Isolation of Group Specific Products from *Lactobacillus casei* and *L. casei* var. *rhamnosus*

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SUMMARY

An examination has been made of serologically reactive products obtained from four strains of *Lactobacillus casei* (serological groups B and C) and two strains of *Lactobacillus casei* var. *rhamnosus* (serological group C). Cell wall preparations were hydrolysed with a preparation of *Streptomyces muralytic* enzyme and the soluble indiffusible products isolated. Rhamnose is the major component of both the cell wall and soluble extracts from group B organisms, whereas glucose is the major component of preparations from *L. casei* group C. Two products differing considerably in composition have been isolated from each strain of *L. casei* var. *rhamnosus*.

The serological properties of the preparations have also been studied and the results confirm that strains of *Lactobacillus casei* can be divided into two serological groups. It is concluded that group B specificity is primarily dependent on rhamnose and group C specificity on glucose, which is probably joined by a 1–6 β linkage to the adjacent sugar. The two fractions isolated from *L. casei* var. *rhamnosus* differ in their serological properties; antibodies formed on injection of this organism are predominantly directed against one component (in fraction I) whereas extracts react with sera against strains of *L. casei* group C because of the presence of a second component (in fraction II).

INTRODUCTION

In an extensive serological study of lactobacilli Sharpe (1955*a*) found that 70% of the strains tested could be classified into six groups and one subgroup. It was shown that a serological group may contain more than one species and further that organisms classified as *Lactobacillus casei* could be divided into two serological groups, one of which also contained organisms designated *L. helveticus*. Subsequently Sharpe & Wheeler (1957) proposed that the species previously defined as *L. helveticus* be renamed *L. casei* var. *rhamnosus* and that the serological groups be referred to by letters, group B including *L. casei* and group C including *L. casei* and *L. casei* var. *rhamnosus*. Sharpe used hot dilute acid extracts of lactobacilli for her serological studies. However, to investigate the chemical basis of the serological specificity of *L. casei* a milder technique for obtaining soluble extracts was considered desirable and a previous report (Knox & Brandsen, 1962) described the isolation of serologically active soluble products from autolysed cell wall of a strain of *L. casei* (group B). This technique was found to have restricted application and the present report describes the properties of products released by *Streptomyces albus* enzymes from the cell wall of *Lactobacillus* of serological groups B and C. This

streptomyces enzyme system was shown by McCarty (1952*b*, 1956, 1958) to dissolve the cell wall of *Streptococcus* of Lancefield group A, the soluble products including the group-specific carbohydrate hapten.

METHODS

Organisms. Cultures of *Lactobacillus* used by Sharpe (1955*a*) were obtained from the National Collection of Type Cultures, Colindale, London (designated NCTC), the National Institute for Research in Dairying, Reading, England (NIRD) and the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland (NCIB). These organisms were *Lactobacillus casei* NIRD strain H 831 and *L. casei* NIRD DECP belonging to group B, and *L. casei* NIRD R 094 and *L. helveticus* NCTC 6375 of group C. A strain of *L. delbrueckii* (NCIB 7473) has also been included, since the wall of this strain was shown by Cummins & Harris (1956) to contain rhamnose, a characteristic component of the cell wall of *L. casei*. However, on the basis of physiological and serological tests this strain is considered to be *L. casei* var. *rhamnosus* (group C). The laboratory strain 55 of *L. casei* (group B) used in previous studies (Knox & Brandsen, 1962) was also included in the current investigation.

Culture media. Cultures for inoculation into rabbits and for the preparation of HCl extracts were grown at 37° in the appropriate media described by Sharpe (1955*b*). Washed organisms were suspended in 0.4% formaldehyde + 0.9% NaCl solution and injected intramuscularly in rabbits every 3–4 days for a 4-week period. Organisms for disintegration and isolation of cell wall were grown in the medium described by Agren & de Verdier (1958).

Preparation of cell wall. Suspensions of organisms (equivalent to about 25 mg. dry wt./ml.) were shaken with Ballotini No. 12 glass beads in a Mickle disintegrator for 1 hr. (Salton & Horne, 1951). The cell-wall residue was suspended in M/15 phosphate (pH 7.8) heated at 100° for 20 min. (to inactivate cell-wall autolysing enzymes) and then digested with crystalline trypsin and ribonuclease (Armour Laboratories, Eastbourne, England) as described by Cummins & Harris (1956). The wall preparation was subsequently washed repeatedly with M/15 phosphate buffer (pH 7.2) then with water and dried from the frozen state.

Analytical methods. Methods for the determination of nitrogen, glucose, rhamnose, hexosamines and amino acids were as described previously (Knox & Brandsen, 1962). The galactose content of preparations was calculated from the total hexose value, determined by the basic cysteine reaction (Dische, 1949). Galactose and rhamnose gave 90 and 5%, respectively, of the amount of colour given by an equal weight (200 µg.) of glucose. The reproducibility of the results for wall preparations was improved by hydrolysing samples for 2 hr. at 100° in N-H₂SO₄ before carrying out the estimations.

Preparation of the Streptomyces albus muralytic enzyme. Murphy (1960) suggested that 'muralytic enzyme' be used as a generic term for those enzymes which lyse bacteria by dissolving the cell wall. McCarty (1952*a, b*) showed that certain strains of *Streptomyces albus* possessed a muralytic enzyme acting on streptococci; cultures of *S. albus* with such activity were provided by Dr M. McCarty (Rockefeller Institute, New York, U.S.A.) and Dr J. A. Hayashi (College of Medicine, University of Illinois, Chicago, U.S.A.). Other strains of *Streptomyces* isolated from soil by the technique described by Salton (1955) were less effective.

The *Streptomyces albus* enzyme was prepared by the method described by McCarty (1952*a, b*). After precipitation by 70 % saturation with $(\text{NH}_4)_2\text{SO}_4$ in the presence of Filter-Cel (Johns-Manville, Lompoc, California, U.S.A.), the enzyme was dissolved in $\text{m}/15$ phosphate buffer (pH 8); this solution was used for dissolving cell wall without further fractionation. As measured by the decrease in optical density (at $620 \text{ m}\mu$) the enzyme was capable of dissolving the cell wall of each of the strains of *Lactobacillus* examined, although there were differences in the speed.

RESULTS

Properties of the streptomyces muralytic enzyme

A preparation of wall from *Lactobacillus* strain NCTC 6375 (40 mg. in 20 ml. water) was incubated at 37° with 4 ml. of dialysed enzyme (dialysis at 2° against distilled water did not affect the activity of the enzyme preparation). The rate of

Fig. 1. The lysis of cell wall of *Lactobacillus* strain NCTC 6375 by streptomyces enzyme. The decrease in optical density (ϕ) is expressed as percentage of the starting value. The amount of rhamnose ($\square$), glucose ($\circ$) and hexosamine ($\bullet$) in the soluble fraction is expressed as percentage of the total amount in the sample of cell wall.

decrease in optical density at $620 \text{ m}\mu$ was followed (using $\frac{1}{2}$ in. tubes in a Unicam SP 1400 Absorptiometer) and at appropriate intervals a 2 ml. sample was removed and added to 2 ml. $\text{N-H}_2\text{SO}_4$. The cell wall residue was removed by centrifugation, the supernatant fluid heated at 100° for 4 hr. to hydrolyse the polysaccharides, and the rhamnose, glucose and hexosamine contents determined. In Fig. 1 the rate of release of these components is compared with the rate of decrease in optical density.

The optimal pH value for enzyme activity was determined by following the rate of decrease in optical density in $\text{m}/15$ phosphate buffer at pH values between 5.3 and 8.0. (At pH values below 5.0 the wall flocculated.) The maximum rate of enzyme action was observed between pH 5.3 and 6.5, with decreasing activity at

higher values. For instance, in 2 hr. at pH 5.9 the optical density decreased by 36 % as compared with 27 % at pH 8.0; incubation for 3 hr. at pH 8 was required to decrease the optical density by 36 %.

After incubation for 3 hr. the suspensions were heated to inactivate the muralytic enzyme (100° for 20 min.), centrifuged and the supernatant fluid dialysed. The rhamnose and glucose contents of the indiffusible fraction were then determined. The results supported the conclusion that maximum enzymic activity was obtained between pH 5.3 and 6.5. Again, comparing the results at pH 5.9 and 8.0, the amounts of soluble rhamnose and glucose at pH 8 represented 57 and 62 %, respectively, of the values obtained at pH 5.9. At all pH values between 5.3 and 8.0 there was no significant change in the relative molar proportions of rhamnose and glucose present in the indiffusible fraction.

Most of the soluble polymers containing rhamnose and glucose were indiffusible. A preparation of wall from *Lactobacillus* strain NIRD H 831 was suspended in water and incubated for 18 hr. at 37° with dialysed streptomycetes enzyme. The cell wall residue was then removed by centrifugation and the supernatant fluid dialysed at 2°. Of the total soluble rhamnose, glucose and glucosamine, only 2–4 % was present in diffusible polymers, whereas 27 % of the total muramic acid was diffusible. No monosaccharides were detectable.

Analysis of cell wall preparations

As shown previously (Knox & Brandsen, 1962), the wall of *Lactobacillus* strain 55 contains rhamnose, galactose and glucose in addition to the characteristic components of the cell wall (glucosamine, muramic acid, glutamic acid, aspartic acid, lysine and alanine).

The cell wall preparations from each of the five additional strains also contain rhamnose, glucose, glucosamine, galactosamine and muramic acid. All except *Lactobacillus* strain NIRD DECP contain galactose and strain NCIB 7473 in addition contains mannose. The results of the quantitative analyses for these components are given in Table 1. The major difference in the preparations from the different strains lies in the relative molar proportions of rhamnose and glucose; the values for this ratio are given in Table 2.

The major amino acids of the cell wall of each strain were: glutamic acid, aspartic acid, lysine, alanine. In contrast to the wide range of rhamnose and glucose values there was a much greater constancy in the molar proportions of the amino acids present in the wall. The molar ratios of glutamic acid, aspartic acid, lysine and alanine for the six strains fell within the range 1.0:0.6–1.2:0.7–1.0:1.6–2.0.

Preparation of cell wall carbohydrates

In earlier experiments, the mixture of cell wall and muralytic enzyme was incubated at pH 8 as suggested by McCarty (1952*b*). When it was observed that a more rapid dissolution of cell wall occurred at or below pH 6.5 experiments were made at pH 5.3. To illustrate the procedure for obtaining soluble cell wall products the results of a typical experiment at pH 8 will be described. A preparation of cell wall from *Lactobacillus* NIRD H 831 (900 mg. in 50 ml. M/15 phosphate buffer) was incubated at 37° with an equal volume of streptomycetes enzyme preparation. To

follow the rate of cell wall lysis, a sample (0.3 ml.) was removed and diluted with 4.0 ml. M/15 phosphate buffer (pH 8.0). The optical density of this suspension fell from 0.62 to 0.28 in 18 hr. and to 0.26 in 24 hr. After 24 hr. the remainder of the suspension was centrifuged and $(\text{NH}_4)_2\text{SO}_4$ added to the supernatant fluid to 70 %

Table 1. *Comparison of the composition of cell wall preparations and soluble products liberated by streptomyces enzyme*

Lactobacillus strain	Fraction	Rhamnose (%)	Glucose (%)	Galactose (%)	Hexosamine* (%)	Muramic acid (%)	Nitrogen (%)
<i>L. casei</i> NIRD H 831	Wall	33	2.0	7	16.2	9.8	7.6
	I	31	4.2	—	15.0	6.5	6.4
	II	48	3.6	7	24.2	5.2	3.6
<i>L. casei</i> NIRD DECP	Wall	26	11.5	0	11.2	3.8	6.6
	I	18.5	6.1	—	9.6	3.8	8.5
	II	39	17	0	18.4	2.0	4.2
<i>L. casei</i> strain 55	Wall	28	11	14	16.0	8.2	4.8
	I	16	4.2	—	7.4	1.0	9.9
	II	41	13	16	18.8	2.2	2.3
<i>L. casei</i> NIRD R 094	Wall	1.5	12.5	13	17.6	12.0	8.2
	I	3.0	11	—	8.6	2.8	9.1
	II	1.7	27.5	23	21.2	5.8	3.8
<i>L. helveticus</i> NCTC 6375	Wall	24	22.5	12	5.0	1.0	4.8
	I	46	33	18	1.0	0.0	1.9
	II	2.0	28	27	14.0	1.0	3.9
<i>L. delbrueckii</i> NCIB 7473	Wall	28	23	18†	6.8	4.0	4.5
	I	56	35	0‡	1	0.0	1.2
	II	9.1	34	24§	20.0	2.8	3.5

* Glucosamine + galactosamine; † includes mannose; ‡ contains 9 % mannose (determined by basic cysteine reaction); § includes trace of mannose.

Table 2. *Molar ratios of rhamnose and glucose in cell wall preparations and isolated soluble products of certain strains of Lactobacillus*

Serological group	Lactobacillus strain	Molar ratio of rhamnose to glucose		
		Wall	Fraction I	Fraction II
B	NIRD H 831	18.4	8.6	14.8
	NIRD DECP	2.5	3.4	2.6
	strain 55	2.8	4.4	3.5
C	NIRD R 094	0.13	0.30	0.09
	NCTC 6375	1.19	1.54	0.08
	NCIB 7473	1.34	1.77	0.30

saturation. At this level, the enzyme and possibly the cell wall components would be precipitated. The precipitate was dissolved in distilled water, and both the precipitate (fraction I) and the supernatant fluid (fraction II) from the $(\text{NH}_4)_2\text{SO}_4$ fractionation dialysed and the non-diffusible portions dried from the frozen state. The yields of cell wall residue, fraction I and fraction II, were 120 mg., 60 mg. and 550 mg. respectively. Fraction II was also the major product obtained from strains NIRD DECP, 55 and NIRD R 094; for the other two strains (NCTC 6375 and

NCIB 7473) fraction I was the major component. For each of the six strains under examination the composition of fractions I and II has been compared with that of the wall (Table 1).

Analysis of cell wall carbohydrates

Fraction I. For four strains (*Lactobacillus* NIRD R094 and the three strains of group B) the fraction precipitated by 70 % $(\text{NH}_4)_2\text{SO}_4$ represented only a small amount of the total soluble material; the analyses (Table 1) indicated that the products contained all the components of the cell wall. Enzyme protein would be contributing to the total nitrogen, which in two instances was considerably higher than the nitrogen content of the wall.

The preparations of fraction I from *Lactobacillus* NCTC 6375 and NCIB 7473 differed in composition from those obtained from the other strains, the nitrogen and hexosamine values being low and muramic acid absent. Rhamnose and glucose were the major components in each case, the remaining sugar being either galactose (strain NCTC 6375) or mannose (strain NCIB 7473). The nitrogen content of fraction I from these two strains could be lowered by further fractionation. The preparation was dissolved in ethylene glycol (1 % w/v) and acetone added; 50 % (v/v) acetone precipitated a small amount of material, the bulk of the material (fraction Ib) being precipitated by 55 % (v/v) acetone. For instance, fraction Ib from strain NCTC 6375 contained 51 % rhamnose, 37 % glucose, 20 % galactose and 0.4 % nitrogen (1.9 % nitrogen in fraction I).

Fraction II. Preparations of fraction II contained all the cell wall components including the amino acids, glutamic acid, aspartic acid, lysine and alanine; quantitative analyses for carbohydrates and nitrogen are given in Table 1. Preparations from *Lactobacillus* strains in serological group B had rhamnose:glucose ratios comparable to those of the cell wall preparations. Preparations from strains in serological group C were similar in composition with low rhamnose:glucose ratios (Table 2).

Three observations on the results in Tables 1 and 2 are relevant to the subsequent serological studies. First, organisms in serological group B have a higher rhamnose:glucose ratio than those in group C; this applies both to the cell wall preparations and to the soluble fractions. Secondly, preparations of fraction II from *Lactobacillus* strains of serological group C have a low rhamnose:glucose ratio. Thirdly, for each of the strains of *Lactobacillus casei* var. *rhamnosus* in serological group C (i.e. strains NCTC 6375 and NCIB 7473) fractions I and II differ considerably in composition—fraction I being composed almost entirely of rhamnose and glucose and fraction II containing much less rhamnose but the carbohydrate components of the mucopeptide.

Further fractionation of fraction II

In previous work (Knox & Brandsen, 1962) it was shown that the soluble material obtained on autolysis of cell wall from *Lactobacillus* strain 55 could be fractionated into products of different composition by dissolving in ethyleneglycol and precipitating with increasing amounts of acetone; the major difference between the fractions was in the relative amounts of rhamnose and glucose, the molar ratios varying between 0.9:1 and 5.0:1. In the present work it was found that fraction II from strain 55 also yields products of differing composition. From 100 mg. of

material was obtained (a) 28 mg. fraction IIa, precipitated by 50% (v/v) acetone, and containing 50% rhamnose and 12% glucose (ratio 4.6:1); (b) 40 mg. fraction IIb, precipitated by 60% (v/v) acetone, and containing 50% rhamnose and 10.5% glucose (ratio 5.3:1); and (c) 18 mg. fraction IIc, precipitated by 65% (v/v) acetone + 1% (w/v) NaCl, and containing 19% rhamnose and 16.5% glucose (ratio 1.3:1). Fraction II from strain NIRD DECP was also fractionated in a similar manner but a separation into products of different rhamnose:glucose ratios was not achieved.

Serological examination of soluble cell wall products

Sharpe (1955a) studied the antigenic relationship between strains of *Lactobacillus casei* and *L. helveticus* by the qualitative precipitin test. Sera prepared against *L. casei* NIRD H831 and *L. casei* NIRD DECP were used for classifying organisms in the *L. casei* group (subsequently called group B) and sera prepared against *L. casei* NIRD R094 and *L. helveticus* NCTC 6375 for classification in the *L. casei-helveticus* group (group C). *L. casei* strain 55 and *L. delbrueckii* NCIB 7473 were examined by this procedure and, as stated previously, it is concluded that these two strains belong to serological groups B and C respectively. The division of these organisms into two groups has been confirmed by studying the serological properties of the products liberated by the Streptomyces enzyme.

Table 3. *Cross reaction between antisera to three strains and the soluble products (fraction II) isolated from cell wall preparations of certain strains of Lactobacillus*

In each case 10 µg. of preparation was mixed with 0.1 ml. of serum in a final volume of 1.0 ml. The values are expressed as % of amount of precipitate obtained in the homologous reaction. When results are not listed the values were not significantly greater than those given by the blank containing serum and saline.

Lactobacillus source of Fraction II	Antiserum		
	H831	DECP %	R094
NIRD H831	100	73	—
NIRD DECP	70	100	—
Strain 55	110	90	—
NIRD R094	—	—	100
NCTC 6375	—	—	84
NCIB 7473	—	—	98

Fraction I. The preparation of fraction I from *Lactobacillus* NCTC 6375 and NCIB 7473 reacted strongly with antisera to strain NCTC 6375 and also to strain NCIB 7473 but gave no detectable precipitate with antiserum to strain NIRD R094. An indication of the extent of cross reaction with antiserum to strain NCTC 6375 was obtained by the quantitative precipitin test. Solutions of fraction I from each strain were diluted to 20 µg./ml. in 0.9% NaCl and 0.5 ml. mixed with 0.5 ml. serum which had been diluted fivefold. Experiments were set up in triplicate. After 1 hr. at 37° and 2–3 days at 4° the protein content of the precipitate was determined by the method of Heidelberger & MacPherson (1943) by using Folin & Ciocalteu's phenol reagent. It was found that the preparation from strain NCIB 7473 gave 98% of the amount of precipitate given by NCTC 6375. A comparison of

the two preparations by the Ouchterlony method (Ouchterlony, 1953) showed the presence of a single serologically identical component.

Fraction II. Preparations were tested against each of the four group sera by the qualitative precipitin test. The results agreed with those obtained for acid extracts, although only weak cross reactions were obtained between antisera to *Lactobacillus* NCTC 6375 and the organisms of group C. An indication of the extent of cross reaction between the six strains was obtained by the quantitative precipitin test. Experiments were set up in triplicate against antisera to strains NIRD H831, NIRD DECP and NIRD R094, using the same conditions as described above for fraction I. In Table 3 the amount of precipitin obtained is expressed as a percentage of that given in the homologous reaction.

The observations were extended to include a study by the Ouchterlony technique (Ouchterlony, 1953), each of the six preparations of fraction II being tested against antisera to *Lactobacillus* NIRD H831 (serological group B) and NIRD R094 (serological group C). The test revealed only one component; within each serological group the fractions were shown to be serologically identical and no cross reaction between the groups was detectable.

Inhibition of precipitin reaction by component sugars

The effectiveness of 25 μ moles of rhamnose, galactose and glucose in inhibiting the precipitin reaction between antisera and fractions I and II was studied (Table 4). The amounts of antiserum and polysaccharide used were found suitable by preliminary studies in which the proportions of antiserum and polysaccharide were varied. The antiserum (0.2 ml. for group B organisms and 0.1 ml. for group C organisms) was mixed with 25 μ moles of rhamnose or glucose in a final volume of 0.5 ml. After 1 hr. at 37°, 0.5 ml. of solution containing the appropriate amount of polysaccharide was added and the amount of precipitate formed on standing was estimated as described previously.

Table 4. *Inhibition of precipitin reaction between antisera and preparations of fractions I and II by 25 μ moles of rhamnose, glucose or galactose*

Sero- logical group	Lactobacillus strain	Amount of component added		Inhibition (%)		
		Fraction	Serum	Rhamnose	Glucose	Galactose
B	NIRD H 831	20 μ g. II	0.2 ml. H 831	55	0	4
	NIRD DECP	10 μ g. II	0.2 ml. DECP	60	7	—
	Strain 55	25 μ g. II	0.2 ml. 55	47	14	25
		25 μ g. II _b	0.2 ml. 55	50	14	—
		25 μ g. II _c	0.2 ml. 55	30	27	—
C	NIRD R094	5 μ g. II	0.1 ml. R094	11	25	3
	NCTC 6375	10 μ g. I	0.1 ml. 6375	10	24	4
		10 μ g. II	0.1 ml. R 094	0	26	2
	NCIB 7473	10 μ g. I	0.1 ml. 6375	7	19	0
		10 μ g. II	0.1 ml. R094	4	34	7

Fraction I. For examining preparations of fraction I from *Lactobacillus* NCTC 6375 and NCIB 7473, antiserum to strain NCTC 6375 gave satisfactory results. Although fraction I from strain NCIB 7473 reacted strongly with the homologous antiserum

it was not possible to obtain more than 4% inhibition of the precipitin reaction, even with 100 μ moles of rhamnose or glucose.

Fraction II. All preparations of fraction II from organisms of group B were tested using the homologous antiserum. Antiserum to *Lactobacillus* NIRD R094 was used for studies on fraction II from group C organisms. The study of strain 55 was extended to include fractions II*b* and II*c*, the products separated by acetone precipitation from ethyleneglycol in which the rhamnose:glucose ratios were 5.1:1 and 1.3:1 respectively.

Further studies on the glucose specificity of group C organisms

The ability of a number of glucosides to inhibit the precipitin reactions between antisera to organisms of group C and corresponding fractions has been studied, using the same conditions as those described above. The sugars employed were methyl- α -D-glucopyranoside, methyl- β -D-glucopyranoside and all the reducing disaccharides containing glucose units joined by β linkages—sophorose (1 $\rightarrow$ 2), laminaribiose (1 $\rightarrow$ 3), cellobiose (1 $\rightarrow$ 4) and gentiobiose (1 $\rightarrow$ 6).

Fraction I. The inhibition of the reaction of fraction I from *Lactobacillus* NCTC 6375 with homologous antiserum was studied. Methyl- β -D-glucoside was more effective than methyl- α -D-glucoside, the respective values for 25 μ moles being 28 and 20% compared with 24% for glucose. None of the disaccharides was more effective than methyl- β -D-glucoside.

Table 5. *Effect of periodate oxidation for 8 min. at room temperature on the component sugars and serological activity of fractions I and II*

Lactobacillus strain	Fraction	Amount of component remaining (expressed as % of starting value)		Serological reactivity (amount of precipitate expressed as % of starting value)
		Rhamnose	Glucose	
NIRD H831	II	54	—	26
NIRD R094	II	—	81	62
NCIB 7473	II	100	78	48
NCIB 7473	I	109	84	69
NCTC 6375	I	108	83	74

Fraction II. Methyl- β -D-glucoside was also more effective than methyl- α -D-glucoside in inhibiting the reaction between fraction II from *Lactobacillus* NIRD R094 and homologous antiserum, the inhibition by 25 μ moles being 42% compared with 12% (25% by glucose). The extent of inhibition by 10 μ moles of disaccharide has been studied; gentiobiose gave 33% inhibition, whereas the remaining disaccharides, as well as methyl- β -D-glucoside (10 μ moles) gave 21–24% inhibition. With smaller amounts (4 μ moles) of carbohydrate the corresponding values for gentiobiose and methyl- β -D-glucoside were 24 and 14% respectively.

Action of periodate on group specific products

Under the same conditions as those adopted by Foster, Davies & Crumpton (1958) preparations were oxidized for 3 and 8 min. and subsequently reduced by sodium borohydride. Samples were then analysed for component sugars, and for serological activity by the quantitative precipitin test; the amounts of components used in the precipitin test were the same as those given in Table 4. The results of the effect of periodate for 8 min. are summarized in Table 5.

Serological group B. Rhamnose is responsible for the serological specificity of *Lactobacillus* strains of group B, and for a strain in this group (NIRD H831) it was found that almost half the rhamnose was oxidized in 8 min. This result indicates that the rhamnose units are joined by either a 1 → 2 or a 1 → 4 linkage.

Serological group C. Glucose is responsible for the serological specificity of both products which have been isolated from group C organisms. The glucose units in fraction II from *Lactobacillus* NIRD R094 and NCIB 7473 were oxidized by periodate, oxidation being accompanied by reduction in serological reactivity. In the absence of more complete analyses, it is only possible to state that glucose units responsible for serological specificity are joined by 1 → 2, 1 → 4 or 1 → 6 linkages. The glucose units in fraction I from strain NCIB 7473 and NCTC 6375 were also oxidized by periodate. Rhamnose is a major component of fraction I from these two strains but in each case the rhamnose was stable to periodate, the oxidation of other sugars actually resulting in an increase in the rhamnose content of the remaining residue. This result suggests that either the rhamnose units are joined by 1 → 3 linkages or, alternatively, that two of the three hydroxyl groupings (on positions 2, 3, 4) of rhamnose are substituted.

DISCUSSION

The work described in the present paper was undertaken in an attempt to establish a relationship between components of the cell wall of *Lactobacillus casei* and the serological properties of the particular organism. The characteristic components of the cell wall of Gram-positive bacteria include mucopolysaccharides, mucopeptides and teichoic acids. Only some species of lactobacilli contain teichoic acid in the cell wall, although in others glycerol teichoic acid has been detected within the cells (Baddiley & Davison, 1961). Further, the presence of glycerol teichoic acid or ribitol teichoic acid in the wall could be related to the serological grouping of the particular organism, those in groups A and E containing glycerol and those in group D containing ribitol. Organisms of groups B, C and F contained no wall teichoic acid but an intracellular glycerol teichoic acid. This compound has been isolated from a strain of *L. casei* and its structure elucidated (Keleman & Baddiley, 1961). The results of Baddiley & Davison (1961) suggest that the teichoic acids participate in the serological reactions of some strains of lactobacilli, but not those of groups B and C, i.e. those designated *L. casei* or *L. casei* var. *rhamnosus*; in these organisms therefore another component of the cell wall must be associated with serological specificity.

Cummins & Harris (1956) studied the cell wall composition of lactobacilli (and other organisms) and their results showed that only two of the strains examined contained rhamnose, one designated *Lactobacillus casei* (NCIB 8079) and the other

L. delbrueckii (NCIB 7473). The latter has subsequently been shown to be a strain of *L. casei* var. *rhamnosus* (group C) so that it seemed likely that a knowledge of the occurrence of cell wall polysaccharides containing rhamnose might be valuable in the designation of organisms as *L. casei*. Examination of a laboratory strain 55 of *L. casei* showed that rhamnose was a major component of the wall and was, together with galactose and glucose, associated with the serological specificity (Knox & Brandsen, 1962). In the present investigation, cell wall components of five other strains of *L. casei* were studied, four of these having been used by Sharpe (1955*a*) to distinguish between organisms of groups B and C. To investigate the group specific products of these strains of *L. casei* the technique developed by McCarty was used. McCarty (1952*b*) incubated organisms of group A streptococci with *Streptomyces albus* enzyme and obtained soluble products containing the group specific carbohydrate. The preparation also contained the components of the cell wall mucopeptide, but subsequently it was shown that formamide would separate the polysaccharide from the mucopeptide (Krause & McCarty, 1961).

The analyses of the soluble products obtained from cell wall following digestion with the streptomyces muralytic enzyme are given in Table 1. Fraction I in each case was the product precipitated by 70% saturation with $(\text{NH}_4)_2\text{SO}_4$ (this was the concentration of $(\text{NH}_4)_2\text{SO}_4$ required to precipitate the enzyme), and fraction II was the material then remaining in the supernatant fluid. When the analyses for the wall and these two fractions from each of the four strains designated *Lactobacillus casei* are examined, three observations emerge: (i) for any one strain, the relative proportion of rhamnose and glucose in the two fractions is similar to that in the wall; (ii) each preparation of fraction II contains more hexosamine (glucosamine+galactosamine) and less muramic acid than the corresponding wall; (iii) from the analyses for nitrogen, fraction II preparations contain only a minor peptide component, whereas fraction I preparations contain less carbohydrate and a greater proportion of peptide. As it has also been observed that the diffusible fraction following enzyme action contains relatively large amounts of muramic acid, the results suggest the streptomyces enzyme is hydrolysing specific linkages in the cell wall mucopeptide. Krause & McCarty (1961) have reached the same conclusion from their studies on the action of the streptomyces enzyme on streptococcal cell wall.

With the two strains of *Lactobacillus casei* var. *rhamnosus* a different pattern is obvious. In each case fraction I differed significantly in composition from fraction II, fraction I having a much greater content of rhamnose and virtually no hexosamine or peptide component (following further fractionation from ethylene glycol with acetone). The difference is even more pronounced for strain NCIB 7473; fraction I contained mannose but not galactose, whereas galactose was a major component of fraction II and mannose only a minor component. The results indicate that the wall of strains of *L. casei* var. *rhamnosus* contains two polysaccharide components. One component, in fraction II, is almost devoid of rhamnose and is also present in strain NIRD R 094 (group C). The other component, fraction I, contains most of the rhamnose, and it may be concluded that a polysaccharide composed of galactose, glucose and rhamnose represents about half the weight of the wall. The presence of this major cell wall polysaccharide in strains of *L. casei* var. *rhamnosus* is associated with a decrease in the mucopeptide fraction, the hexosamine, muramic acid

and nitrogen values being considerably less than those for the other group C organism, strain NIRD R094. Fractions I and II differ considerably in composition but from studies on the rate of dissolution of the wall (Fig. 1) there is no evidence for the two polysaccharide fractions being released at different rates; further, the rate of release was not affected by changes in pH between 5.3 and 8.0. There is no evidence, therefore, that enzymes of different specificity are acting on the cell wall to release the polysaccharide components. The small amount of hexosamine and nitrogen in preparations of fraction I may be indicative that this fraction is also released from the cell wall by hydrolysis of specific linkages in the mucopeptide.

From the analytical figures it is possible to calculate the percentage of the dry weight of the fractions which has been accounted for. This has been done for the six preparations of fraction II and the preparations of fraction I from the two strains of *Lactobacillus casei* var. *rhamnosus*. Assuming that the hexosamines are acetylated and that the total nitrogen is accounted for by amino acids and hexosamines, the percentage recovery lies between 91 and 98 %, with an average of 95 % (allowing for water of hydrolysis). Thus it seems unlikely that any of the preparations contain significant amounts of undetected components.

Qualitative and quantitative precipitin studies have confirmed the conclusion of Sharpe (1955*a*) that strains of *Lactobacillus casei* can be divided into two groups, subsequently designated B and C (Sharpe & Wheeler, 1957). Rhamnose is the major component of the products obtained from group B organisms and it is also the most effective inhibitor of the precipitin reaction between the corresponding fraction II preparations and homologous antiserum. Further, the periodate oxidation of rhamnose residues in fraction II is accompanied by a decreased serological reactivity. Galactose and glucose contribute to the serological specificity of strain 55, and the cell walls apparently contain a polysaccharide component (isolated as fraction IIc) in which these two sugars are important components. A fraction with similar properties has also been isolated from autolysed cell walls of strain 55 (Knox & Brandsen, 1962). However, when the analytical and serological results for the two strains NIRD DECP and NIRD H831 are compared, it is apparent that galactose and glucose could not be contributing to their cross reaction. It may therefore be concluded that the classification of organisms as 'group B' depends on specific carbohydrate components which contain rhamnose, with some strains (such as 55) containing galactose and/or glucose as additional antigenic determinants.

Group C organisms comprised one strain of *Lactobacillus casei* NIRD R094, containing only a small amount of rhamnose in the cell wall, and two strains of *L. casei* var. *rhamnosus*, in which rhamnose is a major component of the wall. Fractionation of the soluble products, released from each of the latter two strains by the streptomycetes muralytic enzyme, showed most of the rhamnose was present in fraction I (precipitated by $(\text{NH}_4)_2\text{SO}_4$) whereas fraction II contained a much smaller amount of rhamnose and was very similar in composition to fraction II from *L. casei* NIRD R094. The preparations of fraction II from strains of *L. casei* var. *rhamnosus* cross react with antisera to NIRD R094 but do not give a precipitate with homologous antiserum, this reaction being given only by fraction I. It is concluded therefore that strains of *L. casei* var. *rhamnosus* cross react with strains of *L. casei* in group C because of the presence of one specific antigenic determinant with a low rhamnose to glucose ratio (in fraction II), whereas antibodies produced on

injection of these organisms are predominantly specific for another antigenic determinant with a greater rhamnose to glucose ratio (in fraction I). Sharpe (1955*a*) observed that cultures of *L. casei* var. *rhamnosus* grown at 37° stimulated the production of predominantly type serum, whereas with *L. casei* incubation at 37° gave organisms which favoured the formation of group antibodies. It therefore appears probable that fraction I contains the type specific product and fraction II the group specific product.

The major components of fraction II from group C organisms are galactose and glucose, but only glucose is a significant inhibitor of the precipitin test. Additional studies indicate that the specific determinant contains glucose joined to the adjacent sugar by a β linkage, the adjacent sugar probably being glucose substituted in the 6 position. Rhamnose is the major component of fraction I from the two strains of *Lactobacillus casei* var. *rhamnosus* but again glucose is the most efficient inhibitor. However, studies on the inhibition of glucosides gave results which do not clearly define the specificity in more detail, although it is probable that once again glucose is joined to the adjacent sugar by a β linkage. Periodate oxidation studies showed that the rhamnose units in fraction I from *Lactobacillus casei* var. *rhamnosus* and fraction II from *L. casei* group B are joined by different linkages, probably 1 $\rightarrow$ 3 for the former and either 1 $\rightarrow$ 2 or 1 $\rightarrow$ 4 for the latter. The results for the preparation from *L. casei* var. *rhamnosus* could also be accounted for by a highly branched structure in which every rhamnose unit is substituted on two of the three hydroxyl groups; such a structure, however, would appear less probable.

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