ACETYLHEXOSAMINE COMPOUNDS ENZYMICALLY RELEASED FROM *MICROCOCCUS LYSODEIKTICUS* CELL WALLS

I. ISOLATION AND COMPOSITION OF ACETYLHEXOSAMINE AND ACETYLHEXOSAMINE–PEPTIDE COMPLEXES

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SUMMARY

Some of the dialyzable products of the digestion of *Micrococcus lysodeikticus* cell walls by lysozyme and by a similar enzyme secreted by a *Streptomyces* have been isolated and their compositions determined. The two simplest substances released by both enzymes are (a) a di-saccharide of N-acetylmuramic acid-N-acetylglucosamine and (b) an N-acetylmuramic acid-N-acetylglucosamine complex. Seven peptide-acetylamino sugar complexes have been isolated. All seven compounds contain lysine, glutamic acid, glycine, alanine in the same molecular proportions as found in the original cell wall and a di-saccharide moiety of N-acetylmuramic acid and N-acetylglucosamine. One of the peptide-amino sugar complexes contains in addition a polysaccharide moiety [N-acetylmuramic acid-N-acetylglucosamine]$_{10}$.

INTRODUCTION

As reported earlier[1], the dialyzable fraction of lysozyme-digested *Micrococcus lysodeikticus* cell walls, contains at least one "small fragment" distinguishable by its R_F value and by its positive MORGAN AND ELSON reaction. It was also shown that this "small fragment", possibly di-saccharide, is composed of N-acetylglucosamine and an unknown N-acetylamino sugar, subsequently shown to be muramic acid[2,3]. Muramic acid was first detected in hydrolysates of spore peptides[4,5] and later found in many bacterial cell walls[6–9]. The structure of 3-O-α-carboxethyl-D-glucosamine has been proposed for it by STRANGE AND KENT[10].

This paper describes the isolation and the composition of several compounds giving a positive MORGAN AND ELSON reaction, which are released from *Micrococcus lysodeikticus* cell walls during digestion with egg-white lysozyme and with the F_1 fraction obtained from a bacteriolytic *Streptomyces* culture[11–13].

Abbreviations: AG = N-acetylglucosamine; DMAB = *p*-dimethylaminobenzaldehyde.

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MATERIALS AND METHODS

Cell walls preparations

Micrococcus lysodeikticus (NCTC 2665) was grown in vigorously aerated nutrient broth supplemented with 1 % glucose and 0.1 % mannitol. The cell walls were made by the method of SALTON AND HORNE[14].

Enzymes

Crystalline egg-white lysozyme (Armour Laboratories) was used throughout these studies. The *Streptomyces* bacteriolytic enzyme was isolated by one of us (J.M.G.) from culture filtrates (280 L) by a selective adsorption[13] at pH 7 on an Amberlite IRC 50 column. The resin was then washed three times with a *N* KCl solution, suspended in a buffer 0.08 *M* KOH–0.08 *M* boric acid–0.42 *M* NaCl and treated with 100 ml (per 200 g of resin) of a 4 *N* KOH–*N* KCl solution. The supernatant, dialyzed and lyophilized (2.1 g) was readsorbed on the minimum of Amberlite IRC 50 XE 64, previously equilibrated against a 0.0125 *M* veronal buffer pH 7, and eluted at the same pH by a 0.05 *M* veronal–0.2 *M* NaCl buffer. This elution was carried out four times by stirring the resin with the selected buffer system. The extracts were dialyzed together against distilled water and the white insoluble material (0.195 g) was collected. This last step of purification by precipitation at zero ionic strength was repeated three times and finally the insoluble purified enzyme (0.086 g) was dissolved in the buffer system KH_2PO_4–K_2HPO_4, μ 0.01–pH 7, 0.1 *M* NaCl (2 mg of dry weight enzyme/ml of solution), divided in lots of 1 ml each and freeze-dried. This final "F_1 preparation", at a concentration of 10 μg/ml, is without any detectable activity upon heated *Escherichia coli* cells, casein and keratin[11,13] and reduces the turbidity of a staphylococcal cell suspension[13,14] (lab. strain SA_3) to 50 % of its original value in 1 h.

Preparation of the dialyzable fractions of enzyme-digested walls

1 g of cell walls (dry wt.) was incubated for 20 h at 37° in the presence of 1 mg enzyme (lysozyme or F_1 preparation) in 40 ml 0.05 *M* ammonium acetate. The clear solution was dialyzed against 150 ml distilled water at 4° for 24 h. Dialysis was repeated three times and the dialyzable fraction freeze-dried *in vacuo* over H_2SO_4. Under these conditions, the dialyzable fraction accounted for approx. 35 % of the original weight of cell walls after F_1 treatment, and approximately 45 %, after lysozyme treatment.

Chromatography

Chromatography was performed on Whatman No. 1 paper in one dimension (descending in *n*-butanol–acetic acid–water (3 : 1 : 1, v/v/v)) or in two dimensions at right angles (ascending in pyridine–water (4 : 1, v/v) and descending in the butanol–acetic acid solvent).

Electrorheophoresis

Electrorheophoresis was carried out without avoiding the evaporation of the solvent from the papers by supporting them on a frame 34 cm/20 cm. Whatman 3MM paper and glass fiber filter paper (No 934-AH; Hurlbut Paper Co.) were used

under a constant voltage of 700 V with the following buffer systems: 2 *N* acetic acid (pH 2.35); glacial acetic acid–pyridine–water (2:4:1000) (pH 5.5); 0.02 *N* acetic acid + NH_3 (d 0.88) to pH 8.9. The material to be separated by electrorheophoresis on 3MM paper was deposited either at the equilibrium position near the center of the sheet (when performed at pH 2.35), or close to the positive electrode (for pH 5.5) or to the negative electrode (for pH 8.9). For all the experiments on glass paper, the substances were deposited at the center of the sheet.

Sprays and color reactions

On the chromatograms and electrorheophoreograms, the amino acids and amino sugars were detected with ninhydrin (0.2 % in acetone) and estimated by extracting the spots in 5 ml of acetone–water (3:1, v/v) and measuring the O.D. of the solution at 570 mμ. The reducing substances were detected with ammoniacal silver nitrate reagent and the acetylamino sugar-type substances by SALTON's method[15].

Hexosamines in solution were determined by a modification[16] of the method of ELSON AND MORGAN.

The technique used by REISSIG *et al.*[17] for the MORGAN-ELSON acetylhexosamine reaction was applied to the purified acetylamino sugar complexes as follows: 1 ml of sample, in 0.05 *M* $Na_2B_4O_7$, was heated for an adequate period of time (see EXPERIMENTAL) in a boiling water bath, and, after cooling to room temperature, mixed with 2.5 ml of EHRLICH's reagent (1 g DMAB [*p*-dimethylaminobenzaldehyde] in 100 ml glacial acetic acid containing 2 ml of conc. HCl). After 20 min at 37°, the O.D. was read at 585 mμ.

EXPERIMENTAL

Paper chromatographic properties

After digestion by lysozyme or by F_1, the dialyzable material corresponding to 5 mg of original *Micrococcus lysodeikticus* cell walls was examined by paper chromatography (Fig. 1).

The chromatograms obtained from both enzymic digests showed the presence

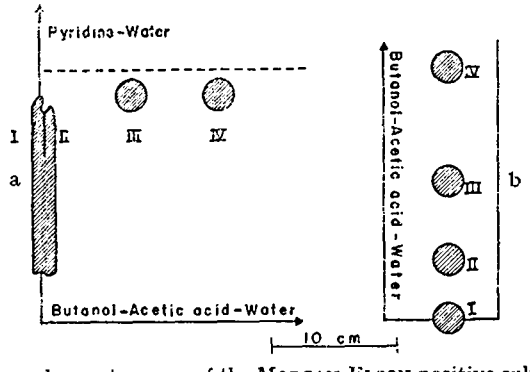

Fig. 1. Diagram of paper chromatograms of the MORGAN-ELSON positive substances released from *Micrococcus lysodeikticus* cell walls by lysozyme and F_1 digestion. (a) Two-dimensional chromatography for 12 h in pyridine–water (ascending) and 18 h in butanol–acetic acid–water (descending). (b) One-dimensional chromatography for 30 h in butanol–acetic acid–water (descending). IV and III components: DMAB and $AgNO_3$ positive; ninhydrin negative. II and I components: DMAB, $AgNO_3$ and ninhydrin positive.

of four spots detected by the modified MORGAN-ELSON spray reaction[15]. The components were called respectively LI, LII, LIII, LIV (after lysozyme digestion) or F_1I, F_1II, F_1III, F_1IV (after F_1 digestion), the index I to IV indicating an increasing migration in the butanol–acetic acid solvent. In pyridine–water, components III and IV migrated together and were very close to the solvent front, while components I and II showed considerable tailing.

Chromatograms were also sprayed with ammoniacal silver nitrate and ninhydrin reagents. Compounds I, II, III and IV showed a positive reaction with silver nitrate, but only compounds I and II were ninhydrin positive. Additional silver nitrate positive and ninhydrin positive spots could be detected but were not further examined*.

Electrorheophoretic properties

For the studies of the electrorheophoretic properties of the four MORGAN-ELSON positive substances liberated by lysozyme and F_1 from *Micrococcus lysodeikticus* cell walls, the dialyzable, digested fractions were applied to Whatman No. 1 paper as a band (400 μg/cm) and irrigated with the butanol–acetic acid system. After the four spots were located by spraying a narrow strip with the modified MORGAN-ELSON spray reagent[15], the bands were eluted with water and the solution evaporated to dryness.

After electrorheophoresis on Whatman 3MM paper under the conditions described in MATERIALS AND METHODS, the substances were located by their positive reactions with silver nitrate and DMAB reagents (components III and IV) and, in addition, with ninhydrin (components I and II).

The migrations of the main substances are illustrated in Fig. 2. This figure also shows the behavior of a neutral substance (N-acetylglucosamine).

At pH 5.50 and 8.90, all the substances were found to have a negative charge with a migration decreasing in the following order: LIII and F_1III; LIV and F_1IV; LI and F_1I; LII and F_1II. At pH 2.35, LIII and F_1III, and LIV and F_1IV were neutral while LI and F_1I, and LII and F_1II behaved as basic substances, the former pair being less positively charged than the latter pair.

These electrorheophoretic properties and color reactions strongly suggested that the components LIII and F_1III, LIV and F_1IV, were two different types of N-acetylamino sugar complexes, with free carboxylic groups belonging to muramic acid. On the other hand, the behavior of the components LI and F_1I, LII and F_1II showed that they were probably peptide-N-acetylamino sugar complexes.

The electrorheophoretic properties of these complexes were also examined on glass fiber paper with the same buffer systems. These studies were undertaken with fractions already purified by chromatography in butanol–acetic acid and electrorheophoresis on Whatman 3MM paper. Since SALTON's modified MORGAN-ELSON spray reagent gave irregular and generally rather weak reactions on this glass paper, the substances were detected only with the ninhydrin and silver nitrate reagents. The substances migrated more readily on the glass paper than on Whatman paper. Therefore, the time of electrorheophoresis on glass paper was less and the various

* It is worth noting that the silver nitrate reagent showed a fifth well-separated spot, ninhydrin and MORGAN-ELSON negative, with a R_F 0.95 in pyridine–water and 0.50 in butanol–acetic acid. It originated from the dialysis tubing and appeared to be glycerol or a related substance.

substances could not be applied close to the electrode to which they were attracted by their electrical charges (*e.g.*, applied at pH 5.5 near the positive electrode, components III and IV migrate off the glass paper).

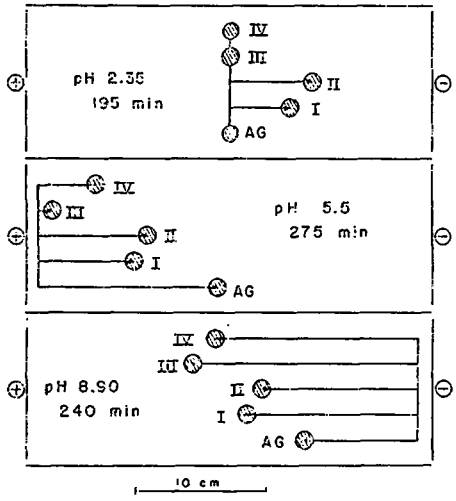

Fig. 2. Diagram of electrorheophoretic behavior on Whatman 3MM paper of the MORGAN-ELSON positive substances released from *Micrococcus lysodeikticus* cell walls by lysozyme and F_1 digestion. IV and III components: DMAB and $AgNO_3$ positive; ninhydrin negative. II and I components: DMAB, $AgNO_3$ and ninhydrin positive. AG: N-acetylglucosamine.

At pH 5.5 and after 1 h of electrorheophoresis, components III and IV migrated 12 cm from the center of the sheet towards the positive electrode. Although no further separation of these components occurred under these conditions, this electrorheophoresis was a useful step in the purification of both compounds because of the removal of one or more contaminating reducing substances (with a migration of 3 to 15 cm).

The situation was the same for the components I and II. Contaminating substances were removed by further electrorheophoresis on glass fiber at pH 2.35 (2 h) or at pH 5.5 (1 h). In addition, a further separation was achieved with the F_1II fraction. This fraction, separated after a 5-cm migration on Whatman 3MM at pH 2.35, was resolved by electrorheophoresis on glass fiber, at the same pH (2 h), into four components with a migration from the center of the sheet of 0; 4.5; 7 and 13 cm. Only two intermediate fractions F_1II B and F_1II C were both silver nitrate and ninhydrin positive.

Separation and purification

The technique was based on the chromatographic and electrorheophoretic properties described above. In every case, the dialyzable, digested fractions were first separated by chromatography in the butanol–acetic acid system and the four MORGAN-ELSON positive areas eluted and taken to dryness.

Fractions III and IV were successively submitted to electrorheophoresis at pH 5.5, first on 3MM paper (deposited near the positive electrode) and afterwards on glass fiber paper, the transfer of the substances from one paper to the other being made by applying the band of 3MM paper to the center of the glass fiber paper. The

substances were finally eluted from the glass paper, filtered through a sintered glass filter and evaporated to dryness by lyophilization.

The purification of fractions I and II was more complicated since, unlike fractions III and IV, each of them contained several compounds, with different electrorheophoretic properties. Furthermore, the number and behavior of the compounds in fractions I and II varied in different enzymic digests. These variations perhaps suggest some difference in the degree of the enzymic digestion of the preparations or in the efficiency of the dialysis. Whatever the explanation, the purifications were carried out in the manner reported above for the components III and IV, by successive electrorheophoresis at pH 2.35 and 5.5 on Whatman 3MM paper and on glass fiber paper.

Composition of the purified compounds LIII, F_1III, LIV and F_1IV

Preliminary experiments showed that these purified complexes consisted only of N-acetylglucosamine and N-acetylmuramic acid, with no free amino groups. Estimations of the two hexosamines liberated by acidic hydrolysis and of the purity of the complexes III and IV were performed as indicated below. 800 μg of each compound were hydrolyzed with 1 ml 2 *N* HCl, 2 h, 100° and each hydrolysate divided into four aliquots called series A, B, C, D and treated as follows.

(A) Aliquots were chromatographed in two dimensions and the ninhydrin positive spots, corresponding to glucosamine and muramic acid, were eluted with the acetone–water solvent and the O.D. determined at 570 mμ. (B) Aliquots were chromatographed in the butanol–acetic acid solvent and the areas corresponding to glucosamine and muramic acid were eluted, lyophilized and analyzed[16]. (C) The same procedure as (B) was carried out but the analyses were performed on cut-out areas of the paper placed directly in test tubes. In this case, the proportion H_2O–acetylacetone buffer was 2/1.5 ml. (D) The ELSON-MORGAN reaction[16] was directly performed on $NaHCO_3$ neutralized hydrolysates. For these hexosamine analyses, standard N-acetylglucosamine was also hydrolyzed and treated as above.

Table I shows the O.D. of glucosamine/O.D. of muramic acid obtained with ninhydrin (series (A)) and the ELSON-MORGAN tests (series (B), (C), (D)). In series (D), muramic acid concentrations could be calculated since the amount of glucosamine was known by the above procedures ((A), (B), (C)).

TABLE I

RATIO GLUCOSAMINE/MURAMIC ACID, IN TERMS OF OPTICAL DENSITY, OF THE HYDROLYZED AMINO SUGAR COMPLEXES RELEASED FROM *Micrococcus lysodeikticus* CELL WALLS BY LYSOZYME AND F_1 DIGESTION

Fraction	*Ratio estimated by*			
	Ninhydrin	ELSON-MORGAN *Reaction*		
	Series (A)	*Series (B)*	*Series (C)*	*Series (D)*
F_1III	1.13	2.40	3.28	2.30
L III	1.02	2.30	2.98	2.50
F_1IV	1	2.26	3.20	2.35
L IV	1.03	2.25	3.03	2.42
Average	1.04	2.30	3.14	2.39

Both ninhydrin and ELSON-MORGAN ratios showed that the same proportion of glucosamine and muramic acid occurred in the four amino sugar complexes. By ultracentrifugation (1.5 %; ammonium acetate buffer 0.5 *M*; 238,300 × *g*), amino sugar LIV gave an *S* value of 0.3 S which indicated that it was a di-saccharide (P.M. about 500). Therefore, a 1 to 1 proportion of N-acetylglucosamine to N-acetylmuramic acid was assigned to this compound as well as to the three other amino sugar complexes.

The ELSON-MORGAN ratio varied from 2.30 to 3.14 according to the technique. However, it is known[5] that muramic acid cannot be estimated with reference to glucosamine unless the conversion factor is known for the method used. STRANGE AND KENT[10] reported that the ratio glucosamine/muramic acid, expressed in terms of ELSON-MORGAN reaction coloration, was respectively 1.78, 1.85 and 1.56 when the

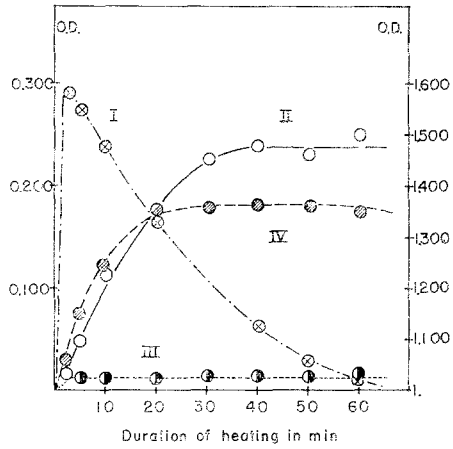

Fig. 3. Effect of duration of heating of the borate solution on color development, at 585 mμ, in MORGAN-ELSON reaction of the amino sugar complexes IV and III and of the amino sugar-peptide complexe LII, released from *Micrococcus lysodeikticus* by enzymic digestion. Right ordinate: I; N-acetylglucosamine 100 μg; left ordinate: II: F_1IV 75 μg (corrected weight); III: F_1III 75 μg (corrected weight); IV: LII B (preparation 4b) 200 μg.

estimation was made by the methods of ELSON AND MORGAN[18], DISHE AND BORENFREUND[19] and TRACEY[20]. The method of NEUHAUER *et al.*[16] was selected for the present studies because it minimized interferences by sugars and amino acids, but the effectiveness of the extraction procedure for the different chromogens is not known.

The purity of each amino sugar complex was estimated by referring to a standard glucosamine curve. It was 75 % for F_1III, LIII and F_1IV and 90 % for LIV.

Acetylhexosamine MORGAN-ELSON *reaction*: This reaction was applied to the disaccharides LIV and F_1IV using the modified method of REISSIG *et al.*[17]. As shown in Fig. 3, heating the alkaline solution for 3 min provided a maximum coloration for the N-acetylglucosamine while the reaction was only slightly positive for the disaccharides. Heating for a longer time resulted in an increased coloration until a maximum was finally reached after 40 min. The absorption spectrum of the colored complexes was identical to that given by N-acetylglucosamine or by the "small fragment" previously isolated[1] from *Micrococcus lysodeikticus* cell walls, with two peaks at 545 and 585 mμ and a minimum at 565 mμ.

By this technique, the di-saccharides were differentiated from the N-acetylglucosamine and from the amino sugar complexes III which as shown in Fig. 3 gave no appreciable color (a faint color was, however, obtained after heating 1 h at 120°).

Fig. 4 shows the relationship between the O.D. and the concentration of di-saccharides. As the proportionality coefficient was exactly the same for both LIV and F_1IV, we used this property for their estimation. On the basis of a molecular weight of 497, the molar extinction coefficient was found to be 4870 at 585 mμ.

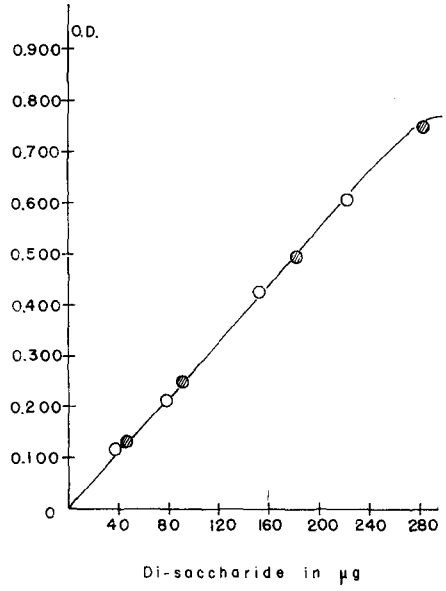

Fig. 4. Relationship between the O.D. (585 mμ) and the amount of di-saccharides LIV (○) and F_1IV (●) released from *Micrococcus lysodeikticus* by enzymic digestion, using the MORGAN-ELSON reaction. The weight of the amino sugar complexes are corrected on the basis of a purity of 75 % for F_1IV and 90 % for LIV. Calculation of the molar extinction coefficient: $\frac{497 \cdot 10^6 \times 3.5 \times 0.0028}{1000} = 4870$.

P.M. of the di-saccharide, 497; volume of the solution, 3.5 ml; angular coefficient, 0.0028.

Composition of the purified compounds F_1I, F_1II, LI and LII

Seven amino sugar-peptide complexes were isolated from three different preparations (called 3, 4a and 4b) of the dialyzable fractions: F_1I, F_1II B and F_1II C (preparation 3), LI, LII (preparation 4a) and F_1I, F_1II (preparation 4b). As had been found by STRANGE AND POWELL[4] with the *Bacillus* spore peptides, complete hydrolysis of the different compounds I and II demanded fairly strong conditions, so that the hydrolyses were carried out in 6 *N* HCl, for 20 h at 100°.

600 μg of each compound, as well as synthetic control mixtures of equal quantities of lysine, glutamic acid, glycine, alanine and N-acetylglucosamine, were hydrolyzed and divided into three aliquots. These aliquots were further treated exactly in the same way as described above for the series (A) (two-dimensional chromatography, detection with ninhydrin and estimation of amino acids and amino sugars at 570 mμ), C (one-dimensional chromatography; ELSON-MORGAN reaction on the separated

TABLE II

COMPOSITION OF *Micrococcus lysodeikticus* CELL WALLS AND OF THE PURIFIED AMINO SUGAR-PEPTIDE COMPLEXES RELEASED BY ENZYMIC DIGESTION

Results expressed in μmoles of each component in 200 μg of compound. The rough estimate of purity (right column) was obtained by adding the weight of each component in 200 μg of compound.

No. dialyzable fraction	Complexes	Ninhydrin estimation						Elson-Morgan estimation		Morgan-Elson estimation	Purity
		Lysine	Glutamic acid	Glycine	Alanine	Glucosamine	Muramic acid	Glucosamine and muramic acid		Di-saccharide	
								Test series C	Test series D		
3	F_1I	0.170	0.132	0.140	0.406	0.156	0.120	0.150	0.160	0.122	80%
	F_1II B	0.034	0.030	0.030	0.106	0.348	0.282	0.352	0.355	0.026	89%
	F_1II C	0.206	0.200	0.116	0.534	0.174	0.152	0.136	0.150	0.154	92%
4a	F_1I	0.147	0.144	0.158	0.361	0.136	0.153	0.130	0.127	0.133	78%
	F_1II	0.155	0.144	0.174	0.363	0.149	0.149	0.163	0.157	0.160	88%
4b	L I	0.130	0.125	0.145	0.241	0.101	0.101	0.124	0.144	0.085	77%
	L II	0.220	0.182	0.166	0.545	0.164	0.197	0.158	0.210	0.180	106%
Intact cell walls		0.176	0.146	0.133	0.436	0.132	0.157				

glucosamine and muramic acid) and D (ELSON-MORGAN reaction on the neutralized hydrolysates).

The content of glucosamine and amino acids in each compound was obtained with reference to glucosamine and amino acids controls. The content of muramic acid was calculated from the O.D. of the colored solution on the basis of a ratio O.D. glucosamine/O.D. muramic acid equal to 1 in series (A) and to 3.14 and 2.39 in series (C) and (D).

Moreover, each unhydrolyzed compound gave a positive MORGAN-ELSON reaction when the duration of heating of the alkaline solution was increased to 40 min as had been the case for the di-saccharides F_1IV and LIV (Fig. 3). Also, there was a direct relationship between the coloration and the amount of compound so that the amino sugar moieties were considered to be, at least partially, of the same type as the free di-saccharides and therefore could be accurately estimated by applying the molar extinction coefficient of 4870.

The results of these analyses are shown in the Table II and expressed in μmoles of amino acids and amino sugars found in 200 μg of each compound. Although no structural inferences should be drawn from the following formula, the molecular compositions, at least for five of these compounds were identical within the limits of experimental error ($\pm$ 20 %): lysine$_1$–glutamic acid$_1$–glycine$_1$–alanine$_{2-3}$–[N-acetylglucosamine-N-acetylmuramic acid]$_1$. The molar proportion of alanine varied with the different preparations: 2 for LI (preparation 4b); 2.50 for F_1I and F_1II (preparation 4a); 3 for F_1I and F_1II C (preparation 3) and LII (preparation 4b).

LI (preparation 4b) may also have this composition but its relative molar proportion of di-saccharide (0.70) was slightly beyond the limits of error.

F_1II B (preparation 3) showed a much more complex amino sugar structure bonded to a di-saccharide-peptide moiety of the same type as the preceding one: [(N-acetyl)-glucosamine-(N-acetyl)-muramic acid]$_{10}$-[lysine$_1$-glutamic acid$_1$-glycine$_1$-alanine$_3$]-[di-saccharide].

The μmoles of various amino acids and amino sugars estimated by the elution of ninhydrin-stained spots from the hydrolysate of 200 μg of intact *Micrococcus lysodeikticus* cell walls are also given in Table II. It should be noted that the molar proportion of amino acids and amino sugars found in the original cell walls was that found in the amino sugar-peptide complexes isolated from the enzymic digests.

CONCLUSIONS

Among the small fragments released from the walls during their digestion with F_1 or lysozyme, two types of amino sugar complexes [N-acetylglucosamineN-acetylmuramic acid]$_n$ were isolated: a di-saccharide (n = 1) previously detected by SALTON[1] and an oligo-saccharide with n unknown. They could be differentiated on the basis of the positive MORGAN-ELSON acetylhexosamine reaction (with a molar extinction coefficient of 4870) given, in solution, by the di-saccharide. It should be recalled however that a positive MORGAN-ELSON reaction is given by the oligo-saccharide when the test is done on paper by SALTON's technique.

Several amino sugar-peptide complexes were also isolated. Their peptide moieties were always of the same type: lysine$_1$–glutamic acid$_1$–glycine$_1$–alanine$_{2-3}$. It is noteworthy that the same proportion of amino acids was found in the original cell

walls. These results suggest that the wall polymer is made up of peptide sub-units of the same type, although there is as yet no information on the actual sequences of amino acids in such peptides. On the other hand, their amino sugar moieties usually consisted of the di-saccharide itself, and, in one case, of an additional residue consisting of ten molecules of glucosamine and ten of muramic acid, both very probably acetylated.

It is now possible to give some idea of the relative quantities of the various dialyzable complexes which are formed as a result of the enzymic digestion of the cell walls. After a lysozyme-digestion of 100 mg of *Micrococcus lysodeikticus* cell walls, it was found that the dialyzable fraction contained 7.2 mg of free di-saccharide, 10.2 mg of oligo-saccharide and 3.2 mg of bond di-saccharide. This latter figure would correspond to 8 mg of peptide-di-saccharide complexes. Thus the substances which have been investigated represent at least 25 % of the weight of the original cell walls and 50 % of the dialyzable material released during their enzymic digestion.

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REFERENCES

1 M. R. J. SALTON, *Biochim. Biophys. Acta*, 22 (1956) 495.
2 L. H. KENT, *Biochem. J.*, 67 (1957) 5P.
3 M. R. J. SALTON, *Bacteriol. Rev.*, 21 (1957) 82.
4 R. E. STRANGE AND J. F. POWELL, *Biochem. J.*, 58 (1954) 80.
5 R. E. STRANGE AND F. A. DARK, *Nature*, 177 (1956) 286.
6 C. S. CUMMINS AND H. HARRIS, *Biochem. J.*, 57 (1954) XXXII.
7 C. S. CUMMINS AND H. HARRIS, *J. Gen. Microbiol.*, 14 (1956) 583.
8 W. VON WEIDEL AND J. PRIMOSINGH, *Z. Naturforsch.*, 12 (1957) 441.
9 H. R. PERKINS AND H. J. ROGERS, *Biochem. J.*, 69 (1958) 15P.
10 R. E. STRANGE AND L. H. KENT, *Biochem. J.*, 71 (1959) 333.
11 M. WELSCH, *Rev. belge pathol. et med. expil.*, 28 (1947) *suppl.* 2.
12 M. R. J. SALTON AND J. M. GHUYSEN, *Biochim. Biophys. Acta*, 24 (1957) 160.
13 J. M. GHUYSEN, *Arch. internat. physiol. et biochim.*, 65 (1957) 173.
14 M. R. J. SALTON AND R. W. HORNE, *Biochim. Biophys. Acta*, 7 (1951) 177.
15 M. R. J. SALTON, *Biochim. Biophys. Acta*, 34 (1959) 308.
16 O. W. NEUHAUER AND M. LETZRING, *Anal. Chem.*, 29 (1957) 1230.
17 J. L. REISSIG, J. L. STROMINGER AND L. F. LELOIR, *J. Biol. Chem.*, 217 (1955) 959.
18 L. A. ELSON AND W. T. J. MORGAN, *Biochem. J.*, 27 (1933) 1824.
19 Z. DISHE AND E. BORENFREUND, *J. Biol. Chem.*, 184 (1950) 517.
20 M. V. TRACEY, *Biochem. J.*, 52 (1952) 265.