SUMMARY

1. An oligosaccharide has been isolated from lysozyme digests of the cell walls of *Micrococcus lysodeikticus*, and has been shown to contain only *N*-acetylglucosamine and *N*-acetylmuramic acid, thus confirming the work of Salton (1956).

2. Oxidation of this oligosaccharide with alkaline iodine solution, followed by acid hydrolysis, has shown that it is a disaccharide, the reducing terminus of which is *N*-acetylmuramic acid.

3. A study of the Morgan-Elson chromogens formed by heating the disaccharide with alkaline solutions has shown that its structure is 6-*O*-(*N*-acetylglucosaminy)-*N*-acetylmuramic acid.

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Substances Reacting as Hexosamine and as *N*-Acetylhexosamine Liberated from Bacterial Cell Walls by Lysozyme

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It has long been known that egg-white lysozyme acts upon certain Gram-positive micro-organisms and their isolated cell walls to yield substances giving the Morgan & Elson (1934) reaction for *N*-acetylhexosamine (Epstein & Chain, 1940; Salton, 1956). Salton (1956) also demonstrated that at least some of the colour given in this reaction was due to the presence of an oligosaccharide of *N*-acetylglucosamine and *N*-acetylmuramic acid (see Perkins, 1960), but he did not give any quantitative figures for the amount of '*N*-acetylhexosamine' that he found. It has also been reported by Brumfitt, Wardlaw & Park (1958; cf. Table 1 in their paper) that material reacting in the Elson & Morgan (1933) test as 'glucosamine' is liberated by lysozyme from the cell walls of *Micrococcus lysodeikticus*. This result seemed to contradict the work of Ingram & Salton (1957), who treated lysozyme digests of bacterial cell walls with fluorodinitrobenzene and found no dinitrophenylglucosamine and only traces of dinitrophenylmuramic acid after acid hydrolysis of the product. In the work re-

ported here these conflicting reports have been further examined. It has been found that direct measurements of '*N*-acetylhexosamine' and 'hexosamine' are an unreliable guide to the composition of lysozyme digests. In order to obtain quantitative information about the various fragments liberated by lysozyme, the mixture of compounds produced by the action of lysozyme on the cell walls of *M. lysodeikticus* has been fractionated and, after acid hydrolysis, the distribution of hexosamine between the fractions has been studied.

METHODS AND MATERIALS

Micro-organisms. The organisms used were *Micrococcus lysodeikticus* (N.C.T.C. 2665), *Bacillus subtilis* (N.C.T.C. 6346) and *Staphylococcus* strain 11 which was partially sensitive to lysozyme (Rogers & Perkins, 1959).

Preparation of cell walls. Cell walls were prepared essentially according to the method of Cummins & Harris (1956) as described by Perkins & Rogers (1959).

Action of lysozyme. Isolated cell walls were incubated at 37° with lysozyme (crystalline; The Armour Laboratories)

at a final concentration of 50 $\mu\text{g./ml.}$ unless otherwise stated, at pH 6.1 in 0.1M-buffer, which was either ammonium acetate or in some cases sodium and potassium phosphate, and the reaction was stopped by cooling to 0°. Estimations of 'hexosamine' and 'N-acetylhexosamine' were performed on the supernatant solutions after centrifuging the lysozyme digests in the cold at 11 000 g. When lysozyme digests were to be employed for fractionation and other special procedures, incubation was continued for 3 hr., by which time the digestion was essentially complete. Soluble lysozyme digests were sometimes separated into diffusible and non-diffusible portions as described elsewhere (Perkins, 1960).

Estimation of hexosamine. Hexosamine was estimated by the procedure of Rondle & Morgan (1955), in some cases after preliminary separation of glucosamine and muramic acid as described by Perkins & Rogers (1959). In certain experiments designed to test the possibility that the 'hexosamine' colour obtained was an artifact, the concentration of acetylacetone in the reaction mixture and its pH value were varied as described by Immers & Vasseur (1950).

When the Rondle & Morgan (1955) procedure was carried out in the presence of material which had been treated previously with fluorodinitrobenzene as described below, it was necessary to correct for the absorption at 530 $m\mu$ which was due to the dinitrophenyl (DNP) derivatives. These compounds still absorb at 700 $m\mu$, at which wavelength glucosamine in the Elson & Morgan (1933) test gives no absorption. Measurements were therefore made at a wavelength of 700 $m\mu$, in order to obtain an indication of the amount of DNP compounds present. A correction factor was calculated and subtracted from the absorption value obtained at 530 $m\mu$.

Estimation of N-acetylhexosamine. The colorimetric method of Reissig, Strominger & Leloir (1955), which employs borate buffer, pH 9.1, in its initial phase, and that of Aminoff, Morgan & Watkins (1952), which uses sodium carbonate, pH 10.8, were both employed in various stages of the work and comparisons were made between them.

Reaction of cell walls and lysozyme digests with fluorodinitrobenzene. Cell walls were treated with 1-fluoro-2,4-dinitrobenzene (FDNB) as described by Ingram & Salton (1957). Lysozyme digests of walls were prepared as described above and, after removal of the ammonium acetate buffer *in vacuo*, were allowed to react with FDNB, the solution being made alkaline by the addition of trimethylamine. After reaction for 2 hr. at room temperature the mixture was extracted repeatedly with benzene, and then the aqueous phase was dried in a vacuum desiccator over NaOH. The walls and digests after treatment with FDNB were hydrolysed for only 4 hr. in 4N-HCl at 105° in sealed ampoules, so that destruction of acid-labile DNP derivatives, such as those of glycine, glucosamine and muramic acid, would be limited. In recovery experiments 55% of DNP-muramic acid and 45% of DNP-glucosamine remained after hydrolysis. Compounds present in these hydrolysates were identified on two-dimensional chromatograms, the buffered 2-methylbutan-2-ol (*tert.*-pentanol) solvent of Blackburn & Lowther (1951), followed by the phosphate buffer described by Levy (1954), being used. Marker spots of the DNP derivatives of all the relevant compounds were run in both dimensions.

In some cases lysozyme digests were treated under

alkaline conditions similar to those used in the Rondle & Morgan (1955) procedure (i.e. heated for 20 min. at 100° in 0.167N-Na₂CO₃ before reaction with FDNB). In these experiments the pH of the solution was then lowered with CO₂ to 8.2 before addition of ethanol and FDNB in the usual way. After acid hydrolysis the solution was extracted with ether, which would remove DNP-muramic acid along with non-basic DNP-amino acids. The DNP compounds remaining in the aqueous phase were subjected to zone electrophoresis in Na₂CO₃ solution (0.1M), which was found to separate DNP-glucosamine, ϵ -DNP-lysine and dinitrophenol. This procedure avoided undue interference by the salt which was inevitably present under these conditions.

Acetylation of lysozyme digests of cell walls. In some experiments examination of lysozyme digests of cell walls by the Rondle & Morgan (1955) method for hexosamine and the Reissig *et al.* (1955) method for N-acetylhexosamine was preceded by acetylation procedures. Lysozyme digests of cell walls prepared as previously described were freed as far as possible from ammonium acetate, and the dry material was treated with acetic anhydride containing 10% (v/v) of pyridine, at 37° for 4 hr., a procedure which should acetylate free amino groups and possibly some hydroxyl groups. The product was extracted several times with ether and finally dried in a vacuum desiccator over NaOH and H₂SO₄. In order to remove any O-acetyl residues which had been introduced the acetylated material was allowed to stand overnight in 0.01N-NaOH at room temperature.

Paper chromatography and zone electrophoresis. These techniques were performed as described by Perkins (1960).

Turbidity measurements. The turbidity of cell-wall suspensions was measured in the Hilger Spekker photoelectric absorptiometer.

Dinitrophenylglucosamine. This (m.p. 196°, uncorr.) was a crystalline sample prepared essentially according to the method of Annison, James & Morgan (1951).

Dinitrophenylmuramic acid. This was prepared by the action of fluorodinitrobenzene on muramic acid which had been isolated from cell walls. It was isolated from a paper chromatogram, and the solution used as a marker and in recovery experiments from acid hydrolysates.

RESULTS

Lysozyme digestion of cell walls and the liberation of materials reacting as 'hexosamine' and 'N-acetylhexosamine'. Isolated cell walls of *M. lysodeikticus* were incubated with lysozyme (10 $\mu\text{g./ml.}$) at 37° and samples were taken at intervals for measurement of turbidity, 'N-acetylhexosamine' by the procedure of Reissig *et al.* (1955) and 'hexosamine' by the method of Rondle & Morgan (1955). The result of a typical experiment is given in Fig. 1, which shows that the amount of apparent N-acetylhexosamine liberated and estimated under these conditions represents a very small proportion of the wall as a whole. Perkins & Rogers (1959) have indicated that the wall contains about 15% of N-acetylglucosamine, and an approximately equimolecular proportion of N-acetylmuramic acid, so that the maximal liberation of N-acetylhexosamine

according to Fig. 1 would correspond to only about 0.7% of the *N*-acetylglucosamine present in the wall. On the other hand, the amount of 'hexosamine' estimated is equal to about ten times the '*N*-acetylhexosamine' and is in good agreement

Fig. 1. Action of lysozyme on the isolated cell walls of *M. lysodeikticus*. Reduction in turbidity and liberation of materials reacting as 'glucosamine' (Rondle & Morgan, 1955) and '*N*-acetylglucosamine' (Reissig *et al.* 1955). □, Decrease in turbidity; △, 'glucosamine'; ○, '*N*-acetylglucosamine'.

Fig. 2. Absorption curves in hexosamine test (Rondle & Morgan, 1955). △, Muramic acid; ○, glucosamine; □, 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid; ●, lysozyme digest of isolated cell walls of *M. lysodeikticus*.

with the value found by Brumfitt *et al.* (1958). In parallel experiments conducted with the isolated cell walls of *B. subtilis* and a strain of *Staphylococcus* (strain 11), a similar relationship was observed between the amounts of 'hexosamine' and '*N*-acetylhexosamine' liberated. In the Rondle & Morgan (1955) test glucosamine gives an absorption maximum at 530 mμ, whereas muramic acid produces a colour with a maximum at 510 mμ (Crumpton, 1959). The 'hexosamine' colour given by lysozyme digests of cell walls has an absorption maximum at about 525 mμ, that is, not far removed from the glucosamine peak, and quite unlike that of muramic acid (Fig. 2). Since it appeared possible that lysozyme had liberated from cell walls some kind of substituted hexosamine having a free amino group, which would react in the Rondle & Morgan test, an attempt was made to identify any such free amino groups.

Examination for free amino groups. The isolated cell walls of *M. lysodeikticus* were treated with FDNB and, after acid hydrolysis, the only DNP derivatives which could be found by two-dimensional paper chromatography were DNP-alanine (in small yield) and ε-DNP-lysine (in large yield), a result which confirms that of Ingram & Salton (1957). When the cell walls were first digested with lysozyme and then treated in the same way, the result was qualitatively the same; in contrast with the findings of Ingram & Salton (1957) not even a trace of DNP-muramic acid could be detected. From the results given above it seemed possible that the amino groups of glucosamine or muramic acid in lysozyme digests of cell walls might be set free under the alkaline conditions of the Rondle & Morgan (1955) hexosamine test. Accordingly, digests were heated under these conditions for 20 min. at 100°, before being treated with FDNB. After acid hydrolysis the ether-soluble derivatives were examined by paper chromatography and the water-soluble portion by zone electrophoresis in Na₂CO₃ buffer. Once more the DNP derivatives of glucosamine and muramic acid could not be found. It seemed therefore that the explanation for the 'hexosamine' reaction observed in lysozyme digests of cell walls must be sought elsewhere.

Investigation of 'hexosamine' colour reaction. The best-known artifact colour in the Elson & Morgan (1933) reaction is given by mixtures of glucose with glycine or lysine (Sideris, Young & Krauss, 1938; Immers & Vasseur, 1950). The latter authors showed that, compared with the colour given by glucosamine, the artifact colour was only slightly diminished by decreasing the amount of acetylacetone used in the alkaline reagent from 2% (v/v) to 0.75% (v/v). On the other hand, when a higher concentration of acetylacetone was used at a lower pH value, it was the development of artifact colour

Table 1. *Variation of colour development in the Rondle & Morgan (1955) test with altered acetylacetone concentration*

Acetylacetone concentration in alkaline reagent (% v/v)	Alkaline buffer used	Extinction at 530 m μ (1 cm. cell)	
		25 μ g. of glucosamine hydrochloride	Lysozyme digest of 1.3 mg. of cell wall
0.75	Na ₂ CO ₃ (0.5 N)	0.081	0.284
2.0	Na ₂ CO ₃ (0.5 N)	0.245	0.292
5.0	Na ₂ CO ₃ -NaHCO ₃ (M), pH 9.5	0.214	0.210

which was adversely affected. Furthermore, when mixtures of glucose and glycine or lysine were treated by the Elson & Morgan (1933) procedure, but with the acetylacetone omitted, then unlike glucosamine they gave a coloured product with maximal absorption at about 560 m μ .

In the present experiments cell wall isolated from *M. lysodeikticus* was incubated with lysozyme (final concentration 50 μ g./ml.) at 37° in phosphate buffer (0.1 M, pH 6.5) for 2 hr. Samples of digest were treated by the Rondle & Morgan procedure, except that the acetylacetone concentration and the pH value were varied in the manner described by Immers & Vasseur (1950). The results given in Table 1 show that the colour developed by lysozyme digests was relatively unaffected by the changed conditions, whereas lowered acetylacetone concentration produced a considerable diminution in colour yield from glucosamine. In a similar experiment acetylacetone was omitted altogether, and a colour appeared which was shown within the range 500–600 m μ to have a single absorption maximum at 555 m μ . In these respects the behaviour of the digests resembled that described by Immers & Vasseur (1950) for mixtures of glucose and lysine. It seemed possible therefore that the colour given in the Rondle & Morgan test by lysozyme digest of cell walls might have been an artifact due to the free ϵ -amino groups of lysine (known to be present from the work with FDNB described above) and the reducing groups of some sugar. The sugar need not have been glucose, since as mentioned earlier the same effect could be observed with the cell walls of *Staphylococcus* (strain 11), which had been shown on hydrolysis to contain glucosamine and muramic acid as their only carbohydrate components.

Reduction of production of artifact colour in the Elson & Morgan (1933) test. It was found that if lysozyme digests of cell walls were treated in such a way as to mask the free amino groups of lysine, then the colour development in the Rondle & Morgan test could be reduced by 60 %. Cell walls of *M. lysodeikticus* were digested with lysozyme in ammonium acetate buffer as previously described, and the whole digest was freeze-dried under high vacuum. One sample was treated with FDNB in

Table 2. *Effect of masking the ϵ -amino groups of lysine on the reaction given by lysozyme digests of cell walls in the N-acetylhexosamine and hexosamine tests*

Treatment of lysozyme digest of cell walls	Percentage of cell wall estimated	
	As N-acetylglucosamine*	As glucosamine†
None	0.178	1.62
Acetylated (20°; 50 min.)	0.163	0.99
Acetylated (37°; 4 hr.)	0.142	0.63
Fluorodinitrobenzene	—	0.88

* Reissig, Strominger & Leloir (1955).

† Rondle & Morgan (1955).

order to prepare the DNP derivative of the ϵ -amino groups of lysine, and another was acetylated with acetic anhydride and pyridine. The latter procedure was followed by treatment with NaOH (0.01 N) to remove any *O*-acetyl groups which might have been introduced. Samples of the products were then treated by the Rondle & Morgan (1955) hexosamine procedure, and also by the Reissig *et al.* (1955) method for *N*-acetylhexosamine, with the result shown in Table 2. There was a slight reduction in the amount of apparent *N*-acetylhexosamine estimated, but the 'hexosamine' colour produced could be decreased to about 40 % of its initial value. This result suggests that the ϵ -amino group of lysine, known to be the only free amino group present in any quantity in these digests, is involved in the production of a large proportion of the colour obtained in the Rondle & Morgan test.

Production of 'hexosamine' colour by a disaccharide isolated from lysozyme digests of cell walls. The preceding paper (Perkins, 1960) describes the isolation from lysozyme digests of the cell walls of *M. lysodeikticus* of a disaccharide characterized as 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid. Although under conditions of the *N*-acetylhexosamine tests employed this material yields a colour characteristic of *N*-acetyl amino sugars, when treated by the Rondle & Morgan procedure it also yields a colour with an absorption maximum at 525 m μ (Fig. 2), presumably due to complex reactions caused by the alkaline treatment (see

below). It seems probable therefore that the presence of this material may have accounted for the residual colour production, which could not be eliminated by masking the free amino groups of lysine (Table 2).

N-Acetylhexosamine reaction of lysozyme digests of cell walls. In their work upon blood-group substances, which like bacterial cell walls contain bound *N*-acetyl amino sugars, Aminoff *et al.* (1952) showed that when these substances were heated with Na_2CO_3 solution under the conditions of their *N*-acetylhexosamine test, the amount of colour produced on addition of Ehrlich's reagent varied with the time of heating. Maximum colour production was not necessarily obtained after the same time interval as that required for the free *N*-acetylhexosamines. Such differences are related to the position of substitution of the *N*-acetyl amino sugar which is giving the reaction (see Buchanan, Crumpton & Morgan, 1957). The *N*-acetylhexosamine reaction of lysozyme digests of cell walls behaved similarly to that of the blood-group substances when tested under varying conditions of treatment with alkali.

Lysozyme digests of the isolated cell walls of *M. lysodeikticus* were prepared as described earlier. In the particular experiment discussed here the diffusible portion of the digest was employed, but whole digests gave essentially similar results. Samples of the diffusible material were heated at 100° for various times under the conditions of Reissig *et al.* (1955), who used borate buffer at about pH 9.0, or those of Aminoff *et al.* (1952), who used carbonate, pH 10.8. After the addition of the acetic acid solution containing *p*-dimethylamino-benzaldehyde in the usual way, the light absorp-

tion was measured at $585\text{ m}\mu$. The results given in Fig. 3 are expressed in terms of the apparent amount of *N*-acetylglucosamine present, i.e. the absorption at $585\text{ m}\mu$ of each sample is compared with that given by *N*-acetylglucosamine heated in the corresponding conditions for the time necessary to give maximal colour yield. In most cases the absorption curve of the final solution was essentially the same as that given by *N*-acetylglucosamine, but, after heating for 60 min. in the carbonate solution, the peak at $585\text{ m}\mu$ had disappeared and a single peak at $555\text{ m}\mu$ had arisen. This latter peak resembled the one obtained in the Rondle & Morgan test without acetylacetone, described above.

Evidently the higher pH value of the carbonate solution used in the method of Aminoff *et al.* (1952) facilitated the formation of chromogens from the wall digests, probably by accelerated alkaline hydrolysis. It is apparent that the quantity of '*N*-acetylhexosamine' measured in lysozyme digests of cell walls is largely a function of the conditions used in the test, and is no direct indication of the number of *N*-acetylhexosamine-reducing groups set free by the enzyme.

Distribution of hexosamine amongst fractions of a lysozyme digest of cell walls. Although Salton (1956) discussed the presence of materials reacting as *N*-acetylhexosamine in the diffusible and non-diffusible fractions of lysozyme digests of cell walls, so far no quantitative figures have been available for the distribution of these components. From the foregoing results it was evident that the ordinary reactions for hexosamine or *N*-acetylhexosamine were not likely to provide reliable information on this point. It was therefore decided to separate the wall digests into various fractions, and to estimate the total amount of hexosamine present in each fraction after acid hydrolysis.

A lysozyme digest of the cell walls of *M. lysodeikticus* was separated into diffusible and non-diffusible fractions as described by Perkins (1960). Each fraction was freeze-dried in a high vacuum, but it was difficult to remove ammonium acetate completely from the diffusible fraction. The percentage of the whole material in this portion was therefore taken as the difference between the original weight and that of the non-diffusible material. When samples of the diffusible material were run on paper chromatograms in butanol-pyridine-water, material reacting with aniline phthalate and giving a purple with the hexosamine spray reagent (Partridge, 1948) could be found in two regions only, one running from the origin (fraction A), and the other at $R_{N\text{-acetylglucosamine}} 0.85$. The latter spot was found to be a disaccharide of *N*-acetylglucosamine and *N*-acetylmuramic acid corresponding to the oligosaccharide described by Salton (1956). It is discussed in detail in the

Fig. 3. Development of colour in *N*-acetylhexosamine tests given by the diffusible fraction of a lysozyme digest of isolated cell walls (*M. lysodeikticus*). Δ , Borate, pH 9.1 (Reissig *et al.* 1955); $\circ$, carbonate, pH 10.8 (Aminoff, Morgan & Watkins, 1952).

preceding paper (Perkins, 1960). A sample of the diffusible fraction of a lysozyme digest was separated in this way on washed paper (Hanes, Hird & Isherwood, 1952). The two regions were eluted and samples were hydrolysed in acid before separation and estimation of glucosamine and muramic acid. Other regions of the chromatogram were not found to contain any hexosamine, and it was therefore assumed that the whole of the hexosamine of the diffusible material was contained in these two fractions. In all fractions studied the glucosamine was accompanied by an approximately equimolecular proportion of muramic acid. The distribution of glucosamine among the various fractions is shown in Table 3. Lysozyme converts about 20% of the cell-wall amino sugars into the disaccharide and about 50% into other compounds diffusible through cellophan. Neither portion of the diffusible fraction was found to contain any of the glucose present in the whole cell wall.

Composition of fraction A. The portion of the diffusible material which moved slowly on butanol-pyridine-water chromatograms (fraction A) was examined further. After zone electrophoresis in acetic acid (2N), three well-defined bands were observed (one moved 0.8 cm. towards the anode and the others 4.8 and 6.8 cm. towards the cathode), all of which reacted to a greater or lesser extent with ninhydrin, aniline phthalate and *N*-acetylhexosamine reagent. Samples of these components eluted from washed paper were hydrolysed in 4N-HCl for 4 hr. and the hydrolysates were examined by two-dimensional paper chromatography in butanol-pyridine-water and butanol-acetic acid-water. Each fraction was composed of all the components of whole cell walls with the exception of glucose, which was found in a separate experiment to be absent. The anionic material contained a preponderance of glucosamine and muramic acid with only small amounts of the four amino acids and hence may not have been homogeneous, but no accurate quantitative analysis of the fractions has yet been made. Apart from the disaccharide therefore the diffusible fraction of lysozyme digests of cell walls contains compounds in which the amino sugars are bound to amino acids.

Table 3. *Distribution of bound glucosamine in fractions of a lysozyme digest of cell walls of Micrococcus lysodeikticus*

Fraction of digest	Percentage (by wt.) of whole cell	Glucosamine (% of total)
Non-diffusible	56.5	28.5
Diffusible	Disaccharide	19.9
	Remainder	51.6
	Total	71.5

DISCUSSION

The action of lysozyme on certain bacterial cell walls was studied by Salton (1956), who described the liberation of materials giving the Morgan & Elson (1934) reaction for *N*-acetylhexosamines, although he did not give any quantitative estimate of the amount. Although the exact nature of the groups set free by lysozyme was not known, he was able to show that some proportion of the *N*-acetylhexosamine reaction he obtained was due to an oligosaccharide, possibly a disaccharide, of *N*-acetylglucosamine and *N*-acetylmuramic acid.

In the present work it has been shown that the quantity of reactive *N*-acetylhexosamine groups measured in the usual tests is no direct indication of the number of such groups present in the digests, since the intensity of colour produced is a function of the alkalinity of the reaction mixture and the time of heating. The Elson & Morgan (1933) reaction for hexosamine, as measured by Brumfitt *et al.* (1958), has been shown not to be due to the presence of non-acetylated hexosamine end groups. In all probability it is an artifact produced partly by the simultaneous presence of carbohydrate-reducing groups and the free ϵ -amino groups of lysine, and partly by alkaline decomposition of the chromogen initially formed from *N*-acetylmuramic acid end groups (see also Perkins, 1960). It is not possible therefore to deduce anything about the proportion of the amino sugar end groups of cell walls set free by lysozyme from the amount of colour developed either in the Elson-Morgan (hexosamine) or in the Morgan-Elson (*N*-acetylhexosamine) reaction.

Brumfitt *et al.* (1958) proposed a simple repeating unit as the structural basis of the cell walls of lysozyme-sensitive organisms, and suggested that C-1 of *N*-acetylmuramic acid was set free by lysozyme. Their evidence for the latter assertion was based on the relative amount of colour developed in *N*-acetylhexosamine tests in the presence of borate as opposed to carbonate (Park, 1956). As Reissig *et al.* (1955) and Cifonelli & Dorfman (1958) have shown, the colour yield of 3-substituted *N*-acetylhexosamines such as *N*-acetylmuramic acid is unaffected by the use of borate, which with *N*-acetylglucosamine itself gives a greatly enhanced colour value. It can be seen from Fig. 3, however, that interpretation of the amount of colour yielded by lysozyme digests of cell walls in these tests must necessarily be difficult. Nevertheless, the evidence given by Perkins (1960) shows that lysozyme does indeed liberate the C-1 of *N*-acetylmuramic acid, which at least in some parts of the wall structure is linked by C-6 to C-1 of *N*-acetylglucosamine.

On the basis of their experiments Brumfitt *et al.* (1958) suggested that lysozyme acts on only about

one in ten of the *N*-acetylmuramic acid glycosidic bonds in their proposed repeating structure. It can be seen from Table 3 that lysozyme does not set free from the wall a number of essentially similar fragments, but rather that the product is a heterogeneous mixture which can be divided into three fractions.

The smallest molecule found in the digests is the disaccharide, 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid. Unless the enzyme causes extensive transglycosidation, about 20% of the hexosamines of the wall must occur as this disaccharide, in which the muramic acid carboxyl group is not linked to amino acids. The reducing group of the muramic acid may be linked either to the *N*-acetylglucosamine part of a second molecule of disaccharide, or to some other unknown point in the wall structure.

The second fraction, forming the remainder of the diffusible portion of the wall digests, consists of larger molecules in which the amino sugars are linked to amino acids. It is probable that in these compounds the amino acids are in the form of peptide chains bound in amide linkage to the carboxyl group of muramic acid, since it has been possible to isolate this type of compound from partial acid hydrolysates of the cell wall of *M. lysodeikticus* (Perkins & Rogers, 1959). The fact that this second group of products from lysozyme digestion contains at least three distinct fragments separable by electrophoresis suggests a heterogeneity in the wall from which they come. Probably the peptide chains attached to the muramic acid do not all have the same composition.

The third fraction comprises the non-diffusible portion of the digest, which is apparently resistant to further attack by lysozyme, and contains the bulk of the glucose present in the wall. It seems possible either that the lysozyme-sensitive linkage does not occur in this fraction, or that attack by the enzyme is inhibited by the presence of some other substituent on one of the hexosamine moieties, rather in the manner that the presence of the *O*-acetyl group can be shown to inhibit its action (Brumfitt *et al.* 1958).

Assuming that only one type of bond is split by lysozyme, the variability in composition of the fragments which can be found in lysozyme digests of the walls of *M. lysodeikticus* suggests once more that the mucopeptide of these structures may be heterogeneous, as was indicated by Rogers & Perkins (1959).

SUMMARY

1. Lysozyme digests of the cell walls of *Micrococcus lysodeikticus* were prepared, and their reactions in the Elson-Morgan (hexosamine) and Morgan-Elson (*N*-acetylhexosamine) tests examined.

2. The Elson-Morgan reaction given for 'hexosamine' was shown to be an artifact, since no free amino sugar amino groups could be demonstrated.

3. The amount of colour given by lysozyme digests in the *N*-acetylhexosamine reaction depended upon the pH of the alkaline solution used in the Morgan-Elson test and upon the time of heating.

4. Cell-wall digests were fractionated, and the distribution of wall components amongst the fractions was studied: 20% of the hexosamine appeared as 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid and 50% as diffusible material in which the amino sugars were bound to amino acids.

5. The significance of these results in connexion with cell-wall structure has been discussed.

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