40% sulfur and samples 2 and 3 were sulfur-free.

Synthetic samples were made by mixing weighed amounts of *p,p'*-DDT, α-BHC, γ-BHC, sulfur, and talc. These were then extracted and analyzed. The results of single determinations are shown in Table II. The average errors for γ-BHC and for *p,p'*-DDT were again nearly equal and were slightly less than ±0.05%.

The band of DDT at 9.8 microns was selected because it is a strong absorption band, it is well isolated, and it is produced by both the para-para' isomer and the ortho-para' isomer. The presence of *o,p'*-DDT may be detected by the absorption band at 12.3 microns. If this isomer is present, one can still use the 9.8-micron band for the determination of total DDT (*5*), as both isomers absorb to nearly the same extent. Although these isomers are not equal in their insecticidal properties, no distinction is made between them in the guaranteed analysis of present samples in Alabama. Several compounds related to DDT also absorb at 9.8 microns and the spectrum should be checked qualitatively to assure the absence of significant quantities of these interfering substances. Spectra for the several commonly found related compounds are given by Downing (*5*). In only a few of the samples analyzed to date have significant amounts of these compounds been detected.

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OLIGOSACCHARIDE PRODUCTION

Concentration Effects in the Enzymatic Conversion of Lactose to Oligosaccharides

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A study was made of hydrolyzing conditions conducive to high oligosaccharide yields by the action of *Saccharomyces fragilis* lactase on lactose. With pH, temperature, and enzyme concentration held constant, lactose substrates from 5 to 50% were hydrolyzed. The percentage of lactose converted to oligosaccharides increased as the starting lactose concentration increased. This relationship held up to a limiting lactose concentration of 35% (w./v.), at which a maximum conversion of 44.6% was obtained. At starting lactose concentrations of 22 to 50%, the quantity of oligosaccharides present (at the time at which the oligosaccharide concentration reached a maximum value) was linearly related to the sum of the galactose and glucose concentrations as well as to the starting substrate concentration.

THE HYDROLYSIS OF LACTOSE by acid was first reported in 1812, and its hydrolysis by a lactase preparation in 1883 (*9*). That lactase is a hydrolyzing enzyme capable of splitting the glycosidic bond of lactose to form galactose and glucose is common knowledge. That lactase is also capable of synthesizing di- and oligosaccharides was not known until recently.

The advent of paper chromatography permitted broad advances in elucidating the action of lactase on lactose. The report in 1951 by Wallenfels (*7*), the first on the presence of oligosaccharides, was followed by the contributions of Aronson (*1*), Pazur (*4*), and Roberts and McFarren (*5*).

The formation of 11 galactosyl oligosaccharides (Nos. 1 to 11, Figure 1) is the result of a transgalactosidation reaction in which galactose in the form of a galactosido-enzyme complex reacts

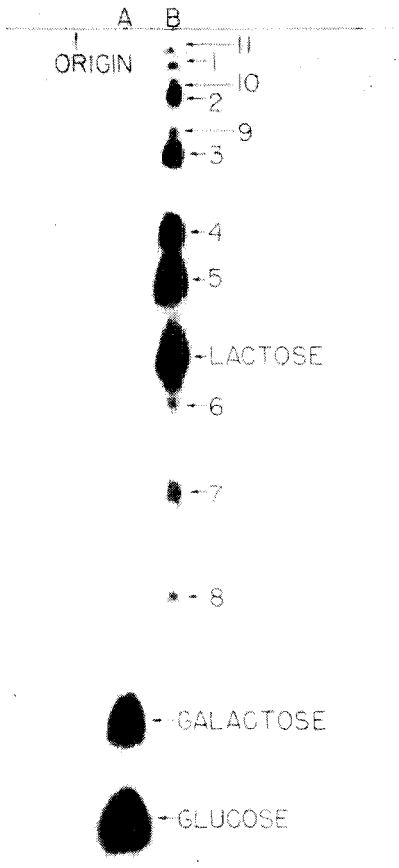


Figure 1. One-dimensional descending paper chromatogram of demonosed oligosaccharide preparation showing presence of 11 oligosaccharides formed by action of *S. fragilis* lactase on lactose

as a donor molecule with various acceptors.

A is a galactose-glucose standard, and *B* is a demonosed oligosaccharide preparation. The oligosaccharides were separated using two 24-hour developments in the solvent system ethyl acetate–pyridine-water (2.5:1.0:3.5). The color reagent was ammoniacal silver nitrate. The monoses were removed from the oligosaccharide preparation by the charcoal column technique of Whistler and Durso (*8*). Equations illustrating the reactions involved have been discussed by Wallenfels (*7*) and Pazur (*3*).

For evaluation purposes, large quantities of oligosaccharides were desirable. Therefore a study was undertaken to determine what conditions would provide substantially large quantities of galactosyl oligosaccharides by the action of *Saccharomyces fragilis* lactase on lactose. This paper reports the effect of varying the lactose concentration on the production of oligosaccharides.

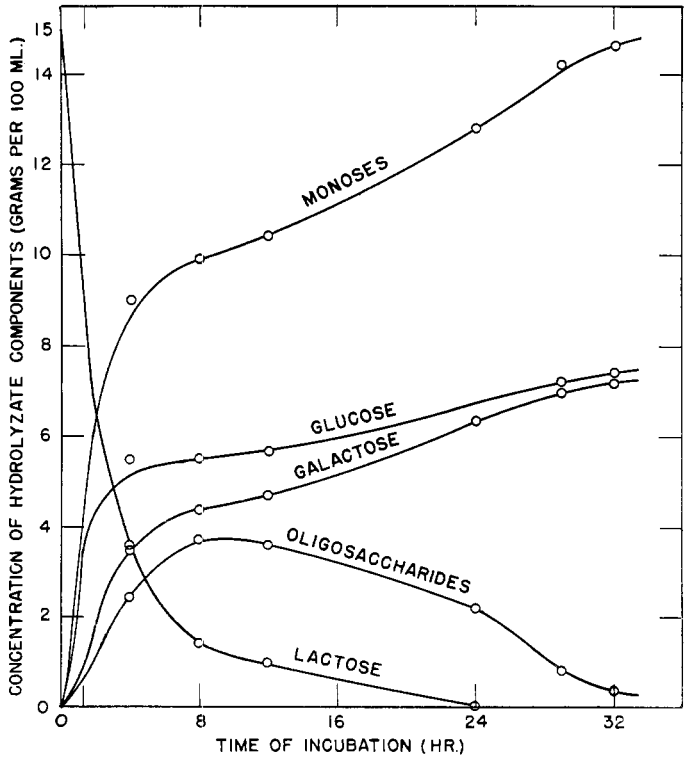
Experimental

Hydrolysis Procedure. Oligosaccharides were formed in the reaction in buffer at 35° C. between measured quantities of lactose and lactase. The buffer, pH 6.2, contained 8 parts of 0.067*M* potassium monobasic phosphate and 2 parts of 0.067 *M* sodium dibasic phosphate. The preparation of *S. fragilis* lactase has been described (*5*). A 0.5% (w./v.) slurry of the lactase preparation was made separately with the phosphate buffer and added to the reaction flask (Table I). A preservative containing 7 parts of toluene and 3 parts of chloroform was added so as to make up 5% of the final volume. In reactions of only 6 hours' duration, the use of this preservative is optional. The reaction mixture was mixed thoroughly by hand every 20 minutes during the course of the reaction at 35° C. Samples were taken every hour; the enzyme was inactivated by heating at 77° C. for 30 minutes, deproteinized with equivalents of barium hydroxide and zinc sulfate (*6*), and diluted to a volume, an aliquot of which could be satisfactorily spotted on filter paper chromatograms by the method of McFarren, Brand, and Rutkowski (*2*).

Determination of Oligosaccharides. Paper chromatography made it possible to determine not only the presence but the amount of oligosaccharides in the hydrolyzates. As the lactase preparation was zymase inactive (*5*), the quantity of oligosaccharides synthesized was calculated by subtracting the sum of the lactose, galactose, and glucose content of each hydrolyzate as determined by paper chromatography (*2*) from the quantity of lactose used for the hydrolysis. With a few exceptions, only those samples which by a visual examination of the chromatogram appeared to contain oligosaccharides at maximum concentration were analyzed quantitatively.

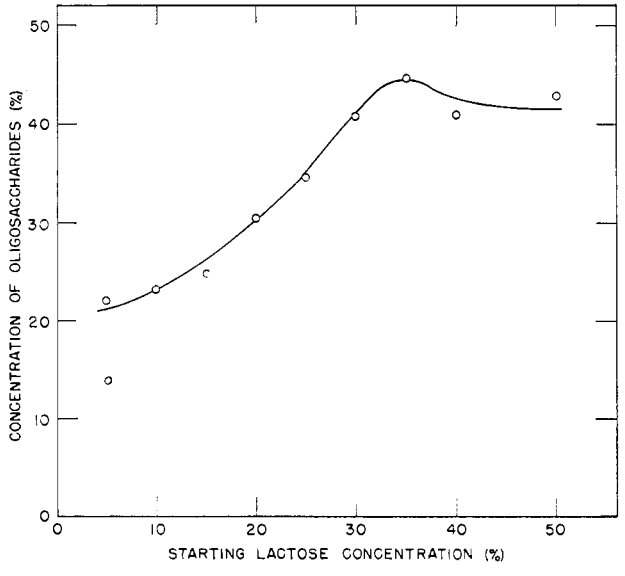
Results and Discussion

The data obtained from analyses of samples taken during a 15% lactose



◀ Figure 2. Hydrolysis of 15% lactose with 0.5% *S. fragilis* lactase at 35° C. in 0.067*M* phosphate buffer, pH 6.2

▼ Figure 3. Maximum conversion of lactose to oligosaccharides by *S. fragilis* lactase



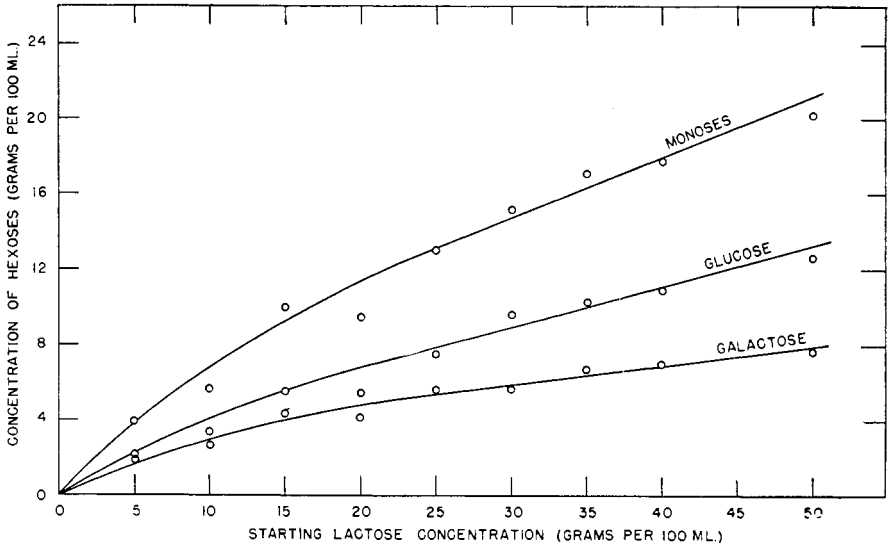


Figure 4. Concentration of hexoses at time oligosaccharide concentration reaches maximum value

hydrolysis are presented in Figure 2. The general form of these curves is typical of lactase hydrolysis at the various concentrations of lactose studied. The curves for galactose and glucose reflect the fact that less free galactose is present, because of its incorporation into the oligosaccharide molecules in the transgalactosidation reaction. The lactose curve illustrates the rapid hydrolytic activity of the enzyme in the early stages of the reaction. The oligosaccharides increased to a maximum of 24.6% of the total sugars at 8 hours and then decreased rapidly as they were converted to monoses. The oligosaccharides were still present well after the time at which all the lactose had been utilized.

Lactose concentrations ranging from 5 to 50% were studied and the maximum conversion of lactose to oligosaccharides at each substrate level is shown in Table I. The value representing the oligosaccharides in each determination is the sum of 11 different oligosaccharides tabulated as a single quantity. The percentage of lactose converted to oligosaccharides increased as the starting lactose concentration increased up to a limiting concentration of 35% (w./v.). Maximum conversion of lactose to oligosaccharides was 44.6% obtained in 5 hours at the 35% starting lactose level.

The oligosaccharide data shown in Table I are presented graphically in Figure 3. The maximum conversions of lactose to the oligosaccharides at each substrate level are plotted independently of the time required in each reaction to obtain maximum oligosaccharide conversion. Hydrolysis of lactose substrates greater than 35% failed to increase the percentage of lactose converted to oligosaccharides. However, at initial lactose concentrations greater than 35%, the oligosaccharide concentration remained at its maximum for periods varying up to 6 hours before decreasing.

Figure 4 depicts the relationship among galactose, glucose, and lactose when the oligosaccharide concentration reaches a maximum value. Because the lactase enzyme functions as a transgalactosidase, the synthesis of oligosaccharides involves the transfer of galactose to suitable acceptor molecules. As a result, free glucose was always present in greater quantities than free galactose. The difference in concentration of the two monoses at the time at which the oligosaccharide concentration reached a maximum value increased with increasing original substrate. This was the result of greater galactose utilization as oligosaccharide formation became more efficient. Between 22 and 50% substrate levels, the concentration of monoses at the time at which the oligosaccharide concentration reaches a maximum value is linearly related to the original lactose concentration.

Further interpretation of the enzyme action can be made when the per cent lactose converted to monoses and oligosaccharides at the time at which the concentration of oligosaccharides reaches a maximum value is plotted against the original lactose concentration (Figure 5). As the substrate concentration increased, the percentage of lactose converted into monoses decreased, until at an initial lactose concentration of 50% the concentration of oligosaccharides was greater than the concentration of monoses. Lower substrate levels favored the for-

Table I. Chromatographic Analyses of Lactase Hydrolyzed Lactose Solutions at Time of Maximum Oligosaccharide Production

Lactose, %	Lactase, %	Time, Hr.	Hydrolyzote Components, Grams/100 Ml.				Lactose Converted to Oligosaccharides, %
			Lactose	Galactose	Glucose	Oligosaccharides	
5.0	0.5	12.0	...	1.9	2.0	1.1	22.0
10.0	0.5	1.0	2.0	2.6	3.1	2.3	23.0
15.0	0.5	8.0	1.4	4.4	5.5	3.7	24.6
20.0	0.5	4.0	4.5	4.1	5.4	6.0	30.0
25.0	0.5	8.0	3.4	5.5	7.5	8.6	34.4
30.0	0.5	5.0	2.6	5.6	9.6	12.2	40.7
35.0	0.58	5.0	2.3	6.8	10.3	15.6	44.6
40.0	0.5	5.0	5.9	7.0	10.8	16.3	40.8
50.0	0.5	6.0	8.6	7.6	12.6	21.2	42.4

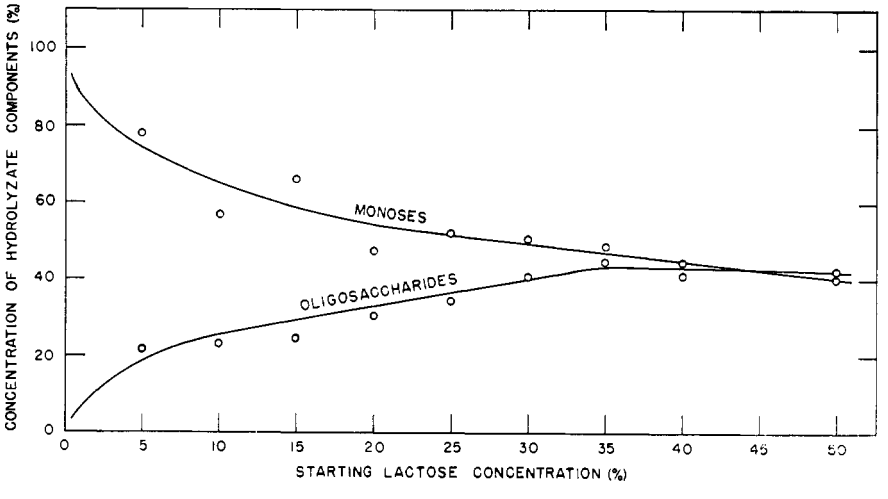


Figure 5. Per cent lactose converted to oligosaccharides and monoses at time oligosaccharide concentration reaches maximum value

mation of monoses rather than oligosaccharides.

The data obtained in this study show that enzyme efficiency (per cent lactose converted to oligosaccharides) falls off at substrate levels above 35%. However, when the concentration of hydrolyzate components, at the time at which the oligosaccharide concentration reaches a maximum value, is plotted against the starting lactose concentration, the curve shows that the quantity of oligosaccharides formed increases with increasing substrate concentrations (Figure 6). Starting at 22% lactose, which is the solubility limit of lactose at 35° C., the quantity of oligosaccharides formed was linearly related to the concentration of lactose. The concentration of oligosaccharides at a 50% starting lactose concentration exceeded the quantity of monoses present at the time at which the oligosaccharide concentration reached a maximum value.

In each curve of Figure 7, the concentrations of hexoses are plotted as ordinates, and the maximum concentrations of oligosaccharides formed in the substrate range, 5 to 50%, are plotted as abscissas. In solutions containing originally 22% or more substrate, the concentration of monoses was a linear function of the concentration of oligosaccharides at the time at which the oligosaccharide concentration reached a maximum value. This relationship existed for all subsequent lactose concentrations studied up to and including the 50% level. It appeared that once a saturated solution of lactose was employed at the start of the hydrolysis, the concentration of the products of enzymatic action (monoses and oligosaccharides), at maximum oligosaccharide concentration became linearly related to one another.

Addition-Type Hydrolysis. The experiments in which 20% lactose was used initially showed that if sufficient lactose was available during the reaction, the enzyme continued to form the oligosaccharides, as well as galactose and glucose. When the lactose concentration became negligible, the oligosaccharides formed were hydrolyzed to galactose and glucose at a faster rate.

During the hydrolysis of a 15% lactose solution, portions of the hydrolyzing mixture were analyzed quantitatively. The course of lactose depletion was consequently known (Figure 2). After 12 hours the oligosaccharide concentration began to decrease. The hydrolysis was repeated, but during the first 6.5 hours, 100 ml. of a 15% lactose solution in pH 6.2 phosphate buffer were added continuously to the reaction flask which originally contained 15 grams of lactose plus 0.5 gram of lactase in the phosphate buffer. After 10.5 hours, a chromatographic analysis showed a 34.1% conversion of lactose to oligosaccharides —a 9.5% increase in yield beyond that

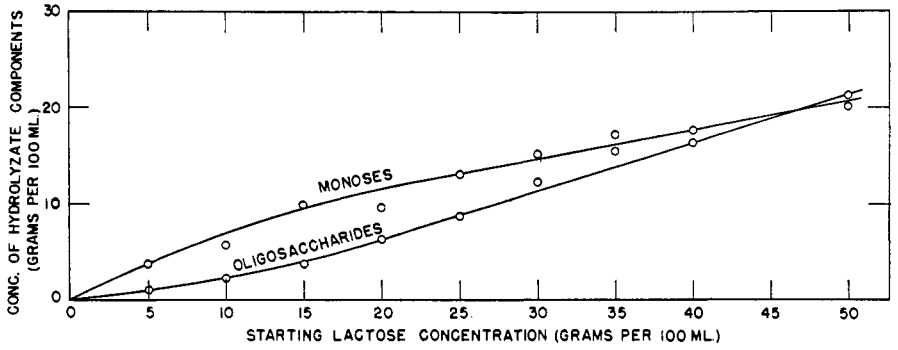


Figure 6. Effect of lactose concentration on production of hydrolyzate components at time oligosaccharide concentration reaches maximum value

obtained in the 15% lactose control experiment.

Comparable experiments using 25 and 35% lactose solutions were performed. Although the addition-type hydrolysis yielded larger amounts of oligosaccharides than the corresponding batch-type hydrolysis with solutions under 35% lactose (Table II), they failed to increase the 44.6% yield obtained at 35% lactose, which was the maximum conversion obtained in this study. A 35% addition-type hydrolysis yielded 44.0% of oligosaccharides. The yield of oligosaccharides in the 35% addition-type hydrolysis (Table III) reached 42.6% and remained in the range 42.6 to 44.0% from 5 to 12 hours. After reaching this limit, apparently an equilibrium condition was established between the formation and hydrolysis of the oligosaccharides. However, when the lactose concentration diminished to a point where the necessary bond energy for the synthesis of oligosaccharides was depleted, the oligosaccharides were hydrolyzed to the monose at an increased rate.

Hydrolysis of Supersaturated Solutions. To take advantage of the optimum rate occurring during the first few hours of hydrolysis and thereby to increase the oligosaccharide yield, supersaturated lactose solutions were employed. In this manner advantage was also taken of the decrease in water concentration, inasmuch as water functions as a competitive inhibitor. Reactions were run employing 30, 35, and 50% solutions of lactose in which all of the lactose was dissolved initially. The quantity of lactose converted to oligosaccharides by this modification did not exceed the quantities obtained in those hydrolyses in which lactose went into solution as it was utilized.

Hydrolysis of Milk Products. In addition to crystalline lactose, other

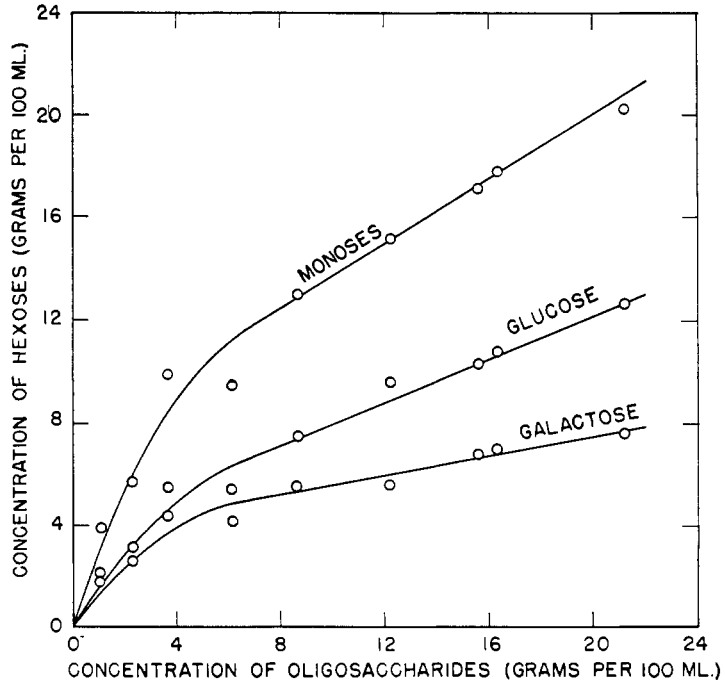


Figure 7. Relationship of hexoses to oligosaccharides at time oligosaccharide concentration reaches maximum value

sources of lactose were employed. A deproteinized casein whey containing 13.8% lactose was hydrolyzed at 35° C. with the 0.5% lactase preparation, but excluding the phosphate buffer. A 36.2% conversion of lactose to oligosaccharides was obtained in 5 hours, an increase of 11.6% over that obtained with a 15% solution of U.S.P. lactose.

The hydrolysis of a milk product with a lactose concentration above 25% was effected using 50% solids whey containing 35.5% lactose. The maximum conversion of lactose to oligosaccharides in the hourly fractions analyzed was 43.6% at 7 hours. This served as additional evidence that under the set of hydrolysis conditions employed in this study, a conversion of 44.6% (or approximately 45%) lactose to oligosaccharides was the limit.

Table II. Influence of Addition-Type Hydrolyses on Oligosaccharide Production

Lactose, %	Lactase, %	Time, Hr.	Hydrolyzate Components, Grams/100 Ml.				Lactose Converted to Oligosaccharides, %
			Lactose	Galactose	Glucose	Oligosaccharides	
15.0	0.5	8.0	1.4	4.4	5.5	3.7	24.6
15.0	0.5[a]	10.5	1.1	3.9	4.9	5.1	34.1
Add	0.25[b]						
25.0	0.5	8.0	3.4	5.5	7.5	8.6	34.4
25.0	0.5[a]	8.0	2.3	5.9	7.7	9.2	36.9
Add	0.25[b]						
35.0	0.58	5.0	2.3	6.8	10.3	15.6	44.6
35.0	0.58[a]	10.0	2.5	6.7	10.4	15.4	44.0
Add	0.29[b]						

[a] Starting concentration.
[b] Concentration at end of reaction.

Table III. Chromatographic Analysis of a 35% Lactose Addition-Type Hydrolysis

Time, Hr.	Hydrolyzate Components, Grams/100 Ml.				Lactose Converted to Oligosaccharides, %
	Lactose	Galactose	Glucose	Oligosaccharides	
5.0	8.1	4.5	7.5	14.9	42.6
6.0	5.9	4.7	9.4	15.0	42.9
8.0	3.7	5.7	10.5	15.1	43.1
10.0	2.5	6.7	10.4	15.4	44.0
12.0	1.8	6.9	11.1	15.3	43.7

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CAROTENE ASSAY

Determination of Carotene in Silages and Forages

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A rapid chromatographic method for determination of carotene in silages and forages is described. Necessary conditions for separation of carotene from impurities on magnesium oxide–Celite columns in the presence of small amounts of alcohol have been established. The analysis combines the advantages of splash-free blending extraction afforded by alcohol–Skellysolve B foaming mixtures and the elimination of epiphasic washing to remove alcohol. Additional advantages include faster and more compact elutions gained by the presence of alcohol on the column. Direct collections in smaller volumes eliminate concentration and transfer operations.

THE EXTRACTION OF CAROTENE from moist samples of forages and silages is usually effected by solvents which extract water as well. Methods in current use employ such extractants as 85% acetone (*1*), alcohol–petroleum ether mixtures (*6*), or acetone–petroleum ether mixtures (*9*). Generally, the extracts are made into two-phase systems by addition of water and petroleum ether—a hypophase of water plus acetone, or water plus alcohol, and an epiphase of petroleum ether. The two phases are allowed to settle before separation, carotene being extracted into the epiphase.

Because chromatographic separation of carotene from impurities is adversely affected by rather small amounts of alcohol, epiphases from alcohol–petroleum ether blendings are first made free of alcohol by water-washing, an operation often attended with troublesome emulsions. Zscheile (*9*) eliminated the need for washing by selecting acetone plus Skellysolve B as a blending liquid—acetone having much less effect than