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The Linkage between the Polysaccharide and Mucopeptide Components of the Cell Wall of *Lactobacillus casei*

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1. The linkage between the polysaccharide and mucopeptide components of the cell wall of *Lactobacillus casei* is rapidly hydrolysed under mild acid-hydrolysis conditions. 2. The release of the polysaccharide is accompanied by the hydrolysis of an *N*-acetylhexosaminide linkage. The *N*-acetylhexosamine residue readily forms chromogen and it is concluded that it is substituted on $C_{(3)}$ by the adjacent sugar. 3. Continued heating of the polysaccharide in acid results in a slower release of reactive *N*-acetylhexosamine due to the hydrolysis of glycosidic linkages within the polysaccharide. 4. After the linkage between the polysaccharide and mucopeptide has been hydrolysed, acid phosphatase will release approx. 40% of the total phosphorus as inorganic phosphate. 5. It is concluded that the polysaccharide component of the cell wall is joined through its reducing end group to a phosphate grouping in the mucopeptide.

Although there is evidence indicating that the cell walls of certain bacteria contain polysaccharide components, the structural relationship between the polysaccharide and mucopeptide components is not clear (Perkins, 1963). Krause & McCarty (1961, 1962) have studied the polysaccharide–mucopeptide complex in the cell wall of streptococci and have shown that heating in formamide resulted in almost complete separation of the polysaccharide and mucopeptide. However, the procedure has the disadvantage that there is considerable destruction of sugars.

Before the introduction of formamide for extracting serologically active material from streptococci (Fuller, 1938), it had been shown by Lancefield (1933) that heating streptococci in hot dilute acid yielded an active extract. However, Krause & McCarty (1961, 1962) found that acid was less effective than formamide in removing polysaccharide from the streptococcal cell wall.

The heating of cells in dilute acid to extract serologically active material was also used by Sharpe (1955) in her studies on the classification of the lactobacilli. The effect of hot dilute acid on the cell wall and soluble cell-wall products from *Lactobacillus casei* has been studied and the results are described in this and the succeeding paper (Hall & Knox, 1965). The rate of hydrolysis of the linkage between the polysaccharide and mucopeptide has been followed, and evidence is presented that the linkage hydrolysed is between the potential reducing end group of *N*-acetylgalactosamine and a phosphate grouping that remains attached to the mucopeptide. Under appropriate conditions a complete separation of the polysaccharide and mucopeptide can be achieved, and the properties of the isolated components have been studied (Hall & Knox, 1965).

MATERIALS AND METHODS

Carbohydrates. *N*-Acetylgalactosamine (grade B) was obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. *O*-β-D-Glucopyranosyl-(1→3)-*N*-acetyl-D-galactosamine was isolated from the products of mild acid hydrolysis of the cell-wall polysaccharide from *L. casei* NIRD RO 94 (Knox & Hall, 1965).

Enzymes. Wheat-germ acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Phosphoprotein phosphatase from ox spleen was prepared as described by Hofman (1958). The fraction designated B_1 was used.

Column chromatography. DEAE-Sephadex A-50 (medium grade) was obtained from Pharmacia, Uppsala, Sweden. It was prepared for use by repeated alternate washing with 0·5N-HCl and 0·5N-NaOH, and washed to neutrality with water, followed by 0·2M-tris–HCl buffer, pH 7·6. The DEAE-Sephadex was then suspended in 0·05M-tris–HCl buffer, pH 7·6, and packed into a column of appropriate size. The rate of elution of carbohydrate-containing material from the column was followed with the primary cysteine–H_2SO_4 reaction (Dische, 1953).

Organisms. Cultures of *Lactobacillus* were obtained from the National Collection of Type Cultures, Colindale, London (designated NCTC), and the National Institute for

Research in Dairying, Reading (designated NIRD). These organisms were *L. casei* NIRD strain H831 and *L. casei* NIRD DECP belonging to serological group B, and *L. casei* NIRD RO94 and *L. helveticus* (*L. casei* var. *rhamnosus*) NCTC 6375 of group C (Sharpe, 1955; Sharpe & Wheater, 1957).

Organisms were grown at 37° for 2 days in the medium described by Ågren & de Verdier (1958).

Preparation of cell wall. Suspensions of washed cells (equivalent to 40mg. dry wt./ml.) were shaken at 5° for 1hr. with Ballotini no. 12 beads (100g. of beads/100ml. of suspension) in the attachment for the International PR1 centrifuge described by Shockman, Kolb & Toennies (1957). The method previously employed for isolating the cell wall (Knox & Brandsen, 1962) has been modified by substituting 0·1M-tris–HCl buffer, pH7·8, for phosphate buffer, as this led to a lower phosphate content of the cell-wall preparation: cell wall from strain NIRD RO94 contained 0·30% of total phosphorus when prepared in phosphate buffer and 0·20% of phosphorus when prepared in tris–HCl buffer. When the product prepared in phosphate buffer was heated in 0·1N-H_2SO_4 at 60° for 3hr., phosphorus equivalent to 0·10% was released as inorganic phosphate and the isolated cell wall now contained 0·20% of phosphorus. When the cell wall prepared in tris–HCl buffer was heated in 0·1N-H_2SO_4 no inorganic phosphate was released.

Preparation of soluble cell-wall polymers. Preparations of cell wall were incubated with the muralytic enzyme preparation of *Streptomyces albus* and the soluble cell-wall components isolated. These products were fraction I from strain NCTC 6375 and fraction II from strains NCTC 6375, NIRD RO94, NIRD H831 and NIRD DECP (Knox, 1963).

Fraction II from *L. casei* NIRD RO94 was purified by applying a solution containing 500mg. in 10ml. of 0·05M-tris–HCl buffer to a column (30cm.×2cm.) of DEAE-Sephadex. The material was eluted with 0·05M-tris–HCl buffer containing increasing amounts of NaCl. A small amount of material was eluted with 0·05M-tris–HCl buffer made 0·02M with respect to NaCl. The major fraction (390mg.) was eluted with 0·05M-tris–HCl buffer made 0·05M with respect to NaCl and has been designated fraction III; higher concentrations of NaCl eluted only small amounts of material. Fraction II contained 0·34% of phosphorus whereas fraction III contained 0·17% of phosphorus. Spectrophotometric examination of fraction III (0·5% solution) from 230 to 300mμ showed no detectable nucleic acid. The possibility that the phosphorus is present as glycerol teichoic acid is unlikely as glycerol could not be detected in hydrolysates of 5mg. of material (Ellwood, Kelemen & Baddiley, 1963). The properties of fraction III were further examined by applying 50mg. in 1ml. of 0·05M-tris–HCl buffer to a column (50cm.×1·5cm.) of DEAE-Sephadex and eluting with 0·05M-tris–HCl buffer made 0·05M with respect to NaCl. Evidence for heterogeneity was obtained but the degree of separation of the components was only slight.

Fraction II from *L. casei* NIRD DECP contained 0·41% of phosphorus. In previous studies (Knox, 1963) this product had been precipitated from ethylene glycol by acetone; this procedure lowered the phosphorus content to 0·29%. Elution of this material from DEAE-Sephadex did not lower the phosphorus content further.

Analytical methods. Glucose, galactose, rhamnose, muramic acid, total hexosamine and total nitrogen were estimated by the methods previously described (Knox, 1963; Knox & Brandsen, 1962). Galactosamine was determined by the method of Cessi & Serafini-Cessi (1963).

Phosphorus was estimated by the method of Chen, Toribara & Warner (1956). Samples for total phosphorus were digested with H_2SO_4 followed by $HClO_4$ before estimation; those for inorganic phosphorus were mixed with an equal volume of 20% (w/v) trichloroacetic acid and allowed to stand for 4hr. at 2° before being centrifuged, and a portion of the supernatant was removed for estimation. Interference in the method for estimating inorganic phosphate was observed when cell-wall products were being analysed, especially in those cases where the release of inorganic phosphate by phosphatase was to be determined.

Because of the low phosphate content of the preparation, it was necessary to use several milligrams of material for each estimation. The preparation is not precipitated by adding 1vol. of 20% trichloroacetic acid, even on standing for several hours at 2°, but a faint opalescence develops on adding fraction III to the reagents for determining phosphate (Chen *et al.* 1956). The opalescence was more obvious with 4mg. of material than with 2mg., but even for 4mg. the extinction at 820mμ was only 0·015 when read in a 1cm. cell against a reagent blank.

It has also been shown that the polysaccharide and polysaccharide–mucopeptide complex partially inhibit the development of the colour obtained in the phosphate estimation. To a solution containing 3μg. of inorganic phosphate and various amounts of cell-wall products in a total volume of 1·0ml. was added 1·0ml. of 20% trichloroacetic acid, and the phosphorus was estimated. The extinction in the presence of 1, 2·5, 5 and 10mg. of polysaccharide–mucopeptide complex was depressed by 7, 22, 38 and 46% respectively. The inhibition remained proportional when the amount of phosphate was varied; for instance, the extinction values for 3 and 12μg. of phosphate in the presence of 5mg. of material were 0·35 and 1·40 respectively. The method for estimating phosphate is therefore applicable to a study of phosphatase action if internal standards are used.

Determination of $C_{(3)}$*-substituted* N*-acetylhexosamine.* *N*-Acetylhexosamine substituted on $C_{(3)}$ is readily degraded under mild alkaline conditions, chromogen being formed at room temperature and pH10·8 (Kuhn, Gauhe & Baer, 1954). In order to have a method for estimating *O*-β-D-glucopyranosyl-(1→3)-*N*-acetyl-D-galactosamine and other related products, the rate of chromogen formation at lower pH values has been investigated; of the conditions examined the most convenient was to heat the solution at pH7·0 and 100°.

A solution containing 20μg. of *O*-β-D-glucopyranosyl-(1→3)-*N*-acetyl-D-galactosamine and 0·1ml. of 0·5M-phosphate buffer, pH7·0 (Huggett & Nixon, 1957), was diluted to 1·0ml. with water and heated in a boiling-water bath. Tubes were removed at appropriate intervals and then cooled, and chromogen was estimated by the method of Aminoff, Morgan & Watkins (1952). The maximum was obtained after 45min.; in contrast, the maximum chromogen content from *N*-acetylgalactosamine was not reached until 6–7hr., the value after 45min. being only 30% of the maximum (Fig. 1). The amount of chromogen produced by 100μg. of the disaccharide in 45min. is 209% of that given in the same time by 500μg. of *N*-acetyl-D-galactos-

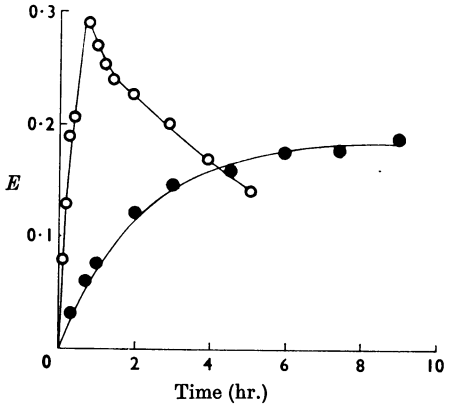

Fig. 1. Rates of chromogen formation from 100μg. of *N*-acetylgalactosamine (●) and 20μg. of *O*-β-D-glucopyranosyl-(1→3)-*N*-acetyl-D-galactosamine (○). The preparations were heated at 100° and pH 7·0 for appropriate intervals and chromogen was estimated as described in the text. Extinctions were read at 550mμ.

amine. Extinction values were read at intervals of 10mμ from 500 to 630mμ and the shapes of the curves were found to be identical.

It has also been shown that glucose (estimated with glucose oxidase) is released from the disaccharide concomitant with chromogen formation, the maximum obtained after 45min. being 75% of the theoretical value; on more prolonged heating the glucose value decreased. Control experiments with glucose showed that, after 45min. at pH 7·0 and 100°, 85% of the starting material was estimated by glucose oxidase. After 3hr. this value had fallen to 51%.

The method has been applied to a study of the rate of acid hydrolysis of the polysaccharide–mucopeptide complex. To minimize variations in pH, which could occur on neutralizing 0·2ml. of 0·1N-H_2SO_4 with 0·2ml. of 0·1N-NaOH, the possibility of adding an increased amount of phosphate buffer was investigated. To a series of tubes containing 20μg. of disaccharide in 0·2ml. of water was added 0·05, 0·10, 0·15, 0·20, 0·30, 0·40 and 0·80ml. of 0·5M-phosphate buffer, pH 7·0. Solutions were made up to 1ml. with water and heated at 100° for 45min. The maximum amount of chromogen was detected with 0·05 and 0·10ml. of buffer. With 0·15, 0·2, 0·3 and 0·4ml. (i.e. 0·2M) the amount of chromogen detected was decreased by 9, 35, 72 and 87% respectively; no chromogen was detectable in the presence of 0·8ml. of buffer. A similar effect was obtained when *N*-acetylgalactosamine (100μg.) was heated for 6hr. with increasing amounts of phosphate buffer. Heating *N*-acetylgalactosamine in 0·05M-phosphate buffer and subsequently increasing the buffer concentration also decreased the amount of the carbohydrate detected; a final concentration of 0·2M caused 87% inhibition, the same effect as was obtained on heating the disaccharide in 0·2M-buffer. It is concluded therefore that the higher concentrations of phosphate buffer inhibit the detection of chromogen but not its formation.

The following procedure has been employed for estimating the rate of hydrolysis of *N*-acetylhexosaminide linkages in which the hexosamine is substituted on $C_{(3)}$ by another sugar: 0·2ml. of 0·1N-H_2SO_4 containing 1mg. of soluble material was heated in small glass stoppered tubes under appropriate conditions and then cooled; 0·1ml. of 0·5M-phosphate buffer was added followed by 0·2ml. of 0·1N-NaOH. The contents of the tube were immediately mixed and transferred to a test tube (8in.×$\frac{5}{8}$in.) graduated at 10ml.; the small tube was washed twice with 0·25ml. of water and the washings were transferred to the test tube. The mixture was then heated at 100° for 45min., cooled and Ehrlich's reagent added according to the procedure of Aminoff *et al.* (1952). For experiments on cell-wall preparations larger amounts were heated in 0·1N-H_2SO_4 and then centrifuged. A suitable portion of the supernatant solution was then neutralized as described above.

N-Acetyl-D-galactosamine, *N*-acetyl-D-glucosamine and *O*-β-D-galactosyl-(1→6)-*N*-acetyl-D-glucosamine, which are among the products of acid hydrolysis of the polysaccharide (Knox & Hall, 1965), would yield some chromogen under these conditions, but the amount would be relatively small compared with that obtained from $C_{(3)}$-substituted *N*-acetylhexosamine. The observed yield of chromogen from $C_{(3)}$-substituted *N*-acetylgalactosamine is 18 times that for free *N*-acetylgalactosamine and 8 times that for free *N*-acetylglucosamine; further, on a molar basis *O*-β-D-galactosyl-(1→6)-*N*-acetylglucosamine yields the same amount of chromogen as *N*-acetylglucosamine (Knox & Hall, 1965).

RESULTS

Conditions for the acid hydrolysis of the polysaccharide–mucopeptide complex from L. casei *NIRD RO* 94

Heating at 100° *in* 0·1N-*sulphuric acid.* Previous studies on the partial acid hydrolysis of cell-wall components showed that the major products comprise a series of oligosaccharides with a terminal $C_{(3)}$-substituted *N*-acetylgalactosamine residue (Knox & Hall, 1965). To follow the rate of hydrolysis of *N*-acetylhexosaminide linkages containing this substituent a series of tubes containing 1mg. of fraction II in 0·2ml. of 0·1N-sulphuric acid was heated at 100° for periods up to 6hr., and the *N*-acetylhexosamine content then estimated. The maximum was reached after 45min. and was followed by a decrease to 65% of the maximum after 6hr. When readings for the rate of *N*-acetylhexosamine release were taken at 2min. intervals up to 40min. an inflexion at 20min. became apparent. The inflexion was small but reproducible, and in an attempt to make it more pronounced milder conditions of hydrolysis were examined. Heating in 0·1N-acid at a lower temperature (60°) proved more satisfactory than heating at 100° in more dilute acid (pH 2·0).

Heating at 60° *in* 0·1N-*sulphuric acid.* A series of tubes containing 1mg. of fraction II in 0·2ml. of 0·1N-sulphuric acid was heated at 60° for periods up to 24hr. Experiments were carried out in duplicate and the amounts of terminal reducing

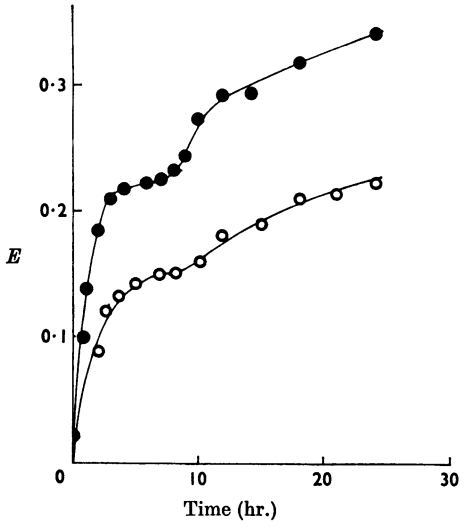

Fig. 2. Rates of release of 'reactive *N*-acetylhexosamine' on heating 1mg. of cell wall (○) and 1mg. of fraction III (●) from *L. casei* NIRD RO94 at 60° in 0·1N-H_2SO_4 for appropriate intervals. 'Reactive *N*-acetylhexosamine' is defined as *N*-acetylhexosamine that forms chromogen on heating at pH7·0 and 100° for 45min. Details are given in the text.

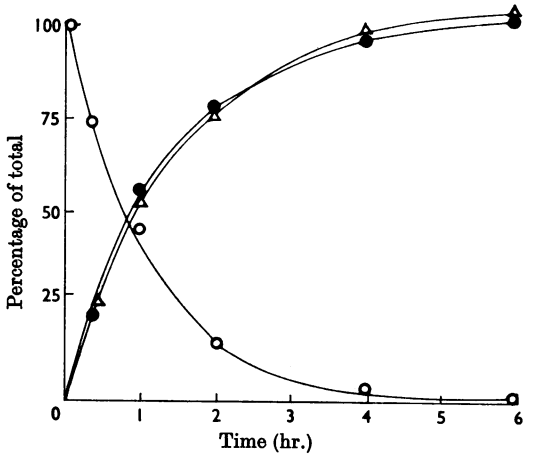

Fig. 3. Rates of release of reactive *N*-acetylhexosamine (△) and soluble hexose (●) from cell wall of *L. casei* NIRD RO94 on heating at 60° in 0·1N-H_2SO_4 for appropriate intervals. The amount of hexose (○) remaining in the insoluble residue is also shown. The results are expressed as a percentage of the total released in 6hr. for reactive *N*-acetylhexosamine, and as a percentage of the total cell-wall content for hexose.

N-acetylhexosamine estimated. A rapid initial release of *N*-acetylhexosamine was obtained within 3hr.; an inflexion at 7hr. was followed by a further slower release of *N*-acetylhexosamine (Fig. 2). Subsequent experiments showed that the *N*-acetylhexosamine content continued to rise slowly until a maximum was reached after 8 days; the extinction value after 8 days was 3·0 times that after 5hr. A curve of similar shape was obtained when the experiment was repeated on 1mg. of cell wall (Fig. 2).

Rate of release of polysaccharide from the cell wall. The initial rate of release of terminal reducing *N*-acetylhexosamine has been compared with the rate of dissolution of the cell-wall polysaccharide. A series of tubes containing 10mg. of cell wall in 1·0ml. of 0·1N-sulphuric acid was heated at 60° for periods up to 6hr After cooling, the contents were diluted to 5ml. and centrifuged at 10000*g* for 15min. *N*-Acetylhexosamine and hexose were estimated on portions of the supernatant equivalent to 1mg. and 200μg. respectively of the cell wall. The residue was washed with water, centrifuged, resuspended and hexose estimated on the equivalent of 200μg. of wall. The results (Fig. 3) indicated a rapid and complete release of cell-wall carbohydrate concomitant with the release of terminal reducing *N*-acetylhexosamine.

The amount of hexose present in the supernatant after 1hr. represented 56% of the total; the corresponding value found for galactosamine was also 56%. Further analyses showed that the cell-wall residue obtained after heating for 6hr. was devoid of galactosamine but contained all the phosphorus and muramic acid originally present in the preparation.

In another experiment, in which 50mg. of wall was heated for 1hr. at 60° in 0·1N-sulphuric acid, it was shown that only 4% of the soluble carbohydrate was diffusible (as determined by the primary cysteine–sulphuric acid reaction).

Rate of hydrolysis of hexose 1-phosphate in 0·1N-sulphuric acid at 100°. The initial rapid release of terminal *N*-acetylgalactosamine has been compared (Fig. 4) with the rate of hydrolysis of glucose 1-phosphate (estimated with glucose oxidase). The results for the cell-wall product (fraction III) are consistent with the hydrolysis of a phosphate grouping joining the potential reducing end group of a polysaccharide to another component of the wall.

Action of phosphatase on the polysaccharide–mucopeptide complex. The purified polysaccharide–mucopeptide complex (fraction III) contained 0·17% of organic phosphorus, and evidence has been sought that the potential reducing end group of the polysaccharide is joined to another component (probably the mucopeptide) by an orthophosphate group. Removal of the polysaccharide by dilute acid hydrolysis would yield a phosphomonoester, and the action of wheat-germ acid phosphatase has therefore been examined.

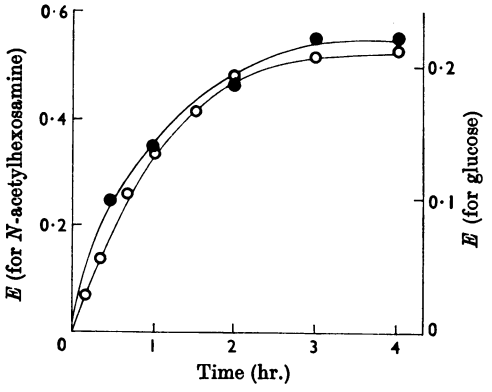

Fig. 4. Comparison of the rate of release of reactive *N*-acetylhexosamine from fraction III from *L. casei* NIRD RO94 (●, left-hand ordinate) with the rate of release of glucose from glucose 1-phosphate (○, right-hand ordinate) on heating in 0·1N-H_2SO_4 at 60° for appropriate intervals. Experimental details are given in the text.

Preliminary experiments showed that incubation of the enzyme with the polysaccharide–mucopeptide complex did not liberate detectable amounts of inorganic phosphate. For studying the action of phosphomonoesterase on hydrolysed samples, the periods of preliminary acid hydrolysis chosen were 40min. and 3hr. Previous results on the rate of release of terminal *N*-acetylhexosamine (cf. Fig. 4) had indicated that the initial stage of hydrolysis would be virtually complete in 3hr. and have reached approximately half this value in 40min.

Portions of a 5% solution of fraction III in 0·1N-sulphuric acid were heated at 60° for 40min. and 3hr., cooled and neutralized with an equal volume of 0·1N-sodium hydroxide. The concentration of the hydrolysates was now 5mg./0·2ml. and a portion of each was diluted to 2mg./0·2ml. A series of tubes was then set up in duplicate containing 2mg. of material previously heated for 40min., 2mg. heated for 3hr., 5mg. heated for 40min. and 5mg. heated for 3hr. Water (0·1ml.), enzyme (6mg./0·6ml. of 0·2M-acetate buffer, pH5·4) and a drop of toluene were added, and the mixture was incubated at 37° for 16hr. An equal volume of 20% trichloroacetic acid was then added and after standing for 4hr. at 2° the solution was centrifuged at 10000*g* for 15min. Phosphate was estimated on 1·5ml. of the supernatant. Control experiments consisted of tubes to which trichloroacetic acid was added before the enzyme and tubes to which 2μg. of phosphorus (in 0·1ml.) were added followed by trichloroacetic acid and enzyme. Extinctions were read at 820mμ against a reagent blank, and are given in Table 1. The extinction for the control

Table 1. *Action of phosphatase on the polysaccharide–mucopeptide complex after mild acid hydrolysis*

The preparation (fraction III from *L. casei* strain NIRD RO94) was hydrolysed for 40min. and 3hr. at 60° in 0·1N-H_2SO_4. After neutralization, solutions containing 2 and 5mg. of material were incubated with 6mg. of enzyme at pH5·4 and 37° for 16hr. Appropriate controls and internal phosphate standards were also included. Experimental details are given in the text. The amount of inorganic phosphate present was estimated by the method of Chen *et al.* (1956).

Experiment	$E_{820m\mu}$ Hydrolysed for 40min.	$E_{820m\mu}$ Hydrolysed for 3hr.
Control (2mg. of fraction III)	0·13	—
Control (5mg. of fraction III)	—	0·12
Enzyme+2mg. of fraction III	0·23, 0·24	0·30, 0·30
Enzyme+5mg. of fraction III	0·27, 0·28	0·41, 0·42
2μg. of P added to 2mg. control	0·41	0·41
2μg. of P added to 5mg. control	0·35	0·35

(equivalent to 0·015% of phosphorus in the enzyme preparation) has been subtracted from the experimental results in determining the extent of hydrolysis of phosphomonoester linkages. From 5mg. of material that had been subjected to preliminary acid hydrolysis for 40min. the enzyme released 15% of the total phosphorus, and 30% of the total phosphorus from material that had undergone acid hydrolysis for 3hr. From 2mg. of material the values were 21 and 37% of the total phosphorus content respectively; on repeating the experiment the amount of phosphorus released from 2mg. of material that had undergone acid hydrolysis for 3hr. was 43%. The maximum amount of inorganic phosphate released by ox-spleen phosphatase from material hydrolysed for 3hr. was 29%.

Results with other strains of L. casei

Rate of acid hydrolysis of cell-wall products. The rates of acid hydrolysis of preparations of fraction II from other strains of *L. casei* have been compared with results for strain NIRD RO94. The new strains examined were NCTC 6375 (serological group C), NIRD H831 (group B) and NIRD DECP (group B). Solutions of the preparations in 0·1N-sulphuric acid (1mg./0·2ml.) were heated at 60° for periods up to 5hr. and the rates of hydrolysis of *N*-acetylhexosaminide linkages followed. In each instance there was a rapid release within 3hr. of terminal reducing *N*-acetylhexosamine, similar to

that shown previously for strain NIRD RO 94 (Fig. 2). The studies with strain NIRD RO 94 had shown that heating for 8 days at 60° was required to reach the ultimate maximum value. To attain the maximum more quickly a series of tubes that had been heated at 60° for 5 hr. was then heated at 100°; this resulted in a rapid release of reactive *N*-acetylhexosamine, the maximum being reached in 3 hr. For each of the group C strains (NIRD RO 94 and NCTC 6375) the maximum value at 100° was approx. 3·7 times the value reached at 60° in 3 hr.; for the group B strains (NIRD H 831 and DECP) the maximum value at 100° was approx. 2·2 times the value at 60°. When fraction I from strain NCTC 6375 was heated in 0·1N-sulphuric acid for 3 hr. at 60° no reactive *N*-acetylhexosamine was detectable.

The cell wall of *L. casei* var. *rhamnosus* contains two polysaccharide components (Knox, 1963; Hall & Knox, 1965), one of which contains rhamnose, glucose and galactose, the other containing glucose, galactose and hexosamine. The rate of release of the two polysaccharide components on heating cell wall in dilute acid has been followed by determining the amount of soluble rhamnose and hexosamine. The cell-wall preparation (150 mg.) was suspended in 30 ml. of 0·1N-sulphuric acid and 3 ml. portions were heated at 60° with intermittent shaking. Tubes were removed after suitable intervals up to 6 hr. and then after 22 hr., cooled and centrifuged and the supernatants removed. For the determination of total hexosamine a portion of the solution was hydrolysed in 4N-hydrochloric acid at 100° for 1 hr. The rates of release of rhamnose and hexosamine were similar, approx. 50% of the total being in the supernatant after 1 hr., and 98% of the total after 6 hr.

Action of phosphatase. The action of wheat-germ acid phosphatase on the purified fraction II from *L. casei* NIRD DECP has also been studied. After hydrolysis of 2 mg. amounts for 3 hr. at 60° in 0·1N-sulphuric acid, wheat-germ acid phosphatase released 45% of the total phosphorus as inorganic phosphate.

A preparation of fraction I from *L. casei* var. *rhamnosus* NCTC 6375 (Knox, 1963) contained 0·08% of phosphorus. After hydrolysis of 2 mg. amounts for 3 hr. at 60° in 0·1N-sulphuric acid, wheat-germ acid phosphatase released 48% of the total phosphorus as inorganic phosphate; ox-spleen phosphatase released 60% of the total phosphorus.

Examination of other conditions of acid hydrolysis of cell wall

Heating at 100° *and* pH 2·0. For extracting serologically reactive material from strains of *L. casei*, Sharpe (1955) heated cells at 100° and pH 2·0 for 15 min. (cf. Lancefield, 1933). Cell-wall preparations from strains NIRD RO 94, NIRD H 831 and NCTC 6375 were heated at 100° in hydrochloric acid, pH 2·0, for appropriate intervals and the amounts of soluble carbohydrate estimated. The results indicated that 50–60% of the cell-wall polysaccharide was soluble after 15 min., and approx. 90% after 1 hr.

Heating at 60° *in* 10% (*w/v*) *trichloroacetic acid.* Strominger & Ghuysen (1963) heated preparations of cell wall from *Staphylococcus aureus* for periods up to 5 hr. in 10% trichloroacetic acid and obtained evidence for the hydrolysis of a phosphodiester linkage between teichoic acid and mucopeptide. When preparations of cell wall from strain NIRD RO 94 were heated under these conditions the polysaccharide component was completely released within 2 hr.

Extraction with sodium hydroxide followed by trichloroacetic acid. Ikawa (1961) extracted the cell wall of a strain of *L. casei* (ATCC 7469) with 0·1N-sodium hydroxide at 25° and subsequently heated both the residue and extract in 5% (w/v) trichloroacetic acid for 15 min. at 90°. From the results obtained it was concluded that the cell wall contained two polysaccharide components, one containing rhamnose, glucose and hexosamine (fraction VIII) and the other containing rhamnose and glucose (fraction X). The procedure of Ikawa (1961) has been used for extracting products from the cell wall of three strains of *L. casei*: strain 55 (Knox & Brandsen, 1962), NIRD RO 94 and NCTC 6375. [Sharpe (1955) states that strain NCTC 6375 is identical with strain ATCC 7469.] Fractions VIII and X were obtained from each strain and analysed for rhamnose, glucose and hexosamine. For strain NCTC 6375 the amounts of rhamnose, glucose and hexosamine in fraction VIII were 40, 23 and 5·2%, and for fraction X were 31, 25 and 3·0% respectively. The hexosamine contents of fractions VIII and X from strain NIRD RO 94 were 19 and 5·2% respectively, and from strain 55 were 8·5 and 27% respectively. Thus, in contrast with the conclusion of Ikawa (1961), each preparation of fraction X contains hexosamine.

DISCUSSION

Evidence has been obtained that the cell-wall polysaccharides of *L. casei* are joined through their reducing end groups to the mucopeptide component. Mild conditions of acid hydrolysis are sufficient to hydrolyse the linkage in the wall and yield soluble polysaccharides without causing detectable hydrolysis of the mucopeptide, which remains insoluble. This procedure has been employed for preparing the polysaccharide and mucopeptide components of the cell wall from strains of *L. casei* and these

results are described in the succeeding paper (Hall & Knox, 1965).

The release of polysaccharide from the cell wall is accompanied by the hydrolysis of an *N*-acetylhexosaminide linkage, as a terminal reducing *N*-acetylhexosamine residue can now be detected. Heating of soluble cell-wall products (fractions II and III) also results in the rapid appearance of terminal reducing *N*-acetylhexosamine, presumably due to the hydrolysis of the linkage between the polysaccharide and mucopeptide components. From the rate of chromogen formation from the *N*-acetylhexosamine residue, it is also concluded that the *N*-acetylhexosamine is substituted on the $C_{(3)}$-position by the adjacent sugar. A disaccharide with such a substituent (namely *O*-β-D-glucopyranosyl-(1→3)-*N*-acetyl-D-galactosamine) has been isolated from the products of acid hydrolysis of the polysaccharide from *L. casei* of serological group C (Knox & Hall, 1965), and evidence has been obtained that the terminal reducing hexosamine of the polysaccharide is galactosamine (Hall & Knox, 1965).

The initial rate of release of reactive *N*-acetylhexosamine from fraction III is comparable with the rate of hydrolysis of glucose 1-phosphate, suggesting that the terminal reducing *N*-acetylhexosamine residue is substituted by a phosphate grouping on $C_{(1)}$. This rapid release of reactive *N*-acetylhexosamine is complete in 3–4hr. at 60° in 0·1N-acid, and is followed by a much slower release over several days at 60° (or 3hr. at 100°), presumably due to the hydrolysis of *N*-acetylhexosaminide linkages within the polysaccharide. The products of acid hydrolysis include *N*-acetylglucosamine, *N*-acetylgalactosamine and *O*-β-D-galactopyranosyl-(1→6)-*N*-acetyl-D-glucosamine as well as *O*-β-D-glucopyranosyl-(1→3)-*N*-acetyl-D-galactosamine (Knox & Hall, 1965), but, under the conditions employed for forming chromogen, products other than $C_{(3)}$-substituted *N*-acetylhexosamine would yield relatively small amounts.

A comparison has been made between the rates of release of reactive *N*-acetylhexosamine from strains of *L. casei* belonging to both serological groups. In each case the amount of chromogen formed in the initial stage is similar, though during the second stage less chromogen is formed from strains of *L. casei* of group B. This is not unexpected as polysaccharides from organisms of groups B and C differ considerably in composition (Knox, 1963). However, the results do suggest that the polysaccharide from group B organisms also contains *N*-acetylhexosamine residues substituted on $C_{(3)}$ by the adjacent sugar, and evidence has been obtained that the products of mild acid hydrolysis include a disaccharide with such a substituent (Hall & Knox, 1965).

The cell wall of *L. casei* var. *rhamnosus* contains two serologically distinct products, one of which contains rhamnose, glucose, galactose and a small amount of hexosamine (fraction I), and the other containing glucose, galactose and hexosamine (fraction II) (Knox, 1963). When the cell wall is heated in acid, both polysaccharides are released at approximately the same rate, suggesting that the rhamnose-containing polysaccharide is also substituted on the $C_{(1)}$-position of the terminal sugar by a phosphate grouping. However, when fraction I, which contains this polysaccharide component, is heated in acid, the products of hydrolysis do not contain a reactive *N*-acetylhexosamine. Studies on the isolated rhamnose-containing polysaccharide confirm the absence of a terminal $C_{(3)}$-substituted *N*-acetylhexosamine (Hall & Knox, 1965).

Cell-wall preparations contain approx. 0·2% of phosphorus, and similar low values have been found for wall preparations from 12 species (Archibald, Armstrong, Baddiley & Hay, 1961). These species lacked teichoic acid as a cell-wall component, although *L. casei* does contain intracellular glycerol teichoic acid (Kelemen & Baddiley, 1961). Purified soluble cell-wall products from strain NIRD RO 94 (fraction III) also contain phosphorus, but as the preparation is free from nucleic acid and glycerol it is unlikely that the phosphorus is a contaminant. It is possible that the phosphorus is present as phosphorylmuramic acid, a compound isolated from *L. casei* by Ågren & de Verdier (1958).

When the cell-wall preparation from strain NIRD RO 94 was heated for 6hr. in dilute acid, the residue contained all the muramic acid and phosphate originally present in the cell wall, but was devoid of the hexose components. The residue is therefore considered to be the mucopeptide component of the wall. As the rate of release of the reducing end group of the polysaccharide was comparable with the rate of hydrolysis of glucose 1-phosphate, and as the mucopeptide component contained phosphorus, it was considered probable that the polysaccharide and mucopeptide components were joined through a phosphate grouping. Evidence for such a linkage has been obtained from a study of the action of phosphatases. Wheat-germ acid phosphatase had no detectable action on fraction III from strain NIRD RO 94, whereas the enzyme released approx. 40% of the phosphate as inorganic phosphate after the linkage between the polysaccharide and mucopeptide components had been hydrolysed by acid. Further, after acid hydrolysis for a shorter period, which was expected to release only half of the polysaccharide, a correspondingly smaller amount of phosphate was released by the enzyme. Wheat-germ acid phosphatase had a similar effect on the products of mild

acid hydrolysis from fraction II of strain NIRD DECP and fraction I from strain NCTC 6375. On the other hand, ox-spleen phosphoprotein phosphatase was less effective on fraction III from strain NIRD RO 94 but more effective on fraction I from strain NCTC 6375.

The action of phosphatases on fraction III after mild acid hydrolysis contributes added evidence for a phosphate linkage between the polysaccharide and mucopeptide. The wheat-germ preparation contains an orthophosphoric monoester phosphohydrolase, so that its action on the presumptive phosphate linkage to the mucopeptide provides evidence of a phosphomonoester. The fact that only 40% of the phosphorus is released by this enzyme could be due to the presence of another type of phosphate linkage, or alternatively could be accounted for by differences in substrate specificity. The action of ox-spleen phosphoprotein phosphatase, which does not act on mononucleotides or glycerol phosphate (Hofman, 1958), renders it unlikely that nucleic acids and teichoic acids are present in the mucopeptide fraction. This enzyme is very active towards phosphate residues in compounds of higher molecular weight (phosphorylated proteins and peptides), and compounds in which the phosphate group is esterified with a phenolic hydroxyl group (Hofman, 1958; Glomset, 1959). As both enzymes tested showed activity towards the phosphorylated compound, it is probably that the linkage of phosphate to mucopeptide is by a phosphomonoester group.

The ease of hydrolysis of the linkage between the reducing end group of the cell-wall polysaccharide and the phosphorylated mucopeptide accounts for the efficacy of Lancefield's (1933) method for obtaining serologically active extracts from *L. casei*. Such a linkage may also occur in other species that contain a polysaccharide as a component of the cell wall, and where classification has depended on the use of mild acid-extraction techniques.

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