ACETYLHEXOSAMINE COMPOUNDS ENZYMICALLY RELEASED FROM *MICROCOCCUS LYSODEIKTICUS* CELL WALLS

III. THE STRUCTURE OF DI- AND TETRA-SACCHARIDES RELEASED FROM CELL WALLS BY LYSOZYME AND *STREPTOMYCES* F_1 ENZYME*

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SUMMARY

Two compounds, both of which have been isolated from cell walls of *Micrococcus lysodeikticus* digested with lysozyme or *Streptomyces* F_1 enzyme, have been identified as di- and tetra-saccharides. The reducing groups of the di- and tetra-saccharides and those of the high-molecular weight, non-dialysable compounds belong to muramic acid. β-glucosidase yields free N-acetylglucosamine and N-acetylmuramic acid from the di- and tetra-saccharides. The proposed structure for the di-saccharide liberated by lysozyme and F_1 is: 6-O-β-N-acetylglucosaminyl-N-acetylmuramic acid. The tetra-saccharides isolated from lysozyme and F_1 digests appear to be identical. The structure proposed for the tetra-saccharide isolated from lysozyme digested walls is: O-β-N-acetylglucosaminyl-(1→6)-O-β-N-acetylmuraminyl-(1→4)-O-β-N-acetylglucosaminyl-(1→6)-β-N-acetylmuramic acid.

Both lysozyme and *Streptomyces* F_1 enzyme degrade di- and tetra-chitobiose, indicating their β(1→4) N-acetyl hexosaminidase activity.

INTRODUCTION

It has been known for some time that the enzyme lysozyme, brings about a liberation of N-acetylamino sugar compounds from the "mucopolysaccharide" substrates isolated from bacterial cells[1,2]. Subsequent investigations with isolated cell walls as "substrate" established that lysozyme liberated N-acetylamino sugars but none of the amino acids present in the cell-wall mucopeptides[3]. SALTON[3] presented evidence for the release of a di-saccharide of N-acetyl-glucosamine and N-acetylmuramic acid as the simplest product of digestion of the substrate with lysozyme. BERGER AND WEISER[4] found that lysozyme degraded chitin [aβ(1→4)N-acetylglucosamine polymer]. Thus it became apparent that lysozyme belonged to the N-acetylhexosaminidase group of enzymes,

Abbreviations: AG, N-acetylglucosamine; AMA, N-acetylmuramic acid; DNP, dinitrophenyl-; FDNB, 1, fluoro-2,4-dinitrobenzene; NDF, non-dialysable fraction.

* A preliminary account of this work appeared[11].

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and it seemed likely from the results of BERGER AND WEISER[4], that the enzyme is specific for the β-glycosidic bond. It will be recalled that MEYER, PALMER, THOMPSON AND KHORAZO[5] had concluded a long time ago that the linkages broken by lysozyme were definitely not α-glycosidic bonds. BRUMFITT, WARDLAW AND PARK[6] have also advanced the hypothesis that lysozyme is a $\beta(1 \longrightarrow 4)$ N-acetyl hexosaminidase and from a differential test for the reaction of N-acetylamino sugars suggested that the reducing group liberated by the enzyme was that of AMA.

With the development of a more sensitive reaction for detecting N-acetylamino sugars[7] additional products in the dialysable fractions of lysozyme and Streptomyces enzyme F_1, digests of *M. lysodeikticus* walls have been detected[7,8]. This paper presents the details of attempts to establish the structure of a compound corresponding to the di-saccharide reported earlier[3,7,8] and an oligo-saccharide[8]. Independent confirmation of the existence of the disaccharide has been reported by PERKINS[9,10] and the structure he has recently suggested is in complete agreement with that proposed by SALTON AND GHUYSEN[11].

EXPERIMENTAL

Unless otherwise specified, the compounds used throughout these studies were those isolated as described by GHUYSEN AND SALTON[8], from lysozyme and Streptomyces F_1 enzyme digests of *Micrococcus lysodeikticus* (NCTC 2665) cell walls.

Preparation of ^{14}C compounds

Cell walls containing ^{14}C in glucosamine and muramic acid were prepared from *M. lysodeikticus* grown with aeration at 30° on a medium containing 0.4% Difco casamino acids, 0.05% Difco yeast extract and [^{14}C] fructose, or [^{14}C] glucose. The organism was cultivated on a medium of the same composition with non-radioactive fructose or glucose (0.8%) prior to washing (with the casamino acids–yeast extract mixture) and replacement in the radio-active medium. The walls were isolated and radioautographs of the hydrolysed walls (6 *N* HCl, 16 h, 105°) showed that labeling of the wall compounds was almost exclusively confined to glucosamine, muramic acid and glucose with only very faint traces in wall amino acids such as glutamate and lysine.

Di- and oligo-saccharides were isolated from [^{14}C] wall material after digestion with lysozyme (Armour Laboratory, crystalline egg-white lysozyme) for 24 h at 37°, pH 7, 0.1 *M* ammonium acetate buffer. The dialysable fraction (material diffusable on dialysis for 48 h at room temperature) was then placed as a band on Whatman paper No. 1 and using *n*-butanol–acetic acid–water (3:1:1, v/v) the compounds suspected of being di- and tetra-saccharides separated cleanly from one another, and from the slower moving, peptide-amino sugar complexes. On the elution of the two compounds and re-running on paper with the same solvent systems, they were found to be homogeneous—each compound gave a single spot on radioautographs.

Reaction with $NaBH_4$

Reduction of amino sugar compounds with $NaBH_4$ has been successfully used by DAVIDSON AND MEYER[12,13] in their studies on the structure of mucopolysaccharides. We have investigated the reaction of our compounds with $NaBH_4$ as well as treating the NDF of lysozyme-digested walls and the untreated walls with this reagent.

The oligo-saccharides, the walls and NDFs were treated with an excess of $NaBH_4$ (Metal Hydrides, Inc., Beverly, Mass.) at 0° for 2 h. The reaction mixtures were then

warmed to 20° and held at that temperature for periods varying from 30 min to 90 min, prior to decomposition of the reagent with acetic acid in the cold and adding sufficient acid to bring the pH to 5. Walls were washed free of the reagents by centrifugation and the NDF was dialysed against several changes of distilled water.

The di- and oligo-saccharide were reduced with $NaBH_4$ in the following media: (a) distilled H_2O; (b) 0.1 *M* borate buffer, pH 8.0; (c) 0.05 *M* borate buffer, pH 9.2. The reaction was allowed to proceed at 0° and samples were taken at intervals and tested for loss of ability to give a MORGAN AND ELSON reaction on paper (Whatman, No. 1) using the conditions described by SALTON[7]. Loss of MORGAN AND ELSON reaction occurred most rapidly in unbuffered solution. Weak positive reactions for N-acetylamino sugars were obtained even after 2 h reduction with $NaBH_4$ in borate buffer at both pH 8.0 and pH 9.2, indicating incomplete reaction under these conditions. In one experiment, reduction was prolonged to 12 h at 0° and a small amount of residual material was still detectable with the sensitive spray reagent. Reaction products were generally held for 90 min at 20° prior to destruction of the reagent. [^{14}C] compounds were treated in an identical manner.

Examination of the products of reduction with $NaBH_4$

All preparations reduced with $NaBH_4$ were hydrolysed with 2 *N* or with 6 *N* HCl (for 3 or 16 h respectively) at 105°, the HCl removed by drying *in vacuo* over H_2SO_4 and NaOH pellets. The hydrolysates were examined by two-dimensional paper chromatography using pyridine–water (4:1, v/v) in one direction and *n*-butanol–acetic acid–water (3:1:1, v/v) in the second direction. Paper chromatograms were sprayed with ninhydrin, the $AgNO_3$ reagents of TREVELYAN, *et al.*[14], and the Schiff's reagents as described by BUCHANAN *et al.*[15].

With the exception of the intact, untreated cell-wall preparation, all other reduced fractions showed the appearance of a new compound on examination of the hydrolysates by paper chromatography. This substance reacted more weakly with ninhydrin than the parent amino sugars, it reacted with $AgNO_3$ and gave a white spot with the Schiff's spray reagent[15]. The new compound was well separated from both glucosamine and muramic acid on paper chromatograms and did not correspond to the position of a sample of glucosaminitol prepared by reduction of AG with $NaBH_4$ (the reduced AG was de-ionized and then de-acetylated by hydrolysis). That the new substance was the reduction product of muramic acid was supported by its separation in the neutral fraction when the hydrolysis products of the reduced fractions were submitted to electrophoresis at pH 6.4 (pyridine–acetic acid buffer[16]), 10 V/cm. This evidence indicated the presence of both amino and carboxyl groups. The substances therefore possessed all the properties of the amino sugar hexitol of muramic acid. A sample of muramic acid isolated by paper chromatographic separation of hydrolysed *M. lysodeikticus* wall, upon reduction with $NaBH_4$ gave as a major product, a compound of similar behaviour on electrophoresis and on paper chromatography as that detected in the reduced substances under investigation. The compound will thus be referred to subsequently as "muramicitol" (the amino sugar hexitol of muramic acid).

Neither muramicitol nor glucosaminitol was detected in hydrolysates of walls treated with $NaBH_4$. The products detected in the oligo-saccharides, NDF and cell walls submitted to reduction, are summarized in Table I.

A determination of the ratios of the products would give confirmatory evidence

TABLE I

PRODUCTS OF THE REDUCTION OF WALLS, NDF AND OLIGOSACCHARIDES WITH $NaBH_4$

Preparation	*Enzyme used*	*Products of acid hydrolysis*			*Other compounds*
		Glucosamine	*Muramic acid*	*Muramicitol**	
Walls	None	+	+	—	Amino acids, glucose[3]
NDF	Lysozyme, F_1	+	+	+	Amino acids, glucose[3]
Di-saccharide	Lysozyme, F_1	+	Trace	+	±**
Tetra-saccharide	Lysozyme, F_1	+	+	+	±**

* Muramicitol — $R_{F\text{muramic acid}} = 0.75$ } *n*-butabol–acetic acid–water (3:1:1)
Glucosaminitol — $R_{F\text{muramic acid}} = 0.35$ }

** Small amounts of what is believed to be de-acetylated disaccharide which has resisted hydrolysis; $R_{F\text{muramic acid}} = 0.22$.

as to the structure of the compounds. The ratio of muramicitol to glucosamine in the di-saccharides (from both lysozyme and F_1 enzyme digests) was determined by conversion of the hydrolysis products of the reduced compound to the DNP derivatives by reaction with FDNB under the conditions successfully applied to the determination of amino sugars in blood group substances by LESKOWITZ AND KABAT[17]. The DNP compounds were separated on paper chromatograms (Whatman 3MM paper saturated with phthalate buffer and irrigated in one direction with *tert.*-amyl alcohol as described by BLACKBURN AND LOWTHER[18] and using the 1.5 *M* phosphate buffer of LEVY[19] for the second dimension). The DNP compounds were eluted in 1 % w/v $NaHCO_3$ and the extinctions measured at 360 mμ. A ratio of 1:0.8 was obtained for the proportion of DNP-glucosamine to DNP-muramicitol. The DNP-glucosamine and DNP-muramicitol could also be separated by electrophoresis on 3MM paper (10 V/cm, 2-3 h, pH 6.4). The products from the oligosaccharide could not be determined by this method, as the conditions used did not separate the DNP derivatives of muramic acid and muramicitol.

The ratios of muramicitol to glucosamine and muramic acid were therefore determined with the use of ^{14}C-labeled compounds. After reduction, the compounds were hydrolysed (2 *N* HCl, 3 h, 105°) and the three substances glucosamine, muramic acid and muramicitol were separated on Whatman paper No. 1 with *n*-butanol–acetic acid–water (3:1:1, v/v), the compounds were eluted, concentrated and then the radioactivity in the fractions determined. The results for the lysozyme di- and

TABLE II

RATIOS OF ACID HYDROLYSIS PRODUCTS IN $NaBH_4$-TREATED [^{14}C] DI- AND TETRA-SACCHARIDES ISOLATED FROM LYSOZYME-DIGESTED [^{14}C] WALL

	Di-saccharide		*Tetra-saccharide*	
	Counts/min	*Ratio**	*Counts/min*	*Ratio**
Glucosamine	261	1.6	302	2.5
Muramicitol	162	1	115	1
Muramic acid	70	0.4	121	1.1

* Relative to muramicitol.

tetra-saccharide are presented in Table II, adding further confirmation as to the identity of one compound as a di-saccharide and supporting the impression gained from paper chromatography that the other oligo-saccharide is a tetra-saccharide.

The experiments with the [^{14}C] compounds confirm the presence of some muramic acid after reduction, thus indicating incomplete reaction. This would in part explain the lower yields of muramicitol in the reduced di- and tetra-saccharides. Recent experiments performed by one of us (M.R.J.S.) has revealed the presence of an additional product in the reduced preparations which may also account for the low values for muramicitol. Thus it is likely that some degradation of the muramic acid occurs under conditions leading to reduction and such compounds are readily detectable when radioautographs of the hydrolysed products are examined (SALTON, unpublished observations).

Oxidation with $NaIO_4$

The linkages between the N-acetylamino sugars in the di- and tetra-saccharides were investigated by attempting to determine the liberation and possible origin of formaldehyde on oxidation with $NaIO_4$. As the isolated di- and tetra-saccharides were not 100 % pure[8], ambiguous results on periodate consumption and formaldehyde liberation were anticipated. This was confirmed with some preliminary experiments using the spectrophotometric method of determining periodate consumption and determining the H·CHO colorimetrically[21]. To avoid the difficulties of determing the origin of any H·CHO formed, [^{14}C] di- and tetra-saccharides (from lysozyme and F_1 digests) were used.

Oxidation with $NaIO_4$ was performed at pH 7.5, at 2° for 30 h under conditions giving maximum H·CHO liberation from AG[22] and from blood group substances[23]. At the end of oxidation, carrier H·CHO was added, followed by an excess of 5,5-dimethyl-1,3 cyclohexandione (dimedon) and the ^{14}C contents of the insoluble H·CHO-dimedon compounds were determined. A preliminary experiment in which a large excess (5 mg) of carrier H·CHO was added showed a small amount of radioactivity in the dimedon precipitates. With [^{14}C] di-saccharide (3,260 counts/10 min) and [^{14}C] tetra-saccharide (3,060 counts/10 min) [both from wall isolated from cells grown on [^{14}C] fructose and digested with lysozyme] the radioactivity in the H·CHO-dimedon precipitates was 7 counts/10 min and 19 counts/10 min respectively; a control sample of [^{14}C] di-saccharide treated in an identical manner, but without $NaIO_4$, showed a similar level of activity (18 counts/10 min) in the precipitates. [^{14}C] glucose oxidized under these conditions gave 75 % of the theoretical yield of [^{14}C] formaldehyde which on adjustment for absorption of the radioactivity (determined by mixing [^{14}C] glucose with an equivalent weight of non-radioactive H·CHO-dimedon) indicated a 97 % liberation of formaldehyde.

[^{14}C] di- and tetra-saccharides isolated from walls derived from *M. lysodeikticus* grown on [^{14}C] glucose were dissolved in 0.3 ml H_2O and 0.3 ml $NaHCO_3$ buffer containing $NaIO_4$ was added and oxidation allowed to proceed for 30 h at 2° in the dark[22]. [^{14}C] glucose was treated in the same manner, and controls of di- and tetra-saccharides together with a [^{14}C] glucose control were treated under the same conditions but in the absence of $NaIO_4$. At the end of oxidation the following reagents were added to each tube: 3 drops *N* HCl; 1 ml 0.1 *N* Na_2AsO_3; 0.14 ml formaldehyde solution containing 500 μg H·CHO; 1 ml *M* sodium acetate; 1 ml 5 % w/v dimedon

in ethanol. The contents of the tubes were mixed thoroughly and allowed to stand at room temperature for complete formation of the H·CHO–dimedon complexes. The H·CHO–dimedon precipitates were collected on membrane filters, washed several times with ice-cold distilled water, dried and the ^{14}C contents determined. The controls showed no activity in the precipitates. The results for the $NaIO_4$-treated compounds are summarized in Table III.

TABLE III

DETERMINATION OF THE [^{14}C] FORMALDEHYDE LIBERATED ON PERIODATE OXIDATION OF [^{14}C] GLUCOSE AND [^{14}C] DI- AND TETRA-SACCHARIDES

^{14}C Compound	Initial radio-activity	[^{14}C] in H.CHO-dimedon	Theoretical ^{14}C when C_6 available for oxidation	
	Counts/min	Counts/min	Counts/min	% theory
Di-saccharide	6,080	0	405*	0
Tetra-saccharide	3,670	0	122	0
Glucose	11,900	1,930	1,980	97.5

* Values expected for a 1 → 4 linkage with C_6 of the reducing sugar free to react with $NaIO_4$.

The action of β-glucosidase on di- and tetra-saccharides

The nature of the linkages between AG and AMA has been investigated by examining the products formed after incubation with β-glucosidase (Mann Research Laboratories Inc., salt-free, almond β-glucosidase). Approximately 400 μg of each compound (lysozyme and F_1 di- and tetra-saccharides in 0.1 ml 0.1 *M* sodium acetate buffer, pH 5.0, were added to 0.1 ml of the enzyme solution (500 μg enzyme in 0.1 ml 0.1 *M* sodium acetate buffer). The mixtures were incubated at 30° for 48 h, together with control solutions of the four compounds (no enzyme added). The reaction mixtures were examined by paper chromatography (Whatman No. 1, *n*-butanol–acetic acid–water; 3:1:1, v/v) using the N-acetylamino sugar spray described by SALTON[7]. All four compounds incubated with the enzyme gave substances separating in an identical manner to free AG and free AMA. Only the original di- and tetra-saccharides were detectable in the absence of β-glucosidase. In addition to the free acetylamino sugar constituents, residual di- and tetra-saccharides were detectable on the chromatograms, indicating incomplete cleavage of the compounds by β-glucosidase. Elution of the free AG and AMA from the paper chromatograms as described by SALTON[7] and estimation of the colours at 585 mμ gave ratios of AG to AMA of 1:1.3 for both lysozyme and F_1 disaccharides (the greater colour of the AMA is probably due to the presence of borate in the spray reagent, an effect used by PARK[24] for a differential test for AMA). That the compounds believed to be free AG and AMA were indeed these substances was further substantiated by their behaviour on finger-printing (electrophoresis followed by chromatography) under the conditions used by SALTON[7]. Electrophoresis at pH 6.4 indicated the presence of a free carboxyl group in the compound corresponding to AMA.

Incubation of the NDF from lysozyme digested walls of *M. lysodeikticus* with β-glucosidase under the conditions used above, also liberated free AG and AMA from the 10,000–20,000 molecular weight[3] mucopeptides. Untreated cell walls incubated similarly did not show the presence of free acetylamino sugars.

The action of lysozyme and Streptomyces F_1 enzyme on di- and tetra-chitobiose

In view of the further degradation of the tetra-saccharides liberated from walls by lysozyme and F_1 upon further incubation of the isolated compounds with the enzymes, it was of interest to determine whether these enzymes were capable of degrading N-acetylglucosaminides of known structure. Di- and tetra-chitobiose, kindly provided by Professor S. ROSEMAN, were incubated with lysozyme and F_1 enzyme using the following conditions: 500 μg each compound in 0.05 ml H_2O were added to enzyme (25 μg) dissolved in 0.05 ml 0.2 *M* ammonium acetate buffer, pH 7, and the mixtures were incubated for 48 h at 37°.

Examination of the products on Whatman paper No. 1 irrigated with *n*-butanol–acetic acid–water (3:1:1) and spraying for acetylaminosugars[7], revealed the presence of two compounds in the enzyme-treated di-chitobiose (original material and a substance separating very closely to AG) and three compounds in the tetra-chitobiose incubation mixture (original tetra-chitobiose, a compound with an R_F identical to that of di-chitobiose and a third substance separating as AG). Whether the substance separating closely to AG, is in fact AG or chitobiose (4-O-β-N-acetylglucosaminyl-N-acetylglucosamine) has not been established. These results do show, however, that both enzymes possess $\beta(1 \rightarrow 4)$-N-acetylglucosaminidase activity.

DISCUSSION

The results of our investigations and those of PERKINS[9,10] have established the simplest product of the action of lysozyme and Streptomyces F_1 enzyme as a di-saccharide of AG and AMA. The isolation of a tetra-saccharide, the structure of which we would like to propose as: O-β-N-acetylglucosaminyl-$(1 \rightarrow 6)$-O-β-N-acetyl-muraminyl-$(1 \rightarrow 4)$-O-β-N-acetylglucosaminyl-$(1 \rightarrow 6)$-β-N-acetylmuramic acid provides experimental verification of earlier suggestions by BRUMFITT, WARDLAW AND PARK[6] and BERGER AND WEISER[4] as to the nature of the linkages split by lysozyme. We can conclude that the enzymes lysozyme and Streptomyces F_1 break similar glycosidic bonds, although it is likely, as GHUYSEN[25] has pointed out, that there are differences in affinities for the substrate. Further structural evidence for the existence of the $(1 \rightarrow 4)$ linkage between the two di-saccharide residues of the tetra-saccharide is being sought, although the important discovery that both lysozyme and F_1 degrade the isolated tetra-saccharides to the di-saccharide[25] together with the above results of their action on known $\beta(1 \rightarrow 4)$-N-acetylglucosaminides and the action of β-glucosidase strongly support the proposed structure.

It is of interest to note that both the di- and tetra-chitobioses can be detected on paper with the spray reagent described by SALTON[7], despite the fact that substitution in the C_4 position usually abolishes or greatly reduces the MORGAN AND ELSON reaction as shown by JEANLOZ AND TRÉMÈGE[26]. Thus detection of the products of digestion of walls with the MORGAN AND ELSON reagents did not preclude the possibility of $1 \rightarrow 4$ linkages. The results of PERKINS[10] and our evidence clearly establish $1 \rightarrow 6$ linkages in the di-saccharide. It may be argued that the absence of [^{14}C] formaldehyde from the $NaIO_4$-treated compounds is not conclusive evidence of a $1 \rightarrow 6$ linkage. However, although experiments with reduced [^{14}C] compounds do yield some radio-activity in the H·CHO–dimedon complexes, the amounts are not compatible

with a $1 \rightarrow 4$ structure and it seems likely that they originate from degradation products formed on treatment with $NaBH_4$ (SALTON, unpublished data).

The type of structure constituting the cell walls of certain lysozyme-sensitive bacteria was originally proposed by SALTON[27] from the results of the investigation of the products of digestion of the walls by lysozyme. It was thus suggested that the walls possessed a structure "in which peptides and peptide-amino sugar complexes may be glycosidically linked through terminal amino sugars to other amino sugar and sugar residues of polysaccharide components"[27]. With the characterization of muramic acid[28,29] and the muramic acid-nucleotide-peptide[30] a more detailed structure for the wall was proposed by BRUMFITT, WARDLAW AND PARK[6]. The results of PERKINS[10] together with the above investigations and those of GHUYSEN AND SALTON[8] and GHUYSEN[25] provide experimental evidence for the existence of an AG–AMA backbone in the wall and suggest the following type of structure for the wall of *M. lysodeikticus* (Fig. 1).

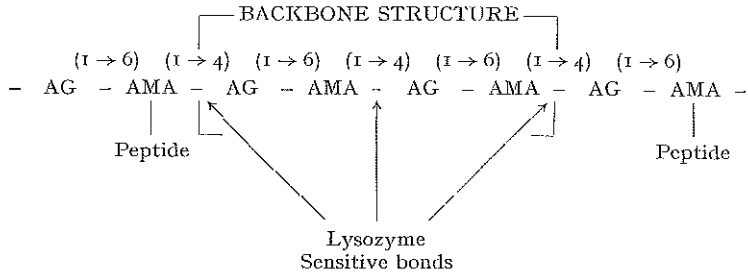

Fig. 1.

Little can be said about the structure of the peptide part of the wall mucopeptide. Although the amino acid ratios in some of the diffusable compounds isolated by GHUYSEN AND SALTON[8] are identical with those for the whole cell wall, the analysis of the products of partial acid hydrolysis of the wall of *M. lysodeikticus* by PERKINS AND ROGERS[31] shows a more complicated variety of peptides. With the isolation of a compound containing glucosamine, muramic acid and glycine in approximately equimolar proportions[31], it is conceivable that some glycine residues may be present as substituent groups and not in a peptide structure. The detection of substantial amounts of "C-terminal" glycine residues in the walls of *M. lysodeikticus* submitted to hydrazinolysis would be compatible with this view (SALTON, unpublished results).

NOTE ADDED IN PROOF

Although specific activities of ^{14}C compounds were not determined, it is evident from the results of RICHMOND AND PERKINS[32] that under our growth conditions the casamino acids would depress the labelling of the lactyl residue of muramic acid to give ratios observed in Table II.

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