Extracellular Polysaccharide Synthesis by Members of the Genus *Lactobacillus*: Conditions for Formation and Accumulation

BY L. K. DUNICAN* AND H. W. SEELEY, JUN.

*Division of Bacteriology, Department of Dairy and Food Science,
College of Agriculture, Cornell University,
Ithaca, New York*

(Received 6 July 1964)

SUMMARY

Members of the genus *Lactobacillus* were examined for their ability to synthesize extracellular polysaccharide from sucrose. Strains physiologically similar to *L. pastorianus* synthesized a glucan. Other strains liberated a complex polysaccharide which contained mannose and glucose. Variants of the glucan-producing strains occurred spontaneously and these lacked the capacity to synthesize glucan; however, extracts of these bacilli contained glucansucrase and invertase. Manometric studies indicated that the latter enzyme functioned in the sucrose metabolism of these non-glucan producing variants.

INTRODUCTION

The extracellular dextransucrases of *Leuconostoc mesenteroides* (Hehre, 1951; Tsuchiya *et al.* 1956) and of *Streptococcus bovis* (Dain, Neal & Seeley, 1956; Bailey, 1959) have been described. A number of reports have also shown that some lactobacilli produce extracellular polysaccharides which yield glucose on hydrolysis. These investigations, some of which were reviewed by Perquin (1940), did not attempt to identify fully or compare the polysaccharides produced by lactobacilli with the dextrans of leuconostoc and streptococcus. In addition, little information is available on the conditions under which lactobacilli produce extracellular polysaccharides except that the synthetic capacity is unstable and cultures stored in the laboratory lose the ability to produce the extracellular material after a period of time.

Pederson & Albury (1955) reported that certain non-dextran-forming leuconostocs and certain lactobacilli could be 'trained' to produce dextran by serial transfer in tomato or orange juice broths at low pH. More recently, Langston & Bouma (1960) found that isolations from silage, which they designated *Lactobacillus brevis* 'variable', produced extracellular polysaccharides when transferred by the method of Pederson & Albury (1955). Niven & Evans (1957) described *L. viridescens* from spoiled meat products which also produced an extracellular polysaccharide from sucrose.

A number of additional reports have dealt with lactobacilli which produce a heterogeneous polysaccharide material from various sugars. Millis (1951) described a slime-forming heterofermentative rod from 'ropy' beer. Shimwell (1949) had

*Present address: Department of Bacteriology, University College, Galway, Ireland.

found an organism earlier which had similar properties. Barker, Bourne, Salt & Stacey (1958) found that the organism which Millis had isolated produced a polysaccharide which contained several sugars. Williamson (1959) also found a slime-producing organism in beer which grew on maltose and produced a heteropolysaccharide.

This paper reports the results of an investigation in which slime-forming lactobacilli from several sources were examined for purposes of comparison. The chemical compositions of the extracellular materials produced by various lactobacilli were studied as well as the conditions under which they were produced. A previous report stemming from the study described the relationship between temperature and dextran production in one of the strains (Duncan & Seeley, 1963).

METHODS

Micro-organisms and media. The lactobacilli used were those which had, at some time, produced extracellular polysaccharide from sucrose. The group contained eleven strains used by Pederson & Albury (1955), *Lactobacillus viridescens* of Niven & Evans (1957), a rosy beer lactobacillus from Carr (1959), five rosy beer strains, including Walker's strain from a collection of Dr M. E. Sharpe (Shinfield), six silage strains from Dr T. Gibson (Edinburgh), five strains from Dr Naylor (Cornell University); nine strains were isolated by the authors.

Culture medium. The medium used was that of Man, Rogosa & Sharpe (1960) without peptone. The basal medium contained no carbohydrate and is referred to as 'MRS' medium. The concentrations of carbohydrates used in various experiments are included in the legends of the Figures or in the text. Stock cultures were maintained by freezing in skim milk or in the lyophilized state. Because of the inhibitory effects of elevated temperatures of growth on the synthesis of polysaccharides, all cultures were incubated at 30° (Duncan & Seeley, 1963).

Purification of polysaccharides. The polysaccharides were precipitated from the cell-free supernatant culture fluid by the addition of 2 vol. chilled 95% ethanol. When low yields of glucan were encountered the flasks containing ethanol were stored overnight in the refrigerator to allow precipitation of polysaccharide. Two additional precipitations with ethanol and one with 2 vol. acetone were sufficient to give relatively protein-free samples of dextran.

The heteropolysaccharide was produced in very low yields and its isolation necessitated the concentration of the cell-free spent media to one-third its original volume by distillation under vacuum at 50° before the addition of the ethanol.

Analysis of polysaccharides. The partially purified polysaccharides were hydrolyzed by the method of Williamson (1959). The hydrolysates were neutralized by the addition of saturated Ba(OH)₂ and the solid BaSO₄ was removed by centrifugation. Paper chromatograms were prepared by standard techniques with the following solvents: (1) butanol+ethanol+water (2+1+1, by vol.) descending (Chou & Tobias, 1960); or (2) butanol+acetic acid+water (5+1+4, by vol.), ascending (Lederer & Lederer, 1955). Sugars were detected by methods using (a) aniline+diphenylamine (Smith, 1960), (b) naphthoresorcinol (Partridge, 1948) and (c) *p*-anisidine (Hough, Jones & Wadman, 1950). The reagents (a) and (c) were of particular value because of the different colours developed with the different sugars.

Serological detection of glucan. The supernatant cell-free spent culture fluid was adjusted to pH 6.0–6.2. A portion of this culture fluid was layered over type II pneumococcus serum (Burroughs Wellcome) in a vial. The formation of a ring at the interface within 15 min. constituted a positive test. Types I and III sera were used as controls.

Preparation of cell-free extracts. Organisms were deposited by centrifugation and were washed and suspended in acetate buffer (0.1 M, pH 5.6). The organisms were broken in a Raytheon sonic oscillator, Model DF 101, 10 kcy. for 30 min. The unbroken organisms and large particles were removed by centrifugation (800g) for 30 min. Glucan sucrose and invertase were assayed as described in Dunican & Seeley (1963); protein was determined by the biuret method (Gornall, Bardawill & David, 1949); nucleic acids were measured by the spectrophotometric method of Mitchell (1950).

Manometric studies. These studies were done by conventional techniques. Organisms in the logarithmic phase of growth were washed and suspended in sugar acetate buffer (0.1 M, pH 5.6). Each cup contained 2 ml. organisms, 10 μ mole of the sugar in acetate buffer. Total volume was 3.0 ml. The centre well had 0.3 ml. 20% (w/v) KOH. The temperature of the bath was 30.2°.

RESULTS

Two types of extracellular polysaccharides were produced during growth of the cultures. Table 1 shows the distribution of the two types among some of the strains studied. Four organisms which were physiologically similar to *Lactobacillus pastorianus* formed a glucan from sucrose, whereas the majority of the strains produced a polysaccharide which contained mannose and glucose. The latter polysaccharide was designated as 'heteropolysaccharide'.

Table 1. *Extracellular polysaccharides of various lactobacilli*

Organism	No. of strains	Glucan producing	Heteropolysaccharide producing
<i>L. brevis</i>	5	None	None
<i>L. pastorianus</i>	6	4	None
Unidentified*	26	None	18

* Exhibit similarities to *L. buchneri* and *L. cellobiosus* (Rogosa *et al.* 1953).

Substrates for polysaccharide production

The glucan-producing strains RWM-13, RWM-1, RW-119 used sucrose as the sole substrate for glucan formation. Polysaccharide was not produced from glucose, maltose, cellobiose, trehalose, mannose or fructose. These three strains varied in their ability to grow on lactose, melibiose, arabinose, galactose, α -methylglucoside and starch, but none produced glucan from any of these substances.

In contrast to the specificity of sucrose as the substrate for glucan formation, the heteropolysaccharide was produced from several sugars, including maltose, sucrose, glucose, galactose and lactose, but not from fructose. Furthermore, fructose with sucrose prevented the formation of the polysaccharide.

Chemical composition of the polysaccharides formed

The isolated glucans were purified as noted previously by ethanolic precipitation; they were then hydrolysed, and examined by chromatography to identify the component sugars. Glucose was the only sugar found. Its identity was confirmed by reaction with glucose oxidase. The unhydrolysed polysaccharide did not give a colour with iodine and yielded a precipitate with type II pneumococcus serum confirming the view that the material was a glucan.

The purified heteropolysaccharides were examined chromatographically after hydrolysis. Table 2 shows the composition of these materials obtained from several strains; mannose and glucose were the component sugars except in two strains in which rhamnose also occurred. The unhydrolysed material contained substantial amounts of nucleic acid material and protein. The heteropolysaccharide did not give a precipitate with type II pneumococcus serum or stain with iodine.

Table 2. *Chemical composition of heteropolysaccharide synthesized by various strains of lactobacilli*

Strain	Mannose	Glucose	Rhamnose
rw-11	+++	+	—
5-144	+++	+++	—
s-88A	+++	+++	—
D-18	+++	+	—
5-49	+++	+	—
D-11	+++	+++	—
D-16	+++	+++	—
B-ver	+++	+++	—
B-g	+++	+++	—
L-15	+++	+++	+
2	+++	+	+

+++ = major component; + = minor component; — = absent

It was apparent that while relatively few strains of lactobacilli examined produce a glucan serologically similar to that of *Leuconostoc mesenteroides* and *Streptococcus bovis*, the majority of the strains produced a material which contained mannose and glucose, and so resembled the material reported by Williamson (1959) and Barker *et al.* (1958). The heteropolysaccharide was so similar to the gross chemical composition of the cell walls (Cummins & Harris, 1956) that the material was considered to be the lytic product of the bacteria and was not studied further. This conclusion was supported also by the low yields of heteropolymer found, viz. 250 mg./l. in contrast to the high yields of glucan observed, viz. 15 g./l. by the glucan-producing strains.

Effect of sucrose concentration on the production of glucan

The relationship between the quantity of glucan produced by *Lactobacillus* strain RWM-13 grown at 30° and concentrations of sucrose is shown in Fig. 1. The results differ from those of Neely & Nott (1962) who observed that *Leuconostoc mesenteroides* did not produce dextran in media with sucrose concentrations below 2%.

Effect of initial pH of the medium on the production of glucan

Adequate control of pH value has been shown to be a requirement for dextran production by cultures of *Streptococcus bovis* (Bailey, Barker, Bourne & Stacey, 1957) and *L. mesenteroides* (Tsuchiya *et al.* 1952). Figure 2 shows that when the initial pH of media was between pH 5.0 and 7.0 there was no profound effect on the glucan production by *Lactobacillus* strain RWM-13. There was a marked decrease in the production of glucan, however, in flasks adjusted to pH values outside this range.

Fig. 1

Fig. 2

Fig. 1. The effect of sucrose concentration on glucan production by *Lactobacillus* strain RWM-13 after 48 hr of growth.

Fig. 2. The effect of initial pH value on the yield of glucan by *Lactobacillus* strain RWM-13, after 48 hr of growth.

The effect of the omission of various components of the medium on glucan production

The medium of Man *et al.* (1960) was used in this investigation. It contained sodium acetate, diammonium citrate, Tween 80 and Mn^{2+} , in addition to the normal components of a medium for lactic acid bacteria. The results (Fig. 3) show the effects of the omission of each of these components on growth and glucan production by *Lactobacillus* strain RWM-13. The acetate, citrate and Mn^{2+} were required for optimal growth and glucan production. The effect with citrate was especially noteworthy since the omission of citrate led to a lag in growth and no glucan was produced in the first 24 hr of growth.

The lag in growth due to the omission of citrate was further investigated and the results are presented in Fig. 4. The broken lines show that the omission of citrate had a twofold effect, decreased cell and glucan yield and a greater lag before appreciable growth and glucan production occurred.

The stimulatory nature of these components of the medium on growth and glucan production corresponds to the observations of the requirements for citrate and manganese (Evans & Niven, 1951; MacLeod & Snell, 1947) and acetate (Guirard, Snell & Williams, 1946). The effect of the citrate may be due to its effect as an additional energy source (Gunsalus & Campbell, 1944).

There was no requirement for Tween 80 or carbon dioxide for either growth or glucan production. *Streptococcus bovis* has been shown to require CO₂ for good dextran production (Dain, *et al.* 1956; Oxford, 1958).

The production of glucan by Lactobacillus strain RWM-13 in relation to cell growth

It was observed early in this work that there was a large increase in the production of glucan towards the end of cell growth. This is shown in Figs. 3 and 4 where it may be seen that the greater part of the glucan was synthesized in the second day of growth.

Fig. 3

Fig. 4

Fig. 3. The effect of the absence of certain components of the medium on growth and polysaccharide production by *Lactobacillus* strain RWM-13. Columns represent the total yields of cells or polysaccharide after 48 hr growth. Horizontal shading represents cell yield in 24 hr. Diagonal shading represents polysaccharide yield in 24 hr. Medium contained 8% sucrose.

Fig. 4. The effect of the omission of citrate from the medium on growth and polysaccharide production by *Lactobacillus* strain RWM-13. Solid lines = media with citrate; dotted lines = media without citrate. Sucrose concentration, 8%.

In order to study the relationship of polysaccharide formation to growth over the entire growth cycle, portions of the culture were removed at intervals and the quantities of bacteria and glucan were measured gravimetrically. The results are shown in Fig. 5 in which the plots of both entities are made on a semilogarithmic scale. The bacteria dry weight shows the development of a typical exponential growth curve between 5 and 15 hr. During the same interval the production of glucan is also exponential, suggesting that the processes of glucan production and growth were dependent on one another and that the enzyme glucansucrase was perhaps liberated in relation to the need of the organism to hydrolyse sucrose. The decrease in the quantity of bacteria during the late stationary phase of growth is correlated with increased glucansucrase activity although no agent capable of lysing *Lactobacillus* strain RWM-13 was observed in the spent medium or in cell extracts of this organism.

The results shown in Fig. 5 indicate that the synthesis of the enzyme or its release were related to growth. Efforts to locate the enzyme in the organism were only partially successful since methods of disintegration of the bacteria by means other than sonic treatment failed so that extracts free from excessive breakage of the particulate fractions could not be obtained. However, the centrifugal fractionation of such extracts of *Lactobacillus* strain RWM-13 in a Spinco preparative centrifuge into $105,000g \times 90$ min. soluble and particulate fractions showed that 85% of the activity was contained in the soluble fraction. There was little activity in the cell-wall fraction. The data do not identify the enzyme glucansucrase with any structure but do indicate that the enzyme is located within the organism, necessitating the liberation of the enzyme during growth.

Activity of glucansucrase in cell extracts

Glucansucrase was assayed in extracts of *Lactobacillus* strain RWM-13 by the method of Dunican & Seeley (1963). Replicate assays were prepared in $10^{-4}M$ - $AgNO_3$ and invertase activity was computed as reported previously. Figure 6 shows the activity of glucansucrase as a function of time of incubation and a 6 hr period was selected for future experiments as a suitable time to incubate the enzyme assays. This preparation showed negligible invertase activity and this activity was not recorded.

Fig. 5

Fig. 6

Fig. 5. The synthesis of glucan in relation to bacterial growth of *Lactobacillus* strain RWM-13. Yields of polysaccharide and cells are expressed on the same logarithmic scale.

Fig. 6. The activity of glucansucrase from extracts of *Lactobacillus* strain RWM-13 with respect to time incubation of reaction mixture. Temperature of assay, 37° .

Effect of incubation in glucose broth on the content of glucansucrase in cell extracts

Lactobacillus strain RWM-13 was grown to the early logarithmic phase in MRS medium containing 4% glucose. Organisms were removed by centrifugation, washed in acetate buffer (0.1M, pH 5.6) and suspended in MRS medium containing 2% sucrose. Portions of the culture were removed at intervals after the addition of the sucrose and were quickly chilled to 0–5° at which temperature the bacteria were collected by centrifugation. The bacteria were then washed in acetate buffer, broken in the sonic oscillator, and assayed for glucansucrase and invertase. The content of glucan was also determined in each of the portions of the culture. Figure 7 shows the results obtained. There was a significant amount of glucansucrase at zero time which decreased as the time of incubation in the sucrose increased. From the data shown in Fig. 7 it was concluded that the glucansucrase was constitutive in *Lactobacillus* strain RWM-13 since it was present in the bacteria in the absence of its substrate, sucrose. This enzyme, therefore, resembles that of *Streptococcus bovis* which has been reported to be constitutive (Bailey, 1959). The reservations placed by Bailey on the constitutive nature of the *S. bovis* enzyme apply also in the present investigation since it was not possible to rule out the presence of minute amounts of sucrose in the components of the medium or to exclude the possibility of synthesis of trace amounts of sucrose by the bacteria sufficient to induce glucansucrase. The presence of glucansucrase in extracts of *Lactobacillus* strain RWM-13 grown on glucose has been reported before (Duncan & Seeley, 1963) where extracts of bacteria grown on 4% glucose or sucrose were shown to contain equivalent amounts of glucansucrase. These findings may be contrasted with those of Neely & Nott (1962) who showed that *Leuconostoc mesenteroides* did not form glucansucrase on a glucose medium, but began to form the enzyme upon the addition of sucrose.

*Spontaneously occurring, non-glucan-forming variants of
Lactobacillus strain RWM-13*

Mayer (1938) first noted variation in the colonies of dextran-forming lactobacilli grown on glucose agar. Smooth colonies which did not grow aerobically were found in the matrix of rough colonies. Perquin (1940) also observed smooth colonies in cultures of *Betabacterium vermiforme* and, in addition, found that the smooth colonies failed to produce dextran. Spontaneously occurring variants of *Lactobacillus* strain RWM-13 appeared frequently when cultures of this organism were streaked on 8% sucrose agar. The variants were initially very sensitive to oxygen and colonies picked from the agar failed to grow in glucose or sucrose broths unless the broth was boiled before use and the medium was covered by a vaspar seal.

Properties of the variants

The variants existed as long rods in chains. Many organisms in a culture were 10–20 μ long in contrast to the parents which ranged from 1.5 to 3 μ .

Although studies to compare the biochemical characteristics of the parent and the variant strains are not yet completed, it was observed that the variant had a strongly fermentative type of growth since gas production by this organism was vigorous as compared with the nominal amount produced by the parent.

Utilization of sucrose by the variant

Some reports on dextran formation by members of the lactobacilli contain the observation that the capacity of organisms to produce dextran was transient and was often lost when organisms (after isolation from natural sources) were cultivated in the laboratory (Shimwell, 1949; Carr, 1959). The effect of increasing the temperature on the production of dextran by lactobacilli has been discussed (Dunican & Seeley, 1963). Since the variants of *Lactobacillus* strain RWM-13 were characterized

Fig. 7

Fig. 8

Fig. 7. Polysaccharide production and glucansucrase activity of *Lactobacillus* strain RWM-13 in a sucrose-containing medium as a function of time. Time 0 is the point at which sucrose was added.

Fig. 8. Oxygen uptake by glucose-grown organisms of the variant strain of *Lactobacillus* strain RWM-13 on different sugars. Corrected for endogenous activity.

Table 3. *Sucrose hydrolysis of cell-free extracts of variant of Lactobacillus strain RWM-13*

Organisms grown in MRS 4% sucrose broth for 48 hr at 30°. Assays carried out for 6 hr at 30°. AgNO₃ was used at 10⁻⁴M; pH of assay mixture was 5.6.

Extract	Reducing sugars in assay mixture	
	mg. net activity	% activity
Without AgNO ₃ (total activity)	3.07	100
With AgNO ₃ (glucansucrase)	2.55	83
Difference (invertase)	0.52	17

by their inability to synthesize glucan, it was apparent that a study of the utilization of sucrose, in the absence of glucansucrase, might contribute to the understanding of the loss of the ability of the micro-organism to form glucan.

The variant strain of *Lactobacillus* strain RWM-13 produced no glucan when grown on sucrose in air, when grown anaerobically, or in various atmospheres of

CO₂ and N₂. Since invertase has been shown to function in sucrose utilization by dextran-forming lactobacilli under certain conditions (Duncan & Seeley, 1963) it was considered probable that invertase was the means by which sucrose was utilized by the variants. To test this hypothesis the variant was grown on an 8% sucrose medium and extracts prepared and assayed for glucansucrase and invertase. The data obtained are shown in Table 3. The content of glucansucrase of the extract was found to be higher than was expected, considering the failure of the whole organisms to synthesize glucan. However, these extracts contained a small amount of invertase (Table 3). Manometric studies (Fig. 8) indicated that the invertase present in the organism was the enzyme primarily acting on the sucrose. The results show that oxygen uptake from sucrose was approximately one-third greater than that from equimolar glucose or fructose. Such a result would be expected from invertase activity which provides both monosaccharide moieties for oxidation, since glucansucrase activity would provide only the fructose portion for oxidation while the glucose was polymerized.

The failure of the variant to form glucan cannot be attributed to the absence of the enzyme but apparently to its unavailability. The enzyme could only be demonstrated with broken organisms, indicating that some liberating mechanism was not functional in the variant. The mechanism for the liberation of the glucansucrase was not elucidated beyond the demonstration that the small invertase content of the organisms was functioning during growth. The slow growth of the variant may result from the inadequate supply of this enzyme.

DISCUSSION

The observations reported here confirm a number of reports that some heterofermentative lactobacilli accumulate two types of extracellular polysaccharide in the culture media in which they are growing (Perquin, 1940; Niven & Evans, 1957; Millis, 1951). Several strains which were physiologically similar to *Lactobacillus pastorianus* produced a glucan which was serologically similar to that produced by *Leuconostoc mesenteroides* and *Streptococcus bovis*. The majority of the cultures studied produced a heteropolysaccharide which contained mannose, glucose, protein and nucleic material, and resembled the substance reported by Williamson (1959). The composition of this material and the small amount produced led to the conclusion that the material was not produced extracellularly but was an autolytic product. No physiological basis could be found to explain why cultures of these particular strains did not always contain this lytic material or why it was not formed on fructose-containing media.

One of the objectives of this investigation was to explain the cause of the instability of glucan production. Although Pederson & Albury (1955) had found that dextran-producing strains of lactobacilli which had lost their ability to produce dextran could be 'trained' to synthesize this polysaccharide by serial transfer in fruit juice media, no explanation has yet been found to explain this behaviour. It was assumed in the present work that the ability to lose and regain the capacity to synthesize glucan was a variation of a single process. This assumption was not borne out experimentally. However, two events which resulted in the loss of glucan synthesis were established. The loss of dextran synthesis at temperatures above the

optimum for growth has been reported (Dunican & Seeley, 1963); also, naturally-occurring variants of *Lactobacillus* strain RWM-13 were found which did not produce glucan. These two events are related in that invertase activity accounted for sucrose hydrolysis in the absence of glucansucrase activity. Glucansucrase was not synthesized at the elevated temperatures but it could be detected in the broken variant. The failure of glucansucrase to be liberated or to function in variant organisms in which it was shown to be present could not be explained.

The loss of the ability of *Lactobacillus* to synthesize glucan can be related to conditions which (1) inhibit the synthesis of the enzyme or (2) inhibit the liberation of the enzyme into the medium where its activity is necessary for sucrose utilization. Under laboratory conditions, it is improbable that the elevated temperatures necessary to cause the inhibition of glucan sucrose synthesis are a factor in the routine growth of glucan-producing *Lactobacilli*. The variability of glucan synthesis would then seem to lie in the accumulation of the spontaneously occurring variants which lacked the synthetic capacity and which utilized sucrose by an invertase-type hydrolysis.

This work was supported in part by Training Grant 5TI AI 144(01-04) from the National Institutes of Allergy and Infectious Diseases, U.S. Public Health Service.

REFERENCES

- BAILEY, R. W. (1959). Transglucosidase activity of rumen strains of *Streptococcus bovis*. *Biochem. J.* **72**, 42.
- BAILEY, R. W., BARKER, S. A., BOURNE, E. J. & STACEY, M. (1957). Immunopolysaccharides. Part IV. The isolation and properties of dextranucrase of *Betacoccus arabinosus*. *J. chem. Soc.* p. 3580.
- BARKER, S. A., BOURNE, E. J., SALT, E. & STACEY, M. (1958). Production of mannitol by *Lactobacillus* causing ropiness in cider. *J. chem. Soc.* p. 2736.
- CARR, J. G. (1959). Some special characters of the cider *Lactobacilli*. *J. appl. Bact.* **22**, 377.
- CHOU, T. C. & TOBIAS, J. (1960). Quantitative determination of carbohydrates in ice cream by paper chromatography. *J. Dairy Sci.* **43**, 1031.
- CUMMINS, C. S. & HARRIS, H. (1956). The chemical composition of the cell wall in some Gram-positive bacteria and its possible value as a taxonomic character. *J. gen. Microbiol.* **14**, 583.
- DAIN, J. A., NEAL, A. L. & SEELEY, H. W., JUN. (1956). The effect of carbon dioxide on polysaccharide production by *Streptococcus bovis*. *J. Bact.* **72**, 209.
- DUNICAN, L. K. & SEELEY, H. W., JUN. (1963). Temperature sensitive dextranucrase synthesis by a *Lactobacillus*. *J. Bact.* **86**, 1079.
- EVANS, J. B. & NIVEN, C. F., JUN. (1951). Nutrition of the heterofermentative *Lactobacilli* that cause greening of cured meat products. *J. Bact.* **62**, 599.
- GORNALL, A. G., BARDAWILL, C. J. & DAVID, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. biol. Chem.* **177**, 751.
- GUIRARD, B. M., SNELL, E. E. & WILLIAMS, R. J. (1946). The nutritional role of acetate for lactic acid bacteria. I. The response to substances related to acetate. *Arch. Biochem.* **9**, 361.
- GUNSALUS, I. C. & CAMPBELL, J. J. R. (1944). Diversion of the lactic acid fermentation with oxidized substrates. *J. Bact.* **48**, 455.
- HEHRE, E. J. (1951). Enzymatic synthesis of polysaccharides. *Advanc. Enzymol.* **11**, 297.
- HOUGH, L., JONES, J. K. N. & WADMAN, W. H. (1950). Quantitative analysis of mixtures of sugars by the method of partition chromatography. V. Improved methods for the separation and detection of the sugars and their methylated derivatives on the paper chromatogram. *J. chem. Soc.* p. 1702.

- LANGSTON, G. W. & BOUMA, C. (1960). A study of the microorganisms from grass silage. II. The lactobacilli. *Appl. Microbiol.* **8**, 223.
- LEDERER, E. & LEDERER, M. (1955). *Chromatography*. New York: Elsevier Publishing Co.
- MACLEOD, R. A. & SNELL, E. E. (1947). Some mineral requirements of the lactic acid bacteria. *J. biol. Chem.* **170**, 351.
- MAN, J. C. DE., ROGOSA, M. & SHARPE, M. E. (1960). A medium for the cultivation of lactobacilli. *J. appl. Bact.* **23**, 130.
- MAYER, H. D. (1938). Dissertation. Delft. Cited by Perquin (1940).
- MILLIS, N. F. (1951). Thesis, University of Bristol. Cited by Carr (1959).
- MITCHELL, P. (1950). Spectrophotometric estimation of nucleic acid in bacterial suspension. *J. gen. Microbiol.* **4**, 399.
- NEELY, W. B. & NOTT, J. (1962). Dextranucrase, an induced enzyme from *Leuconostoc mesenteroides*. *Biochemistry*, N.Y. **1**, 1136.
- NIVEN, C. F., JUN., & EVANS, J. B. (1957). *Lactobacillus viridescens* nov. spec., a heterofermentative species that produces a green discoloration of cured meat pigments. *J. Bact.* **73**, 758.
- OXFORD, A. E. (1958). The nutritional requirements of rumen strains of *Streptococcus bovis* considered in relation to dextran synthesis from sucrose. *J. gen. Microbiol.* **19**, 617.
- PARTRIDGE, S. M. (1948). Filter-paper partition chromatography of sugars. *Biochem. J.* **42**, 238.
- PEDERSON, C. S. & ALBURY, M. N. (1955). Variation among the heterofermentative lactic acid bacteria. *J. Bact.* **70**, 702.
- PERQUIN, L. H. C. (1940). On the incidental occurrence of rod-shaped dextran producing bacteria in a beet-sugar factory. *Antonie van Leeuwenhoek*, **6**, 227.
- ROGOSA, M., WISEMAN, R. F., MITCHELL, J. A., DISRAELY, M. N. & BEAMEN, A. J. (1953). Species differentiation of oral lactobacilli from man including descriptions of *Lactobacillus salivarius* nov. spec. and *Lactobacillus cellobiosus* nov. spec. *J. Bact.* **65**, 681.
- SHIMWELL, J. (1949). A study of ropiness in beer. Part III. Ropiness due to lactobacilli. *J. Inst. Brewing*, **46**, 26.
- SMITH, I. (1960). Ed. *Chromatographic and Electrophoretic Techniques*. London: Heineman.
- TSUCHIYA, H. M., KOEPEL, H. J., CORMAN, J., BRYANT, G., BOGARD, M. O., FEGER, V. H. & JACKSON, R. W. (1952). The effect of certain cultural factors on production of dextranucrase by *Leuconostoc mesenteroides*. *J. Bact.* **64**, 521.
- WILLIAMSON, D. H. (1959). Studies on lactobacilli causing ropiness in beer. *J. appl. Bact.* **22**, 392.