The Isolation of Oligosaccharides from the Cell-Wall Polysaccharide of *Lactobacillus casei*, Serological Group C

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1. A number of disaccharides and oligosaccharides have been isolated from the products of mild acid hydrolysis of the specific substance from *Lactobacillus casei*, serological group C. 2. The major disaccharide is *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosamine (*B*4) and evidence is presented for the structure of a tetrasaccharide composed of *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-galactose (*B*1) joined through its reducing end group to *B*4. 3. Disaccharide *B*1 is also a component of a trisaccharide *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- β -D-galactopyranosyl-(1 $\rightarrow$ 6)-*N*-acetyl-D-glucosamine (*A*7). 4. A number of other oligosaccharides have been shown to be related structurally. 5. The ability of certain of the oligosaccharides to inhibit the precipitin reaction has been studied. The disaccharide *B*1 is more effective as an inhibitor than gentiobiose and the trisaccharide *A*7 is considerably more effective than *B*1. 6. These results have been compared with those obtained previously for the composition of the cell wall.

Strains of lactobacilli can be divided into a number of serological groups (Sharpe, 1955; Sharpe & Wheeler, 1957). The characteristic components of the cell wall of Gram-positive bacteria include polysaccharides, teichoic acids and mucopeptides, and the results of Baddiley & Davison (1961) suggest that the teichoic acids of the cell wall participate in the serological reactions of many strains of lactobacilli. Organisms classified as *Lactobacillus casei* belong to one of two serological groups, designated B and C (Sharpe & Wheeler, 1957), but as teichoic acids are not present as components of the cell wall of *L. casei* (Baddiley & Davison, 1961) another component must be associated with serological specificity.

Previous studies have shown that the cell wall of *L. casei* contains a polysaccharide component the composition of which is related to the serological grouping of the strain (Knox, 1963; Glastonbury & Knox, 1963). The group-specific product from strains of *L. casei* in serological group C contains considerable amounts of glucose and galactose, whereas rhamnose is the major component of the specific product from strains in group B.

The present paper is concerned with the serological specificity of the product from group C strains. A number of oligosaccharides have been isolated from the products of mild acid hydrolysis, and evidence for their structure has been obtained. One trisaccharide is a very effective inhibitor of the precipitin reaction, and evidence is presented that

its structure is *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- β -D-galactopyranosyl-(1 $\rightarrow$ 6)-*N*-acetyl-D-glucosamine. In addition, there is a series of oligosaccharides which possess a terminal *N*-acetylgalactosamine residue with the adjacent sugar joined to C₍₃₎ of the galactosamine.

MATERIALS AND METHODS

Group-specific substance. Cultures of *L. casei* were obtained from the National Collection of Type Cultures, Colindale, London, and the National Institute for Research in Dairying, Reading, and were designated NCTC 6375 and NIRD R094 respectively. Organisms were grown in the medium described by Ågren & de Verdier (1958). The procedures for preparing cell wall and the subsequent isolation of the specific products (designated fraction II) have been described (Knox, 1963). The material contains glucose, galactose and galactosamine in addition to the components of the mucopeptide. Most of the studies have been carried out on material isolated from strain NIRD R094. Antiserum to strain NIRD R094 was prepared by the procedure previously described (Knox & Brandsen, 1962).

Carbohydrates. *O*- β -D-Glucopyranosyl-(1 $\rightarrow$ 6)-D-galactose was prepared enzymically by the procedure described by Knox (1965). Dr W. M. Watkins, Lister Institute of Preventive Medicine, London, kindly provided samples of the α - and β -anomers of D-galactopyranosyl-(1 $\rightarrow$ 6)-*N*-acetyl-D-glucosamine; the β -anomer had been prepared by Professor R. Kuhn (Kuhn, Baer & Gauhe, 1955).

Enzymes. Almond emulsin was purified by acetone fractionation followed by ammonium sulphate precipitation,

as described by Edman & Jorpes (1941). The enzyme preparation had β -glucosidase and β -galactosidase activity but no detectable α -glucosidase or α -galactosidase activity.

The enzyme preparation from *Aspergillus niger* E19 (Rohm and Haas Co., Philadelphia 5, Pa., U.S.A.) had been further purified by Krishna Murti & Stone (1961) and was kindly provided by Dr B. A. Stone, University of Melbourne, Australia. The enzyme preparation had α - and β -glucosidase activity and α - and β -galactosidase activity. In testing the glycosidases of almond emulsin and *A. niger*, the disaccharides used were maltose, cellobiose, melibiose and lactose.

α -Galactosidase from *Streptococcus bovis* was kindly provided by Dr G. J. Walker of this Institute. The properties of the enzyme have been described by Bailey (1963).

Glucose oxidase (pure) and horse-radish peroxidase were obtained from Nutritional Biochemical Corp., Ohio, U.S.A. Galactose oxidase was prepared from *Dactylium dendroides* (previously called *Polyporus circinatus*) by the method of Avigad, Amaral, Asensio & Horecker (1962); a culture of the organism was kindly supplied by Dr B. L. Horecker, New York University School of Medicine.

Column chromatography. Sephadex G-25 was obtained from Pharmacia, Uppsala, Sweden, acid-washed charcoal from British Drug Houses Ltd., Poole, Dorset, and Celite 535 from Johns-Manville Co., Calif., U.S.A.

Paper chromatography. The following solvent systems were used: A, butan-1-ol-pyridine-water (6:4:3, by vol.); B, ethyl acetate-pyridine-water (10:4:3, by vol.); C, pentan-1-ol-dioxan-water-acetic acid (43:34:23:0.6, by vol.) (Watkins, 1958); D, butan-2-one-acetic acid-water saturated with boric acid (9:1:1, by vol.) (Rees & Reynolds, 1958). Chromatograms were run in a descending direction on Whatman no. 3MM paper. Where components were to be eluted from the chromatograms, the sheets were previously soaked in 1% (v/v) acetic acid, washed with water until the washings were neutral, and then dried.

Sugars were detected with alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950), and hexosamines with alkaline acetylacetone followed by Ehrlich's reagent (Partridge, 1948). For *N*-acetylhexosamine and oligosaccharides containing *N*-acetylhexosamine, acetylacetone was omitted from the alkaline reagent, and the colours obtained on spraying with Ehrlich's reagent were allowed to develop at room temperature.

Paper electrophoresis. Electrophoretic mobilities were determined on strips (64 cm. $\times$ 16 cm.) of Whatman no. 3MM paper between horizontal glass plates, 1.4 cm. thick, in 0.05 M-sodium tetraborate (Frahn & Mills, 1959). A potential difference of 800–900 v was maintained for 5 hr.; cooling was achieved by carrying out the experiment in a refrigerator. Carbohydrates were detected with alkaline silver nitrate.

In addition to employing electrophoresis as a criterion of purity, electrophoretic mobilities may also provide evidence on the linkage between the component sugars. Foster (1953) has shown that, when two D-glucose residues are joined by a (1 $\rightarrow$ 2) or (1 $\rightarrow$ 4)-linkage, the M_R value is much lower than when the linkage is (1 $\rightarrow$ 3) or (1 $\rightarrow$ 6). As the point of attachment of the remainder of the molecule to the reducing moiety is the most important structural feature in determining electrophoretic mobility, these observations would probably apply to disaccharides containing other sugars and to oligosaccharides in general (Foster, 1957).

Analytical methods. Hexosamine was estimated by the method of Rondle & Morgan (1955), and glucose by the method of Huggett & Nixon (1957); the modifications described by Knox & Brandsen (1962) were employed. D-Galactose was determined with galactose oxidase (Avigad *et al.* 1962). An indication of the hexose content of eluates was obtained by the primary cysteine-sulphuric acid reaction (Dische, 1953), in which galactose gives 60% of the colour of an equal weight of glucose; the secondary cysteine-sulphuric acid reaction enabled the detection of galactose.

Formaldehyde determination. The procedure described by Kabat (1961b) has been followed for the determination of the yield of formaldehyde on periodate oxidation of carbohydrates. A solution containing 3 μ moles of carbohydrate and 40 μ moles of sodium metaperiodate in 5 ml. of water was allowed to stand in the dark at room temperature (20°); periodate consumption was followed spectrophotometrically (at 225 m μ). The formaldehyde content of a portion of the solution was determined at appropriate intervals by the chromotropic acid method. The method was standardized by periodate oxidation of serine.

Borohydride reduction. For reduction of oligosaccharides before periodate oxidation, a sample containing 6 μ moles/ml. was reduced with 1 ml. of a freshly prepared ice-cold solution containing 50 mg. of sodium borohydride (tetrahydroborate). After 4 hr. at room temperature, the solution was acidified with 0.1 vol. of 4N-hydrochloric acid (Kabat, 1961b) and a sample taken for periodate oxidation. A similar procedure was followed for the reduction of oligosaccharides where the terminal alcohol was to be subsequently detected by paper chromatography. In these cases the acidified solution was passed through a short column of Bio-Deminolit (CO₃²⁻ form) (The Permutit Co Ltd.), and borate removed by the repeated addition and evaporation (*in vacuo* at 35°) of methanol.

RESULTS

Acid hydrolysis of specific substance

After preliminary experiments on the rate of liberation of glucose from the specific substance by dilute acids, hydrolysis for 6 hr. at 100° in 0.1 N-sulphuric acid was chosen as the most suitable procedure; 26% of the total glucose content was present as monosaccharide after this period. Other conditions examined included hydrolysis at 80° with polystyrenesulphonic acid (Painter, 1960). This procedure was not satisfactory as short-chain oligosaccharides comprised only a very small proportion of the products diffusible through Visking dialysis tubing (size 18/32).

The specific substance (2.0 g. of fraction II from *L. casei* NIRD RO94) was dissolved in 200 ml. of 0.1 N-sulphuric acid and heated under reflux in a boiling-water bath for 6 hr. The solution was cooled, neutralized with a saturated solution of barium hydroxide and centrifuged. The supernatant was concentrated *in vacuo* at 35°, applied to a column containing 125 g. of Sephadex G-25 (35 cm. $\times$ 4 cm.) and fractions (7 ml.) were eluted

with water. The oligosaccharide content of certain of the eluates was estimated by applying 0.1 ml. to a column containing 75 mg. of charcoal and 75 mg. of Celite 535, and eluting with water containing increasing amounts of ethanol (Whistler & Durso, 1950). From the analyses of these eluates for total hexose by the primary cysteine-sulphuric acid reaction (Dische, 1953), an indication was obtained of the rate of elution of oligosaccharides from Sephadex G-25. Fractions containing appreciable amounts of sugars eluted by 5% and 15% ethanol were pooled. The remainder of the eluates (except those containing only monosaccharides) were pooled, concentrated *in vacuo*, heated in 0.1 N-sulphuric acid (total volume 100 ml.) at 100° for a further 2 hr. period, and neutralized as described above.

The combined concentrated solutions were applied to a column containing 25 g. of charcoal and 25 g. of Celite 535, and fractions eluted with 500 ml. of 1% (v/v) ethanol, 700 ml. of 5% (v/v) ethanol, 1200 ml. of 15% (v/v) ethanol and 1000 ml. of 30% (v/v) ethanol. Preliminary experiments had indicated that only small amounts of carbohydrate were eluted by intermediate concentrations of ethanol; as determined by the primary cysteine-sulphuric acid reaction, the greatest amount of oligosaccharide was eluted by 15% ethanol.

Eluates were concentrated *in vacuo* and examined by paper chromatography. The 1% ethanol eluate contained glucose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine. Chromatography of the 5% ethanol eluate in solvent *B*, followed by development with alkaline silver nitrate, showed four distinct components. In order of increasing chromatographic mobility (relative to glucose) these components were designated *B*1 (R_{Glc} 0.37), *B*2 (0.47), *B*3 (0.54) and *B*4 (0.62); *B*1 and *B*4 were the major components; *B*2 and *B*4 gave a positive reaction for *N*-acetylhexosamine.

Chromatography of the 15% ethanol eluate in solvent *B* for 4 days, followed by development with alkaline silver nitrate, indicated eight components. Chromatographic mobilities were determined by comparison with *B*1 and from these the R_{Glc} values were calculated. In order of increasing mobilities the oligosaccharides were designated *A*1 (R_{Glc} 0.04), *A*2 (0.06), *A*3 (0.09), *A*4 (0.12), *A*5 (0.14), *A*6 (0.17), *A*7 (0.19) and *A*8 (0.23). All except *A*3 and *A*6 gave a positive reaction for *N*-acetylhexosamine.

The 5% and 15% ethanol eluates were chromatographed (separately) as bands on sheets of washed Whatman no. 3MM paper, and the oligosaccharide components eluted with water. Eluates from each paper were examined for chromatographic purity before being pooled with other eluates of the same oligosaccharide; each pooled eluate was passed

through a short column of Bio-Deminrolit (CO_3^{2-} form).

From an estimation of the hexose and hexosamine contents of the fractions, together with information subsequently obtained on the component sugars, the approximate yields of the oligosaccharides have been determined: *B*1, 10 mg.; *B*2, 3 mg.; *B*3, 3 mg.; *B*4, 21 mg.; *A*1, 7 mg.; *A*2, 10 mg.; *A*3, 3 mg.; *A*4, 7 mg.; *A*5, 2 mg.; *A*6, 1.5 mg.; *A*7, 2.5 mg.; *A*8, 2 mg.

Interrelationships of the oligosaccharides

A study of the products obtained after the action of glycosidases, dilute acid and pyridine has shown that a number of the oligosaccharides are inter-related.

Properties of *A*4. The oligosaccharide *A*4 moved as a single component on chromatography (R_{Glc} 0.12 in solvent *B*) and on electrophoresis in borate (M_{G} 0.33). Component sugars detected on acid hydrolysis (0.5 N-hydrochloric acid for 16 hr. at 100°) were glucose, galactose and galactosamine. The preparation (500 μg .) was partially hydrolysed with 0.33 N-sulphuric acid at 100° for 30 min. After treatment with Bio-Deminrolit (CO_3^{2-} form) the hydrolysate was examined in solvent *B*; products indistinguishable from *A*6, *A*8, *B*1 and *B*4 were detected.

A 0.2% solution of *A*4 (0.1 ml.) was incubated with an equal volume of 1% (w/v) of almond emulsin in 0.01 M-acetate buffer, pH 5.0, at 37° for 18 hr. The products of enzyme action were glucose and a component chromatographically identical with *A*8 (in solvent *B*). A further portion of *A*4 (0.1 ml. of 0.2% solution) was heated with an equal volume of 0.2 N-pyridine at 65° for 30 min. (Painter, Cheese & Morgan, 1962). The products obtained were free *N*-acetylhexosamine chromogen (R_F 0.64 in solvent *A*) and a component indistinguishable from *A*6 in solvent *B*.

Properties of *A*6. The oligosaccharide moved as a single component on chromatography (R_{Glc} 0.17 in solvent *B*) and on electrophoresis in borate (M_{G} 0.65). The component sugars detected on acid hydrolysis were glucose and galactose. A solution of *A*6 (1 ml. containing 250 μg .) was reduced with sodium borohydride. After removal of borate, the residue was heated in 0.33 N-sulphuric acid for 30 min. at 100° and examined in solvent *B*; a disaccharide chromatographically identical with *B*1 was present. Incubation of *A*6 with almond emulsin under the conditions described above yielded glucose and a component designated *B*5 which could be distinguished from *B*1. This disaccharide (R_{Glc} 0.40 in solvent *B*) was also obtained from *A*8 by heating in pyridine (see below). With the *A*.

niger enzyme under the same conditions the products were glucose and galactose, glucose being the major component.

The terminal linkage of *A6* has been investigated by estimating the products of periodate oxidation before and after reduction with sodium borohydride. The yields of formaldehyde were 0.05 mole/mole of trisaccharide before reduction and 0.8 mole/mole after reduction. A hexose substituted on either C₍₂₎ or C₍₆₎ would yield 1 mole of formaldehyde after reduction. To distinguish between these possibilities, the triphenyltetrazolium reaction of Wallenfels (1950) was used, as modified by Schiffman, Kabat & Leskowitz (1960). The trisaccharide formed a red formazan, thus eliminating the possibility of a (1→2)-linkage.

Properties of *A8*. The oligosaccharide moved as a single component on chromatography (R_{Glc} 0.23 in solvent *B*) and on electrophoresis in borate (M_{G} 0.27). The component sugars detected on acid hydrolysis were glucose, galactose and galactosamine. On heating *A8* in pyridine under the conditions described above the products obtained were free chromogen (R_{F} 0.64 in solvent *A*) and the disaccharide *B5* (R_{Glc} 0.40 in solvent *B*). *A8* is not hydrolysed by almond emulsin; it does undergo partial hydrolysis with *A. niger* enzyme, yielding glucose as the major component and smaller amounts of the other component sugars.

The interrelationships of the oligosaccharides *A4*, *A6* and *A8* and the three disaccharides *B1*, *B4* and *B5* are shown in Scheme 1. The results indicate that *A4* is a tetrasaccharide and that *A6* and *A8* are trisaccharides. The component sugars obtained on acid hydrolysis of *B1* and *B5* were glucose and galactose, and on hydrolysis of *B4* were glucose and galactosamine. Further evidence for these monosaccharide components is provided below. Evidence

will also be presented that *B4* contains a terminal alkali-labile *N*-acetylhexosamine residue, the same structure as is present in *A4* and *A8*.

Properties of *A7*. The oligosaccharide moved as a single component on chromatography (R_{Glc} 0.19 in solvent *B*) and on electrophoresis in borate (M_{G} 0.29). A solution of *A7* (1 mg./ml.) was examined by the same procedure as described for *A4*. Partial acid hydrolysis (0.33 *N*-sulphuric acid for 30 min. at 100°) yielded two disaccharides, one of which was indistinguishable from *B1* on electrophoresis and chromatography (in solvent *B*). The other disaccharide was indistinguishable from *B2* on electrophoresis and chromatography (in solvent *B*), and also gave a positive reaction for *N*-acetylhexosamine. The components obtained on complete acid hydrolysis of *A7* were glucose, galactose and glucosamine. Almond-emulsin β -glycosidase gave no detectable hydrolysis. However, under comparable conditions, the enzyme preparation from *A. niger* hydrolyses *A7* to *B2* and glucose. The component *B1* has been shown to be *O*- β -D-glucopyranosyl-(1→6)-D-galactose (see below) and further confirmation that *B1* is a component of the trisaccharide has been obtained by studying the action of almond-emulsin β -glycosidase on the products of mild acid hydrolysis. The neutralized solution was incubated with the enzyme preparation at 37° for 16 hr. at pH 5.0, and the products of enzyme action were detected by electrophoresis and chromatography in solvent *B*; the *B1* component was no longer detectable whereas the amounts of glucose and galactose had increased. These results are summarized in Scheme 1.

Properties of *A2* and *A3*. The component *A2* had R_{Glc} 0.06 (in solvent *B*) and M_{G} 0.27.

On heating in 0.1 *N*-pyridine for 30 min. at 65°, *A2* yielded a product chromatographically identical with *A3* (R_{Glc} 0.09 in solvent *B*). On partial acid hydrolysis of *A2* (0.33 *N*-sulphuric acid for 30 min. at 100°) components chromatographically identical with *A4* and *A7* were obtained, in addition to products previously obtained from *A4* and *A7*. The component sugars of *A2* are glucose, galactose, glucosamine and galactosamine, and of *A3*, glucose, galactose and glucosamine. These results suggest that *A2* is a heptasaccharide in which *A7* is joined to *A4*, the terminal unit being the alkali-labile galactosamine of the *A4* component (Scheme 1). It would then follow that *A3* is *A7* joined to *A6* containing three residues of glucose, two of galactose and one of *N*-acetylglucosamine. For such a compound, containing a terminal glucose residue, reduction with borohydride would decrease the colour produced in the primary cysteine-sulphuric acid reaction to 76% of the value for the unreduced oligosaccharide (*N*-acetylglucosamine does not react and galactose gives 60% of the colour

Scheme 1. Interrelation of the heptasaccharide (?) *A2*, hexasaccharide (?) *A3*, tetrasaccharide *A4*, trisaccharides *A6*, *A7*, *A8*, and the disaccharides *B1*, *B2*, *B4*, *B5*.

of an equivalent weight of glucose). A solution of A3 (0.1 ml.) was mixed with an equal volume of 5% sodium borohydride and allowed to stand at room temperature for 4 hr. In duplicate experiments, the glucose equivalent of 0.1 ml. of A3 decreased from 58.5 μ g. and 60.5 μ g. to 45.5 μ g. and 47.0 μ g. on reduction. This represents a mean decrease to 78% of the initial value and is consistent with the proposed structure. Evidence that glucose, and not galactose, had been reduced was obtained from the secondary cysteine-sulphuric acid reaction (Dische, 1953), in which the extinction for galactose at 600 $m\mu$ is considerably greater than that for glucose. The ratio of the extinction at 600 $m\mu$ to that at 400 $m\mu$ increased from 0.59:1 before reduction to 0.64:1 after reduction.

Effect of hot dilute pyridine on A1 and A5. On heating in 0.1 N-pyridine at 65° for 30 min., A1 and A5 both yielded a faster-moving oligosaccharide which was not detectable among the products of mild acid hydrolysis of the cell-wall polysaccharide. These oligosaccharides have not been examined in detail.

Identification of disaccharides

Identification of B1 as O- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-galactose. Only one component was detected by chromatography in solvent A (R_{Glc} 0.46), solvent B (R_{Glc} 0.37) and on electrophoresis in borate (M_{G} 0.76). Acid hydrolysis followed by chromatography in solvent A showed the presence of glucose and galactose in approximately equal amounts; the disaccharide was also hydrolysed to these components by almond-emulsin β -glycosidase. The presence of equimolar amounts of glucose and galactose was indicated by the secondary cysteine-sulphuric acid reaction, and also by the analyses for the component sugars in an acid hydrolysate by the specific glucose and galactose oxidases; the reaction with these enzymes also indicates that each sugar was present as the D-isomer. Galactose oxidase did not act on the disaccharide.

Evidence for the terminal reducing sugar was obtained by reduction with sodium borohydride followed by hydrolysis in 0.5 N-hydrochloric acid for 1 hr. at 100°: chromatography in solvent D showed the presence of glucose and galactitol. In another similar experiment the estimation of glucose after hydrolysis of the reduced disaccharide gave 90% of the expected value.

A 4 ml. sample of B1 (containing 3 μ moles of disaccharide) was oxidized with 1 ml. of 0.04 M-sodium metaperiodate at room temperature. The rate of oxidation was followed spectrophotometrically, the amount of periodate consumed being 4.8 moles/mole of disaccharide after 2.5 hr.

Samples were removed for formaldehyde estimation, the maximum (0.1 mole/mole) being reached within 1 hr. In a comparable experiment in which the disaccharide had previously been reduced with sodium borohydride the yield of formaldehyde on oxidation for 1 hr. was 0.9 mole/mole of disaccharide. The yield of formaldehyde is consistent with the presence of a hexose substituted on either C₍₂₎ or C₍₆₎ by a pyranose residue. The presence of a (1 $\rightarrow$ 2)-linkage is excluded by the results of periodate consumption of the unreduced disaccharide, and by the formation of a red formazan (Schiffman *et al.* 1960).

The disaccharide is identical in chromatographic and electrophoretic mobility with O- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-galactose, which has been synthesized enzymically (Knox, 1965).

Identification of B2 as O- β -D-galactopyranosyl-(1 $\rightarrow$ 6)-N-acetyl-D-glucosamine. Only one component was detected on chromatography in solvent B (R_{Glc} 0.47), solvent C (R_{Glc} 0.62) and on electrophoresis in borate (M_{G} 0.31). Acid hydrolysis followed by chromatography in solvent A indicated the presence of galactose and glucosamine. The presence of galactose was confirmed by oxidation with galactose oxidase. The identification of glucosamine was supported by its conversion with ninhydrin into a pentose chromatographically identical in solvent D with arabinose (Stoffyn & Jeanloz, 1954).

When a solution of the disaccharide was heated in 0.05 N-sodium carbonate for 4 min. (Aminoff, Morgan & Watkins, 1952) the amount of chromogen formed was 97% of that expected from the calculated N-acetylglucosamine content. The yield of chromogen suggested that the disaccharide contained a terminal N-acetyl-D-glucosamine residue substituted on C₍₆₎ (cf. Jeanloz & Trémège, 1956); the preparation was therefore compared with authentic samples of the α - and β -anomers of D-galactosyl-(1 $\rightarrow$ 6)-N-acetyl-D-glucosamine. In solvent C, R_{Glc} values for the α - and β -anomers and B2 were 0.68, 0.62 and 0.62 respectively (cf. Watkins, 1958). Further, when a solution of B2 was heated in 0.1 N-pyridine at 65° for 4 hr. the chromogen complex which was formed was chromatographically identical in solvent A with that formed from the authentic disaccharide (Kuhn, Gauhe & Baer, 1954b).

Identification of B3 as O- β -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucose. The disaccharide was hydrolysed by acid and almond-emulsin β -glucosidase to yield D-glucose as the only component. On chromatography in solvent B (R_{Glc} 0.54) and electrophoresis in borate (M_{G} 0.23), the disaccharide was indistinguishable from authentic O- β -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucose (cellobiose).

Identification of B4 as O- β -D-glucopyranosyl-

(1→3)-*N*-acetyl-D-galactosamine. Only one component was detected by chromatography in solvent *B* (R_{Glc} 0.62) and solvent *C* (R_{Glc} 0.75) and on electrophoresis in borate (M_{G} 0.26); $[\alpha]_{\text{D}}^{20} + 21 \pm 2^\circ$ (c 0.6 in water). For the dihydrate of the disaccharide Wolfrom & Juliano (1960) report $[\alpha]_{\text{D}} + 19^\circ$ (final). Acid hydrolysis followed by chromatography in solvent *A* indicated the presence of glucose and galactosamine. The identification of galactosamine was supported by its conversion with ninhydrin into a pentose of the same mobility in solvent *D* as the pentose (i.e. lyxose) formed under the same conditions from authentic galactosamine (Stoffyn & Jeanloz, 1954). Further, the hexosamine component was oxidized with galactose oxidase (Avigad *et al.* 1962). Quantitative analysis of the component sugars gave a ratio of 1 mole of D-glucose (by the glucose oxidase method) to 0.90 mole of galactosamine (Rondle & Morgan, 1955).

When the disaccharide was heated in pyridine for 30 min. under the conditions described previously the products detected were glucose and free chromogen (R_{F} 0.64 in solvent *A*). Estimation of the *N*-acetylhexosamine content of the disaccharide by heating the preparation in 0.05 *N*-sodium carbonate for 4 min. at 100° (Aminoff *et al.* 1952) gave a value 3.0 times that expected from the calculated *N*-acetylgalactosamine content. Chromogen was also readily formed at room temperature in 0.05 *N*-sodium carbonate, the colour intensity after 30 min. being 23% of that obtained after 4 min. at 100°.

These reactions are characteristic for a hexosamine residue substituted on C₍₃₎ (Kuhn, Gauhe & Baer, 1954a), indicating that the disaccharide is glucosyl-(1→3)-*N*-acetyl-D-galactosamine; the low optical rotation suggests a β -linkage. As the disaccharide is the major product of mild acid hydrolysis of the cell-wall polysaccharide, it is assumed that glucose is present as the pyranose.

Identification of B5 as 6-O- α -D-galactopyranosyl-D-glucose. On the basis of the inter-relationship of the products obtained from A4, B5 is assumed to be galactosylglucose. A solution of A4 (1 mg./0.5 ml.) was incubated at 37° for 18 hr. with 0.1 ml. of 1% (w/v) almond emulsin in 0.01 *M*-acetate buffer, pH 5. After passage through a short column of Bio-Deminrolit (CO₃²⁻ form), the solution was concentrated and then heated in 0.1 *N*-pyridine for 1 hr. at 65°. The solution was applied as a band on a washed sheet of Whatman no. 3MM paper, the chromatogram developed in solvent *B* and the band corresponding to B5 eluted.

The product obtained by this procedure was hydrolysed by α -galactosidase (from *Streptococcus bovis*) to galactose and glucose. Further, it was indistinguishable from 6-*O*- α -D-galactopyranosyl-D-glucose (melibiose) by paper chromatography in

solvent *B* (R_{Glc} 0.40) and electrophoresis in borate (M_{G} 0.78).

Isolation of disaccharide components from group-specific substance of L. casei NCTC 6375

A preparation (800 mg.) of fraction II from strain NCTC 6375 was hydrolysed with 0.1 *N*-sulphuric acid under the same conditions as those employed for strain NIRD RO94. Disaccharides were isolated which had properties identical with B1 and B4.

Serology

The effectiveness of certain of the oligosaccharides in inhibiting the precipitin reaction between the specific substance (cell-wall fraction II) and homologous antiserum was studied. Glucose and gentiobiose were also included as previous experiments (Knox, 1963) had shown these to be the most effective monosaccharide and disaccharide then available. Table 1 shows the increasing effectiveness of A4, A7 and A2 as inhibitors of the precipitin reaction. For the experiment recorded in Table 1 enzymically synthesized *O*- β -D-glucopyranosyl-(1→6)-D-galactose was used. However, in a subsequent experiment in which B1 (duplicate amounts) was compared with gentiobiose, a similar result was achieved.

Table 1. *Inhibition of the precipitin reaction between the group-specific substance and antiserum by oligosaccharide components*

An appropriate amount of carbohydrate in a total volume of 0.6 ml. of 0.9% (w/v) NaCl was mixed with 0.2 ml. of antiserum to *L. casei* NIRD RO94. After 1 hr. at 37°, 10 μ g. of group-specific substance (fraction II) in 0.2 ml. of 0.9% (w/v) NaCl was added and the solutions were mixed. After further standing for 1 hr. at 37° and 2–3 days at 2° the amount of the antibody in the precipitate was determined by the method of Heidelberger & McPherson (1943). The degree of inhibition was determined by comparing the results with those obtained in the absence of added carbohydrate; the results represent the mean of triplicate experiments.

Amount of carbohydrate added (μ moles)	Percentage inhibition				
	25	5	0.5	0.1	0.01
D-Glucose	25	—	—	—	—
Gentiobiose	—	17	—	—	—
<i>O</i> - β -D-Glucosyl-(1→6)-D-galactose	—	28	—	—	—
Oligosaccharide A4	—	—	80	30	—
A7	—	—	—	76	22
A2	—	—	—	—	50

DISCUSSION

The soluble cell-wall products used in this investigation contain a polysaccharide and mucopeptide component. Acid hydrolysis showed the presence of glucose, galactose and galactosamine in addition to the expected components of the mucopeptide: glucosamine, muramic acid and amino acids (Knox, 1963). The component sugars of the disaccharides and oligosaccharides which have been isolated are glucose, galactose, glucosamine and galactosamine, and it is therefore concluded that glucosamine is a component of both the polysaccharide and mucopeptide moieties. This has subsequently been confirmed by analysing the polysaccharide obtained free from mucopeptide (E. A. Hall & K. W. Knox, unpublished work).

A characteristic feature of six of the eight oligosaccharides eluted with 15% ethanol is the presence of a terminal reducing *N*-acetylhexosamine, indicating a preferential acid hydrolysis of *N*-acetylhexosaminide linkages in the polysaccharide. Eight of the twelve hexosamine-containing oligosaccharides isolated from human blood-group substances also contain a terminal reducing *N*-acetylhexosamine residue (Rege, Painter, Watkins & Morgan, 1963). Wolf from & Juliano (1960) have observed the preferential hydrolysis of the *N*-acetylgalactosaminide linkage in reduced chondroitin, obtaining only one of the two possible disaccharides, namely *O*- β -D-glucosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosamine. In support of this observation they record that the rate of hydrolysis of methyl β -*N*-acetyl-D-glucopyranoside is 9–18 times that of methyl β -D-glucopyranoside.

For all of the hexosamine-containing oligosaccharides, except A7, the *N*-acetylhexosamine residue was readily degraded under mild alkaline conditions, indicating that the adjacent sugar is joined to C₃ of the hexosamine. Three of the oligosaccharides with this property (A2, A4 and A8) are related to one another and to three other oligosaccharides, A3, A6 and A7 (Scheme 1). Further, four of the five disaccharides identified are obtained on hydrolysis of these oligosaccharides, suggesting that the products examined represent a significant part of the cell-wall polysaccharide. The remaining disaccharide, B3, has been identified as cellobiose.

The major disaccharide is B4, identified as *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosamine. The only previous known report of this disaccharide is that of Wolf from & Juliano (1960), who obtained it from reduced chondroitin.

The isolation of *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-galactose (B1) from the cell wall is relevant to previous serological studies (Knox, 1963), which indicated that the polysaccharide contained a

glucosyl residue joined by a β -(1 $\rightarrow$ 6)-linkage to the adjacent sugar. *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-galactose was first described by Freudenberg, Wolf, Knopf & Zaheer (1928), and has been synthesized enzymically (Knox, 1965). The product isolated from the cell wall was indistinguishable in its properties from that prepared enzymically. D-Galactose oxidase had no detectable action on either preparation, whereas the enzyme oxidized the galactose component obtained on hydrolysis of the disaccharide. Avigad *et al.* (1962) isolated D-galacto-hexodialdose as the product of enzyme action on D-galactose, indicating oxidation of the C₆ hydroxyl group; the failure of the enzyme to oxidize the disaccharide is consistent with their conclusion.

The disaccharide B2 has been identified as *O*- β -D-galactopyranosyl-(1 $\rightarrow$ 6)-*N*-acetyl-D-glucosamine. The small amount of material available limited the number of confirmatory tests which could be performed but the preparation was chromatographically indistinguishable from an authentic sample of the disaccharide (Kuhn *et al.* 1955).

The disaccharide B5 is obtained on removing both the terminal non-reducing and reducing sugars from A4, and is therefore considered to be galactosylglucose. The action of α -galactosidase on the disaccharide and the chromatographic and electrophoretic properties suggest that the structure of B5 is *O*- α -D-galactopyranosyl-(1 $\rightarrow$ 6)-D-glucose. As described below, the studies on A6 also support this conclusion.

Chromatographic and electrophoretic evidence indicates that *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-galactose (B1) arises from two different oligosaccharides, A4 and A7. The preparation A7 gives B1 and *O*- β -D-galactopyranosyl-(1 $\rightarrow$ 6)-*N*-acetyl-D-glucosamine (B2) on mild acid hydrolysis, and glucose and B2 on hydrolysis with the *A. niger* enzyme preparation. On the basis of this evidence A7 is considered to be *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- β -D-galactopyranosyl-(1 $\rightarrow$ 6)-*N*-acetyl-D-glucosamine. Almond emulsin does not cause detectable hydrolysis of A7, although it does hydrolyse the B1 component obtained on mild acid hydrolysis of A7. The serological studies also provide evidence for a β -glucosidic linkage in A7.

The tetrasaccharide A4 yields three disaccharides, B1, B4 and B5, on partial hydrolysis. Thus the properties of A4 are consistent with this product being *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 6)-*O*- β -D-glucopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosamine. Removal of the glucosyl residue at the non-reducing end by β -glucosidase would then give A8, and of the terminal reducing *N*-acetylgalactosamine residue by heating in pyridine would give A6. A6 is therefore

Fig. 1. Proposed structures of the oligosaccharides.

considered to be *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 6)-D-glucose. The results of periodate oxidation support the conclusion that the galactosyl residue in A6 is joined to glucose by a (1 $\rightarrow$ 6)-linkage. The electrophoretic mobility of the trisaccharide is also consistent with such a structure.

Almond emulsin will hydrolyse the β -glucosidic linkage in A6 but not the galactosidic linkage, whereas the *A. niger* enzyme preparation will hydrolyse both linkages. The observation that the almond-emulsin preparation did not hydrolyse melibiose whereas the *A. niger* enzyme did hydrolyse melibiose supports the conclusion that an α -galactosidic linkage is present in A6. Such a linkage could also account for the observed differences in action of the two enzyme preparations on A8.

The conclusions regarding the structure of the oligosaccharides related to A4 and A7 are summarized in Fig. 1.

Partial hydrolysis of A2 yielded products chromatographically identical with A4 and A7, in addition to the hydrolysis products of A4 and A7. If it is assumed that A4 and A7 are obtained from the one oligosaccharide, then A2 is a heptasaccharide in which A7 is joined through its reducing end group to A4. Removal of the terminal *N*-acetyl-galactosamine residue of A2 yields A3, and the results of carbohydrate determination before and after reduction of A3 are consistent with the proposed structure.

The serological studies support the previous conclusion (Knox, 1963) that serological specificity probably depended on a glucosyl residue joined by a β -(1 $\rightarrow$ 6)-linkage to the adjacent sugar. This conclusion was based on the observation that

gentiobiose was the most effective inhibitor then available; in the present investigation it has been shown that *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-D-galactose is even more effective. The trisaccharide A7 and the tetrasaccharide A4 contain this disaccharide component and both are more effective inhibitors of the precipitin reaction than the disaccharide. The trisaccharide is significantly more effective than the tetrasaccharide, suggesting that an essential part of the antigenic determinant of *L. casei*, serological group C, is *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)-*O*- β -D-galactopyranosyl-(1 $\rightarrow$ 6)-*N*-acetyl-D-glucosamine. The product A2, which is presumed to be a heptasaccharide containing A7 as a component at the non-reducing end, is twice as effective as A7. Kabat (1961a) has concluded that 'an area complementary to six or seven units is probably very close to the maximum size of the combining site' of an antibody.

From the results of this and previous studies (Knox, 1963; Glastonbury & Knox, 1963) it is concluded that glucose and galactose are important components of the cell-wall polysaccharide of strains of *L. casei* in serological group C, and that organisms belong to this group because of a specific structure containing these sugars.

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