coli *K12*

Experimental conditions are described in the text and analytical methods were as given in Table 1. Results are given in μmoles/g. of peptidoglycan

	Peptidoglycan	Solubilized peptidoglycan
Muramic acid	435	250
Glutamic acid	630	630
Alanine	1210	1160
Glucosamine	485	250
DAP	630	270
NH_2-Alanine	Trace	0
NH_2-DAP	280	0

Table 8. *Action of DMH on the peptidoglycan of* S. aureus *H*

Experimental conditions are described in the text and analytical methods were as given in Table 1. Results are given in μmoles/g. peptidoglycan.

	Peptidoglycan	DMH-solubilized material	Residue
Muramic acid	325	—	225
Glutamic acid	490	75	410
Glycine	2420	345	2170
Alanine	1010	150	910
Glucosamine	410	—	275
Lysine	550	75	425

Table 9. *Analysis of fractions obtained by ion-exchange chromatography of the DMH extract from* L. arabinosus

	Fraction 1	Fraction 2	Fraction 3
Weight (mg.)	77·8	5·6	97·5
Phosphate content (% P)	1·8	11·7	7·1
% of total phosphate in original extract	16·2	7·2	77·0

for 20 hr. The residual material was washed with water and then freeze-dried; a sample (2·6 mg.) was homogenized in water and treated with DMH for 5 hr. as before, when the extinction had decreased from 0·68 to 0·38. The wall residue was removed by centrifugation and portions of residue and of supernatant were analysed (Table 8).

Action of DMH on walls of L. arabinosus. The action of the DMH reagent on walls of *L. arabinosus* (400 mg.) is shown in Fig. 1. During the digestion, the extinction decreased from 0·665 to 0·195 and 86% of the phosphate was extracted. The residual material (194 mg., containing 0·64% of phosphorus) was peptidoglycan and this, on examination under the electron microscope, retained the shape of the original walls. The weights and analyses of material corresponding to fractions 1, 2 and 3 are given in Table 9.

Fraction 1 on chromatographic analysis on paper was shown to represent a polysaccharide of rhamnose, glucose and glucosamine; it did not give anhydroribitol, ribitol or ribitol phosphates on acid hydrolysis. Fraction 2 gave on acid hydrolysis ribitol, ribitol phosphates and anhydroribitol, but only traces of glucose. Fraction 3 analysed similarly to fraction 2 but also gave substantial amounts of glucose; its characterization as teichoic acid is given below.

The phosphate analyses of fraction 3 and the products of acid hydrolysis are similar to those reported previously for the wall teichoic acid

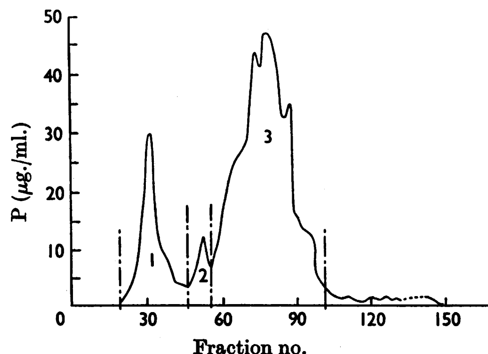

Fig. 1. Fractionation of walls of *L. arabinosus* with DMH. Walls (400mg. containing 2·4% of P) in water (320ml.) were heated with vigorous stirring for 2hr. at 80° with 2% (v/v) DMH solution (80ml.) adjusted to pH 7 with formic acid. Insoluble peptidoglycan was centrifuged at 25000*g* for 30min. The supernatant, diluted with an equal volume of water, was applied to a column (2cm. × 30cm.) of DEAE-cellulose (CO_3^{2-} form), which was then washed with water (500ml.) followed by a linear gradient of water (500ml.) and 0·5M-$(NH_4)_2CO_3$ (500ml.). Fractions (7ml.) were collected at the commencement of gradient elution and samples (0·2ml.) were analysed for P (see Table 9). Fractions corresponding to peaks 1, 2 and 3 were rotary-evaporated and then freeze-dried.

extracted with trichloroacetic acid (Archibald *et al.* 1961). Moreover, hydrolysis in alkali [2mg. in M-sodium hydroxide (100μl.) at 100° for 3hr.], then passage through Dowex 50 (NH_4^+ form) resin (1ml.), freeze-drying and complete dephosphorylation of the resulting phosphomonoesters with phosomonoesterase (0·1mg. in 0·05M-ammonium carbonate) during 16hr. at 37°, gave anhydroribitol, ribitol and major amounts of 2-*O*-D-glucopyranosyl-L-ribitol, 3-*O*-D-glucopyranosylribitol and 2,3-*O*-diglucopyranosyl-L-ribitol. These products were identified by comparison with degradation products of the teichoic acid extracted from walls of this organism by the action of dilute alkali, as described by Hay, Archibald & Baddiley (1965).

The teichoic acid in fraction 3 reduced 3·3mol. of periodate/mol. of phosphate during 24hr. with 0·2M-sodium periodate at room temperature in the dark; oxidation was complete during that time. It yielded 10% of its phosphate as inorganic phosphate when a sample (10mg.) was treated at 37° for 16hr. with phosphomonoesterase (0·01mg.) in 0·05M-ammonium carbonate.

DISCUSSION

We have previously reported briefly (Archibald & Baddiley, 1965) that treatment of the walls of several bacteria with dilute aqueous solutions of phenylhydrazine leads to solubilization of teichoic acid, and that DMH is also effective and in some cases completely dissolves the walls. Isolation of heteropolymers after extraction with phenylhydrazine is complicated by tar formation during the reaction, so we now report more extensive studies on the much clearer action of dilute aqueous DMH on walls of a number of bacteria. Early work was characterized by a marked lack of reproducibility, the origin of which is twofold. A requirement for oxygen, suggested by the finding that consistently greater proportions of teichoic acid were extracted from the walls of *L. arabinosus* when reactions were carried out in large conical flasks rather than in test tubes, was directly demonstrated for *M. lysodeikticus* by attempting a reaction under nitrogen when no dissolution of the walls occurred. A further source of variation was eliminated after it was observed that the fractions having high b.p. (>64°) obtained during distillation of DMH contain a powerful inhibitor; addition of this fraction to purified reagent completely suppressed its action on cell walls. Under the conditions now described reproducible results were obtained, and the procedure is valuable for solubilization of teichoic acids and polysaccharides under relatively mild conditions. This permits the isolation and determination of the proportion of various heteropolymers in walls. Walls of *M. lysodeikticus* and *B. megaterium* dissolved completely on treatment with the reagent, in contrast with the behaviour of walls of *S. epidermidis* I 2 and *S. aureus* H, the peptidoglycan fractions of which were largely unaffected although teichoic acid was solubilized. Dissolution of the walls of *M. lysodeikticus* and *B. megaterium* resulted in the formation of largely non-diffusible material; thus, after treatment with the reagent for 6·5hr. at 80°, 89% (w/w) of the original wall of *M. lysodeikticus* was recovered as non-diffusible material, and on similar treatment of walls of *B. megaterium* less than 10% (w/w) of the peptidoglycan was diffusible (Table 6). These findings suggest that relatively few covalent bonds are broken under reaction conditions sufficient to destroy the wall structure, since only small amounts of material of low molecular weight were formed. The requirement for oxygen and the inhibitory effect of the high-boiling fraction of commercial DMH suggest that the reaction may involve free radicals.

Depolymerization of various biological macromolecules by free-radical oxidation has been reported (cf. Herp *et al.* 1967) as has the generation of free radicals by oxidation of DMH (Scott & Barry, 1968); although the present study has not established the nature of the linkages attacked, it seems likely that degradation is due to a reaction of this type. Salton (1964) has reported the dis-

solution of walls by sodium hypochlorite, and it seems likely that this reaction also involves radical oxidation. Although small (diffusible) fragments were not produced in significant amounts on prolonged treatment of walls with the reagent, it is clear that further degradation of peptidoglycan occurred after its initial solubilization. Thus brief treatment of *M. lysodeikticus* walls gave substantial proportions of material of high molecular weight that was deposited as a clear viscous gel on centrifugation at 30000*g*. Treatment of this viscous material with lysozyme, or further treatment with reagent, caused depolymerization to material with greatly decreased viscosity. Gel-like fractions were also observed after incomplete digestion of walls of *B. subtilis* and *L. arabinosus* 17-5. These fractions contained both peptidoglycan and teichoic acid, presumably attached to each other.

A sample of solubilized *M. lysodeikticus* walls was analysed for amino compounds and for *N*- and *C*-terminal amino acids (Table 4). Although differences were observed in the values for certain amino compounds no substantial differences in the number of free amino or carboxyl groups was detected other than a decrease of 20% in the amount of free ϵ-amino group of lysine. After dialysis, the non-diffusible fraction of the extract was similar in composition, although the value for ammonia was similar to that of untreated walls. Dissolution of the walls of *B. megaterium* was accompanied by substantial loss of DAP, particularly of DAP with a free amino group; this was decreased from 190 to 34μmoles/g. of wall (Table 5). This destruction of the free amino group of DAP was even more marked on walls of *E. coli* K12, where the total DAP was decreased from 630μmoles/g. to 270μmoles/g. and the ϵ-amino group of DAP was decreased from 280μmoles/g. to zero (Table 7). The nature of the degradation products, which presumably arise from the DAP possessing a free ϵ-amino group, is unknown. It is noteworthy that free DAP is less rapidly destroyed by treatment with the reagent.

In contrast with the complete solubilization of walls of *M. lysodeikticus* and *B. megaterium*, the peptidoglycan fraction from *S. aureus* H, prepared by extraction of the teichoic acid from intact walls with trichloroacetic acid, was largely unaffected by the reagent, less than 10% (w/w) of the peptidoglycan components being dissolved. On the other hand, treatment of the intact wall with the reagent solubilized over 80% of the phosphate (teichoic acid), but again little peptidoglycan was removed (unpublished observation). Walls of *S. epidermidis* I 2 behaved similarly (Archibald *et al.* 1968) whereas with walls of *B. subtilis* not only teichoic acid but most of the peptidoglycan was dissolved. With *L. arabinosus* 17-5 walls almost all of the teichoic acid and polysaccharide were extracted, together with a little peptidoglycan. The extracted polymers were conveniently separated on DEAE-cellulose by using gradient elution with ammonium carbonate, when quantitative recovery of the applied material was obtained (Fig. 1). Results were highly reproducible, and it was shown that 16–20% of the phosphate in the wall represents a phosphorylated polysaccharide and the remainder is teichoic acid. A polysaccharide containing rhamnose has previously been reported in walls of *L. arabinosus* (Ikawa & Snell, 1960; Archibald *et al.* 1961), but has not hitherto been obtained free of teichoic acid. Polysaccharide obtained here did not, on acid hydrolysis, give products characteristic of teichoic acid, and the purified material contained rhamnose, glucose, glucosamine and phosphate but no ribitol. The amount of teichoic acid in walls has frequently been calculated on the basis of the phosphate content; the present results demonstrate that any such calculations are invalid when applied to *L. arabinosus* 17-5; however, the new procedure does permit the determination of the proportions of these phosphate-containing polymers in the wall.

Chemical studies on the teichoic acid fraction isolated from walls of *L. arabinosus* 17-5 showed that it resembled material isolated (in much lower yield) by extraction with trichloroacetic acid. Thus alkali hydrolysis followed by enzymic dephosphorylation gave the same products from material prepared by both procedures. Comparable amounts of inorganic phosphate were produced when each preparation was incubated with phosphomonoesterase, and the amount of periodate reduced by each sample was identical.

Although the concomitant formation of tar led us to discontinue studies with phenylhydrazine, the use of this reagent under conditions previously described has been applied successfully by Grov & Rude (1967) to the fractionation of walls of strains *S. aureus* and the separation of various antigenic materials. Thus whereas complete removal of teichoic acid from the wall by trichloroacetic acid was achieved only under conditions so drastic that almost complete loss of serological activity of the teichoic acid was observed, with phenylhydrazine all of the teichoic acid was extracted without loss of activity.

Lack of understanding of the mechanism of action of dilute aerated solutions of DMH on bacterial walls restricts its use at present in structural work on peptidoglycans. Nevertheless, the procedure may be used for the isolation of various heteropolymers from walls and may be valuable in studies on the nature and amounts of such polymers by both chemical and immunochemical techniques.

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