ENZYMIC SYNTHESIS OF OLIGOSACCHARIDES FROM SUCROSE AND LACTOSE BY *ASPERGILLUS FLAVUS*

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Summary

The synthesis of oligosaccharides from sucrose and lactose by the mould *Aspergillus flavus* has been investigated.

Two trisaccharides synthesised from sucrose and lactose by the action of the enzyme extract of the mould have been separated in substantially pure form using cellulose and charcoal columns, and their composition and physical characteristics have been studied.

Recent investigations on carbohydrate transferring enzymes in moulds have resulted in the synthesis of a number of new oligosaccharides using disaccharides as substrates. Yeast invertase has been studied extensively during recent years because of its rapid hydrolysis of sucrose with the production of a number of oligosaccharides (Blanchard and Albon, 1950; Bacon and Edelman, 1950; Fischer, Kohtes and Fellig, 1951; White and Secor, 1952; White, 1952; Aronoff and Bacon, 1952; Albon, Bell, Blanchard, Gross and Rundell, 1953; Whelan and Jones, 1953; Edelman, 1954; Gross, 1954; Gross, Blanchard and Bell, 1954; Bacon, 1954). The hydrolysis of sucrose by mould invertase was described independently by Pazur (1952) and by Bealing and Bacon (1953) using enzyme preparations from *Aspergillus oryzæ*, *Penicillium spinulosum* and *Aspergillus niger*. Both the workers attributed the breakdown of sucrose to the transference of fructose residues to suitable acceptors by a β-fructofuranosidase. This theory also explains the synthesis of oligosaccharides other than sucrose as intermediates during the reaction.

The formation of oligosaccharides during the lactase hydrolysis of lactose was first reported by Wallenfels (1951) using an enzyme preparation from *Aspergillus oryzæ*, and independently by Aronson (1952) using *Saccharomyces fragilis* lactase. Roberts and McFarren (1953) confirmed Aronson's (1952) observation and showed that lactose hydrolysis by *S. fragilis* is accompanied by the formation of at least ten additional substances including some oligosaccharides. In a later publication (Roberts and McFarren, 1953), they reported the separation of seven oligosaccharides in pure form using

an activated charcoal-celite column. During the progress of our work on the transferring enzymes of *Aspergillus flavus*, Wallenfels *et al.* (1953) have reported the isolation of some of the carbohydrates formed during lactose hydrolysis by an enzyme preparation from a mould of *Aspergillus flavus* group. Pazur (1953, 1954) has recently described the formation and mechanism of synthesis of galactosyl oligosaccharides by using an enzyme preparation from lactose fermenting yeasts.

In continuation of our studies on transferring enzymes in this laboratory we have shown the presence of a *trans*-α-glucosidase in *Penicillium chrysogenum* (Giri *et al.*, 1953) and a *trans*-β-glucosidase in *Aspergillus flavus* (Giri *et al.*, 1954), which are able to transfer glucose units to glucose, maltose, cellobiose and other oligosaccharides formed during the course of enzyme action.

This paper reports the action of the enzymes of *Aspergillus flavus* on lactose and sucrose. By the use of chromatography on cellulose powder and on charcoal, we have isolated a trisaccharide from the reaction mixture of sucrose and another trisaccharide from lactose, in amounts sufficient to determine the general composition and other characteristics.

EXPERIMENTAL

Preparation of enzyme extract.—The enzyme preparation was obtained from *Aspergillus flavus*. The mould was grown in Czapeck-Dox medium. The pH of the medium was adjusted to 6·5. Quantities of 150 ml. of the medium were distributed in a number of Roux-bottles. The medium was inoculated with spores of *Aspergillus flavus* and incubated at room temperature (24–26°). After about four days the mycelial mats were harvested. 5 grams of the mycelium was crushed in a porcelain pestle and mortar to a fine suspension with 15 ml. of cold distilled water. The suspension was centrifuged and the supernatant liquid was dialysed against distilled water for 24 hrs. at 0–5° C. under toluene. A flocculant precipitate formed during dialysis was removed by centrifugation and the light yellow coloured supernatant liquid was stored in a refrigerator and used as a source of the enzyme.

Paper partition and adsorption chromatography.—The circular paper chromatographic technique (Giri and Rao, 1952) according to the procedure reported for separation of sugars (Giri and Nigam, 1953, 1954) was employed for the study of the course of the synthesis of oligosaccharides. Butanol : pyridine : water (60 : 40 : 30) and butanol : acetone : water (20 : 70 : 10) were used as developing solvents. Triphenyl tetrazolium chloride reagent for detecting the presence of reducing sugars, α-naphthylamine phosphoric acid reagent for ketoses and aniline diphenylamine phosphoric acid as general

reagent for all sugars were used. For quantitative estimation, the paper chromatographic procedure as described by Giri and Nigam (1954) was used.

The charcoal column of Whistler and Durso (1950) for the isolation of lactose oligosaccharide and a powdered cellulose column of Hough, Jones and Wadman (1949) for the sucrose oligosaccharide were employed.

The mould extract (4 c.c.) was added to 1 c.c. of buffered sucrose and lactose solutions respectively to give a final concentration of 5% (W/V) of the sugar in ·02 M acetate buffer at pH 5·0. The solution was incubated at room temperature (24–26° C.) and aliquots were removed at intervals and spotted on the circumference of a circle drawn at the centre of a circular filter-paper of 30 cm. diameter. The chromatograms were developed with acetone-butanol-water and after drying at room temperature each developed spot area on the chromatogram was divided into two sectors and treated with α-Naphthylamine phosphoric acid and diphenylamine phosphoric acid reagents respectively with the help of a brush.

Results

Action of mould extract on sucrose.—It was observed that the new oligosaccharides were produced during the 15 minutes of incubation followed by rapid disappearance of sucrose. At later stages sucrose started gradually disappearing and the oligosaccharides were hydrolysed to glucose and fructose which formed the final products. The formation and disappearance of the oligosaccharides is shown in Fig. 1. A similar reaction was also observed during the growth of the mould in Czapeck's medium. A maximum production of the oligosaccharides was obtained during the third day of incubation which was followed later by their disappearance. Both the oligosaccharides formed gave positive keto group test and were non-reducing. The order of the circular R_f values of the oligosaccharides from sucrose is given in Table I.

Isolation of oligosaccharide 1 (*from sucrose*).—24 ml. of sucrose solution (pH 5·0) containing 6 gm. of sugar was incubated with 96 ml. of the enzyme extract for 45 minutes, when the production of the oligosaccharide 1 was maximum. The enzyme was inactivated by boiling the solution in a water-bath for a few minutes and the digest was transferred to a cellulose column of 4 × 32·5 cm. dimensions. The column was developed with the solvent acetone : butanol : water (70 : 20 : 10), which was allowed to drop through a dropping funnel attached to the column. The rate of flow was adjusted to 50 ml. per hour, and 100 ml. fractions in the initial stages, and 25 ml. fractions in the later stages were collected. Acetone was evaporated from different fractions and the water layer was separated from butanol and

TABLE I

R_f values of sugars and oligosaccharides in two solvents on Whatman No. 1 paper (30 cm. diam.)

Sugars and oligosaccharides	Acetone 70 Butanol 20 Water 10	Butanol 60 Pyridine 40 Water 30
Fructose	0·62	0·49
Glucose	0·53	0·47
Sucrose	0·45	0·40
Lactose	0·36	0·31
Oligosaccharide 1 (from Sucrose) ..	0·37	0·33
Oligosaccharide 2 (from Sucrose) ..	0·33	0·28
Oligosaccharide 1 (from Lactose) ..	0·28	0·21
Oligosaccharide 2 (from Lactose) ..	0·20	0·12

analysed chromatographically. Initially glucose and fructose were eluted out in the first 1·5 litres of the solvent followed by the appearance of sucrose in the later stages. Complete removal of sucrose was achieved by passing 1·5 litres more of the solvent followed by the appearance of oligosaccharide 1. The next 750 ml. portion contained the pure trisaccharide. Acetone was removed from this portion by evaporation under vacuum and the water layer separated from butanol. The butanol layer was further extracted with small quantities of water to take away the trisaccharide and finally the combined solution was evaporated *in vacuo* over calcium chloride in a desiccator. The thick syrup remaining after 5 days was precipitated with alcohol and again evaporated. The colourless precipitate was further shaken with acetone to give an amorphous white powder, which was chromatographically pure oligosaccharide 1. The final yield was 450 mg., *i.e.*, about 6% of sucrose used. It had a melting point 81–85° C. $[\alpha]_D$ + 30·2 (C—5 in H_2O). These constants agreed closely with those given by Barker and Carrington (1953) and Bacon and Bell (1953) for trisaccharides from *Aspergillus niger* and Taka diastase respectively. On acid and enzymic hydrolysis with invertase it was completely hydrolysed to glucose and fructose. Heating above 60° C. for a short time resulted in the partial decomposition of the sugar to glucose and fructose.

Partial hydrolysis of the oligosaccharide 1 (50 mg.) with 1 c.c. of 20% acetic acid for 8 hrs. at 40° C. showed the presence of large quantities of sucrose together with small amounts of glucose, fructose and the remaining oligosaccharide.

For finding the units of fructose and glucose, a 1% solution of the sugar was hydrolysed with invertase, completely to glucose and fructose. The solution was spotted on the filter-paper and the amounts of fructose and glucose were determined colorimetrically after spraying with triphenyl tetrazolium chloride reagent keeping controls having different amounts of fructose and glucose for comparison. The results showed the ratio of fructose to glucose as 2: 1. The sugar is a trisaccharide composed of two fructose and one glucose units. The inverted solution also gave the same reducing value with Somogyi's reagent as a mixture of fructose and glucose in the ratio 2: 1.

Action of the mould extract on lactose.—Unlike the action of the extract on sucrose, lactose was hydrolysed only slowly. Though there was formation of two new oligosaccharides during the 24 hr. incubation, complete hydrolysis of lactose was only possible after prolonged period of incubation. The disappearance of lactose was accompanied by the simultaneous accumulation of the oligosaccharides 1 and 2 (Fig. 2) with R_f values less than that of lactose (Table 1). The sugars had reducing action with triphenyl tetrazolium chloride and silver nitrate reagents. Attempts to increase the production of one of the oligo-saccharides by suppression of the other by addition of glucose or galactose as reported by Aronson (1952), in *S. fragilis*, did not result in any marked increase in our case, thereby showing that they are of a different nature.

Isolation of the oligosaccharide 1 (from lactose).—A digest consisting of 5 gm. of lactose in 20 ml. of 0·02 M acetate buffer of pH 5 and 80 ml. of enzyme extract of *Aspergillus flavus* was incubated for 3 days and added to a charcoal column of 4 × 40 cm. dimension. Monosaccharides, glucose and galactose were removed by eluting with water and the disaccharide by 5% alcohol, the trisaccharide was obtained in the 15% alcohol eluate. It was concentrated to a small volume and the sugar was precipitated with absolute alcohol. After the evaporation of alcohol *in vacuo* over calcium chloride in a desiccator it was obtained as a white amorphous powder. It had a melting point 120–25° C. $[\alpha]_D + 27{\cdot}6$ (C— 4 in H_2O); the reducing value = 36·8 (glucose = 100).

On hydrolysis with acids the sugar was converted to glucose and galactose. Partial hydrolysis with dilute hydrochloric acid gave 3 bands representing glucose, galactose and lactose together with the unreacted trisaccharide.

Estimation of glucose and galactose units after complete acid hydrolysis showed it to be composed of 2 units of galactose and one of glucose by a procedure as described in the case of sucrose.

DISCUSSION

In the aforementioned investigation of the action of enzymes of *Aspergillus flavus* on sucrose and lactose, it was found that at least two oligosaccharides were formed from both the substrates. One of the oligosaccharides synthesised from sucrose (oligosaccharide 1) was isolated in pure form (not crystallised) by fractionation on a cellulose column. The saccharide was non-reducing and homogeneous by paper chromatography as well as by the method of isolation. On hydrolysis with acid and invertase it gave fructose and glucose, the ratio of fructose to glucose being 2·0:1·0. Partial hydrolysis yielded glucose, fructose, sucrose and unhydrolysed trisaccharide. The saccharide had $(\alpha)_D + 30·2°$. From the optical rotation it appears to be identical with that of O—α—D glucopyranosyl- (1 → 2) -O-β-D-fructofuranosyl (1 → 2)-β-D-fructo furanoside isolated by Bacon and Bell (1953) and Barker and Carrington (1953) from the products of the action of Takadiastase and *Aspergillus niger* respectively on sucrose. The synthesis of this trisaccharide is probably due to the enzymic transfer of a β-fructofuranosyl radical to sucrose as suggested by Bacon and Bell (1953).

One of the two oligosaccharides synthesised from lactose by the mould enzyme was isolated in pure form by fractionation on charcoal column. The results of analysis of the oligosaccharide indicate that it is composed of two galactose and one glucose units which are linked as galactose-galactose-glucose. Recently French and Wild (1953) have suggested a method of correlating the carbohydrate structure with their paper gram mobilities. They have shown that the paper gram mobilities of homologous oligosaccharides fall into a regular series. On plotting the logarithm of the partition function α' against the molecular size a straight line characteristic of each series of oligosaccharides is obtained. By increasing the size of a saccharide by a single hexose unit, the paper gram mobility was found to decrease by an amount depending on the type of the hexose unit being added and its mode of linkage. This method was used for the determination of the structural features of the oligosaccharides synthesised from sucrose and lactose by the mould extract. Fig. 3 shows the relationship between the logarithm of partition function α' and the number of hexose unit per molecule of the sugar and oligosaccharides. In the case of oligosaccharide 1 of sucrose and lactose series, it was found that they contain three hexose units; while those present in oligosaccharide 2 are assumed to be four in number. It may be seen from Fig. 3 that the two oligosaccharides 2 (from sucrose and lactose) fall into a series with glucose, sucrose and oligosaccharide 1, and glucose, lactose and oligosaccharide 1 respectively. The regularity

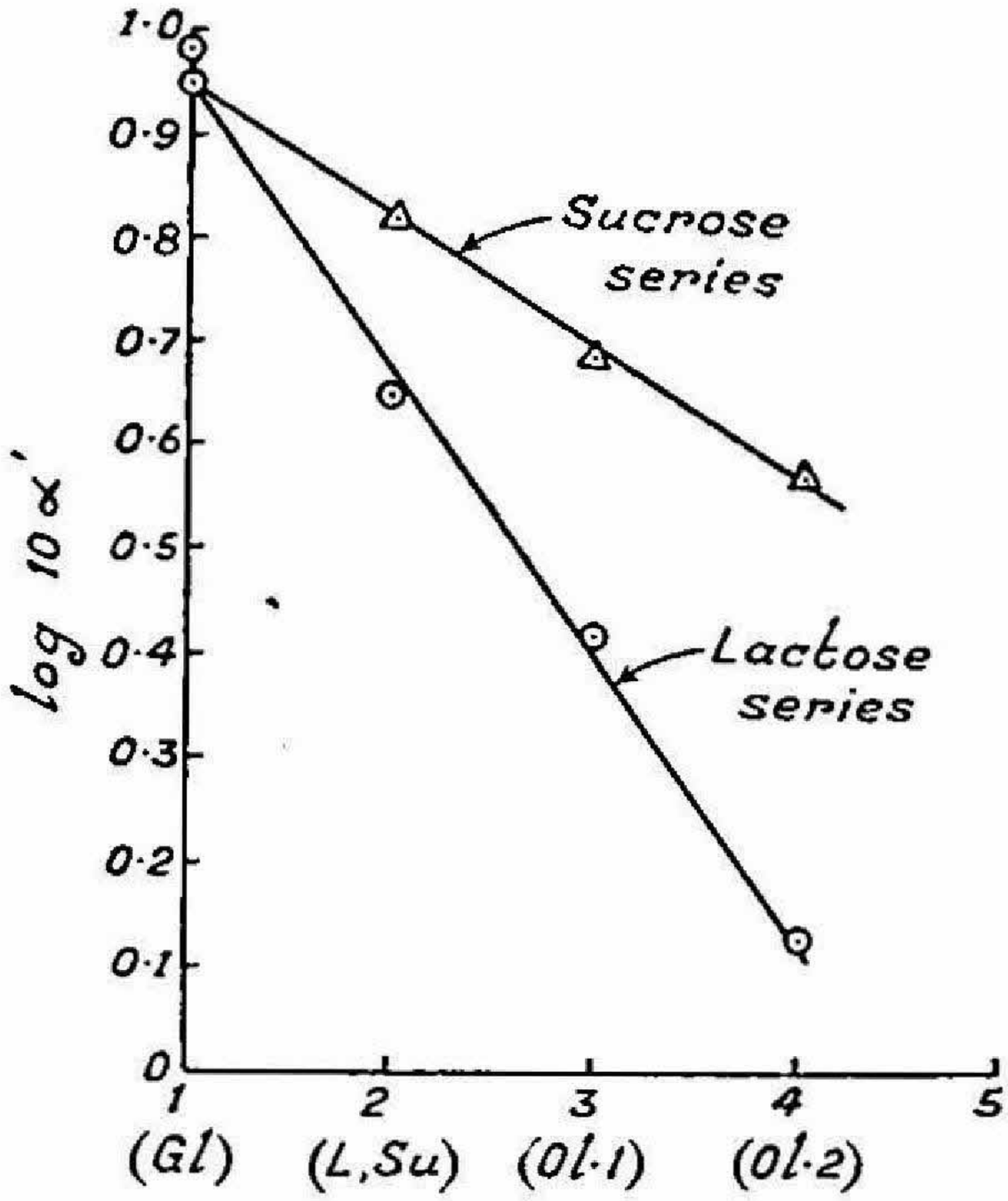

FIG. 3. Paper gram mobility of the oligosaccharides produced by the action of *Aspergillus flavus* enzyme on sucrose and lactose.

Gl—glucose: L—lactose: Su—sucrose; Ol. 1—oligosaccharide 1 from sucrose and lactose; Ol. 2—oligosaccharide 2 from sucrose and lactose.

with which the two oligosaccharides fall into a chromatographic series with other sugars of the same series shows that the oligosaccharides 2 may be composed of four hexose units. As the oligosaccharide 1 of lactose series contains three hexose units—galactose-galactose-glucose, the oligosaccharide 2 probably contains four units joined together as galactose-galactose-galactose-glucose.

In a similar manner the oligosaccharide 2 synthesised from sucrose may also contain four hexose units. Further work on the structure of the two oligosaccharides by the study of their physical and chemical properties, after isolation in pure form, is in progress.

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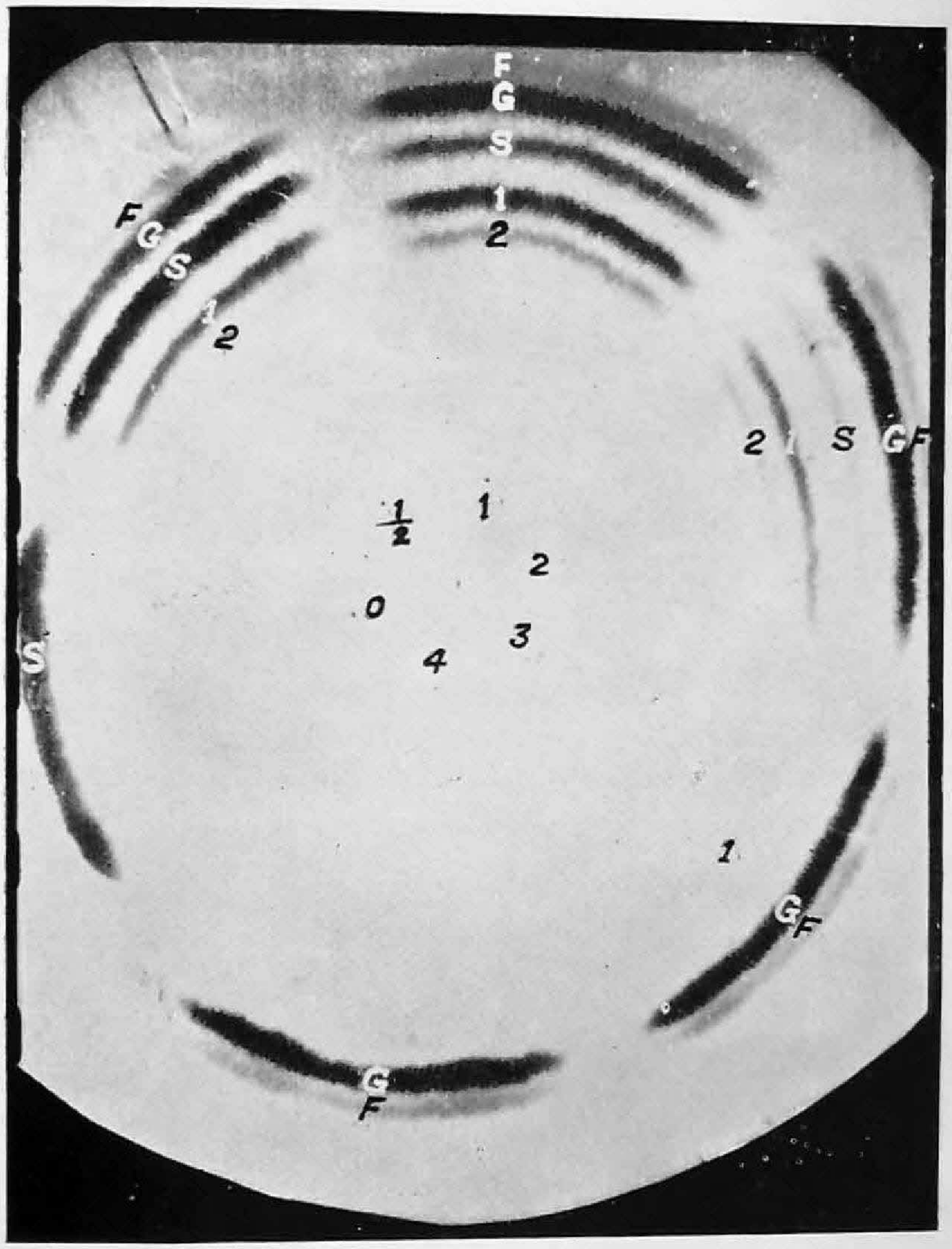

FIG. 1. Multi-sector chromatogram showing the formation of sugars and oligosaccharides from sucrose by the action of the enzyme extract of *Aspergillus flavus*.

The numbers on the circumference of the inner circle indicate the hours of incubation.
F—Fructose; G—Glucose · S—Sucrose; 1—Oligosaccharide (1); 2—Oligosaccharide (2).
Whatman No. 1, 30 cm. diam. Developing Solvent—*n*-butanol-acetone-water (20 : 70 : 10).
Reagent—Aniline-Diphenylamine phosphoric acid. Double development.

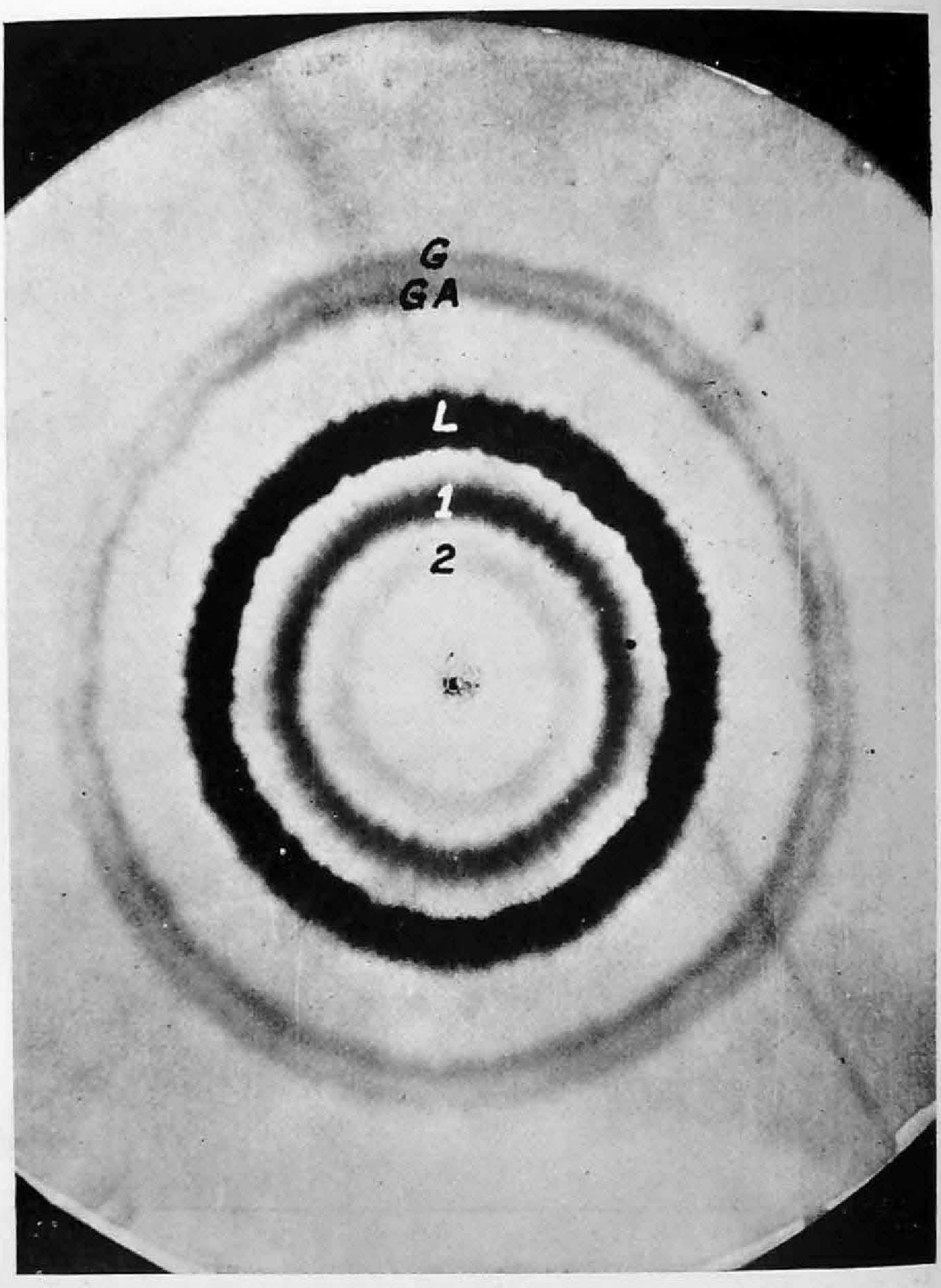

FIG. 2. Circular chromatogram showing the formation of sugars and oligosaccharides from lactose by the action of the enzyme extract of *Aspergillus flavus* after three days of incubation at 24–26° C.

G—Glucose ; GA—Galactose ; L—Lactose ; 1 and 2—Oligosaccharides.
Developing solvent—*n*-Butanol-pyridine-water (60 : 40 : 30).
Reagent—Aniline-Diphenylamine phosphoric acid. Whatman No. 1, 30 cm. diam. Double development.