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The Cell Wall of *Bacillus licheniformis* N.C.T.C. 6346

BIOSYNTHESIS OF THE TEICHURONIC ACID

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1. Particulate fractions prepared from disrupted cells of *Bacillus licheniformis* N.C.T.C. 6346 catalyse the uptake of radioactivity from UDP-[^{14}C]glucuronic acid or UDP-*N*[^{14}C]-acetylglucosamine. Maximal uptake requires the presence of both nucleotides and Mg^{2+} ions. The reaction is inhibited markedly by high concentrations of novobiocin and, to a certain extent, by vancomycin and by methicillin. 2. The radioactive product formed is resistant to Pronase and is soluble in 5% (w/v) trichloroacetic acid. It is of high molecular weight, from its behaviour on columns of Sephadex G-50 or G-200, and behaves during paper electrophoresis in N-acetic acid and chromatography on DEAE-cellulose in a manner similar to teichuronic acid. 3. Both teichuronic acid and the synthesized material are resistant to testicular hyaluronidase and to *Flavobacterium heparinum* heparinase. 4. The specific activity of suspensions of broken cells or of washed particulate fractions is greatest when they are prepared from exponentially growing cells. Fractions obtained from late exponential-phase or stationary-phase cells have very low activity. 5. The galactosamine content of *B. licheniformis* N.C.T.C. 6346 cell walls increases during the exponential phase and decreases during the stationary phase.

The cell wall of *Bacillus licheniformis* N.C.T.C. 6346 contains a polysaccharide, teichuronic acid, which consists of equimolar amounts of glucuronic acid and *N*-acetylgalactosamine (Janczura, Perkins & Rogers, 1961; Hughes, 1965*a*). The exact structure of the polysaccharide is unknown but several properties suggest that the glycosidic bonds are α in configuration. The glycosidic linkage between glucuronic acid and *N*-acetylgalactosamine is most probably on C-3 of the amino sugar. The biosynthesis of teichuronic acid has been studied by using a particulate enzyme fraction prepared from disrupted cells of *B. licheniformis*. Enzyme preparations obtained from cells harvested in the exponential phase of growth catalyse an uptake of radioactivity from UDP-[^{14}C]glucuronic acid in the presence of UDP-*N*-acetylgalactosamine and a similar uptake from UDP-*N*[^{14}C]-acetylgalactosamine when UDP-glucuronic acid is present; Mg^{2+} ions are essential for the reaction. The radioactive product has many of the properties of teichuronic acid. A preliminary account of this work has been previously published (Hughes, 1965*b*).

MATERIALS AND METHODS

Materials. UDP-glucuronic acid was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. UDP-[^{14}C]-glucuronic acid, uniformly labelled in the glucuronic acid group, was prepared enzymically (Strominger, Maxwell, Axelrod & Kalckar, 1957) from UDP-[^{14}C]glucose (Glaser, 1958). The UDP-glucose dehydrogenase was purchased from Sigma Chemical Co. The purified UDP-[^{14}C]glucuronic acid contained $6{\cdot}9 \times 10^6$ counts/min./μmole. UDP-*N*[^{14}C]-acetylgalactosamine and UDP-*N*-acetylgalactosamine were gifts from Dr G. W. Jourdian and Dr S. Roseman. The labelled material used contained $2{\cdot}4 \times 10^6$ counts/min./μmole. Pronase, *Streptomyces griseus* protease, was purchased from California Biochemical Corp., Los Angeles, Calif., U.S.A. Bovine testicular hyaluronidase was obtained from Evans Medical Supplies, Ruislip, Middlesex, and contained 1500 units/ml. Dried cells of *Flavobacterium heparinum* (Korn & Payza, 1956; Linker, Hoffman, Meyer, Sampson & Korn, 1960) were kindly given by Dr A. Linker. The cells were suspended at 4 mg./ml. in 0·1M-sodium phosphate buffer, pH 7·0, and broken by treatment with ultrasound for 10 min. at low temperature. The suspension of broken cells was centrifuged at 10000 *g* for 20 min. and the supernatant used as the source of heparinase. Methicillin was from Beecham Research Laboratories, Brentford, Middlesex. Novobiocin, vancomycin and heparin were given by the Division of Biological Standards at this Institute.

Analytical procedures. Protein was determined with the biuret method (Layne, 1957) with a bovine serum albumin standard. Glucuronic acid was estimated with the method of Dische (1947). Cell-wall amino acids and hexosamines were kindly analysed on the Beckman Spinco autoanalyser by Mr R. A. Foulkes and Dr S. Jacobs of this Institute, as previously described (Hughes, 1965*a*). Counting of radio-

active materials was carried out in a Packard Tri-Carb liquid-scintillation spectrometer. Aqueous solutions were counted in a mixture that contained 0·5 ml. of sample, 10 ml. of dioxan, naphthalene (1·8%), 2,5-diphenyloxazole (0·4%) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene(0·01%). Paper strips were assayed in 10 ml. of the counting medium. Samples were counted for times sufficient to collect at least 1000 counts above background.

Preparation of cell walls and teichuronic acid. Cell walls were prepared essentially by the method of Salton & Horne (1951) and included a heating step to eliminate autodigestion of the wall during isolation. Teichuronic acid was isolated from cell walls by extraction with trichloroacetic acid followed by chromatography on DEAE-cellulose (Hughes, 1965*a*).

Preparation of enzyme fractions. B. licheniformis N.C.T.C. 6346 was grown at 35°, usually to the beginning of exponential phase (5 hr.), when the E_{675} value of the culture fluid reached 0·1–0·2. The cultures were quickly cooled and all subsequent steps were carried out below 5°. The cells from 11. cultures were collected by centrifugation at 5000*g* for 10 min. and washed with 50 mM-tris–chloride buffer, pH 7·1, containing cysteine (6 mM). The washed cells were suspended at 10 mg./ml. in buffer and disrupted by treatment with ultrasound for 5 min. Intact cells and large fragments were removed by centrifugation at 2000*g* for 5 min. and the supernatant fluid was centrifuged at 30000*g* for 40 min. The sediment was washed twice by centrifugation in 50 mM-tris–chloride buffer, pH 7·1, containing cysteine (6 mM) and finally suspended in the same buffer (5·0 ml.). The protein content of the 30000*g* particulate fraction was usually 2–4 mg./ml.

Assay of enzyme fractions. A typical reaction mixture consisted of the enzyme fraction (0·3 ml.) in 50 mM-tris–chloride buffer, pH 7·1, containing cysteine (6 mM), UDP-glucuronic acid (15 mμmoles), UDP-*N*-acetylgalactosamine (15 mμmoles) and $MgCl_2$ (10 μmoles) in a final volume of 0·5 ml. After incubation at 37° for 2 hr. the reaction was stopped by heating at 100° for 3 min. Carrier teichuronic acid (equivalent to 0·1 mg. of glucuronic acid) and Pronase (0·05 ml.; 5 mg./ml.) were added and the mixture was left at 37° for 2 hr. Then trichloroacetic acid (0·05 ml.; 50%, w/v) was added and the mixture was kept at 37° for 12–15 hr., conditions that are known to extract quantitatively the teichuronic acid from isolated cell-wall preparations (Hughes, 1965*a*). The mixture was neutralized and centrifuged, and a known volume of the clear supernatant was placed on to a column (1·2 cm. × 20 cm.) of Sephadex G-50 that had been equilibrated with 0·1 M-pyridine–acetate buffer, pH 5·1. Authentic teichuronic acid was eluted quantitatively between 7 and 12 ml.; nucleotides and salts were eluted between 16 and 21 ml. The eluate containing carrier teichuronic acid was collected and dried from the frozen state. The dry material was dissolved in water (5·0 ml.) and samples were assayed for radioactivity and for uronic acid, as an index of carrier teichuronic acid content.

RESULTS

Properties of the enzyme system. Incubation of ultrasonically prepared extracts of *B. licheniformis* N.C.T.C. 6346 with UDP-*N*[^{14}C]-acetylgalactosamine, UDP-glucuronic acid and Mg^{2+} ions resulted in incorporation of radioactivity into an acid-soluble product that was resistant to proteolysis and that was eluted together with carrier teichuronic acid from a column of Sephadex G-50. Reaction mixtures that were heated at the beginning of the incubation period contained negligible radioactivity in the isolated teichuronic acid fraction.

Table 1. *Localization of enzymic activity*

B. Licheniformis was grown and the cells were broken as described in the Materials and Methods section. Whole cells and large fragments were sedimented by centrifugation at 2000*g* for 5 min. and the supernatant was centrifuged at 30000*g* for 40 min. The particulate fractions were suspended in 50 mM-tris–chloride buffer, pH 7·1, containing cysteine (6 mM). Reaction mixtures contained UDP-*N*[^{14}C]-acetylgalactosamine (15 mμmoles; $3·6 \times 10^4$ counts/min.), UDP-glucuronic acid (15 mμmoles), $MgCl_2$ (10 μmoles) and enzyme fraction in 50 mM-tris–chloride buffer, pH 7·1, containing cysteine (6 mM). The total volume was 0·5 ml. and incubation was at 37° for 2 hr. Incorporation of radioactivity into polysaccharide was measured as described in the Materials and Methods section. The polysaccharide fraction isolated from control reaction mixtures that had been heated at 100° for 3 min. at the beginning of the incubation period contained less than 50 counts/min./mg. of protein.

Enzyme fraction	Radioactive product formed (counts/min./mg. of protein)	Precursor incorporated (mμmoles/mg. of protein)
Whole ultrasonically prepared extract	980	0·41
2000*g* sediment	640	0·27
30000*g* sediment	2400	1·00
30000*g* supernatant	140	0·06

The distribution of incorporating activity in suspensions of broken cells is shown in Table 1. The cells were harvested in early exponential phase and disrupted by treatment with ultrasonic oscillations for 5 min. in the cold. Treatment of the cells for 10 min. did not release more activity. Most of the activity (70%) was located in the particles sedimenting between 2000 and 30000*g*. The specific activity of this fraction expressed as mμmoles of precursor incorporated into the teichuronic acid fraction/mg. of protein was more than twice that of whole ultrasonically prepared extracts. Negligible activity (7%) was found in the supernatant obtained after centrifugation at 30000*g* for 40 min.

Incorporation of radioactivity from UDP-[^{14}C]-glucuronic acid catalysed by the 30000*g* particulate fraction in the presence of 20 mM-magnesium chloride was increased about 14-fold by the addition of UDP-*N*-acetylgalactosamine (Table 2). Maximal incorporation of radioactivity from UDP-*N*[^{14}C]-acetylgalactosamine required the simultaneous presence of UDP-glucuronic acid. The amount of

Table 2. *Substrate requirements for incorporation of radioactivity into polysaccharide*

The particulate fraction that sedimented between 2000 and 30000*g* was prepared as described in Table 1. All reaction mixtures contained $MgCl_2$ (10 μmoles) and 0·30 ml. of the same freshly prepared enzyme fraction in 50 mM-tris–chloride buffer, pH 7·1, containing cysteine (6 mM). Where indicated the mixtures also contained: UDP-[^{14}C]glucuronic acid (15 mμmoles; $10{\cdot}4 \times 10^4$ counts/min.), UDP-*N*[^{14}C]-acetylgalactosamine (15 mμmoles; $3{\cdot}6 \times 10^4$ counts/min.), UDP-glucuronic acid (15 mμmoles), or UDP-*N*-acetylgalactosamine (15 mμmoles). The final volume was 0·5 ml. and incubation was at 37° for 2 hr. The radioactive polysaccharide fraction was isolated as described in the Materials and Methods section. Control reaction mixtures were heated at 100° for 3 min. at the beginning of the incubation period and contained less than 50 counts/min./mg. of protein.

Nucleotides added	Radioactive product formed (counts/min./mg. of protein)	Precursor incorporated (mμmoles/mg. of protein)
UDP-*N*-acetylgalactosamine; UDP-[^{14}C]glucuronic acid	6300	0·92
UDP-[^{14}C]glucuronic acid	450	0·07
UDP-*N*[^{14}C]-acetylgalactosamine; UDP-glucuronic acid	3200	1·32
UDP-*N*[^{14}C]-acetylgalactosamine	390	0·16

either radioactive precursor taken up by the same enzyme fraction in the presence of the other substrate and Mg^{2+} ions was quantitatively similar (Table 2).

The incorporation of radioactivity increased with time up to 2 hr. and then stopped. The limited incorporation of precursors into teichuronic acid is due in part to the breakdown of the substrates under the assay conditions and also to the lability of the enzyme system. Chromatography of assay mixtures after incubation at 37° for 2 hr. showed that UDP-*N*-acetylgalactosamine was completely destroyed. The precursor was stable under the same conditions in control mixtures that had been heated for 3 min. at 100° at the onset of incubation. However, addition of fresh nucleotides to assay mixtures after incubation for 2 hr. did not stimulate further incorporation. The 30000*g* particulate fraction was rapidly inactivated in the absence of substrates at 37° and lost about one-half of the original activity after overnight storage at 5°. Addition of magnesium chloride (20 mM) did not stabilize the enzyme fraction.

The effect of variation in the amount of the 30000*g* particulate fraction on incorporation of radioactivity into teichuronic acid was measured with UDP-[^{14}C]glucuronic acid as the radioactive precursor. The incorporation varied approximately proportionately over the concentration range 0·05–0·70 mg. of protein/reaction mixture. Incorporation of radioactivity from UDP-[^{14}C]-glucuronic acid into the teichuronic acid fraction was maximal at pH 7·1–7·5. The 30000*g* particulate fraction was about 50% as effective at pH 6·2 or pH 9·0 as at pH 7·1.

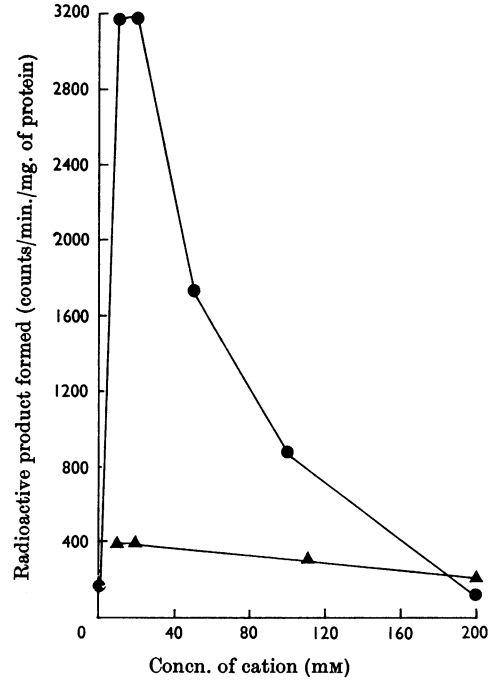

Fig. 1. Effect of Mg^{2+} (●) and Ca^{2+} (▲) concentration on enzymic activity. The reaction mixtures contained UDP-*N*[^{14}C]-acetylgalactosamine (15 mμmoles; $3{\cdot}6 \times 10^4$ counts/min.), UDP-glucuronic acid (15 mμmoles), freshly prepared 30000*g* particulate fraction in 50 mM-tris–chloride buffer, pH 7·1, containing cysteine (6 mM), and various amounts of $MgCl_2$ or $CaCl_2$ in final volume of 0·5 ml. The cation concentrations do not include the contributions of cations from the enzyme preparation itself. Incubation was at 37° for 2 hr. and the radioactive polysaccharide was isolated as described in the Materials and Methods section.

The effect of three antibiotics on incorporation of radioactivity from UDP-*N*[^{14}C]-acetylgalactos-

Table 3. *Effect of antibiotics on teichuronic acid synthesis*

Reaction mixtures, containing UDP-*N*[^{14}C]-acetylgalactosamine as radioactive precursor, were made up as described in Table 2. Antibiotics were added at the indicated concentrations. The results shown were obtained in several experiments and are expressed as percentages of the activity of the enzyme system in the absence of antibiotics.

Antibiotic	Concn. (mg./ml.)	Enzymic activity (% of control)
None	—	100
Methicillin	0·01	79–86
	1·0	36–67
Novobiocin	0·5	18
	1·0	7–10
Vancomycin	1·0	77
	2·0	28–43

amine into teichuronic acid are shown in Table 3. The inhibitors have been shown to interfere with synthesis of the bacterial cell wall (Davis & Feingold, 1962). Methicillin (1mg./ml.), when added in high concentrations to reaction mixtures, gave values 36–67% of those of control mixtures. Insufficient work has been done to explain the wide variation in the effects observed. Particulate enzyme fractions prepared from growing cultures that had been exposed for 30min. to low concentrations of methicillin (10μg./ml.) showed, in two separate experiments, 28 and 37% of the incorporating activity of fractions obtained from control cells. Similar effects on particulate enzyme fractions after treatment of growing cultures with antibiotics have been reported on mucopeptide biosynthesis in *Staphylococcus aureus* (Chatterjee & Park, 1964). Addition of novobiocin (1mg./ml.) to reaction mixtures effectively stopped incorporation (Table 3). A less marked effect was given by a similar concentration of vancomycin (Table 3).

Examination of the radioactive product. To characterize the polysaccharide formed by the 30000*g* particulate fraction, material isolated in several experiments was pooled. A product of closely similar properties was synthesized when the source of radioactivity was UDP-[^{14}C]glucuronic acid or UDP-*N*[^{14}C]-acetylgalactosamine in the complete assay systems described in Table 2.

The pooled radioactive product was eluted in 80–90% yield with carrier teichuronic acid in the void volume (130–160ml.) of a large column (1·8cm. × 130cm.) of Sephadex G-50. The elution pattern of the radioactive product and carrier teichuronic acid was not changed after overnight treatment at 37° and pH 5·5 with testicular hyaluronidase (150i.u.). The resistance of teichuronic

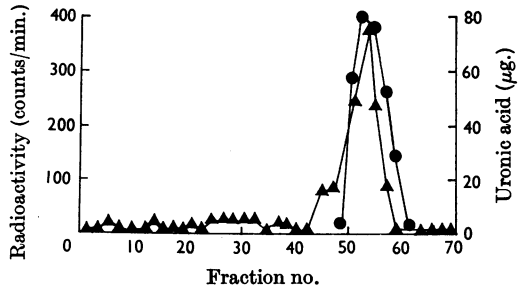

Fig. 2. Chromatography on DEAE-cellulose of radioactive polysaccharide. The radioactive polysaccharide fraction was isolated as described in the Materials and Methods section from reaction mixtures containing UDP-*N*[^{14}C]-acetylgalactosamine as the source of radioactive precursor. The pooled material (4910counts/min.) contained carrier teichuronic acid (equivalent to 0·3mg. of glucuronic acid) and was applied to a DEAE-cellulose column (15cm. × 1·5cm.) that had been equilibrated with 0·01M-pyridine–acetate buffer, pH 5·1. The column was developed with a linear gradient established by running 5M-pyridine–acetate buffer, pH 5·1, into 1l. of 0·01M-pyridine–acetate buffer, pH 5·1. Fractions (4·5ml.) were collected and analysed for glucuronic acid (●) and for radioactivity (▲).

acid to hyaluronidase was described by Janczura *et al.* (1961) and is consistent with the polysaccharide having α-glycosidic linkages. The heparinase present in cells of *Flavobacterium heparinum* (Korn & Payza, 1956) had no effect on teichuronic acid or on the radioactive product. Treatment with the heparinase was for 24hr. at 37°.

A portion of the pooled radioactive product was applied to a DEAE-cellulose column and eluted with a salt gradient. As shown in Fig. 2, all of the recovered radioactivity was eluted in the region associated with the uronic acid-containing material that is probably mainly carrier teichuronic acid and the small amount of teichuronic acid initially present in the 30000*g* particulate fraction. The recoveries of radioactivity and uronic acid from the column were 70 and 78% respectively. The labelled polysaccharide was examined by paper electrophoresis in N-acetic acid. After electrophoresis for 3hr. the radioactive product had migrated to a position similar to that of carrier teichuronic acid (Fig. 3).

The radioactive product and carrier teichuronic acid were eluted together in the included volume of a column of Sephadex G-200 (Fig. 4).

Activity of enzyme preparations from cultures grown for different times. The results of two separate experiments are shown in Fig. 5. The specific activity of the 30000*g* particulate fraction was greatest when prepared from cells harvested in early exponential phase and decreased sharply during the

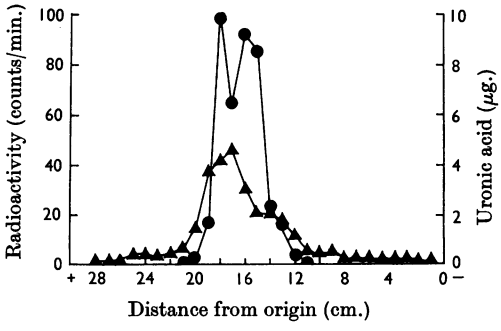

Fig. 3. Paper electrophoresis of radioactive polysaccharide. Material obtained as described in Fig. 2 and containing 1500 counts/min. and 0·12 mg. of glucuronic acid equivalent was applied to Whatman no. 3 paper. Electrophoresis was carried out in N-acetic acid at 2000 v for 3 hr. The paper was cut into strips 1 cm. in width. One half of each strip was counted directly for radioactivity (▲) and the other half was eluted with water and analysed for glucuronic acid (●).

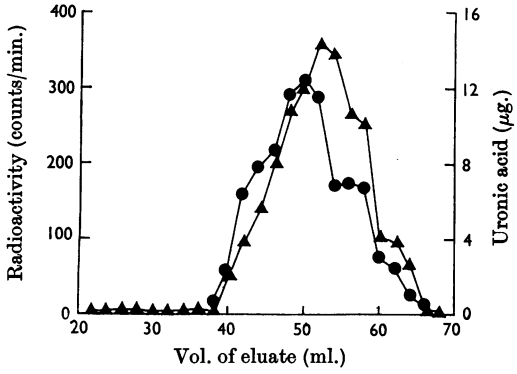

Fig. 4. Elution of the radioactive polysaccharide from Sephadex G-200. Material obtained as in Fig. 2 contained 3540 counts/min. and 0·14 mg. of glucuronic acid equivalent, and was applied to a column (1·4 cm. × 47 cm.) of Sephadex G-200. The column was equilibrated and eluted with 0·1M-pyridine–acetate buffer, pH 5·1. Fractions (1 ml.) were analysed for radioactivity (▲) and for uronic acid of the carrier teichuronic acid (●). Salts were eluted at 65–80 ml.

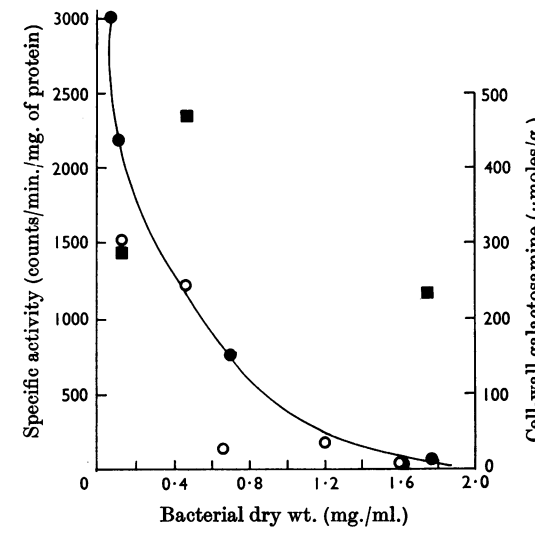

Fig. 5. Effect of age of culture on the activity of particulate fractions and on the galactosamine content of cell walls. In Expt. 1, cultures (200 ml.) were incubated at 35° until the cell density had reached the values shown and then quickly cooled. Washed cells were suspended at 10 mg./ml. in 50 mM-tris–chloride buffer, pH 7·1, containing cysteine (6 mM), broken by ultrasonic oscillations and fractionated by centrifugation as described in the Materials and Methods section. The 30000 *g* particulate fractions were assayed for activity in a reaction mixture (0·5 ml.) containing UDP-*N*[^{14}C]-acetylgalactosamine (15 mμmoles; $3{\cdot}6 \times 10^4$ counts/min.), UDP-glucuronic acid (15 mμmoles) and $MgCl_2$ (10 μmoles). Incubation was at 37° for 2 hr. The results of two independent experiments are shown (○ and ●). In Expt. 2 cell walls were prepared from cultures at the cell densities indicated. The galactosamine content of the wall (■) was determined as described in the Materials and Methods section. Under the growth conditions used, exponential phase started at 5 hr., when the cell opacity was about 0·04 mg. dry wt./ml., and continued to about 0·6 mg. dry wt./ml. In the stationary phase the cell opacity had reached about 1·3 mg. dry wt. of bacteria/ml., which was maintained until at least 28 hr.

period of exponential growth. Fractions prepared from cells grown to the stationary phase showed very low enzymic activity. Similar results were obtained when unfractionated suspensions of broken cells were examined.

Particulate fractions prepared from cells grown for 28 hr. were added to assay mixtures containing enzyme preparations of high specific activity obtained from early exponential-phase cells. The incorporation after 2 hr. at 37° of radioactivity from UDP-*N*[^{14}C]-acetylgalactosamine into the teichuronic acid fraction was thereby inhibited by 60–80%. The inhibition was not found when the particulate fraction prepared from 28 hr. cells was heated at 100° for 3 min. before addition to the reaction mixtures.

Variation in the teichuronic acid content of cell walls during growth. The amount of teichuronic acid in isolated cell-wall preparations of *B. licheniformis* N.C.T.C. 6346 was estimated by analysis for galactosamine. It has been previously shown that teichuronic acid after purification contains equimolar amounts of glucuronic acid and galactosamine (probably as the *N*-acetyl derivative), and it is

likely that all of the galactosamine of the cell wall, at least of stationary-phase cells, is present in the teichuronic acid component (Hughes, 1965*a*). As shown in Fig. 5, the amount of galactosamine rose from 290 μmoles/g. of cell walls in cells harvested during the early exponential phase ($6\frac{1}{2}$ hr.) to 470 μmoles/g. of cell walls in late exponential-phase (9 hr.) cells. Cell walls isolated from cells in late stationary phase (28 hr.) contained 230 μmoles of galactosamine/g. of cell walls. The yield of cell walls obtained from 1 l. cultures grown for $6\frac{1}{2}$, 9 and 28 hr. were 19, 88 and 266 mg. respectively. The absolute amounts of galactosamine in the cell walls isolated from cells grown for $6\frac{1}{2}$, 9 and 28 hr. are calculated to be 5·5, 41·3 and 61·0 μmoles/l. of culture respectively. The absolute values are very approximate since the recovery of cell wall is certainly not quantitative and is probably not the same in every preparation.

DISCUSSION

Teichuronic acid in *B. licheniformis* N.C.T.C. 6346 is found in firm association with the cell wall. The polysaccharide is present in small amounts in the extracellular fluid of cultures of the organism, particularly in the late stationary phase of growth, but this is most probably due to enzymic degradation of the cell wall. It has been shown that teichuronic acid is removed *in vitro* quantitatively from the cell wall into a soluble form on treatment of cell-wall preparations with egg-white lysozyme (Janczura *et al.* 1961; Hughes, 1965*a*). The structurally analogous polysaccharide, hyaluronic acid, is well known as the capsular material of a strain of group A *Streptococcus* (Rogers, 1961), and net synthesis of bacterial hyaluronate has been demonstrated in a cell-free system (Markovitz, Cifonelli & Dorfman, 1959). In the present work, with the complete systems described in Table 1, up to 10% of the total precursor radioactivity was incorporated into the isolated teichuronic acid fraction. However, the total amount of radioactive precursor incorporated was always small in proportion to the total endogenous polysaccharide present in the particulate enzyme fraction. This made attempts to demonstrate net synthesis of polysaccharide difficult. Incorporation of radioactive precursors into teichuronic acid was limited by the rapid degradation of the precursors in assay mixtures on incubation at 37°. The destruction occurred in mixtures containing 30 000 *g* particulate fractions prepared from both exponential-phase and stationary-phase cells. The decrease in specific activity of enzyme fractions prepared from cells grown for longer times (Fig. 5) may therefore be a reflection of two independent phenomena: a real decrease in the ability of fractions prepared from stationary-phase cells to form teichuronic acid from the activated precursors, as well as an increase with time of growth in the rate of enzymic degradation of substrates by the particulate fractions.

The amount of galactosamine in the cell wall increases in cells growing exponentially (Fig. 5), when the apparent ability of the cell-free system to form teichuronic acid is high, and is low in cell walls isolated from stationary-phase cells. In the biosynthesis of bacterial hyaluronate, cells harvested after 5–7 hr. growth were found to form polysaccharide at a maximal rate. Suspensions of resting cells of these organisms in a medium of glucose and glutamine formed hyaluronic acid, whereas cells grown for 12–14 hr. did not (Lowther & Rogers, 1956; Cifonelli & Dorfman, 1957).

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